



Published in final edited form as:

Horm Behav. 2016 February ; 78: 168–177. doi:10.1016/j.yhbeh.2015.11.007.

Two-hit exposure to polychlorinated biphenyls at gestational and juvenile life stages: 1. Sexually dimorphic effects on social and anxiety-like behaviors

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Abstract

Endocrine disrupting chemicals (EDCs) are widespread environmental contaminants that affect many neuroendocrine functions. The brain is particularly vulnerable to EDCs during critical periods of gestational development when gonadal hormones exert organizational effects on sexually dimorphic behaviors later in life. Peripubertal development is also a time of continued neural sensitivity to organizing effects of hormones, yet little is known about EDC actions at these times. We sought to determine effects of prenatal or juvenile exposures to a class of EDCs, polychlorinated biphenyls (PCBs) at human-relevant dosages on development, physiology, and social and anxiety-related behaviors later in life, and the consequences of a second juvenile “hit” following prenatal treatment. We exposed male and female Sprague-Dawley rats to PCBs (Aroclor 1221, 1mg/kg/day, ip injection) and/or vehicle during prenatal development (embryonic days 16, 18, 20), juvenile development (postnatal days 24, 26, 28), or both. These exposures had differential effects on behaviors in sex and age-dependent ways; while prenatal exposure had more effects than juvenile, juvenile exposure often modified or unmasked the effects of the first hit. Additionally, females exhibited altered social and anxiety behavior in adolescence, while males displayed small but significant changes in sociosexual preferences in adulthood. Thus, the brain continues to be sensitive to organizing effects of EDCs through juvenile development. As humans are exposed to EDCs throughout multiple periods in their life, these findings have implications for our understanding of EDC effects on physiology and behavior.

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Keywords

Endocrine-disrupting chemical; PCB; Aroclor 1221; adolescence; ultrasonic vocalization; social behavior; anxiety

Introduction

Endocrine disrupting chemicals (EDCs) are ubiquitous in our environment and alter a range of behaviors and physiological functions (Zoeller et al., 2012). One class of EDC is polychlorinated biphenyls (PCBs); while banned in the U.S. in the 1970's, PCBs are detectable in virtually all humans (Agency for Toxic Substances and Disease Registry, 2000). PCBs, along with other environmental EDCs, have been well-studied for how early life exposures (usually prenatal or early postnatal) lead to a latent dysfunction in the adult. Nevertheless, there are other life stages when there is high vulnerability to EDCs, as well as times in life other than adulthood when outcomes should be assessed. A case in point is juvenile and adolescent development, when the brain is sensitive to organizational effects of gonadal hormones, and during which time pubertal increases in these hormones activate, and continue to organize, neural circuits that were established earlier (Ahmed et al., 2008; Cooke and Woolley, 2009; De Lorme and Sisk, 2013; Markham et al., 2007; Mohr and Sisk, 2013; Primus and Kellogg, 1990; Schulz et al., 2004; 2009; Sisk and Foster, 2004; Sisk et al., 2003). This life stage is characterized by age-specific behaviors that may be uniquely affected by prior EDC exposure; and exposure during which can lead to alterations in the adult behavioral phenotype (DellaSeta et al., 2006; Yu et al., 2011).

Initial behavioral work on developmental PCB exposures in rats showed that perinatal exposure reduced the performance of sexual behavior in adulthood (Chung and Clemens, 1999; Chung et al., 2001; Cummings et al., 2008; Steinberg et al., 2007; Wang et al., 2002). However, these behaviors are part of a much broader suite of behaviors used to communicate affective and social information to a conspecific, and which have been sparsely studied for PCB effects (Jolous-Jamshidi et al., 2010). The current study extended prior work by assessing sexually dimorphic and hormone-dependent adolescent and adult behaviors in response to PCBs. In adolescent rats, play behavior and ultrasonic vocalizations (USVs) are important features of the behavioral repertoire and communicate social status, hedonia, and social interest and memory (Auger and Olesen, 2009; Knutson et al., 1998; Meaney and Stewart, 1981; Moles et al., 2007; Scattoni et al., 2009). In adult rats, hormone-dependent sociosexual partner preference and the production of USVs can also reflect sociosexual choice and motivation distinct from overt sexual behaviors (Harding and McGinnis, 2003; Henley et al., 2011; McGinnis and Vakulenko, 2003). At both ages, anxiety-like behaviors are also hormone sensitive and sexually dimorphic (Adler et al., 1999; Bitran, 1993; Johnston and File, 1991; Marcondes et al., 2001; Mora et al., 1996; Nomikos and Spyraiki, 1988) and therefore may also be targets for PCB disruption.

Humans and animals are exposed to PCBs and other EDCs throughout the lifespan, and there is evidence in humans that PCB exposure is tied to cognitive and neurodevelopmental problems when evaluated in infants and young children (Engel and Wolff, 2013). It is not

possible in humans, though, to differentiate between various sensitive periods such as prenatal and adolescence on neurobehavioral outcomes. The current study directly addressed this by exposing rats to PCBs in a “two hit” model, whereby rats were exposed prenatally, in adolescence, or both, the latter enabling elucidation of the consequences of a second hit on the first. We ascertained outcomes on a range of social and anxiety-like behaviors in adolescence and adulthood. The hypothesis tested was that a second PCB hit would change the neurobehavioral developmental trajectory in a manner not predicted by either hit alone, and that due to natural sex differences in gonadal hormones, the sexes would differ in their outcomes.

Methods

PCB Treatment

The PCB mixture used in this study was Aroclor 1221 (A1221, AccuStandard, New Haven, CT, catalog number: C-221N-50MG, Lot: 23683) dissolved in vehicle (Veh, 4% dimethylsulfoxide, Sigma number D4540; Sigma, St Louis, Missouri in sesame oil). A1221 is a mix of ~45 congeners, but predominantly composed of PCBs 1, 3, 8, 4, 15, 6, and 2 in order of greatest (~%40) to least (~3%) amount (Frame et al., 1996). These congeners are lightly chlorinated (66% mono- and 26% di-chlorinated), with chlorine substitutions predominantly at ortho and para positions. For prenatal exposure, dams were injected with Veh or A1221 solution (1mg/kg, ip) on E16, E18, and E20. For juvenile exposure, pups were given an additional set of injections (1 mg/kg, ip) on P24, 26, and 28. This mixture, dosage, and injection paradigm was selected so that outcomes of the current study could be compared with findings from several other previous studies using a very similar exposure regime. This dose is not toxic to dams and does not cause fetal loss (Dickerson et al., 2011; Reilly et al., 2015; Steinberg et al., 2008; Walker et al., 2014), and is estimated to be within the range of human exposure to PCBs (Agency for Toxic Substances and Disease Registry, 2000; Centers for Disease Control, 2009; Matthews and Anderson, 1975; Patterson et al., 2009; Steinberg et al., 2008; Takagi et al., 1976).

Animal care and physiological measures

All animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by The University of Texas at Austin Institutional Animal Care and Use Committee. Sprague Dawley rats were purchased from Harlan Laboratories (Houston, Texas) and were housed in a humidity- and temperature- controlled room with a 12:12 reversed light cycle (lights off at noon) at 21–23C. 2–3 animals were group housed in polycarbonate cages (43 × 21 × 25cm) with aspen bedding (PJ Murphy Forest Products, Sani-Chip), a PVC tube for enrichment, and weekly cage changes. Rats were fed low phytoestrogen Harlan-Teklad 2019 Global Diet (Harlan-Teklad, Indianapolis, Indiana) ad libitum for the duration of the experiment. Upon arrival, rats were handled daily to acclimate them to their new housing conditions, and mating began at least two weeks later.

Females (3–4 month old, virgin) were mated with sexually experienced male rats (~6 months old); each male sired 2 litters, one with a Veh and one with an A1221 dam. The

morning after successful mating (indicated by a sperm positive vaginal smear), termed embryonic day (E) 1, dams were singly housed. Dams were randomly assigned to either Veh (n = 6) or A1221 (n = 6), and each litter contributed no more than two animals per group. On E16, E18, and E20, during the period of sexual differentiation of the rat brain (Breedlove, 1992; Ramaley, 1979; Rhees et al., 1990; Tobet and Fox, 1989; Wagner et al., 1998), dams were weighed and injected with 0.1 mL of Veh or A1221 solution (ip, 3 hours prior to onset of dark stage). Dams were provided with nesting materials several days prior to expected parturition on E23. On the day after birth [postnatal day (P) 1], litters were culled to 6–8 pups of equal sex ratios by removing individuals with extreme anogenital index (anogenital distance/³ body weight) measures. Pups were weaned on P21 and housed with same sex littermates (2–3 per cage), and were weighed and handled for at least 5 minutes weekly. For juvenile exposure, rats were given an additional set of injections on P24, 26, and 28, when puberty is beginning and the brain is highly sensitive to organizing effects of testosterone (Döhler and Wuttke, 1975; Saksena and Lau, 1979; Schulz et al., 2009; Smyth and Wilkinson, 1994; Vetter-O'Hagen and Spear, 2011). Cagemates were given the same treatment to prevent cross-contamination.

With both gestational and juvenile exposures, there are four experimental groups per sex, in a 2×2 balanced design (first hit prenatal, second hit adolescent): Veh-Veh; A1221-Veh; Veh-A1221; A1221-A1221 (Figure 1). Final Ns per group were between 9 and 12. The experimenters were blind to treatment throughout the duration of the experiment. Developmental and physiological measures were taken throughout animals' lives. Small but significant effects of PCBs were found on body weight, age of puberty onset in females, and testes weight at time of sacrifice (described in Supplemental Materials).

Behavioral testing

All behaviors were run in a consistent order at least one hour after lights-out under dim red light (Table 1). Adolescents were tested for social interactions with same-sex and age conspecifics and anxiety-related behaviors over 7 days, while they were between P30-39 (details in Supplemental Materials). On testing days (T)1 through T3, animals were tested for affiliative behaviors and USVs during social interactions. Adolescent rats show play behaviors and emit 50kHz USVs in anticipation of, and during, the interaction (Auger and Olesen, 2009; Knutson et al., 1998; Moles et al., 2007; Scattoni et al., 2009). These USVs are produced in multiple rewarding contexts and can be roughly divided into flat and frequency modulated (FM) calls thought to serve as communication and indicate positive affect, respectively (Burgdorf et al., 2011; Knutson et al., 2002; Wöhr and Schwarting, 2007). In addition to standard play behaviors, a previously undefined behavior was also observed and called 'hopping'. It was frequently performed by animals immediately prior to a play bout, and consisted of an animal quickly jumping around the cage. On T4 or T5, they were tested for sociability, with half of the animals run on each day. Rats tend to show a preference for a novel over a familiar conspecific stimulus animal, and tests of this behavior can be used to indicate general social interest and memory. On T6 and T7, animals were tested for elevated plus maze (EPM) and light dark box (LD) performance, respectively. In these test, rats can chose to explore the entire novel environment, including the more exposed open arm or light side of the apparatus, or remain more hidden in the closed or dark

side. Greater time spent in the exposed areas is an indicator of reduced anxiety. Females were not cycling regularly yet, and testing was not synchronized to estrous cycle.

As adults, these same rats were tested for sociosexual interactions with receptive opposite-sex conspecifics and anxiety-related behaviors over 4 days between P90-110 (details in Supplemental Materials). Behavior testing began on the evening of proestrus in females, and receptivity was confirmed with a stimulus male animal. On T1 sociosexual USVs and partner preference were tested, in that order. As with adolescent tests, flat and FM calls in the 50 kHz range may serve to communicate with the conspecific and indicate hedonia, respectively (Burgdorf et al 2011). Preference for spending time near a hormone- over a non-hormone treated potential mate is also indicative of sociosexual motivation. Two and three days after sociosexual testing, on T3 and 4, animals were tested for EPM and LD performance, respectively (during diestrus in females). Sexes and litters were balanced across apparatus, testing time, and stimulus animal.

Analysis and Statistics

Final Ns were as follows: for males, Veh-Veh (12); A1221-Veh (10); Veh-A1221 (10); A1221-A1221 (9) and for females, Veh-Veh (10); A1221-Veh (9); Veh-A1221 (12); A1221-A1221 (10). No effects of birth litter were observed in any of the significant effects, so individual rats were used as the unit of analysis for statistical purposes, and there were no cohort effects. Any outliers were identified via Grubbs test (maximum of one per group) and were removed. Within each test, 0–2 animals were identified as outliers across all groups; in the sociosexual test, 4 animals were excluded from analysis because they did not leave the center chamber. Individual behavioral and physiological measures were analyzed using a 2×2 analysis of variance (ANOVA) within each sex to determine any main effects of prenatal or juvenile treatment and interactions, with appropriate follow-up t-tests to identify the source of detected interactions. If measures failed to meet normality or homogeneity assumptions (as indicated by Shapiro Wilks and Levene's tests), a non-parametric Kruskal-Wallis test was used and is indicated in the results as a 'KW'. In this case, an interaction was identified by testing for effects of one treatment while holding the other constant and *vice versa*.

Effect sizes of main effects and interactions from ANOVAs are indicated with Eta squared (η^2 = treatment variance/corrected total variance); when multiplied by 100, η^2 indicates the percent of variance in the dependent variable that is attributable to each effect. Effect sizes of follow-up t-tests were determined by Cohen's d (d = the difference between two means divided by a standard deviation for the data). Analysis was completed using SPSS, GraphPad Prism and <http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php>, with significant differences demarcated in figures and tables as *, $p < 0.05$ and **, $p < 0.01$.

Results

Juvenile Behavior

PCB exposures primarily affected female, but not male, adolescent animals, and were dominated by interactions between prenatal and juvenile exposure (Table 2).

Affiliative USVs—PCB treatments did not affect the numbers of flat or frequency modulated (FM) USVs produced while interacting with stimulus animals on any day of testing (Supplemental Figure 2). However, they did affect calling in experimental female, but not male, animals in a subset of tests (Fig 2). On Day 1 of testing (T1), prenatal and juvenile exposure interacted to affect the number of FM calls following the first conspecific interaction (KW, $p < 0.05$, $\eta^2 = 0.17$, Fig 2a), such that females exposed to two hits called less than those exposed to a single prenatal ($t_{(16)} = 2.62$, $p < 0.05$, $d = -1.24$) or single juvenile ($t_{(19)} = 2.93$, $p < 0.01$, $d = -1.29$) hit. On Day 2, a main effect of prenatal exposure was found on flat calls in the Pre-stimulus session (KW, $p < 0.05$, $\eta^2 = 0.12$, Fig 2b), with more calls in the prenatal A1221 than the Veh females.

Affiliative behavior—PCBs tended to reduce play behavior, significantly so in females (Fig 2). The number of wrestles was affected in females, but not males (Fig 2c). An interaction between prenatal and juvenile exposure was found ($F_{(1,36)} = 4.14$, $p < 0.05$, $\eta^2 = 0.10$) such that females exposed to A1221 during both prenatal and juvenile development showed more wrestles than those only exposed as juveniles ($t_{(19)} = 3.28$, $p < 0.01$, $d = 1.70$). While the number of hops was not affected by PCBs (data not shown), the latency to perform the behavior was modified, in both males and females (Fig 2d). In males, an interaction between prenatal and juvenile exposure was found ($F_{(1,25)} = 4.36$, $p < 0.05$, $\eta^2 = 0.14$), such that males exposed during both prenatal and juvenile development showed a longer latency than those only exposed as juveniles ($t_{(10)} = 2.26$, $p < 0.05$, $d = 0.36$). In females, a main effect of juvenile exposure was found (KW, $p < 0.05$, $\eta^2 = 0.15$), such that latency to hop was longer in juvenile A1221 compared to Veh females, regardless of their prenatal exposure. No effects were found on nape contacts (data not shown). Positive correlations between USV production and the performance of play behaviors are discussed in the Supplemental materials.

Sociability—In juveniles, no effects of PCBs were seen on time spent in the area immediately proximate to the stimulus animal in the first stage of the test where they were given a choice between sides with or without a stimulus animal in it (Fig 2e). However, females (but not males) exposed to juvenile PCBs showed a longer latency to reach that area near the stimulus animal (KW, $p < 0.05$, $\eta^2 = 0.13$, Fig 2f). On the second stage of the test, animals were given a choice between the familiar and a novel stimulus animal. In this stage, prenatal and juvenile exposures did not affect time spent in, or latency to reach, the area immediately proximate to either stimulus animals or both stimulus animals combined (data not shown).

Anxiety-like Behavior—Prenatal and juvenile PCB exposure interacted to affect anxiety behavior in females, but not males (Fig 3). In the elevated plus maze, prenatal x juvenile

exposure interactions were found for time in the closed arm ($F_{(1,36)} = 4.71$, $p < 0.05$, $\eta^2 = 0.10$), such that females exposed to PCBs during both prenatal and juvenile development showed less anxiety than those exposed only as juveniles ($t_{(15)} = 2.58$, $p < 0.05$, $d = -0.81$). The expected inverse was also found with time in the open arm. A prenatal x juvenile exposure interaction was found for this measure, ($F_{(1,35)} = 8.00$, $p < 0.01$, $\eta^2 = 0.17$, Fig 3a), however this dataset failed Levene's test of homogeneity, so a Kruskal-Wallis analysis was used to determine where effects of one exposure depended on the level of the other. In that measure, females exposed to PCBs during both prenatal and juvenile development showed less anxiety than those exposed only as juveniles (KW, $p < 0.01$, $d = 1.24$). In addition, females exposed to PCBs during both prenatal and juvenile development showed less anxiety than those exposed only prenatally (KW, $p = 0.05$, $d = 1.01$). A similar effect was found in open arm entries ($F_{(1,36)} = 8.22$, $p < 0.01$, $\eta^2 = 0.17$, Fig 3b), such that the double-hit females entered the open arm more than females only exposed as juveniles ($t_{(19)} = 3.39$, $p < 0.01$, $d = 1.48$). In the light/dark box, no effects of PCBs were observed on the time spent in the light side of the box (Fig 3c). However, females exposed to PCBs during prenatal development showed more entries into the light side than females not exposed at that time, independent of any subsequent juvenile exposure ($F_{(1,36)} = 7.86$, $p < 0.01$, $\eta^2 = 0.18$, Fig 3d).

Adult Behavior

PCB effects on adult behavior were observed in males, and were dominated by main effects of the single prenatal and juvenile hits that affected behavior in opposite directions (an increase or decrease in performance, Table 3).

Sociosexual USVs—Prenatal exposure increased the number of flat calls in the session preceding the opposite sex interaction in males, independent of juvenile exposure ($F_{(1,37)} = 5.82$, $p < 0.05$, $\eta^2 = 0.13$, Supplemental Figure 3). Other aspects of USV behavior with an opposite sex conspecific were unaffected by treatments.

Sociosexual Choice—PCBs altered the preference for an opposite sex stimulus animal in males but not females. Males exposed to PCBs prenatally spent less time near a no-hormone stimulus animal than animals not exposed prenatally, independent of juvenile exposure ($F_{(1,34)} = 15.55$, $p < 0.01$, $\eta^2 = 0.28$, Fig 4b). As no significant effects were seen on time spent near a hormone stimulus animal (Fig 4a), this effect drove the preference for a hormone-treated stimulus animal in prenatally exposed males ($F_{(1,34)} = 10.17$, $p < 0.01$, $\eta^2 = 0.17 = 0.22$, Fig 4c). A separate main effect of juvenile exposure was found on time spent near the stimulus animals. Juvenile exposed males spent more time with the no-hormone female than did unexposed males ($F_{(1,34)} = 5.37$, $p < 0.05$, $\eta^2 = 0.10$, Fig 4b). This effect, together with the nonsignificant increase in time spent with the hormone-treated stimulus females, resulted in the observation that males with juvenile exposure spent more time with both stimulus animals than males not exposed at that time ($F_{(1,34)} = 8.65$, $p < 0.01$, $\eta^2 = 0.19$, Fig 4d).

Anxiety-like Behavior—No effects of PCBs were found on adult anxiety behavior, in either elevated plus maze or light dark box tests (Supplemental Figure 4).

Discussion

The current study utilized a two-hit model of exposure to PCBs to differentiate between different critical stages of exposure, ages at analysis, and sexes of subject on behavioral outcomes. The most consistent effects of PCBs were disruptions of female adolescent social and anxiety-like behavior. These results add to the literature on EDC effects on behavior in several novel and important ways. First, animals exposed during prenatal or juvenile development had unique sets of behavioral phenotypes. Second, the age at which testing was conducted and the sex of the animal also played important roles in the measured outcomes: effects of developmental PCB exposures on behaviors were predominantly manifested in females when tested in adolescence, while adult behaviors were affected in males. Third, the two-hit model revealed that the effects of prenatal exposure could be modified by juvenile exposure, suggesting that early exposure changes the developmental trajectory of the brain in ways that may not be discernible unless animals experience an additional exposure later in life. Fourth, only a subset of behaviors was affected, underscoring the importance of thorough behavioral testing to assess neurobehavioral outcomes. Humans are exposed to PCBs and other EDCs at multiple stages of development, and these results suggest that it is important to consider these factors in assessing exposures and potential neurobiological effects.

Effects of A1221 on Adolescent Social Behavior

Females exposed to prenatal PCBs produced more USVs in the session preceding introduction of the playmate on Day 2 than animals not exposed at that time. As the short-term isolation and placement into the recording chamber could serve as cues for the impending play session, this finding could indicate an increased anticipation of play after prenatal exposure. While no other main effects of prenatal exposure were found in additional measures of play or social interest or memory, juvenile exposure revealed additional prenatal effects. Specifically, prenatal exposure to PCBs was associated with more wrestling during play and fewer USVs post-play, but only in females that were also exposed as juveniles. The ethological meaning of post-play USV production is unclear but, considering the other prosocial effects of prenatal exposure, perhaps it indicates reduced arousal after a period of heightened engagement. Juvenile exposure also revealed an effect of prenatal exposure on the latency to hop during play interactions in adolescent males; however, the behavioral significance of this effect is limited, as no other measures of prosocial behavior were affected in male adolescents.

Prenatal PCB exposure in humans, as indicated by levels in umbilical cord blood, has been correlated with sex-dependent shifts in the performance of masculine and feminine play behavior in children (Vreugdenhil et al., 2002; Winneke et al., 2014). To our knowledge, no other studies have assessed the effects of prenatal PCBs on the performance of play behavior in rodents. In a related measure, prenatal exposure to other PCBs (PCB 47 and 77 mix, 2–4mg/kg, from mating until weaning) altered olfactory investigations of conspecifics in juvenile males (Jolous-Jamshidi et al., 2010). Although the PCB literature is limited, comparisons to results found using another EDC, bisphenol A (BPA) are illuminating. While PCBs have multiple mechanisms of action, both A1221 and BPA can be estrogenic in

some contexts. As in our study, BPA (~150 ug/kg oral to dam E0-P1) altered specific aspects of social interactions in juvenile male and female mice (Wolstenholme et al., 2011). However, a longer exposure period (E0-P21) of high and low doses in mice and rats shows inconsistent results (Dessi-Fulgheri et al., 2002; Ferguson et al., 2014; Porrini et al., 2005). Therefore, it appears that effects of prenatal exposure to these EDCs on adolescent social behavior are moderate and depend on the exposure period, the sex of the animal tested, and the specific behavior that was tested.

In contrast to prenatal exposure, juvenile exposure tended to reduce social motivation in adolescent females. Specifically, it increased the latency to hop in play interactions and reach the social stimulus in sociability tests in adolescent female animals. Thus, the timing of the PCB exposure has major implications for the direction of the effect on social behavior. Sex and age-dependent effects of postnatal EDC exposure on social behavior are also found in the literature. For example, juvenile female voles exposed to BPA from P8-P14 spent more time investigating a novel same-sex stimulus animal, while exposed juvenile males spent less time performing the same behavior than controls (Sullivan et al., 2014). However, juvenile BPA exposure (P23-30) had no effect on play and social interaction in juvenile male or female rats (Yu et al., 2011). Again, the choice of EDC and species differences may explain the opposite direction of the effects. We are unaware of any studies on links between PCBs in juveniles on social behavior in adolescent humans.

Effects of A1221 on Adult Sociosexual Behavior

Unlike adolescent behaviors, which were primarily affected in female rats, changes in adult behaviors were observed only in males. Sociosexual interest and motivation, as assessed in tests of USV production in response to a receptive opposite sex stimulus animal, was not affected by 1mg/kg prenatal A1221. This is in agreement to findings with a similar PCB exposure, where only a 0.5mg/kg (but not 1mg/kg) dose affected flat USVs produced after a sociosexual interaction (Davaeva et al., 2013). In preference tests, prenatal exposure reduced the amount of time spent by adult males near the no-hormone-treated stimulus animal. While this was associated with an increased preference for the hormone-treated animal, the meaning of this effect is difficult to interpret because even vehicle-treated animals did not show the expected preference for hormone-treated stimulus animals over non-hormone animals. Perhaps this is because of experimental factors such as time of day tested, or that rats were sexually naïve. In contrast to the current study of preference behavior, studies on the performance of sexual behavior have found that A1221 and mixes of indicator congeners tend to increase the latency to perform sex behavior (Chung and Clemens, 1999; Colciago et al., 2009; Faass et al., 2013; Steinberg et al., 2007). Therefore, it appears that prenatal PCBs affect specific aspects of sexual behavior.

Juvenile exposure tended to affect general partner preference. Juvenile PCB-exposed males spent more time with the no-hormone and (non-significantly) hormone treated stimulus animals, resulting in an overall increase in time spent with both social stimuli. To our knowledge, no other studies have assessed effects of juvenile PCB exposure on sociosexual behavior in adulthood. While lactational PCB exposure decreased typical partner preference (Cummings et al., 2008), adult exposure had no effect on sex behavior in female rats (Chung

et al., 2001), suggesting the importance of developmental stage in efficacy of exposure. An increase in adult same-sex sociability was found in response to peripubertal BPA exposure (P23-30); however the effect was present in female, but not male mice (Yu et al., 2011). Together, these studies suggest that the juvenile brain remains sensitive to EDCs, but effects are manifested only for certain measures of sociosexual behavior and motivation.

Effects of A1221 on Anxiety Behavior

When tested in adolescence, females but not males showed consistent effects of prenatal PCBs on anxiety. As a main effect in the LD box and an interaction in the EPM test, adolescent females exposed to PCBs prenatally showed a reduction in anxiety-like behavior. This reduction in anxiety in prenatally exposed adolescent females could underlie the increased social motivation observed in the same group. This is in contrast to anxiogenic effects reported after exposure of adolescent male and female mice to a mixture of non-dioxin like PCBs containing PCB 153 (10 or 100 ng/kg oral to dam P1-21)(Elnar et al., 2012). In humans, Inuit preschoolers with relatively higher levels of PCB 153 in umbilical cord blood had greater levels of anxiety and reduced positive affect. However, A1221 does not contain any PCB 153, so these studies suggest congener-specific effects of PCBs on adolescent anxiety.

Unlike in adolescence, no effects of exposure were found in tests of anxiety-like behaviors in adult animals in the current study. Age specific effects of PCBs are consistent with the human literature as well; heavily chlorinated PCBs increase depressive symptoms in older adults (Fitzgerald et al., 2008) but not young adults (Strøm et al., 2014). Therefore, some effects of PCBs on affective behavior may be missed if testing only occurs at a single age. Alternatively, it is possible that the previous adolescent testing in the same environment prevented us from observing effects in adulthood. Indeed, a low dose lactational exposure to a non-dioxin like mix of PCBs resulted in an increase in anxiety behavior in adult male and female mice (Elnar et al., 2012). More experiments are needed to determine if these discrepancies are the result of species, dose, or mixture. No main effects of juvenile exposure were seen in tests of anxiety in adolescent or adult animals.

A unique phenotype emerges from exposure to PCBs during juvenile development

Juvenile PCB exposure had effects that were different from those induced by prenatal exposure, so we must consider the possibility that the juvenile brain is uniquely sensitive to some effects of A1221. PCBs are known to act on several different receptors and enzymes, and these systems undergo developmental change. Therefore, it is not surprising that effects of prenatal exposure are unique when compared to juvenile exposure, and are modulated by a second hit. Interactions between prenatal and juvenile exposure were observed in female adolescent affiliative and anxiety-like behaviors and, in all cases, were the result of differences between animals exposed to a single juvenile hit compared to those exposed to the double hit. As such, *juvenile exposure unmasked an effect of prenatal exposure*. Therefore, we can conclude that prenatal PCB exposure changes the developmental trajectory of the brain in some ways that are undetectable except when animals experience an additional exposure later in life.

Conclusion

This is the first study to demonstrate that the behavioral phenotype is uniquely affected by a single hit of PCBs given either prenatally or during juvenile development. Moreover, in the two-hit PCB model, the second juvenile hit sometimes unmasks effects of a single prenatal hit to reveal an emergent phenotype. The most consistent effects of PCBs were an increase in prosocial and reduction in anxiety-like behavior in adolescent females. However, we should not conclude that PCBs affect behavior in a positive way, but instead, that exposure caused the behaviors to be performed in an atypical manner. The performance of these behaviors must be considered from the perspective of a conspecific rat, a process that we cannot do well as humans. Therefore, although effects were moderate and specific to certain behaviors, they highlight the need for continued testing across a range of behaviors, ages, and in both sexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Bethany Hart, Isaac Miller-Crews, Spurthi Tarugu, Michael Reilly, Viktoria Topper, Alexandra Garcia, and Weiling Yin for their help with data analysis and tissue collection. This work was funded by grants from NIEHS: RO1-ES020662 (ACG), T32-ES07247 (MRB), F32-ES023291 (MRB).

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Highlights

- Prenatal PCB exposure alters social and anxiety behaviors, especially in females.
- Juvenile PCB exposure affects male sociosexual preference behaviors.
- Prenatal and juvenile PCB exposures have both individual and interacting effects.
- Adolescence may be a second critical period for PCB exposure effects.

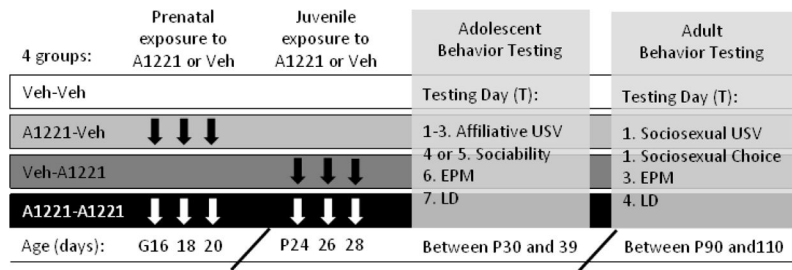


Figure 1. Experimental Design. Animals were exposed to either Vehicle (Veh) or Aroclor 1221 (A1221) prenatally and/or during juvenile development, as indicated by arrows. Behaviors were tested in adolescence (over 7 days between P30-39) and adulthood (over 4 days between P90-110). More test details are also included in Table 1. Abbreviations: G (gestational day); P (postnatal day); USV (ultrasonic vocalization); EPM (elevated plus maze); LD (light dark).

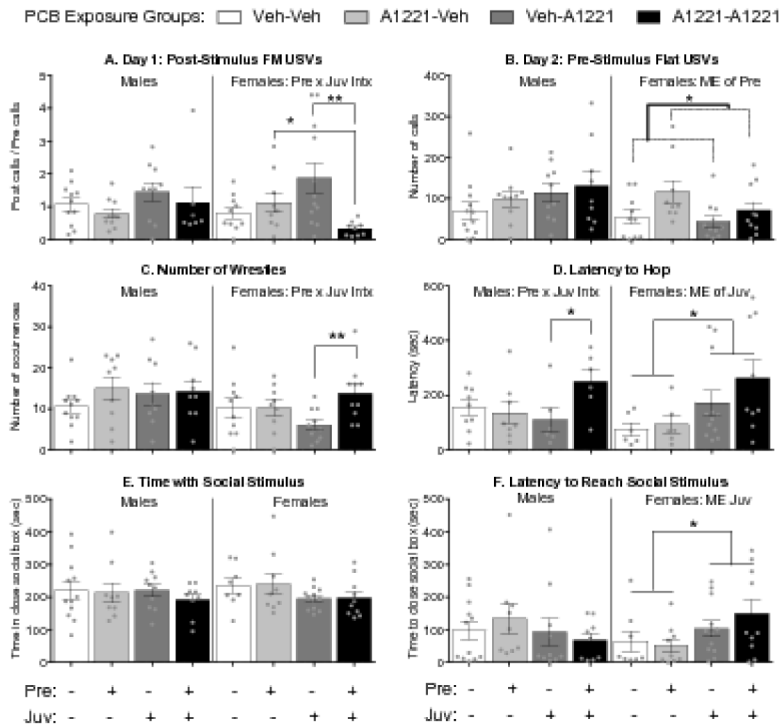


Figure 2. Effects of PCBs on adolescent affiliative and sociability behavior with a same-sex and age conspecific. Frequency modulated (FM) or flat ultrasonic vocalization (USV) production (A, B); play behaviors (C, D); and time spent with, and latency to reach, the stimulus animal in Stage 1 of the sociability test (E, F) are shown. Bars are mean \pm SEM, with dots indicating individual data points. Within-sex main effects (ME) of, or interactions between, prenatal (Pre) or juvenile (Juv) exposure are described in each subtitle, with specific group differences indicated by *, $p < 0.05$ or **, $p < 0.01$.

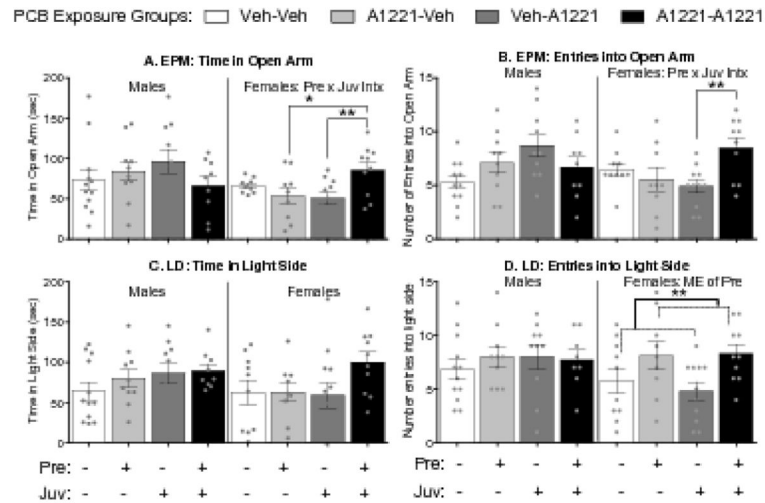


Figure 3. Effects of PCBs on adolescent anxiety-like behavior. Results from elevated plus maze (EPM, A and B) and light dark box (LD, C and D) are shown (mean ± SEM), with dots indicating individual data points. Within-sex main effects (ME) of, or interactions between, prenatal (Pre) or juvenile (Juv) exposure are described in each subtitle, with specific group differences indicated by *, $p < 0.05$ or **, $p < 0.01$.

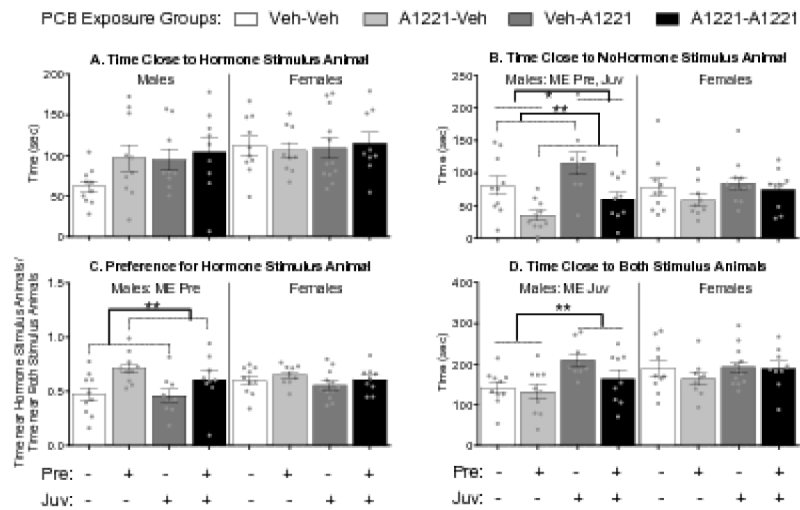



Figure 4. Effects of PCBs on sociosexual choice. Time near (A, B, D) and preference for (C) stimulus animals are shown (mean ± SEM), with dots indicating individual data points. Within-sex main effects (ME) of prenatal (Pre) or juvenile (Juv) exposure are described in each subtitle, with specific group differences indicated by *, $p < 0.05$ or **, $p < 0.01$.

Table 1

Behavior Testing Procedures

Adolescent (Adol.) animals were between 30 and 39 days old, and adult animals were between 90 and 110 days old.

Test Name (&interpretation)	Test Design	Age at day of test (T)	Stages of Test	Duration of Test	Outcomes Measured
Affiliative Behavior (motivation for playful social interactions)		Adol. (T1-3)	Isolation Pre-Stimulus Stimulus Post-Stimulus	4 hours 5 min (T1, 2) 10 min (T1-3) 5 min (T1,2)	T1, 2, and 3: Number of frequency modulated (FM) and Flat Ultrasonic vocalizations (USVs). T3: Number, duration, and latency to nape contact, hop, and wrestle
Sociability (attraction to and memory of same-sex social interactions)		Adol. (T4 or 5)	Habituate to chamber 1: Familiar vs Empty 2: Familiar vs Novel	5 min 10 min 10 min	Time and latency to reach area proximate to stimulus chamber
Sociosexual USVs (motivation for sexual interactions)		Adult (T1)	Pre-Stimulus Stimulus Post-Stimulus	5 min 10 min 5 min	Number of FM and Flat USVs
Sociosexual Choice (preferences for opposite-sex interactions)		Adult (T1)	Habituate to chamber Hormone/No-Hormone	5 min 10 min	Time in and latency to reach area proximate to stimulus chamber



Test Name (&interpretation)	Test Design	Age at day of test (T)	Stages of Test	Duration of Test	Outcomes Measured
Elevated Plus Maze (EPM) (anxiety-like behavior)		Adol. (T6) & Adult (T3)	Habituate to room Test	30 min 5	Time and number of entries into open arms
Light Dark Box (LD) (anxiety-like behavior)		Adol. (T7) & Adult (T4)	Habituate to room Test	30 min 5	Time and number of entries into light side

Table 2

Summary of Adolescent Results

	Males			Females		
	Effect of Prenatal A1221	Effect of Juvenile A1221	Effect of Prenatal (in intx)	Effect of Prenatal A1221	Effect of Juvenile A1221	Effect of Prenatal (in intx)
Affiliative Behavior						
T1, Pre Stimulus, FM USV	-	-	-	-	-	-
T1, Pre Stimulus, Flat USV	-	-	-	-	-	-
T1, Stimulus, FM USV	-	-	-	-	-	-
T1, Stimulus, Flat USV	-	-	-	-	-	-
T1, Post/Pre Stimulus, FM USV	-	-	-	-	-	↓*** -1.29
T1, Post/Pre Stimulus, Flat USV	-	-	-	-	-	-
T2, Pre Stimulus, FM USV	-	-	-	-	-	-
T2, Pre Stimulus, Flat USV	-	-	↑* 0.12	-	-	-
T2, Stimulus, FM USV	-	-	-	-	-	-
T2, Stimulus, Flat USV	-	-	-	-	-	-
T2, Post Stimulus, FM USV	-	-	-	-	-	-
T2, Post Stimulus, Flat USV	-	-	-	-	-	-
T3, Stimulus, FM USV	-	-	-	-	-	-
T3, Stimulus, Flat USV	-	-	-	-	-	-
T3, Number of Hops	-	-	-	-	-	-
T3, Number of Nape Contact	-	-	-	-	-	-
T3, Number of Wrestle	-	-	-	-	-	↑*** 1.70
T3, Latency to Hop	-	-	↑* 0.36	-	↑* 0.15	-
T3, Latency to Nape Contact	-	-	-	-	-	-
T3, Latency to Wrestle	-	-	-	-	-	-
Sociability						
Stage 1, Time in Social Zone	-	-	-	-	-	-
Stage 1, Latency to Social Zone	-	-	-	-	↑* 0.13	-
Stage 2, Time in Novel Zone	-	-	-	-	-	-

	Males			Females		
	Effect of Prenatal A1221	Effect of Juvenile A1221	Effect of Prenatal (in intx)	Effect of Prenatal A1221	Effect of Juvenile A1221	Effect of Prenatal (in intx)
Stage 2, Latency to Novel Zone	-	-	-	-	-	-
Stage 2, Time in Familiar Zone	-	-	-	-	-	-
Stage 2, Latency to Familiar Zone	-	-	-	-	-	-
Anxiety-like Behavior						
Elevated Plus, Time in Open	-	-	-	-	-	↑ ** 1.23
Elevated Plus, Entries into Open	-	-	-	-	-	↑ ** 1.48
Light Dark Box, Time in Light	-	-	-	-	-	-
Light Dark Box, Entries into Light	-	-	-	↑ ** 0.18	-	-

The direction (↑ increase or ↓ decrease in measure), significance (*p < 0.05, **p < 0.01), and size (η² for main effects or d for pair-wise interaction follow ups) of effects are shown. Interactions are due to an effect of prenatal exposure detected only in animals also exposed as juveniles; for simplicity, only this effect is shown in the interaction (intx) column. Abbreviations: T (testing day); FM (frequency modulated); USV (ultrasonic vocalization); A1221 (aroclor 1221)

Table 3

Summary of Adult Results

	Males			Females		
	Effect of Prenatal A1221	Effect of Juvenile A1221	Prenatal x Juvenile Intx	Effect of Prenatal A1221	Effect of Juvenile A1221	Prenatal x Juvenile Intx
Sociosexual USVs						
Pre Stimulus, FM USV	-	-	-	-	-	-
Pre Stimulus, Flat USV	↑* 0.13	-	-	-	-	-
Stimulus, FM USV	-	-	-	-	-	-
Stimulus, Flat USV	-	-	-	-	-	-
Post Stimulus, FM USV	-	-	-	-	-	-
Post Stimulus, Flat USV	-	-	-	-	-	-
Sociosexual Choice						
Time in Hormone Stim Zone	-	-	-	-	-	-
Time in No Hormone Stim Zone	↓** 0.28	↑* 0.10	-	-	-	-
Time in Either Stim Zones	-	↑** 0.19	-	-	-	-
Percent Time with Hormone Stim	↑** 0.22	-	-	-	-	-
Anxiety-like Behavior						
Elevated Plus, Time in Open	-	-	-	-	-	-
Elevated Plus, Entries into Open	-	-	-	-	-	-
Light Dark Box, Time in Light	-	-	-	-	-	-
Light Dark Box, Entries into Light	-	-	-	-	-	-

The direction (↑ increase or ↓ decrease in measure), significance (*p < 0.05, **p < 0.01), and size (η² for main effects) of effects are shown. Abbreviations: FM (frequency modulated); USV (ultrasonic vocalization); A1221 (aroclor 1221); Intx (interaction)