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## Effects of polychlorinated biphenyl (PCB) exposure on response perseveration and ultrasonic vocalization emission in rat during development

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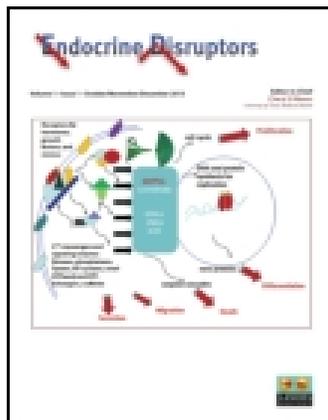
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# Effects of polychlorinated biphenyl (PCB) exposure on response perseveration and ultrasonic vocalization emission in rat during development

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**Keywords:** animal model, autism, environmental toxins, PCB, t-maze, ultrasonic vocalizations

**Abbreviations:** ANOVA, analysis of variance; ASD, autism spectrum disorder(s); PBDE, polybrominated biphenyl ether(s); PCB, polychlorinated biphenyl(s); PND, postnatal day(s); USV, ultrasonic vocalization(s)

The 3 major symptoms of autistic spectrum disorders include 1) social behavioral alterations, 2) problems in communication and 3) higher-order motoric deficits of perseveration and stereotyped movements. Previous work has shown that early developmental exposure to polychlorinated biphenyls (PCBs) alters rat pup social motivation and juvenile rat social recognition/investigation. The present work extends this previous research by examining how perinatal PCB exposure alters motoric functions and communication abilities at different stages of development. Action perseveration was examined using performance measures from a T-maze environment. Communication abilities were evaluated by monitoring ultrasound emission in rat pups during a brief isolation from the litter. T-maze learning and performance were significantly impaired in PCB exposed animals. Additionally, PCB exposure led to reduced ultrasound emission rates during brief isolation from the natal group. When combined with the previous work using the same developmental exposure regimen, it seems clear that PCB exposure at moderate doses can lead to alterations in 1) social behavior, 2) action choice and perseveration, and 3) communication abilities making it a potential candidate as an endocrine disruptor involved in the production of autistic spectrum disorder in the human population.

## Introduction

Research using animal models and attempting to replicate impairments of autism spectrum disorders (ASDs) is difficult but crucial to advance the search for treatments and causal factors. There are diverse ways to model autism, including, 1) perturbing behavioral/emotional experience (e.g., gestational stress); 2) producing brain lesions (e.g., early amygdala damage) or 3) exposing the organism to some form of xenobiotic (e.g., valproic acid exposure). Each of these methods has its advantages and can yield important information. The majority of the animal model work focuses on a subset of autistic-like alterations. For example, studies using early brain lesions have focused on social isolation by disrupting the hippocampus,<sup>1</sup> the amygdala,<sup>2</sup> or the orbitofrontal cortex.<sup>3</sup> Although these models mimic well several of the specific ASD social deficits, they do not account for the comorbid motor deficits seen in the clinical population. Genetic approaches often

use targeted mutations to define mechanisms regulated by genes considered important for ASD.<sup>4</sup> For example, the *Engrailed 2* mouse,<sup>5</sup> the *reeler* mouse,<sup>6</sup> and the *Dischevelled 1* mouse may capture the changes in social attachment and communication,<sup>4</sup> but again, they do not typically incorporate the developmental motor deficits seen in the clinical population. A move toward developing and using models with greater comprehensive validity in terms of modeling symptomology is an important goal in biomedical research.

Several studies have implicated environmental factors as acting synergistically with genetic factors to play a role in the production of the broad autistic phenotype.<sup>7-9</sup> There is a growing interest in possible links between certain environmental contaminants and autism.<sup>10</sup> A focus has been on heavy metals such as mercury and lead and commercial chemicals such as polychlorinated biphenyl (PCB) because of their established endocrine and neural disrupting potential.<sup>11-13</sup> Different studies have found exposure to contaminants to produce diverse and in most cases

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non-overlapping alterations that include emotional, behavioral, or communicative impairments.

Polychlorinated biphenyls (PCBs) are environmental toxicants that remain pervasive in the soil, water and air.<sup>14,15</sup> Exposure in work environments has been reduced since the industrial use of PCBs was banned in 1976; however, the threat of exposure persists particularly from ingestion of certain food items (i.e., fish and other marine organisms) and breast milk.<sup>16-18</sup> Exposure early in development has been shown to have the greatest potential to alter important neural and endocrine systems.<sup>19</sup> Impairments in fundamental functions related to growth, metabolism and hormone mechanisms have been characterized and replicated in basic animal research and human health surveys.<sup>20</sup> PCB is a powerful endocrine disruptor that alters thyroid status,<sup>21</sup> sex hormone functions<sup>22,23</sup> and other neuroendocrine processes.<sup>24-26</sup> These effects can lead to significant and long-lasting changes in neurochemistry and brain morphology.<sup>27,28</sup> Several brain regions including areas of the hypothalamus<sup>29,30</sup> and the basal ganglia<sup>31</sup> have been found to be deleteriously impacted by PCB exposure during development.

Our recent work has found that perinatal exposure to a simple mixture of 2 tetrachlorinated PCB congeners (PCB 47 - 2,2',4,4'-tetrachlorobiphenyl ortho-substituted, and PCB 77 - 3,3',4,4'-tetrachlorobiphenyl non-ortho-substituted) leads to social deficits in the young rat pup.<sup>32</sup> These deficits in social motivation include altered approach and preference for cues related to maternal care and suckling found in 2 week old rat pups using a conditioned place preference technique.<sup>32</sup> In adult animals, social recognition expressed typically by habituation to a familiar conspecific over repeated trials was found to be altered in juvenile rats after exposure to PCBs during development.<sup>33</sup> In addition, stress-induced social investigation was also found to be impaired in adult rats following earlier perinatal exposure to PCBs.<sup>33</sup> A goal of the present study was to expand on this previous work by examining the impact of PCB exposure on higher order motor functions and on early vocal responses emitted by the animals. The results would provide a more comprehensive picture for the role of PCB exposure in the development of each of the major symptom groups (Social, Motor and Communication Subgroups) observed in ASD. Though several studies have documented motor deficits as a result of perinatal PCB exposure,<sup>34-36</sup> few have examined possible perseverative motor deficits using a motor learning task or have followed the development of a wide battery of motor skills and retested to see if these skills improve or resolve with age. The present study measured the effect of 2 maternal dietary concentrations of PCB, 12.5 ppm (PCB 12.5) or 25 ppm (PCB 25) as compared to controls (PCB 0) on diverse motor skills at 3 stages of development (pup,

juvenile, adult), including repetitive behavior in the t-maze (post-natal days - PND 24–28), and isolation ultrasonic vocalizations (PND 10) to test the hypothesis that PCB exposure will result in a range of motor and social autistic-like behavioral dysfunctions.

## Results

### No significant impact of PCB on weight or food intake

Litter sizes were similar among the 3 conditions. Weight changes for the dam were calculated for the 2 weeks prior to parturition and during the 21-day pre-weaning period. No significant differences were found among weights of the pregnant females in the different groups 2 weeks prior to parturition. Similarly, the amount of weight gained by rat dams between parturition and weaning of the pups at 21 d of age was not significantly influenced by PCB. Changes in litter weight were compared during the pre-weaning period. The mean weight gain for rat pups exposed to PCB was not significantly less than that of controls over that 21-day period. There were no differences among the groups in daily rat chow consumption by the dam prior to parturition or dam and pups during the 21 day pre-weaning period. Thus, PCB exposure had no significant effect on measures related to food intake or weight gain over time (Table 1).

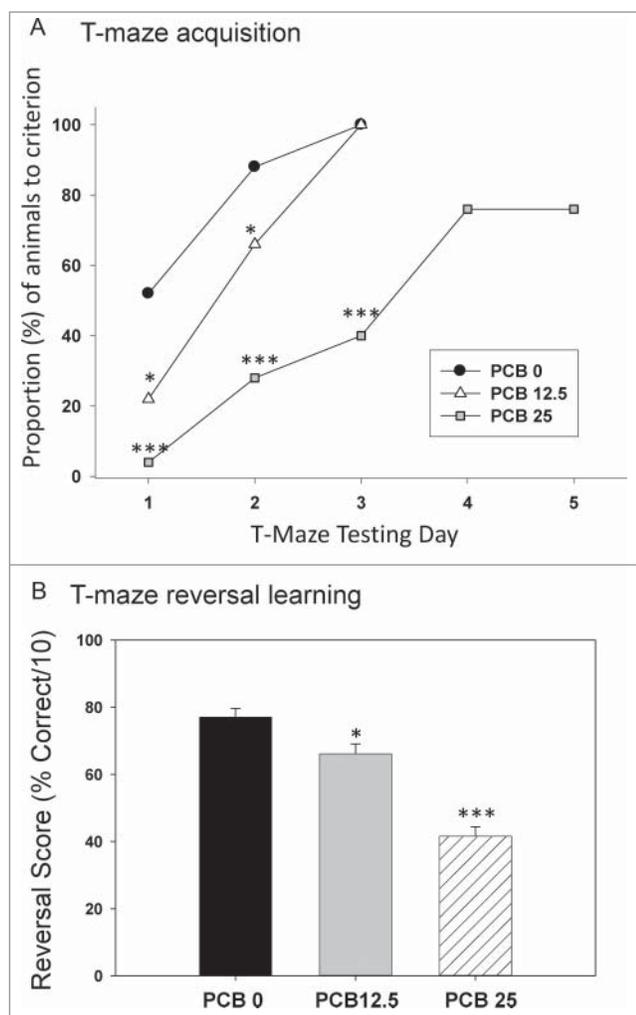
### Behavioral Measures

PCB effects on learning and behavioral flexibility: PCB exposure led to a significant increase in latency to learning criterion (80% correct trials) in t-maze performance in a dose dependent manner [ $C(4) = 32.98$ ,  $P < 0.001$ ]. Mann Whitney comparison showed that rats exposed to PCB 12.5 ( $P = 0.027$ ) and PCB 25 ( $P < 0.001$ ) differed significantly from controls (Fig. 1A). Significantly fewer PCB 25 animals were able to meet the 80% criterion to move on to the reversal task in comparison to the 12.5 and control groups ( $P < 0.01$ ) (Table 2). Correspondingly, PCB exposed rats showed significantly less competency in the reversal task in a dose dependent manner [ $F(2,61) = 39.22$ ,  $P < 0.001$ ]. Pairwise comparisons showed PCB 12.5 rats ( $P = 0.026$ ) and PCB 25 rats ( $P < 0.001$ ) were significantly less able to adapt to a reversal task than control rats (Fig. 1B). These data suggest that animals exposed to PCB have impaired ability to acquire discriminatory behavior in a 2 choice task. In addition, the results point to an alteration in the ability to shift responses to new behavior-outcome associations.

PCB effects on open field and motor testing: A 3-factor mixed ANOVA revealed a main effect for the within variable of developmental time for mean horizontal counts [ $F(2,74) = 18.22$ ,

**Table 1.** Basic measures of food intake over time in control and PCB groups

Group	Litter size (pups)	Gestational weight (grams)	Pre-weaning weight change	Rat pup weight gain	Dam food intake	Dam and litter food intake
PCB 0	14 ± 3.0	129 ± 6.0 g	29 ± 6.5 g	19 ± 0.1 g	25.5 ± 2.5 g	60 ± 3.0 g
PCB 12.5	13.8 ± 1.2	128 ± 2.0 g	34 ± 9.1 g	16 ± 1.7 g	24 ± 0.4 g	57 ± 2.8 g
PCB 25	10.3 ± 2.3	102 ± 9.3 g	33 ± 8.1 g	16 ± 3.1 g	23.3 ± 0.8 g	44 ± 5.5 g



**Figure 1.** Maze learning. (A) T-maze learning acquisition was the mean number of days the rat needed to establish a habit (8/10 trials correct) of obtaining a food reward from one of the t-maze arms. (B) The T-maze reversal task assessed adaptability with a reversal score of the number of correct choices out of 10 when the food reward was moved to opposite goal arm of the t-maze. Juvenile rats PND 25–29. Significantly different from control; \* $P < 0.05$ , \*\*\* $P < 0.001$ .

$P < 0.001$ ). There was an interaction between developmental time and condition [ $F(4,74) = 2.67$ ,  $P = 0.039$ ]. Mean horizontal activity level showed no difference in the pup and juvenile rats. Adult rats exposed to PCB demonstrated a significant decrease in horizontal movement [ $F(2,42) = 18.80$ ,  $P < 0.001$ ] (Fig. 2A). Pairwise comparisons showed that PCB 12.5 pups differed significantly from control rats ( $P = 0.001$ ), and PCB 25 pups differed significantly from controls ( $P < 0.001$ ). A 3 factor mixed ANOVA revealed a main effect for the within variable of developmental time for mean rear counts [ $F(2,74) = 37.79$ ,  $P < 0.001$ ]. Mean rear count did not differ between the pups and adult rats. Juvenile rats exposed to PCB demonstrated a significant increase in mean rear count in comparison to controls [ $F(2,46) = 3.87$ ,  $P = 0.028$ ] (Fig. 2B). Pairwise comparisons showed PCB 12.5 differed significantly from control rats

**Table 2.** % Meeting competency criteria percent of animals meeting criterion (8/10 correct trials in one test session) within the 5 d of testing

Treatment Group	% Meeting Competency Criteria
PCB 0	100 n = 26
PCB 12.5	100 n = 18
PCB 25	76 <sup>a</sup> n = 25

Values represent mean% meeting competency  $\pm$  standard error of the mean

<sup>a</sup> Significantly different from control group ( $P < 0.05$ )

( $P = 0.024$ ). A 3 factor mixed ANOVA revealed a main effect for the within variable of developmental time for mean time spent rearing [ $F(2,72) = 21.61$ ,  $P < 0.001$ ].

A 2-factor between subjects ANOVA revealed that PCB rats were significantly impaired in hang ability in comparison to controls [ $F(2,60) = 3.29$ ,  $P = 0.044$ ]. Pairwise comparisons showed that adult rats exposed to PCB 25 were significantly impaired in comparison to controls ( $P = 0.047$ ) (Fig. 3A).

A 2-factor between subjects ANOVA revealed that PCB rats had significantly delayed negative geotaxis responses in comparison to controls [ $F(2,90) = 8.75$ ,  $P < 0.001$ ]. Pairwise comparisons showed that juvenile rats exposed to PCB 12.5 ( $P < 0.001$ ) and PCB 25 ( $P = 0.004$ ) were significantly impaired in comparison to controls (Fig. 3B).

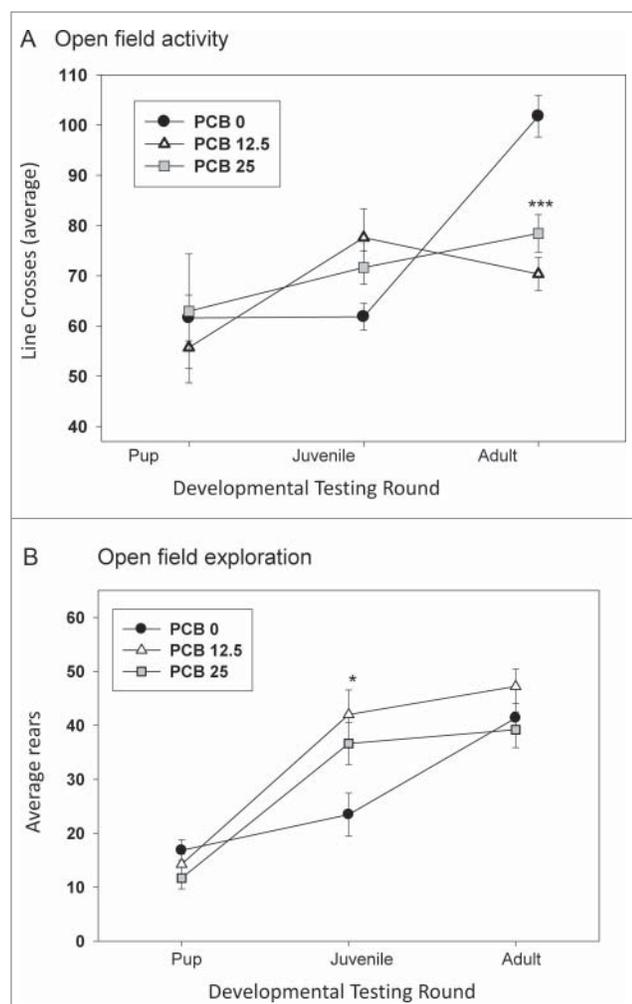
Taken together, the motor skills results indicate that animals exposed to PCB experience a dose-dependent alteration in basic locomotion, exploration and motor skills. At moderate doses, PCB exposure led to greater amounts of activity and exploration. At increased amounts of PCB, animals showed deficits in motor skills.

## Communication and Emotional State

### PCB effects on ultrasonic vocalization

PCB appears to modify the production of separation induced vocalizations in young pups, with the more elevated of the 2 doses we used decreasing ultrasonic signaling in those pups. Analysis of the isolation distress data provided surprising results (Fig. 4). Unexpectedly, the ANOVA conducted did not yield significance but a trend between the conditions of PCB exposure and the mean USVs at an  $\alpha$  level of 0.05, [ $F(2) = 2.726$ ,  $P = 0.071$ ].

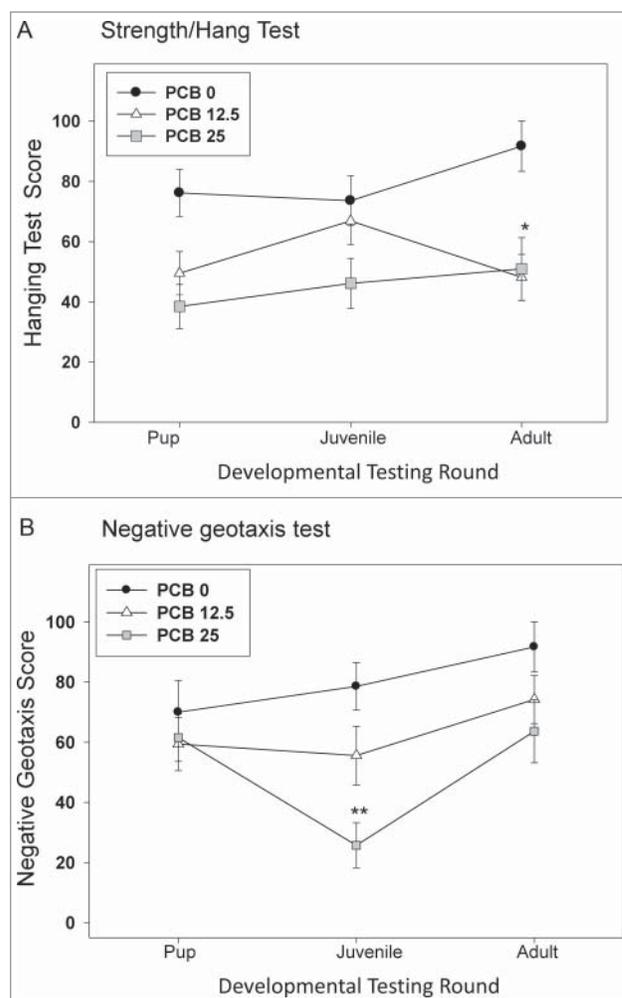
The indication of a trend ( $P = 0.071$ ), of PCB exposure affecting USVs, lead to the use of post hoc t-tests between each condition and the mean number of USVs per minute even after not obtaining a main effect from the 2-way ANOVA. The mean number of USVs emitted by control animals proved to be significantly different ( $p = 0.034$ ) compared to the number of USVs emitted by PCB 25 animals. The large amount of variance within the PCB 12.5 group, gives reason for the lack of significance between 12.5 ppm and 25 ppm, despite their obvious difference in quantity of USVs.



**Figure 2.** General motor activity. (A) Open Field Mean Horizontal Crosses is the number of times the rat crossed a line into a new square in a 10 minute open field test session. (B) Open Field Mean Rear count (exploration) is the number of times the rat's 2 front paws came off the ground in a 10 minute open field test session. Developmental Time: Pup = PND 14–16, Juvenile = PND 28–32, Adult = PND 60–64. Significantly different from control; \* $P < 0.05$ , \*\*\* $P < 0.001$ .

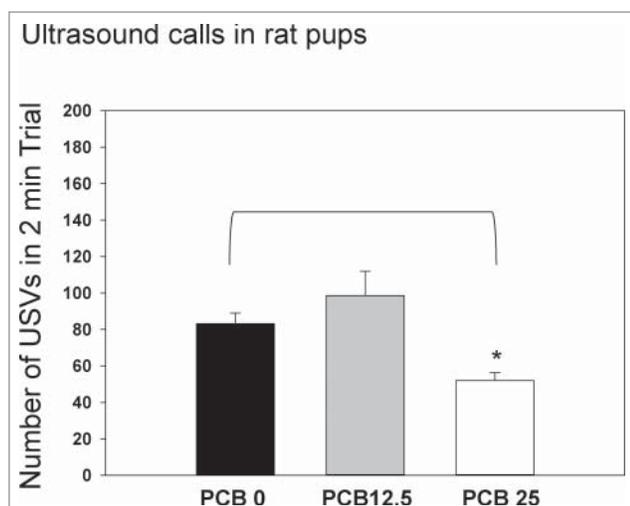
## Discussion

Overall, the results of this study support the idea that exposure to PCB can lead to significant changes in behavioral and emotional measures which are similar to deficits seen in ASD. The T maze task has been used abundantly to explore response perseveration following neural and pharmaceutical perturbations.<sup>37–39</sup> In most cases the problems in learning the task and in reversing response-outcome associations have been related to a lack of behavioral flexibility and an interruption in typical error correction. The task is relevant to autistic motor deficits because it forces the subject to inhibit a well learned response while activating the previously incorrect response. Autistic individuals show a variety of motor problems which involve inability to switch modes of responding, and the problems



**Figure 3.** Coordination and strength. (A) Mean Hang Score was determined by averaging the rats scores on the hang test. The rat was given a 100 if it reached the top of the incline grid, a 0 if it fell off, or a 60 if it could hang on but do not reach the top within 60 seconds. (B) Mean Negative Geotaxis Score was determined by averaging the rats scores on its negative geotaxis response. The rat was given a 100 if it could turn 180° when placed head down on an incline grid, a 0 if could no turn was observed or if it fell off within 60 seconds. Developmental Time: Pup = PND 14–16, Juvenile = PND 28–32, Adult = PND 60–64. Significantly different from control; \* $P < 0.05$ , \*\* $P < 0.01$ .

are more intense when previous responses have to be overridden.<sup>40,41</sup> Others have used a similar paradigm to examine motor problems in mouse models of developmental disorders.<sup>42</sup> In the present study, PCB exposed 25–29 day old male rats tested in the t-maze were significantly delayed in acquisition learning and significantly less able to reverse the task in a dose dependent manner in comparison to control rats. Twenty 4 percent fewer PCB 25-exposed rats met the 80% criterion to move on to the reversal task in comparison to the 100% competency of the PCB 12.5 treatment and control groups (Table 2), suggesting that the PCB 25 rats were having difficulty learning the correct location of the food reward. Then, once having learned that location, the PCB exposed rats had



**Figure 4.** Communication and emotional state. Mean USVs emitted by the rats after being separated from their mother. Pups PND 9–10. \* $P < 0.071$ .

difficulty adapting to a new food location. We were interested in examining general motor and reflex abilities in similarly treated animals in order to address whether or not these more basic deficits could be playing a role in motor perseveration.

Results from a general battery of motor tests uncovered deficits that appeared at various times in development, with some deficits becoming more pronounced with age, while others improved and eventually were resolved (Table 3). These results support several other studies that have reported PCB disruption of general activity and developing motor skills. Similar to the younger rats in the present study, Bowers and colleagues<sup>35</sup> found that a PCB mixture, Aroclor 1254, reduced grip strength (analogous to the present hang test) in rat pups at PND 10–14. Interestingly, the present study found the more elevated dose of PCB 25 decreased hang ability in the rat pups tested. As expected, hang strength improved with age in control rats, and PCB-exposed rats were impaired at every time point tested, but only significantly so with the greater dose at the adult age. This

**Table 3.** Summary of behavioral results in rats exposed to PCB 47/77 at 0 ppm (PCB 0), 12.5 ppm (PCB 12.5) and 25 ppm (PCB 25)

	Juvenile	Adolescent	Adult
Open Field Activity	∅	↑ <b>12.5 Rear Count/Time</b>	↓ <b>12.5/25 Horz</b>
Hang Test	∅	∅	↓ <b>25</b>
Negative Geotaxis	∅	↓ <b>12.5/ 25</b>	∅
USVs	∅	ND	ND
T-maze Reversal	ND	↓ <b>12.5/ 25</b>	ND

↓ = significant decrease relative to controls, ↑ = significant increase relative to controls, ∅ = no significant difference from controls, Horz = mean horizontal open field activity, Rear # = mean number of rears, Rear time = mean length of time spent rearing and grooming, ND = not done.

Bold headings represent means of diagnosing autism spectrum disorders and the specific relevant investigations in rodents.

difference is most likely the result of the considerably greater dose (15 mg/kg/b wt) of a more complex PCB mixture (Aroclor 1254) given in the previous study<sup>35</sup> as opposed to the simple 2 congener mixture at lower doses (PCB 47/77 at 1.04 and 2.08 mg/kg/b wt) used in the present study. Another study revealed decreased walking speed in the open field in PND 60 PCB-exposed mice relative to control mice.<sup>36</sup> The present study also observed decreased walking speed in similar aged adult rats as indicated by significantly fewer mean horizontal crosses over a constant time.

The observed motor alterations could arise from a number of PCB-induced changes to neural or hormonal function. Three candidate regions/hormones are proposed including: 1) the basal ganglia region including dopamine input from the midbrain to forebrain regions (mesolimbic and mesostriatal connections), 2) cerebellar functions, and 3) hormones including thyroid hormone function during development. The use of different in vitro and in vivo assays has shown PCB exposure to alter dopamine function. Dopamine input to forebrain sites has been demonstrated to mediate the acquisition of incentive value by cues and in error learning.<sup>43</sup> The present t-maze PCB dose dependent deficits warrant further investigation into the cortico-limbic-striatal circuitry that is potentially being disrupted.<sup>44</sup> Additionally, as expected, these data are consistent in modeling both the challenge of learning tasks (particularly those that require sensory input) and the rigid habit formation characteristic of the ASD population.<sup>45</sup>

It has been reported that cerebellar mass was more greatly suppressed in male rodents perinatally exposed to PCB than in PCB-exposed females.<sup>46</sup> Differential changes in the behavior such as righting reflex and negative geotaxis of PCB pups were associated with alterations in cerebellar structure and protein expression, with greater effects in males. The importance of euthyroidism for normal morphological, neurochemical, and functional development of many areas of the brain, including the cerebellum, has been established (for a review see<sup>47</sup>). Although our previous PCB studies have focused primarily on the hippocampus and basal forebrain, thyroid status disruption in these studies has been correlated with alterations of morphological,<sup>48</sup> neurochemical,<sup>21,49</sup> and biobehaviorally functional<sup>25,32,33,50</sup> aspects of these areas, as well as impacting development of stress response mechanisms.<sup>26</sup> Thus it is likely that the altered thyroid status induced by PCB exposure has ancillary effects on cerebellar function. Though significant depression in righting (pup) and negative geotaxis response (adolescent) were seen in the present study, sex differences were not evaluated. However, the difference may also be attributed to the much greater dose (10 mg/kg/b wt) and more complex mixture (Aroclor 1254) administered by the latter study<sup>46</sup> in comparison to the present study (PCB 47/77 at 1.04 and 2.04 mg/kg/b.wt.) as well as to sex difference.

USVs are important indicators of normal emotional states and are involved in socialization and pup-dam communication; therefore, abnormalities in the occurrences of these calls could be indicative of adverse effects caused by direct exposure to toxic substances and indirect exposure to these substances through environmental contamination. In the present study, PCB 25

**Table 4.** PCB exposure as a possible animal model for ASD

Clinical ASD Diagnosis	Investigations carried out in the PCB-exposed rodent model
Social	Behavioral Paradigms: ultrasonic vocalizations <sup>a</sup> , social port <sup>b</sup> , conditioned odor preference <sup>c</sup> , play behavior <sup>b</sup> , social recognition <sup>d</sup> , Endocrine Status: oxytocin <sup>d</sup> and vasopressin <sup>b</sup>
Motor	General Motor Activity: open field <sup>a</sup> , hang test <sup>a</sup> , negative geotaxis <sup>a</sup> , righting reflex <sup>a</sup> Stereotypic Repetitive Behavior: T-maze learning and reversal, <sup>a</sup> grooming fixed action patterns <sup>b</sup>
Communication	Behavioral paradigms: ultrasonic vocalizations <sup>a</sup>

<sup>a</sup>Present Study<sup>b</sup>Unpublished preliminary findings<sup>c</sup>Cromwell et al., 2007<sup>32</sup><sup>d</sup>Jolous-Jamshidi et al., 2010<sup>33</sup>

animals emitted the fewest isolation calls per unit time (Fig. 3). Studies have shown that an auditory component of pup behavior is important in the initiation and regulation of maternal behaviors.<sup>51</sup> The lesser number of isolation USVs seen in the PCB 25 pups can be an indication of a communication deficit.

The importance of data and implications for an adequate animal model can be expanded to the clinical setting for comparison. The results discussed in this study demonstrate that PCB exposure is capable of causing disruption to similar core domains affected in the ASD population (Table 4). Specifically, the different results in animals given PCB 12.5 ppm and PCB 25 ppm may allow use of this system to serve as a model to observe a range of behavioral severity much like that seen in the broad autistic phenotype. Since PCB compounds are such ubiquitous environmental endocrine disruptors capable of affecting the developing brain,<sup>53</sup> the present study encourages further investigation into the possibility of exogenous contaminants like PCB as potential environmental triggers for ASD.

While development of an animal model for the induction of neurological and behavioral disorders like autism by an environmental toxicant like PCB is an appropriate motivation for collecting the data presented in this paper, the question remains: does PCB “cause” autism in humans? A problem with trying to answer this question directly by means of a study administering PCB to rodents is that exposed humans are rarely (if ever) exposed to only one toxicant, but rather to a “witches brew” that contains PCB, PBDE, dioxin, lead, mercury, tin, pesticides, herbicides and the like. Nonetheless, studies in which PCB alone is administered to experimental animals during various stages of development have revealed modifications of a number of behavioral endpoints, including learning and memory,<sup>21,25,49,50</sup> recognition,<sup>32</sup> affiliation,<sup>33</sup> behavioral flexibility and general motor activity (the presents study), similar to those that occur in autism. Endocrine connections between PCB and genetics impacting behavior can be made through thyroid<sup>21,25,49,50</sup> and/or glucocorticoid<sup>26</sup> status alterations by PCB, each of which hormones interact with the genome and are important players during specific developmental windows. Thus, there are a number of ways that PCB could contribute development of autism and other social behavioral impairment. Our work has attempted to tease out its influence from that of other factors, and has revealed it to play a role in such disorders.

## Methods

### Animals and perinatal PCB administration

Animals (Sprague-Dawley rats; Harlan, Indianapolis, IN, USA) were kept in a temperature- and humidity- controlled room (70°F ± 2 and 30–70%, respectively) with a 12-hour light-dark cycle (lights on 0700, lights off 1900) throughout the studies. Female rats weighing 225–275 g were mated to males of the same strain. In order to accurately expand the previous work that found social behavioral changes, the methods of PCB administration and the dose and type of PCB congeners used are identical to the previous work.<sup>32,33</sup> Both PCB 47 and PCB 77 were chosen for our investigations because they are structurally similar to thyroid hormones (e.g., the same degree of halogenation). Additionally, both congeners represent archetypes of different modes of action, and, at the same time, they possess structural comparability. Once females were determined to be pregnant as confirmed by a sperm positive vaginal smear, they were caged separately, and fed ad libitum either standard rat chow for control groups or chow with PCB 47/77 added in equal amounts at a total concentration of 12.5 ppm (PCB 12.5) or 25 ppm (PCB 25) (w/w). PCB 47 and PCB 77 congeners were obtained from AccuStandard, Inc., New Haven, CT, USA. Stock PCB was dissolved in absolute ethanol, mixed with 100 g of rat chow (Mowlan Teklad, Madison, WI, USA), and the ethanol was allowed to evaporate. Equal amounts of PCB 47 - and PCB 77 - containing diet were mixed together and formulation of 12.5 ppm and 25 ppm doses was done by adding the appropriate weight of this concentrated mixture to sufficient unaltered diet to give a weight of 1000 g, which was thoroughly mixed by prolonged tumbling of the sealed container. Control animals (PCB 0) were continued on standard rat chow after conception. Food consumption was measured daily to determine amount of PCB ingested. For PCB 12.5, the calculated mean value was 1.04 mg/kg/day, and for PCB 25, 2.04 mg/kg/day.

Four to 10 litters of rats were generated for each of the treatment groups (PCB 0, PCB 12.5, PCB 25). The majority of litters used for the present study was standardized to 10 pups when possible (5 male, 5 female when possible) on PND 3. All pups were housed in the maternal cage until weaning at PND 21, and then housed in same sex pairs. Only male offspring were used in the present study (no more

than 6 nor fewer than 3 from the same litter) with the remainder being used for tests of conditioned odor preference at PND 13–15,<sup>32</sup> and of social recognition at PND 20–21.<sup>33</sup> The specific number of pups used for each behavioral test is indicated below.

#### Behavioral Measures: T-maze Acquisition and Reversal

The effort-based decision-making t-maze task,<sup>53,54</sup> consisted of an approach arm and 2 goal arms (77 × 135 × 14 cm). Animals were first given a 25 mg sucrose pellet in the home cage, habituated to the t-maze arena for 3–5 min, and then food deprived for 12–14 hr before day one of testing. At the beginning of each test session, the rat was placed in the start box at the bottom of the approach arm. The start box door was then opened, and the rat (PCB 0: n = 26, PCB 12.5: n = 18, PCB 25: n = 25) was given a choice of entering either arm. In this manner, the rats were trained, with 10 trials on 2 d of testing, to establish a habit of finding a food reward at the assigned goal arm. The reinforced arm (left versus right) was randomly assigned. A correct attempt was defined as when the rat entered the reinforced arm and consumed the sucrose pellet, while an incorrect attempt was defined as when the rat crossed the marked line (40 cm into the arm) in the non-goal arm. If the rat made a correct choice, it was confined to the correct arm by closing a door, and given time to consume the pellet before being guided back to the start box. If the rat made an incorrect choice, the door was closed as soon as the forepaws crossed the marked line, and the rat was confined to the incorrect side with no reinforcement for 5 seconds before being guided back to the start box.

After a minimum of 2 d of training, those rats meeting 80% criterion, 8/10 trials correct (PCB 0: n = 26, PCB 12.5: n = 18, PCB 25: n = 19), were tested with the reversal task. The food reward was changed to the arm opposite that used in learning acquisition, and the number of correct choices of 10 trials was determined for each rat. Those rats who did not meet 80% criterion within the 5 d of testing (only PCB 25: n = 6) were not used in the reversal task.

#### Behavioral measures: general motor skills

The following 4 behavioral measures of general motor skills were carried out at similar times of day (8:00 am–4:00 pm), for 2 consecutive days, at 3 different developmental stages: pups = PND 14–16, juvenile = PND 28–32, and adult = PND 60–64.

**Open-Field Activity:** General activity was tested in pups and juvenile rats using a 40 × 50 × 20 cm square open-field apparatus with a 9 square grid drawn on the floor. Each rat was transferred from the home cage directly into the center of the open field and observed for 10 min. Locomotor activity was recorded, and later scored for horizontal movement (crossing a line into a new square), vertical rear counts (number of times the 2 front paws came off the ground), and rear time (length of time spent with 2 front paws off the ground). Correspondingly, a larger open-field apparatus, 61 × 62 × 31 cm, was used for the adult rats at round 3, PND 60+. (Pup PCB 0: n = 16, PCB 12.5: n = 28, PCB 25: n = 22; juvenile PCB 0: n = 12, PCB 12.5: n =

28, PCB 25: n = 16; adult PCB 0: n = 16, PCB 12.5: n = 16, PCB 25: n = 16).

**Hang Test:** The hang test measured the animal's grip strength by its ability to climb a 27 × 16 cm grid on a 30° incline within 60 sec. The animal was given a score of 0 if it fell off the grid, 100 if it reached the top within 60 sec, or 60 if it hung on but did not reach the top within 60 sec. A proportionately larger, 61 × 44 cm, grid at the same angle was used for the adult rats. (Pup PCB 0: n = 26, PCB 12.5: n = 40, PCB 25: n = 38; juvenile PCB 0: n = 28, PCB 12.5: n = 32, PCB 25: n = 36; Adult PCB 0: n = 12, PCB 12.5: n = 32, PCB 25: n = 22.)

**Negative Geotaxis:** Negative geotaxis measured the animal's ability to turn 180° when placed head facing downward on a 30° inclined 27 × 16 cm grid. The animal was scored 100 if they were able to turn around or a 0 if they could not. A proportionately larger, 61 × 44 cm, grid at the same angle was used for the PND 60+ rats. (Pup PCB 0: n = 26, PCB 12.5: n = 40, PCB 25: n = 32; juvenile PCB 0: n = 28, PCB 12.5: n = 32, PCB 25: n = 36; adult PCB 0: n = 10, PCB 12.5: n = 32, PCB 25: n = 22.)

#### Communication and emotional state: ultrasonic vocalizations

USVs were recorded during isolation at PND 9–10. The isolation chamber was located in a room separate from the housing room, and no other animals were present in the isolation room at the time of testing. Pups were placed in a 500 mL glass beaker inside of a small cage with clean bedding. The ultrasonic microphone was placed above the pup approximately 12 cm above the base of the beaker. USVs were recorded using a high frequency bat detector (Pettersson D230 ultrasound).

On PND 9, pups were separated from the dam and placed in a separate cage before habituation. They were individually habituated in the testing chamber for one minute, during which vocalizations were recorded. Pups were then returned to the home cage. On PND 10, pups were once again separated from the dam and placed in a separate cage immediately prior to testing. Ultrasonic vocalizations were recorded during 2 minutes of isolation in the testing chamber. Testing order was random. Ultrasonic vocalizations were manually counted using Avisoft Bioacoustic software for isolation distress testing. Isolation calls within the 30–45 kHz range were counted regardless of duration. (PCB 0: n = 29, PCB 12.5: n = 25, PCB 25: n = 47).

#### Statistical Analysis

Basic litter statistics (litter size, weights of dams and litters, and food/PCB consumption) were completed using a 2-factor (Condition × Sex) between groups analysis of variance (ANOVA). A subset of the rats/litters from each group was chosen for weight and PCB consumption determination. Open field parameters (horizontal, rear counts, rear time) were completed using a mixed design ANOVA with 2 between group factors (Condition × Sex) and one within group factor (Developmental

Stage). The hang test, negative geotaxis response, and righting reflex were analyzed using a 2-factor between subjects ANOVA. T-maze learning was analyzed using a nonparametric Kruskal-Wallis ANOVA and t-maze learning competency was assessed using Chi-square. T-maze reversal tasks were analyzed using one-way ANOVA. Each ANOVA assessment was run both with and without litter as a covariate. Since inclusion of litter did not alter level of significance, values reported are the result of ANOVA without litter covariate. For all comparisons, significance was set at  $p < 0.05$ . All numerical data with error terms are presented as mean  $\pm$  standard error of the mean.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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