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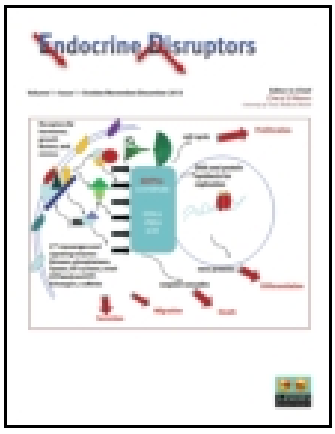

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ORIGINAL RESEARCH PAPER

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Polychlorinated biphenyl exposure alters oxytocin receptor gene expression and maternal behavior in rat model

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Polychlorinated biphenyl (PCB) is a persistent organic pollutant known to induce diverse molecular and behavioral alterations. Effects of PCB exposure could be transmitted to future generations via changes in behavior and gene expression. Previous work has shown that PCB-exposure can alter social behavior. The present study extends this work by examining a possible molecular mechanism for these changes. Pregnant rats (Sprague-Dawley) were exposed through diet to a combination of non-coplanar (PCB 47 - 2,2′,4,4′-tetrachlorobiphenyl) and coplanar (PCB 77 - 3,3′,4,4′-tetrachlorobiphenyl) congeners. Maternal care behaviors were examined by evaluating the rate and quality of nest building on the last 4 d of gestation and dam/pup interactions on postnatal days 1, 2, 4 and 6. On postnatal day 17, dams were euthanized and hypothalamic tissue was removed for expression analyses of the oxytocin receptor (OXTR) and cytochrome P450 1a1 (Cyp1a1). PCB altered nest building and maternal care behaviors. Specifically, there was a significant increase in time spent in low crouch and high crouch nursing posture on PND 4 and PND 6 respectively. Molecular analysis revealed that PCB exposure upregulated OXTR expression in the hypothalamus of dams. These results provide a possible molecular mechanism for PCB-induced changes in social interactions during early development.

Introduction

Persistent organic pollutants, such as polychlorinated biphenyls (PCBs) are pervasive in the environment and pose a threat to health and the ecosystem.[1,2] Exposure in human populations is likely to occur through absorption, inhalation, or ingestion, with the ingestion most prevalent in populations with high fish consumption.[3-5] Although PCB production in the United States was discontinued in 1976, significant amounts remain in the environment resulting from the long half-life of the toxicant.[6-8] There are 2 main classifications of PCB molecules, coplanar (non-ortho substituted), which bind to the aryl hydrocarbon receptor and non-coplanar (ortho-substituted), which can alter hormone homeostasis as well as binding to the gamma amino butyric acid receptor.[9,10] Exposure to a combination of these PCB congeners can alter physiological processes including reproductive development, immune function, growth, and brain function.[9-12]If PCB exposure occurs during gestation, alteration of these physiological processes can manifest as altered psychological and behavioral development.[13,14]

One route of PCB exposure is from mother to offspring, which can occur throughout gestation and in the mother's milk during nursing.[15-19] In animal models, the level of PCB in offspring is correlated with the amount of maternal PCB exposure.[20-22] Recently, connections have been made between early exposure to endocrine disrupting compounds (EDCs) and developmental disorders.[23-25] EDC exposure, especially from PCBs, can alter gene expression profiles that could lead to harmful effects on social behavior, development, and health.[26] Previous work has shown that PCB exposure during the perinatal period can impact many interactions in rats including reproductive behaviors and other social behaviors.[27-30] Exposure to a simple mixture of 2 PCB congeners (non-coplanar 47 and coplanar 77) through diet diminished pup conditioned preferences for maternally associated cues, which supports the hypothesis that low dose exposure during early development can be harmful to complex psychological processes.[30,31] In addition to maternal cue alterations, PCB administration led to reduced social recognition in juvenile rat pups indicating that early PCB exposure can produce long-term behavioral modifications.[32] These studies

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highlight the importance of examining early exposure to PCB and other environmental contaminants, but do not highlight the behavioral changes that can manifest from altered maternal behavior.

Maternal care is critically important to the social development of offspring, as reduced maternal care quality can manifest as increased anxiousness and fearfulness even through adulthood.[33] The quality of maternal care in rats is determined by 2 distinct behaviors including licking/grooming and arched back nursing.[34] Dependent on the amount of time spent in these behaviors dams are classified as high or low licking/grooming and arched back nursing (LG-ABN) with high LG-ABN dams reported as the more effective mothers because of increased milk letdown during this nursing posture.[34-36] Studies have shown that the initial 6 d postnatal were sufficient in order to characterize maternal care groups into different categories (for example[37]). High and low LG-ABN nursing dams in different experimental groups remained distinct and recognizable from each other during most of the observation time periods, specifically during PND 2–4; however, after 6 d of observations, observed maternal care behaviors were no longer significantly different between the 2 groups. This strongly suggests that the first week postpartum is crucial in terms monitoring maternal care and then generalizing to other care/social behaviors and long-term influences on behavior, physiology and gene expression regulation.

Interestingly, a cross-fostering study with PCB 77 exposure demonstrated that maternal behavior is altered by pup PCB exposure with increased attentiveness of the mothers, characterized by increased nursing.[38] Other maternal behaviors, such as amount of time on the nest, were correlated to maternal PCB exposure.[38] The cross-fostering paradigm is essential to understanding this mechanism as it enables a dissociation between maternal and pup PCB exposure, and allows a glimpse into the complex interactions that occur between mother and pup to produce maternal care behaviors.[39,40]

While behavioral examination is an indicator of harmful PCB exposure, it is necessary to understand the molecular mechanism behind behavioral modification, which can include both endocrine and neural systems. Given the permissive nature of euthyroid status on developmental processes, our research group has focused on PCB-related changes in thyroid hormones and has shown that early exposure reduces thyroxine levels in rat pups, which is alleviated by thyroxine replacement.[41-44] The interaction between PCB and thyroid function is complex with both antagonistic and agonistic effects, thus PCB-thyroid interaction could contribute to diverse behavioral and psychological changes after exposure to PCB.[45] A potential genetic candidate for this interaction is Cyp1a1 because it is a known target gene that is upregulated in endothelial cells[46] and hepoatocytes,[47] but this effect has not been observed in nervous tissue. Thus, a more promising candidate for PCB altered behavior is oxytocin, which is known to be involved in mediating social behavior, with an emphasis on maternal care.[48-52] Not surprisingly, PCB 77 exposure is known to increase oxytocin secretion and expression in luteal cells in different animal models.[53-55] However, no study has analyzed the link between PCB mediated alteration in oxytocin function and changes in maternal care behaviors.

The alteration of oxytocin function by PCB could occur through changes in oxytocin secretion and expression or through changes in the expression of the receptor. Environmental contaminants, toxins, and EDCs can influence gene expression, which could be the molecular mechanism of PCB effect on oxytocin function as it has been previously shown to alter gene expression.[56-60] Maternal care behaviors are also strong mediators of gene expression[61,62] and the differences in maternal care in typical circumstances are associated with expression of oxytocin receptor genes.[63] The present study extends previous behavioral work by examining possible molecular mechanisms involved in altering early social behavior. A thorough monitoring of maternal care by rat dams was completed prior to the molecular investigation. For the molecular portion of the study, we focused on gene expression of the oxytocin receptor gene in the hypothalamus because this particular gene in this brain region has been found to be important in the production of typical maternal care behaviors.[64-66] PCB congeners 47 and 77 were chosen to use in the present study because a mixture of those 2 allows exposure to both coplanar and non-coplanar congeners and advances our previous work using those 2 congeners.

Results

Maternal weight and PCB consumption

In order to determine the effect of PCB on maternal body weight and food consumption, these measures were evaluated throughout the gestational period. Maternal weight gain during the first week of gestation was depressed in the PCB treatment group ($F(1,17) = 4.55$, $p < 0.05$) compared to the controls (**Table 1**). The weight of the dams was not significantly altered by dietary group in subsequent weeks (**Table 1**). PCB consumption was measured as micrograms consumed per gram of body weight and did not vary significantly across the 3 weeks of gestation (**Table 1**). Litter weight and size were not significantly altered by PCB exposure (data not shown).

Nest building

Nest building was examined as a measure of maternal instinct and care behavior in the PCB ($n = 6$) and control ($n = 5$) groups for each gestational day. There was a significant main effect for

Table 1. Basic measures of food intake over time in PCB and Control Groups

Group	Litter Size (pups)	Gestational weight (grams)	Pre-weaning weight change	Rat pup weight	Dam food intake	Litter food intake
Controls	14 ± 3.0	129 ± 6.0 g	29 ± 6.5 g	19 ± 0.1 g	25.5 ± 2.5 g	60 ± 3.0 g
PCB12.5	13.8 ± 1.2	128 ± 2.0 g	34 ± 9.1 g	16 ± 1.7 g	24 ± 0.4 g	57 ± 2.8 g
PCB25	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

day of observation ($F(3,27) = 11.7$, $p < 0.001$). This reflects the increase in the nesting strip number used by animals in both groups from gestational day (GD) 20 to 23 (**Fig. 1A**). There was a significant day x diet interaction ($F(3,27) = 3.29$, $p < 0.05$). We examined this interaction effect in more detail with pairwise comparisons and found a significant increase in the number of nesting strips used on GD20 in the PCB-exposed dams compared to controls ($t(9) = 2.40$, $p < 0.05$). The quality of the nest was also assessed and there was a significant main effect for day of observation that the nest was scored ($F(3,27) = 81.03$, $p < 0.001$). This finding is related to the increase in nest quality rating over the course of gestational days (**Fig. 1B**) but was not modified by PCB exposure. The mean quality of nests built by PCB fed dams was lower than that of controls on each gestational day (**Fig. 1B**), but these differences did not reach significance.

Maternal care: Nursing behaviors

Dietary consumption of PCB during gestation led to alterations in maternal care behaviors thought to be crucial for rat pup development. We examined each maternal care variable (**Fig. 2A**, B) and found that PCB exposure significantly altered 2 nursing behaviors. The other behaviors including time-off-nest appeared to be the same in the 2 groups. In addition, we did not find a significant difference related to cross-fostering. The amount of time spent in low crouch nursing was significantly different between PCB-exposed and control dams ($F(1,12) = 8.55$, $p < 0.05$). We found a 140.7% increase in low crouch nursing in PCB-exposed dams compared to controls on post-natal day 4 (see **Fig. 3A**; $t(11) = 6.34$, $p < 0.01$). We also found a difference for high crouch nursing between groups ($F(1,12) = 3.43$, $p < 0.05$). Specifically, on post-natal day 6, PCB-exposed animals showed a greater proportion of time in high crouch nursing behavior compared to control dams (**Fig. 3B**; $t(11) = 2.01$, $p < 0.05$).

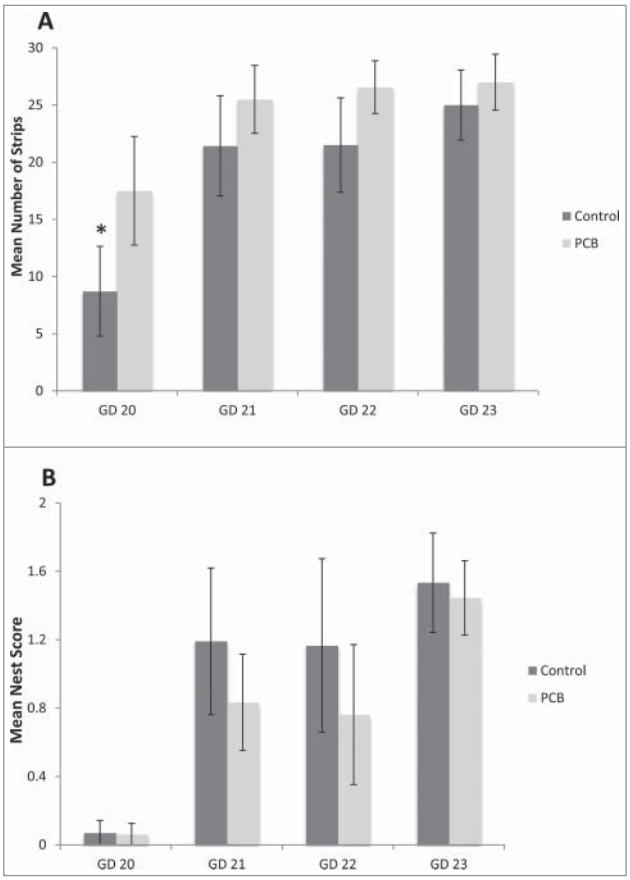

Figure 1. Nest building measures. (**A**) Average number of strips taken into the maternal cage by PCB and control treatment groups over gestational range, GD 20-23 (mean ± SEM; $n \geq 5$). Significant day effect revealed by pairwise day comparison on GD 20 in both groups (* $p < 0.05$). (**B**) Average nest quality score per gestational day (mean ± SEM) for the PCB and control treatment groups ($n \geq 5$).

Quantitative real time RT-PCR analysis

Expression of the Cyp1a1 and OXTR genes in the hypothalamus was examined in the dams by qRT-PCR in order to assess the molecular mechanisms underpinning maternal care behavior. Results were normalized to β-actin and relative fold change was obtained from the normalized Ct values between PCB treatment and mock. While Cyp1a1 expression within the hypothalamus was not effected by diet or foster status and revealed no significant difference (data not shown), a significant increase in OXTR expression in the hypothalamus was observed in the PCB treatment groups compared to the controls ($U = 5.50$, $p \leq 0.05$) (**Fig. 4A**). There was an increase in the expression of maternal hypothalamic OXTR gene regardless of whether pups were fostered or non-fostered (**Fig. 4B**).

Discussion

The results of the present study did not follow our expectations for how this PCB mixture would alter maternal behavior. Instead of a clear decrement in maternal care, we obtained a mixture of decreased and elevated maternal care. There could be a possible influence of the soy content of diet used in the present study, since this commercial diet has been used in previous studies to illustrate that high soy intake can influence behavior.[67] However, the behavioral modifications have been developmental ones, rather than occurring in adult animals. Additionally, this potential confound was addressed in the present study by feeding it both as control diet and diet containing PCB. Rat dams consuming PCB weighed less during the first week of gestation, but then gained more weight than controls and this has been documented in previous work using the same administration procedure and PCB congener mixture.[30] Prior to birth, the pregnant rats exposed to PCB prepared for the birth by utilizing a greater number of nesting strips in the cage but after parturition generally built nests of lesser quality. Surprisingly, the rat dams exposed to PCB expressed longer periods of nursing behavior during the first 6 postnatal days and this included the higher quality care of high crouch nursing on postnatal day 6. Amounts of high crouch and low crouch nursing vary dependent upon the procedures to acquire the behavioral data. Previous studies have found similar proportions of HCN and LCN that range from

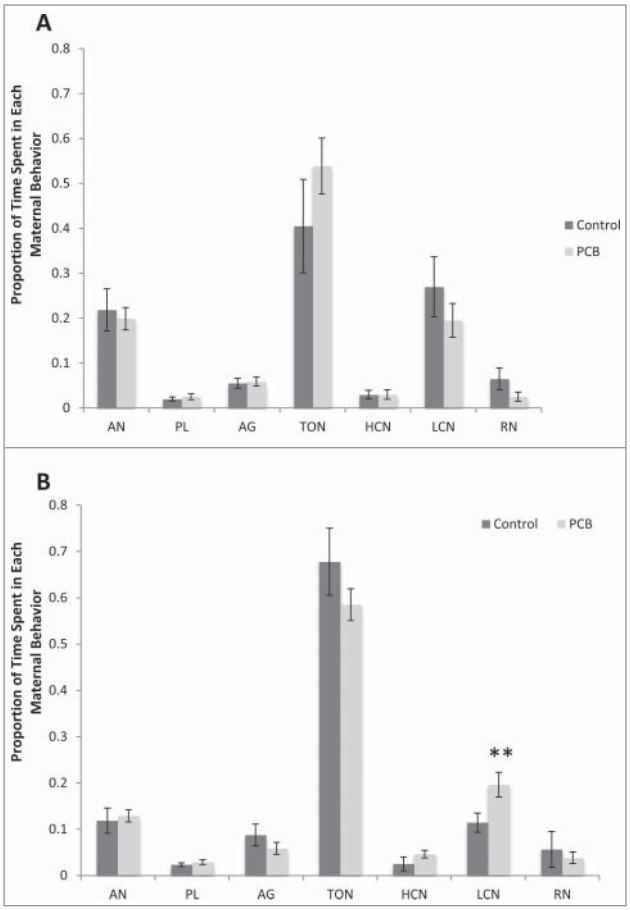

Figure 2. Comparison of PCB effects on maternal behavior over the first six postnatal days; (**A**) mean ± SEM for days PND 1, 2 combined; (**B**) mean ± SEM for PND 4, 6 combined. AN: active nursing; PL: pup licking; AG: autogrooming; TON: time off nest; HCN: high crouch nursing; LCN: low crouch nursing; RN – resting nursing. ** $p \geq 0.01$.

0.05 to 0.1 for HCN and 0.25 to 0.3 for LCN.[68,69] Procedural changes in the way maternal care is measured can dramatically shift the levels sampled. Cummings and colleagues[38] had a significantly greater proportion of HCN (0.25 for oil-injected controls) but measured the behavior 2 hours before lights turned off on a 12:12 light:dark cycle. The work done on gene expression effects related to maternal care has typically measured the nursing using different sampling intervals during the day and night periods (3-6 time windows) with very short periods of behavioral measurement (2-5 minutes).[37] These procedural differences could influence the proportions of the behaviors acquired. High crouch nursing is a high energy cost behavior and is thought to be the most effective at providing milk letdown and cannot be maintained for an extended period of time. Finally, we found a robust and consistent elevation of OXTR gene expression in the hypothalamus in these same rat dams, although the expression of Cyp1a1, a gene known to be altered by PCB in other tissues,[46,47] was not different from controls in this area of the brain. Elevated levels of OXTR gene expression have been found in previous work to be related to enhanced maternal care[70,71] so this result could be expected given the behavioral findings of this study. Additional work has shown that oxytocin receptor concentrations are high within specific hypothalamic subregions such as the medial preoptic area and the ventromedial nucleus.[72] Oxytocin receptor levels increase typically after parturition[73] and infusion of the neurohormone into the medial preoptic area facilitates maternal care expressed by the dam.[74-76] More recent work has shown that variations in maternal care are related to oxytocin receptor levels in hypothalamus (MPOA) as well as other brain regions such as the lateral septum and bed nucleus of the stria terminalis.[77,78] The results have shown that higher levels of maternal care coincide with greater amounts of oxytocin receptor.

The present results differ from previous work examining maternal care changes following PCB exposure.[33,38] This could arise from several methodological differences in the previous work including the use of a single congener (PCB 77) and administration by way of injection. Despite this previous work finding a reduction in nursing in the PCB-exposed group, those investigators did find an elevation in other maternal behaviors (e.g., licking

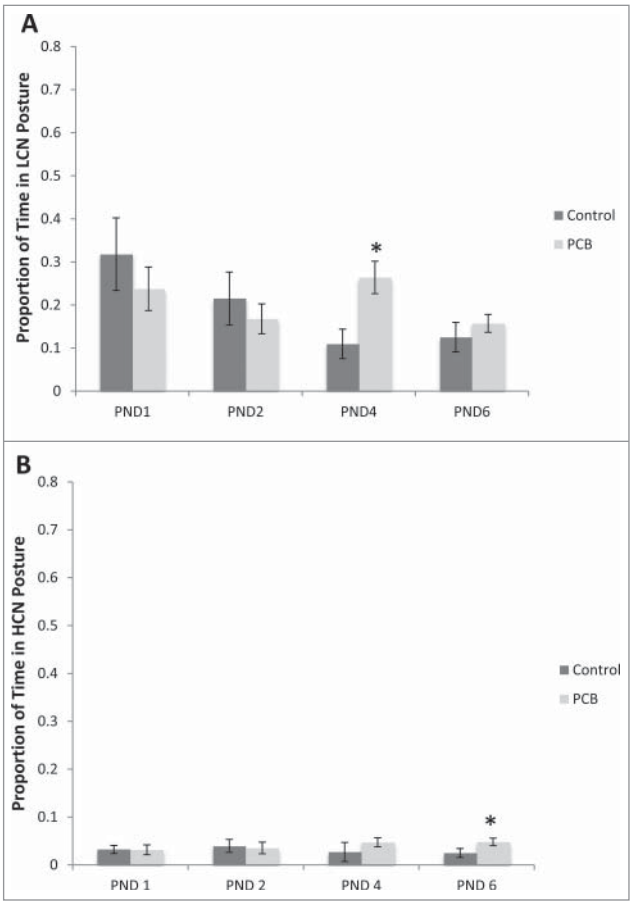

Figure 3. Effect of gestational PCB exposure on Low and High Crouch Nursing behavior as a proportion of all other maternal behaviors. (**A**) Low crouch nursing (LCN) behavior on postnatal days 1, 2, 4, and 6 (PND1, PND2, PND4, and PND6). One-way ANOVA revealed a significant effect on PND 4 (*p £ 0.05). (**B**) High crouch nursing (HCN) behavior on PND1, PND2, PND4, and PND6. Mann-Whitney non-parametric analysis revealed a significant effect on PND 6 (*$p < 0.05$).

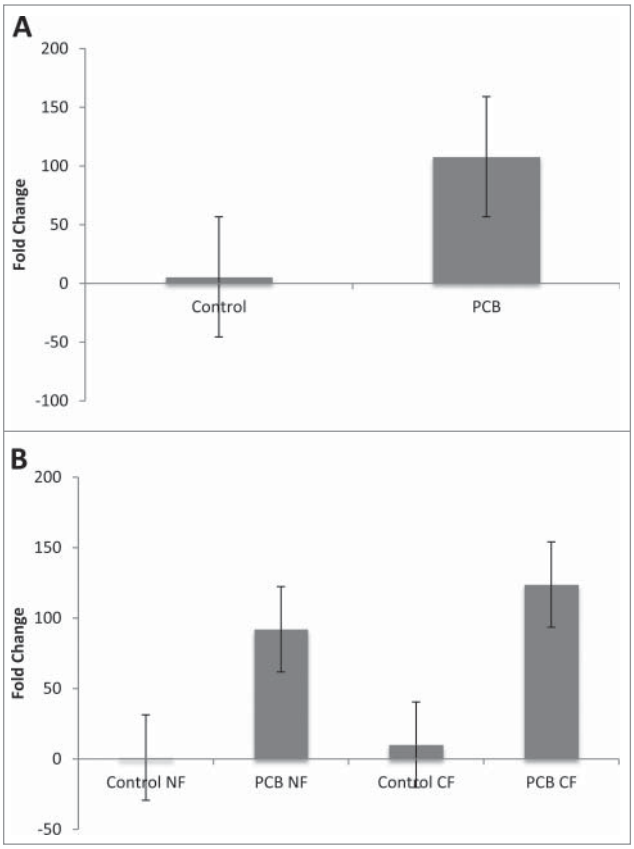

Figure 4. qRT-PCR analysis of OXTR expression in the hypothalamus. (**A**) Significant increase in expression of OXTR mRNA was observed in response to PCB regardless of pup foster status ($p < 0.05$). (**B**) Increase in expression of OXTR was observed when examining both maternal PCB treatment and pup fostering treatment but no significant differences were observed among groups. NF = non-cross foster and CF = cross foster.

and grooming) leading to a similar general notion that PCB-exposure can increase early social interactions in the rat model.

One way that animals can respond to toxin exposure is via compensatory behaviors that work to reduce harmful effects. This type of 'wild health' or 'animal medicine' is well known in behavioral ecology or physiological psychology[79-81] but not well studied in behavioral toxicology. Most of the work in this area focuses on detoxification through shifts in diet after experiencing a toxic or poisonous substance.[82-84] A related area is the shift in behavior to combat infections from parasites or other pathogen.[85] Animals exposed to harmful environments make adjustments in diverse ways including reactions to urban stress or crowding.[86] Exposure to endocrine disruptors could trigger behavioral compensation in animals, and enhancements of maternal care actions might be one form of compensatory act used to reduce harmful effects of exposure. Future work must address the sequence of PCB-OXTR-maternal care effects in order to understand more fully the causal relationship between these levels of PCB influence. Since we did not find a main effect or interaction for the cross-fostering, it is likely that direct exposure to PCB is involved in producing the behavioral changes. Control females combined with PCB-exposed rat pups did not express the same type of behavioral or molecular changes. Our previous work has focused on pup social motivation[87,88] and emphasized the important dynamic between care-seeker and care-giver.[89] The present set of results supports the idea that direct PCB exposure that leads to hormonal and neural changes in the care-giver is paramount and that alterations in rat pup behavior alone do not lead to the same effect. Another important issue to explore is the order of effects between OXTR gene expression upregulation and shifts in behavior. Direct manipulation of OXTR levels can dramatically shift early behavioral responses of rats and specifically alter social behavior in different context.[90] There is recent work supporting this relationship in human clinical research.[51] Despite this strong directional evidence, it is known that shifts in behavior can act as strong mediators of hormone gene expression as well.[52] Overall, attempting to determine an initial role for either PCB-related gene expression or behavioral changes may not be fruitful because the 2 processes are clearly intimately intertwined.[91]

The means by which PCB disrupts endocrine function is becoming ever clearer. Modification of OXTR gene regulation in the hypothalamus is one of many molecular/endocrine alterations following PCB exposure. We examined Cyp1a1 gene expression in the hypothalamus and found no differences between the exposed and unexposed animals. This is contrary to other studies, which have found that exposure to coplanar PCB molecules, like PCB 77, can and does induce Cyp1a1 gene expression after exposure.[46] However, in the present study, PCB 47/77 administration was discontinued after parturition. This means that during the 17 subsequent postnatal days through the testing period, the expression of Cyp1a1 might have been restored to normal levels in the hypothalamus of exposed dams. So far, no studies have considered the length of time post-PCB exposure Cyp1a1 remains elevated, especially in brain regions. It is possible that PCB is still inducing Cyp1a1 expression in other areas of the body, such as the liver, that are critically important in eliminating the toxicant from the body. Alterations in these enzymes provide a possible biomarker for toxicity of the compound and absence of effect in the present study indicates a relatively lower general toxicity of the PCB exposure.[47] Other work that has found alterations in these enzymatic pathways has examined exposure at greater doses of PCB or mixtures of PCB with other halogenated organic compounds.[60,92,93]

PCB-related modifications of other hormone systems could be involved in alteration of social behaviors during early development. PCB exposure significantly alters estrogen, progesterone and glucocorticoid function.[93-95] Each of these steroid hormones has been shown to be important in regulating maternal care in the rat.[35,96] PCB has been shown to have both estrogenic and antiestrogenic properties.[97-99] For example, PCB has led to an increase in estrogen receptors in the hippocampus[100] and can act as an estrogen hormone agonist as well.[101] PCB exposure has been shown to alter stress hormone responses.[102] Exposure to gestational stress alters maternal care and in a few studies stress exposure has been shown to increase certain maternal care actions.[36,48,103,104] PCB-related shifts in glucocorticoids such as

corticosterone could be an important influence in shifts in early social behavior, and future work is required to understand better the mechanisms by which PCB alters both maternal and pup hormone levels in relationship to behavioral changes.

In conclusion, our data add an important finding to the growing literature on PCB related social behavior changes. We demonstrate that certain key actions are altered as part of the early maternal care behavior sequence and that these same female rats have a robust upregulation in OXTR gene expression. This link focuses on hypothalamic levels of oxytocin because of previous work showing that oxytocin in the hypothalamus is critical to maternal behavior prior to and following parturition. The results add to our understanding of possible epigenetic influences of PCB with implications that PCB exposure can have lasting effects on critical behaviors over multiple generations.

Materials and Methods

Animals and PCB exposure

Care and use of animals were performed in accordance with the Bowling Green State University Institutional Animal Care and Use Committee (Protocol # 09-008). Female Sprague-Dawley rats weighing 200-250 g (10-12 weeks of age, virgin) (Harlan Sprague-Dawley Indianapolis, IN) were mated to males of similar age. Pregnancy was confirmed by a sperm positive vaginal smear, with that day designated gestational day 0 (GD 0). Following the positive smear, females were placed in individual cages and fed either control (Harlan Teklad Rodent Diet 8604, mash form, Madison, WI) or the same diet with PCB added. The PCB diet contained equal parts of 2 moderately chlorinated PCB congeners obtained from AccuStandard Inc.. (New Haven, CT) 47 (2, 2′, 4, 4′-tetrachlorobiphenyl, non-coplanar) and 77 (3, 3′, 4, 4′-tetrachlorobiphneyl, coplanar). The rationale for choosing these 2 PCB congeners was to provide a simple mixture, but one that contained both non-dioxin-like (PCB 47) and dioxin-like (PCB 77) toxicant exposure. The 2 congeners were dissolved in ethanol and thoroughly mixed with 1000 g of rat chow mash for a final concentration of 25 ppm (25 mg/kg w/w). The control diet consisted of rat chow mash mixed with ethanol (the vehicle for added PCB) that was allowed to evaporate entirely. Water and diet (control or PCB) were provided ad libitum. Rat dams were provided with 100 g of diet daily in a spill deterrent feeding container, and daily consumption was monitored by weighing remaining food each morning. All rats were weighed daily at the time of feeding.

Cross-Fostering

On PND 0, PCB diet was removed from the PCB exposed dams, and they were continued on standard diet without PCB. On that same day litters were culled to 8 pups (4 males, 4 females) and assorted in accordance with the following schematic: control non-fostered, control pups with PCB exposed dams, PCB non-fostered, and PCB exposed pups with control dams. Litters were coded (A or B) in terms of diet exposure so that the individuals collecting the data or scoring the behavior were blind to the actual group membership of the animals until the end of the project.

Maternal behavior analysis

Nest building

Nest building behavior was analyzed beginning on gestational day 20. Thirty brown paper towel strips, measuring 3 cm wide by 20 cm long were placed atop the wire metal cages on gestational day 20 at 3:00 p.m. The nest building behavior was analyzed at 6:00 p.m. on GD 20. The basis of nest analysis consisted of the latency to begin pulling strips into the cage, the total number of strips the dam used per day, and the quality of the nest built. Then, the nest quality in maternal cages was scored daily at 8:00 am, 12:00 pm, and 6:00 p.m. until the birth of the pups. The quality of the nest was scored based on the schematic set forth by Beach[105] using the following categories for nest score: **0 Point Nest:** Paper strips remain on the top of the cage, no nest construction, and any paper strips that are moved into the cage are scattered; **1 Point Nest:** No sides, no flooring, serves no practical purpose, and offers no protection ; **2 Point Nest:** No sides, hardly any flooring, and only a small number of the 16 paper strips are utilized ; **3 Point Nest:** Lacks sides, relatively thin flooring, and all paper strips are utilized ; **4 Point Nest:** Lacks the high sides of a 5 point nest, has relatively thick flooring, and all paper strips are utilized; **5 Point Nest:** Approximately 5 inches deep, floor composed of several thicknesses of paper, is compactly constructed in a cage corner, and all of the paper strips are utilized. The nest remained in the cage until postnatal day 6 so that the dam would not be disturbed by nest removal during filming.

Maternal care evaluation

Maternal care behaviors were investigated on postnatal days one, 2, 4, and 6. Video recordings began one hour before lights out, and continued for 2 hours total. The following behaviors were scored from these video recordings using OD Log Software (Macropad Software Inc..): time off nest, pup licking, autogrooming, active nursing, low crouch nursing, high crouch nursing, and supine (resting) nursing. These behaviors were hand scored using a maternal behavior check list by an investigator blind to the experimental condition of the animal being scored. Time spent in each category as well as the proportion of total time spent in each behavior was recorded. High crouch nursing was evaluated as the proportion of time spent in this behavior over all the other nursing behaviors.

Maternal hypothalamic gene expression

Sprague-Dawley dams were euthanized by a sub-lethal dose of sodium pentobarbital based solution and decapitated on PND 17. Hypothlamic tissue consisted of a square punch through the median eminence at the base of the brain, using the mammillary bodies as the posterior marker and the optic chiasma as the anterior marker. Dimensions of the punch were approximately 4 mm by 4 mm at the surface, and extended 5 mm into the brain. Tissues were collected from the hypothalamus and flash-frozen with liquid nitrogen. Total RNA was extracted using Qiagen RNeasy kit (Qiagen, CA) and overall quantity and quality of RNA was determined using a Nanodrop Spectrophotometer (NanoDrop Technologies, DE) and 2%

agarose gel electrophoresis. cDNA was made using random nonamer and SuperScriptII reverse transcriptase (Invitrogen, CA) according to manufacturer's specifications. Quantitative real time RT-PCR analysis was performed on the MiniOpticon (BioRad, CA) using Dynamo SYBR qPCR (Thermo Scientific, PA). Forward and reverse primers for OXTR gene were 5′-gtcaatgcgcccaaggaag -3′ and 5′- gtcaatctacccccgaagcagct -3′, respectively. Forward and reverse primers for Cyp1a1 gene were 5′- CAAAGCCCATGTTCCTGTTT-3′ and 5′-GCGGTCATGACTGTACCCT-3′, respectively. Beta actin gene was used as reference with forward primer, 5′-caaccttcttg-cagctcctc-3′; reverse primer, 5′-ttctgacccatacccaccat-3′. The PCR cycle included initial denaturation (10 min, 95°C), followed by 35 cycles of denaturation (94°C, 10s), annealing (58°C, 30s [OXTR], 52°C, 30s [B-actin] and 52°C, 30s {Cyp1a1]), extension (72°C, 30s). Melt curve analysis was performed from 68°C–90°C, 3s hold, 0.5°C interval. The expression quantification was analyzed with the ΔC(t) method. Each data represents the average of 3 PCR with 3 replications per PCR.

Statistical analysis

Statistical analysis was performed on behavioral observations and gene expression using the SPSS statistical analysis software (SPSS Inc., Chicago, IL). ANOVA and multifactorial ANOVA tests were performed with significance having a p value < 0.05. If significant main effects or interactions were obtained, pairwise comparisons for each developmental day were completed (t-tests). Significance was represented by having a p value < 0.05. If data showed a violation of normal distribution, Friedman's ANOVA coupled with Mann-Whitney non-parametric statistical analysis was used and significance was noted as having a $p < 0.05$.

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