Influence of Perinatal Exposure to a Polychlorinated Biphenyl Mixture on Learning and Memory, Hippocampal Size, and Estrogen Receptor-Beta Expression

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Abstract. Perinatal exposure to PCB has been reported to cause a variety of health effects including endocrine disruption, and immunologic, reproductive, neurologic, and behavioral deficits. In the present study, a mixture of two PCB congeners, one non-coplanar (PCB 47) and one coplanar (PCB 77), were administered to young female Sprague-Dawley rats by route of maternal dietary consumption (either 12.5 ppm or 25.0 ppm, w/w). Impact on learning and memory were examined by radial arm maze on postnatal day 24-27. After behavioral tests were completed, the rats were transcardially perfused, and brains were excised. Immunohistochemistry for ER- β was carried out on free-floating sections. Sections were stained with cresyl violet stain, and hippocampal area was measured. A subjective comparison of staining density suggested a greater intensity of ER- β staining in female rat hippocampus exposed to PCB 47/77 at 25 ppm concentration. A decrease in the hippocampal area measurement was observed in the case of 25 ppm PCB exposed rats. Significant behavioral effects involving spatial learning and memory were not observed. However, animals exposed to PCB 47/77 at 25 ppm displayed a trend toward improved performance. Taken together, the combination in PCB exposed rats of reduced hippocampal size, increased ER-β concentration, and unaltered behavior suggests the existence of compensatory mechanisms in the animals.

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INTRODUCTION

Polychlorinated biphenyls (PCB) are among the most persistent contaminants in our ecosystem (Safe 1994), having been demonstrated to cause a variety of adverse health effects. Concern over the toxicity and persistence (chemical stability) of PCB in the environment led U.S. Congress in 1976 to enact prohibitions of manufacture, processing, and distribution in commerce. Perinatal exposure to PCB either directly or through maternal exposure causes alterations in hippocampal formation processing and neurochemical status (Provost and others 1999), auditory and hormonal deficits (Goldey and others 1995), depressed immunocompetence (Tryphonas and others 1998) and decreased reproductive success (Donahue and others 2002). PCB exposed animals showed persistent and significant deficits in neurological development, including visual cognition, short-term memory, and learning (Carpenter and others 2002). Besides direct alteration of hormones, many of the other PCB-induced physiological alterations appear to be linked to endocrine alteration as well. The exposure to a mixture of one *ortho*-substituted (non-coplanar) congener and one non-*ortho* substituted (coplanar) tetrachlorinated PCB congener significantly depressed thyroid hormone level and hippocampal choline acetyltransferase activity (ChAT), and thereby caused depression of long-term memory in the Morris water maze test (Provost and others 1999). Endocrine disruption in animals can have long-lasting, life altering effects. PCB 77 has been reported to possess both estrogenic and antiestrogenic actions, depending on the observed end points, tissues examined, and timing of exposure (Nesaretnam and others 1996). PCB 77 is listed in the priority group of PCB of highest concern as environmental contaminants (McFarland and Clarke 1989) and is the most common congener present in the environment.

A recent study reported that disruption of ER-β gene impairs spatial learning in female mice (Rissman and others 2002). In this study, ER-β knockout (ER-β KO) and wild-type littermates were tested for spatial learning in the Morris water maze after ovariectomy, followed by appropriate control treatment, or physiological doses of estrogen. These data show that ER-β is required for spatial learning. The importance of estrogen in learning and memory has been reported (Good and others 1999) where induction of long-term potentiation (LTP) during the proestrous stage of the estrous cycle has been demonstrated. However, no previous studies have investigated the effect of PCB on estrogen receptor-β in hippocampus, and its related effects on hippocampal morphology, learning, and memory.

In the present study, 27-day-old female Sprague-Dawley rats were exposed perinatally to a mixture of PCB 77 and PCB 47 to determine a potential mechanism of estrogen disruption by carrying out immunohistochemical analysis of estrogen receptor-β density in hippocampus. A morphological evaluation of the hippocampus was carried out by measurement of stained hippocampal sections at approximately -3.30, -3.60, -3.80 mm from bregma point. Since, estrogen plays a crucial role in the development of brain, disruption of the action of this hormone during prenatal development may lead to neurological and behavioral deficits influencing learning and memory. Hence, the effect of PCB on learning and memory were studied using a simple four-arm radial arm maze.

MATERIALS AND METHODS

Animals

All procedures relating to the animals including husbandry were performed according to a protocol approved by Bowling Green State University Institutional Animal Care and Use Committee (IACUC Protocol # 04-015). To generate offspring for investigation of the effect of PCB during development, female Sprague-Dawley rats were mated to males of the same strain. From the day of conception, determined by a sperm positive vaginal washing, females were fed diets containing either 0, 12.5, or 25 ppm of an equal mixture of two PCB congeners: PCB 77 (3,3',4,4'-tetrachlorobiphenyl) and PCB 47 (2,2'4,4'-tetrachlorobiphenyl) obtained from AccuStandard, Inc. (New Haven, CT) mixed homogeneously into standard rat

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chow mash (Mowlan Teklad, Madison, WI). This exposure was provided to rat pups through maternal diet during gestation and lactation. Control pups were produced by feeding diet with no PCB added. Litters were standardized to 10 pups (five males and five females when possible) on postnatal day (PND) three. Females were used in the present study and males were used in a separate study in the lab. On PND 23-27 female rat pups were food deprived for 14 hours daily and trained for behavioral analysis (radial arm maze). At the end of PND 27, the rat pups were anesthetized by intraperitoneal injection of Nembutal® (Pentobarbital, 50 mg/kg, Abbott Laboratories, N. Chicago, IL) and perfused transcardially with 0.9 percent saline and four percent paraformaldehyde. Brains were removed and postfixed in four percent paraformaldehye at 4°C overnight. Following postfixation, brains were cryoprotected with 30 percent sucrose in phosphate buffer overnight until the brain settled to the bottom of the tube. Following the cryoprotection, every alternate 30 μm thick section of the rat brain was taken from -3.30 to 3.80 mm from the bregma point. Randomly selected sections were incubated in 0.1M phosphate buffered saline (pH-7.4) for 30 minutes and processed for immunohistochemistry of ER-β. The remaining sections were placed on gelatin-coated slides, allowed to dry overnight, and processed for cresyl violet acetate staining for measuring total hippocampal area.

Radial Arm Maze

From PND 23-27 female rat pups were food deprived for 14 hours with access to water, trained and tested in a radial arm maze. The maze was made of plywood and consisted of a central arena 50 cm in diameter, with eight arms 60 cm long by 10 cm wide, radiating equi-angularly from the central arena. Only four of the arms, each extending at right angles to the adjacent arms, were used in the present study. The room where the maze was located provided external visual cues, allowing and animal in the maze to establish its spatial position. On the first and second days (PND 24 and 25), which constituted training/habituation days, a sucrose pellet was placed in the central arena with all the four doors to the arms closed. A reward pellet was also placed at the end of each of the four arms. Each rat pup was placed individually in the central arena. Once the rat pup consumed the center pellet, one of the doors was randomly opened, and the rat pup was allowed to enter that arm. This was repeated for the three remaining arms and time required for the rat to consume the pellet from each of four arms was noted. On the third and fourth days (PND 26 and PND 27), which constituted testing days, doors to all four arms containing sucrose pellets remained open. Each rat pup was placed individually in the center arena and a timer was started. The time taken for each rat to enter and consume the sucrose pellet from each of the four arms was noted. On the testing days, error scores were noted as an additional parameter of consideration. If a rat consumed a pellet from one arm and re-entered to the same arm then it was marked as an error. An entry was considered when all four limbs of the rat were inside the arm. The session was terminated when the rat had retrieved and eaten all the sugar pellets, or after five minutes had elapsed. Total time to complete the maze was recorded on each of the four days, and an average of the numbers of errors (re-entries into arms from which the sucrose pellet had already been consumed) on day three (PND 26) and day four (PND 27) were recorded for statistical analysis.

Immunohistochemistry

This procedure was adapted from Alves and others (1998). Free-floating 30 μm sections were washed in ice-cold 0.1 M phosphate buffered saline (PBS: pH7.4) to thoroughly remove cryoprotectant. To deter nonspecific staining, sections were washed in one percent sodium borohydride ($NaBH_4$) in PBS for 30 minutes and rinsed eight to 10 times with PBS to remove all $NaBH_4$. Endogenous peroxidase activity was inhibited by washing tissue with 0.3 percent H_2O_2 in 40 percent methanol in PBS for 30 minutes. Following several washes in PBS, tissue was blocked with two percent normal goat serum in PBS with 0.3 percent Triton X-100 (PBST) for one hour. Tissue sections were incubated with an affinity-purified rabbit polyclonal antibody to ER-β (2.0 μg/ml; Affinity BioReagents, Golden, CO) in PBST and one percent goat serum for 72 hours at 4°C. This antibody (PA1-312) detects estrogen receptor (ER) beta from rat tissues. This immunizing peptide corresponds to amino acid residues 467-485 from mouse ER-β. PA1-312 has been characterized and demonstrated to be specific for ER-β; in the rat it shows cross reactivity with only ER-β and no western blot cross reactivity has been observed with ER-α (Affinity Bioreagents, Package Insert). Following primary antibody incubation, all sections were washed in PBS and exposed to a biotinylated secondary anti-rabbit IgG antibody made in goat (1:600, Vector Laboratories) in PBST and one percent normal serum, for one hour at room temperature. Following several PBS washes, sections were exposed to the avidin-biotin complex (ABC, 1:100, Vector Elite kit) in PBS for 45 minutes. Following several additional washes, sections were treated for 10 minutes with freshly prepared reaction mixture, containing a 10 mg tablet of the substrate 3,3'-diaminobenzidine tetrachloride (DAB, Sigma, St. Louis, MO) dissolved in 10 ml of 0.1 M sodium acetate buffer (pH 5-6) containing 0.1 ml three percent H_2O_2. Following several washes with water, the sections were mounted onto gelatin-coated slides, and air-dried in the dark overnight. Sections were dehydrated and cleared in increasing concentrations of ethanol and finally xylene, coverslipped with DePex (Electron Microscopy Sciences, Ft. Washington, PA), and observed at 40X and 100X magnification using a light microscope. Sections were photographed using Motic Image Analysis and scored subjectively with regard to staining density (density scale 1+-3+).

Measurement of Total Hippocampal Area

A light microscope in conjuction with an image analysis system (Motic Image Analysis) was employed for morphological quantification. A total magnification of 10X was used to view individual sections, and the image analysis system was calibrated to produce area measurements in mm^2. Area measurements were taken for sections at approximately -3.30, -3.60, and -3.80 mm from bregma point. Hippocampal area for each section was determined in triplicate, and an average of the three measurements was recorded for statistical analysis.

Data Analysis

Statistical analysis was performed using the SPSS statistical software package. Differences between groups for the hippocampal area measurement and radial arm maze parameters (time taken, working memory errors) were analyzed by means of analysis of variance (ANOVA). Levene's test was used to evaluate the homogeneity of variances, and Tukey HSD post hoc tests were used to compare individual treatment means. When ANOVA indicated significant differences, nonparametric analysis using the Kruskal-Wallis ANOVA by ranks was applied. Categorical data such as the range of time taken in radial arm maze were analyzed by Chi-square analysis.

All behavioral measures except range of time taken by litters were presented as mean values. In all cases, the litter (treatment) was

the independent experimental unit and data from individual female offspring were assumed representative for the litter. When more than one measurement was taken for each offspring (hippocampal area measurement), the results were averaged to give an individual mean. In all cases, significance was set at $p \leq 0.05$. In order to reduce error, self-deception, and bias the investigator was blind to animal treatment throughout the study.

RESULTS

Radial Arm Maze (Time)

The performance of each female pup in the radial arm maze (RAM) was determined. Rats from control and both PCB-exposed groups learned the task, as illustrated by a progressive statistically significant reduction in time taken from the first session through the fourth session [$F(3,189) = 60.34$, $p = 0.001$]. As described in the materials and methods, the first and second sessions involved habituation/training in the radial arm maze, and the third and fourth sessions constituted test days. Time taken to complete the RAM was recorded on all four days, but error numbers were recorded on the third and fourth days only. There were no significant differences between the control and PCB exposed groups for the time taken to successfully complete the RAM on the test day (fourth session) [$F(2, 69) = 0.968$, $= 0.385$]. However, on day four the PCB 25 exposed animals completed the task in less time then PCB 12.5 and control pups, indicating a trend towards better performance compared to the other groups (Table 1, Fig.1). On average, the time taken by pups to complete the RAM on day four was 10.4 percent of that on day one.

Radial Arm Maze (Working Memory Errors)

A comparison of working memory errors (re-entries into arms from which the reward/sucrose pellet had already been retrieved) measured on day three and day four (described as test days in materials and methods) did not reveal a significant difference among the treatment groups [*Day 3* $F(2, 69) = 1.366$, $p = 0.262$; *Day 4* $F(2, 69) = 0.036$, $p = 0.965$] (Fig. 2). However, on an average the PCB 12.5 exposed rats made fewer errors on day three than did controls or PCB 25 animals.

Hippocampal Area Measurement

A comparison of hippocampal area measured in μm^2 at -3.30 mm from the bregma point between different treatment groups did not reach significance [$F(2,31) = 2.109$, $p = 0.140$]. While the hippocampal area measured at -3.60 mm from bregma point in either PCB 12.5 ppm or PCB 25 ppm was significantly less than the control female rats [$F(2,31) = 4.751$, $p = 0.016$]. Further analysis showed that the hippocampal area measured at -3.80 mm from bregma point in PCB 12.5 and PCB 25 ppm exposed rats was not significantly less than the control, but it nearly reached significance [$F(2,31) = 2.980$, $p = 0.067$] (Fig. 3). There was a general trend for less hippocampal area in all sections of PCB exposed pups.

ER- β Immunohistochemistry

The endocrine disruptive properties of a mixture of PCB 47 and 77 at a concentration of PCB 12.5 ppm and 25 ppm were studied by immunohistochemistry for ER-β in randomly selected coronal hippocampal sections from -3.30 to -3.80 mm bregma point. ER-β positive cells were observed throughout the hippocampal section.

A subjective comparison for the immunohistochemical staining revealed greater intensity of staining in PCB 25 ppm (Fig. 4C, 100X microscope magnification) exposed female rats in comparison to controls (Fig. 4A, 100X microscope magnification), whereas, the PCB 12.5 ppm (Fig. 4B, 100 X microscope magnifications) exposed rat hippocampal sections showed no qualitative difference in staining intensity from that in controls. Subjective evaluation of ER-β staining density of controls and PCB exposed rat hippocampal sections (Table 2) revealed strongest intensity (+++) observed for all brains examined in PCB 25 ppm exposed female rats (Fig. 4C). Negative control slices processed in the same manner as all other sections except with omission of the primary ER-β antibody revealed absence of non-specific staining (Fig. 4D).

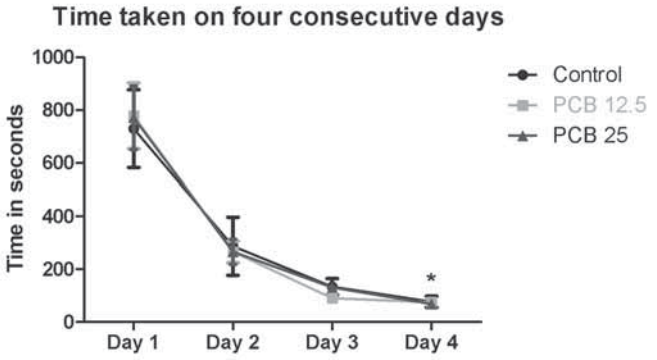

Figure 1. Amount of time taken to successfully complete the radial arm maze. Each value represents the Mean ± SEM (sec) within groups for female pups of control dams (N =21) or dams consuming PCB 12.5 ppm (N =24) or 25 ppm (N =25). Day 1 and Day 2(PND 24, PND 25) comprising the habituation days, and Day 3 and Day 4 (PND 26, PND 27) comprising test days. *Significantly different from day one ($p = 0.001$).

Table 1

Mean time to complete the radial arm maze (sec).

Groups	Day 1	Day 2	Day 3	Day 4
Control	729.82	286.94	133.64	77.17*
PCB 12.5	778.20	266.04	90.37	75.54*
PCB 25	771.10	266.12	131.00	69.16*

Each value represents the Mean ± SEM (sec) for female pups of control dams (N = 21) or dams consuming PCB 12.5 ppm (N = 24) or 25 ppm (N = 25). Day 1 and Day 2 (PND 24, PND 25) comprising the habituation days, and Day 3 and Day 4 (PND 26, PND 27) comprising test days.
*Significantly less from day one ($p = 0.001$).

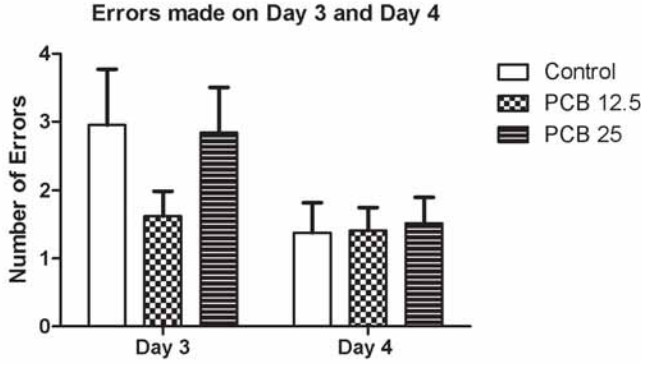

Figure 2. Number of radial arm maze errors on Day 3 and Day 4 sessions (PND 26 and PND 27). Each bar represents Mean ± SEM for female rats born to control dams (N =21) or dams consuming PCB 12.5 ppm (N =24) or PCB 25 ppm (N =25).

DISCUSSION

In the present study, the PCB 25 ppm exposed rats demonstrated a trend toward better performance in the maze than 12.5 ppm and controls as determined by the mean time taken to successfully complete the radial arm maze (Table 1). In addition, the mean of working memory errors did not reveal significant differences among treatment groups (Fig. 2). An enhanced performance by juvenile PCB 25 ppm female rats in time taken in radial arm maze could be the result of hyperactivity as observed previously using young rats exposed to PCB (47/77) mixture in water maze test (Donahue and others 2004). This is likely, as prenatal stress can have long lasting effects on offspring hypothalamus-pituitary-adrenal (HPA) axis, in generally programming a persistently hyperactive system. Another reason for enhanced maze performance by PCB exposed animals could be their use of a response strategy of entering adjacent arms to complete the task. By entering adjacent arms it is theoretically possible for the rat to complete the maze without the use of any memory system, other than memory of the response strategy and direction of turning. It has been hypothesized that rats with hippocampal lesions employ such response strategies in the RAM, because the lesions remove the memory system that would otherwise mask the animal's innate tendency to pattern (Lanke and others 1993). Similarly, in the present study, enhanced performance in the RAM could be the result of adjacent arm entries made by female rat pups. Hence, in the future monitoring of adjacent arm entries should be done to control for the possibility of such a response strategy.

Table 2

Subjective visual analysis of staining density for immunohistochemically localized estrogen receptor-β.

Groups	No. of Brains	Staining Intensity	Mean Value
Control	1	++	
	2	+	
	3	+	1.2
	4	+	
	5	+	
PCB12.5	1	++	
	2	+	
	3	+	1.3
	4	+	
PCB25	1	+++	
	2	+++	
	3	+++	
	4	+++	3.0
	5	+++	
	6	+++	

+ = weak staining; ++ = moderate staining; +++ = strong staining.

Moreover, age could be a factor of consideration since the performance of the female rat pups in the present study was only observed for the age range PND 24-27. A study carried out on Rhesus monkeys has shown that PCB exposure makes them hyperactive when they are young. However, with age, these monkeys became hypoactive, and their performance significantly declined (Bowman and Heironimus1981). Hence, in the future age dependent effects of PCB exposure on learning and memory should be determined.

A significant PCB-related depression in the area of hippocampal sections taken from -3.60 mm from bregma point was observed. There was also a trend toward smaller hippocampal area measurement in PCB exposed rats at -3.30 mm and -3.80 mm, but this was not significantly less than controls (Fig. 3). Apoptosis (programmed cell death) is essential to normal brain development (Martin, 2001). An exposure to PCB causes an increase in the density of apoptotic neurons from a background of two to five percent to levels as high as 18 percent (Howard and others 2003). However, in the present study this morphological abnormality is not found to result in a significant difference in radial arm maze performance. The RAM performance did not correspond with the reduction in hippocampal area perhaps because rats are known to acquire a spatial navigation task if small tissue blocks are spared in lesions of the dorsal hippocampus (Moser and Moser, 1998). Hence, it is possible that the remaining plasticity in the hippocampus is sufficient to support the processing demands inherent in a simple version of radial arm maze (four-arm) used in the present study.

A subjective visual comparison of hippocampal sections immunohistochemically stained for localization of estrogen receptor-β revealed a greater intensity of staining in PCB 25 ppm than in control or 12.5 ppm PCB animals (Table 1). Several studies indicate a protective role of estrogen that is mediated by ER-β. Similarly, upregulation of ER-β in cellular and extracellular localizations in the hippocampus was observed in Alzheimer's patients (Savaskan and others 2001). Hence, it is likely that in the present study an increase in the intensity of ER-β staining is indicative of a compensatory mechanism opposed to the neurotoxic effects of PCB.

In summary, perinatal exposure to PCB in juvenile female rats results in enhanced staining of estrogen receptor-β in the hippocampus of PCB 25 pups suggesting the possible presence of a compensatory mechanism. In addition, a PCB-induced depression of hippocampal area supports the notion of developmentally altered morphology of this brain region in rats exposed perinatally.

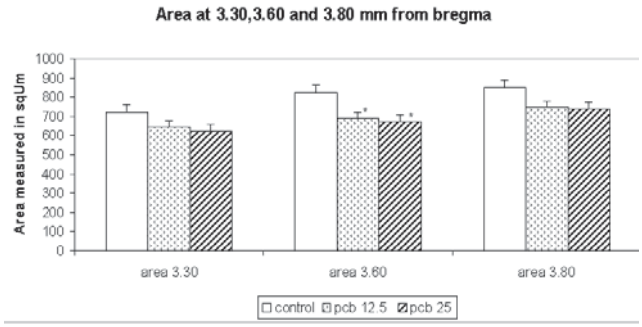

Figure 3. Area of hippocampus measured in μm^2 at -3.30, -3.60 and -3.80 mm from bregma point. Each bar represents the Mean ± SEM (area in μm^2) for female rats born to control dams or dams consuming PCB 12.5 ppm or PCB 25 ppm (N= 10 for each treatment group). * Significantly different from control ($p = 0.016$).

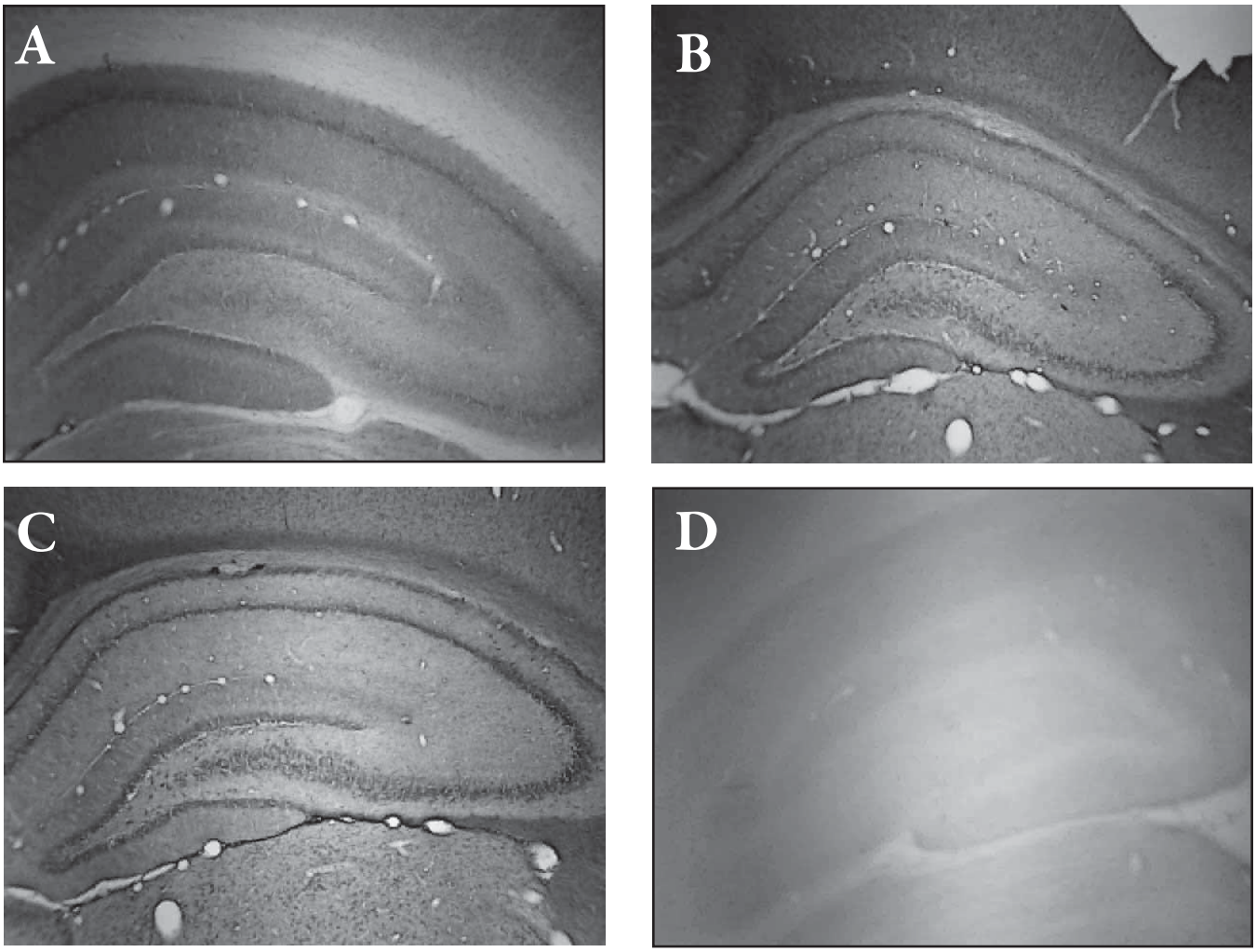

Figure 4. Immunohistochemistry for ER-β. A) Control. B) PCB 12.5. C) PCB 25. D) Negative Control without primary antibody. (100X microscope magnification)

Moreover, the mixture of PCB 47/77 used in the current study did not significantly alter radial arm maze learning and memory, regardless of dose. In the future, a western blot analysis, or a quantitative mRNA assay should be carried out to ascertain the nature of the enhanced staining of estrogen receptor- β revealed in the present study.

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