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Purkinje cell and cerebellar effects following developmental exposure to PCBs and/or MeHg

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Abstract

We recently reported that rats exposed to PCBs and MeHg during development were impaired on the rotating rod, a test of balance and coordination that is often indicative of cerebellar damage. In addition, developmental PCB exposure is known to dramatically reduce circulating thyroid hormone concentrations, which may have a negative impact on cerebellar development. Therefore, we investigated the effects of combined PCB and MeHg exposure on Purkinje cells and the cerebellum. The serum and brains from littermates of the animals tested on the rotating rod were collected at weaning, and we also collected brains from the adult animals at the end of motor testing. Four groups were studied: 1) vehicle controls, 2) PCBs only (Aroclor 1254, 6 mg/kg/d, oral), 3) MeHg only (0.5 ppm, in dams' drinking water), and 4) PCB+MeHg (at the same doses as in individual toxicant exposures). Female Long–Evans rats were exposed beginning 4 weeks prior to breeding with an unexposed male and continuing until postnatal day (PND) 16. There was a significant reduction in serum T4 and T3 concentrations in the PCB and PCB+MeHg pups on PND21. Golgi-impregnated Purkinje cells were examined in PND21 brains, but there were no significant exposure-related effects on primary dendrite length, branching area, or structural abnormalities. However, all three male exposure groups had a marginally significant increase in Purkinje cell height, which may suggest a subtle thyromimetic effect in the cerebellum. Cresyl-violet stained sections from the adult brains showed no exposure-related effects within paramedian lobule in Purkinje cell number, total lobule volume or layer volumes (molecular, granule cell and white matter layers). Evidence is provided for the dysregulation of expression of cerebellar ryanodine receptor (RyR) isoforms in PCB-exposed brains, and this could contribute to the rotating rod deficit by changing critical aspects of intracellular calcium signaling within the cerebellum.

Keywords: Cerebellum; Polychlorinated biphenyls (PCBs); Methylmercury (MeHg); Purkinje cells; Paramedian lobule (PML); Thyroid hormones; Ryanodine receptor

1. Introduction

We recently reported a behavioral deficit on the rotating rod task in rats exposed to polychlorinated biphenyls (PCBs) and methylmercury (MeHg) during development [84]. PCB or MeHg exposure alone did not significantly impair rotating rod performance, but PCB+MeHg exposure resulted in a significant increase in the number of slips from the rod, especially at the highest rpms (25 and 30 rpms). In addition, a recent study found that perinatal exposure to PCBs impaired performance on the righting response, negative geotaxis, the startle response, and rotorod, more severely affecting the PCB-exposed male rat neonates than females [66].

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Rotating rod deficits are known to be indicative of cerebellar damage [14,50,51,73]. Rats neonatally exposed to alcohol were impaired as adults on the rotating rod [50] and also had changes in the cerebellum, including decreases in paramedian lobule (PML) Purkinje cell number, molecular layer volume and total volume [51]. Therefore, as a follow-up to the rotating rod results, we examined the structure of the cerebellum in PCB- and/or MeHg-exposed rats. We focused on the Purkinje cells of the cerebellum because they play a crucial role in the cerebellum as the only source of output from the cerebellar cortex. In addition, recent studies have shown that hydroxylated PCBs have a significant inhibitory effect on T4-induced dendritic growth in cultured Purkinje cells [48,54]. There have been two in vivo studies to date investigating the effects of PCBs on cerebellar structure. One reports a reduction in cerebellar mass, with a greater effect in the PCB-exposed males than females [66]. The other reports that developmental PCB exposure decreased Purkinje cell branching area in male rats on postnatal day (PND) 22 (female rats were not examined) [59]. However, the decreased branching area in the males recovered by PND60 [59].

PCBs could affect cerebellar development through their complex actions on the thyroid hormone system (reviewed in [12]). Although developmental PCB exposure significantly reduces circulating thyroid hormone concentrations [24,40,63,101], it also causes elevations in the expression of RC3/neurogranin and myelin basic protein, two key thyroid hormone responsive genes in the developing brain [101]. Other chemical goitrogens, such as propylthiouracil and methimazole, reduce the expression of these same genes [42,43]. In contrast, calcineurin, a calmodulin-regulated phosphatase that is increased in the cerebellum following neonatal hypothyroidism [86], is also increased in the cerebellum of female rats exposed to PCBs during development [64]. Thus, some effects of PCBs in the developing brain are consistent with a hyperthyroid-like state, whereas others are consistent with a hypothyroid-like state.

Both neonatal hypo- and hyperthyroidism have been shown to change Purkinje cell structure. Hypothyroidism severely reduces dendritic arborization of Purkinje cells (reviewed in [62]) and increases the length of the primary dendrite ([80] and [82] cited in [23]), while hyperthyroidism results in Purkinje cell dendritic trees that are taller and narrower [67]. In addition, both hypo- and hyperthyroidism cause reductions in the molecular layer [79]. Thus, if PCBs change thyroid hormone action in the brain during development, there may be structural changes in the cerebellum that are similar to those of neonatal hypo- and/or hyperthyroidism.

Various studies have shown that high doses of MeHg cause structural abnormalities in the cerebellum. In humans, MeHg poisoning damages the cerebellum causing general tissue loss and abnormal Purkinje cell migration [16,26,32,33]. In rodent studies, MeHg exposure has caused Purkinje cell and granule cell degeneration [15], decreased dendritic arborization of Purkinje cells [17], and reduced the thickness of the granular and molecular layers [88]. In the present study, the estimated total dose of MeHg was 1.20 mg, which is 2–25-fold lower than the doses used in previous rat studies. It was not known if this lower dose of MeHg would cause detectable structural changes within the cerebellum, or if combined exposure to PCBs and MeHg would magnify the effects seen with either chemical alone.

In the current study, rats were exposed to PCBs and/or MeHg during gestation and lactation. At weaning on PND21, serum was collected for analysis of total triiodothyronine (T3) and thyroxine (T4) levels. Brains were also collected on PND21 which was just after exposure ended. In addition, this is the same age at which changes in Purkinje cell structure in PCB-exposed rats were previously reported [59]. The PND21 brains were stained with a Golgi solution to allow for visualization of individual Purkinje cells within the cerebellum. Parasagittal sections of the cerebellum were used to examine the Purkinje cell structure for: dendrite tree height and branching area, primary dendritic length, and ratings of structural abnormalities. Adult brains were also collected at the conclusion of motor testing [84] for Nissl staining. The PML of the cerebellum, which is vital for limb control [19,89], was examined in these adult brains, including Purkinje cell number, total volume, and layer volumes. Ryanodine receptor (RyR) expression and binding was also assessed on PND63 in a separate set of PCB-exposed rats.

2. Methods

2.1. PND21 Thyroid hormone and Purkinje cell endpoints

2.1.1. Animals and exposure

The animals used in this study were littermates of rats tested for motor function [84]. Briefly, female Long–Evans rats were purchased from Harlan Sprague Dawley (Madison, WI) in 3 cohorts spaced approximately 6 months apart. Three to four rats from each cohort were assigned to each of four exposure groups: 1) vehicle control; 2) PCBs only; 3) MeHg only; or 4) PCB+MeHg, resulting in 8, 11, 11, and 10 litters, respectively. Exposure began 4 weeks prior to breeding and continued through PND16 for all groups. The animal facility was AAALAC-approved and all procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The PCB-exposed groups received a commercial mixture of PCBs known as Aroclor 1254 (A1254; Accustandard, Lot #124–191) at a dose of 6 mg/kg/day. A1254 was diluted in corn oil, pipetted onto one half of a vanilla wafer cookie (Keebler Golden Vanilla Wafers®), and these treated cookies were fed to the female rats daily throughout the exposure period. The dose was adjusted daily to account for weight gain in the dams. MeHg-exposed groups received methylmercuric chloride (Alfa Aesar Chemicals, Ward Hill, MA) dissolved at a concentration of 0.5 μg/mL (0.5 ppm) in their drinking water. Females were bred with unexposed males. Pups were weaned on PND21. One male and one female from each litter were randomly selected at weaning for collection of trunk blood for thyroid hormone assays and organ weights (organ weights presented in [84]). Another male and female

from each litter in cohorts 2 and 3 were perfused as described below for Golgi staining. Further, on PND28, one male and one female were randomly selected for motor testing (see [84]), and another male and female were randomly selected for cognitive testing and hearing assessments [97,96].

2.1.2. Thyroid hormone assays

Total T3 and T4 were determined using commercially available radioimmunoassay kits (Coat-A-Count®, Diagnostic Products Corporation (DPC), Los Angeles, CA). The kit uses ^{125}I-labelled tracers. Serum controls were used to evaluate inter-assay variability. Inter-assay %CVs were 6.8% and 6.6% for T3 and T4, respectively. Sample and control values were calculated based on weighted log–logit standard curves. Intra-assay coefficients of variation (%CVs) were 5.5% and 3.0% for T3 and T4, respectively. The limit of detection for T4 was 1×10^6 μg/dL. For T4, any samples below the level of detection were assigned the limit of the assay (1×10^6 μg/dL).

2.1.3. Golgi staining

Whole brains from 1 male and 1 female were collected on PND21 for Golgi impregnation from cohorts 2 and 3, resulting in 6 controls litters, 8 MeHg litters, 7 PCB litters, and 7 PCB+MeHg litters. Again, these were littermates of rats tested for balance and coordination [84]. Rats were anesthetized with sodium pentobarbital and perfused with saline. Brains were immediately removed from the skull and placed in a Golgi–Cox solution [38]. Using test sections, staining was determined to be complete after 15 days in the Golgi–Cox solution. The Golgi–Cox solution was poured off, and the cerebellar hemispheres were dissected. The cerebellar hemispheres were then rapidly dehydrated in 1:1 ethanol:acetone, ethanol, and 1:1 ether:ethanol before submersion in 5.3% celloidin for 7 days. The blocks were then transferred to 10.6% celloidin for another 7 days, transferred to fresh 10.6% celloidin, and hardened over chloroform. Parasagittal sections (120 μm) were taken with a microtome starting at the midline for both the hemispheres. Sections were submerged in butanol and then dehydrated further with ethanol, washed with deionized water, submerged in ammonia, washed with deionized water, submerged in diluted Kodak rapid fixer, washed with deionized water, dehydrated with ethanol, placed in xylene, and mounted and coverslipped using Permount.

2.1.4. Purkinje cell height measurements

The Golgi-stained slides were coded so that experimenters would be blind to exposure conditions during height measurements of Purkinje cells. Only vermal sections in which the lobules could be identified were used. The criteria for selecting Purkinje cells for height measurements were that the cell had to span the molecular layer, be well-stained, and be unobscured by adjacent cells. The lobule within which the Purkinje cell was found was recorded, and the data were grouped into anterior (lobules I–IV), dorsal (lobules V–VII), or posterior (lobules VIII–X) areas of the cerebellum. The scale on the Olympus BH-2 light microscope reticule was used to take height measurements at 60× magnification, and calibration measurements of the reticule scale were taken regularly. The cell body was not included in the height measurements. The scale was consistently placed at the point where the cell body thinned into the primary dendrite. Two measurements of height were taken from the Purkinje cells: perpendicular height (measuring from the cell body in an axis perpendicular to the edge of the molecular layer) and long-axis height (measuring in a direct line from the cell body along the axis of the longest dendrite). On average, 40 Purkinje cells per rat were used in the height measurements.

2.1.5. Purkinje cell primary dendrite length

The length of the Purkinje cell primary dendrite was also measured in the Golgi-stained slides from the PND21 rats. The length was measured from the top of the cell soma to the first bifurcation of the dendrite.

2.1.6. Purkinje cell branching area and stained dendrite area

The branching area of the dendritic domain of the Purkinje cells was measured using a Zeiss Axiovert microscope (magnification ×60) with an attached camera projecting to a computer to create a digitized image of the Golgi-stained Purkinje cells. Images of complete, fully-stained Purkinje cells were focused on the computer screen, and the outer extent of the Purkinje cell dendritic tree was traced using the mouse. MicroComputer Imaging Device (MCID) software calculated the branching area of the dendritic field. The stained dendritic area within the traced outline was also calculated by MCID.

2.1.7. Purkinje cell structural abnormalities

Two vermal sections per rat were examined for structural abnormalities of the Purkinje cells, one from each hemisphere when possible. Only intact Purkinje cells were assessed. Intact cells were defined as those in which the dendritic tree was: 1) sufficiently stained, 2) not significantly truncated, and 3) not obscured by other cells. There was one exception: ectopic Purkinje cells were recorded even if they were not intact as long as they were still identifiable as Purkinje cells. One person who was blind to the exposure groups assessed the Purkinje cells on several abnormalities that included items such as small, narrow, missing dendrites, and abnormal organization.

2.1.8. Data analysis

All data were analyzed using SPSS 12.0.1 for MS Windows. For the thyroid hormone assays, T3 and T4 were analyzed via separate 3-way ANOVAs (exposure (4) by cohort (3) by sex (2)), using the litter as the unit of analysis nested within exposure group, and sex was treated as a nested within litter variable. For the Purkinje cell Golgi data, 14 of 56 brains could not be used because there were too few complete, well-stained Purkinje cells, and this left only 14 of 28 complete male/female littermate pairs. Thus, to maximize the available data, the data from the males and females were analyzed separately. For the Purkinje cell height measurements, the data for the males and females were analyzed separately using three-way ANOVAs (exposure (4) by cohort (2) by cerebellar area (3)) for each of the following: perpendicular height, long-axis height, and the

primary dendrite length. The Purkinje cell branching area and stained dendritic area were analyzed separately for males and females using two-way exposure (4) by cohort (2) ANOVAs. The Purkinje cell structural abnormalities, including the percent of ectopic cells, were also analyzed via separate two-way exposure (4) by cohort (2) ANOVAs for males and females separately. Significant effects were further analyzed via tests for simple main effects and/or planned comparisons of the exposed groups to the control group, as appropriate [47]. Statistical significance was ascribed as $p < 0.05$.

2.2. Adult brains

2.2.1. Preparation of adult brains

Following motor testing [84], the adult rats (PND90–100) were anesthetized with sodium pentobarbital (100 mg/kg) and intracardially perfused with 0.1 M sodium phosphate buffer followed by 4% paraformaldehyde fixative. Brains were immediately removed and stored in the paraformaldehyde fixative. The whole brain was later weighed, and then the cerebellum was dissected and also weighed. Sagittal 40-μm serial cryostat sections were taken through the entire right hemisphere of the cerebellum and mounted directly onto chromalum gelatin-coated slides maintaining their order. Resulting sections were stained with a 1% cresyl-violet solution, dehydrated, and coverslipped. A quantitative stereological evaluation of the PML was performed to obtain the total Purkinje cell number, the total volume, and the volumes of the cortical layers.

2.2.2. Total Purkinje cell number and PML volumes

The boundaries of PML were defined medially as beginning after the disappearance of the lobulae IX and X and proceeding laterally until the PML's disappearance at the level of the flocculus/paraflocculus, using Refs. [72] and [74] as guides. StereoInvestigator (version 6.0.1; MicroBrightField, Colchester, VT) was used to count the number of Purkinje cells in a known fraction of the PML using the optical disector method, as described in Ref. [95]. For each brain, every ninth section within the PML was examined, starting with a randomly selected section within the first nine sections. For each section, the boundary of the PML was traced on the computer screen using the software. The software then randomly placed a 200×200 μm grid on each section, and at each (x, y) position of the grid an unbiased sampling frame (80×80 μm) was determined for Purkinje cell counting (see [51] for further illustration of the method). Purkinje cells were counted under 60× oil magnification. The total Purkinje cell count for each rat was calculated using the following formula:

$$\text{Total Purkinje cell count} = Q^{-} \; X \; (1/f_1) \; X \; (1/f_2) \; X \; (1/f_3)$$

where

Q^{-} = sum of all Purkinje cells counted within all sampling frames

f_1 = sampling fraction of all sections (every ninth) = 1/9

f_2 = sampling fraction of each section area = area (counting frame)/area (grid) = 6400/40,000 μm^2

f_3 = sampling fraction of section thickness = 1

The total volume of the PML was calculated using the estimated areas given by the StereoInvestigator's optical fractionator probe. These areas were summed and multiplied by the approximated intervening thickness of 360 μm, which was calculated by multiplying the number of intervening sections (9) by the sectioning thickness (40 μm). The areas of the cortical layers were determined using the StereoInvestigator's Cavalieri Estimator probe, in which intersections of an 80×80 μm grid were assigned separate markers for molecular, granule cell, and white matter layers depending on the location of each crosshair. These section areas were again multiplied by the approximated intervening thickness of 360 μm to calculate the layer volumes.

2.2.3. Western blot and [^{3}H] ryanodine binding analyses

Ryanodine receptors (RyR) have been shown to be a target of non-coplanar PCBs [75]. Cerebella from additional control and PCB-exposed groups, whose dams were exposed using the same methods described above, were removed on PND63, and a whole membrane fraction was isolated. Cerebellar proteins were denatured in SDS-PAGE sample buffer (Bio Rad, Hercules, CA) with 2.5% 2-mercaptoethanol at 60 °C for 5 min. 10–50 μg of protein from whole membrane homogenates isolated from control and PCB groups were loaded onto 4–15% gradient SDS-PAGE gels (Ready Gels, Bio Rad, Hercules, CA) and electrophoresed at 150 V for 75 min, before transfer to PVDF membranes at 200 V for 1 h. Membranes were blocked with 5% nonfat dry milk in TBST buffer (150 mM NaCl/2.5 mM KCl/25 mM Tris, pH 7.4/0.1% tween-20) for 1 h, rinsed 3×5 min with TBST, and incubated with primary antibody diluted in 0.1% nonfat dry milk/TBST for at least 1 h. Two monoclonal antibodies were utilized in this study: 34C specific for RyR1/3 subtypes (Univ. Iowa Hybridoma Bank, culture media) at 1:2000 dilution, or C3-33 specific for RyR2 subtype (Alexis Biochemicals, San Diego, CA) at 1:5000 dilution. Following incubation, membranes were washed 3×5 min with TBST and incubated with horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Sigma, St. Louis, MO, diluted 1:20,000) for 1 h and visualized with an enhanced chemiluminescent (ECL) reagent kit per manufacturer's instructions (Perkin Elmer, Boston, MA) on film. Developed films were imaged using a flatbed scanner in transmissive mode and pixel densities were determined from scanned images using Scion Image software (Scion Corporation, Frederick, MD). Density values obtained for a given primary antibody were determined from at least 4 blots. Values are presented as the percent change relative to the respective control density (means ± SEM), with 4–5 measurements per group. [^{3}H] ryanodine binding analysis was performed as previously describe [99] in the presence of 5 nM radioligand.

2.2.4. Data analysis

The adult brain and cerebellar weights, total Purkinje cell number, and PML total and layer volumes were all analyzed via separate three-way ANOVAs (exposure (4) by cohort (3) by sex (2)). Significant differences in RyR expression and [^{3}H] ryanodine binding between groups were detected by one-way

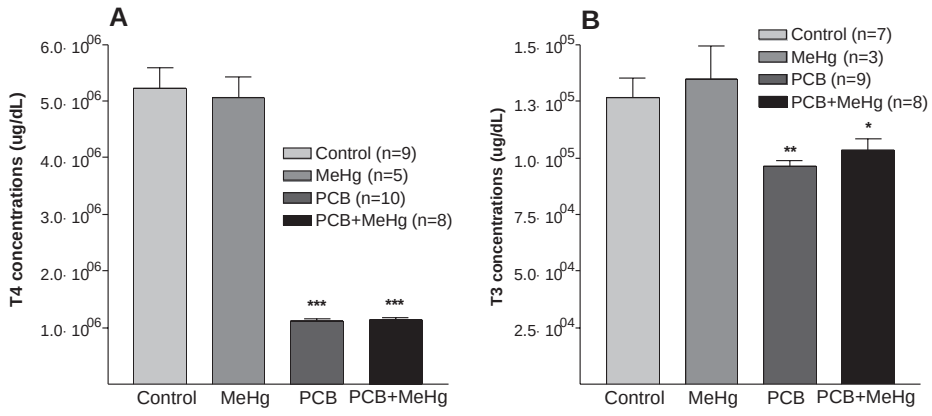

Fig. 1. A) Mean T4 concentrations (±SEM) are graphed according to exposure group. T4 concentrations on PND21 were significantly reduced in the PCB and PCB+MeHg groups. Samples below the limit of detection of the assay were conservatively assigned the detection limit, which was 1×10^6 μg/dL. B) Mean T3 concentrations (±SEM) are graphed according to exposure group. Note the difference in scale on the *y*-axis from A). T3 concentrations on PND21 were significantly reduced in the PCB and PCB+MeHg groups. * denotes a group that differs from controls at $p < 0.05$ and ** denotes a group that differs from controls at $p < 0.01$. *** denotes a group that differs from controls at $p < 0.001$.

ANOVA analysis and Bonferroni post hoc testing ($p < 0.05$), and individual '*p*-values' were determined by use of unpaired *t*-tests.

3. Results

3.1. PND21 thyroid hormone and Purkinje cell endpoints

3.1.1. Developmental outcomes and body weights

As previously published [84], there were no overt signs of clinical toxicity in the dams from any of the treatment groups. Dams in all exposure groups had gestational lengths, gestational weight gains, liver weights, litter sizes, percent male pups, and percent live births similar to controls. We observed a PCB-induced decrease in body weights in both the PCB and PCB+MeHg pups that began shortly after birth and persisted into adulthood. As adults MeHg exposure alone did not change body weight postnatally. MeHg-exposed rats weighed on average 7% less than controls, while PCB rats weighed on average 8% less and PCB+MeHg rats on average 11% less than controls.

3.1.2. Thyroid hormone assays

3.1.2.1. T4. There was a highly significant main effect of exposure for serum T4 concentrations [$F(3,20) = 93.601$, $p < 0.001$]. PCB exposure dramatically reduced T4 concentrations when measured on PND21 ($p < 0.001$ for both the PCB and PCB+MeHg groups) (Fig. 1A). In fact, two-thirds of the samples from PCB-exposed rats were below the limit of detection for the assay and were conservatively assigned the limit of detection (1×10^6 μg/dL) for analysis. MeHg exposure had no effect on T4 concentrations ($p = 0.948$).

3.1.2.2. T3. There was also a significant main effect of exposure for serum T3 concentrations [$F(3,16) = 4.126$, $p = 0.024$]. T3 concentrations were significantly reduced in the PCB ($p = 0.004$) and PCB+MeHg groups ($p = 0.026$) (Fig. 1B). PCBs alone caused a 24% reduction in T3 from controls, while PCB+MeHg caused an 18% reduction in T3. Comparison of Fig. 1A and B illustrates that T4 serum concentrations were more affected than T3 concentrations following PCB exposure. MeHg exposure had no effect on serum T3 concentrations ($p = 0.784$).

3.1.3. Purkinje cell height measurements

For males, there were marginally significant main effects of exposure for both Purkinje cell perpendicular [$F(3,15) = 3.158$, $p = 0.056$] and long-axis heights [$F(3,15) = 3.036$, $p = 0.062$]. All exposed males had slightly taller Purkinje cell dendritic trees than control males (Table 1), but none of the post hoc comparisons were significant. For females, the main effect of exposure was not significant for either perpendicular [$F(3,14) = 0.537$, $p = 0.664$] or long-axis height [$F(3,14) = 0.672$, $p = 0.583$] (Table 1).

Table 1
Purkinje cell height measurements and primary dendrite length are presented by exposure (means ± SEM)

Exposure	Perpendicular Height (mm)	Long-axis Height (mm)	Primary dendrite Length (mm)
Males			
Control	0.127 ± 0.004[a]	0.142 ± 0.005	0.021 ± 0.001[c]
MeHg	0.132 ± 0.003	0.149 ± 0.004	0.023 ± 0.001
PCB	0.136 ± 0.004	0.151 ± 0.004	0.024 ± 0.001
PCB+MeHg	0.131 ± 0.003	0.145 ± 0.002	0.024 ± 0.002
Females			
Control	0.128 ± 0.007[b]	0.138 ± 0.008	0.020 ± 0.001
MeHg	0.124 ± 0.004	0.141 ± 0.004	0.021 ± 0.001
PCB	0.131 ± 0.004	0.144 ± 0.003	0.021 ± 0.001
PCB+MeHg	0.129 ± 0.004	0.144 ± 0.004	0.021 ± 0.001

[a] For males, there were marginally significant main effects of exposure for both Purkinje cell perpendicular ($p = 0.056$) and long-axis heights ($p = 0.062$). All exposed males had slightly taller Purkinje cell dendritic trees than control males, but none of the post hoc comparisons were significant.

[b] For females, the main effect of exposure was not significant for either perpendicular or long-axis height.

[c] There were no significant exposure-related differences in primary dendrite length.

Table 2
Purkinje cell branching area, stained dendrite area, and percent of ectopic Purkinje cells are presented by exposure (means ± SEM)

Exposure	Purkinje Cell Branching area (μm²)	Stained Purkinje Cell dendritic area (μm²)	Ectopic Purkinje cells (%)
Males			
Control	13429.5 ± 817.7[a]	9170.7 ± 719.8	0.63 ± 0.63
MeHg	13852.6 ± 501.4	10129.9 ± 523.8	0.00 ± 0.00
PCB	12970.5 ± 627.2	9183.9 ± 703.7	0.85 ± 0.58
PCB + MeHg	12468.4 ± 726.3	8925.8 ± 195.0	0.77 ± 0.77
Females			
Control	11375.5 ± 1168.0	8684.4 ± 921.6	0.00 ± 0.00
MeHg	13081.5 ± 1053.4	9682.4 ± 858.4	0.29 ± 0.29
PCB	12354.9 ± 460.7	8575.4 ± 729.6	0.63 ± 0.63
PCB + MeHg	12375.9 ± 958.1	8582.7 ± 658.8	1.26 ± 0.92

[a] There were no significant exposure-related differences.

For both sexes, there were highly significant main effects of cerebellar area for both perpendicular ([$F(2,30)=28.314$, $p<0.001$] and [$F(2,28)=64.505$, $p<0.001$], for males and females, respectively) and long-axis heights ([$F(2,30)=18.484$, $p<0.001$] and [$F(2,28)=14.391$, $p<0.001$], for males and females, respectively). For both measurements, Purkinje cell heights significantly increased from anterior to posterior.

3.1.4. Purkinje cell primary dendrite length

The main effect of exposure on primary dendrite length was not significant for males [$F(3,15)=1.007$, $p=0.417$] or females [$F(3,14)=0.145$, $p=0.931$] (Table 1). For males, there were no other significant effects. For females, there was a significant three-way area by exposure by cohort interaction [$F(6,28)=3.863$, $p=0.006$]. However, the simple area by exposure interactions for each cohort were both non-significant.

3.1.5. Purkinje cell branching area and stained Purkinje cell dendrite area

For the Purkinje cell branching area and stained dendritic area, the main effects of exposure were not significant for the males ([$F(3,11)=0.357$, $p=0.785$] and [$F(3,11)=0.041$, $p=0.988$], respectively) or the females ([$F(3,13)=0.558$, $p=0.652$] and [$F(3,13)=0.399$, $p=0.756$], respectively) (Table 2). There were no significant exposure-related interactions and no non-exposure related significant effects.

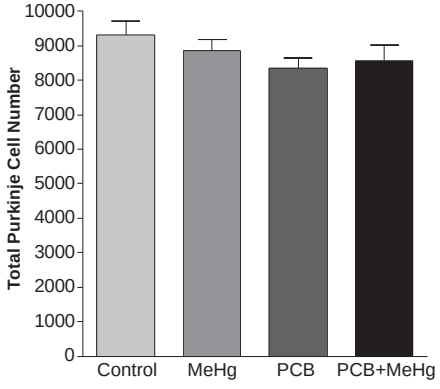

Fig. 2. The mean total number of Purkinje cells is graphed by exposure. Error bars denote the SEM. Although the main effect of exposure was not significant, all exposure groups seem to have a trend toward less Purkinje cells than controls.

3.1.6. Purkinje cell structural abnormality ratings

There were no significant differences in the percent of ectopic Purkinje cells among the groups, [$F(3,14)=0.509$, $p=0.682$] and [$F(3,12)=0.249$, $p=0.860$], for males and females, respectively (Table 2). Nor were there any group differences for the rest of the structural abnormalities that were examined.

3.2. Adult brains

3.2.1. Adult brain and cerebellar weights

PCB and/or MeHg exposure did not result in differences in adult brain [$F(3,28)=1.749$, $p=0.180$] or cerebellar [$F(3,28)=1.941$, $p=0.146$] weights. However, there were significant sex differences in the adult brain [$F(1,28)=94.797$, $p<0.001$] and cerebellar [$F(1,28)=66.683$, $p<0.001$] weights, with males having heavier whole brain (2.162 ± 0.017 vs. 1.986 ± 0.012 g) and cerebellar weights (0.553 ± 0.005 vs. 0.503 ± 0.004 g) than females.

3.2.2. Total Purkinje cell number

The main effect of exposure on total Purkinje cell number was not significant [$F(3,28)=0.215$, $p=0.885$] (Fig. 2). However, there was a trend for all exposed groups to have fewer Purkinje cells than controls. There was a significant exposure by cohort interaction for the total Purkinje cell number [$F(6,28)=2.476$, $p=0.048$]. However, the simple main effects of exposure were not significant for either cohort. The main effect of sex was marginally significant [$F(1,28)=3.891$, $p=0.058$], with females (8924.1 ± 225.0) having more Purkinje cells than males (8562.7 ± 260.7).

3.2.3. PML total and layer volumes

There were no significant exposure-related effects for total PML [$F(3,28)=0.210$, $p=0.888$], molecular layer [$F(3,28)=0.055$, $p=0.983$], granule cell layer [$F(3,28)=0.354$, $p=0.787$], or white matter layer [$F(3,28)=0.187$, $p=0.904$] volumes (Table 3).

3.2.4. Expression of RyR isoforms in PND63 cerebellum

We examined the level of RyR expression by Western blot analysis using two monoclonal antibodies (mAb). mAb 34C selectively binds to type 1 and type 3 isoforms (RyR1 and

Table 3
PML volumes (in mm³) are presented by exposure as means ± SEM

Exposure	Total PML Volume	Molecular layer Volume	Granule cell Layer volume	White matter Layer volume
Control	3.53 ± 0.17[a]	1.83 ± 0.10	1.53 ± 0.08	0.17 ± 0.01
MeHg	3.40 ± 0.13	1.75 ± 0.08	1.47 ± 0.05	0.17 ± 0.01
PCB	3.44 ± 0.13	1.76 ± 0.06	1.50 ± 0.05	0.17 ± 0.01
PCB + MeHg	3.47 ± 0.17	1.80 ± 0.09	1.51 ± 0.07	0.17 ± 0.01

[a] There were no significant exposure-related differences in PML volumes.

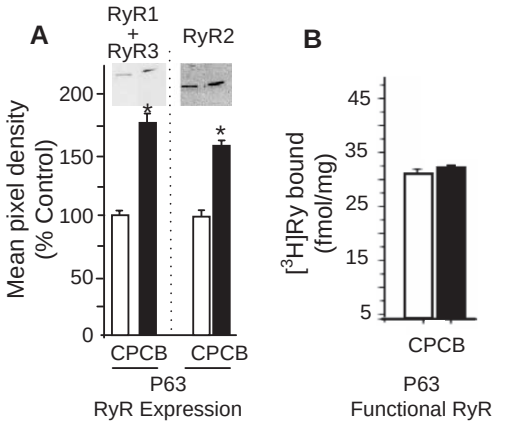

Fig. 3. Expression profiling of RyR isoforms by Western blot and [^{3}H] ryanodine binding analyses. Whole membrane fractions were prepared from P63 animals exposed to perinatal corn oil (C) or PCB (6 mg/kg/day) as described in Methods. (A) Membrane samples (10–50 μg/lane) were size separated by SDS-PAGE and Western blots probed with anti-RyR1/3 mAb 34C or anti-RyR2 mAb C3–33 (top panels show sample blots). Note that only expression of RyR1 was detected with 34C in all preparations. Normalized density data are plotted as the mean percent change relative to the density of the respective control group for RyR1 (left panel) and RyR2 (right panel) with combined SEM. Measurements were made from at least 4 blots with non-saturated signals as determined by loading multiple quantities of protein. Significance was tested by one-way ANOVA ($p<0.05$), followed by unpaired t-tests to determine individual p-values. (B) Specific binding of [^{3}H] ryanodine (5 nM) to rat whole particulate fraction isolated from cerebellum of corn oil control (white bar) and PCB (dark bar) exposed rats at PND63. Data is mean ± SD of $n=4$ replicates. Non-specific binding was measured in the presence of 10 μM unlabelled ryanodine.

RyR3) [2]. We found detectable levels of RyR1, whereas RyR3 was below detection limits in the membranes isolated from PND63 rats (Fig. 3A). At this age, the levels of RyR1 protein were significantly higher in membranes isolated from PCB-exposed rats compared to controls. mAb C3-33 selectively binds to the type 2 isoform (RyR2), and, similar to RyR1, was found to be up-regulated in PCB-exposed rats relative to the corn oil controls (Fig. 3A). We also measured the density of functional RyR channels in cerebellum preparations using [^{3}H] ryanodine binding analysis [77,98]. Fig. 3B shows that there is no significant difference in PND63 membranes isolated from control and PCB-exposed cerebella when measured at near-saturating (5 nM) radioligand.

4. Discussion

PCBs alone or in combination with MeHg caused significant reductions in the circulating T3 and T4 levels when measured on PND21. There were no statistically significant exposure-related effects on the Purkinje cell and volume measurements, but there were some non-significant trends in the data. In addition, there were two interesting effects not related to exposure. First, we observed a significant increase in Purkinje cell height across the cerebellum from the anterior to the posterior lobules, and second, we observed a nearly significant sex difference in Purkinje cell number, with females having more Purkinje cells than males. To our knowledge, there have been no previous reports regarding differences in Purkinje cell heights across the lobules of the cerebellum. We also found RyR dysregulation in adult rats exposed perinatally to PCBs.

4.1. Thyroid hormones

The reduction in circulating thyroid hormone concentrations (T3 and T4) on PND21 is in agreement with previous reports of developmental exposure to Aroclor 1254 [24,40,63,101]. Serum samples were not collected at later ages in this study, but circulating thyroid hormone concentrations have been shown to return to control levels by PND45 following developmental Aroclor 1254 exposure in rats [40].

Because PCBs dramatically reduced serum T4 to below the detection limit of the assay, it was impossible to determine whether combined exposure to PCBs and MeHg had any additional effect. However, it is unlikely that combined exposure would exacerbate the effect of PCBs on serum T4 concentrations, since the MeHg alone did not alter T4 concentrations, nor does the literature suggest it would. Both PCB and combined PCB and MeHg exposure significantly reduced serum T3 concentrations. However, the effect appeared to be somewhat larger with PCBs alone.

4.2. Purkinje cell height and primary dendrite length

PCB and/or MeHg exposure did not significantly alter Purkinje cell height, but we did observe a non-significant trend for PCB exposed rats to have slightly taller Purkinje cells, especially in the male rats. One study indicates that PCBs may have greater cerebellar effects in male rats than females [66]. Neonatal hyperthyroidism has been shown to make Purkinje cells taller yet narrower [67], so this may be an indication of a thyromimetic effect within the cerebellum following PCB exposure. Other studies [6,37,101] have also indicated that PCBs may mimic the effects of thyroid hormones in the brain. However, in vitro studies have shown that hydroxylated PCBs inhibit T4-dependent dendritic growth in Purkinje cells. Given that this effect was only marginally significant and was present only in the male rats, it should be interpreted with caution.

4.3. Purkinje cell branching area

PCB and/or MeHg exposure did not significantly change the total branching area or the stained dendritic area of the Purkinje cells. There was a slight increase in the percent of ectopic Purkinje cells in the PCB-exposed rats, but this was not a significant effect. A previous study [59] examined the cerebellum of males exposed to PCBs perinatally, and they also observed the occurrence of some ectopic Purkinje cells within the molecular layer (personal communication with Ron Mervis). In that study, male rats exposed to 6 mg/kg/d Aroclor 1254 from gestation day (GD) 6 to PND21 were reported to have a smaller Purkinje cell branching area on

PND22, which recovered by PND60 [59]. However, there were not any differences in the branching density of Purkinje cell dendrites using an ocular grid. There are several differences between the earlier study and the current study that could explain the differing results. Although both studies used the same dose of Aroclor 1254, the earlier study [59] used Sprague–Dawley rats, whereas the current study used Long–Evans rats. Also, the two studies differed in the timing of PCB exposure. In the current study PCB exposure began 4 weeks prior to breeding, while the previous study [59] began PCB exposure on GD6. Beginning PCB exposure prior to gestation may have allowed for protective mechanisms such as enzyme induction to be initiated, facilitating the metabolism and excretion of PCBs. This may have resulted in less toxicity than in the previous study [59] where PCB exposure began during gestation. Previously, we have observed impaired radial-arm maze performance if exposure began on GD6 [83] but not if exposure began prior to breeding (Medora et al., unpublished data).

4.4. Adult brain and cerebellar weights

Both neonatal hypo- and hyperthyroidism can reduce cerebellar weight [58,68]. Although PCBs dramatically reduced T4, PCB exposure did not reduce adult brain or cerebellar weight in this study. PCB exposure did significantly reduce pup brain weights on PND21, and a recent study reports reduced cerebellar mass in rat neonates exposed to a slightly higher dose of A1254 [66]. PCB exposure did cause a significant decrease in body weight that persisted into adulthood, and similar weight deficits have been reported previously [40]. However, the body weight decrease was only about 10%, which is considerably less than that typically observed with neonatal hypothyroidism [21].

4.5. Total Purkinje cell number in adult rats

Neither neonatal hypo- nor hyperthyroidism results in changes in Purkinje cell number [18,67]. In addition, two studies of MeHg exposure found no changes in Purkinje cell number [55,88]. Thus, it is not surprising that PCB and MeHg exposure did not reduce Purkinje cell number.

There was a nearly significant sex difference in Purkinje cell number with females having approximately 4% more Purkinje cells than males. Previous reports of significant sex differences in Purkinje cell number have found that males have 7–8% more Purkinje cells than females [30,100], but these sex differences were from across the whole cerebellum and not just one specific lobule. Although the adult female rats had significantly smaller whole brain weights and cerebellar weights, they did not have smaller PML total or layer volumes than the males. Thus, it seems that the sex difference in cerebellar weight is attributable to some part of the cerebellum other than the PML, and within the same approximate PML volume, the number of Purkinje cells in females was increased compared to adult male rats.

4.6. PML total and layer volumes in adult rats

MeHg exposure has been shown to reduce the thickness of the granular and molecular layers in mice following a single 4 mg dose [88]. The lack of a similar effect in this study could be due to the fact that the estimated total dose of MeHg was only around 1.20 mg, and that rats were used rather than mice. It is also possible that PCB and/or MeHg exposure changes the volumes of other cerebellar lobules. However, the PML, which is important for limb control [19,89], seemed the most obvious area of the cerebellum to investigate given the rotating rod deficits.

4.7. RyR expression and binding

RyR1 and RyR2 protein was significantly upregulated in the cerebellum of PCB-exposed rats on PND63. However, the density of specific high-affinity [^{3}H]Ry binding sites, a measure of the density of functional RyR channels [76,77], in the cerebellum remained unchanged compared to controls. These data suggest that changes in RyR expression may represent a transcriptional response to compensate for loss of functional RyR protein. Previously, we have shown that the single PCB congener 95 significantly increased [^{3}H] ryanodine binding in PND181 cerebella following an 8 mg/kg/d dose given gestational days 10–16 in Sprague–Dawley rats, while the higher dose of 32 mg/kg/d had no significant cerebellar effects [91]. The differences between studies could be due to age, rat strain, or the use of a single congener vs. a PCB mixture.

Considering RyR channels are highly regulated by non-genetic [75] and transcriptional mechanisms, including thyroid hormone [45], one plausible explanation of our current results is that by PND63 dynamic regulation has restored functional RyR protein to control level. However, given the critical contributions made by RyRs to neurodevelopment and plasticity, it is possible that the compensated CNS may be changed in subtle ways that alter behavior [84,91,99] but eluded our morphological analysis in the present study. Clearly, more work is needed understand how RyR dysregulation relates to neurodevelopmental perturbations.

4.8. General conclusions

We previously tested rats exposed to PCBs and/or MeHg during development on three motor tests-vertical rope climb, traversing parallel bars, and crossing the rotating rod [84]. We observed no significant deficits following PCB and/or MeHg exposure on the rope climb or parallel bars tasks, but combined exposure to PCBs and MeHg caused significant rotating rod deficits [84]. The present study set out to investigate possible underlying sources of the rotating rod impairments. However, we did not observe any changes in Purkinje cells or PML volume that would explain the rotating rod deficit. We did observe RyR dysregulation in the cerebellum of PCB-exposed rats on PND63.

As for the lack morphological findings, it is possible that our Golgi studies did not have enough statistical power due to

the small number of rats representing each group. Also, this was not an exhaustive study of cerebellar morphology. Purkinje cell dendritic spine density and morphology were not examined, which are important for the functioning of Purkinje cells [56]. In addition, other measures of dendritic plasticity have not been evaluated. There were no changes in granule cell layer volume in the PML, but we do not know if there are changes in granule cell number, density, or function. Further, there could be changes in cerebellar function that were not detected with these methodologies, such as changes in neurotransmission.

It has been recently reported that combined exposure to PCBs and MeHg in vitro in cerebellar granule cells causes synergistic and antagonistic effects on calcium release [8], and these interactive effects may be occurring at the RyR. In addition, a study in cultured Purkinje cells suggests that Ca^{+2} release from internal stores, particularly from ryanodine-sensitive stores, is necessary for the induction of long-term depression (LTD) within the cerebellum [52], and LTD has been proposed as a possible cellular substrate of motor learning [44]. Thus, if combined PCB and MeHg exposure results in inactivation of RyR calcium release channel, then this would likely impair LTD induction within the cerebellum as well as motor learning, including rotating rod performance.

It is possible that we did not observe any morphological changes to the cerebellum because it is not the site of action for the rotating rod deficits. The rotating rod is a complex task that requires both fine motor coordination and precise postural control [3]. To successfully cross the rotating rod, the rats must detect the speed of rotation of the rod and adapt, which involves both somatomotor and vestibular components. So, despite previous reports implicating the cerebellum in rotating rod deficits [14,50,51,73], we cannot rule out the possibility that PCB and MeHg exposure damages the somatosensory or vestibular systems. PCB exposure has complex effects on the thyroid hormone system. Some PCB effects resemble neonatal hypothyroidism, while other effects indicate that PCBs may be thyromimetic. Table 4 compares the currently known effects of PCB exposure to those of neonatal hypo- and hyperthyroidism. The vestibular system is a likely target of PCBs given that the vestibular apparatus is dependent on thyroid hormones for normal development [9,27,28,90]. Perinatal hypothyroidism results in immature vestibular ganglia lacking myelin sheaths around the cell soma, delays in type I hair cell innervation, and persistent immature synaptic structures within the type I hair cells [28]. PCB-induced reductions in circulating thyroid hormones are known to damage cochlear hair cells and hearing in rats [25,39]. Thus, reductions in circulating thyroid hormones following PCB exposure could also damage the peripheral vestibular system, including the type I hair cells.

It is difficult to compare the doses used in laboratory rats to the exposure levels observed in humans. In humans, serum PCB concentrations are known, but the daily intake resulting in those concentrations can only be estimated. Conversely, in

Table 4
Comparison of effects of developmental PCB exposure with those of neonatal hypo- and hyperthyroidism

Endpoint	Developmental PCB exposure	Neonatal Hypothyroidism	Neonatal Hyperthyroidism
Serum T3 and T4	↓[a] [c.s.[b]], [24,29,40,63,101]	↓ [11,49,93]	-[11], ↑ [94]
Serum TSH	-[10,53]	↑ [4,49,93]	↓ [94]
Dam gestational weight gain	-[84]	-[93]	
Litter size	-[84]	↓[4,11,34]	-[11,34], ↓[11]
Pup body weight	↓, 10% [84]	↓, 25–50% [4,11,58,65,81,93] [5,81,85,94]	-[11,57], ↓ ↑ [11]
Brain weight	↓ [84]	↓ [65]	-[57], ↓ [5]
Cerebellar weight	-[c.s.]	↓ [58,65,68]	↓ [5]
Eye opening accelerated	-[24,70] Accelerated [10,39,40]	Delayed [4,11,93]	[5,11,57,85]
Cochlear structure and hearing	Damaged [25,39]	Damaged [9,61,60]	Damaged [13,36]
Vestibular apparatus		Damaged [1,9,27,28,61,60,90]	
Adult testis size	↑ [22]	↑ [20,21,49]	
RC3/neurogranin expression	↑ [37,101]	↓ [43]	
Cerebellar MBP expression	↑ [101]	↓ [35,42]	Transient ↑ [35]
NSP-A expression	↑ [37]	↑ [31]	
Oct-1 expression	↑ [37]		
Cerebellar calcineurin	↑ Females only [64]	↑ [86]	
HES mRNA in fetal cortex	↑ [6]	-[6]	↑ [6]
Basal forebrain ChAT[c]	-[29], ↓ [46,78]	↓ [71]	↑ [69]
Hippocampal ChAT	↓ [29,46,78]	↓ [71]	↑ [69]
Purkinje cell height	Slight ↑ males only [c.s.]	↓ [62,67]	↑ [67]
Purkinje cell primary dendrite length	-[c.s.]	↑ [23,80,82]	
Purkinje cell dendritic branching area	-[c.s], ↓ males only [59]	↓ [62]	↓ [67]
Purkinje cell number	-[c.s.]	-[18,67]	-[67]

[a] Symbols used: ↑ indicates an increase, ↓ indicates a decrease, and - indicates no change.
[b] c.s. stands for current study.
[c] ChAT stands for choline acetyltransferase.

animal models exposure levels are accurately known, but serum concentrations are generally lacking. The doses of PCBs used in this and most other animal studies would likely result in body burdens above the high end of what can be found in environmentally exposed humans. Nevertheless, animal studies have provided valuable information about the types of cognitive and behavioral effects can be expected in environmentally exposed humans (e.g. [87]). The MeHg dose used in this study would likely also result in a MeHg exposure that is higher than that observed in exposed human populations. However, as mentioned previously, the estimated total MeHg dose in this study is lower than those used in most other animal studies. Further, it is important to understand the effects of co-exposure to PCBs and MeHg because they are widespread contaminants found in the same food sources (i.e. fish and seafood products), and there have been recent reports that co-exposure may result in interactive effects [7,8,41,84,92].

In conclusion, in a previous study we found that combined PCB and MeHg exposure during development significantly impaired rotating rod performance, but here we report that changes in Purkinje cells or cerebellar structure do not seem explain the deficit. Alternative hypotheses, such as changes in intracellular calcium signaling due to RyR dysregulation and/or vestibular system development, should be investigated.

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