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Comparative analyses of the 12 most abundant PCB congeners detected in human maternal serum for activity at the thyroid hormone receptor and ryanodine receptor

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Supporting Information:

Detailed methods of PCB synthesis and thyroid hormone receptor (THR) activity assays using transiently transfected GH3 cells, NMR spectra of individual PCB congeners (24 figures), supplemental THR activity data (5 figures), and references.

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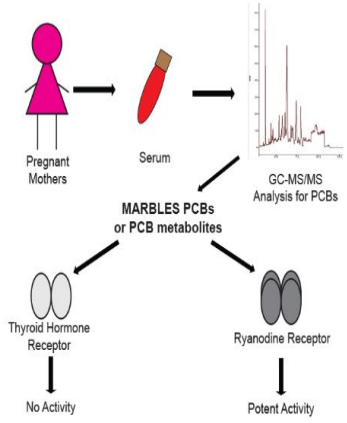


Keywords: In vitro toxicology; MARBLES; neurotoxicology; PCBs; thyroid hormone receptor; risk assessment; ryanodine receptor

Abstract

Polychlorinated biphenyls (PCBs) pose significant risk to the developing human brain; however, mechanisms of PCB developmental neurotoxicity (DNT) remain controversial. Two widely posited mechanisms are tested here using PCBs identified in pregnant women in the MARBLES cohort who are at increased risk for having a child with a neurodevelopmental disorder (NDD). As determined by gas chromatography-triple quadruple mass spectrometry, the mean PCB levels in maternal serum was 2.22 ng/mL. The 12 most abundant PCBs were tested singly and as a mixture mimicking the congener profile in maternal serum for activity at the thyroid hormone receptor (THR) and ryanodine receptor (RyR). Neither the mixture nor the individual congeners (2 fM to 2 μM) exhibited agonistic or antagonistic activity in a THR reporter cell line. However, as determined by equilibrium binding of [^{3}H]ryanodine to RyR1-enriched microsomes, the mixture and the individual congeners (50 nM to 50 μM) increased RyR activity by 2.4-19.2-fold. 4-Hydroxyl (OH) and 4-sulfate metabolites of PCBs 11 and 52 had no TH activity; but 4-OH PCB 52 had higher potency than the parent congener towards RyR. These data support evidence implicating RyRs as targets in environmentally-triggered NDDs and suggest that PCB effects on the THR are not a predominant mechanism driving PCB DNT. These findings provide scientific rationale regarding a point of departure for quantitative risk assessment of PCB DNT, and identify *in vitro* assays for screening other environmental pollutants for DNT potential.

Introduction

Polychlorinated biphenyls (PCBs) are a class of persistent organic pollutants that were synthesized for multiple industrial and commercial applications. Despite the worldwide ban on their production in the early 2000's, there continues to be widespread human exposure to PCBs,[1, 2] including women of child-bearing age and children.[3, 4] An important health concern for human PCB exposure is developmental neurotoxicity (DNT). The evidence from epidemiological studies and animal studies strongly supports the contention that PCBs are developmental neurotoxicants.[5-7] Emerging epidemiological evidence suggests that PCBs increase risk for neurodevelopmental disorders (NDDs), including autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD).[8-10] The mechanism(s) by which PCBs disrupt neurodevelopment remain controversial. Two prevailing hypotheses include disruption of thyroid hormone (TH) signaling[11-13] and modulation of calcium signaling in developing neurons.[14-16]

The scientific premise underlying the hypothesis that PCBs cause developmental neurotoxicity by interfering with the thyroid system is that TH signaling is critical for normal neurodevelopment. Congenital hypothyroidism, if untreated, causes severe adverse neurodevelopmental outcomes, [17-19] and while conflicting, there is some evidence that TH insufficiency may increase the risk of ASD[20, 21] and is related to ADHD.[22, 23] Thus, there is apparent overlap of neurodevelopmental deficits related to TH insufficiency and developmental PCB exposure. In addition, multiple studies have demonstrated that developmental PCB exposures decrease serum T4 levels in preclinical models,[24-28] although these relationships are less consistent in humans.[29, 30] Collectively, these observations have led many to posit that disruption of TH signaling contributes to neuropsychological deficits associated with developmental PCB exposures.[11-13] However, this hypothesis is not supported by all studies. Outcomes of studies exploring relationships between PCB exposures and serum thyroid hormone levels are inconsistent and reviews of these studies generally do not support the concept that PCBs affect neurodevelopment by reducing serum thyroid levels.[26] An alternative hypothesis is that individual PCB congeners or their metabolites interfere with thyroid hormone receptors, but these data are also inconsistent.[31, 32]

Calcium signaling is also critical to normal neurodevelopment and synaptic plasticity.[33-35] Of the various mechanisms implicated in PCB effects on calcium signaling, the most sensitive is ryanodine receptor (RyR) sensitization.[16, 36] The RyR is a Ca^{2+}-regulated Ca^{2+} ion channel localized to the endoplasmic reticulum, and RyR sensitization by a subset of non-dioxin-like (NDL) PCBs stabilizes the ion channel in its open configuration, which increases release of Ca^{2+} from intracellular stores.[16, 37] PCB effects on RyR are causally linked to enhanced dendritic arborization *in vitro* via activation of Ca^{2+}-dependent signaling pathways that normally function to link neuronal activity to dendritic growth.[38, 39] Effects of RyR-active PCBs on dendritic growth and plasticity have been confirmed *in vivo*,[25, 38] and shown to coincide with deficits in cognitive behaviors.[25]

The relative contribution of THR *vs*. RyR-dependent mechanisms to PCB DNT remains an outstanding question in the field, and one that has been a point of discussion in terms of risk assessment. Multiple groups have argued for the need for risk assessment guidelines other than dioxin toxic equivalents or total sum PCBs, and the proposal to integrate either changes in TH signaling and/or RyR activity is a recurring theme.[16, 40, 41] This debate is driven in part by the fact that only a small subset of individual PCB congeners have been tested for THR and RyR activity, and the relevance of the congeners that have been tested to current human exposures is uncertain. To address this controversy, we sought to identify PCBs relevant to contemporary human exposures, and to examine the influence of not only the individual congeners but also a mixture on THR and RyR activity. To this end, we leveraged samples from the Markers of Autism Risk in Babies-Learning Early Signs (MARBLES) study. MARBLES is a prospective study of pregnant women in northern California at increased risk for having a child with a NDD.[42] The congener profile of PCBs in serum from pregnant women in the MARBLES cohort was determined, and these data were used to develop a mixture, referred to as the MARBLES mix, comprised of the 12 most abundant congeners in maternal serum. This mixture, as well as the individual congeners comprising the mixture and metabolites of two of the congeners in the MARBLES mix, PCB 11 and PCB 52, were tested for agonistic and antagonistic activity at the THR using a luciferase reporter cell line,[43, 44] and for activity at the RyR1, as determined by radiolabeled ryanodine (Ry) binding studies.[45, 46]

Materials and Methods

Materials

Triiodo-L-thryonine (T3) and L-thyroxine (T4) were purchased from Sigma-Aldrich (≥ 95%; St. Louis, MO). PCB 11, 28, 52, 84, 95, 101, 118, 135, 138, 149, 153 and 180, as well as the hydroxylated and sulfated metabolites of PCB 11 and 52, were synthesized and authenticated by the Synthesis Core of the University of Iowa Superfund Research Program (The University of Iowa, Iowa City, IA). Synthesis methods for all congeners are detailed in the supplemental material except for PCB 11 and its metabolites, which have been reported previously.[47-49] All synthesized PCBs were > 99% pure as determined by ^{1}H-NMR, ^{13}C-NMR, and GC-MS (Supplemental Material). THR antagonist, NH-3, was synthesized by Dr. Heike Wulff (University of California Davis, Davis, CA) as previously described.[50] All stock solutions for *in vitro* experiments were made in dry sterile dimethylsulfoxide (DMSO, Sigma-Aldrich). PCB standards (PCB 11, 28, 52, 77, 84, 91, 95, 101, 118, 131, 132, 135, 136, 138, 149, 153, 174, 175, 176, 180, and 196) for the analysis of PCBs in maternal serum were purchased from AccuStandard, Inc. (New Haven, CT, USA). Stock solutions for analytical techniques were made in isooctane (Thermo Scientific, Waltham, MA).

Analysis of PCBs in maternal serum

Human maternal plasma samples (n = 241) were obtained from the MARBLES study at the University of California, Davis.[42] Women recruited into the MARBLES study lived within a 2.5 hour drive of Sacramento, CA, were currently pregnant, and had a biological child diagnosed with ASD, which significantly increased their risk for having a second child with a NDD. All maternal blood samples were collected into sodium citrate Vacutainer® tubes post venipuncture. Whole blood samples were processed within 12 h of collection to separate plasma, which was stored at -80 °C until thawed on ice for PCB analysis.

All PCB analyses were conducted using a validated standard operating procedure to extract, separate, and detect PCBs by gas chromatography coupled with triple quadruple mass spectrometry (GC/MS/MS, Scion TQ triple quadruple mass spectrometer, Bruker, Fremont, CA, USA). The detailed description for sample preparation and GC-MS/MS parameters is reported elsewhere.[51] In summary, 500 µL of plasma were extracted by solid phase extraction, further purified by silica cartridges and analyzed for PCB 11, 28, 52, 77, 84, 91, 95, 101, 118, 131, 132, 135, 136, 138, 149, 153, 174, 175, 176, 180, and 196. $^{13}C_{12}$ labeled PCB 97 was used as surrogate internal standard throughout the extraction and analytical procedures (Cambridge Isotope Laboratories, Inc, Tewksbury, MA, USA). Another internal standard, Mirex, was added after extraction to monitor any shifts in instrument performance during analysis. Standard reference material (SRM1957) was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). Human control serum (DDC Mass Spect Gold®, MSG 3000, Golden West Biologicals, InC. Temecula, CA, USA) was fortified with all PCB standard solutions (AccuStandard, Inc., New Haven, CT, USA) at concentrations of 0.1, 0.8 and 4 ng/mL for quality control (QC) purposes. The analytical laboratory participates in the Arctic Monitoring and Assessment Program (also known as AMAP Ring Test of Persistent Organic Pollutants in Human Serum) [52] for PCB analyses with z-scores between -2< Z'-score < 2. Limit of quantification (LOQ) and limit of detection (LOD) were determined by the concentrations where the signal to noise ratios were 10: 1 and 3:1, correspondingly. For concentrations below LOQ, but above LOD, we used the reported values provided by the GC-MS/MS analysis software, Bruker MSWS 8.0.1 (Bruker, CA, U.S.A.). For statistical analysis, a non-detected congener was assigned a value of the corresponding LOD divided by $\sqrt{2}$. PCBs were then ranked for abundance in maternal serum and the top 12 most abundant congeners were selected to comprise the MARBLES mix (Table 2).

Analyses of TH activity

GH3.TRE-Luc[43] cells, which were generously provided by Dr. J. David Furlow (University of California, Davis, CA) were derived from a pituitary tumor of a 7-month-old female rat. These cells were grown in DMEM/F12 (Thermo Scientific) supplemented with 10% fetal bovine serum (GIBCO/Thermo

Scientific) in T75 flasks (Thermo Scientific) at 37 ºC in a 5% CO_2 humidified incubator. GH3.TRE-Luc cells tested negative for mycoplasma at an early and late passage as determined using the MycoAlert™ Plus Mycoplasma Detection Kit (Lonza, Basel, Switzerland). GH3.TRE-Luc cells were passaged every 4-5 days at a 1/8 split. All experiments were performed using cells between passage numbers 5-12. To assess the activity of PCBs at the THR, a 24-well plate format assay was used.[44] GH3.TRE-Luc cells were trypsinized and plated in 24-well cell culture plates (Thermo Scientific) at a density of 150,000 cells/well in DMEM/F12 supplemented with 10% FBS. Cells were rinsed with phosphate buffered saline (PBS) 24 h after plating, and media was changed to serum-free PCM medium.[53] At 24 h after the PCM medium change, medium was exchanged to either PCM + vehicle (0.1% DMSO), or PCM + PCB (1:1000 dilution from stocks) in the absence or presence of T3 (0.2 nM) or T4 (2 nM). We included T4 in this experiment because it is converted to T3 by the type 1 deiodinase (Dio1) in these cells prior to its action. Therefore, if PCB congeners interfered with Dio1, it would be revealed if a PCB congener suppressed THR activation in response to T4 but not T3. The MARBLES mix was prepared by creating 10 millimolar (mM) stocks of each individual congener and then mixing the appropriate volume of each individual congener to approximate proportions found in the maternal serum resulting in a 10 mM stock of the MARBLES mix that was then serially diluted for experimentation. The MARBLES mix was tested at concentrations ranging from 2 femtomolar (fM) to 2 micromolar (μM). Individual PCB congeners were tested at 10 nanomolar (nM), 100 nM and 1 μM. After a 24 h exposure, cells were washed with PBS and lysed with 100 μL of Reporter Lysis Buffer (Promega, Madison, WI). Plates containing the lysed cells were immediately frozen at -80 ºC. Lysate was then thawed to room temperature, and 5 μl of lysate was combined with 20 μl of Luciferase assay reagent (Promega). Luciferase activity was measured in a Synergy H1 hybrid microplate reader (BioTek Instruments, Winooski, VT), and normalized to lysate protein concentrations determined using a BCA assay (Thermo Scientific). To account for plate-to-plate variability, each sample was normalized to the vehicle control wells within that plate. Each duplicate assay was independently repeated 4-6 times.

Analyses of RyR activity

RyR1-enriched microsomal membrane fractions were prepared from rabbit skeletal muscle by differential centrifugation.[54] Equilibrium binding of [^{3}H]ryanodine ([^{3}H]Ry; 56.6 Ci/mmol; Perkin Elmer Life, Bellerica, MA) to microsomes (0.05 mg/mL) was measured in tightly sealed test tubes at 37 °C after 3 h with constant shaking in binding buffer consisting of 2nM [^{3}H]Ry (in mM), 250 KCl, 14 NaCl, 20 HEPES, pH 7.4, and 2 μM free Ca^{2+} (obtained by the addition of EGTA calculated according to the software Bound-and-Determined 6).[55] DMSO was used as a vehicle at final concentrations ≤1% and had no influence on basal RyR1 activity.[56, 57] Non-specific [^{3}H]Ry binding was measured as the residual binding measured in the presence of a 1,000-fold excess of unlabeled ryanodine.[54] Each radioligand–receptor binding experiment was performed in triplicate and repeated 2-5 times on separate days. The EC_{50} and maximal activation parameters were determined by nonlinear regression equations using Origin 9.1 (OriginLab). The equations used for the best fit are as follows:

Sigmoidal Logistic: $$y = \frac{A_1 - A_2}{1 + (X - X_0)^p} + A_2$$

Sigmoidal Boltzmann: $$y = \frac{A_1 - A_2}{1 + e^{(X - X_0)/\Delta X}} + A_2$$

Where y is the variable corresponds to the bound [^{3}H]Ry (fold increase); A_1= initial value (Bound); A_2 = final value (Bound); X = the independent variable (total concentration of PCB congener(s)); X_o = center; p = power; ΔX = slope at X_o.

Results and Discussion

Concentration of PCBs in serum from MARBLES Mothers

Mass spectrometry was used to analyze PCB content in plasma samples collected from women enrolled in the MARBLES study at UC Davis.[42] MARBLES is a longitudinal study of pregnant women

who have a biological child diagnosed with ASD, and thus are at increased risk for having a second child diagnosed with a NDD. PCBs were detected in all of the 241 samples that were analyzed. The sum total PCBs present in human maternal serum samples ranged from 0.74 ng/mL to 12.58 ng/mL with a mean value of 2.22 ng/mL (Table 1). The top 12 congeners in order from most abundant to least abundant was: PCB 28, 11, 118, 101, 52, 153, 180, 149, 138, 84, 135, and 95. The relative proportion of the top 12 congeners that comprised the MARBLES mix is listed in Table 2 (% of Σ_{12}PCBs).

Table 1. Total sum PCBs detected in serum from women enrolled in the MARBLES study

Sample Type	Minimum	25th Percentile	Mean	75th Percentile	Maximum
Maternal Plasma	0.74 ng/mL	1.66 ng/mL	2.22 ng/mL	2.37 ng/mL	12.58 ng/mL

N = 241 serum samples from 126 women at varying stages of pregnancy ranging from 1st to 3rd trimester

Table 2. The 12 most abundant congeners detected in serum of MARBLES subjects

PCB Congener	Proportion (%) of the sum of the 12 most abundant PCB congeners in maternal serum
PCB 28	48.2
PCB 11	24.3
PCB 118	4.9
PCB 101	4.5
PCB 52	4.5
PCB 153	3.1
PCB 180	2.8
PCB 149	2.0
PCB 138	1.7
PCB 84	1.5
PCB 135	1.3
PCB 95	1.2

A simplified mixture comprised of the 12 most abundant PCB congeners identified in the MARBLES samples was chosen for THR and RyR activity assays in part to avoid confounding by non-PCB contaminants present in Aroclors, and because the two most abundant congeners, PCB 28 and PCB 11, which together comprised almost 75% of the PCB burden in the maternal serum samples, are not present in the Aroclors.[43] The caveats of this approach include the lack of consideration of enantiomeric enrichment and the possibility that congeners important in PCB DNT may have been overlooked. Moreover, the MARBLES mixture is representative of a specific at-risk population in northern California, and other PCB profiles may be present in other populations, or even of individuals within any given population. However, an independent study that assessed all 209 PCB congeners in samples obtained from mothers and their children living in Midwestern United States detected the 12 congeners included in the MARBLES mix at relatively high frequencies,[4, 58] thus, the 12 congeners included in the MARBLES mix are not unique to the MARBLES cohort.

A key observation from the analyses of the MARBLES samples was the abundance of the lightly chlorinated congeners, PCB 11 and PCB 28. This observation raises questions regarding the common practice of using PCB 153 as a general marker for overall PCB exposure and for comparison of PCB burdens amongst various populations.[59] PCB 153 was among the 12 most abundant congeners detected in the pregnant women assessed in our study; however, PCB 28 and PCB 11 were present at quantities 16 and 8 times greater than that of PCB 153, respectively (Table 2). One other study has similarly demonstrated an abundance of lower chlorinated PCBs in serum samples from mothers and their children.[3] In light of recent reports indicating the potential for PCB 11 to interfere with neuronal morphogenesis,[60, 61] these observations warrant rethinking of traditional exposure assessment strategies to quantify lower chlorinated congeners in addition to PCB 153 in human biomonitoring studies.

The MARBLES mix, its individual congeners and metabolites lack activity at the THR

We first tested whether the MARBLES mix possesses agonistic activity at the THR using a sensitive THR reporter cell line[43] that expresses a luciferase reporter gene under the regulation of thyroid

hormone response elements. Exposure to T3 (0.2 nM) significantly increased luciferase activity as expected; however, the MARBLES mix had no effect on luciferase activity at concentrations ranging from 2 fM to 2 µM (Figure 1A). Since TH effects can vary depending on the PCB congener,[62] we next tested each of the 12 PCB congeners making up the MARBLES mix individually. None of the 12 individual congeners displayed agonistic activity at the THR at any concentration tested (10 nM, 100 nM and 1 µM) (Figure 1B-E).

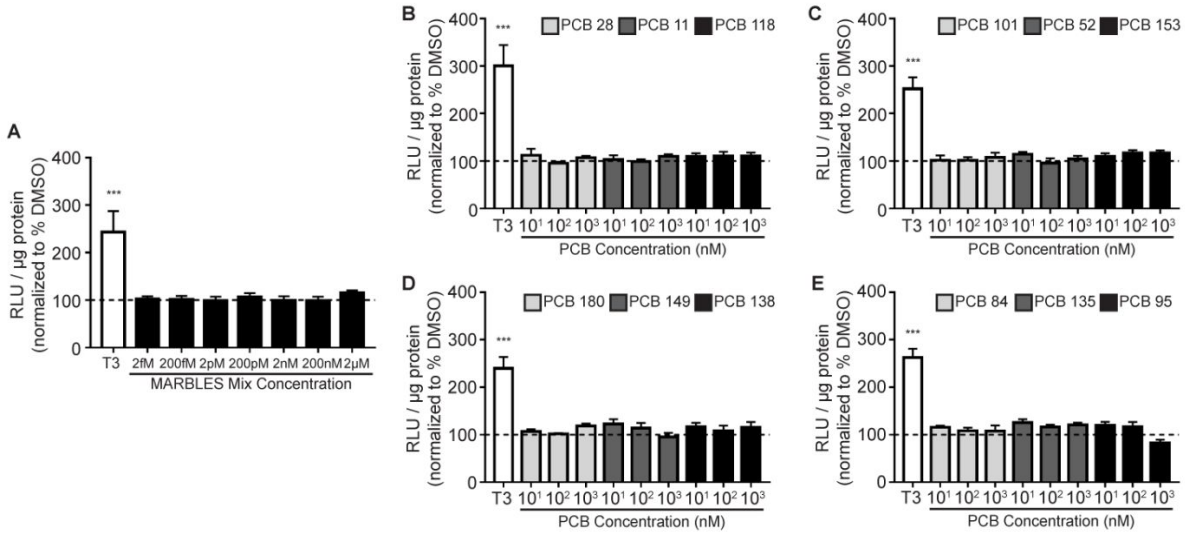


Figure 1. The MARBLES mix and its individual components do not activate the THR. Luciferase activity was measured in GH3.TRE-Luc cells treated with either T3 (0.2 nM) or (A) MARBLES mix, (B) PCB 28, 11, 118, (C) PCB 101, 52, 153, (D) PCB 180, 149, 138, or (E) PCB 84, 135, 95. Luciferase activity is expressed as relative light units (RLU) normalized to total protein concentration in the same sample. Data presented as the mean ± SE (n = 5-6 independent experiments). *Significantly different from vehicle (0.1% DMSO) control at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined using a one-way ANOVA ($p < 0.05$) followed by a Holm-Sidak's multiple comparisons test.

PCBs have also been reported to block THR signaling,[11] so GH3.TRE cells were exposed to the MARBLES mix in the presence of T3 (0.2 nM) or T4 (2 nM) to test for antagonism of THR signaling. Co-exposure to the MARBLES mix had no effect on T3- (Figure 2A) or T4- (Figure 3A) induced

luciferase activity. Similarly, none of the 12 congeners of the MARBLES mix blocked the luciferase activity induced by T3 (Figure 2B-E) or T4 (Figure 3B-E) when tested individually. Co-treatment with NH-3, a THR antagonist,[50] decreased the T3 and T4 responses to levels that were not significantly different from vehicle controls (Figures 2F and 3F).

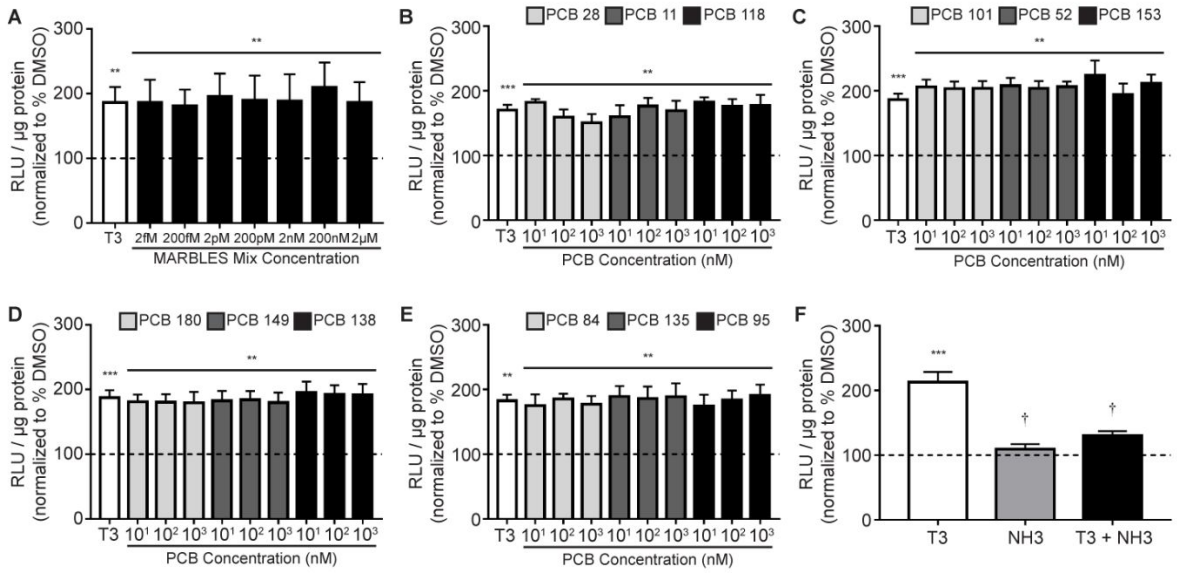
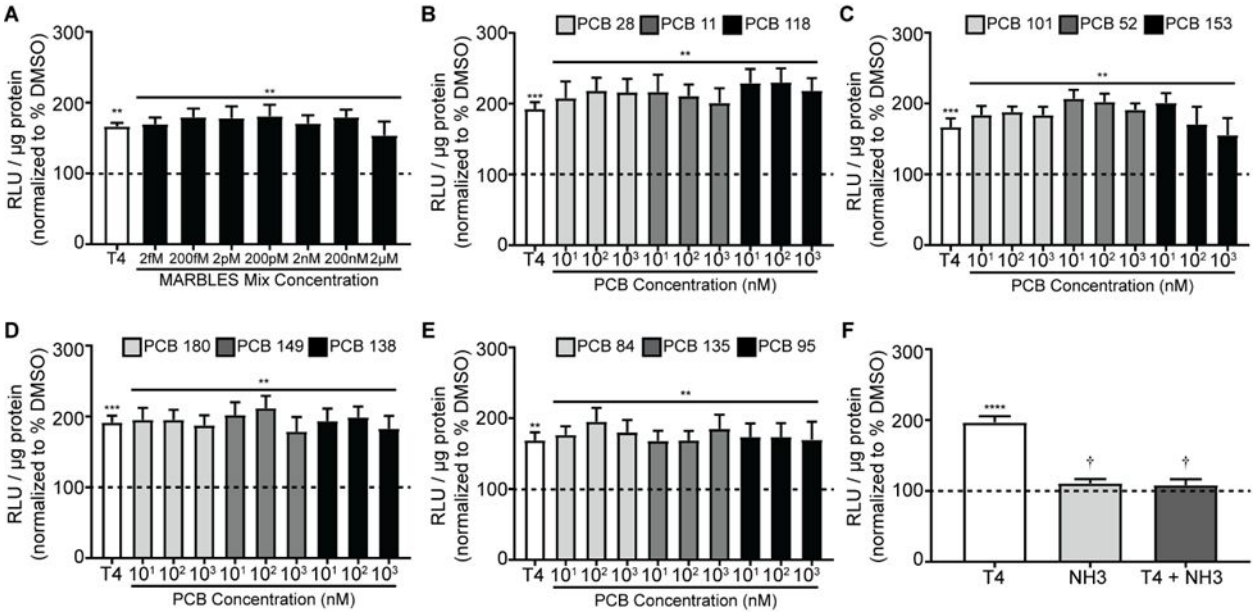


Figure 2. The MARBLES mix and its individual congeners do not block T3 activation of the THR. Luciferase activity was measured in GH3.TRE-Luc cells treated with T3 (0.2 nM) in the absence or presence of the MARBLES mix **(A)** or the individual congeners in the mix **(B-E)**. **(F)** Luciferase activity in cells treated with T3 (0.2 nM) in the absence or presence of the THR blocker, NH-3 (100 pM). Luciferase activity is expressed as relative light units (RLU) normalized to total protein concentration. Data presented as the mean ± SE (n = 6 independent experiments). *Significantly different from vehicle (0.1% DMSO) at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, †Significantly different from T3 at $p < 0.01$ as determined using a one-way ANOVA ($p < 0.05$) and *post hoc* Holm-Sidak's multiple comparisons test.



289 **Figure 3.** The MARBLES mix and its individual components do not antagonize T4-induced THR
290 signaling. Luciferase activity was measured in GH3.TRE-Luc cells treated with T4 (2 nM) in the absence
291 or presence of the MARBLES mix **(A)** or the individual congeners in the mix (**B-E**). **(F)** Luciferase
292 activity in cells treated with T4 (2 nM) in the absence or presence of the THR blocker, NH-3 (100 nM).
293 Luciferase activity is expressed as relative light units (RLU) normalized to total protein concentration of
294 each sample. Data presented as the mean ± SE (n = 5-6 independent experiments). *Significantly different
295 from vehicle (0.1% DMSO) at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, †Significantly different from T4 at p
296 < 0.0001 as determined using a one-way ANOVA ($p < 0.05$) and *post hoc* Holm-Sidak's multiple
297 comparisons test.
298
299 The lack of effect of the MARBLES mix and its individual congeners on THR-dependent
300 signaling is consistent with an earlier study that also failed to detect a direct interaction between PCBs
301 and the THR.[31] More recent screening studies using reporter cell lines that express only the THR alpha
302 isoform, including one study of 25 individual PCBs that included 5 of the PCBs we examined here,[63] and
303 another study of lower chlorinated PCBs,[64] also reported no agonistic effects of PCBs. These findings
304 from THR reporter assays are at odds with the observation that PCB 118 mimics TH in an

oligodendrocyte differentiation assay.[62] These discrepancies may reflect cell-context differences; however, the possibility that the effects of PCB 118 on oligodendrocyte differentiation are mediated by THR-independent mechanisms cannot be ruled out because early in development, there is a THR-independent pathway to oligodendrocyte differentiation.[65]

Our observations that neither the MARBLES mix nor the individual congeners block T3 or T4 induced THR activity are inconsistent with previous reports that PCBs interfere with THR-mediated signaling.[32, 66] This may reflect the fact that these prior results were obtained using cell lines that expressed only the THR beta 1 isoform, in contrast to the GH3.TRE-Luc cell line that expresses THR alpha1, THR beta1 and THR beta 2, as well as their heterodimer partners and respective cofactors.[67] Interestingly, in the studies that used the THR beta 1-expressing cell lines, THR activity was significantly suppressed not by the parent PCBs, but by their corresponding hydroxyl metabolites (OH-PCBs).[32, 66] In contrast, other studies using the GH3 cell line detected agonistic activity of OH-PCBs at the THR.[68, 69] While the reason for the lack of consistent outcomes in tests of OH-PCB activity at the THR remains unknown, these observations suggest that metabolism may contribute to the influences of PCBs on TH signaling. Consistent with this suggestion, more pronounced effects of PCBs on TH signaling during development were observed in animals exposed to a PCB congener that upregulated the cytochrome P450 enzymes that metabolize PCBs.[70]

To determine whether human-relevant metabolites of the PCB congeners in the MARBLES mixture had activity at the THR, we tested hydroxylated and sulfated metabolites of PCB 11 and 52 using the GH3.TRE-Luc cell line. While metabolites of eight (PCB 11, 28, 52, 101, 118, 138, 153, 180)[3, 71-73] of the twelve PCB congeners in the MARBLES mix have been detected in human serum, we were only able to obtain purified metabolites for PCB 11 and 52. Neither the 4-hydroxy nor 4-sulfate metabolites of PCB 11 or PCB 52 exhibited agonistic (Figure 4A) or antagonistic (Figure 4B-C) activity at the THR. In a separate set of studies we screened the parent, 4-OH and 4-sulfate metabolites of PCBs 3, 8, 11 and 52 at concentrations ranging from 10 pM – 10 μM using a different model: GH3 cells transiently transfected with a THR reporter construct (see Supplemental materials for detailed description). Consistent with

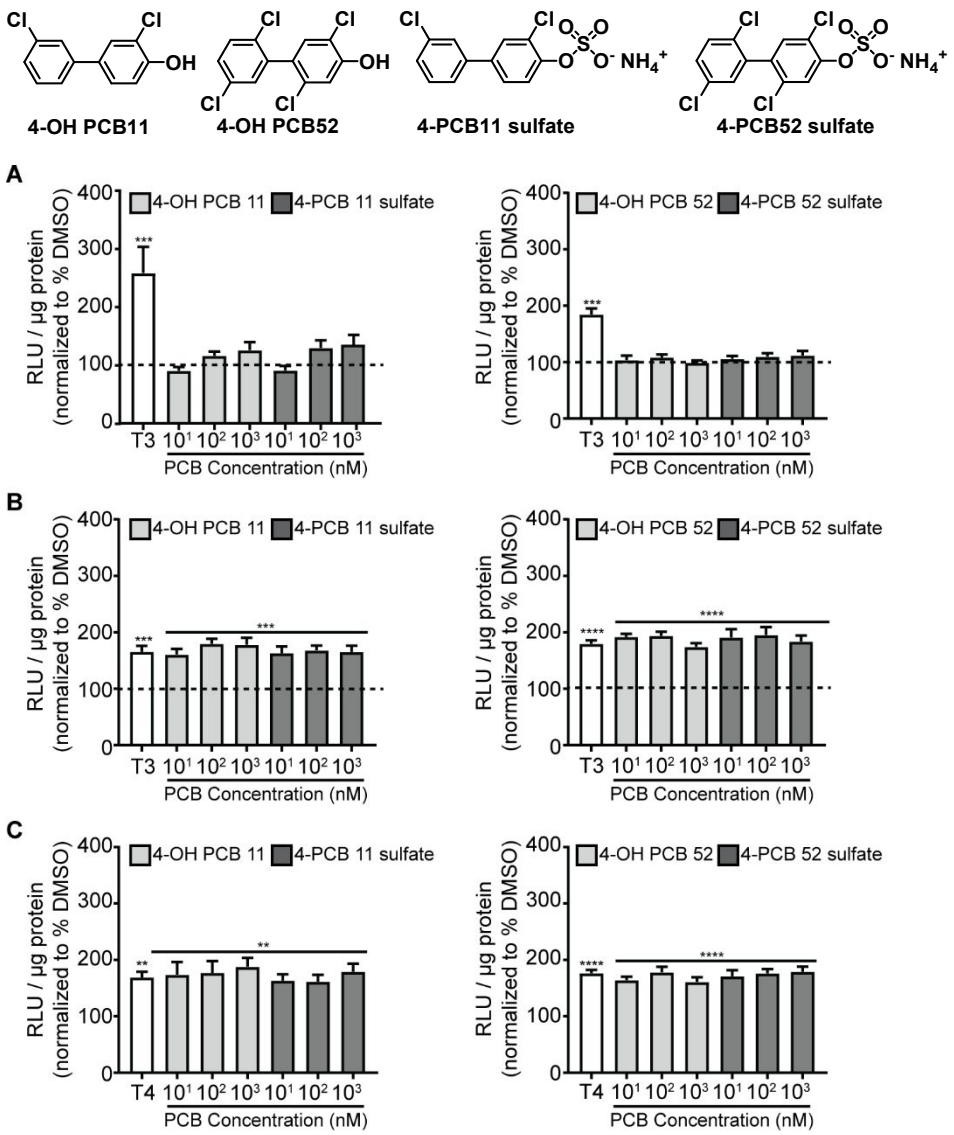


Figure 4. PCB 11 and 52 metabolites exhibit no agonistic or antagonistic activity at the THR. **(A)** To test for THR agonism, luciferase activity was measured in GH3.TRE-Luc cells treated with either T3 (0.2 nM) or metabolites of PCB 11 or 52. **(B-C)** To test for THR antagonism, luciferase activity was measured in cells treated with 0.2 nM T3 (B) or 2 nM T4 (C) in the absence or presence of PCB 11 or 52 metabolites. Luciferase activity is expressed as relative light units (RLU) normalized to total protein concentration. Data presented as the mean ± SE (n = 4-6 independent experiments). *Significantly different from vehicle (0.1% DMSO) at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined using one-way ANOVA ($p < 0.05$) with *post hoc* Holm-Sidak's multiple comparisons test.

findings generated using the GH3.TRE-Luc cell line, at concentrations ≤ 1 μM, none of these PCB congeners (parents or metabolites) had agonistic or antagonistic effects on THR activity (Supplemental materials, Figures S26-S29). At 10 μM, PCB 11, 4-OH PCB 3, PCB 3 sulfate and PCB 8 sulfate significantly altered THR activity, with PCB 11 inhibiting T3 activation of THR, 4-OH PCB 3 increasing THR activity in the absence or presence of T3 or T4, and the 4-sulfate metabolites of both PCB 3 and PCB 8 increasing THR activity in the presence of T4 (Supplemental materials, Figures S26-S28). The relevance of these observations is not clear since 10 μM is significantly higher than PCB concentrations documented in human tissues.

Our current data do not support the hypothesis that PCBs in combination or individually interact with the THR in a manner that could explain PCB developmental neurotoxicity. Although GH3 cells cannot be considered a model of all neuronal cell types, there is sufficient evidence in the literature at large to indicate that THRs are not obvious targets of PCB congeners of relevance to contemporary human exposures. However, these data do not preclude the possibility that PCBs or their metabolites interfere with the delivery of T3 to target cells during critical windows of neurodevelopment.[74]

MARBLES mix and each of its individual congeners stabilize the open state of RyR1

As previously reported, RyR channels are a direct and highly sensitive target of PCBs,[16, 36] and RyR sensitization by PCBs has been linked to adverse effects on neuronal Ca^{2+} dynamics that alter dendritic arborization.[25, 38, 54] Here, we report the structure-activity relationship of the 12 PCB congeners comprising the MARBLES mix using high affinity [^{3}H]Ry binding to RyR1-enriched microsomal preparations as a quantitative biochemical indicator of PCB-induced modification of channel conformations previously demonstrated in muscle and brain.[46, 75] Figure 5 shows that in the presence of the MARBLES mix (Fig 5A) or individual PCB congeners (Fig 5 B-E), specific [^{3}H]Ry binding increased in a concentration-dependent manner. The MARBLES mix had an EC_{50} = 4.46 ± 0.88 μM, reaching a maximal efficacy of 3.96 ± 0.25-fold increase from the baseline of control (Figure 5A). Among the 12

individual congeners, PCB 95, PCB 135, and PCB 149 were the most efficacious of the parent congeners, enhancing [^{3}H]Ry binding ~12-19 fold over baseline (Figure 5D, E). Although PCB 135 and PCB 149 showed slightly higher efficacy than PCB 95 (54% and 15% more efficacious; $p<0.001$ and 0.05, respectively; using one-way ANOVA followed by Tukey means comparison test) under the experimental conditions used, they are 5- and 3-fold less potent than PCB 95, respectively (Figure 5E; Table 3).

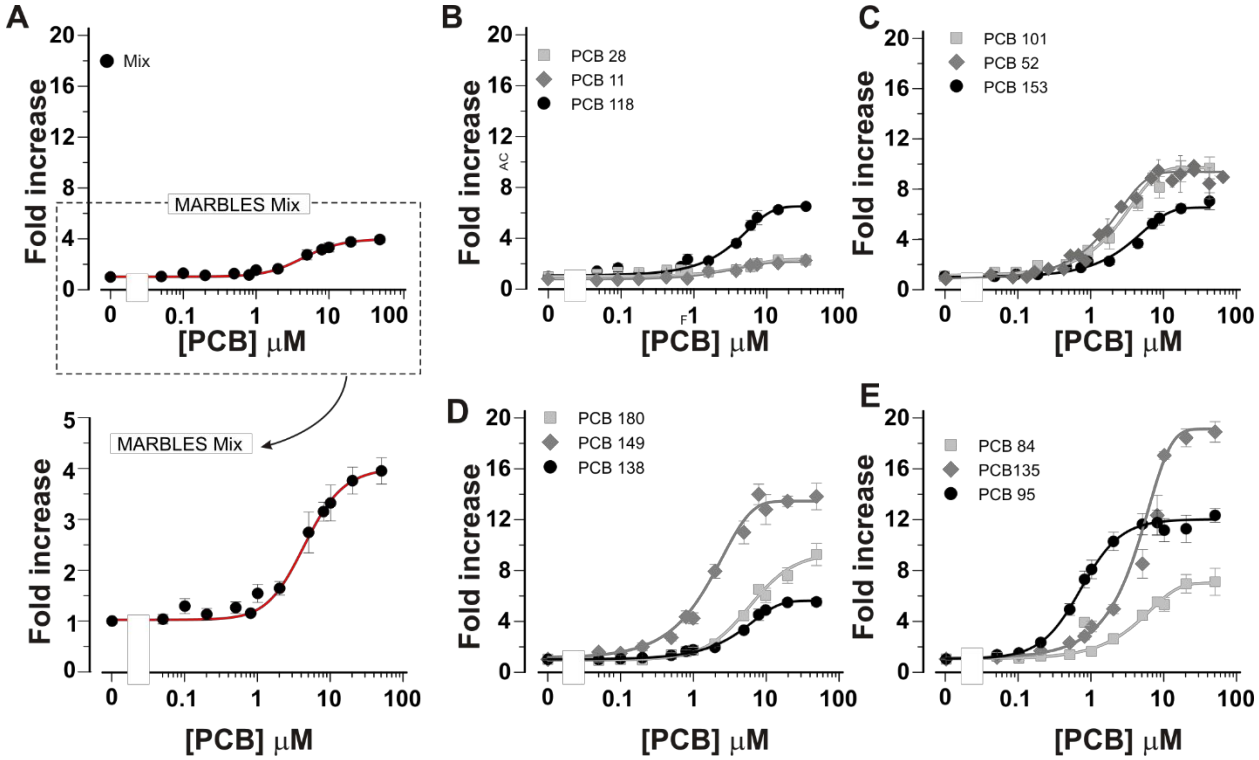


Figure 5. The MARBLES mix and its 12 individual congeners allosterically enhance the binding of [^{3}H]Ry to RyR1 in its high affinity open conformational state. Specific [^{3}H]Ry binding in the presence of the MARBLES mix (A), individual PCB congeners (B-E) or vehicle (DMSO, 1%). DMSO concentrations in PCB samples varied from 1% to 0.2%. Data was plotted as fold increase from respective baseline binding. Baseline binding in the presence of DMSO (1%) averaged 0.16±0.06 pmol/mg protein across experimental trials. Each data point represents the mean ± SE of triplicate determinations repeated 2-5 times under identical experiment conditions. Concentration-effect data were fitted using nonlinear regression Sigmoidal Boltzmann or Logistic with Origin 9.1, and the latter was used to obtain EC_{50} values and their maximal activation levels (a measure of efficacy), which is summarized in Table 3.

Table 3. Summary of EC_{50} values and maximal activation of [^{3}H]ryanodine ([^{3}H]Ry) binding to the RyR1 as fold increase from the baseline by the MARBLES mix and its 12 individual congeners.

PCB Congener(s)	[^{3}H]Ry Binding	
	EC_{50} (μM)	$Activity_{max}$ (Fold Control)
MARBLES mix	4.5 ± 0.9	4.0 ± 0.2
PCB 28	7.1 ± 5.2	2.4 ± 0.8
PCB 11	5.1 ± 6.1	2.1 ± 0.2
4-OH PCB 11	ND	ND
4-PCB 11 sulfate	ND	ND
PCB 118	5.2 ± 1.8	6.8 ± 0.1
PCB 101	4.7 ± 3.3	6.5 ± 0.6
PCB 52	1.9 ± 0.6	9.4 ± 0.3
4-OH PCB 52	0.6 ± 0.2	9.4 ± 0.2
4-PCB 52 sulfate	17.3 ± 6.6	2.4 ± 0.3
PCB 153	3.0 ± 1.3	9.7 ± 0.7
PCB 180	6.0 ± 1.4	9.4 ± 1.1
PCB 149	2.2 ± 2.5	13.8 ± 2.0
PCB 138	4.9 ± 1.4	5.6 ± 2.2
PCB 84	5.3 ± 0.9	7.0 ± 2.4
PCB 135	3.6 ± 0.7	19.2 ± 0.8
PCB 95	0.7 ± 0.2	12.0 ± 3.8

ND, Not significantly different from DMSO control up to 50 μM.

In our previous studies, we found negligible RyR activity of highly purified PCB 11[37, 76]. However, due to recent appreciation of the relatively high volatility of lightly chlorinated PCBs, including PCB 11,[77-79] we reassessed the activity of PCB 11 and tested its 4-hydroxy and 4-sulfated metabolites of PCB 11 using tightly sealed test tubes to minimize loss through air-water repartitioning. Using this experimental approach, PCB 11 showed consistent activity toward RyR1 with an EC_{50} of 5.1 μM and efficacy 2-fold that of the DMSO baseline (Figs 5B; Fig 6A; Table 3). Moreover, neither 4-OH nor PCB

11 sulfate had any detectable activity at the maximum concentration tested (50 μM) (Fig 6A; Table 3). We believe the divergent results from earlier studies were caused by the loss of PCB 11 from the aqueous assay buffer over the 3 hr incubation at 37ºC, as predicted by its high Henry's Law Constant compared to higher chlorinated PCBs.[80] Likewise, lower chlorinated PCB 28 was also active towards RyR1, reaching 2.4-fold increase over baseline and an EC_{50} = 7.1 ± 5.2 μM. Importantly, as determined using a previously described model,[37] the activity of the MARBLES mix at the RyR is consistent with a purely additive model in which the sum of the relative RyR activity of the individual congeners predicts the RyR activity of the mixture.

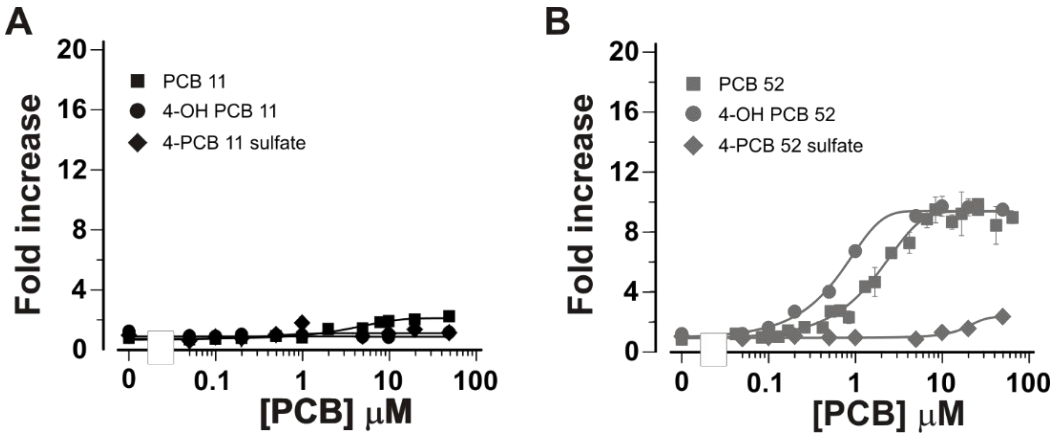


Figure 6: The parent congener and 4-OH and sulfate metabolites of PCB 11 (A) and PCB 52 (B) were tested using [^{3}H]Ry binding analysis as described in Figure 5. Data represent 12 replicates performed on at least 4 independent measurements and are plotted as fold-increase from DMSO control. Baseline binding average is 0.14±0.01pmol/mg protein.)

In contrast, 4-OH PCB 52 exceeded the potency of PCB 52 by ~3-fold (EC_{50} 0.6 μM; p<0.01) without change in efficacy (Fig 6B; Table 3). PCB 52 sulfate showed much lower potency and efficacy than its parent PCB (EC_{50} 17.3 μM; and $Activity_{max}$ 2.4-fold of DMSO baseline; p<0.001; Fig 6B and Table 3). These results reaffirm the activity of lightly chlorinated PCBs and the importance of the bulk of *para*-substitutions in determining both potency and efficacy of more highly chlorinated PCBs.[37, 81] We

acknowledge that EC_{50} values reported from [3H]Ry binding analysis are higher than the highest concentration tested in the THR cell reporter assay. Based on alternative electrophysiological methods we have previously used to investigate direct modulation of RyR1 single channel gating kinetics by PCBs, we concluded that [^{3}H]Ry binding assays likely underestimate the true potency of active PCBs due to the high lipid content of the microsomes used to measure equilibrium receptor binding.[37] Partitioning of PCBs with the lipid phase during the 3 h incubation undoubtedly reduces the free PCB concentration available to interact with binding sites on the RyR.

The two major genetic RyR isoforms, RyR1 and RyR2, have been established as among the most sensitive targets of NDL-PCBs both *in vivo*[25, 82, 83] and *in vitro*.[37, 38, 84] Samso and coworkers provided direct evidence that PCB 95 interacts with RyR1 using two complementary approaches: (1) single channel voltage clamp, and (2) cyroEM reconstruction of open and closed conformations of RyR1 at ~10 angstrom resolution in the presence and absence of PCB 95.[85] Interactions of NDL-PCBs with RyRs meet several criteria for specificity, including a stringent structure-activity relationship where potency and efficacy vary with the position and degree of chlorination.[16] Atropisomers of chiral PCB 136 and PCB 95 have clearly shown consistent stereoselectivity with higher activity for the (-)-atropisomer.[54, 86] Predictions from biochemical and biophysical studies of stereoselectivity of PCB 136 were largely validated by their stereoselective influences on dendritic arborization in primary neuronal cell cultures.[39] Moreover, the RyR is necessary for the effects of PCBs on neuronal connectivity in vitro.[25, 38, 84] Changes in either expression and/or activity of RyRs *in vivo* have also been associated with deficits in the development of primary auditory cortex[82] and deficits in spatial memory the Morris water maze, the latter indicative of cognitive deficits.[25, 87] Interestingly, PCB-associated deficits in the Morris water maze correlated more closely with PCB effects on RyR expression and activity in the brain than with changes in circulating TH levels.[25] A novel finding in this study was the high efficacies of PCB 135 and PCB 149 towards RyR1, as well as the relative potency of PCB 52 compared to its known metabolite 4-OH-PCB 52, which not only maintained high efficacy but ~3-fold greater potency. The fact that the MARBLES mix is dominated by lightly chlorinated PCBs from contemporary sources, exemplified by PCB 11 and

461 PCB 28, and that this complex mixture maintains significant RyR1 activity warrant the further study of
462 how complex environmentally relevant mixtures and their constituent congeners modify
463 neurodevelopmental profiles and behavioral outcomes.

464 To our knowledge, this study is the first to test for direct effects on THR and RyR1 activities of a
465 PCB mixture representative of PCBs in pregnant women at increased risk for NDDs. The relevance of
466 these findings to human health is underscored by recent epidemiologic studies linking PCBs to increased
467 risk for NDDs, including ADHD and ASD.[8-10] While altered TH signaling has been hypothesized to play
468 a role in ADHD and ASD, the human data are conflicting.[20-23] In contrast, heritable mutations in Ca^{2+}
469 signaling are strongly associated with increased risk of NDDs.[16, 36, 88] While a better understanding of the
470 PCB exposure profile, including metabolites, within the developing brain is needed to truly understand
471 the relevance of outcomes from THR and RyR assays, collectively, these observations, together with the
472 findings reported herein, argue that the RyR activity of at least some PCB congeners is more important
473 than their THR activity as a point of departure for risk assessments of the developmental neurotoxicity of
474 human relevant PCB mixtures.

475

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484

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