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Investigating the Cellular and Molecular Mechanisms By Which Lower-Chlorinated Polychlorinated Biphenyls (LC-PCBs) and Their Human-Relevant Metabolites Alter Neurodevelopment Using Primary Rat Neuron-Glia Cocultures

By

JESSIE RENEE BADLEY

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACOLOGY AND TOXICOLOGY

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Pamela Lein, Chair

Allison Ehrlich

Erkin Seker

Committee in Charge

2025

DEDICATION

This work is dedicated to those who have carried me through this journey.

To my Nana: My best friend. Your unwavering love, wisdom, and steady belief in me even when I doubt myself is the only reason I am alive today.

To my siblings: Ruby, thank you for the countless ways you've supported, encouraged, and reminded me that I could keep going. If all I do is be a positive guide in your life, I've succeeded. Hank, I can't believe you are not here to celebrate this, but I like to think you've been with me every step of the way, guiding me to the finish line.

To Dave: Your unexpected presence in my life has guided me in the most positive direction. I am me because of you and I wish you were here to celebrate our victory.

To my close friends and chosen family: you know who you are, thank you for understanding when I disappeared into my work, cried for days straight, for celebrating every small victory, and for lifting me up when the road felt long. I appreciate and love you all so much.

And most especially, to Boscoe, my most loyal companion throughout this crazy life: You finally got your honorary DOGtorate.

#HOODGIRLRISING

ABSTRACT

Polychlorinated biphenyls (PCBs) are persistent environmental pollutants that continue to pose a significant threat to human health despite being banned from production in most regions since the early 2000s. Human exposure occurs not only through legacy PCBs present in commercial mixtures but also through lower-chlorinated (LC) PCBs generated as inadvertent byproducts of modern manufacturing. A major target of concern is the developing brain, where epidemiological and experimental evidence link PCB exposure to deficits across multiple neurobehavioral domains, including cognition, attention, executive function, social behavior, and traits associated with attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), affective disorders, and neurodegeneration. However, much of the existing research has focused on higher-chlorinated (HC) congeners, leaving critical gaps in our understanding of the developmental neurotoxicity (DNT) of LC-PCBs that are increasingly detected in contemporary human tissues, including the neonatal brain.

To address this gap, this dissertation investigated the DNT potential of LC-PCBs and their metabolites using primary sex-segregated rat cortical neuron-astrocyte co-cultures. In Chapter 2, we characterized the effects of PCB 37, demonstrating that environmentally relevant concentrations elicited sex-, age-, and concentration-dependent increases in apoptosis, axonal growth, and dendritic growth without compromising cell viability. Mechanistic analysis revealed that the apoptotic and dendrite-promoting effects of PCB 37 were mediated through CREB signaling, while effects on axonal growth proceeded through CREB-independent pathways. These findings establish PCB 37 as a potential developmental neurotoxicant and highlight CREB as a convergent signaling mechanism underlying PCB-induced neurotoxicity.

In Chapter 3, we extended this work to compare the effects of PCB 2, PCB 3, PCB 28, and their human-relevant metabolites. We found that LC-PCBs and metabolites produced

congener-, structure-, and sex-specific effects on apoptosis and neuronal morphology at concentrations that did not impair viability. PCB 2 and its metabolite 4-OH PCB 2 significantly altered caspase activity and induced opposing effects on axonal and dendritic growth in male versus female neurons. PCB 3 was largely inactive, although its metabolite 3-OH PCB 3 increased apoptosis and decreased axonal growth in male cultures. PCB 28 exerted the most pronounced effects, enhancing axonal growth in both sexes while reducing dendritic complexity in female cultures, whereas hydroxylated and sulfated PCB 28 metabolites decreased both axonal and dendritic growth in a sex-dependent manner. Collectively, these findings emphasize that developmental neurotoxicity of LC-PCBs is highly structure-dependent, that metabolism can qualitatively shift neurotoxic potential, and that sex differences are consistent and biologically meaningful modifiers of response.

Overall, this work demonstrates that LC-PCBs and their metabolites disrupt key processes in neuronal development, providing mechanistic evidence that supports their classification as potential developmental neurotoxicants. This dissertation advances our understanding of how contemporary PCB exposures may contribute to neurodevelopmental disorders. These findings highlight the importance of considering chemical structure, metabolic transformation, and sex as critical determinants in risk assessment and regulatory frameworks for PCBs.

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I would first like to express my deepest gratitude to my mentor, Dr. Pamela Lein: I often struggle to find words that fully capture the love and appreciation I have for you. You took a chance on me when I joined your lab-I came in rough around the edges, with strong technical experience but little direction, and you welcomed me with open arms. What first drew me to you was not only your passion for research, but also your unwavering commitment to mentorship. You have created a lab environment where PhD students, Master's students, undergraduates, and postdocs alike thrive, and I truly believe the productivity and quality of science in the Lein Lab stems from the happiness and support you cultivate. Thank you for always meeting me where I was, for never giving up on me, and for giving me the space to grow. Thank you for listening with patience on difficult days, for allowing me to be vulnerable, and for being one of the most positive female presences in my life. Over the past five years, I have grown not only as a scientist but also as a person because of your mentorship. I will forever be grateful that you believed in me when I needed it most. I have so much love and respect for you, PJL- and I hope you know you are stuck with me for life (sorry, not sorry).

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Chapter 1 Introduction

Adapted from the published book chapter:

1. Badley JR, Wilson RJ, Werner AS, Smiley-Jewell SM, Lein PJ (2023) Chapter 2: The neurotoxicity of polychlorinated biphenyls (PCBs). In: Advances in Neurotoxicology: Neurotoxicity of Halogenated Organic Compounds, volume 10. (Edited by M Aschner, LG Costa and PRS Kodavanti). Elsevier, Amsterdam, Netherlands, pp. 27-79.

Abstract: Polychlorinated biphenyls (PCBs) are pervasive environmental pollutants. Despite having been banned from production in most parts of the world since the early 2000's, PCBs continue to pose a significant risk to human health. Humans are exposed via diet and inhalation to not only legacy PCBs from commercial mixtures synthesized prior to the ban on PCB production, but also PCBs created as inadvertent byproducts of contemporary manufacturing processes. A major target of concern for PCBs is the developing brain; however, adverse effects on the adult and aging brain have also been reported. In this chapter, we summarize epidemiological and experimental animal studies of early-life and mid-life exposures to PCBs, focusing on outcomes across domains related to cognition (e.g., IQ, language, memory, learning), attention, behavioral regulation and executive function, and social behavior, as well as traits related to attention deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), affective disorders and neurodegenerative disease.

Keywords: ADHD, affective disorders, autism, behavior, cognition, epidemiology, neurodegenerative disorders, neurodevelopmental disorders, Parkinson's disease

INTRODUCTION

Epidemiologic (Schantz, Widholm et al. 2003, Boucher, Muckle et al. 2009, Berghuis, Bos et al. 2015, Pessah, Lein et al. 2019) and experimental animal (Ulbrich and Stahlmann 2004, Sable and Schantz 2006, Klocke and Lein 2020) studies have identified the brain as a vulnerable target of polychlorinated biphenyls (PCBs). Much of the scientific literature on PCB neurotoxicity describes the developmental neurotoxicity of PCBs (Figure 1), which are among the most extensively studied developmental neurotoxicants of the nonmetallic persistent organic pollutants (POPs). Both human and experimental animal studies of early-life exposures to PCBs have documented adverse neurodevelopmental outcomes across domains related to cognition (e.g., IQ, language, memory, learning), attention, behavioral regulation and executive function, and social behavior, including traits related to attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) (Carlson, Christensen et al. 2023). While our knowledge of how early or mid-life exposures to PCBs influence the adult brain is not as comprehensive, there is evidence linking PCBs to increased risk of affective disorders and neurodegenerative outcomes, and this literature is also addressed in this chapter.

PCBs are a class of synthetic organic halogenated compounds that were originally manufactured and marketed in the United States (U.S.) from the late 1920's through the late 1970's under the product name "Aroclor" and in Europe until the early 2000's under trade name "Clophen". Because of their incredibly stable chemical properties and ability to dissipate heat, PCBs were used as plasticizers, lubricants, and coolants in many different products and applications, including electrical equipment, hydraulic fluids, printing inks and dyes, building materials like caulking compounds and paints, and fluorescent light ballasts. Unfortunately, the chemical stability that made PCBs so useful also rendered them resistant to environmental and metabolic degradation. These properties underlie the ubiquitous environmental distribution and bioaccumulation of PCBs, which have raised global concerns about adverse health

consequences of widespread human exposures (Domingo and Bocio 2007, Stahl, Snyder et al. 2009, Faroon and Ruiz 2016). These concerns led to restrictions on the production of PCBs imposed by many governments through the Stockholm Convention on POPs signed in 2001 and appended in 2008 and 2014 to include the goal of eliminating the use of existing PCB-containing equipment by 2025 (Guida, Capella et al. 2020). While environmental levels of "legacy" PCBs have declined following these regulatory efforts, legacy PCBs are still routinely detected in the environment and in human tissue. In addition, humans are exposed to PCBs that were not manufactured as Aroclors, but rather are inadvertent byproducts of contemporary pigment production processes (Hornbuckle 2022).

PCBs are a complex mixture of congeners that differ in the number and position of chlorine substituents on the biphenyl backbone (Figure 2). PCB congeners are categorically divided into either lower-chlorinated PCBs (LC-PCBs), which have $\leq$ 4 chlorine substituents, or higher-chlorinated PCBs (HC-PCBs), which contain > 4 chlorine substituents. LC-PCBs, which are the predominant congener type produced inadvertently during pigment production, are less stable, more volatile and more quickly metabolized than HC-PCBs (Li, Hefti et al. 2022). The structure of PCBs influences their biological activity. PCBs with zero chlorines in the *ortho* position assume a coplanar biphenyl orientation in solution, while PCBs with one to four chlorines in the *ortho* position assume increasing degrees of noncoplanar ring orientation (Figure 1). Coplanar congeners can bind to and activate the aryl hydrocarbon receptor (AhR) to cause toxic effects similar to those observed for dioxins; hence, coplanar congeners are known as "dioxin-like" PCB congeners. Of the 209 possible PCB congeners, only 12 are classified as dioxin-like. While low-level exposures to dioxin-like PCBs are associated with a number of adverse outcomes in several organ systems, especially liver, skin, and immune function (Bock 2016, Mellor, Steinmetz et al. 2016, Wheeler, Rothhammer et al. 2017), and are probable carcinogens (Lauby-Secretan, Loomis et al. 2013), there is less evidence from epidemiologic

and experimental animal studies that they are associated with neurotoxic outcomes (Pessah, Lein et al. 2019). In contrast, non-coplanar PCBs have little to no AhR activity and are thus “non-dioxin-like”. Historically, non-dioxin-like PCBs were considered to be biologically inert. However, pioneering research by Richard Seegal of the New York State Department of Health and Prasad Kodavanti and Hugh Tilson from the U.S. Environmental Protection Agency revealed that non-dioxin-like PCBs had considerable biological activity independent of the AhR (Panesar, Wilson et al. 2022). Non-dioxin-like congeners make up most contemporary human PCB exposures, and there is extensive scientific evidence implicating these congeners as predominantly responsible for the developmental neurotoxicity associated with PCB exposures (Klocke and Lein 2020, Montano, Pironti et al. 2022).

Humans are exposed to PCBs via consumption of contaminated foods and inhalation of contaminated air (Figure 3), although exposure via dermal absorption is also possible, particularly in occupational settings. While the consumption of high-fat foods, predominantly meat, fish and dairy products, has long been considered the predominant route of human exposure to PCBs, there is increasing appreciation of the importance of inhalation (Grimm, Hu et al. 2015). Volatilization of PCBs from building materials such as caulking, paints, and varnishes, as well as leaking of fluids from PCB-containing fluorescent light ballasts, can result in significant indoor air levels of PCBs (Hornbuckle 2022). Air sampling from schools built or remodeled prior to the PCB production ban has detected PCB levels far exceeding those measured near some of the largest PCB-contaminated Superfund sites in the U.S. (Hornbuckle 2022). Recent findings from the AESOP (Airborne Exposure to Semivolatile Organic Pollutants) study found that indoor air PCB concentrations were the main factor affecting human blood levels of PCBs, and modeling of dietary intake and inhalation exposures relative to serum PCB levels revealed that for some children, inhalation exposures were greater than their dietary exposure (Ampleman, Martinez et al. 2015). While dietary ingestion is thought to be the primary

route of exposure to HC-PCBs and inhalation the primary route of exposure to the more volatile LC-PCB (Ampleman, Martinez et al. 2015, Grimm, Hu et al. 2015), recent data suggest this may be an oversimplification. The LC-PCB, PCB 11, was detected in commercial milk products in northern California, and PCB 95, a HC-PCB, was the second most abundantly detected PCB congener in school air samples within the U.S. (reviewed in (Klocke and Lein 2020)). Collectively, these observations suggest that human exposure to HC- and LC-PCBs occurs through both diet and inhalation.

PCBs are lipophilic and thus are readily transferred from mother to fetus prenatally via the placenta (Figure 3) and postnatally through breast milk. According to the National Health and Nutrition Examination Survey (NHANES), there is widespread exposure to HC- and LC-PCBs, with U.S. women of child-bearing age having average total PCB blood levels in the 30-200 ng/g range (Panesar, Kennedy et al. 2020). Similar levels are reported in other countries. A recent study investigating the age-, sex-, and brain region-specific distribution of all 209 PCBs using gas chromatography-tandem mass spectrometry (GC-MS/MS) in neonatal (N = 7) and adult (N = 7) postmortem brain samples found that HC-PCBs were observed at higher levels in samples from adult vs. young donors, while LC-PCBs predominated in samples from young vs. adult donors (Li, Hefti et al. 2022). Interestingly, this same study also detected hydroxylated PCB metabolites in human brain samples. The AESOP study had previously detected 58 OH-PCB congeners in serum samples collected between 2010 and 2011 from adolescents and mothers and found that HC-OH-PCBs predominated, and that LC-OH-PCB congeners were rarely detected in the serum samples (Marek et al., 2013). PCBs are hydroxylated by cytochrome P450 monooxygenases. While some OH-PCBs are readily eliminated, most undergo phase 2 metabolism (Grimm, Hu et al. 2015). Levels of hydroxylated and sulfated PCBs found in human blood and tissues, like placenta, can be quite high, and data from both

human and experimental models have implicated these metabolites in PCB neurotoxicity (Panesar, Wilson et al. 2022).

HUMAN STUDIES

Developmental Neurotoxicity

The relationship between PCBs and neurodevelopment has been studied extensively in many different populations around the world, including Europe, Asia, and the U.S. (Table 1). Two accidental mass poisonings that involved PCB-contaminated cooking oil, the first in Yusho, Japan in 1968 and the second in Yu-Cheng, Taiwan in 1979, provided the first human evidence of PCB neurotoxicity (Masuda, Kuroki et al. 1985). In Yusho, more than 1,000 people became ill after ingesting rice oil contaminated with PCBs during the manufacturing process. In Yu-Cheng, more than 2,000 people became ill from consuming PCB-contaminated rice oil. Children born to women exposed through ingestion of PCB contaminated rice oil while pregnant had an increased incidence and severity of cognitive and psychomotor deficits (Rogan 1982).

While the Yusho and Yu-Cheng incidents involved extremely high PCB exposures, subsequent epidemiological studies have identified neuropsychological deficits in populations exposed to much lower levels of PCBs. These latter studies have mostly identified negative associations between prenatal PCB exposure and measures of cognitive function, learning, memory, and IQ (Schantz, Widholm et al. 2003, Boucher, Muckle et al. 2009, Berghuis, Bos et al. 2015, Pessah, Lein et al. 2019). Deficits in these behavioral domains are commonly observed in many neurodevelopmental disorders (NDD), including autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD), and emerging evidence implicates PCBs as environmental risk factors for NDDs (Eubig, Aguiar et al. 2010, Klocke and Lein 2020, Keil-Stietz and Lein 2023).

Below we briefly summarize selected human studies on PCB developmental neurotoxicity published after 1990 that were chosen based on their historical significance and/or rigorous study design with respect to cohort size, PCB measures in relevant biological samples (maternal blood collected during gestation, umbilical cord blood and/or placenta) and assessment of neuropsychological function.

Yu-Cheng Studies: Longitudinal studies of children aged 2 to 12 years old from the Taiwan cohort employed the Bayley Scales of Infant Development-II (BSID-II) to assess the level of cognition, language, and social development, as well as both fine and gross motor function (Nellis and Gridley 1994). The Chinese version of the Stanford-Binet IQ Test, Raven's Colored Progressive Matrices and Raven's Standardized Progressive Matrices were also utilized to assess cognitive development. Children of mothers with PCB exposure had reduced scores on these scales compared to control children (Lai, Guo et al. 2001), indicating developmental delay. The same participants were followed up when they were aged 26-32 using the Wechsler Adult Intelligence Scale [WAIS-III]. The WAIS-III consists of 13 subtests, four index scores, three IQ assessments and two additional measures of incidental memory (Ryan and Lopez 2001). Some additional testing was done in conjunction with a functional MRI (fMRI), including the n-back test and picture rotation test (Chu, Wu et al. 2019). No differences in cognitive and behavioral testing were found between PCB exposed participants and the control group at this more advanced age, but the PCB exposed group had areas of increased cortical thickness and showed altered task deactivation and activation signals in the fMRI. These results suggest that the cognitive effects of developmental PCB exposures may not persist later in life, brain plasticity may be altered at later ages.

Michigan Cohorts: The relationship between low-level maternal PCB exposure via dietary intake of Lake Michigan fish and developmental outcomes in children (Jacobson, Fein et al. 1984, Jacobson and Jacobson 1997) was investigated in two separate cohorts using a

longitudinal study design. More than 8,000 women who delivered babies in western Michigan hospitals during 1980–1981 were interviewed on the day after delivery, and those who had consumed 26 or more pounds of Lake Michigan fish during the preceding 6 years were asked to participate. Women who did not eat Lake Michigan fish were randomly selected and invited to serve as controls. The PCB burden in two birth cohorts was assessed from cord serum and from serum collected from the children at 5 and 7 months and 4 and 11 years of age. Both PCB-exposed cohorts had significant cognitive and psychological deficits relative to control subjects.

Dutch Cohort: After the World Health Organization (WHO) announced that levels of PCBs and dioxins found in breastmilk were higher in Europe than in other parts of the world, the Dutch government responded by forming its own longitudinal study to study adverse effects of PCB and dioxin exposure during development (Vreugdenhil, Lanting et al. 2002). This was the first neurodevelopmental study to use congener-specific analysis to assess PCB exposure. The study included approximately 400 healthy pregnant women, half of whom breast-fed, while the other half formula-fed. The cohort was also separated by geographic location, with half living in Rotterdam, a heavily industrialized area, and the other half in Groningen, a rural area in northern Netherlands. PCB exposure was measured in maternal blood during the last month of pregnancy, in umbilical cord serum, and in breast milk. Four non-dioxin-like PCB congeners, PCBs 118, 138, 153, and 180, were quantified in both maternal and cord serum. The PCB levels in maternal serum were about 4-5 times higher than those in cord serum. However, lipid composition differs greatly between maternal and cord serum, and when expressed on a lipid basis, the PCB levels were similar. ∑PCB levels were 2.21 ng/g, 0.45 ng/g, and 428.5 ng/g in maternal plasma, cord blood, and breast milk, respectively. Neurodevelopment was assessed at 3, 7, 18, 42, and 84 months of age. Many important developmental outcomes were found to be negatively associated with pre- and postnatal exposure to PCBs, but because so many different outcomes were assessed, the results were complex and inconsistent across age. The European

Union (EU) funded an opportunity to expand the Dutch cohort study into a transnational, multicenter study that included a German cohort in Dusseldorf and another in Faroe Islands, which are described below.

German Studies: In 1993, 171 German mother-infant pairs were recruited to study PCB exposure. PCBs 138, 153, and 180 were measured in cord serum and breast milk, and neurodevelopment was assessed at 7, 18, 30 and 42 months of age. Similar to findings from the Michigan and Dutch cohorts, a negative association was found between prenatal PCB exposure and measures of cognitive development. The German cohort study found significantly increased omission errors in the computer-based test battery of attention performance (KITAP) in 8.5-year-old boys and girls with high levels of PCBs found in prenatal maternal blood (Neugebauer, Wittsiepe et al. 2015). However, this study also found a negative association between PCB exposures and ADHD-related behaviors (-10%; 95%-CI: 0.82–0.99). Unlike similar studies (e.g., Michigan, Dutch), the German study identified a relationship between postnatal PCB exposure via breast-feeding and adverse neurodevelopmental outcomes.

Faroe Islands Cohort: PCB exposure was assessed in two cohorts from the Faroe Islands, a territory between Norway and Iceland, that originally were studied to evaluate the effects of methylmercury exposure on neurodevelopment. Whale meat and blubber were a primary dietary source of methylmercury and PCBs. The first cohort of 1,022 singleton births was recruited in 1986-1987. PCBs were measured in samples of umbilical cord from a subset of 443 children. The sum of three major congeners, PCB 138, 153, and 180, was used to estimate total PCB burden. Twelve adolescent boys from this cohort underwent fMRI, and those with high levels of PCB exposure had more activation across brain regions during both visual and motor tasks than those with lower level exposures. A second cohort of 182 singleton infants born at term at the National Hospital in Torshavn, Faroe Islands was recruited in 1994-1995 as part of the EU multicenter study. PCB levels were measured in maternal serum, maternal hair, breast

milk, and cord blood. PCB levels within this cohort were ~3-fold higher than those in the Dutch cohort, yet PCB exposure had no effect on neurological outcome measures. Interestingly, observations from a rat model of developmental PCB exposure demonstrated a non-monotonic dose-response relationship with deficits in spatial learning and memory observed in weanling rats exposed to Aroclor 1254 in the maternal diet at 1, but not 6, mg/kg/d (Yang, Kim et al. 2009).

Oswego, New York Cohort: The Owego Newborn and Infant Development Project was a longitudinal study that took place in 1991 and initially started with 309 mom-child pairs. This was a follow up study to the Michigan cohort studies. Behavioral assessments were done at birth, 6, 12, 36, and 54 months of age, then again when subjects were 8 and 9.5 years old. The primary measure of exposure was PCB levels in umbilical cord plasma at birth. The placenta was also collected, and a small subset of women provided breast milk. The NES3 Extended Continuous Performance Tests (E-CPT) revealed that at 9.5 years old, the children had increased impulsivity due to impaired inhibition (executive functioning), but not impaired sustained attention, and these impairments were correlated with increased PCB levels (Stewart, Reihman et al. 2005).

The New Bedford, Massachusetts Studies: In 1982, the New Bedford harbor in southeastern Massachusetts was designated a Superfund site because of heavy PCB contamination of the sediment. A prospective birth cohort study conducted near this site included 788 mother-infant pairs recruited from 1993-1998. Infants born by Cesarean section or whose mothers did not speak English were excluded. Umbilical cord serum and maternal milk were collected and analyzed for PCBs using a congener-specific technique that quantified 51 individual PCB congeners. The concentrations of key indicator congeners, PCBs 118, 138, 153, and 180, were about one-half those reported in the Dutch cohort, but quite similar to those reported in the Oswego, New York cohort. Attention was assessed when the children were 8

years of age using the Continuous Performance test (CPT) and Wechler Intelligence Scale for Children (WISC-III). Boys with higher exposures to PCBs had more errors of omission, or the number of nonresponses, in the CPT and slower processing speed in the WISC-III than the control population, suggesting an attention deficit. Girls had a less errors of omissions in the CPT than the males (Sagiv, Thurston et al. 2012).

Inuit Cohort: The Inuit people depend on sea mammals (seal, beluga whale, and walrus) as the primary source of protein and fat in their diet. As a result, they have unusually high body burdens of PCBs. In the early 1990s, PCB concentrations in breast milk samples collected from Inuit women were 7 times higher than the PCB concentrations in Caucasian women residing in southern Quebec. Between September 2005 and February 2010, 294 children and their mothers participated in neurocognitive assessments in the three largest Nunavik villages in Canada. The concentrations of PCB 153 in this cohort were similar to those in the Dutch cohort and about 2-fold higher than those in the New Bedford cohort; however, there was no clear evidence of adverse effects of PCB exposure on neurocognitive measures in the Inuit children.

Flemish Environmental Health Survey: Between 2002 and 2006**,** 206 mother-child pairs were recruited and levels of PCBs 118 and 170 in addition to levels of “indicator” PCBs (138, 153, and 180) were measured in cord blood. The children were assessed during the first year of life for crawling and age at which they took their first steps, and higher PCB exposure levels were associated with delayed activity. At 1 year of age, the children were assessed using the Infant Behavior Questionnaire (IBQ), and at 36 months of age, they received the Reynell Taal Ontwikkelings Schalen (RTOS) test for language development. Increased PCB exposures were associated with slower development of language, suggesting early-life PCB exposure reduced language capabilities. The Snijders-Oomen non-verbal intelligence (SON) test, also taken at 36 months of age, showed PCB 118 exposure was associated with lower IQ in males

only, and that sum PCBs was associated with deficits in non-gender specific play in both males and females. These data suggest lower-level PCB exposures adversely affect the neurobehavioral development of young children, especially boys (Vermeir, Covaci et al. 2021).

Rhea Study, Crete, Greece: This study included 689 mother-child pairs, in which the pregnancy occurred within one year of February 2007. PCBs 118, 138, 153, 156, 170, and 180 were measured in maternal serum collected in the first trimester of pregnancy and neurodevelopment was assessed at 4 years. Concentrations of various PCBs correlated with behavioral scores from the McCarthy Scales of Children's Abilities (MSCA), which is a well validated test for assessing delays in verbal information processing, perceptual information processing, numerical abilities, general cognition, memory, and motor skills. Alternate scales for executive function, working memory, memory span, and cognitive functions were also included based on the underlying construct between brain region and behavior (Julvez, Ribas-Fito et al. 2007). High exposure to PCBs during pregnancy (>90th percentile) was associated with deficits in a working memory task and decreased scores in perceptual performance, general cognitive, and executive function (Kyriklaki, Vafeiadi et al. 2016).

Norwegian Study: This study includes 1,024 children enrolled in a longitudinal prospective study of ADHD with participants recruited from The Norwegian Mother and Child Cohort Study (MoBa). Boys and girls aged 3.5 years participated in extensive clinical assessments. Levels of dietary PCB 153 were used as a measure of PCB exposure. Clinical assessments included the preschool version of the Behavior Rating Inventory of Executive Function (BRIEF-P), the Preschool Age Psychiatric Assessment interview (PAPA), Stanford-Binet 5th revision (SB-5), Child Development Inventory (CDI), and Behavior Rating Inventory of Executive Function. No associations were found between low-level exposure to PCB 153 and ADHD-symptoms, verbal/non-verbal IQ, or executive functions, including working memory, in preschoolers. However, the findings did show that maternal dietary exposure to PCB 153 during

pregnancy was significantly associated with poorer expressive language skills in preschool girls (Skogan, Zeiner et al. 2014, Caspersen, Aase et al. 2016). It should be noted that the study population was designed to recruit adolescents with ADHD symptoms, which may have skewed results.

The Collaborative Perinatal Project: The Collaborative Perinatal Project (CPP) was a prospective study designed to identify determining factors of neurodevelopmental deficits (Gray, Klebanoff et al. 2005). More than 56,000 pregnant women were recruited from 12 centers across the United States between 1959 and 1966 and followed longitudinally through pregnancy and after birth. Serum was collected during the third trimester and analyzed for 11 specific PCB congeners: PCBs 28, 52, 74, 105, 118, 138, 153, 170, 180, 194, and 203. Cognitive function was assessed at 7 years of age using the intelligence quotient (IQ) scores on the Wechsler Intelligence Scale for Children. In this study, *in utero* exposure to PCBs was not associated with lower IQ at age 7 years.

PCBs as Environmental Risk Factors for Neurodevelopmental Disorders (NDDs)

Reviews of the human literature on PCB developmental neurotoxicity have consistently concluded that the weight of evidence indicates that PCBs are developmental neurotoxicants (Schantz, Widholm et al. 2003, Boucher, Muckle et al. 2009, Berghuis, Bos et al. 2015, Pessah, Lein et al. 2019), but whether PCBs are associated with an increased risk for specific NDDs is less clear. There has been significant interest in investigating PCBs as a risk factor for autism in particular (Lyall, Croen et al. 2017, Bernardo, Lanphear et al. 2019, Alampi, Lanphear et al. 2021, Mehri, Bashirian et al. 2021). Autism spectrum disorder (ASD) is a complex, multifactorial neurodevelopmental condition that is defined clinically by core deficits in social reciprocity and communication, restrictive interests and repetitive behaviors, although the severity and

symptom profile vary considerably between individuals. Research on ASD has focused primarily on genetic factors, identifying a strong hereditary component and hundreds of genes that influence autism risk; however, solely genetic causes account for approximately 10-30% of all autism cases (Lai, Lombardo et al. 2014, Ansel, Rosenzweig et al. 2016, Masini, Loi et al. 2020). It is now thought that gene-environment interactions underlie most cases of ASD (Lein 2015).

The MARBLES study, a longitudinal study that began in 2006, was designed to identify possible pre- and postnatal biological and environmental risk factors that may contribute to the development of autism. This study recruited pregnant women living in northern California who already had a child with a NDD and, therefore, had an increased risk of having a second child with an NDD (Hertz-Picciotto, Schmidt et al. 2018). A total of 104 mother-child pairs were included in a study to assess association of PCBs with autism. Maternal serum was collected through gestation and analyzed for PCB composition. Interestingly, of the 12 most abundant PCB congeners detected in these samples, LC-PCBs 28 and 11 constituted 70% of the total PCB burden (Sethi, Morgan et al. 2019). Multinomial logistic regression was used to assess the relative risk of a clinical outcome classification of ASD and non-typical development (Non-TD) compared to typically developing (TD) in the children at 3 years of age. There were no significant associations between these outcome measures and total PCB levels; however, there were borderline significant associations between DL-PCBs and decreased risk for Non-TD outcome classification (adjusted OR: 0.41 (95% CI 0.15-1.14)) and between ryanodine receptor-activating PCBs and increased risk for ASD outcome classification (adjusted OR: 2.63 (95% CI 0.87-7.97)). While this study provided modest supporting evidence that PCBs are risk factors for ASD or Non-TD, these analyses suggested the need to investigate associations between neurodevelopmental outcomes and specific PCB congeners based on their biological activities.

A subsequent study of the MARBLES cohort examined the association between maternal PCB levels, placental DNA methylation patterns and child neurodevelopmental outcomes (Mouat, Li et al. 2023). A complete congener profile (all 209 PCB congeners) was assessed in 104 maternal serum samples collected at delivery and DNA methylation networks were identified from 147 placenta samples. PCBs 153, 168, 170, 180, 187 and 193 were detected in over 50% of maternal serum samples and were highly correlated with one another. Maternal age was the strongest predictor of serum PCB levels, with PCB levels also influenced by calendar year in which samples were collected (samples collected in earlier years had higher PCB levels), pre-pregnancy BMI and polyunsaturated fatty acid levels. Twenty-seven modules of placental DNA methylation were identified, including five that significantly correlated with one or more PCBs, and four that correlated with child neurodevelopment. Two modules associated with both maternal PCB levels and child neurodevelopment mapped to two genes, *CSMD1* and *AUTS2*, implicated in ASD and identified as differentially methylated regions in mouse brain and placenta following gestational exposure to a “MARBLES” PCB mixture. These findings suggest that at least some PCB congeners may be associated with altered methylation patterns implicated in autism.

In 2019, a review of the literature (Pessah, Lein et al. 2019) identified human studies of prenatal exposure to PCBs and neurodevelopmental outcomes across domains related to cognition (e.g., IQ, language, memory, learning), attention, behavioral regulation, executive function, and social behavior, including traits related to ADHD and ASD. The authors were able to identify 29 papers that met their inclusion criteria, which included: (1) a sample size of 100(+) subjects; (2) study design that measured prenatal PCB exposure; (3) quantification of PCBs in relevant biological matrices; and (4) subjects tested at 3 years of age or older. Of these 29 papers, 12 focused on impacts of PCB exposure on cognitive function in children aged 3-11, and most (8/12) found associations of developmental exposure and cognitive deficits.

Seventeen studies from 11 different cohorts examined associations of prenatal PCBs with attention, behavioral regulation and social behavior among 3-12-year-old children. The majority (10 of 17) reported PCB-related associations with impulse control, hyperactivity, and attention. Only 2 of the 17 studies examined PCBs in relation to social behavior and autistic traits, with one study reporting that total PCB levels were associated with fewer autistic traits, and the other reporting congener-specific associations with autistic traits.

Overall, the authors (Pessah, Lein et al. 2019) concluded that most studies found prenatal PCB exposures were associated with poorer cognitive function and/or increased risk of behavior problems (Pessah et al. 2019). However, a significant limitation of the available data was that associations with specific PCB congeners or mechanism-based classes of congeners were not evaluated since most epidemiologic studies report total sum PCB levels or levels of indicator PCBs. Moreover, the variety of analytical techniques used to detect PCBs in human tissues and the differing PCB congener profiles analyzed across cohorts made it exceedingly difficult to discern whether adverse neurodevelopmental outcomes were predominantly associated with specific PCB congeners. The significance of this limitation is underscored by a recent study suggesting that NDL PCBs with activity at the ryanodine receptor, but not dioxin-like PCBs or total PCBs, are weakly associated with ASD (Granillo, Sethi et al. 2019). Another complication is that the genetic substrate likely also influences the impact of PCBs on NDD-relevant outcomes (Lein 2015). This is illustrated by a study not included in the Pessah et al. 2019 review that identified a trend towards a positive association between PCB 153 levels and ASD in individuals with a deletion mutation in the gene encoding glutathione-S-transferase (GST) but not in individuals who did not have this mutation (Bach et al. 2020). A 2017 study not included in the summary because it did not specify the age of the child at the time of ASD diagnosis, also found elevated ASD risk associated with specific PCB congeners (Lyall et al. 2017). Specifically, the authors found increased ASD risk for the highest *vs*. lowest quartile of PCB 138/158 and PCB 153, and for the

highest deciles of other congeners examined in secondary analyses. Finally, a 2019 study reported an association between plasma PCB concentrations measured during pregnancy and increased incidence of autistic behaviors in children aged 3-4 years old when the data were analyzed using Bayesian predictive odds ratios (Bernardo et al. 2019). When considering the additional studies that have examined the impact of prenatal PCB on ASD phenotypes together with the two studies included in the focused review, most (5 of 6) found that PCB exposures are associated with increased expression of autistic traits. In line with this simple review of the literature is the conclusion of a meta-analysis of studies published through 2019 that reported odds ratios for PCB exposure and autism from case-control or cross-sectional studies. The authors used random-effects models to examine the association among five different studies representing five independent cohorts from across North America and Finland using pooled odds ratios. A significant association was found between PCB exposure during pregnancy and autism risk (Mehri, Bashirian et al. 2021).

Data Gaps in the Human Literature on PCB Developmental Neurotoxicity

While the majority of human studies suggest adverse effects of PCBs on the developing brain, the epidemiological data are variable, with a small subset of studies reporting no effects of PCBs on neuropsychological outcomes, and an even smaller subset reporting a positive association between PCB exposure and behavioral outcomes (Pessah, Lein et al. 2019, Klocke and Lein 2020). These discrepancies across studies likely reflect differences in study design, including outcome measures and how they were assessed, the age at which the child is tested, and how PCB exposures were determined, as well as differences between cohorts with respect to socioeconomic status or home environment of the child, co-exposures to other neurotoxic compounds (e.g. methylmercury), genetic background and PCB exposures (in terms of both levels and congener profiles). Epidemiological studies of PCB effects on NDD are further

complicated by the fact that many NDDs are phenotypically and genetically heterogeneous, thus masking clear associations between diagnosis and exposure (Lein 2015, Klocke and Lein 2020, Keil-Stietz and Lein 2023).

In addition to the general limitation of publication bias (e.g., positive findings are more likely to be reported than negative findings), a significant challenge in human studies of PCB developmental neurotoxicity is that most epidemiological studies still rely on total PCBs or levels of indicator PCBs when assessing exposure. While this approach may provide reasonable estimates of overall PCB exposure, it may obscure associations because the influence of individual congeners or functional classes of PCBs on neurotoxic outcomes cannot be evaluated (Schantz, Widholm et al. 2003, Panesar, Wilson et al. 2022). Another factor that complicates interpretation of the human literature is the lack of uniformity in both outcome measures and in the tools used to assess any specific outcome, which make it difficult to compare between epidemiological studies. For example, some studies only tested visual-spatial abilities, which have been shown to not be very sensitive to PCBs, where other studies tested IQ or response inhibition, which appear to be more sensitive to PCBs (Boucher, Muckle et al. 2009). A review summarizing the neuropsychological tests that have been used in human studies of PCB developmental neurotoxicity provides a detailed discussion of tests that may be the most beneficial to perform and proposes standardization of test protocols across studies with the goal of enabling more rigorous comparisons across studies (Boucher, Muckle et al. 2009). Another potentially complicating factor is evidence that populations and communities with lower socioeconomic status are more likely to be exposed to higher levels of pollution, but less likely to be recruited or participate in studies looking at population-level health outcomes. That this may be problematic in the PCB literature is suggested by the observation that most of the participants in the above-mentioned studies were middle-to-upper class Caucasian women with unremarkable pregnancies and births (Hajat, MacLehose et al. 2021).

While discrepancies between human studies of PCB developmental neurotoxicity with respect to the spectrum and persistence of adverse neurobehavioral outcomes, confounding co-exposures and differences in congener profiles that comprise the exposure have raised questions concerning the causative role of PCBs in human developmental neurotoxicity (Winneke 2011), extensive experimental findings in non-human primate and rodent models confirm that developmental PCB exposure causes deficits in learning and memory (Ulbrich and Stahlmann 2004, Sable and Schantz 2006, Klocke and Lein 2020). These animal data will be discussed below.

Neurotoxic Outcomes in Adults

PCB exposure has been associated with cognitive deficits, including those in executive functioning, memory, language and communication in a variety of adult populations. Mohawk adults on the Akwesasne Reserve in New York were observed for executive function after being exposed to PCBs, along with dichlorodiphenyldichloroethylene (DDE), mirex, and hexachlorobenzene (HCB) via consumption of local fish. Adults aged 17-79 years old were assessed for cognitive function measured with the digit symbol substitution test. LC- and HC-PCB exposure was found to increase cognitive and executive function decline in the adults aged 47-79 years old (Sasaki, Jones et al. 2023). The association of deficits with DDE and HCB was much weaker, suggesting that PCBs had the greatest adverse effect on executive function at older stages of life.

A limited number of studies have examined PCB effects on learning and memory in adults. The neuropsychological function of Michigan residents, 49- to 86-years old, who regularly ate fish caught from Lake Michigan (>24 pounds eaten per year) was compared to that of residents who ate less than 6 pounds of fish per year. The heavy fish consumers had lower

scores than the low fish consumers on the Wechsler Memory Scale and the California Verbal Learning Test. Consistent with this observation, performance scores were inversely associated with serum PCB levels of participants (Schantz, Gasior et al. 2001).

Language and communication skills may also be negatively affected by PCB exposure in adulthood. A German health program surveillance was done on workers for a transformer and capacitor recycling company who had increased PCB burden (Rengelshausen, Randerath et al. 2023). Workers with a PCB burden in the 95th percentile rank or higher exhibited reduced verbal fluency regarding letter and semantic word generation as well as word production and fine motor function as shown by the Regensburg Word Fluency Test RWT. More specifically, workers with an increased LC-PCB burden showed a significantly longer duration in the word response task than those with a smaller LC-PCB burden (Fimm, Sturm et al. 2017). However, additional neuropsychological and cognitive analyses done with this group found no PCB effects.

Epidemiological studies of PCB effects on affective disorders in adults is relatively limited, but includes at least one report of a positive association between PCB exposure and risk for bipolar disorder, as well as several others linking adult exposure to higher rates of depression (Table 2). A higher-than-average incidence of dementia as well as a significantly higher rate of bipolar disorder diagnoses was observed in a sample of Yusho poisoning survivors relative to healthy controls (Akahane, Matsumoto et al. 2018). A 1989 cohort study reported that firefighters who sustained acute, high-level dermal and inhalation exposure to PCBs during a transformer fire experienced more depressed moods several months after the fire than firefighters not present at the fire (Kilburn, Warsaw et al. 1989). More recent evidence of an association between PCB exposure and increased prevalence of depressive symptoms emerged from a large cohort study that examined neuropsychological endpoints in adults living along a segment of the Hudson River highly contaminated with PCBs (Fitzgerald, Belanger et al. 2008). These findings are consistent with those of a longitudinal study of the German

workers exposed to PCBs involved in recycling of transformers and capacitors (Gaum, Esser et al. 2014). Participants underwent a psychological screening questionnaire and had blood drawn at three separate timepoints to determine plasma concentrations of individual PCB congeners. Relative to the general German population, PCB-exposed workers had an increased prevalence and severity of depressive symptoms that persisted over multiple years and corresponded with elevated plasma concentrations of HC-, LC-, and dioxin-like PCBs. These findings were replicated in a more recent study involving a separate cohort occupationally exposed to PCBs, that also found a significant positive association between depressive symptoms and plasma concentrations of HC-, LC-, and dioxin-like PCBs (Gaum, Gube et al. 2017). The more recent study also identified associations between PCB exposure and altered dopamine metabolism, as well as between altered dopamine metabolism and depressive symptoms. The authors proposed that PCB-mediated disruption of dopaminergic signaling was the cause of PCB-associated depressive symptoms.

The epidemiological data relating PCB exposure to affective disorders in adults is challenging to interpret because of inconsistent findings, relatively few studies, and the small sample sizes of many of the available studies. Results may also be influenced by reliance on self-reporting and questionnaires, which may be subject to bias. Furthermore, affective disorder presentation can vary widely between individuals, and symptoms are often subjective and difficult to quantify. These characteristics may impose an additional level of uncertainty and further complicate the gathering or interpretation of epidemiological findings.

PCB Effects on Neurodegenerative Disease

PCBs have been identified as a potential environmental risk factor for neurodegenerative diseases such as Parkinson's disease (PD), amyloid lateral sclerosis (ALS), and Alzheimer's

disease (AD), although there is only moderate support from epidemiological studies (Table 3). Interestingly, several studies report sex-specific effects of PCB exposure, with statistically significant findings observed only in females. Two studies, in which brain tissue was used to measure PCB exposure, reported significant associations with PD. In one, subjects diagnosed with PD were found to have higher concentrations of PCB 153 in the anterior caudate nucleus, a region that is commonly implicated in PD pathophysiology (Corrigan, Murray et al. 1998). In agreement with this finding, a case-control study reported higher concentrations of PCB congeners 153 and 180 in occipital cortex samples obtained from PD patients (Hatcher-Martin, Gearing et al. 2012). When stratified by sex, their analysis revealed significant elevations in total PCBs and congeners 138, 153, and 180 in female PD patients only. Hatcher-Martin et al. also examined substantia nigral tissue from a separate cohort of females who had not displayed any observable clinical symptoms of PD. However, higher concentrations of total PCBs and of individual congeners 138, 170, and 180 were detected in brains exhibiting greater reductions in neuromelanin pigmentation in nigral neurons, a common neuropathological feature of PD.

Earlier findings of electrical capacitor manufacturing plant workers employed between 1939 and 1977 similarly reported sex-specific effects of PCBs. This study investigated the prevalence of neurodegenerative disease in workers who had sustained varying degrees of occupational exposure to PCBs. Compared to the U.S. general population, female plant workers estimated to have higher levels of PCB exposure also experienced higher rates of PD and AD (Steenland, Hein et al. 2006).

In the Faroe Islands, whale meat constitutes a considerable portion of the traditional Faroese diet and poses a relatively higher risk for dietary PCB exposure due to the tendency PCBs to bioaccumulate in marine mammals. Compared to healthy residents, Faroese adults who had been diagnosed with PD reported higher lifetime consumptions of whale meat and blubber (Petersen, Halling et al. 2008). In addition, quantification of serum PCB concentrations

in these individuals revealed a significant positive association between PCB 101 exposure and PD risk.

There are also epidemiological data suggesting a link between exposure to certain PCBs, especially PCB congener 151, and ALS. A case-control study that screened blood samples for various POPs detected a significant positive correlation between plasma levels of PCB 151 and risk of ALS (Su, Goutman et al. 2016). Consistent with these findings, a more recent study sampled ALS patients and identified a significant association between elevated plasma concentrations of PCB 151 and increased risk of earlier death after adjusting for patient age at diagnosis (Goutman, Boss et al. 2019).

A small number of studies have identified PCB exposure as a possible environmental risk factor for a general clinical diagnosis of dementia. Approximately 40 years after the Yusho mass-poisoning in Japan, various health outcomes were assessed in a sample of poisoning survivors (Akahane, Matsumoto et al. 2018). Results of this study indicated significantly higher rates of dementia in Yusho survivors when compared to age-matched controls with no history of Yusho poisoning. Further evidence linking dementia with PCB exposure came from a prospective cohort study that took place in a heavily PCB-contaminated town in Italy. Results indicated that the risk of developing dementia was significantly higher for residents whose blood serum contained higher total concentrations of PCBs at the time of their enrollment in the study (Raffetti, Donato et al. 2020).

Despite the epidemiological evidence of PCB exposure as a potential environmental risk factor for neurodegenerative diseases, the limited number of studies and inconsistent findings remain a major shortfall of the literature. Many studies fail to demonstrate an effect. For instance, a 2000 study detected no notable differences in PCB tissue concentrations in brain samples obtained from subjects diagnosed with PD or AD when compared to controls (Corrigan, Wienburg et al. 2000). Inconsistent findings may be due to relatively small sample sizes,

differences in study design, including approaches for assessing PCB exposures. Furthermore, because epidemiological studies of neurodegenerative diseases rely on older populations, it is impossible to rule out survival-imposed selection bias. The higher rate of prescription medication use in older adults presents another complicating factor. The difference in life expectancies for men and women should also be considered, as this may contribute to sex-based differences reported in some studies.

A factor that remains largely unaccounted for in the literature pertains to the question of whether calculated PCB body burden provides an accurate representation of lifetime exposure. The majority of studies incorporate only a single timepoint for biological sample collection and fail to provide estimates of exposure duration or time of occurrence. Furthermore, the effects of LC-PCBs may be under-accounted because they tend to be metabolized faster than HC-PCBs, A small number of longitudinal studies attempt to address this difference but are confounded by few intermittent follow-up assessments and large participant drop-out.

EXPERIMENTAL ANIMAL STUDIES

A large number of experimental animal studies of PCB neurotoxicity have been published in the peer-reviewed literature [reviewed in (Ulbrich and Stahlmann 2004, Sable and Schantz 2006, Faroon and Ruiz 2016, Klocke and Lein 2020). These studies employed various individual PCB congeners and mixtures, including the commercial legacy Aroclor mixtures, to assess the impacts of PCB exposures, which have been predominantly developmental exposures (Carlson, Christensen et al. 2023), on behavioral outcomes of translational relevance to humans. The tightly controlled exposures that are feasible in experimental animal studies are critically important for (1) corroborating human epidemiological data; and (2) providing mechanistic insights, such as brain regions likely targeted by PCBs and potential cellular and/or

molecular mechanisms. Several reviews of the animal literature on PCB neurotoxicity have previously been published (Faroon and Ruiz 2016, Pessah, Lein et al. 2019, Klocke and Lein 2020). Here, we highlight selected studies published after 2000 that address PCB-induced behavioral changes using rigorous study designs, validated tests of behaviors relevant to human PCB neurotoxicity and PCB exposures relevant to human exposures in terms of both the congener profile and levels.

Learning and Memory

Learning and memory are complex behaviors that combine situational paradigms with information encoding, storage, and retrieval. Memory is further categorized as working memory, short-term memory, and long-term memory (Crowder 2019). Not only are learning and memory critical for survival of the organism, but they are also important in establishing patterns of neuronal connectivity in the developing brain via effects on dendritic morphogenesis and formation/stabilization of synapses (Ma and Zuo 2022). Significant to studies of PCB developmental neurotoxicity, learning and memory are behavioral outcomes with direct translational relevance to behavioral deficits observed in human studies of developmental PCB exposures.

A commonly used test to assess learning and memory in rodent models is the novel object recognition (NOR) task, which engages the hippocampus (CA1 and CA3 regions), insular cortex (IC), perirhinal cortex (PRh), and medial prefrontal cortex (mPFC). Thus, deficits in NOR performance indicate perturbations in one or more of these brain regions (Tanimizu, Kono et al. 2018). NOR has been used to assess PCB developmental neurotoxicity in rodent models. For example, the performance of mouse pups in the NOR task was assessed after lactational exposure to 0, 6 or 8 mg/kg/d of Aroclor 1254 (A1254) in the maternal diet from postnatal day

(PND) 7 to 21 and direct oral exposure from PND 22 to 42. Females, but not males, had significant deficits on this task at PND 35-37, regardless of Aroclor dose (Tian, Hwan Kim et al. 2011), suggesting that developmental exposure to this PCB mixture caused sex-specific deficits in learning and/or memory in mice. In a similar study, CYP transgenic mouse dams were exposed via oral gavage to the non-dioxin-like PCB congeners 105, 118, 138, 153, and 180 and dioxin-like PCB congeners 77, 126, and 169 at gestational day 10.5 and PND 5. CYP1A2 knockout mice pups were significantly impaired in the NOR task (Curran, Nebert et al. 2011).

Deficits in spatial learning and memory have also been measured in rodent models of developmental PCB exposures. Oral gavage of pregnant rats with Aroclor 1254 (A1254) at 5, 10, and 20 mg/kg body weight every three days from gestational day 5 to PND 20 caused significant deficits in spatial learning and memory in their pups as determined using the Morris water maze (Liu, Bai et al. 2015). Pups exposed to 10 or 20 mg/kg/d in the maternal diet had a significantly worse performance compared to controls (0 mg/kg A1254). Impaired spatial learning was associated with increased intracellular calcium concentrations and ultra-micro structural changes in the hippocampus (Liu, Bai et al. 2015). While the majority of studies have examined learning and memory deficits in animals exposed to PCBs during early life, at least one study has examined impacts of PCBs on Morris water maze performance following mid-life exposures. Adult female Sprague-Dawley rats exposed as adults to a PCB mixture simulating the PCB found in school air exhibited modest PCB-induced deficits in spatial learning and memory (Wang, Adamcakova-Dodd et al. 2020). The majority of studies have observed PCB-induced learning and memory deficits; however, a limited number of studies have not observed significant memory impairment as a consequence of developmental PCB exposure. For example, following dietary exposure of Sprague-Dawley rat dams to PCB 47 and 77 at 1.25 ppm, 12.5 ppm or 25.0 ppm (w/w) in rat chow provided *ad libitum*, no effects of PCBs were observed in either short-term or long-term spatial memory as determined using Morris water

maze training in PND30 pups (Donahue, Dougherty et al. 2004). The reason for these discrepancies across studies are not clear, but may be due to differences in species, behavioral testing paradigms, or the PCB dosing paradigm with respect to PCB congener profiles or levels.

Motor Function

Motor function is an umbrella term for locomotion, balance, coordination, limb movement, and other aspects of movement. Motor function generally involves the cerebellum and the nigrostriatal pathways of the brain (Salman and Tsai 2016). Motor function can be readily tested in animal models, and it is an important component of other behavioral tests. For example, changes in motor function can alter performance in cognitive tests that require movement, such as the y-maze and Morris water maze. Thus, many behavioral tests assess motor function as a control (Schönfeld, Dooley et al. 2017).

PCBs have been shown to alter motor function in experimental animals in a congener- and sex-specific manner. Sprague-Dawley rats at an age corresponding to human puberty were given a diet augmented with PCB-contaminated fish from the St. Lawrence River or Aroclor 1248 at a concentration approximating that found in the contaminated fish. After being exposed for 30-days, the rats received operant conditioning training to assess activity level, impulsiveness, and visual discrimination. Both PCB-exposed groups had significantly higher lever pressing rates than controls, suggesting PCB induced hyperactivity (Berger, Lombardo et al. 2001). Similar results were reported in Swiss albino mice exposed to 0, 1, 10, or 100 ng/kg of PCBs 28, 52, 101, 138, 153, and 180 dissolved in rapeseed oil and administered daily by oral gavage from PND 0 until weaning at PND 21. Females exposed to 100 ng/kg exhibited increased latency to turn in the Negative Geotaxis (NG) test at PND 7 and 9 (Elnar, Diesel et al. 2012). In contrast, other studies have found little to no effect of PCBs on motor behavior. For

example, an *ad libitum* diet-based exposure to PCB 153 during gestation and lactation did not change motor function, including grasping reflexes, righting reflexes, and forelimb in BALB/c mice when tested from PND 5 to 15 (Haave, Bernhard et al. 2011). However, the amount of PCB that animals received was not controlled in this study, and the dose was lower than in the other two studies, suggesting PCB-induced motor deficits exhibit a classic dose-response relationship with deficits increasing as PCB dose increases.

Attention

Attention can be defined as behaviors related to obtaining and maintaining the alert state, orienting to sensory stimuli, and resolving conflict among competing responses (Petersen and Posner 2012). Attentional deficits are a primary component of NDDs, particularly ADHD. Attention is mediated by two major networks: (1) the alerting network, which is modulated by the brain's norepinephrine system and involves major nodes in the frontal and parietal cortex; and (2) the executive network, which resolves competing actions during tasks with conflicting demands. This network involves the anterior cingulate cortex, areas of the prefrontal cortex, and the striatum (Soydaner 2022).

Rodent models can be used to assess attention behavior following PCB exposure. Spontaneously hypertensive rats (SHR/NCrl) were exposed to 1, 3, and 6 mg/kg of PCB 153 at PND 8, 14, and 20 using a gastric tube. Compared to controls, the SHR/NCrl rats demonstrated dose-dependent differences in a lever pressing test of attention. Sustained attention was significantly increased in the 1 mg/kg dose group, but significantly decreased in the 3 and 6 mg/kg dose group. The 6 mg/kg dose group also had increased inter-response times (IRT), suggesting reduced attention in the higher dose groups (Johansen, Fonnum et al. 2014). There is also evidence of altered attention following inhalation exposure. Compared to control animals,

rats exposed to air-borne Aroclor 1248 or sediment vapor containing PCBs showed significantly higher rates of bar pressing (indicating hyperactivity and frustration) in a multiple fixed interval extinction operant behavior paradigm (Lombardo, Berger et al. 2015). These results support clinical findings of attentional deficit in PCB-exposed humans.

Social Behavior

Many NDDs are associated with PCB exposures, including ASD (Lyall, Croen et al. 2017, Bernardo, Lanphear et al. 2019, Alampi, Lanphear et al. 2021, Mehri, Bashirian et al. 2021, Keil-Stietz and Lein 2023) and ADHD (Eubig, Aguiar et al. 2010). Impaired social behavior, including poor social cue recognition, reduced social interaction and communication are common to multiple NDDs (Baribeau, Dupuis et al. 2019, Keil-Stietz and Lein 2023). Behavioral tests, such as ultrasonic vocalizations and the social approach test (Rein, Ma et al. 2020), have been used to assess social deficits in rodent models of autism, making them excellent candidates for behavioral tests of PCB exposure.

Multiple studies have examined social behavior following developmental exposure to PCBs (Klocke and Lein 2020). As an example, one study assessed social behavior in pups born to female mice exposed to 0, 0.1, 1, or 6 mg/kg/day of the MARBLES PCB mix during gestation and lactation. The MARBLES PCB mix simulates the relative proportions of the twelve most abundant PCB congeners found in the serum of pregnant women enrolled in the MARBLE cohort who are at increased risk for having a child with a neurodevelopmental disorder (Sethi, Morgan et al. 2019). Ultrasonic vocalizations were measured at PND 7 and social approach was tested at PND 32. Exposure to the MARBLES PCB mix resulted in significantly less ultrasonic vocalizations in male and female pups in all dose groups. In contrast, only the males in the 0.1 mg/kg dose exhibited decreased sociability in the social approach task (Sethi, Stietz et al.

2021). Similar results were observed in rat pups exposed PCBs 47 and 77 at 12.5 mg/kg/d and 25 mg/kg/d in the maternal diet during gestation and lactation (Jolous-Jamshidi, Cromwell et al. 2010). PCB exposure significantly impaired social recognition in PND 21 males (Jolous-Jamshidi, Cromwell et al. 2010). This same group of investigators assessed adult male rats for social investigation behavior using the social port test following exposure to the same doses of PCBs. The adult rats that were isolation housed had decreased social investigation, which reflected the developmental exposure results (Jolous-Jamshidi, Cromwell et al. 2010).

There are also data showing that PCBs affect sociosexual behavior in a sex- and time-dependent manner. For example, Bell et al. (2016) exposed Sprague-Dawley rats to Aroclor 1221 (A1221) prenatally through the dam, who received i.p. injections of 0 or 1 mg/kg A1221 at E16, 18, and 20, and/or via i.p. injections in the pups at PND 24, 26, and 28. Prenatal exposure resulted in more sociosexual behavioral changes than juvenile exposure. Additionally, whereas female rats had significant deficits in social behavior between PND 30 and 39 (juvenile age) following the dual exposure paradigm and the juvenile only exposure, males showed significant alterations in sociosexual choice assessed between PND 90 and 110 in adulthood following prenatal or juvenile, but not the combined exposures (Bell, Thompson et al. 2016).

Insights from Behavioral Studies of Mechanisms of PCB Neurotoxicity

Behavioral studies can provide insights regarding mechanisms by which PCBs cause neurotoxicity. One example is a study that investigated PCB neurotoxicity in transgenic mouse lines expressing high levels of hepatic cytochrome P450 (CYP) 1A2 vs. CYP 1A2 knockout, as well as lines with variable levels of AhR affinity. Behavioral responses were assessed in these mice following exposure to a PCB mixture of dioxin-like (PCBs 77, 126 and 169), and non-dioxin-like (PCBs 105, 118, 138, 153, and 180) congeners found in food, human tissue, and

breast milk mixture. Mice were exposed at gestational day 10 and postnatal day 5 via oral gavage of the dam (Curran, Vorhees et al. 2011). The high-affinity AhR CYP1A2 knockout mice had the greatest impairment in the novel object recognition test and Morris water maze for spatial learning (Curran, Nebert et al. 2011), suggesting that PCB neurotoxicity is related to AhR affinity and that CYP1A2-mediated metabolism is neuroprotective. The assessment of motor behavior in these same mice by rotarod, gait analysis, sticker removal, pole test, and challenging balance beam, revealed that developmental PCB exposure significantly decreased performance on the rotarod test and that the CYP knockout mice were less impaired than the wildtype mice (Colter, Garber et al. 2018). Similar results were seen with the gait analysis. In the challenging balance beam test, the high affinity AhR genotypes exposed to PCBs showed significantly more slips than the PCB-exposed wildtype controls. These findings suggest that CYP metabolism of PCBs negatively affects motor behavior and AhR affinity may influence the magnitude of the PCB effect.

EXPERIMENTAL *IN VITRO* STUDIES

Experimental in vitro studies have been instrumental in elucidating the cellular and molecular mechanisms by which PCBs disrupt neurodevelopment. These studies reveal that both legacy and emerging non-legacy PCBs alter key neurodevelopmental processes, including neuronal apoptosis, and dendritic and axonal growth.

Aroclor mixtures, including Aroclor 1254, induce apoptosis in primary neuronal cultures at sub-cytotoxic concentrations, with evidence pointing to non-dioxin-like (NDL) congeners as primary mediators. For example, PCB 47 (NDL) increases caspase-dependent apoptosis in primary hippocampal neurons, while PCB 77 (dioxin-like) exhibits pro-apoptotic effects only at high, cytotoxic concentrations. Importantly, not all NDL congeners are pro-apoptotic; PCB 104

has no effect on baseline apoptosis, emphasizing that this activity is congener-specific and influenced by structural features such as ryanodine receptor (RyR) sensitization.

In parallel, certain NDL congeners promote neuronal morphogenesis without compromising viability. PCB 95 and PCB 136, for instance, enhance dendritic arborization in primary rat and mouse neurons at picomolar to nanomolar concentrations, with no effect on axonal growth. These effects display non-monotonic dose-responses and are mediated through RyR-dependent calcium release and downstream activation of CREB, a transcription factor essential for dendritic growth. Structure-activity relationship (SAR) studies have shown that PCB-RyR interactions are highly stereoselective, highlighting the specificity of this mechanism.

Calcium dysregulation is a hallmark of NDL PCB exposure. While high concentrations of some PCBs increase extracellular Ca^{2+} influx, more environmentally relevant doses (pM-nM) sensitize intracellular Ca^{2+} channels, particularly RyRs. This leads to sustained calcium release from internal stores, initiating downstream signaling cascades. RyR sensitization by NDL PCBs is the most sensitive and well-characterized calcium-dependent mechanism and is not observed with dioxin-like PCBs.

More recently, however, attention has turned to LC-PCBs, such as PCB 11, which are increasingly detected in human serum. Unlike legacy congeners, PCB 11 and its hydroxylated and sulfated metabolites promote both dendritic and axonal growth at femtomolar concentrations in primary neuronal cultures. These effects are not mediated by RyR, AhR, THR, L-type Ca^{2+} channels, or IP_3 receptors, but are blocked by CREB inhibition or knockdown, suggesting that PCB 11 activates CREB through a distinct, RyR-independent pathway. Thus, despite differing upstream mechanisms, both legacy and non-legacy NDL PCBs appear to converge on CREB signaling to drive aberrant neuronal morphogenesis.

Together, these in vitro findings demonstrate that PCB-induced developmental neurotoxicity involves diverse and congener-specific mechanisms, often acting at environmentally relevant concentrations. They underscore the importance of investigating both legacy and emerging PCBs to fully understand their impact on brain development.

Neurodevelopmental Processes Influenced by PCBs

Understanding how PCBs alter brain development begins with identifying the specific biological processes they disrupt. Evidence from recent human and animal studies shows that PCBs rarely cause obvious malformations in the developing brain (Pessah 2019). Instead, the DNT of PCBs seems tied to the subtle alterations in how neural networks are organized and connected. The formation of synaptic patterns during early life plays a decisive role in shaping cognitive function, and disturbances to these patterns are closely linked with NDDs and impaired cognition (Stamou 2013). Experimental models have repeatedly shown that PCBs can interfere with at least two major processes that influence synaptic wiring: apoptosis and neuronal morphology.

Neuronal Apoptosis

In the developing brain, apoptosis is not necessarily a sign of injury but rather a vital sculpting tool that ensures proper wiring and balanced cell numbers (Fricker, Tolkovsky et al. 2018)(Fricker et al., 2018). It occurs in both actively dividing neural zones and in postmitotic neurons, timed and localized with remarkable precision. Disturbing either the schedule or intensity of apoptosis can shift total neuron counts, alter connectivity patterns, and ultimately impair complex cognitive abilities, even in the absence of visible tissue damage (Dekkers et al., 2013; Martin, 2001; Sastry and Rao, 2000).

Cell culture work reinforces these findings: commercial PCB mixtures can trigger apoptosis in neurons at concentrations that fall below those causing overt cytotoxicity (Klocke 2020), with studies of individual congeners pointing toward NDL-PCBs as the main drivers of this effect (Klocke 2020). For example, PCB 52 induces apoptosis in human SK-N-MC neurons through p53-independent mechanisms. Similarly, PCB 47 (a NDL congener) increased caspase-dependent apoptosis in rat hippocampal neurons, whereas PCB 77 (a DL congener) did not (Howard 2003). However, not all findings are aligned. Another study observed both PCB 153 (NDL) and PCB 77 (DL) increasing apoptosis in rat cortical neurons, with PCB 77 producing the stronger effect (Sanchez-Alonso 2004). These discrepancies in PCB 77 effects most likely stem from methodological differences. One striking difference is culture conditions: serum-free medium in one study (Howard 2003) versus serum-supplemented in the other (Sanchez-Alonso 2004). More likely, the divergence reflects the concentration ranges tested, sub-micromolar levels in first study versus 30–100 μM in the second, with the higher concentrations causing overt cytotoxicity, which raises doubts about whether the apoptotic activity observed with extremely high concentrations is biologically relevant.

Adding another layer of complexity, Howard et al. (2003) also found that PCB 104 (NDL) failed to induce apoptosis in either hippocampal or cortical neurons, highlighting that pro-apoptotic activity is not universal across NDL congeners.

In vivo studies using rat models reiterate this sensitivity. When dams were fed the commercial PCB mixture Aroclor 1254 during pregnancy and nursing, offspring displayed sharply elevated apoptosis in multiple brain regions at postnatal day 1, but by day 21 levels had returned to baseline (Yang 2010). Caspase-3 activation and TUNEL staining confirmed these effects, with the cerebellum showing the largest increase.

Axonal and Dendritic Morphogenesis

The architecture of axons and dendrites is critical to how neurons connect and communicate (Paus 2023). The growth and branching of dendrites determine how many connections, and subsequent input, a neuron can receive, whereas axons determine where those connections land (Hoersting 2021). Alterations in these growth patterns, whether reduced, excessive, or mistimed, are implicated in NDDs and cognitive impairment (Supekar, Uddin et al. 2013, Copf 2016). Many rodent studies have demonstrated that even brief disruptions to axonal or dendritic development can leave lasting behavioral marks.

Direct in vivo evidence of PCB-induced changes to axonal growth is scarce. In the only published example (Pruitt 1999), rats receiving high-dose Aroclor 1254 before and after birth showed a selective shrinkage of II-P mossy fibers in the hippocampus, while other mossy fiber populations and cortical thickness were unaffected. Why this specific subset of granule cells is vulnerable remains unknown. In vitro, most tested congeners, such as PCB 95 and PCB 136, did not alter axonal length in primary rat hippocampal or cortical cultures (Wayman, Bose et al. 2012, Yang 2014). One striking exception is PCB 11, a NDL- LC-PCB congener recently found in the environment, along with its hydroxylated and sulfated metabolites, stimulated axonal growth at femtomolar concentrations (Sethi 2017).

How PCBs alter dendrites is a more developed story. Multiple in vivo studies using Aroclor 1254 have reported altered dendritic arborization in the offspring of exposed rats (Roegge, Morris et al. 2006, Lein 2007, Yang 2009). All used similar exposure windows, beginning before conception, through gestation, and continuing through lactation, and the same high dietary dose of 6 mg/kg/day, though (Yang 2009) also tested a 1 mg/kg/day group. Using Golgi staining, two studies found significant dendritic changes, while one did not. Lein et al. (2007) saw an unusual developmental trajectory: dendrites in hippocampal CA1 and Purkinje cells grew more slowly at postnatal day 22 but caught up or exceeded controls by day 60. Yang et al. (2009) saw increased baseline arborization and an impaired ability to expand

dendrites in response to experience, and Roegge et al. (2006), by contrast, saw no change in Purkinje dendrites at day 21. Differences in the developmental stage at assessment may explain these inconsistencies.

DNT testing of individual PCB congeners has been critical to furthering our understanding. Developmental exposure to PCB 95 at 0.1-1 mg/kg/day mimicked the dendritic effects of Aroclor 1254, producing greater arborization in hippocampal CA1 neurons at postnatal day 38, but showing no enhancement at the highest tested dose, an example of a non-monotonic dose-response (Wayman 2012). In vitro, low-nanomolar to picomolar concentrations of PCB 95 and PCB 136 stimulated dendritic growth in dense neuron-astrocyte cocultures without affecting viability (Wayman 2012, Lesiak, Zhu et al. 2014, Yang 2014). These effects were absent at low-micromolar concentrations, though the reason remains unclear. PCB 95 also increased dendritic spine density in hippocampal neurons (Lesiak, Zhu et al. 2014).

PCB 11 is one of the most notable congeners altering neuronal morphogenesis. In vitro, it and its metabolites enhanced dendritic arborization in hippocampal and cortical neurons at concentrations as low as one femtomolar, a concentration comparable to levels detected in the serum of pregnant women in Northern California (Sethi 2018, Klocke 2020). Unlike legacy mixtures and HC- congeners, PCB 11 also drives axonal growth, suggesting distinct underlying mechanisms (Sethi 2017). This raises urgent questions about whether other common LC-PCB congeners in human blood exert similar effects.

There have been other hydroxylated metabolites that have influenced dendrite development, as well. For instance, 4-hydroxy (para-substituted) derivatives of PCBs 112, 165, and 187 stimulated dendritic growth in cultured Purkinje cells, while metabolites of PCBs 106, 121, and 159 had no such effect but did block thyroid hormone-driven dendritic expansion (Kimura-Kuroda, Nagata et al. 2007). The proposed mechanism involves structural mimicry of thyroid hormones, though direct receptor interaction was not shown. These findings reinforce

that dendrite-promoting ability is not shared by all congeners. For example, PCB 66, despite physicochemical similarities to PCB 95, failed to enhance dendritic arborization at concentrations where PCB 95 was effective (Wayman, Bose et al. 2012). The capacity to modulate RyRs may be a determining factor.

Finally, species and brain-region differences add further complexity. PCB 95 and PCB 11 both enhanced dendritic growth in primary mouse hippocampal and cortical neurons, but the effects were sex-dependent, and the pattern of sex specificity varied by region (Sethi, Keil et al. 2017, Keil 2019, Keil Stietz 2021). These differences may relate to variations in developmental timing of neuronal maturation, consistent with in vivo evidence that dendritic effects change across developmental windows. Whether human neurons show similar sex-specific sensitivity remains an open question, and if confirmed, could help explain the sex bias observed in many NDDs.

Key Data Gaps in PCB Developmental Neurotoxicity Relevant to this Thesis

There is compelling evidence from human epidemiological, experimental animal, and in vitro studies that PCBs are developmental neurotoxicants associated with several neurological deficits in early life. While there are discrepancies across studies, most human epidemiological studies have found an association between exposure to PCBs and detrimental effects on the developing brain across diverse human populations in multiple geographic locations. The developmental neurotoxicity of PCBs has been largely corroborated in experimental animal and *in vitro* models. Although less well-studied, the influence of PCB exposure on the risk, onset, and severity of affective disorders in adults and late-onset neurodegenerative disorders is suggestive. A significant data gap, however, is the lack of animal studies to corroborate the human observations of PCB effects on neurological outcomes in mid- and later life (Hersi, Irvine et al. 2017, Martino, Candundo et al. 2017, Wang, Little et al. 2017).

It is important to note that the diversity of PCB chemistry, including degree and position of chlorination, chirality, and diverse metabolites, makes the design of animal and epidemiological studies challenging. This complication underlies another key data gap in the field: a comprehensive understanding of which PCB congeners influence neurotoxicity, and how their metabolism alters neurotoxic outcomes. Addressing this gap is further hindered by a lack of knowledge regarding the predominant PCB congeners and metabolites present in human tissues, and how this congener profile is impacted by population demographics (age, sex, and socioeconomic status), time of exposure, and changing environmental PCB profiles.

Experiments designed to assess the consequences of PCB exposures on complex neurological conditions present additional challenges. Gene-environment interactions are implicated in many neurodevelopmental disorders (Lein 2015, Hertz-Picciotto 2018, Keil-Stietz 2023) and aging-related declines in cognitive capacity, clinical dementia, and major neurodegenerative disorders such as Parkinson's disease (PD) (Martino, Candundo et al. 2017), Alzheimer's disease (AD) (Hersi, Irvine et al. 2017), and amyotrophic lateral sclerosis (ALS) (Wang, Little et al. 2017) have both genetic and environmental components that contribute to temporal onset, progression, and severity. A major challenge in the field is the identification of specific genetic risk factors that influence neurotoxic responses to PCBs, and the elucidation of the mechanisms by which genetic factors interact with PCBs to determine individual risk for adverse neurological outcomes.

By framing these limitations as critical data gaps, this thesis aims to address how specific PCB congeners and their metabolites influence neuronal development, how sex and modifies these effects, and how these interactions may contribute to the risk of neurodevelopmental disorders.

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DECLARATION OF COMPETING INTERESTS

PJL was hired as an expert witness by lawyers representing a group of plaintiffs alleging they were harmed by exposure to PCBs in school air. In that capacity, she testified as an expert witness on PCB neurotoxicity. The defendant was Pharmacia, a successor company to Monsanto.

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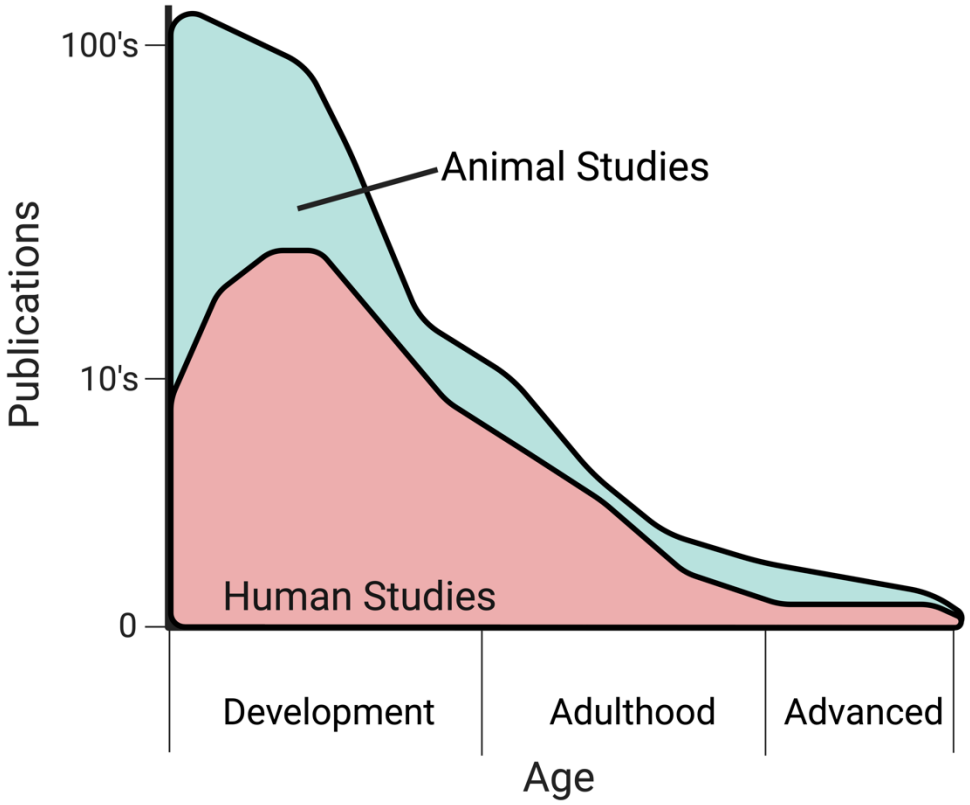


Figure 1.1. Distribution of literature assessing neurotoxic effects of PCB exposures in humans and animal studies. The graph is organized by the magnitude of order of number of publications as a function of the age of exposure. The majority of literature is focused on PCB

effects on the developing brain, with the number of publications decreasing as the age at which neurotoxic outcomes are assessed.

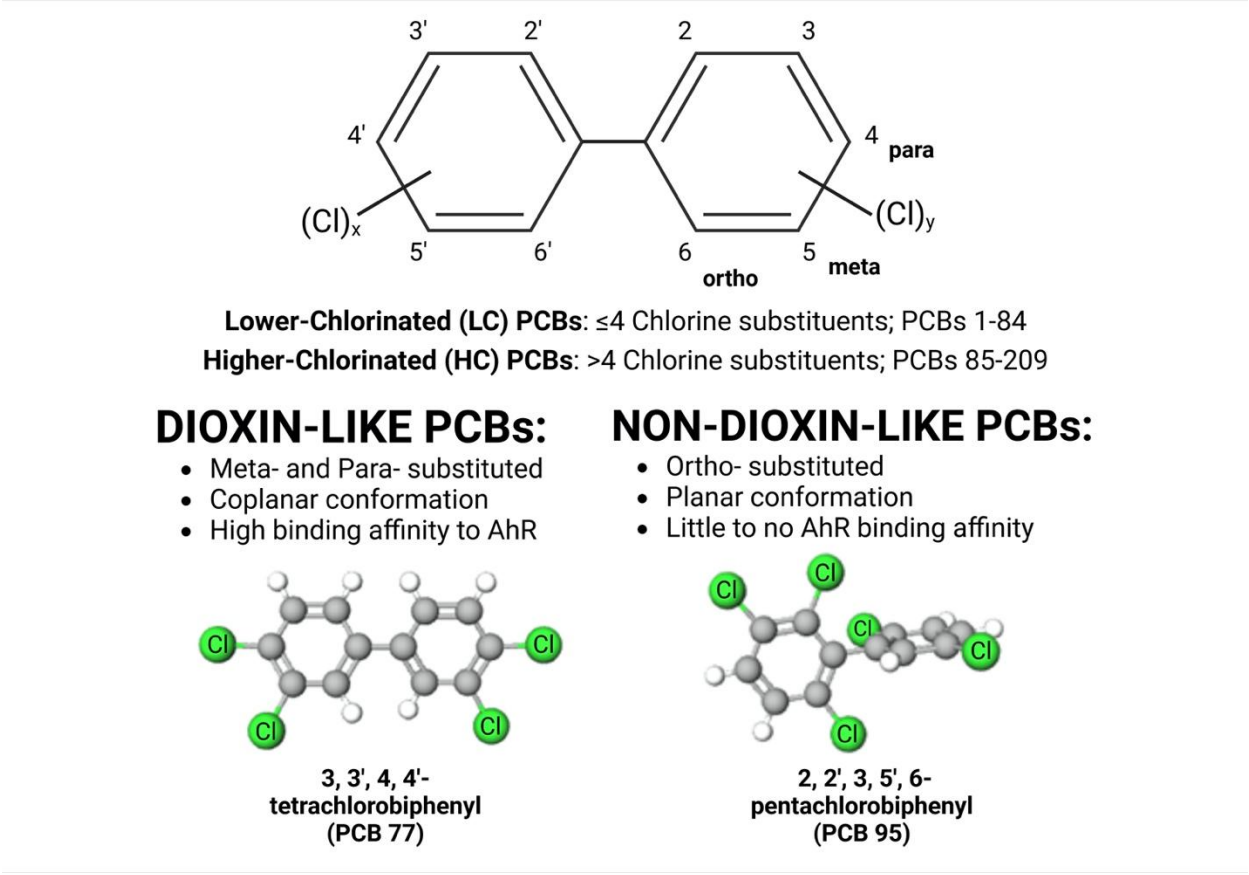


Figure 1.2. General chemical structure, classifications, and examples of PCB congeners. PCBs are a structurally related group of chemicals comprised of a biphenyl structure with varying numbers of chlorine substitutions. There are 209 possible congeners that can be categorized as either lower chlorinated (LC) or higher chlorinated (HC) PCBs depending on the number of chlorine substituents. PCBs are also classified as dioxin-like or non-dioxin-like PCBs. Dioxin-like PCBs, exemplified by PCB 77, assume a coplanar conformation similar to dioxin and bind with relatively high affinity to the aryl hydrocarbon receptor (AhR). In contrast, non-dioxin-like PCBs, such as PCB 95, which typically have >1 ortho-substituted chlorine, have a non-coplanar structure and low to negligible AhR binding.

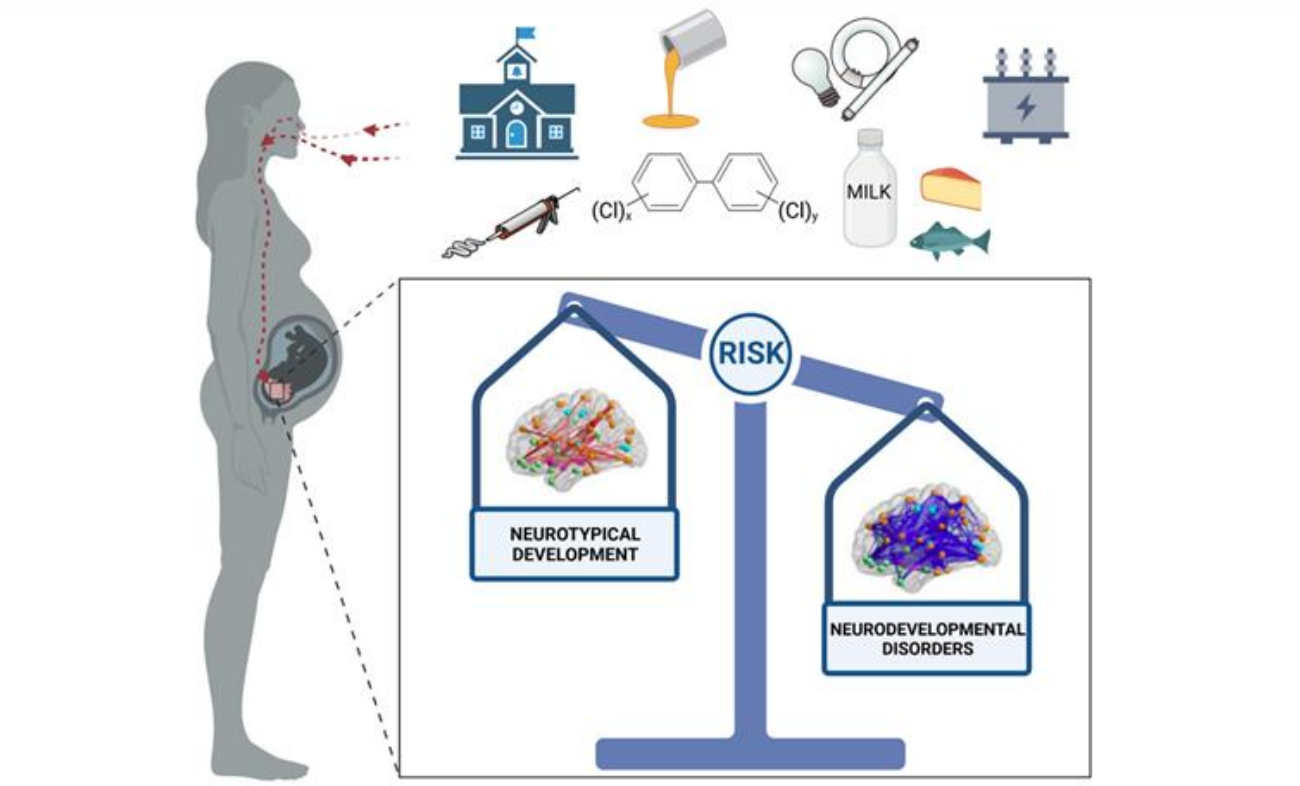


Figure 1.3. Development PCB neurotoxicity. There is documented widespread exposure of pregnant and lactating women to PCBs via ingestion of contaminated foods and inhalation of contaminated air. Sources of PCBs include indoor air, particularly in municipal buildings constructed prior to the ban on PCB production, such as public schools, paints and caulking, fluorescent light ballasts, electrical transformers, and fatty foods, particularly dairy products and fatty fish. Epidemiological and experimental data suggest that exposure to PCBs during critical neurodevelopmental windows may increase risk of neurodevelopmental disorders.

Table 1. Epidemiological studies of PCB effects on the developing brain.

Cohort/Study	Sample Size	Exposure Measure	Median Concentration	Cognitive Tests	Age at Testing	Significant Cognitive Results	Reference(s)
Michigan Cohort	226	Cord serum	0.4 ng/mL	MCSA, Sternberg, Kagan's; WISC- R	4 years; 11 years	↑ Short-term memory task errors ↓ Visual discrimination; ↓ Full-scale IQ	Jacobsen et al. 1992
Michigan Cohort	212	Cord serum	3 ng/mL	WISC-R WRAT-R WRMT- R	11 years	↓ Full Scale IQ ↓ Verbal IQ ↓ Reading comprehension	Jacobsen et al. 1996
The Dutch Cohorts	~400	Maternal blood, cord serum, and breast milk	2.21 ng/g, 0.45 ng/g, 428.5 ng/g	Prechtl; BSID psychomotor development; K-ABC	10 and 21 days; 18 and 42 months	↓ PDI at 7 months; ↓ Neurological optimality at 18 months; ↓ K-ABC cognition and language at 42 months	Koopman-Esseboom et al. 1995; Reviewed in Schantz et al. 2003 and Pessah Et al. 2019
Oswego Cohort	309 mom-child pairs	Cord blood, breast milk, and placenta	0.52 ng/g, 153 ng/g, 1.5 ng/g	MSCA; WISC-IIIa	3-4 years; 9 years	↓ MCSA ↓ Full scale IQ ↓ Verbal IQ	Stewart et al. 2003, 2008; Reviewed in Pessah 2019

The German Cohort	171 mom-child pairs	Cord blood and breast milk	0.55 ng/mL, 426.5 ng/g	BSID-II menta (MDI) l and psychomotor development index; FTII	7 months	↓BSID-II MDI; ↓BNT	Winneke et al.1998; Reviewed in Schantz et al. 2003 Grandjean et al. 1997, 2001, Steuerwald et al. 2000; Reviewed in Schantz et al. 2003 and Pessah et al. 2019
Faroe Islands Cohorts	435		1.88 µg/L	NES-2 Finger Tapping, Hand-Eye Coordination; BVMG; WISC-R; CVLT; BNT	7years		
New Bedford Cohort	393	Cord serum	0.38 ng/g	WRAML	8 years	No significance	Orenstein et al. 2014; Reviewed in Schantz et al. 2003 and Pessah et al. 2019
Flemish Cohort	206 mom-child pairs	Cord blood; PCBs 138, 153, 180, 118, 170	Total PCBs (183+153+180) 87.9 ng/g; PCB 118 14.9 ng/g, PCB 170 8.9 ng/g	RTOS, SON, BSID, NES, IBQ	3 years	↓ Activity, delayed first steps, ↓ Language development, ↓ Non-verbal IQ scores in boys, ↓ Gender-specific play	Vermeir et al. 2021
Greece Cohort	689	Maternal serum; PCBs 118,	20.3, 149.7, 80.0, 8.2, 85.6, 42.3	MSCA	4 years	↓ Working memory	Kyriklaki et al. 2016

		153, 138, 156, 180, 170	pg/mL, respectively				
Norwegian Cohort	1024 children	Dietary exposure; PCB 153	0.81 ng/kg bw/day	D-B Grammar Scale; ASQ	3 years	↑ Instance of incomplete grammar; ↓ Language delay; ↓ Communication score	Casperson et al. 2016
Collaborative Perinatal Project	894	Maternal serum; Total PCB (PCBs 28, 52, 74, 105, 118, 138, 153, 170,180, 194, 203)	2.85 ug/L	WISC; WRAT-R	7 years	No significance	Gray et al. 2005; Reviewed in Pessah et al. 2019

ASQ = Ages and Stages Questionnaire. BNT = Boston Naming Test. BSID=Bayley Scales of Infant Development. CVLT = California Verbal Learning Test. D-B Grammar = Dale and Bishop Grammar Scales. IBQ = Infant Behavior Questionnaire. FTII= Fagan Test of Infant Intelligence. MSCA = McCarthy Scales of Children's Abilities. NES = Neurobehavioral Evaluation System. RTOS = Reynell Taal Ontwikkelings Schalen (language development). SON = Snijders-Oomen Niet (Non-verbal intelligence). WISC – Weschler Intelligence Scale for Children. WRAML = Wide Range Assessment of Memory and Learning. WRAT = Wide Rage Achievement Test.

Table 2. Epidemiological studies of PCB effect on affective disorders in adults.

Reference	Study findings	Cohort Location	Subject age (years)	Median/mean concentrations	Outcome measure	PCB exposure matrix (congeners)	Sample size	Study limitations
Akahane et al., 2018	Increased prevalence of bipolar disorder among survivors of PCB poisoning, relative to individuals with no history of PCB poisoning	Japan	30-100	~	~	~	2329	No quantification of PCBs in biospecimens
Fitzgerald et al., 2008	Increase in depressive symptoms with increasing concentrations of PCBs	United States (New York)	55-74	Mean=3.1 ng/g (wet weight)	NART-R, TOMM, CVLT, WMS, SCWT, WCST, DSST, BDT, SMST, GPT, FOT, BDI, STAI, SIT	Serum (PCBs 28, 74, 99, 105, 118, 138, 153, 170, 180, 183, 187, 194)	253	
Guam et al., 2014	Association between higher PCB concentrations and increases in prevalenc	Germany	Mean= 47.2	Mean: LPCBs=3.18 µg/L; HPCBs=7.78 µg/L; dlPCBs=2.62 µg/L	PHQ-D	Plasma (PCBs 28, 52, 101, 138, 153, 180, 105, 114, 118,	136	Small sample size

	e and severity of depressive symptoms					123, 156, 157, 167, 189)		
Guam et al., 2017	Positive associations between depressive symptoms and concentrations of LPCBs, HPCBs, and dlPCBs; association between increased exposure to LPCBs, HPCBs, and dlPCBs and disrupted dopaminergic signaling; positive association between disrupted dopaminergic signaling and depressive symptoms	Germany	Mean= 46.9	Mean: LPCBs=353.1 ng/g lipid; HPCBs=933.05 ng/g lipid; dlPCBs=329.6 ng/g lipid	BDI	Plasma (PCBs 28, 52, 101, 138, 153, 180, 105, 114, 118, 123, 156, 157, 167, 189)	178	Small sample size
Kilburn et al., 1989	More depressed mood in PCB-exposed participants	United States (New York)	Mean= 34.3	Median=6.0 ppb (serum); 0.7 ppm (body fat)	POMS, CRT, WMS, WAIS	Serum; body fat (Aroclor 1248)	28	Small sample size

dlPCBs: dioxin-like PCBs, LPCBs: lower chlorinated PCBs, HPCBs: higher chlorinated PCBs; ALSFRS-R: ALS Functional Rating Scale Revised, ATC: Anatomical Therapeutic Chemical, BDI: Beck's Depression Inventory, BDT: Block Design subtest, CRT: Choice Reaction Time, CVLT: California Verbal Learning Test, DSST: Digit Symbol Substitution Test, FOT: Finger Oscillation Test, GPT: Grooved Pegboard Test, ICD: International Classification of Diseases, NART-R: New Adult Reading Test-Revised, PHQ-D: Patient Health Questionnaire, POMS: Profile of Mood States, SCWT: Stroop Color-Word Test, SIT: Smell Identification Test, SMST: Static Motor Steadiness Test, STAI: State-Trait Anxiety Inventory, TOMM: Test of Memory Malingering, WAIS: Wechsler Adult Intelligence Scale, WCST: Wisconsin Card Sorting Test, WMS: Weschler Memory Scale

Table 3. Epidemiological studies of PCB effects on neurodegenerative diseases.

Reference	Main findings	Cohort	Subject age (years)	Mean/Median PCB concentration	PCB exposure matrix (congeners)	Sample size	Study limitations
Akahane et al., 2018	Increased prevalence of dementia among survivors of PCB poisoning, relative to individuals with no history of PCB poisoning	Japan	30-100	~	~	2329	No quantification of PCBs in biospecimens
Corrigan et al., 1998	Higher concentrations of PCB-153 in the anterior caudate nucleus of PD patients	United Kingdom (Scotland, England)	50-85	Median=9.70 µg/g lipid (PD); 1.06 µg/g lipid (control)	Brain tissue (PCBs 8, 18, 28, 31, 52, 77, 101, 118, 126, 128, 138, 149, 153, 169, 170, 180)	15	Small sample size
Corrigan et al., 2000	No significant difference in substantia nigra PCB concentrations in PD patients compared to healthy controls	England	~	Mean=1.013 µg/g lipid	Brain tissue (Aroclor 1254 PCB congeners)	28	Small sample size
Goutman et al., 2019	Decreased survival in ALS patients	United States (Michigan)	Median=61.6	Mean (ng/L): PCB 110=2.4; PCB	Plasma (PCBs 110, 118, 138, 151,	167	Small sample size

	with higher PCB concentrations			118=34.5; PCB 138=64.3; PCB 151=50.5 ng/L; PCB 153=238; PCB 174=0.9; PCB 175=1.5; PCB 180=94.9; PCB 202=3.0	153, 174, 175, 180, 202)		
Hatcher-Martin et al., 2012	Higher PCB concentrations associated with increased nigral neuron depigmentation; Increased concentrations of PCBs 153 and 180 in occipital cortex of PD patients	United States	Mean=70.7	Mean=8.65 ng/g (wet weight)	Brain tissue (PCBs 101, 118, 138, 149, 153, 166, 170, 180)	72	Small sample size
	Elevated concentrations of total PCBs and congeners 138, 153, and 180 in females with PD		Mean=95	Mean=2.47 ng/g (wet weight)		40	Small sample size
Koldkjær et al., 2004	No significant difference in PCB concentrations of PD group compared	Greenland	Mean=63	~	Plasma	153	Small sample size; sample size much smaller for PD than control

	to control group						group; far more male than female PD cases
Peterse n et al., 2008	Consumpti on of larger amounts of whale meat and blubber linked to greater risk for PD; Serum concentrati ons of PCB-101 correlated significantly with PD risk	Faroe Islands (Kingdom of Denmark)	Mean=74 .9	Mean=4.96 mg/g lipid	Serum (PCBs 101, 105, 118, 138, 153, 156, 180)	233	Lack of data on other toxins typically present at elevated concentratio ns in whale meat
Prince et al., 2006	Elevated mortality due to diseases of the nervous system and sense organs (including PD and ALS) among women, but not men, who were occupation ally exposed to high levels of PCBs; Increased ALS-related deaths among women, but not men, who were occupation	United States (New York, Massachus etts)	18.5-102; Mean=63 .6	~	Aroclor 1254, 1242, and 1016	2572	No quantificatio n of PCBs in biospecime ns; small cohort size; Low number of deaths; Lack of data on potential confounder s

	ally exposed to high levels of PCBs						
Putschögl et al., 2015	Reduced levels of the dopamine metabolite HVA detected in urine of individuals exposed to high levels of PCBs	Germany	16-73	Mean (µg/L): LPCBs=2.5; HPCBs=7.2; dlPCBs=2.3	Plasma (PCBs 28, 52, 101, 138, 153, 180, 105, 114, 118,123, 156, 157, 167, 189)	177	Relationship between PCB exposure and HVA not held true for all observed timepoints; Large PCB exposure duration range; Neurotransmitter metabolite concentrations in urine may not accurately reflect neurotransmitter concentrations in CNS
Raffetti et al., 2020	Higher concentrations of PCBs linked to greater risk for dementia	Italy	Over 50; Mean=63.2	Mean=5.88 ng/mL Median=5.71 ng/mL	Serum (PCBs 28, 31, 52, 77, 81, 101, 105, 114, 118, 123, 126, 128, 138, 153, 156, 157, 167, 169, 170, 180, 189, 194, 206, 209)	699	
Seegal et al., 2010	Reduced DAT density in caudate, putamen, and combined	United States (New York)	51-85; Mean=63.5	Mean=0.98 ppm	Serum (ΣPCBs)	85	Subjects assessed only at a single timepoint; Small sample size

	caudate and putamen (striatum) of women, but not men, who had higher levels of occupational exposure to PCBs						
Steenland et al., 2006	Increased mortality due to ALS among occupationally exposed women, but not men; Increased numbers of PD- and AD-related deaths among women, but not men, who had the highest levels of occupational PCB exposure	United States (New York, Massachusetts)	Mean=64	~	~	16906	No quantification of PCBs in biospecimens
Su et al., 2016	Plasma concentrations of PCB-151 were elevated and PCB-202 reduced in ALS patients compared to healthy controls	United States (Michigan)	Mean=60.5	Mean (ng/L): PCB 110=2.85, PCB 151=11.05, PCB 135/144=28.55, PCB 118=132.63, PCB 132/153=390, PCB 138/163=238.93, PCB 175=2.61, PCB	Plasma (PCBs 110, 118, 132, 135, 138, 144, 151, 153, 163, 170, 174, 175, 180, 190, 198, 202)	284	

				174=1.37, PCB 202=4.12, PCB 180=166.32, PCB 170/190=12.14, PCB 198=1.98			
Weisskopf et al., 2012	Trend toward decreased risk of PD with increasing serum PCB concentration	Finland	20-79; Mean=52.1	Mean=6.74 ng/g serum (PD); 8.02 ng/g serum (control)	Serum (PCBs 6, 8, 16, 18, 25, 26, 28, 31, 33, 37, 41, 44, 47, 49, 52, 60, 66, 70, 74, 84, 87, 95, 97, 99, 101, 105, 110, 118, 128, 135, 136, 138, 141, 146, 149, 151, 153, 156, 157, 167, 170, 171, 174, 177, 201, 180, 183, 187, 189, 194, 195, 196, 199, 203, 206, 209)	450	

AD: Alzheimer’s disease, ALS: amyotrophic lateral sclerosis, PD: Parkinson's disease; dlPCBs: dioxin-like PCBs, LPCBs: lower chlorinated PCBs, HPCBs: higher chlorinated PCBs; ALSFRS-R: ALS Functional Rating Scale Revised, ATC: Anatomical Therapeutic Chemical, BDI: Beck's Depression Inventory, BDT: Block Design subtest, CRT: Choice Reaction Time, CVLT: California Verbal Learning Test, DSST: Digit Symbol Substitution Test, FOT: Finger Oscillation Test, GPT: Grooved Pegboard Test, ICD: International Classification of Diseases, NART-R: New Adult Reading Test-Revised, PHQ-D: Patient Health Questionnaire, POMS: Profile of Mood States, SCWT: Stroop Color-Word Test, SIT: Smell Identification Test, SMST: Static Motor Steadiness Test, STAI: State-Trait Anxiety Inventory, TOMM: Test of Memory Malingering, WAIS: Wechsler Adult Intelligence Scale, WCST: Wisconsin Card Sorting Test, WMS: Weschler Memory Scale

Chapter 2

Adopted from the publication in Neurotoxicology in Elsevier with the following title and authors:

PCB 37 (3,4, 4'-trichlorobiphenyl) increased apoptosis and modulated neuronal morphogenesis in primary rat cortical neuron-glia cocultures in a concentration-, sex-, age-, and CREB-dependent manner

Authors: Jessie R. Badley[a], Peter M. Andrew[a], Hans-Joachim Lehmler[b], Pamela J. Lein[a]

Abstract

Higher-chlorinated (HC) polychlorinated biphenyls (PCBs) are known developmental neurotoxicants. In contrast, there are limited data regarding the developmental neurotoxicity of lower-chlorinated (LC) PCBs despite the increasing environmental prevalence and detection of LC-PCBs in contemporary human tissues, including the perinatal brain. This study characterized the neurotoxic effects of the LC-PCB congener PCB 37 in primary male and female rat cortical neuron-glia co-cultures. Cultures were exposed to varying concentrations of PCB 37 for 48 h beginning on day *in vitro* 0 or 7. Cell viability was assessed by measuring lactate dehydrogenase release into the culture medium and the percentage of live cells identified using Calcein-AM and Hoechst staining. Apoptosis was measured using fluorometric assays of caspase 3/7 activity and Annexin V binding. Axonal and dendritic growth were quantified in neurons immunostained for Tau-1 or transfected with MAP2B-red fluorescent protein, respectively, using automated image analysis protocols. At environmentally relevant concentrations (0.0001, 0.1, and 100 nM) that did not affect cell viability, PCB 37 caused sex-, age-, and concentration-dependent increases in apoptosis, axonal, and dendritic growth. Pretreatment with the CREB inhibitor 666-15 (500 nM) blocked the effects of PCB 37 on apoptosis and dendritic morphology but not axonal growth. These findings suggest that the pro-apoptotic and dendrite-promoting effects of PCB 37 are mediated by CREB signaling, but that CREB-independent mechanisms underlie PCB 37 effects on axonal growth. Overall, these findings identify PCB 37 as a potential developmental neurotoxicant and further support increasing evidence identifying CREB as a convergent mechanism of developmental neurotoxicity.

Keywords: axonal outgrowth, dendritic arborization, developmental neurotoxicity, in vitro model, PCBs

1. Introduction

Polychlorinated biphenyls (PCBs) remain a significant risk to human health, and the developing brain has been identified as a vulnerable target (Schantz, Widholm et al. 2003, Boucher, Muckle et al. 2009, Berghuis, Bos et al. 2015, Pessah, Lein et al. 2019, Klocke and Lein 2020). Prior studies in both rodent models (Klocke, Sethi et al. 2020, Carlson, Christensen et al. 2023) and humans (Schantz, Widholm et al. 2003, Balalian, Stingone et al. 2024) have reported positive associations between developmental PCB exposures and impaired cognitive function. More recent epidemiological literature suggests that developmental exposure to PCBs may also increase risk for neurodevelopmental disorders (Keil-Stietz and Lein 2023). This epidemiological association is supported by observations from experimental animal studies demonstrating that developmental exposure to PCBs causes adverse neurodevelopmental outcomes that have been implicated in the etiopathogenesis of many neurodevelopmental disorders (Keil-Stietz and Lein 2023).

The mechanism(s) underlying PCB developmental neurotoxicity (DNT) remain speculative. It is known that developmental exposure to PCBs does not cause overt structural damage, but rather induces subtle organizational defects that alter the patterning of synaptic connections in the developing brain (Pessah, Lein et al. 2019, Klocke and Lein 2020). Two critical determinants of synaptic connectivity are neuronal apoptosis and neuronal morphogenesis, the latter encompassing axonal outgrowth and dendritic arborization. Spatiotemporal changes in apoptosis, axonal outgrowth, or dendritic arborization are thought to underlie cognitive deficits and many of the behavioral abnormalities associated with neurodevelopmental disorders (Stamou, Streifel et al. 2013, Guang, Pang et al. 2018, Panesar, Kennedy et al. 2020).

PCBs have been shown to interfere with apoptosis (Howard, Fitzpatrick et al. 2003, Yang and Lein 2010), axonal outgrowth (Sethi, Keil et al. 2017, Pessah, Lein et al. 2019), and dendritic arborization (Yang, Kim et al. 2009, Wayman, Yang et al. 2012, Sethi, Keil et al. 2017).

However, most studies of PCB developmental neurotoxicity have focused on Aroclors, the legacy PCB mixturess produced and commercially marketed in the United States from 1920s-1970s, or the higher-chlorinated (HC)-PCBs found in the legacy PCB mixtures (Klocke and Lein 2020). In contrast, there are limited data available regarding the DNT potential of lower-chlorinated (LC)-PCBs, which are defined as congeners with $\leq$ 4 chlorine substitutions. This is a substantial data gap because, in contrast to HC-PCBs, environmental levels of LC-PCBs, many of which are inadvertent industrial byproducts (Hu 2009), are increasing worldwide (Grimm, Hu et al. 2015, Melymuk, Blumenthal et al. 2022). LC-PCBs are regularly detected in samples of indoor and outdoor air, water, food, and in human tissues, including breast milk and brain (Klocke and Lein 2020, Wang, Adamcakova-Dodd et al. 2020, Li, Hefti et al. 2022) and have been found to comprise more than 70% of the total PCBs in the serum of pregnant women who are at increased risk of having a child with a neurodevelopmental disorder (Sethi, Keil et al. 2017, Sethi, Morgan et al. 2019).

In prior studies, we found that the LC congener PCB 11 and its major human metabolites significantly increased axonal and dendritic growth in primary cortical rat neurons grown in neuron-glia co-cultures (Sethi, Keil et al. 2017) and that the dendrite-promoting activity of PCB 11 was mediated by CREB signaling (Sethi, Keil et al. 2018). However, whether these observations extend to all LC-PCBs is unknown. A recent post-mortem analysis of neonatal and adult brains detected the LC-PCB, PCB 37, in 31-40% of all tissue analyzed (Li, Hefti et al. 2022). PCB 37 has also been found in indoor air samples (Kraft, Sievering et al. 2018). Thus, in this study, we evaluated the developmental neurotoxicity potential of PCB 37 using sex-segregated primary rat cortical neuron-glia cocultures.

2. Materials and Methods

2.1 Materials

PCB 37 (3,4,4'-trichlorobiphenyl; catalog: C-37N, lot#11230-02) was purchased from AccuStandard Inc. (New Haven, CT). Stock solutions of PCB 37 (1000X) were made in dry sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) and stored at room temperature in the dark. Plating and growth media were prepared, as previously described (Sethi, Keil et al. 2017). Briefly, plating medium consisted of Neurobasal Plus medium (Gibco™, Thermo Fisher Scientific, Waltham, MA) supplemented with 2% B27 (Gibco™), 1x GlutaMAX supplement (Gibco™), 10% heat-inactivated horse serum (Gibco™), and 1 M HEPES buffer (pH 7.5). Growth medium consisted of Neurobasal Plus medium supplemented with 2% B27 and 1x GlutaMAX. Feeding medium consisted of growth media supplemented with 5 μM arabinose-C (Thermo Fisher Scientific), an anti-mitotic to halt glial overgrowth. Stock concentrations of PCB 37 were diluted 1:1000 directly into growth medium at 1-2X the final concentration in the well; vehicle controls were were exposed to 0.001% DMSO in growth medium.

2.2 Cell culture

All experiments involving animals were conducted in strict accordance with the ARRIVE guidelines and protocols approved by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC). To account for variability, experiments were repeated using independent cell isolations, with both male and female cultures plated on the same plate to minimize plate effects. Plates included untreated controls, and image analysis was automated with the analyst blinded to treatment conditions until data visualization.Timed-pregnant Sprague Dawley rat dams were purchased from Charles River Laboratory (Hollister, CA) and individually housed in transparent plastic cages with corn cob bedding in a temperature—and humidity controlled vivarium maintained at 22 ± 2°C on a 12-h light-dark cycle. Animals were provided Teklad 2018 18% Rodent Feed (Inotiv, Lafayette, IN) and water *ad libitum*. Dams were imported 5 days before giving birth and allowed time for acclimatation.

Primary cortical neuron-glia co-cultures were dissociated from the neocortices of sex-segregated postnatal day 0 Sprague Dawley rat pups (Fig 1), as previously described (Sethi, Keil et al. 2017). Cell suspensions were plated in plating medium onto glass bottom 96-well plates (CellVis, Sunnyvale, CA) or German glass coverslips (Electron Microscopy Sciences, Hatfield, PA) in plastic Corning 24-well plates (Thermo Fisher Scientific) precoated with 0.5 mg/mL poly-L-lysine (Sigma-Aldrich) in B buffer (3.1 mg/mL boric acid and 4.75 mg/mL borax, pH 7.5, Sigma-Aldrich). To analyze the length of the axonal plexus of an individual neuron (Morgan 2020), cells were plated at 33,000 cells/cm^2; to quantify dendritic arbors, cells were plated at 83,000 cells/cm^2. Cell viability and caspase 3/7 activity were assessed at both cell densities. Cells were allowed to adhere to coverslips in plating medium for 4 h at which time the plating medium was replaced with growth medium. Cultures were maintained at 37°C under 5% CO_2. In cultures used to assess dendritic growth, half the medium in each well was exchanged at day *in vitro* (DIV) 4 with feeding medium.

2.3 PCB 37 exposure

Cortical neurons in culture develop structurally according to a stereotypic morphogenetic program (Fig 1). Within the first 24 h, neurites begin to project from the cell body. By 48 h, a single neurite begins to elongate more rapidly than the others and this differentiates into an axon. On days 3-7, the remaining neurites begin to differentiate into dendrites, and as the culture ages, the dendritic arbor becomes more complex as indicated by increasing length and branching (Flynn 2013, Wilson, Badley et al. 2024). To analyze axonal and dendritic morphology, two different exposure paradigms were used (Fig 1). Axonal outgrowth, cell viability, and caspase 3/7 activity were measured on DIV 2. For these experiments, cultures were exposed to vehicle (0.001% DMSO) or varying concentrations of PCB 37 for 48 h starting 4 h after plating cells. Dendritic arborization, cell viability, and caspase 3/7 activity were measured on DIV 9, in cultures exposed to vehicle (0.001% DMSO) or varying concentrations of PCB 37 for 48 h starting on DIV 7.

2.4 Cell viability

Two different assays were used to assess cell viability: (1) the live/dead assay and (2) the quantification of lactate dehydrogenase (LDH) released from cells into culture media. In the live/dead assay, calcein AM is used to label live cells. This membrane permeable dye is converted by intracellular esterases in live cells from the nonfluorescent parent compound into the fluorescent metabolite; dead cells do not have this metabolic capability. Hoechst 33342, which stains the nuclei of live and dead cells, is used to measure total cell number. To determine the percentage of live cells, cultures were co-stained with 0.5 μM calcein AM (Thermo Fisher Scientific) and 1 μg/ml Hoechst-33342 (Thermo Fisher Scientific) in 1x Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS; Thermo Fisher Scientific). After addition of these reagents, cultures were incubated at room temperature for 30 min covered from light. Cultures were then immediately imaged using an ImageXpress Micro XLS high content imaging system (Molecular Devices, San Jose, CA). The percentage of live cells was determined from the overlay of calcein AM and Hoechst-33342 labeling analyzed with MetaXpress 6.2 software (MetaXpress High Content Image Acquisition and Analysis Software, Live Dead Application Module, Molecular Devices) and was calculated as #calcein AM-labeled cells / #Hoechst-labeled nuclei X 100.

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI) a fluorometric assay, was used to measure LDH in the culture medium; this enzyme is released from cells with damaged membranes, which is a canonical indicator of cytotoxicity. Per the manufacturer's instructions, 100 μL of conditioned medium from each well was mixed with 100 μL CytoTox-ONE™ reagent and incubated at room temperature for 10 min. Stop solution was added, and the fluorescent signal was measured at 560/590 nm using a BioTek Gen5 plate reader (Agilent, Santa Clara, CA). The level of fluorescence is proportional to the number of

lysed, or dead, cells. Data were normalized as a percent of the fluorescent signal measured in vehicle-treated cells.

2.5 Apoptosis

Apoptosis was assessed by measuring two biomarkers of apoptosis: (1) caspase 3/7 activity and (2) annexin V binding. The Caspase-Glo® 3/7 assay (Promega) is a luminescence assay for quantifying caspase-3/7 activity. Per manufacturer directions, 100 μL Caspase-Glo® 3/7 reagent was added to each well followed by incubation at room temperature for 30 min. Staurosporine (10 μM; Millipore Sigma), a known apoptosis inducer, was used as a positive control. The amount of luminescence is directly proportional to caspase 3/7 activity. The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Promega) measures phosphatidylserine (PS), which is expressed on the outer leaflet of cell membranes during the apoptotic process (Schutte, Nuydens et al. 1998). The assay also includes a membrane-impermeant DNA-binding dye to label dead cells as a measure of membrane integrity. The amount of luminescence is proportional to PS and annexin V binding (early apoptosis), and the fluorescent signal is proportional to the loss of membrane integrity (necrosis). For all apoptotic assays, luminescence and fluorescence were quantified using a BioTek Gen5 plate reader (Agilent). Data were normalized as a percentage of the fluorescent signal measured in vehicle-treated cells.

2.6 Visualization of axons

On DIV 2, all culture media were removed, and cells were fixed with 4% (w/v) paraformaldehyde (ThermoFisher Scientific) in sodium phosphate buffer, 0.2M for 1 h. After washing 3 times with 1X DPBS, cells were then permeabilized with 0.5% (v/v) Triton X-100 (MilliporeSigma, Burlington, MA) in 1x DPBS for 5 min. Cells were washed 3 times with 1X DPBS, and then incubated at room temperature with a blocking buffer consisting of 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) in 1X DPBS for 1 h, followed by an overnight

incubation with mouse anti-tau-1 antibody, clone PC1C6 (Sigma-Aldrich # MAB3420, RRID:AB_3068606) diluted 1:1000 in blocking buffer at 4°C with gentle rocking. After a 24 h incubation, the primary antibody was removed, cells were washed 3 times with 1X DPBS, then incubated with a goat anti-mouse IgG2a Alexa fluor 488 (Thermo Fisher Scientific # A-21131, RRID:AB_2535771) diluted 1:1000 in blocking buffer on the rocker at 4°C covered from light for 1 h. Cells were washed 3 times in 1X DPBS, then coverslips were mounted cell-side down on SuperFrost glass slides (Thermo Fisher Scientific) in Prolong Gold Antifade Mounting media with DAPI (Thermo Fisher Scientific) and allowed to dry overnight at room temperature while from light before imaging. Brefeldin A (1 µg/mL;Thermo Fisher Scientific), an inhibitor of vesicular traffic, was used as a positive control (data not shown).

2.7 Visualization of dendrites

The plasmid encoding the MAP2B-pCAG-fusRED cDNA construct (Wayman, Impey et al. 2006), generously provided by Dr. Gary Wayman (Washington University, Pullman, WA), was transformed using One Shot TOP10 chemically competent *E. coli* (Thermo Fisher Scientific). Bacteria were plated on Luria-Bertani broth (Thermo Fisher Scientific) and ampicillin (AMP**,**100 mg/mL; Thermo Fisher Scientific) agar plates (MilliporeSigma) and incubated overnight at 37°C. After 24 h, a single colony was chosen and expanded in sterile Luria-Bertani broth + AMP overnight on the shaker at 220 rpm and at 37°C. On the following day, a Qiagen plasmid Maxiprep (Thermo Fisher Scientific) was performed per the manufacturer's instructions to isolate and purify MAP2B-pCAG-fusRED cDNA. Purified MAP2B-fusRED cDNA concentration was then measured on a Nanodrop 1000 spectrophotometer (ThermoScientific) using an absorbance value of 260 nm.

On DIV 6, 24 h before exposure to PCB 37, cultures were transfected with 0.8 µg of MAP2B-pCAG-fusRED cDNA in 2 µL of Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) per well. The MAP2B fusion protein is selectively localized to the somatodendritic compartment of neurons and does not alter their fundamental dendritic growth patterns in

culture (Wayman 2006). To achieve a low-efficiency (1-10%) transfection, the incubation time was reduced to only 2 h, followed by total replacement of the transfection medium with the conditioned medium. On DIV 7, cultures were exposed to vehicle or PCB 37 at varying concentrations. On DIV 9, media were removed, cells were fixed with 4% paraformaldehyde for 1 h, washed 3 times with 1X PBS, and then mounted cell-side down on glass slides (Thermo Fisher Scientific) with Prolong Gold Antifade Mounting media with DAPI (Thermo Fisher Scientific) and allowed to dry for 24 h at room temperature in the dark before imaging.

2.8 Morphometric analyses of axon outgrowth and dendritic arborization

The Automated ImageXpress high-content imaging system (Molecular Devices), which is interfaced to MetaXpress software (Molecular Devices), was used to acquire images from cell cultures immunostained for tau or transfected with MAP2B-FusRed (Morgan 2020). A total of 25 images per well or coverslip were acquired at 10X magnification, with at least 3 technical replicates per dissection, per experimental group, for a total of 75 images per group per independent dissection. For axon outgrowth, DAPI and FITC channels were used; for dendritic arborization, DAPI and Texas Red channels were used.

To measure axon outgrowth, images of tau-1 immunofluorescence were overlaid on fluorescent images of Hoechst-33342 staining from the same field. Axonal lengths were quantified in tau-1 immunopositive cells using the Neurite Outgrowth application module in the MetaXpress high-content image acquisition and analysis software.

Sholl analysis (Wilson, Sethi et al. 2017) was used to quantify dendritic arborization in cells expressing the MAP2B-FusRed protein. After images of MAP2BFusRed-transfected neurons were acquired on the high-content imaging system, a series of custom ImageJ macros (DOI: 10.5281/zenodo.15749929) (Badley 2025); see supplemental files) were used for automated analysis of dendritic arborization. These macros automatically identify fluorescently labeled neurons associated with a nucleus, extract image data from the background, select center points for the Sholl rings, and subsequently automatically count the number of dendritic

processes of individual neurons that intersect with each of the concentric Sholl rings. Criteria used to include labeled neurons in the automated analysis included: (1) the fluorescence image had only one associated nucleus (e.g., clumps of cells were not included in the analysis); (2) the neuron was isolated (e.g., its processes did not intermingle with processes of another labeled neuron); and (3) the image was not located on the border of the field or cut off by the field edges.

2.9 CREB inhibition

To investigate the role of CREB signaling in mediating PCB 37 effects, CREB was inhibited by pretreating cells with 666-15 (Millipore sigma, 5383410001), a selective CREB inhibitor, at a final concentration of 500 nM. The inhibitor was added to the culture medium 30 min prior to PCB 37 exposure.

2.10 Statistical analysis

Power analysis based on prior data (Sethi, Keil et al. 2018) indicated that a sample size of 4 independent dissections (3 wells/group in each dissection) would provide sufficient statistical power (≥80%) to detect a significant effect size. All statistics were done in GraphPad Prism (RRID:SCR_002798) version 10.0.0. All data, except the Sholl analysis, were analyzed using an ordinary one-way ANOVA with a *post-hoc* Dunnet's test to identify significant differences (p-value <.05) between vehicle and PCB-exposed cultures from both males and females. A mixed-effect analysis with *post-hoc* Dunnet's test was used to identify significant differences in the number of intersections at each set distance between vehicle and PCB-exposed cultures from both males and females (Wilson, Sethi et al. 2017). A one-way ANOVA with a *post-hoc* Šídák's multiple comparisons test was used to identify significant differences between PCB-exposed cultures and cultures co-exposed to 500 nM 666-15.

3. Results

3.1 Cell viability

To identify concentration-dependent effects of PCB 37 on cell viability, dissociated primary rat cortical neuron-glia co-cultures, sex segregated at the time of plating, were grown under the same culture conditions used for apoptosis, axonal growth, and dendritic arborization assays, and exposed to varying concentrations of PCB 37 ranging from 0.0001 nM to 100 μM. Cell viability was determined by quantifying LDH release into the culture media and by determining the percentage of cells stained with calcein-AM, which labels live cells on DIV 2 and DIV 9. Relative to age- and sex-matched vehicle controls, cultures from the same dissections exhibited no significant change in either LDH release (Fig. 2B, C, E, F) or percentage of live cells (Fig. 2B, C, E, F) following a 48-h exposure to PCB 37 at concentrations ≤ 1 μM. At concentrations > 1 μM, there was a significant decrease in cell viability in cultures of both sex (Fig. 2B, C, E, F). Based on this observation, all remaining experiments were conducted using PCB 37 at concentrations < 1 μM.

3.2 PCB 37 induced apoptosis in a time- and sex-dependent manner

Caspases, considered to be the executioners of apoptosis, are known for their critical role in shaping the development of the central nervous system (Lein, Supasai et al. 2018). To determine whether PCB 37 influences apoptosis, we first measured caspase 3/7 activity in cultures exposed to PCB 37 at concentrations ranging from 0.0001 to 100 nM (Fig. 3). Caspase 3/7 activity was significantly increased in female DIV 2 cultures exposed to 100 nM PCB 37 (Fig. 3A). In contrast, significant changes in caspase 3/7 activity were not observed in any of the DIV 2 male cultures exposed to PCB 37 (Fig. 3C). By DIV 9, both female (Fig. 3B) and male (Fig. 3D) cultures exposed to PCB 37 at 100 nM exhibited significant caspase 3/7 activation.

To confirm that PCB 37 effects on caspase 3/7 activity reflect PCB 37-induced apoptosis, we also assessed phosphatidylserine (PS)-annexin V binding in DIV 9 neuron-glia co-cultures exposed to PCB 37 (Fig. 4). PS is normally restricted to the inner leaflet of the

plasma membrane; however, during early apoptosis, PS becomes exposed on the outer leaflet of the plasma membrane. When PS is exposed, annexin V can bind to it freely. Since annexin V can also bind to necrotic cells with ruptured membranes, a DNA-binding dye is used to distinguish between annexin V-binding cells that are apoptotic vs. necrotic. PS externalization was significantly increased in both female and male cultures exposed to 100 nM PCB 37 (Fig. 4 A,C); however, membrane integrity was not altered in any of the cultures exposed to PCB 37 at any of the tested concentrations (Fig. 4 B,D), suggesting that PCB 37 at 100 nM induces apoptosis.

3.3 PCB 37 increased axon length in a sex-dependent manner

Axonal growth is an essential determinant of neuronal connectivity in the developing brain (Desai-Chowdhry, Brummer et al. 2022). Our previous studies demonstrated that the LC-PCB, PCB 11, significantly altered normal patterns of axonal growth in primary cortical neurons (Sethi, Keil et al. 2017). Therefore, we tested whether PCB 37 influenced axonal outgrowth in primary cortical neuron-glia co-cultures. Automated image acquisition and analysis of cultures immunostained for tau-1, a biomarker of axons (Probst, Tolnay et al. 1996), revealed sex-specific effects of PCB 37 on axon length (Fig. 5). In DIV 2 female cortical neurons, PCB 37 had no significant effect on axon length after a 48-h exposure to concentrations ranging from 0.0001 and 100 nM (Fig. 5A). In contrast, the average axon length of DIV 2 male cortical neurons was significantly increased following exposure to 0.0001 nM PCB 37 (Fig. 5C). PCB 37 had no significant effect on average cell body area in female (Fig. 5B) or male (Fig. 5D) cortical neurons cultures at any of the tested concentrations.

3.4 PCB 37 altered dendritic arborization in both male and female cortical neurons

Legacy HC-PCBs and Aroclor mixtures have been shown to increase dendritic growth of rat cortical neurons both *in vitro* and *in vivo* (Yang, Kim et al. 2009, Wayman, Yang et al. 2012) and we previously demonstrated that the LC-PCB, PCB 11, similarly enhanced dendritic arborization in cultured cortical neurons (Sethi, Keil et al. 2018). Sholl analysis was used to

quantify the effects of PCB 37 on dendritic arborization in primary cortical neurons (Fig. 6A). In DIV 9 female cortical neurons, exposure to PCB 37 at 0.0001 or 100 nM significantly decreased the number of Sholl intersections within 50–100 μm from the soma compared to the vehicle control (Fig. 6C). This decrease was confirmed by a significantly reduced area under the curve (AUC) of the Sholl plot in cultures exposed to PCB 37 at 0.0001 or 100 nM (Fig. 6D). In DIV 9 male cortical neurons, exposure to PCB 37 at 0.0001 or 0.1 nM PCB 37 exposure significantly decreased the number of Sholl intersections in proximal dendritic regions (Fig. 6E), which was reflected in decreased AUC values (Fig. 6F). In contrast, exposure to PCB 37 at 100 nM significantly increased dendritic complexity in DIV 9 male cortical neurons, as confirmed by significantly increased AUC values in this exposure group (Fig. 6F).

3.5 CREB inhibition blocked some but not all endpoints of PCB 37 developmental neurotoxicity

Given that PCB 11-induced dendritic growth was unaffected by RyR, AhR, or THR antagonism but was completely blocked by the selective CREB inhibitor 666-15 (Sethi, Keil et al. 2018), we investigated whether CREB signaling also mediates the DNT effects of PCB 37. Pretreatment with the CREB inhibitor 666-15 (500 nM) for 30 min prior to PCB 37 exposure effectively blocked PCB 37 effects on apoptosis and dendritic morphology (Fig. 7). As previously shown in Figure 3, PCB 37 significant increased caspase 3/7 activity in DIV 9 female and male cultures in the absence of 666-15; however, in cultures pretreated with 666-15, a 48-h exposure to PCB 37 at 100 nM had no significant effect on caspase 3/7 activity (Fig. 7A,B). Pretreatment with 666-15 similarly blocked the dendrite-promoting activity of PCB 37 previously observed in DIV 9 female and male cortical cultures (Fig. 7D-G). In contrast, pretreatment with 666-15 failed to block PCB 37-enhanced axonal growth in DIV 2 male cortical neurons (Fig. 7C).

4. Discussion

The data from these studies expands our previous work characterizing the effects of the LC-PCB, PCB 11, on neuronal morphogenesis *in vitro* (Sethi, Keil et al. 2017). The findings

described here suggest that PCB 37 is also a potential developmental neurotoxicant that may interfere with normal neurodevelopment. Specifically, we found that PCB 37 induced significant sex- and concentration-dependent effects on neuronal morphology and apoptosis, independent of cytotoxicity. Caspase 3/7 activity increased in DIV 2 female, but not male neurons exposed to PCB 37 at 100 nM, while neurons from both sexes showed increased caspase 3/7 activity and phosphatidylserine externalization without loss of membrane integrity by DIV 9 when exposed to PCB 37 ata 100 nM. Dendritic complexity decreased at DIV 9 in neurons of both sexes at lower concentrations (0.0001–0.1 nM) but increased in DIV 9 males at 100 nM. Axonal growth was enhanced in DIV 2 male neurons exposed to PCB 37 at 0.0001 nM, with no significant effects in female neurons or on cell body area in either sex. CREB inhibition blocked PCB 37 effects on dendritic complexity and apoptosis but not axonal growth.

Developmental exposure to PCBs, including the commercial Aroclor mixtures and HC-PCBs (Lein, Yang et al. 2007, Yang, Kim et al. 2009, Wayman, Yang et al. 2012, Bell, Dryden et al. 2018, Liberman, Walker et al. 2020, Keil Stietz, Sethi et al. 2021, Wilson, Suh et al. 2024) and the LC-PCB 11 (Sethi, Keil et al. 2017, Sethi, Keil et al. 2018), have previously been shown to disrupt axonal and dendritic morphology *in vitro* and *in vivo*. Our findings extend these previous observations and suggest that changes in neuronal morphogenesis may be a generalized outcome of PCB neurotoxicity. The functional consequences of these changes were not investigated in this study; however, axonal and dendritic morphology are considered major determinants of synaptic connectivity, which in turn, determines neurological function. Deviations in axonal and dendritic number, length, and/or branching patterns are thought to be an underlying cause of human neurodevelopmental disorders (Stamou, Streifel et al. 2013, Guang, Pang et al. 2018), and even subtle changes in the spatiotemporal development of axons and dendrites are associated with altered behaviors in animal models (Berger-Sweeney 1997, Gozzi and Zerbi 2023).

Apoptosis refines and sculpts synaptic networks (Su 2020). Inappropriate modulation of apoptosis can alter neurodevelopmental trajectories, resulting in persistent cognitive and behavioral deficits (Levenson 2005, Yochum, Dowling et al. 2008). *In vitro* studies have demonstrated that Aroclor mixtures and HC-PCBs induce apoptosis in primary rodent neurons at concentrations that do not cause overt cytotoxicity (Howard, Fitzpatrick et al. 2003, Sanchez-Alonso, Lopez-Aparicio et al. 2004, Yang and Lein 2010). Studies of individual congeners indicate that the pro-apoptotic activity of these Aroclor mixtures is largely mediated by the non-coplanar non-dioxin-like (NDL) PCBs, which are thought to be predominantly responsible for PCB DNT (Klocke and Lein 2020), though not all NDL congeners exhibit this property (Howard, Fitzpatrick et al. 2003). The mechanism(s) by which NDL PCBs trigger apoptosis vary between congeners. PCB 52 has been shown to induce apoptosis in human neuronal SK-N-MC cells through a p53-independent mechanism (Hwang 2001), while PCB 47 significantly increased caspase-dependent apoptosis in primary rat hippocampal neurons, but not cortical neurons (Howard, Fitzpatrick et al. 2003). Our findings extend these previous studies by demonstrating that the LC-PCB 37 promotes apoptosis via CREB-dependent activation of caspase 3/7 activity.

The concentration-effect relationships observed in this study varied across neurodevelopmental endpoints. Dendritic arborization was the most sensitive endpoint, demonstrating a nonmonotonic dose-response relationship. In contrast, apoptosis exhibited a monotonic response, while axonal outgrowth was affected at low concentrations in males. In the prior research of PCBs, two frequently identified findings are the nonmonotonicity and low-dose effects of PCBs on dendritic arborization (Yang, Kim et al. 2009, Wayman, Yang et al. 2012). The biological explanation for the non-monotonic dose-related effects of PCBs remains unknown; however, one of the mechanisms that has been implicated in nonmonotonicity is receptor selectivity, where lower doses of the chemical bind with high affinity to one receptor, but at higher doses, the same chemical can bind to multiple receptors, including receptors for which it has relatively low affinity (Mu, Zhang et al. 2009). The divergence in concentration-effect

relationships suggests distinct underlying mechanisms. These findings highlight the complexity of PCB neurotoxicity and the importance of examining multiple endpoints to fully characterize developmental neurotoxic effects.

We also noted sex differences in response to PCB 37. Major sex differences were observed with respect to PCB 37 effects on apoptosis, as evidenced by increased caspase 3/7 activity in DIV 2 female cultures at 100 nM PCB 37, with no corresponding increase in males. Additionally, PCB 37 enhanced axonal growth in DIV 2 male neurons at 0.0001 nM but had effect on axonal growth in female neurons at this concentration. Exposure to PCB 37 at 100 nM increased dendritic complexity in DIV 9 male but not female neurons. There are known sex differences in the developmental trajectory of the brain (Brenhouse and Andersen 2011) that underlie sex differences in response to developmental neurotoxicants (Bell, Dryden et al. 2018, Sethi, Keil Stietz et al. 2021), and this may be responsible for the sex differences observed in this study. The distinct maturation processes in male and female brains may result in varying susceptibility to PCB-induced neurotoxic effects (Hilz and Gore 2022).

CREB is essential for nervous system development and plasticity, particularly in cortical regions, where it predominantly affects pyramidal neurons (Imaki, Yoshida et al. 1994, Pham, Impey et al. 1999, Sakamoto, Karelina et al. 2011, von Bernhardi 2017). Here, we found that pharmacological inhibition of CREB blocked PCB 37 effects on apoptosis and dendritic arborization but did not alter the effects of PCB 37 on axon growth. Whether PCB 37 effects on apoptosis and dendritic arborization are linked or mediated via independent mechanisms is unclear. Certainly, caspase 3/7 activation can lead not only to apoptosis but also to subtle changes in neuronal morphology (Su 2020). This could be due in part by disrupted caspase-mediated cleavage of cytoskeletal or microtubule-associated proteins, such as tau (Fasulo, Ugolini et al. 2000, Chung, Song et al. 2001), or because loss of synapses and retraction of neurites may precede cell death (Luo and O'Leary 2005, Wang, Medress et al. 2012). Disruption in these processes can lead to impaired axonal growth and dendritic branching (Bendiske, Caba

et al. 2002, Marchisella, Coffey et al. 2016, Chang, Yang et al. 2018). This suggests that the effects of PCB 37 on apoptosis and dendritic growth are independent of its effects on axonal outgrowth. Prior research demonstrated that the HC-PCB 95 enhanced dendritic arborization via CREB activation downstream of ryanodine receptor (RyR) sensitization (Wayman, Impey et al. 2006, Wayman, Yang et al. 2012). Subsequent studies found that PCB 11 also promoted dendritic growth via CREB-dependent mechanisms; however, the upstream signaling pathway did not involve RyR activation or signaling via the aryl hydrocarbon receptor or thyroid hormone receptor (Sethi, Keil et al. 2018). The upstream signaling pathways modulated by PCB 37 to activate CREB remain to be determined. Nonetheless, the findings of the current study contribute to the growing body of evidence implicating CREB signaling as a convergent mechanism in the developmental neurotoxicity (DNT) caused by PCBs (Klocke, Sethi et al. 2020).

Epidemiological studies have consistently shown that early-life exposure to PCBs negatively impacts neuropsychological function (Schantz, Widholm et al. 2003, Stolevik, Nygaard et al. 2013, Grandjean and Landrigan 2014, Hertz-Picciotto, Schmidt et al. 2018), and the predominant theory in the field is that they elicit these neurobehavioral deficits by interfering with the establishment of synaptic connectivity (Panesar, Kennedy et al. 2020, Panesar, Wilson et al. 2022). Human neurodevelopmental disorders are thought to arise, in part, from disruptions in synaptic connectivity (Copf 2016, Hoersting and Schmucker 2021, Paus 2023). Additionally, studies in animal models have demonstrated that subtle shifts in the timing and spatial patterns of brain development can lead to behavioral alterations (Berger-Sweeney 1997, Sethi, Keil Stietz et al. 2021, Wilson, Suh et al. 2024). While much of the research has focused on the DNT of legacy PCB mixtures or HC-PCBs, recently, studies have begun investigating the neurodevelopmental impacts of individual PCB congeners or mixtures based on profiles seen in human tissues (Li, Hefti et al. 2022). Our results provide additional insights into PCB-mediated DNT, especially for LC-PCBs, supporting a range of literature.

5. Conclusions

The developmental neurotoxicity profile of PCB 37 does not directly mirror that of PCB 11 (Sethi, Keil et al. 2017, Sethi, Keil et al. 2018). PCB 11 and PCB 37 both modulate dendritic arborization via CREB signaling but differ in their effects and specificity. PCB 11 promotes axonal and dendritic growth across concentrations. In contrast, PCB 37 exhibits variable effects depending on concentration and sex, decreasing dendritic complexity in DIV 9 cortical neurons at lower doses while increasing complexity in male neurons at higher doses (100 nM). PCB 37 also selectively increases axon length in DIV 2 male, but not female, cortical neurons. These findings underscore PCB-specific and context-dependent neurodevelopmental effects, adding to the literature that suggests there are overlapping but also unique toxic effects associated with individual PCB congeners. This underscores the challenges of conducting risk assessments based on individual PCB congeners, as they may not fully capture the developmental neurotoxic potential of other human-relevant PCBs. Further evaluation of human-relevant contemporary PCBs and PCB mixtures in appropriate animal models will be essential to understanding how PCBs interact to influence adverse neurodevelopmental outcomes. Additionally, the development of more complex quantitative structure-activity relationship (QSAR) models will be instrumental in predicting DNT outcomes and improving risk assessment strategies of human exposures to PCB mixtures.

These data also suggest that, despite structural differences, CREB activation may represent a convergent mechanism of PCB developmental neurotoxicity (Klocke, Sethi et al. 2020). Identifying the specific upstream mechanisms by which PCB 37 influences CREB activity will be crucial for understanding the full scope of PCB-induced neurotoxicity and for developing targeted interventions to mitigate these adverse effects.

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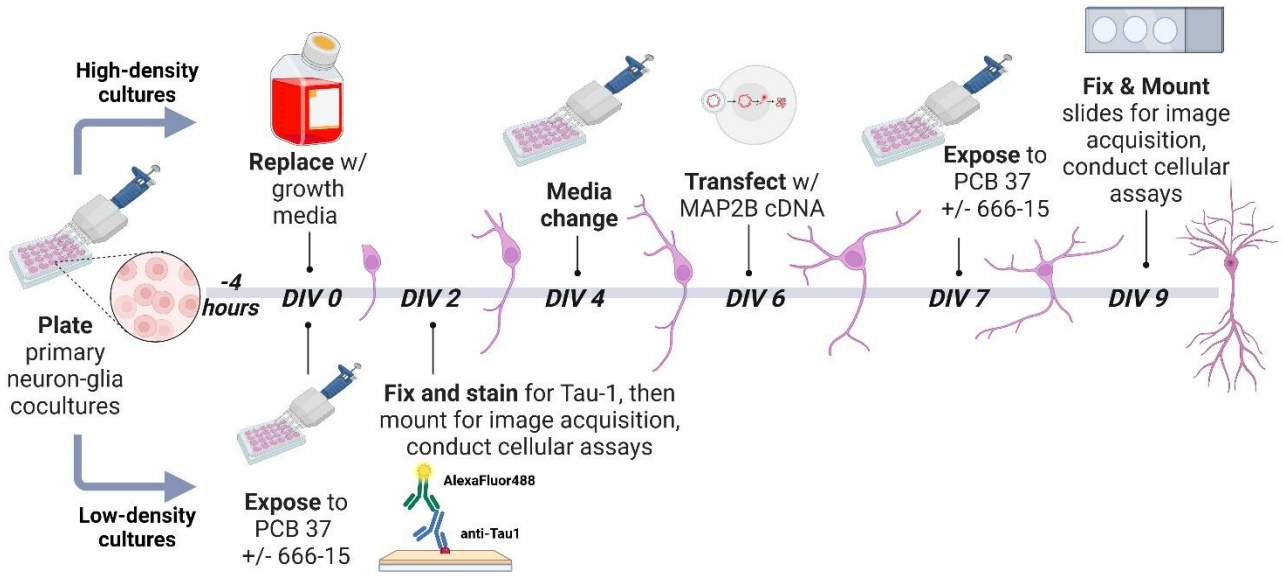


Figure 2.1. Experimental timeline and exposure paradigm. Sex-segregated primary cortical neuron-glia cocultures dissociated from P0 rat neocortices were plated at high or low cell densities for analysis of dendritic and axonal morphology, respectively. On day in vitro (DIV) 0, at approximately 4 h post-plating, the plating medium was replaced with growth medium. Low density cultures, used for axon outgrowth assays, were exposed to vehicle (0.001% DMSO) or varying concentrations of PCB 37 in the absence or presence of the pharmacological inhibitor of CREB, 666-15 (500 nM). On DIV 2, low-density cultures were either used for cellular assays or fixed with 4% paraformaldehyde, immunostained for Tau-1, a protein localized in the somatoaxonal compartment of neurons, and stained for Hoechst 33342, a nuclear stain. In high-density cultures used for dendritic growth assays, a partial media change was performed at DIV 4. On DIV 6, cultures were transfected with MAP2B-RFP cDNA. On DIV 7, cultures were exposed to vehicle (0.001% DMSO) or varying concentrations of PCB 37 with or without 666-15. On DIV 9, cultures were fixed and mounted for image acquisition or used for additional cellular assays.

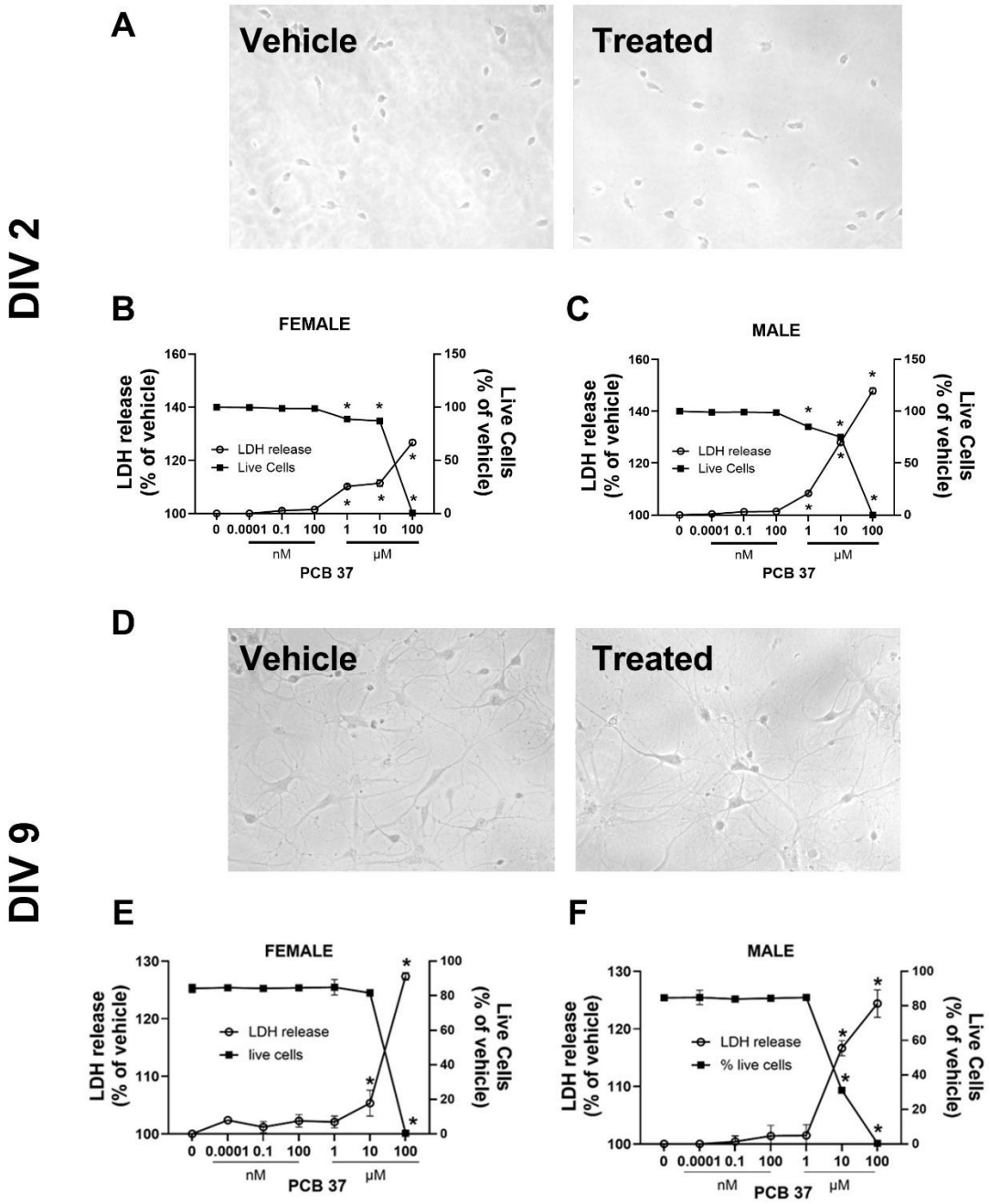


Figure 2.2 At concentrations ≤ 1 µM, PCB 37 was not cytotoxic. Phase contrast images of low-density cultures on DIV 2 (A) and DIV 9 (D). Live cell percentage (black squares, right y-axis) and LDH release (open circles, left y-axis) were quantified in female (B, E) and male (C, F) primary cortical neuron-glia co-cultures at DIV 2 (B, C) and DIV 9(E, F) following a 48-h exposure to vehicle or varying concentrations of PCB 37. Data are expressed as % of vehicle control and presented as the mean ± SD (n = 4 independent dissections). *Significantly different from vehicle control at $p < 0.05$ (one-way ANOVA, *post-hoc* Dunnett's test).

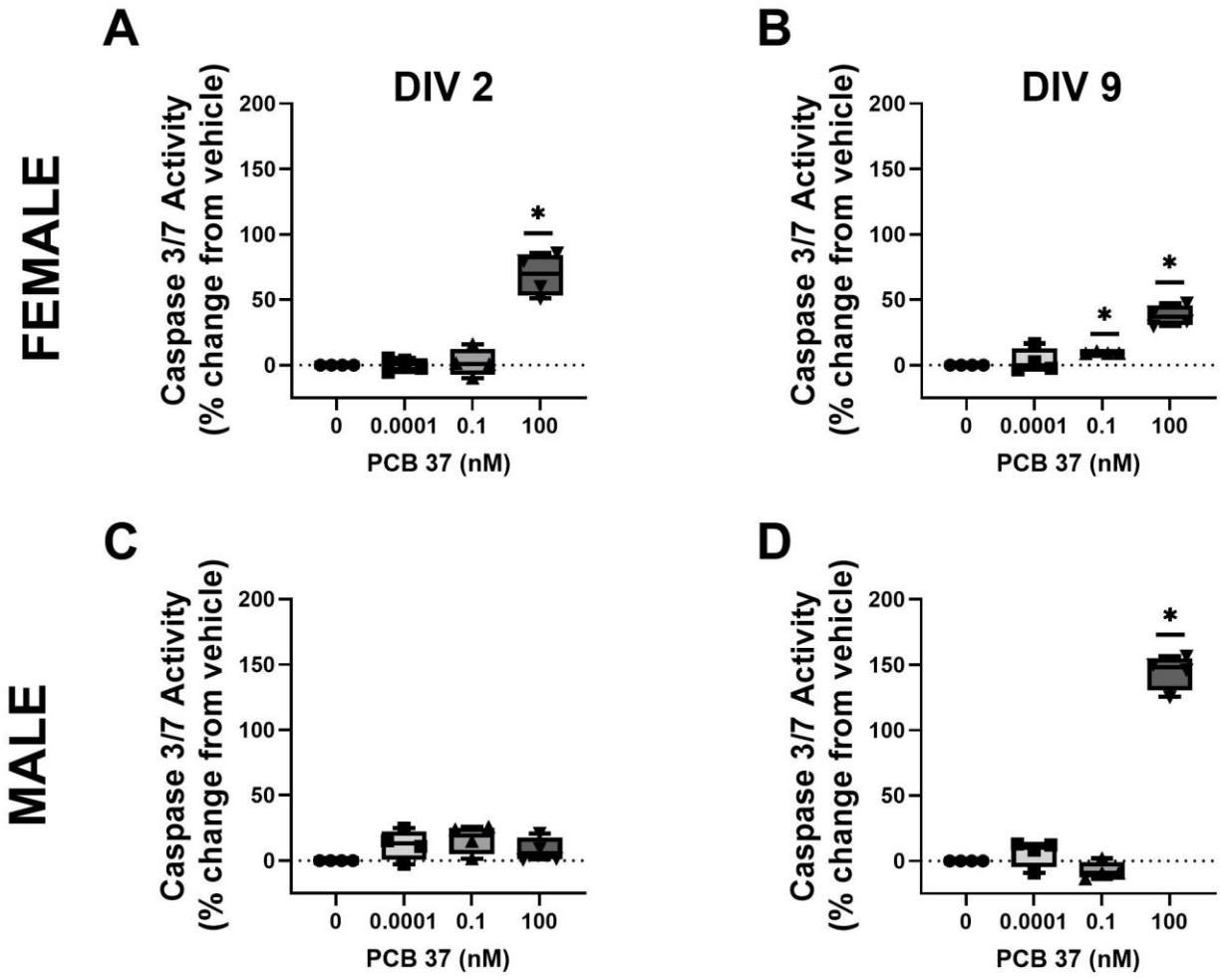


Figure 2.3. PCB 37 increased caspase 3/7 activity in a concentration-, sex-, and age-dependent manner. Caspase 3/7 activity, a marker of apoptosis, was measured in primary female (top) and male (bottom) neuron-glia co-cultures at DIV 2 (left) and DIV 9 (right) following exposure to vehicle or varying concentrations of PCB 37. Data are presented as box-and-whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=4 independent dissections). *Significantly different from vehicle control at $p < 0.05$ (one-way ANOVA, *post-hoc* Dunnett's test)

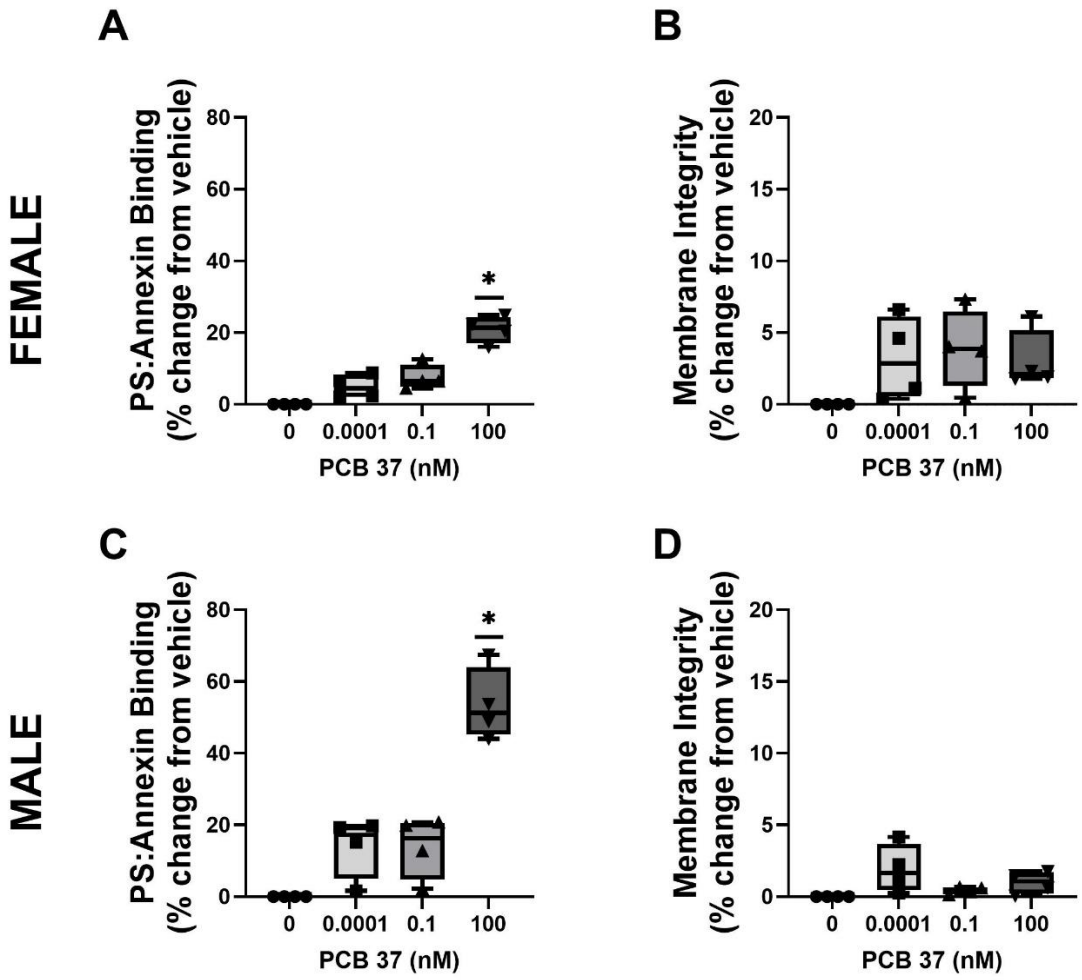


Figure 2.4. PCB 37 increased phosphatidylserine (PS) externalization and Annexin V binding independent of effects on membrane integrity in DIV 9 male and female neuron-glia co-cultures. PS-Annexin binding (left) was measured as an indicator of early apoptosis, while membrane integrity (right) was assessed to determine cytotoxic effects in primary female (top) and male (bottom) cortical cultures following a 48-h exposure to vehicle or PCB 37. Data are presented as box-and-whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=4 independent dissections). *Significantly different from vehicle control at $p < 0.05$ (one-way ANOVA, *post-hoc* Dunnett's test)

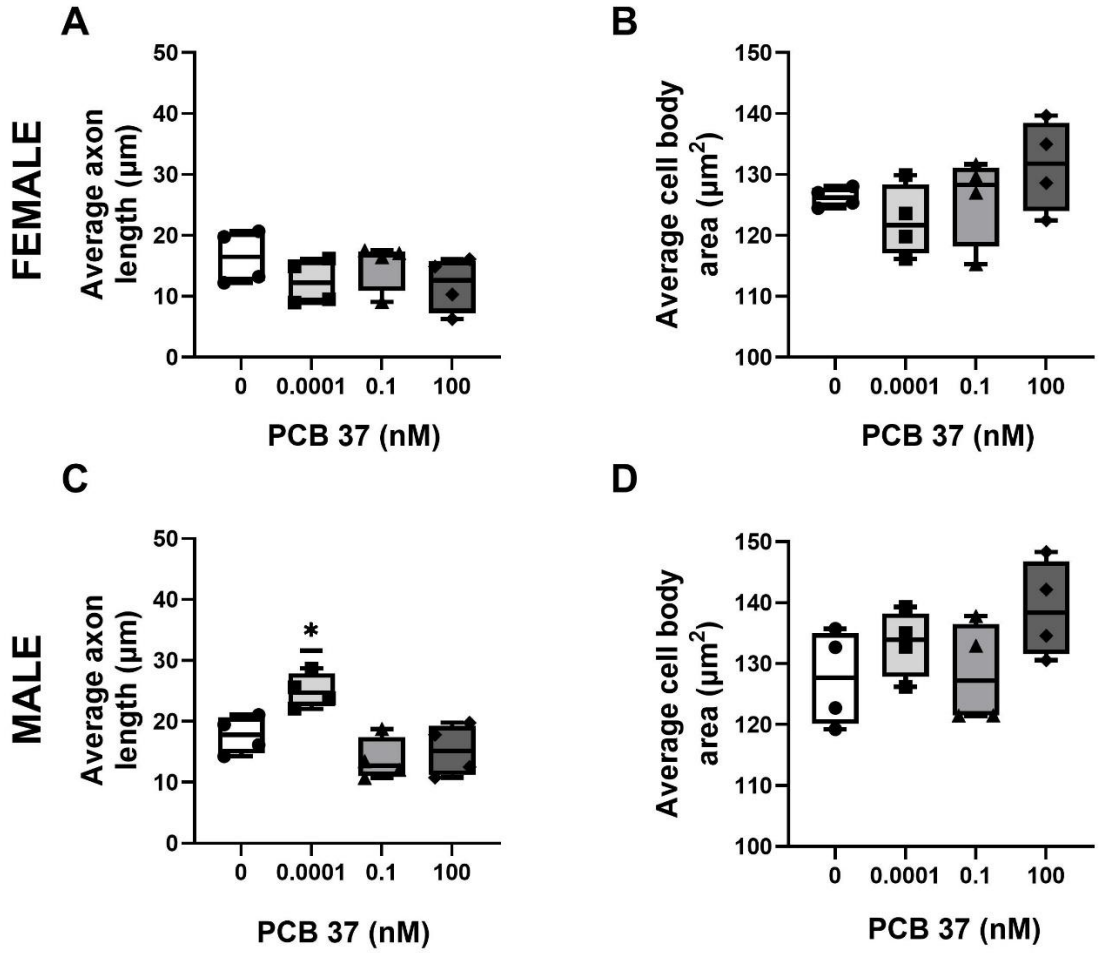


Figure 2.5. PCB 37 non-monotonically increased axon length in male but not female cortical neurons independent of significant effects on cell body area. Primary cortical neuron-glia cocultures were exposed to vehicle or varying concentrations of PCB 37 for 48 h starting 4 h after plating. Quantification of axon length in female (A) and male (C) neurons at DIV 2; quantification of cell body area in female (B) and male (D) neurons at DIV 2. Data are shown as box and whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=4 independent dissections). *Significantly different from vehicle control at $p < 0.05$ (one-way ANOVA, *post-hoc* Dunnett's test)

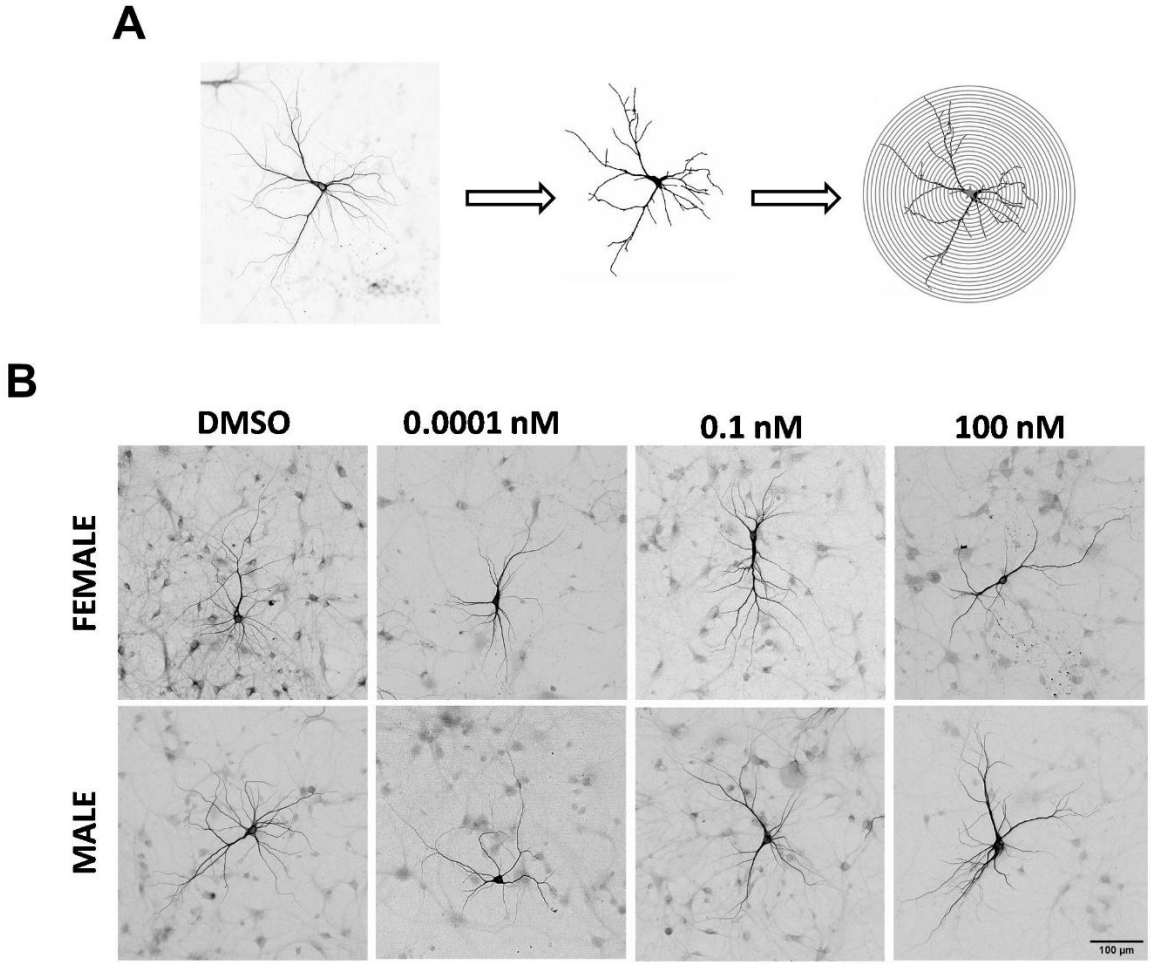

A
B
DMSO
0.0001 nM
0.1 nM
100 nM
FEMALE
MALE
100 µm
C
FEMALE
Avg. # of intersections
distance from soma (µm)
0
0.0001 *
0.1
100 *
PCB 37 (nM)
D
Area Under Curve
PCB 37 (nM)
E
MALE
Avg. # of intersections
distance from soma (µm)
0
0.0001 *
0.1 *
100
PCB 37 (nM)
F
Area Under Curve
PCB 37 (nM)

Figure 2.6. PCB 37 altered dendritic complexity in a sex- and concentration-dependent manner. (A) Representative image of MAP2B-RFP-transfected cortical neuron and automated Sholl analysis pipeline. (B) Representative images of PCB untreated and treated neurons. Sholl plots of female (C) and male (E) cortical neurons at DIV 9 following a 48-h exposure to vehicle or varying concentrations of PCB 37. (D, F) Quantification of total dendritic complexity as area under the curve in the corresponding Sholl plot. Data are presented as mean with standard error. *Statistically different from vehicle control at $p<0.05$ (Sholl: mixed-effects model and post hoc Dunnett's test; Area under the curve: one-way ANOVA and *post hoc* Dunnett's test).

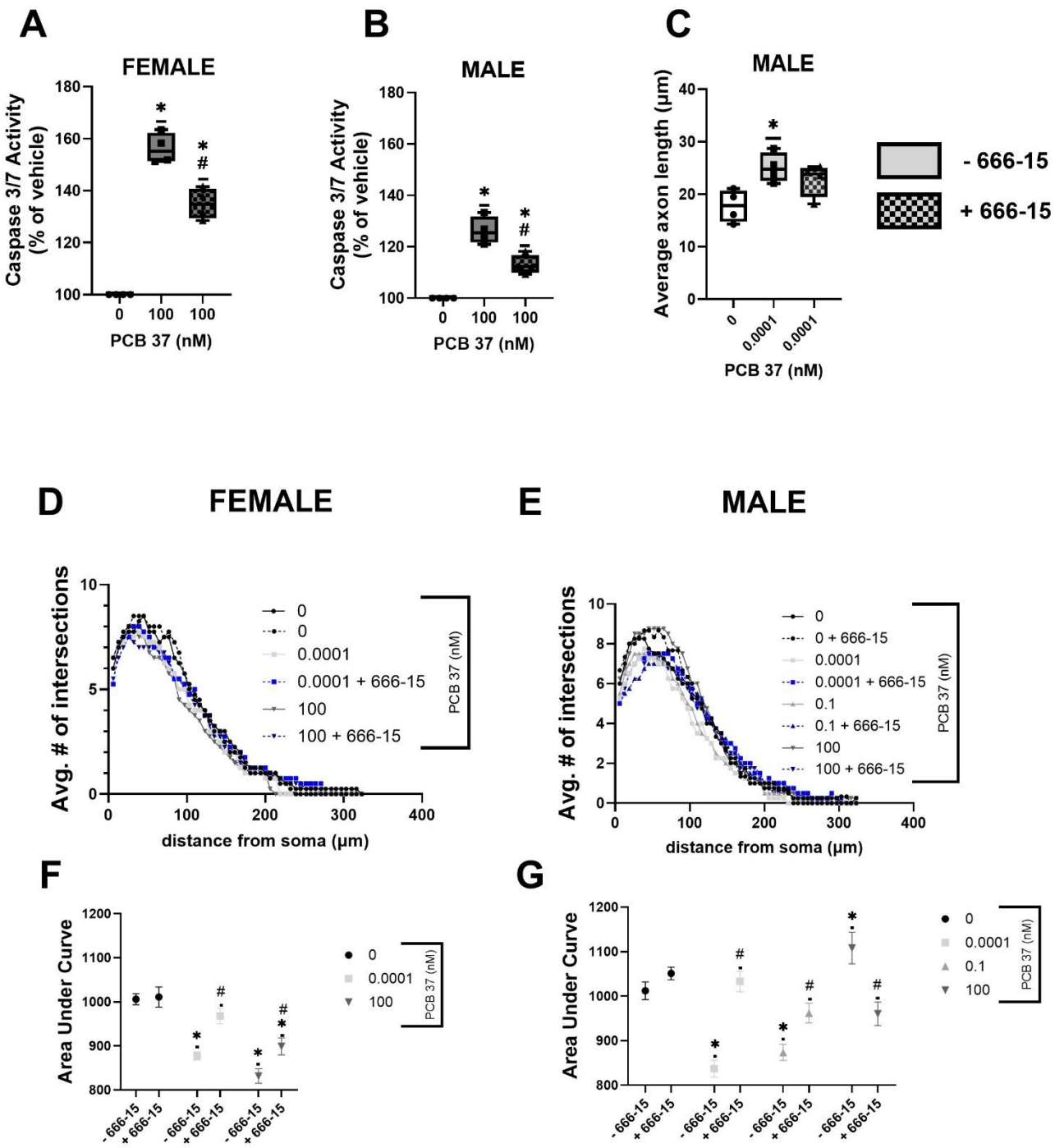


Figure 2.7. Pharmacological inhibition of CREB blocked PCB 37 effects on apoptosis and dendrites, but not axons. Cultures were pretreated with the CREB inhibitor 666-15 (500 nM) for 30 min before PCB 37 exposure. (A, B) Caspase 3/7 activity (% of control levels) in female

(A) and male (B) cultures following a 48-h exposure to PCB 37 (100 nM) in the absence or presence of 666-15. (C) Average axon length per cell (μm) in male cultures exposed for 48-h to PCB 37 (0.0001 nM) with or without 666-15 pretreatment. (D, E) Sholl plots of dendritic complexity in female (D) and male (E) cortical neurons exposed to vehicle or varying concentrations of PCB 37 with or without 666-15. (F, G) Quantification of dendritic complexity as the area under the curve (AUC) in Sholl plots of female (F) and male (G) dendritic arbors. Data are presented as box and whisker plots in which the box indicates the 25^{th} (lower) to 75^{th} (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=4 independent dissections). *Statistically different from vehicle control at $p < 0.05$; #significantly different from PCB 37 without 666-15 pretreatment at $p<0.05$ (Sholl: mixed-effects model and post hoc Dunnett's test; Area under the curve: two-way ANOVA and post hoc Šídák's multiple comparisons test).

Chapter 3

Lower-Chlorinated PCBs and Their Human-Relevant Metabolites Modulate Neurodevelopmental Endpoints in Primary Rat Cortical Neuron-Glia Cocultures in a Sex, Time, and Congener-Dependent Manner

Authors: Jessie R. Badley[a], Caleb Ryce[a], Hans-Joachim Lehmler[b], Pamela J. Lein[a]

[a] Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, 1089 Veterinary Medicine Drive, Davis, CA, 95616 USA

[b] Department of Occupational and Environmental Health, College of Public Health, University of Iowa, 145 N Riverside Drive, Iowa City, IA, 52242 USA

JRB: jrbadley@ucdavis.ed; CR: calebryce22@gmail.com; HJL: hans-joachim-lehmler@uiowa.edu; PJL: pjlein@ucdavis.edu

Corresponding Author:

Pamela J. Lein, PhD

UC Davis School of Veterinary Medicine

1089 Veterinary Medicine Drive

Davis, CA 95616

pjlein@ucdavis.edu

https://leinlab.vetmed.ucdavis.edu/

Abstract

Little is known about the developmental neurotoxicity (DNT) of lower-chlorinated (LC) congeners, despite their increasing prevalence in contemporary human tissues, including the neonatal brain. This study examined the DNT effects of PCB 2, PCB 3, PCB 28, and their human-relevant metabolites in primary male and female rat cortical neuron-glia co-cultures. Cultures were exposed for 48 h to environmentally relevant concentrations (0.0001, 0.1, and 100 nM). Cell viability, assessed by lactate dehydrogenase release and Calcein-AM staining, was unaffected at all concentrations. Apoptosis was quantified using fluorometric caspase 3/7 activity assays, while axonal and dendritic growth were measured in neurons immunostained for Tau-1 or transfected with MAP2B-red fluorescent protein, respectively, and analyzed using automated image analysis protocols. LC-PCBs and their metabolites elicited congener-, concentration-, and sex-specific effects on apoptosis and neuronal morphology. PCB 2 and its metabolite 4-OH PCB 2 significantly altered caspase 3/7 activity and produced opposing effects on axonal and dendritic growth in male versus female neurons. PCB 3 was largely inactive, although its metabolite 3-OH PCB 3 increased apoptosis and reduced axonal growth in males. PCB 28 and its metabolites produced the most pronounced effects, with parent PCB 28 enhancing axonal growth in both sexes but reducing dendritic complexity in females, while its hydroxylated and sulfated metabolites decreased axonal and dendritic growth in a sex-dependent manner. These findings demonstrate that LC-PCBs exert diverse and metabolite-specific effects on apoptosis, axonal growth, and dendritic arborization at concentrations that do not compromise cell viability. Collectively, the results highlight structural and sex-dependent determinants of LC-PCB developmental neurotoxicity and underscore the importance of considering both parent congeners and metabolites in risk assessment.

1. Introduction

Despite a worldwide ban on their production, PCBs remain a critical public health concern (Badley 2023, Carlson 2023). This reflects in part the continued use of equipment containing legacy PCB mixtures, the improper disposal of PCB-containing products, and the environmental persistence of PCBs (Zhang, Saktrakulkla et al. 2021, Hornbuckle 2022, Li 2022). In addition, it is now known that PCBs are formed unintentionally as byproducts in various contemporary manufacturing processes, particularly those involving pigment production (Hannah, Megson et al. 2022). It has also been well established that individual PCB congeners, which vary in structure (Kania-Korwel and Lehmler 2016, Holland, Feng et al. 2017), can interact with different target molecules (Park 2010, Fritsch and Pessah 2013, Milon, Opydo-Chanek et al. 2017), and thus exhibit differing toxicity profiles (Safe 1994). Despite the growing evidence that PCB metabolites are also toxic, significant knowledge gaps remain regarding the DNT potential of LC-PCBs and their metabolites.

Much of the research to date has focused on parent PCB congeners, in part because it was assumed that metabolism was a detoxification process (Esteves, Rueff et al. 2021). However, the toxicities of PCB parent congeners and their metabolites have been shown have diverse mechanisms (Grimm, Hu et al. 2015, Koh, Hornbuckle et al. 2016, Rodriguez, Li et al. 2016, Paranjape, Dean et al. 2023). Decades of studies on hydroxylated PCBs (OH-PCBs) have shown that these metabolites are consistently detected in serum, retained in tissues, and exhibit toxicological properties that often differ from those of their parent PCBs (Koh, Hornbuckle et al. 2016, Quinete, Esser et al. 2017, Sethi 2017, Zhang, Saktrakulkla et al. 2021, Li, Hefti et al. 2022).

PCB 2 (3-chlorobiphenyl) is a component of commercial PCB mixtures, such as Aroclor 1221 and Aroclor 1232 (Frame, Cochran et al. 2005), and has been found in outdoor air and human blood samples (Zhang, Li et al. 2022). Recently, an analysis of PCBs and hydroxylated

PCBs (OH-PCBs) in the postmortem human cerebellum (Li 2022) showed that the PCB congener profiles in the brain of neonates (< 1 year-old; n=7) are different from that of adults (> 58 years-old; n=7). Importantly, the PCB profiles in neonatal brain tissues did not reflect exposure to legacy HC-PCBs. An LC-PCB, PCB 3, was the major PCB congener in the cerebellum of 3 out of 7 neonates but was not detected in any adult brain samples. Analysis also revealed the presence of OH-PCB metabolites of LC-PCBs in human brains. PCB 3 (4-chlorobiphenyl) is also found in high concentration in indoor and outdoor air samples(Kraft, Sievering et al. 2018), and PCB 28 has been found in both indoor and outdoor air samples (Gabrio, Piechotowski et al. 2000) and in the serum of pregnant mothers at risk of their children having a neurodevelopmental disorder (NDD) (Sethi, Morgan et al. 2019). These findings establish that LC-PCBs 3 and 28 are the predominant PCBs present in the gestational environment and the neonatal brain of human populations.

LC-PCBs are mainly oxidized by cytochrome P450 (P450) enzymes into OH-PCBs, which can then undergo sulfation, glucuronidation, or further oxidation to form complex mixtures of metabolites (Grimm, Hu et al. 2015, Zhang, Li et al. 2022). While metabolism is typically thought to enhance excretion, some OH-PCB congeners are known to have especially long biological half-lives. For example, while the half-life of PCB 28 (2,4,4'-trichlorobiphenyl) in humans has been calculated as 4.32 years, its metabolites 3'-OH PCB 28, 4-OH 25, and 3-OH 28 have half-lives of 2.77, 6.46, and 7.55 years, respectively (Quinete, Esser et al. 2017). Such persistence is highly relevant to developmental neurotoxicity, as it extends the duration of exposure across sensitive neurodevelopmental periods and increases the potential for these congeners and/or metabolites to interfere with neuronal growth.

Apoptosis (Lein 2018) and neuronal morphology (Copf 2016, Hoersting 2021) are critical determinants of neuronal connectivity (Paus 2023), making them valuable endpoints for assessing developmental neurotoxicity (DNT) (van Thriel, Westerink et al. 2012, Morgan 2020).

Apoptosis regulates neuronal population size and circuit refinement , and disruptions to apoptotic processes can result in either excessive neuronal loss or inappropriate cell survival, both of which impair functional network formation. Similarly, neuronal morphology, including axonal outgrowth, dendritic arborization, directly governs the capacity of neurons to form and maintain connections essential for higher-order brain function (Desai-Chowdhry 2022). Because alterations in these processes can compromise neuronal circuitry long before overt behavioral deficits manifest, apoptosis and morphological development serve as sensitive and biologically relevant indicators for detecting neurotoxicant-induced perturbations during critical windows of brain development (van Thriel, Westerink et al. 2012) . While the determination of the DNT properties of parent PCB congeners is critically important, the effect of metabolites such as OH-PCBs and PCB sulfates are also critical to a complete understanding of PCB DNT (Duffel and Lehmler 2024).

We previously demonstrated that PCB 11 and its major metabolites exhibit axon and dendrite-promoting activity *in vitro* at environmentally relevant concentrations (Sethi 2017), and that PCB 37 increased apoptosis and modulated neuronal morphogenesis in a concentration-, sex-, age-, and CREB-dependent manner (Badley, Andrew et al. 2025) In this study, we aimed to identify critical neurodevelopmental endpoints effected and directly compare the effects of parent LC-PCBs and their metabolites on critical determinants of neuronal morphology. Apoptosis (Fricker, Tolkovsky et al. 2018), axonal growth (Hoersting 2021), and dendritic arborization (Morgan 2020, Paus 2023) were selected as endpoints because they are fundamental processes that establish neuronal connectivity during development. Dysregulation of apoptosis (Lein 2018), axonal and/or dendritic growth impairs the formation of functional synaptic circuits. Such alterations in connectivity are consistent with the neuropsychological deficits reported in human populations exposed to PCBs, including impairments in attention, memory, processing speed, and executive function (Schantz 2003, Boucher 2009, Grandjean

2014). Thus, evaluating these cellular processes provides a bridge between LC-PCB exposure and the cognitive and behavioral outcomes observed clinically. We found that both parent LC-PCBs and their metabolites in this study (PCB 2, 3, 28) altered neuronal morphology and apoptosis, consistent with evidence (Sethi 2017, Matelski, Keil Stietz et al. 2020, Sethi 2021, Badley, Andrew et al. 2025) that these compounds can disrupt neurodevelopmental processes.

2. Materials and Methods

2.1. Materials

3-chlorobiphenyl (PCB 2) was purchased from AccuStandard, Inc. (New Haven, CT) and previously authenticated (Zhang, Li et al. 2022). 3-chloro-4-hydroxy-biphenyl (4-OH-PCB 2) was synthesized and authenticated as previously described (Espandiari, Glauert et al. 2004, Zhai, Lehmler et al. 2011, Dhakal, He et al. 2012). 4-chlorobiphenyl (PCB 3) and 4-Chloro-3-hydroxy-biphenyl (3-OH PCB 3) were synthesized and authenticated in the Lehmer lab as previously described (Zhai 2011, Dhakal, He et al. 2012) and 2,4,4'-trichlorobiphenyl (PCB 28), 3-OH PCB 28, 3'-OH PCB 28, 4-OH PCB 25, and 4-SO_3 PCB 25 were synthesized and authenticated (unpublished data) in the Lehmer lab, as well. Individual congeners were made up as 1000X stock solutions in dry sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) and stored at room temperature in the dark. Plating and growth media were prepared as previously described (Sethi 2017). Briefly, plating medium was Neurobasal Plus medium (Gibco™, Thermo Fisher Scientific, Waltham, MA) supplemented with 2% (v/v) B27 (Gibco™), 1x GlutaMAX supplement (Gibco™), 10% (v/v) heat-inactivated horse serum (Gibco™), and 1 M HEPES buffer (pH 7.5). Growth medium contained Neurobasal Plus medium supplemented with 2% (v/v) B27, and 1x GlutaMAX. Feeding medium consisted of growth medium supplemented with the antimitotic cytosine arabinoside (Thermo Fisher Scientific) at 5 μM to halt glial overgrowth.

2.2 Cell culture

All experiments involving animals were conducted in accordance with guidelines and protocols approved by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC). Timed-pregnant Sprague Dawley rat dams were purchased from Charles River Laboratory (Hollister, CA). Dams were imported 5 d before the anticipated parturition date and maintained in an IACUC-maintained vivarium. Dams were individually housed with corn cob bedding with their litter in transparent plastic cages in a temperature and humidity-controlled vivarium maintained at 22 ± 2°C on a 12-h light-dark cycle. Animals were provided Teklad 2018 18% Rodent Feed (Inotiv, Lafayette, IN) and tap water *ad libitum*.

Primary neuron-glia co-cultures were dissociated from the neocortices of sex-segregated postnatal day 0 Sprague Dawley rat pups as previously described (Sethi, Keil et al. 2018). Dissociated cells in plating medium were plated onto plastic 96-well plates (CellVis, Sunnyvale, CA) or German glass coverslips (Electron Microscopy Sciences, Hatfield, PA) in plastic Corning 24-well plates (Thermo Fisher Scientific). Both substrates were precoated with 0.5 mg/mL poly-L-lysine (Sigma-Aldrich) in B buffer (3.1 mg/mL boric acid and 4.75 mg/mL borax, pH 7.5, Sigma-Aldrich). For axonal growth assays (Morgan 2020), cells were plated at 33,000 cells/cm^2; to quantify dendritic arborization, cell viability and caspase activity, cells were plated at 83,000 cells/cm^2. Cells were allowed to adhere to coverslips in plating medium for 4 h at which time the plating medium was replaced with growth medium. Cultures were maintained at 37°C under 5% CO_2. In cultures maintained up to day in vitro (DIV) 9, half the medium in each well was exchanged at DIV 4 with fresh feeding medium.

To account for variability, experiments were repeated using cultures from independent dissections, with both male and female cultures plated on the same plate to minimize plate effects. All plates also included vehicle controls; plates used for axon outgrowth assays also included brefeldin A (1 µg/mL;Thermo Fisher Scientific), an inhibitor of vesicular traffic, was used as a positive control of axon inhibition (Jareb 1997).

2.3 PCB exposure

Stock concentrations of PCBs were diluted directly into growth medium at 1-2X the final concentration; vehicle controls were exposed to 0.001% DMSO in growth medium. Axonal outgrowth was measured on DIV 2 in cultures exposed to vehicle or varying PCB concentrations for 48 h starting 4 h after plating. Dendritic arborization, cell viability, and caspase 3/7 activity were measured on DIV 9 in cultures exposed to vehicle or varying PCB concentrations for 48 h starting on DIV 7.

2.4 Cell viability

Two assays were used to measure cell viability on DIV 9: (1) the live/dead assay, and (2) the quantification of lactate dehydrogenase (LDH) which is released from cells with damaged membranes (Korzeniewski 1983). In the live/dead assay, calcein AM (Wang 1993) is used to label live cells while Hoechst 33342, which stains the nuclei of live and dead cells, is used to measure total cell number. To determine the percentage of live cells, cultures were co-stained with 0.5 μM calcein AM (Thermo Fisher Scientific) and 1 μg/ml Hoechst-33342 (Thermo Fisher Scientific) in 1x Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS; Thermo Fisher Scientific). After the addition of these reagents, cultures were incubated at room temperature for 30 min in the dark. Cultures were then immediately imaged using an ImageXpress Micro XLS high content imaging system (Molecular Devices, San Jose, CA). The percentage of live cells was determined from the overlay of calcein AM and Hoechst-33342 labeling, which was quantitated using MetaXpress 6.2 software (MetaXpress High Content Image Acquisition and Analysis Software, Live Dead Application Module, Molecular Devices) and was calculated as number of calcein AM-labeled cells / number of Hoechst-labeled nuclei X 100.

LDH release was quantified using the fluorometric CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI) per the manufacturer's instructions. Fluorescence was measured at 560/590 nm using a BioTek Gen5 plate reader (Agilent, Santa Clara, CA). The amount of fluorescence is proportional to the number of lysed, or dead, cells. Data were normalized to the fluorescent signal measured in vehicle-treated cells in the same multi-well plate.

2.5 Apoptosis

Apoptosis was assessed on DIV 9 by measuring caspase 3/7 activity using the luminescent Caspase-Glo 3/7™ as described by the manufacturer. Per manufacturer directions, 100 µL Caspase-Glo 3/7™ reagent was added to each well followed by incubation at room temperature for 30 min. Staurosporine (10 µM; Millipore Sigma), a known apoptosis inducer, was used as a positive control (data not shown). Luminescence was quantified using a BioTek Gen5 plate reader (Agilent). The amount of luminescence is directly proportional to caspase 3/7 activity.

2.6 Visualization of axons

To visualize axons, cultures were immunostained for tau-1, a cytoskeletal protein localized to the somato-axonal domain of central neurons (Morgan 2020). On DIV 2, all culture media were removed, and cells were fixed for 1 h with 4% (w/v) paraformaldehyde (ThermoFisher Scientific) in 0.2M sodium phosphate buffer. After washing 3 times with 1X DPBS, cells were permeabilized with 0.5% (v/v) Triton X-100 (MilliporeSigma, Burlington, MA) in 1x DPBS for 5 min. Cells were washed 3 times with 1X DPBS, and then incubated at room temperature with a blocking buffer consisting of 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) in 1X DPBS for 1 h, followed by an overnight incubation at 4°C with mouse anti-tau-1 antibody, clone PC1C6 (Sigma-Aldrich # MAB3420, RRID:AB_3068606) diluted 1:1000 in blocking buffer. After a 24 h incubation, the primary antibody was removed, cells were washed 3 times with 1X DPBS, then

incubated with a goat anti-mouse IgG2a Alexa fluor 488 (Thermo Fisher Scientific # A-21131, RRID:AB_2535771) diluted 1:1000 in blocking buffer at 4°C for 1 h in the dark on a rocker. Cells were washed 3 times in 1X DPBS, then coverslips were mounted on SuperFrost glass slides (Thermo Fisher Scientific) in Prolong Gold Antifade Mounting media with DAPI (Thermo Fisher Scientific) and allowed to dry overnight at room temperature in the dark before imaging.

2.7 Visualization of dendrites

To visualize dendrites, cultures were transfected with a plasmid encoding a MAP2B-pCAG-fusRED cDNA construct (Wayman 2006), which was generously provided by Dr. Gary Wayman (Washington University, Pullman, WA). MAP2B is a cytoskeletal protein that is selectively localized to the somatodendritic compartment of neurons; transfection with this construct does not alter their fundamental dendritic growth patterns in culture (Wayman 2006). On DIV 6, 24 h before exposure to PCBs, cultures were transfected with 0.8 µg of MAP2B-pCAG-fusRED cDNA in 2 µL of Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) per well. To achieve a low-efficiency (1-10%) transfection, the incubation time was reduced to 2 h, followed by total replacement of the transfection medium with the conditioned medium. On DIV 7, cultures were exposed to vehicle (0.001% DMSO) or PCBs at varying concentrations. On DIV 9, media were removed, cells were fixed with 4% paraformaldehyde for 1 h, washed 3 times with 1X PBS, and then mounted cell-side down on glass slides (Thermo Fisher Scientific) with Prolong Gold Antifade Mounting media with DAPI (Thermo Fisher Scientific) and allowed to dry for 24 h at room temperature in the dark before imaging.

2.8 Morphometric analyses of axon outgrowth and dendritic arborization

Throughout image acquisition and analysis, the analyst was blinded to treatment conditions. An automated ImageXpress high-content imaging system (Molecular Devices) interfaced to MetaXpress software (Molecular Devices) was used to acquire images from cell cultures

immunostained for tau or transfected with MAP2B-FusRed (Morgan 2020). A total of 25 images per well or coverslip were acquired at 10X magnification, with at least 3 technical replicates per dissection per experimental group for a total of 75 images per group per independent dissection. For axon outgrowth, DAPI and FITC channels were used; for dendritic arborization, DAPI and Texas Red channels were used.

To measure axon outgrowth, images of tau-1 immunofluorescence were overlaid on fluorescent images of Hoechst-33342 staining from the same field. Axonal lengths were quantified in tau-1 immunopositive cells using the Neurite Outgrowth application module in the MetaXpress high-content image acquisition and analysis software.

Sholl analysis (Wilson, Badley et al. 2024) was used to quantify dendritic arborization in cells expressing the MAP2B-FusRed protein. Custom ImageJ macros (Badley 2025) were used for automated analysis of dendritic arborization using images of MAP2BFusRed-transfected neurons acquired on the high-content imaging system. These macros automatically identified fluorescently labeled neurons associated with a nucleus, extracted image data from the background, selected center points for the Sholl rings, and subsequently automatically counted the number of dendritic processes of individual neurons that intersected with each of the concentric Sholl rings. Criteria used to select labeled neurons to be included in the automated analysis were: (1) the fluorescent image had only one associated nucleus (e.g., clumps of cells were not included in the analysis); (2) the neuron was isolated (e.g., its processes did not intermingle with processes of another labeled neuron); and (3) the image was not located on the border of the field or cut off by the field edges.

2.11 Statistical analysis

Power analysis based on prior data (da Costa Souza, Grodzki et al. 2023) indicated that a sample size of 4 independent dissections (3 wells/group in each dissection) would provide

sufficient statistical power (≥80%) to detect a biologically meaningful effect size at α = 0.05. Male and female data were analyzed separately. All statistics were done in GraphPad Prism (RRID:SCR_002798) version 10.0.0. All data, except the Sholl analysis, were analyzed using one-way ANOVA with a *post-hoc* Dunnet's test to identify significant differences (p-value <.05) between vehicle and PCB-exposed cultures. A mixed-effect analysis with *post-hoc* Dunnet's test (Wilson, Sethi et al. 2017) was used to identify significant differences in the number of intersections at each set distance between vehicle and PCB-exposed cultures .

3. Results

Given the detection of these congeners and metabolites in human tissues (Sethi 2019, Li, Hefti et al. 2022), focusing on PCB 2, PCB 3, and PCB 28 allows for a wide-ranging assessment of the DNT potential of LC-PCBs and their metabolites in humans.

3.1. LC-PCBs do not alter cell viability at concentrations ≤ 100 nM

To identify concentration-dependent effects of PCBs on cell viability, dissociated primary rat cortical neuron-glia co-cultures, sex segregated at the time of plating, were grown under the same culture conditions used for apoptosis and dendritic arborization assays, and exposed to varying concentrations (0.0001, 0.1, 100 nM) of the parent PCB congeners or their metabolites for 48 h. On DIV 9, cell viability was determined by quantifying LDH release into the culture media and the percentage of cells stained with calcein-AM, which labels live cells (Sadeh, Oni-Biton et al. 2016). Relative to age- and sex-matched vehicle controls, cultures from the same dissections exhibited no significant change in either LDH release or percentage of live cells following a 48-h exposure to PCBs at all concentrations (Data not shown).

3.2 LC-PCB modulates apoptosis in a congener-, concentration-, and sex-dependent manner

To evaluate apoptotic responses to LC-PCBs, we quantified caspase 3/7 activity in DIV 9 cultures after a 48 h exposure to PCB 2, PCB 3, PCB 28 or their human-relevant metabolites (Figure 3.1). Exposure to PCB 2 or 4-OH PCB 2 did not significantly alter caspase 3/7 activity in female cultures (Figure 3.1A); in contrast, in male cultures, caspase 3/7 activity was significantly increased by PCB 2 at 100 nM and by 4-OH PCB 2 at 0.0001 nM (Figure 3.1B). While exposure to the parent PCB 3 congener did not alter caspase 3/7 activity in either sex, 100 nM 3-OH PCB 3 significantly increased caspase 3/7 activity in both female and male cultures (Figure 3.1C,D). Exposure to the parent PCB 28 congener or four different human-relevant PCB 28 metabolites, 3-OH PCB 28, 3'-OH PCB 28, 4-OH PCB 25 or 4-SO_3-PCB 25, had no significant effect on caspase 3/7 activity in female cultures (Figure 3.1E). In contrast, caspase 3/7 activity was significantly decreased in male cultures exposed to 100 nM PCB 28, 0.1 or 100 nM of 3-OH PCB 28 or 3'-OH PCB 28, or 0.1 nM 4-OH PCB 25, but was significantly increased in male cultures exposed to 4-OH PCB 25 or 4-SO_3 PCB 25 at 0.0001 nM (Figure 3.1F).

3.3 LC-PCBs exert complex effects on axonal growth

The impact of PCBs 2, 3, and 28 and their metabolites on average axon outgrowth per neuron in DIV 2 cultures after a 48 h exposure varied in a congener-, concentration- and sex-dependent manner (Figure 3.2). In female cultures, PCB 2 had no significant effect on axonal growth; however, its metabolite 4-OH PCB 2 significantly increased axon outgrowth at 0.1 and 100 nM (Figure 3.2A). Conversely, in male cultures, PCB 2 significantly reduced axon length at 0.01 and 100 nM, whereas 4-OH PCB 2 had no significant effect (Figure 3.2B). PCB 3 did not significantly alter axonal growth in female (Figure 3.2C) or male (Figure 3.2D) cultures, and while 3-OH PCB 3 also did not significantly affect axon outgrowth in female cultures (Figure 3.3C), it did significantly decrease axonal growth in male cultures exposed to this PCB 3 metabolite at 0.1 or 100 nM (Figure 3.2D). More pronounced effects on axonal morphology were observed in response to PCB 28 and its metabolites (Figure 3.2E, F). In female cultures

exposed to the parent PCB 28 congener at 0.0001, 0.1, or 100 nM, axonal growth was significantly increased; in contrast exposure to 3'-OH PCB 28 at 0.1 nM, 4-OH PCB 25 at 0.001, 0.1 or 100 nM, or 4-SO_3 PCB 25 at all three concentrations significantly reduced axon outgrowth (Figure 3.2E). Similarly, in male cultures, exposure to PCB 28 at any of the three tested concentrations significantly increased axonal outgrowth while male neurons exposed to 0.0001 nM of 3'-OH PCB 28 or 4-SO_3 PCB 25, or 0.1 or 100 nM of any of the four PCB 28 metabolites exhibited significantly decreased axonal outgrowth (Figure 3.2F).

3.4 LC-PCBs modulate dendritic arborization

PCB 2 produced distinct, sex-specific alterations in dendritic architecture. In female cultures, all three concentrations PCB 2 significantly increased proximal dendritic growth (within 50–100 μm from soma) that shifted to a distal reduction in complexity (at distances >100 μm from the soma) across all concentrations (Figure 3.3A). In male cultures, PCB 2 increased dendritic complexity across all concentrations (Figure 3.3B). The hydroxylated metabolite, 4-OH PCB 2, decreased primary dendritic growth, but increased proximal dendritic arborization in female neurons at all concentrations (Figure 3.3C). In male neurons, 4-OH PCB 2 significantly increased dendritic arborization at all concentrations (Figure 3.3D).

PCB 3 had no significant effect on dendritic arborization in female cultures across all concentrations tested (Figure 3.4A) as evidenced by Sholl plots that overlapped the vehicle control. In contrast, dendritic complexity was significantly increased in male cultures exposed to PCB 3 at 0.0001 or 0.1 nM (Figure 3.4B). The hydroxylated metabolite, 3-OH PCB 3, had no significant effect on dendritic complexity in cultures of either sex at any of the concentrations tested in this study (Figure 3.4C,D).

Dendritic effects of PCB 28 showed marked sex differences. In female cultures, PCB 28 significantly decreased dendritic complexity (Figure 3.5A). In contrast, dendritic growth in male

cultures was not significantly altered by PCB 28 (Figure 3.5B). PCB 28 metabolites also elicited sex-specific changes in dendritic arborization. In female cultures, similar to the parent congener, 3-OH PCB 28 (Figure 3.5C) and 3′-OH PCB 28 (Figure 3.5E) significantly decreased dendritic arborization at all three concentrations. In male cultures, in contrast to the parent compound, all three concentrations of 3-OH PCB 28 significantly decreased dendritic arborization (Figure 3.5D) while 3′-OH PCB 28 decreased dendritic complexity at 0.001 and 0.1 nM concentrations, but significantly enhanced dendritic complexity at 100 nM (Figure 3.5F). In female cultures, the 4-OH PCB 25 metabolite produced an increase in complexity at 0.0001 nM and a decrease at 100 nM, whereas in male cultures, a decrease in dendritic complexity was seen at all concentrations. All three concentrations of the metabolite 4-SO_3 PCB 25 significantly reduced dendritic growth in female cultures. In contrast, divergent effects were observed in male cultures, with significantly increased dendritic complexity observed in male cultures exposed to this metabolite at 0.001 and 100 nM.

4. Discussion

Our findings extend previous studies indicating that differences in PCB structure and regiochemistry influence their DNT profiles (Holland, Feng et al. 2017, Sethi 2017), and that these effects are greatly influenced by sex (Sethi, Keil et al. 2017, Matelski, Keil Stietz et al. 2020, Keil Stietz 2021) . Importantly, the concentrations shown to alter key neurodevelopmental endpoints did not affect cell viability, demonstrating that these changes occur in the absence of overt cytotoxicity.

Sex differences were consistently observed across endpoints, aligning with previous studies (Badley, Andrew et al. 2025) (Sethi, Keil et al. 2017). Across sexes, PCB 2 and PCB 3 elicited the strongest responses in males, and PCB 28's effects were strongly metabolite-dependent. These differences likely stem from variations in how key molecular targets are shaped or occupied in each biological context. While many developmental signaling pathways are shared

between sexes (Mank and Rideout 2021), expression or activation can differ substantially due to variations in circulating hormone levels, carrier protein abundance, receptor isoform expression, and metabolic enzyme activity (Bolte, Neufeld et al. 2023). Such differences may alter both the availability and conformational state of binding sites(Milon, Opydo-Chanek et al. 2017), amplifying the biological consequences of even minor structural differences between PCB congeners.

Across all endpoints, dendritic arborization emerged as the most sensitive, with several congeners eliciting significant alterations in dendritic growth in a congener- and sex-specific manner. Dendritic arborization exhibited pronounced sex differences and congener-specific patterns. PCB 2 induced distal reductions in females but global increases in males. 4-OH PCB 2 had male-biased increases in dendritic complexity, whereas PCB 3 showed minimal effects in females and modest increases in males. PCB 28 decreased dendritic complexity in females, while male responses varied with the specific metabolite and concentration. These observations underscore that dendritic growth is a highly structure- and sex-dependent endpoint, influenced by both parent congeners and metabolites. Axonal growth was also affected, but the direction and magnitude of effects varied by congener, metabolite, and sex. PCB 2 had no effect in female cultures but reduced axonal growth in males, however 4-OH PCB 2 decreased growth in female cultures and had no effect in male cultures. PCB 3 had no effect in either sex, and 3-OH PCB 3 decreased growth in male cultures. PCB 28 significantly increased axonal growth in both sexes, whereas its metabolites produced reductions at specific concentrations, highlighting the complex influence of metabolism on endpoint sensitivity. These findings are consistent with previous work showing LC-PCBs such as PCB 11 (Sethi 2017) and PCB 37 (Badley, Andrew et al. 2025), produce subtle, endpoint-specific, and metabolite-dependent effects.

The comparison of parent congeners versus metabolites demonstrates that metabolism can either enhance or qualitatively shift developmental neurotoxicity. Hydroxylation alters polarity

and planarity, influencing interactions with molecular targets such as ryanodine receptors (Fritsch and Pessah 2013, Holland, Feng et al. 2017), and in some cases metabolites can have effects on critical endpoints at lower concentrations than the parent compounds (Dhakal, He et al. 2012, Dhakal, Gadupudi et al. 2018)

Structure clearly modulates DNT outcomes. PCB 2 (meta-Cl) and PCB 3 (para-Cl) adopt more planar conformations relative to ortho-substituted congeners, imparting limited dioxin-like character and reducing RyR activation, whereas PCB 28 (ortho-Cl) adopts a non-planar configuration that favors RyR interactions (Holland, Feng et al. 2017). These structural differences, combined with metabolism, drive the congener- and sex-specific effects observed across apoptosis, axonal growth, and dendritic arborization.

A limitation of this study is the absence of full dose-response analyses for each congener; instead, fixed concentrations were used to allow direct comparisons between parent PCBs and their metabolites across key neurodevelopmental endpoints. This study utilized primary *in vitro* cultures, which are well-suited for mechanistic and high-throughput experiments, however, they do not capture the systemic interactions that would be present in vivo. Future studies should validate these findings in animal models. Further investigation of mechanistic pathways, such as CREB signaling, is also critical to see if previous findings extend to all LC-PCB congeners.

Taken together, these findings demonstrate that LC-PCBs and their metabolites alter apoptosis, axonal growth, and dendritic arborization, endpoints that are critically implicated in neurodevelopmental disorders, supporting their classification as potential developmental neurotoxicants. Importantly, this work underscores that (1) PCB-induced developmental neurotoxicity is highly structure-dependent, (2) metabolism can shift neurotoxic potential, and (3) sex differences modify response. These results have direct human-health relevance, as they suggest that exposure to specific PCB congeners and metabolites during sensitive windows of brain development could contribute to adverse neurodevelopmental outcomes, as the

concentrations tested are within ranges detected in maternal and cord blood (Berghuis, Soechitram et al. 2014, Berghuis, Van Braeckel et al. 2018, Zhang, Saktrakulkla et al. 2021, Balalian 2024). By identifying congener-specific DNT potential, this study also provides insight that can inform risk assessment and regulatory decision-making.

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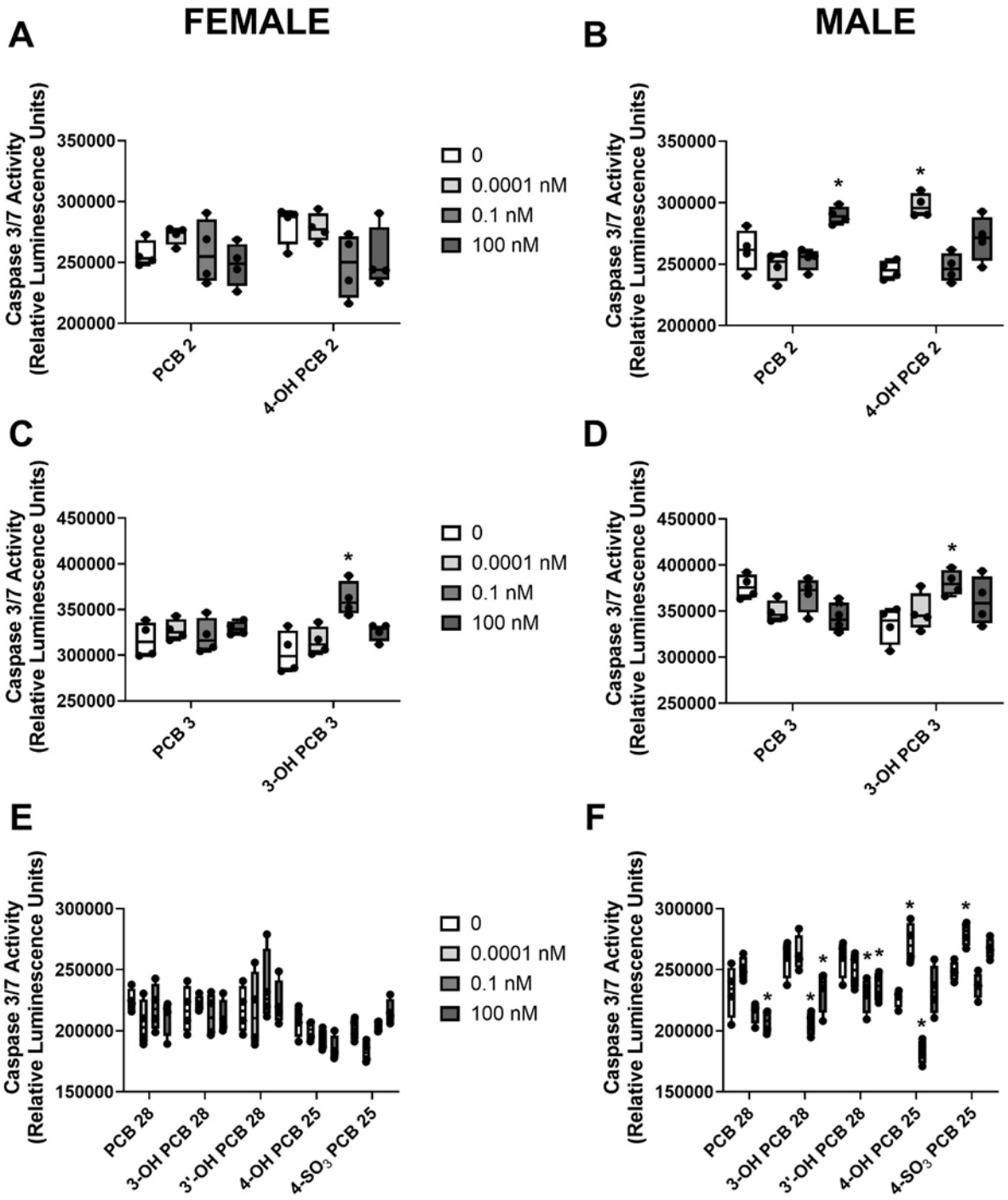


Figure 3.1. **LC-PCBs increased caspase 3/7 activity in primary rat cortical neuron-glia cocultures in a sex- and concentration-dependent manner.** Sex-segregated cultures were

exposed on DIV 7 to vehicle (0.001% DMSO) or 0.0001, 0.1, or 100 nM of parent or human-relevant metabolites of PCB 2 (A, B), PCB 3 (C, D) or PCB 28 (E, F). Caspase 3/7 activity was measured 48 h later. Data are presented as box-and-whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=4 independent dissections). Data for female (A, C, E) and male (B, D, F) cultures were analyzed separately. *Significantly different from vehicle control at $p < 0.05$ (one-way ANOVA, post-hoc Dunnett's test).

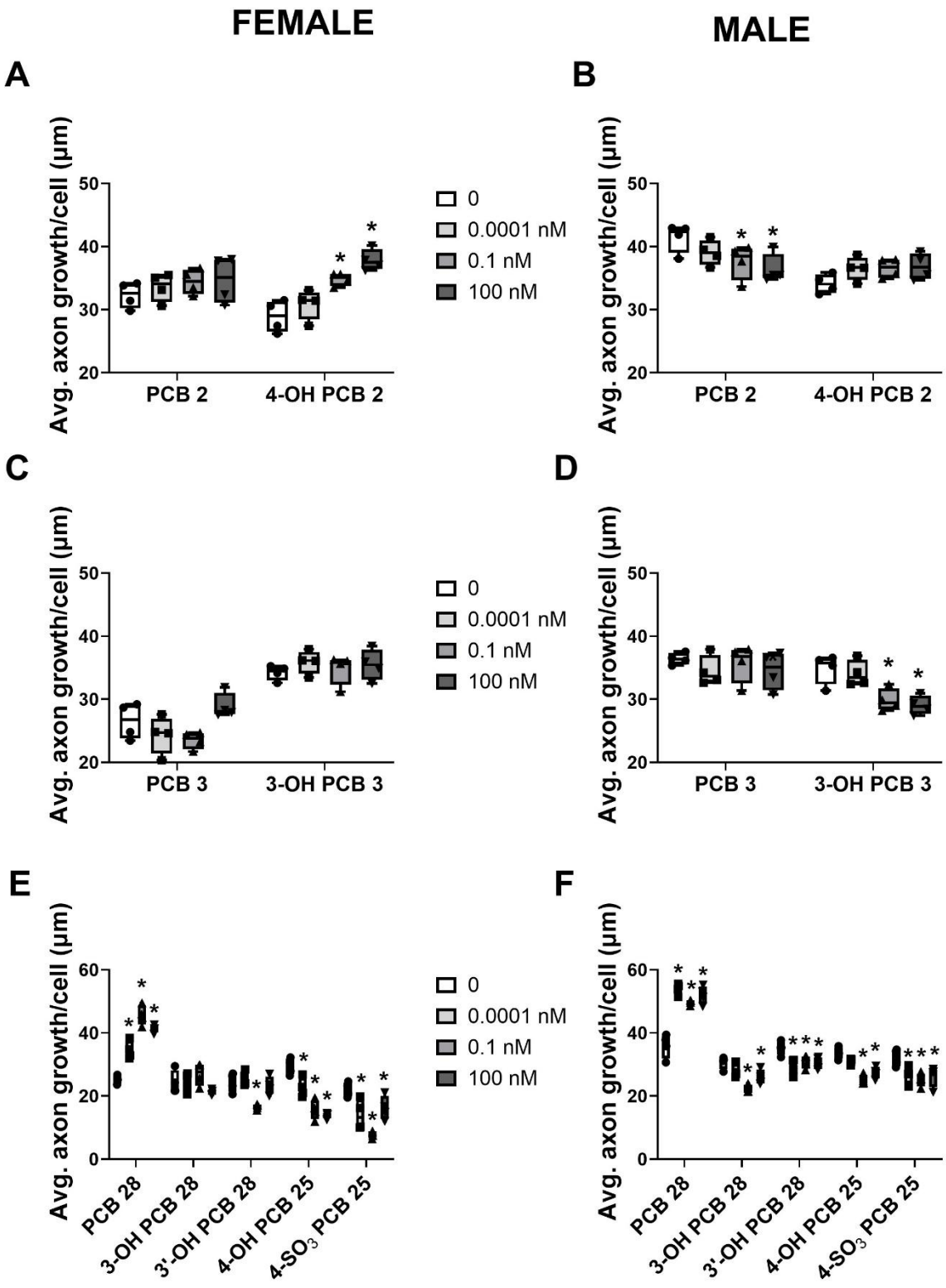

FEMALE
MALE
A
B
C
D
E
F
Avg. axon growth/cell (µm)
0
0.0001 nM
0.1 nM
100 nM
PCB 2
4-OH PCB 2
PCB 3
3-OH PCB 3
PCB 28
3-OH PCB 28
3'-OH PCB 28
4-OH PCB 25
4-SO3 PCB 25

Figure 3.2. Axonal growth in primary cortical cultures following exposure to parent and hydroxylated lower chlorinated PCBs. Primary rat cortical neuron-glia co-cultures were treated on DIV 0 with increasing concentrations (0, 0.0001, 0.1, or 100 nM) of selected parent or hydroxylated PCB congeners. Average axonal outgrowth per cell (μm) was measured after 48 h. Data are shown separately for female (A, C, E) and male (B, D, F) cultures. (A, B) Axonal growth after treatment with PCB 2 and its metabolite 4-OH PCB 2. (C, D) Axonal growth after treatment with PCB 3 and its metabolite 3-OH PCB 3. (E, F) Axonal growth after treatment with PCB 28, 3-OH PCB 28, 3'-OH PCB 28, 4-OH PCB 25, and 4-SO_3 PCB 25. Data are presented as box-and-whisker plots in which the box indicates the 25th (lower) to 75th (upper) quartiles; the middle horizontal bar, the median; whiskers, the minimum and maximum values; and dots, the individual values (n=4 independent dissections). Data for female (A, C, E) and male (B, D, F) cultures were analyzed separately. *Significantly different from vehicle control at $p < 0.05$ (one-way ANOVA, post-hoc Dunnett's test).

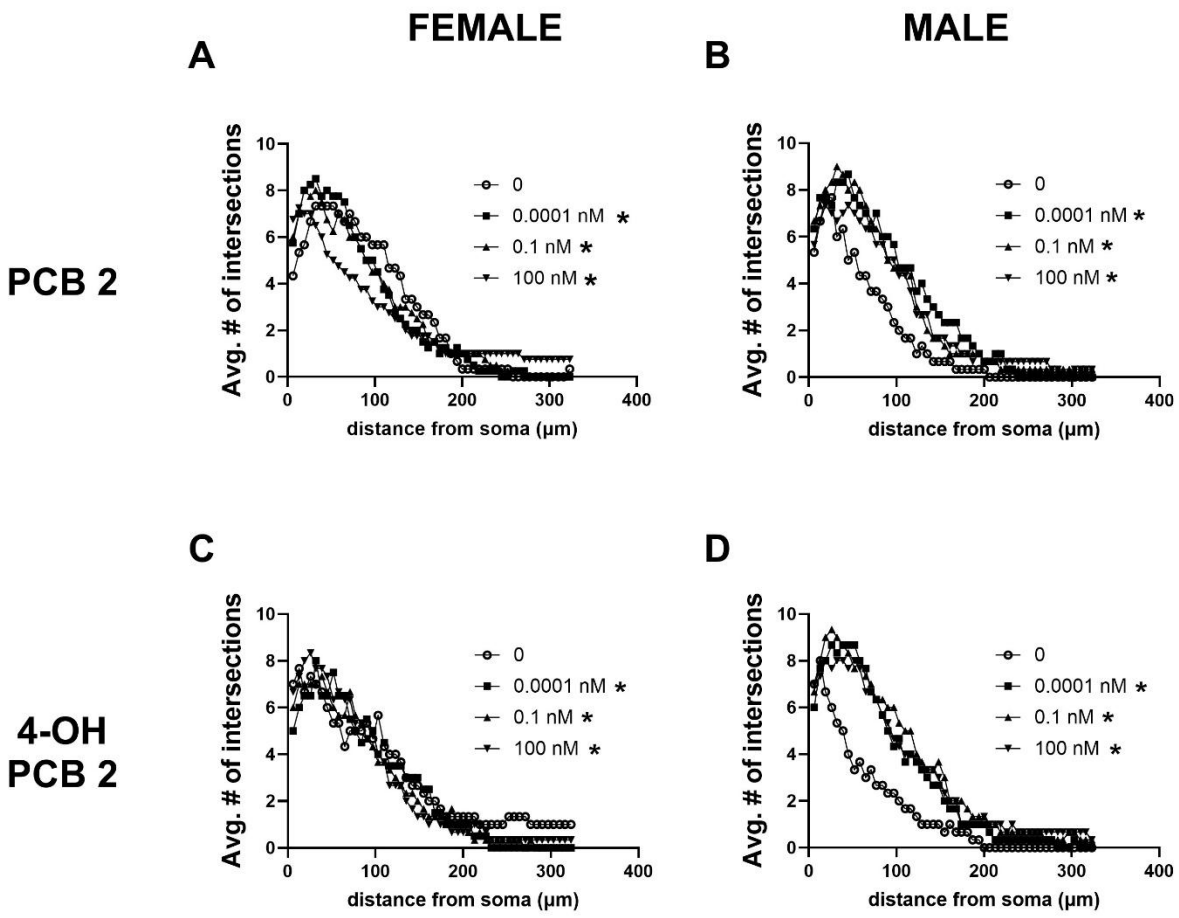


Figure 3.3. **PCB 2 and 4-OH PCB 2 altered dendritic complexity in a sex- and concentration-dependent manner.** Sholl plots of female (A, C) and male (B, D) cortical neurons at DIV 9 following a 48-h exposure to vehicle (0.001% DMSO) or varying concentrations of PCB 2 (A, B) or 4-OH PCB 2 (C, D). Data are presented as the mean # of intersections (n=4 independent dissections). Data for female (A, C) and male (B, D) cultures were analyzed separately. *Significantly different from vehicle control at $p < 0.05$ (mixed-effect analysis, post-hoc Dunnett's test).

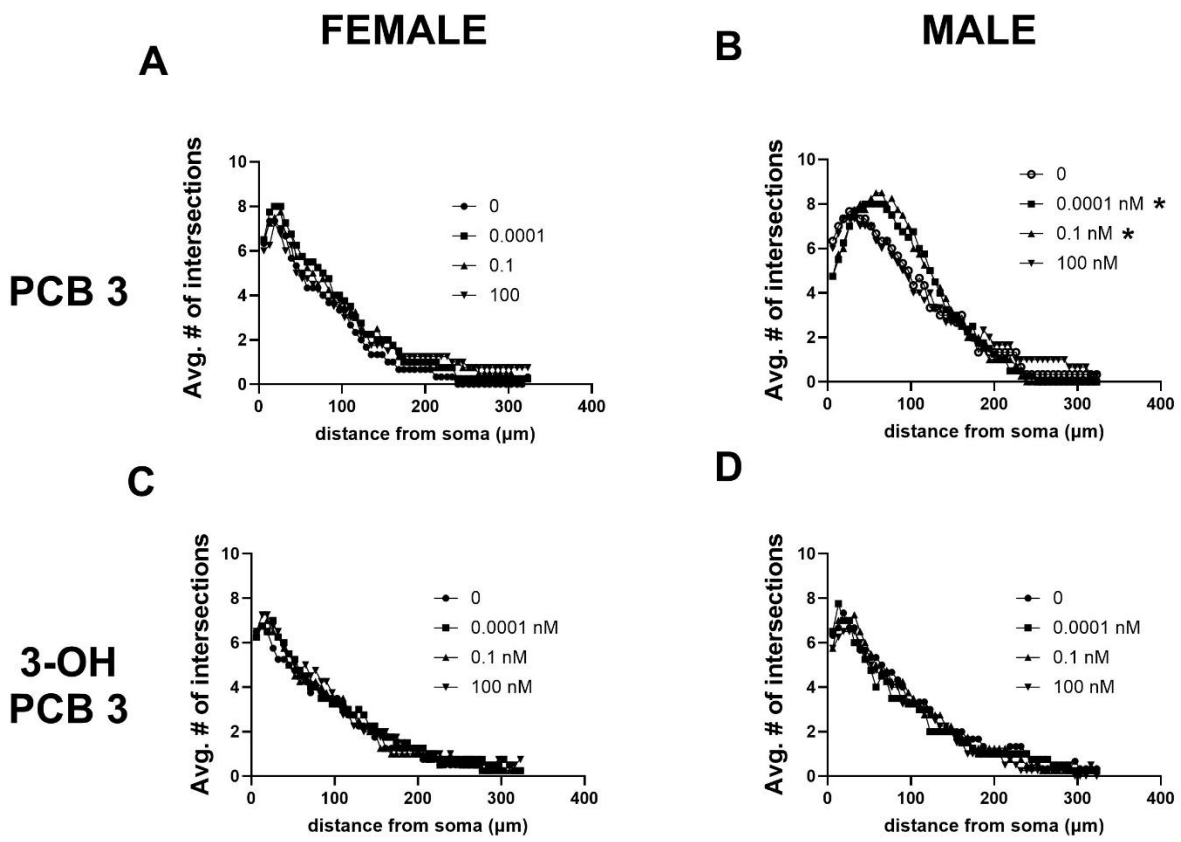


Figure 3.4. **PCB 3 and 3-OH PCB 3 altered dendritic complexity in a sex- and concentration-dependent manner.** Sholl plots of female (A, C) and male (B, D) cortical neurons at DIV 9 following a 48-h exposure to vehicle (0.001% DMSO) or varying concentrations of PCB 3 (A, B) or 3-OH PCB 3 (C, D). Data are presented as the mean # of intersections (n=4 independent dissections). Data for female (A, C) and male (B, D) cultures were analyzed separately. *Significantly different from vehicle control at $p < 0.05$ (mixed-effect analysis, post-hoc Dunnett's test).

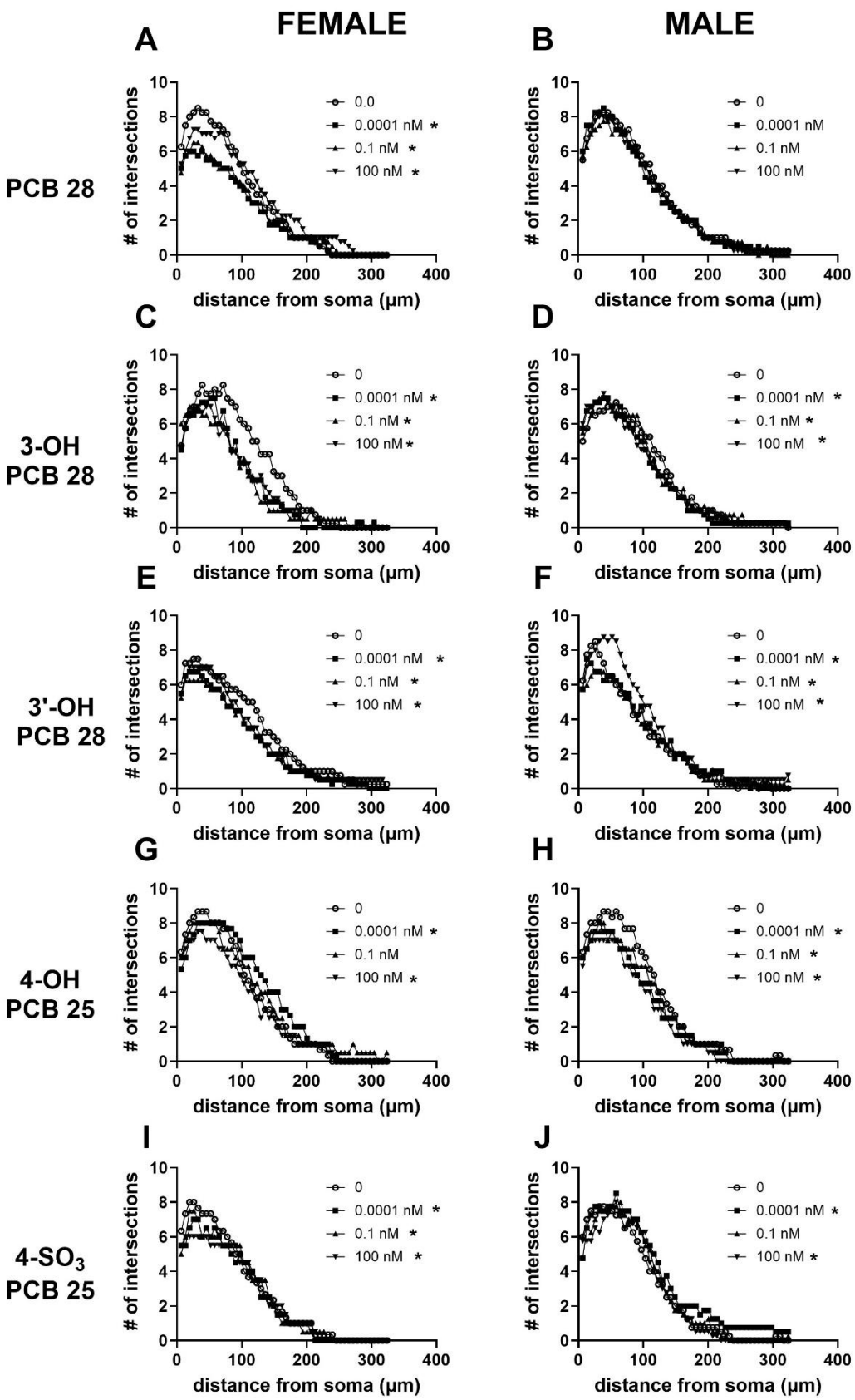

FEMALE
MALE
A
B
C
D
E
F
G
H
I
J
PCB 28
3-OH PCB 28
3'-OH PCB 28
4-OH PCB 25
4-SO3 PCB 25
# of intersections
distance from soma (µm)
0.0
0
0.0001 nM *
0.1 nM *
100 nM *
0.1 nM
100 nM

Figure 3.5. **PCB 28 and its metabolites altered dendritic complexity in a sex- and concentration-dependent manner.** Sholl plots of female (A, C, E, G, I) and male (B, D, F, H, J) cortical neurons at DIV 9 following a 48-h exposure to vehicle (0.001% DMSO) or varying concentrations of PCB 28 (A, B), 3-OH PCB 28 (C, D), 3′-OH PCB 28 (E, F), 4-OH PCB 25 (G, H), or 4-SO_3 PCB 25 (I, J). Data are presented as the mean # of intersections (n=4 independent dissections). Data for female (A, C) and male (B, D) cultures were analyzed separately. *Significantly different from vehicle control at $p < 0.05$ (mixed-effect analysis, post-hoc Dunnett's test).

Chapter 4: Conclusion

The overarching goal of this dissertation was to identify critical endpoints of neurodevelopment altered by lower-chlorinated polychlorinated biphenyls (LC-PCBs) and their human-relevant metabolites in sex-segregated primary rat neuron-astrocyte cocultures. This is important as disruptions in neuronal morphogenesis and apoptosis are implicated in the pathogenesis of neurodevelopmental disorders (Panesar 2020) and identifying specific endpoints altered by LC-PCBs provides mechanistic insight into how these contemporary environmental pollutants perturb brain development while also informing human health risk assessment of environmentally-relevant exposures. Each data chapter provides evidence that exposure to specific LC-PCB congeners alters critical neurodevelopmental processes *in vitro*.

In Chapter 2, I reported that PCB 37 caused sex-, age-, and concentration-dependent changes in apoptosis, axonal growth, and dendritic growth at concentrations (0.001, 0.1, and 100 nM) that did not affect cell viability. Importantly, these concentrations are environmentally relevant (Sethi 2019, Li 2022).

The data presented in Chapter 3 expands our understanding of the potential for LC-PCBs as a general class to interfere with normal neurodevelopment and begins to identify how PCB structure affects the developmental neurotoxicity profile of individual congeners. Specifically, we expanded the list of LC-PCBs with DNT potential from PCB 11 to include not only PCB 37 (Chapter 2), but also PCBs 2, 3, and 28 and their human-relevant metabolites. Our findings also provide new evidence that subtle differences in PCB structure and regiochemistry can strongly influence effects on apoptosis, axonal and dendritic growth, and that these effects are greatly modulated by sex (Cosgrove, Mazure et al. 2007). These LC-PCB congeners were tested at the same concentrations (0.001, 0.1, and 100 nM) to enable direct comparison of their effects under equivalent exposure conditions in order to identify congener-specific differences in neurotoxic potential. Such differences likely arise from variations in the specific molecular

targets that are engaged by each congener and/or sex variations in the spatiotemporal expression patterns of molecular targets (Milon, Opydo-Chanek et al. 2017, Bell 2018, Liberman 2020). These findings are consistent with prior studies of PCB developmental neurotoxicity (Yang 2010, Wayman 2012, Yang 2014, Sethi 2017, Keil 2019), although differences in observed effects likely reflect variations in the specific congeners tested as well as differences in exposure concentrations and developmental timing across *in vivo* and *in vitro* models. Together, Chapters 2 and 3 show that subtle structural differences among PCBs can distinctly influence neurotoxicity. Importantly, these data establish LC-PCBs as developmental neurotoxicants that may be as concerning than HC-PCBs (Klocke 2020).

Findings from Chapter 3 show concentration- and sex-specific trends that align with those described in Chapter 2, reinforcing the hypothesis that current PCB exposures may endanger the developing brain and pointing to possible structure-activity relationships underlying LC-PCB developmental neurotoxicity. Both chapters also reveal non-monotonic dose-response relationships, a phenomenon previously reported in PCB research (Yang 2009, Wayman 2012, Bell 2018) but often overlooked in conventional risk assessment models, which typically assume monotonic effects. This underscores the importance of incorporating non-monotonic dose-response patterns into future PCB risk evaluations (Committee, More et al. 2021).

Sex as a Biological Factor in LC-PCB Developmental Neurotoxicity

This dissertation provides robust evidence that sex is a critical determinant of LC-PCB-induced developmental neurotoxicity. Sex-specific responses were observed for all LC-PCBs across all neurodevelopmental outcomes assessed in this thesis, often with opposite directional changes between male and female cultures for the same endpoint and exposure. For example, PCB 2 increased dendritic complexity in male neurons but increased the proximal dendritic arbor and decreased the distal dendritic arbor in female cultures. Similarly, PCB 28 reduced

dendritic complexity in female cultures while having little to no effect in male cultures, whereas some metabolites of PCB 28 increased dendritic complexity of male neurons. These differences are consistent with a growing body of literature showing that sex influences vulnerability to environmental neurotoxicants, including PCBs (Sethi, Keil et al. 2017, Bell 2018, Liberman 2020), through mechanisms that may include differences in hormone receptors (e.g., estrogen and androgen receptors), neurotransmitter receptors (e.g., GABAergic and glutamatergic receptors), hormone signaling, metabolism, and intracellular signaling cascades (Cosgrove, Mazure et al. 2007, McCarthy 2016, Etchell, Adhikari et al. 2018, Mank and Rideout 2021). These sex-specific responses are relevant because they may underlie differential vulnerability to PCB-induced neurodevelopmental deficits and help explain sex biases in neurodevelopmental disorders (Gore, Krishnan et al. 2019, Keil 2019, Enriquez, Gupta et al. 2021, Keil Stietz 2021, Bolte, Neufeld et al. 2023).

Sex differences in baseline dendritic and axonal architecture (Keil, Sethi et al. 2017, Sethi, Keil et al. 2017) and neuronal signaling (Zhang, Li et al. 2003) could contribute to the observed divergences in response between the sexes. Sex differences in dendritic morphology have been documented, with male hippocampal neurons exhibiting greater dendritic growth than females both *in vivo* and *in vitro*, but female cortical neurons show more greater dendritic growth than male (Keil, Sethi et al. 2017, Sethi, Keil et al. 2017). These effects were partly estrogen-dependent, as blocking estrogen signaling reduced dendritic complexity in male hippocampal neurons.

Sex-dependent differences in neuronal responses to PCB congeners and their metabolites may arise from variations in molecular interactions, such as differential receptor engagement or affinity (Hong, Branham et al. 2015), as well as from divergence in downstream signaling cascades, whereby identical pathway activation leads to distinct cellular outcomes in male versus female neurons. Additionally, sex hormones can regulate metabolic enzyme activity

(Milon, Opydo-Chanek et al. 2017), potentially altering the relative abundance of parent congeners versus hydroxylated or sulfated metabolites, thus influencing potency and direction of effects. Because male neurons carry a Y chromosome while female neurons do not, sex chromosome-encoded genetic determinants inherently differ between the two; moreover, even though primary cultures are isolated from systemic endocrine influences, intrinsic sex differences in hormone receptor expression and downstream signaling within neurons and astrocytes may still contribute to the sex-specific responses we observed. The cumulation of these data in both chapter 2 and 3 emphasize the importance of including both sexes in developmental neurotoxicity testing and of considering sex as a biological variable.

Emerging evidence has also highlighted that sex differences in neurodevelopment may contribute to differential vulnerability to neurodevelopmental disorders (NDDs) such as autism spectrum disorder (ASD) (Enriquez, Gupta et al. 2021, Bolte, Neufeld et al. 2023). Notably, epidemiological studies(Panesar 2020, Keil-Stietz 2023, Balalian 2024) have reported associations between PCB exposure and increased risk of ASD and related neurodevelopmental outcomes, suggesting that environmental toxicants such as PCBs may interact with these sex-dependent developmental pathways. Transcriptomic studies show that genes dysregulated downstream of ASD risk genes exhibit sex-differential expression (Matelski, Keil Stietz et al. 2020), suggesting that male and female brains respond differently to the same genetic risk. There have also been preclinical studies that support a mechanistic role for sex steroids: estrogens can rescue cellular and behavioral phenotypes associated with ASD risk genes, modulate GABAergic and glutamatergic signaling, and promote synaptogenesis and neuronal survival. Collectively, these findings indicate that sex-specific molecular and circuit-level processes shape neurodevelopment.

These data suggest that developmental neurotoxicity testing frameworks should incorporate sex as a variable, evaluate both parent and metabolite congeners, and consider non-monotonic responses in hazard characterization.

PCB Metabolism and Regiochemistry as Determinants of Neurotoxicity

The findings reported in this dissertation reveal that small structural changes, such as changes in the position of a chlorine atom or the site of hydroxylation, can alter a congener's developmental neurotoxicity profile. Regiochemistry influenced both response and direction of dendritic changes. For example, PCB 2 (meta substitution) was associated with female-specific effects, while PCB 3 (para substitution) was associated with male-specific effects. Hydroxylation reduced effects compared to parent congeners, although exceptions were observed (e.g., certain PCB 28 metabolites).

These observations are consistent with structure-activity relationship (SAR) principles in which electron distribution, symmetry, and steric hindrance influence how a molecule interacts with protein targets such as ion channels, nuclear receptors, or cytoskeletal regulators (Akinola, Uzairu et al. 2022, Akinola, Uzairu et al. 2023). Metabolism can either reduce or enhance activity depending on whether the transformation enhances or decreases binding affinity for the target or alters membrane permeability. The divergent effects among PCB 28 metabolites illustrate that different metabolic pathways yield products with distinct neurodevelopmental outcome profiles. SAR studies (Fritsch and Pessah 2013, Holland, Feng et al. 2017) of PCBs have demonstrated that certain ortho-substituted NDL PCBs, such as PCB 95, 136, and 149, exhibit high potency in activating Ryanodine Receptor 1 (RyR1), leading to increased Ca^{2+} release and potential neurotoxic effects. Conversely, non-ortho-substituted congeners like PCB 77 show minimal activity at RyR1, highlighting the importance of specific chlorination patterns in determining receptor interaction and toxicity. In our data, PCB 28 and its metabolites, which were the only ortho-substituted congeners tested in this thesis, may engage RyR-mediated

pathways, whereas PCB 37, which lacks an ortho substitution, would be expected to act through RyR-independent mechanisms. Interestingly, PCB 37's effects on apoptosis and dendritic growth were ameliorated with CREB inhibition, suggesting that it influences these pathways through alternative, CREB-dependent signaling cascades that converge with RyR-mediated calcium signaling. These results highlight that chlorination pattern predicts potential RyR interactions, but even PCBs with negligible RyR activity can still modulate neuronal morphology via alternative molecular pathways.

CREB Signaling as a Potential Mechanism

CREB is a key transcription factor involved in regulating dendritic growth, synapse formation, and neuronal survival during development (Sakamoto 2011). Prior work showed that some NDL-PCBs, such as PCB 95, activated CREB via Ca^{2+}-dependent pathways involving RyR sensitization (Wayman, Bose et al. 2012, Wayman 2012) while others, like PCB 11, engaged CREB through RyR-independent mechanisms (Wayman 2006, Wayman, Bose et al. 2012, Sethi 2018). These findings indicate that structurally diverse congeners can converge on CREB activation through multiple upstream routes (Klocke 2020). Although a role for CREB activity in the neurodevelopmental effects of PCB 2, PCB 3, or PCB 28 or their metabolites was not assessed, CREB inhibition blocked both dendritic and apoptotic effects of PCB 37, supporting a causal role for this pathway in at least some LC-PCB effects. Pretreatment with the CREB inhibitor 666-15 (500 nM) blocked the effects of PCB 37 on apoptosis and dendritic morphology but not axonal growth. These results indicate that PCB 37 alters dendritic growth and induces apoptosis via CREB-dependent mechanisms, while its effects on axonal development are mediated by CREB-independent pathways. Taken together, these findings point to PCB 37 as a potential developmental neurotoxicant and add to the growing body of evidence implicating CREB as a key convergence point in developmental neurotoxicity mechanisms (Sethi 2018, Klocke 2020). The identification of CREB as a convergent molecular

pathway of PCB DNT provides a more receptor-based framework for future risk assessment and therapeutic targeting.

Collectively, these data highlight CREB signaling as a potential unifying mechanism of PCB-induced DNT and a promising focus for future mechanistic studies.

Human Relevance and Risk Implications

These findings have clear human health relevance. All congeners tested here have been detected in human serum (Sethi 2019, Li 2022). LC-PCBs are present in contemporary environmental samples at concentrations overlapping those tested here (Sethi 2019). The concentrations that produced measurable changes in neurodevelopmental endpoints, often in the low nanomolar range, are within the range of human exposure, particularly in populations with elevated PCB burdens due to diet, occupation, or proximity to contaminated sites (Boucher 2009, Park 2010, Quinete, Esser et al. 2017, Wang 2020). The non-monotonic dose-response relationships observed for some congeners underscore the challenge of establishing safe exposure thresholds using traditional models, which assume that effects either consistently increase or decrease with dose.

Apoptosis, axonal growth, and dendritic arborization are essential neurodevelopmental processes that shape the formation of functional neural circuits (Copf 2016, Lein 2018, Hoersting 2021, Desai-Chowdhry 2022). Disruptions to any of these pathways have been linked to the pathogenesis of diverse neurodevelopmental disorders (Supekar, Uddin et al. 2013, Copf 2016, Guang 2018).

In conclusion, this work strengthens previous studies focused on PCB 11 that suggested the developmental neurotoxicity potential of LC-PCBs, by extending the database to include PCBs 2, 3, 28, 37 and their human-relevant metabolites. Specifically, I showed that structurally diverse LC-PCBs altered critical neurodevelopmental endpoints in primary cortical neurons in a

sex-dependent, structure-specific, and concentration-sensitive manner. Subtle alterations in congener regiochemistry and metabolic transformation modulated the developmental neurotoxicity profiles, underscoring the mechanistic complexity of PCB-induced developmental neurotoxicity. The consistent emergence of non-monotonic dose-responses further challenges the use of default monotonic assumptions in regulatory toxicology. My work also adds to the body of literature suggesting CREB-mediated signaling pathways as a convergent mechanism of developmental neurotoxicity. Collectively, these findings refine structure-activity relationship paradigms for LC-PCBs, highlight the necessity of incorporating sex as a biological variable, and argue for the integration of mechanistic endpoints and environmentally relevant exposure ranges into contemporary developmental neurotoxicity risk assessment frameworks.

Future Directions

Building on the findings described in this dissertation, future research should aim to further elucidate the molecular pathways underlying sex-specific LC-PCB neurotoxicity, with particular emphasis on dissecting the upstream regulators and downstream effectors of CREB signaling. Additionally, exploration of how sex- and genetic differences modulate PCB metabolism and receptor interactions will be essential for understanding sex-dependent susceptibility. *In vivo* studies are also needed to validate and extend these *in vitro* observations within the context of behavioral outcomes. Advancing quantitative structure-activity relationship models to integrate regiochemical and metabolic variation alongside non-monotonic dose responses will improve predictive toxicology and risk assessment paradigms. Finally, expanding studies to include mixtures of LC-PCBs at environmentally relevant doses will better approximate real-world exposures and inform regulatory frameworks aimed at protecting neurodevelopment in vulnerable populations.

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