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Abstract

Despite a worldwide ban on their commercial production since the early 2000s, polychlorinated biphenyls (PCBs) continue to pose a significant risk to human health. A primary target of concern is the developing brain. Epidemiological studies have reported positive associations between developmental exposures to PCBs and neuropsychiatric problems in children, including increased risk for neurodevelopmental disorders. However, the relative contribution(s) of individual PCB congeners to PCB developmental neurotoxicity, and the mechanism(s) by which PCBs disrupt

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neurodevelopment remain outstanding questions. Findings from experimental models and recent epidemiological studies suggest that the non-dioxin-like (NDL) PCBs are primarily responsible for the adverse neurodevelopmental outcomes associated with PCBs. Structure–activity relationship analyses indicate that NDL, but not dioxin-like (DL), PCBs increase intracellular calcium concentrations ($[Ca^{2+}]_i$) to alter Ca^{2+} signaling in neurons. NDL, but not DL, PCBs also interact with the ryanodine receptor (RyR) to stabilize this Ca^{2+} ion channel in its open configuration. This interaction is the most sensitive molecular mechanism underlying PCB impacts on Ca^{2} signaling identified to date. Evidence that RyR-dependent mechanisms contribute to PCB developmental neurotoxicity are discussed in this chapter. In addition, the broader implications of these findings for assessing human risk and critical data gaps are addressed.

1. Introduction

Both human and experimental animal studies have identified the developing brain as a vulnerable target of PCBs. Multiple reviews of the epidemiologic literature have concluded that developmental exposures to PCBs are associated with neuropsychological deficits in children, including impaired executive and psychomotor function, as well as deficits in attention, learning, and memory (Berghuis et al., 2015; Boucher et al., 2009; Pessah et al., 2019; Schantz et al., 2003). More recent reviews of the human literature also generally support the hypothesis that developmental PCB exposures increase the risk of neurodevelopmental disorders (NDDs) (Berghuis et al., 2015; Pessah et al., 2019), specifically, autism spectrum disorders (ASD) (Amen et al., 2022; Keil-Stietz and Lein, 2023; Mehri et al., 2021; Pessah et al., 2019; Xu et al., 2023, but see Cunha et al., 2023) and attention-deficit/hyperreactivity disorder (ADHD) (Eubig et al., 2010). A potential caveat of the epidemiological findings is that prenatal exposure to PCBs is also associated with increased risk of low birth weight, defined as <2500 g at birth (Baibergenova et al., 2003; Govarts et al., 2012; Hertz-Picciotto et al., 2005; Patandin et al., 1998; Taylor et al., 1989) and being small for gestational age (Govarts et al., 2018; Lauritzen et al., 2017; Longnecker et al., 2005), both of which are prognostic indicators of poor neurological outcome (Linsell et al., 2015; Linsell et al., 2016; Movsas et al., 2013; Salmaso et al., 2014). However, even after adjusting for birth weight and size, the positive association between developmental PCB exposures and neuropsychological deficits remains.

Experimental animal studies confirm that PCBs cause developmental neurotoxicity independent of adverse effects on reproductive and birth outcomes (Gore et al., 2019; Klocke and Lein, 2020; Sable and Schantz, 2006;

Winneke, 2011; Yang et al., 2009). In agreement with the human literature, experimental animal models of developmental PCB exposure have demonstrated deficits in behavioral domains related to cognition, attention, behavioral regulation and executive function, and social behavior (Carlson et al., 2023; Keil-Stietz and Lein, 2023; Klocke and Lein, 2020) (see Chapter 2 for additional details).

Which of the 209 structurally distinct PCB congeners and their hydroxylated or sulfonated metabolites contribute to PCB developmental neurotoxicity remains an outstanding question. Structurally, PCBs vary according to the number and position of chlorine substituents on the biphenyl backbone, which influences their volatility (volatility decreases with increasing number of chlorine substituents) and their biological activity (Grimm et al., 2015). Extensive experimental evidence has shown that biological targets and modes of action can vary significantly between PCB congeners and between parent congeners and their metabolites (Grimm et al., 2015). A key determinant of biological activity is the planarity of the congener, which is influenced by the position of chlorine substituents on the biphenyl backbone. PCBs with no chlorines in the ortho position assume a coplanar geometry of the rings, while PCBs with one to four chlorines in the ortho position assume increasing degrees of noncoplanar ring geometry. Coplanar congeners can bind to and activate the aryl hydrocarbon receptor (AhR), an intracellular ligand-activated transcription factor that is the canonical receptor for 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD). Therefore, noncoplanar PCBs are classified as dioxin-like (DL) PCBs (Carpenter, 2006; Erickson and Kaley, 2011; Van Den Berg et al., 1998). Of the 209 PCB congeners, twelve (PCBs 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189) are identified as DL-PCBs (Usepa, 2010). In contrast, noncoplanar congeners have little to no AhR binding affinity, and thus are referred to as non-dioxin-like (NDL) PCBs (Carpenter, 2006; Erickson and Kaley, 2011; Van Den Berg et al., 1998). Importantly, NDL PCBs represent a significantly greater percentage of the PCBs detected in human serum, adipose tissue, brain, placenta and breast milk, including brain from children diagnosed with neuropsychological deficits (Beyer and Biziuk, 2009; Granillo et al., 2019; Li et al., 2022; Mitchell et al., 2012; Mouat et al., 2023; Pessah et al., 2010; Sethi et al., 2019).

Although environmentally relevant exposures to dioxin and DL PCBs are associated with adverse outcomes in several organ systems, especially skin, liver and the immune system (Bock, 2016; Mellor et al., 2016;

Wheeler et al., 2017), and some are probably carcinogenic (Lauby-Secretan et al., 2013), there is little evidence that DL PCBs are direct developmental neurotoxicants (but see Hany et al., 1999; Ikeno et al., 2018; Nowack et al., 2015). While the currently available data do not support a major role for DL PCBs in PCB developmental neurotoxicity, they also do not rule out the possibility that DL PCBs play some role, possibly in modifying neurotoxic responses to NDL PCBs (Curran et al., 2012; Klinefelter et al., 2018).

In contrast, human, animal and mechanistic studies confirm the developmental neurotoxicity of at least a subset of NDL PCBs (Klocke and Lein, 2020). For example, perinatal exposure to PCB 95 has been shown to persistently alter cognitive and psychomotor activity in rodent models (Schantz et al., 1997) as well as LTP in hippocampal slice cultures (Wong et al., 1997b), and to alter the ratio of excitatory to inhibitory neurotransmission in the developing auditory cortex (Kenet et al., 2007) and hippocampal slice cultures (Kim et al., 2009). Moreover, congener-specific analyses of brain tissues from human subjects (Corrigan et al., 1998; Li et al., 2022; Mitchell et al., 2012; Mouat et al., 2023; Sethi et al., 2019) and experimental animals (Dziennis et al., 2008; Yang et al., 2009) exhibiting neuropsychological or behavioral impairment associated with exposure to complex PCB mixtures indicate enrichment of NDL ortho-rich congeners. These observations suggest that AhR-independent mechanisms are responsible for much of the developmental neurotoxicity associated with PCBs. The identification of alternative molecular targets and AhR-independent modes of actions by which PCBs promote adverse neurodevelopmental outcomes has been and continues to be the focus of extensive research.

A consistent observation across multiple human and experimental animal studies of PCB developmental neurotoxicity is that even overtly toxic levels of PCBs do not cause significant structural changes in the developing brain (Pessah et al., 2019). This is consistent with evidence suggesting that the neuropsychiatric and neurobehavioral deficits observed in PCB-exposed children and experimental animal models result from subtle organizational defects that alter the patterning of synaptic connections in the developing brain (Chu et al., 2019; Gilbert et al., 2000; Lamoureux-Tremblay et al., 2021; Lein et al., 2007; Migneron-Foisy et al., 2022; Pessah et al., 2019; Seegal, 1996; Sussman et al., 2022; White et al., 2011; Yang et al., 2009). The pattern of synaptic connectivity established during brain development determines neuropsychiatric function later in life, and altered patterns of synaptic connectivity are associated with NDDs and cognitive deficits not associated with NDDs (Alaerts et al., 2016;

Cooper et al., 2017; Coskun et al., 2013; Keown et al., 2013; Khan et al., 2015). The dynamic remodeling of synapses that occurs during development is driven in large part by Ca^{2+} signaling that mediates the influence of neural activity and other environmental factors on synaptogenesis and synaptic plasticity (Chen and Nedivi, 2010; Cline, 2001; Konur and Ghosh, 2005). Many NDD risk genes encode proteins that regulate intracellular Ca^{2+} signaling, are regulated by local fluctuations in Ca^{2+} concentrations and/or are involved in regulating synaptogenesis (Grove et al., 2019; Krey and Dolmetsch, 2007; Pessah et al., 2010).

Reports that NDL PCBs altered Ca^{2+} signaling in neural cells (Kodavanti et al., 1993; Kodavanti et al., 1996a; Kodavanti et al., 1996b; Trilivas and Brown, 1989) were among the first evidence that NDL PCBs were biologically active despite having negligible AhR activity. Subsequently, diverse biochemical, biophysical and cellular studies across many laboratories have consistently shown that NDL PCBs potently alter intracellular Ca^{2+} dynamics (Pessah et al., 2010), and a number of studies have linked PCB-induced Ca^{2+} dyshomeostasis to specific neurodevelopmental deficits (Keil-Stietz and Lein, 2023; Stamou et al., 2013). An early hypothesis was that developmental exposures to NDL PCBs caused cognitive and behavioral deficits by disrupting Ca^{2+}-dependent PKC signaling (Kodavanti and Tilson, 2000; Yang and Kodavanti, 2001). This hypothesis derived in part from observations that PCB disruption of Ca^{2+} fluctuations caused rapid redistribution of protein kinase C (PKC) isoforms in astrocytoma cells (Trilivas and Brown, 1989). Subsequent structure–activity relationship (SAR) studies indicated that NDL PCBs (Kodavanti and Tilson, 2000; Yang and Kodavanti, 2001), but not DL PCBs (Do and Lee, 2012), increased the translocation of PKC to the plasma membrane in cultured cerebellar neurons. In vivo studies demonstrated that developmental exposure to the commercial PCB mixture, Aroclor 1254, which is predominantly comprised of NDL PCBs (Yang et al., 2009), changed the subcellular distribution of PKC isoforms in the brain in a complex region-specific manner (Tilson and Kodavanti, 1997). However, whether influences on PKC signaling represent a primary mechanism driving the behavioral and cognitive impairments observed following developmental PCB exposure remains to be determined. For additional details about this mechanism, please see chapter 7 in this series.

Subsequent studies focused on the primary molecular mechanism(s) responsible for PCB effects on intracellular Ca^{2+} dynamics. In vitro studies using pharmacological blockade of specific Ca^{2+} channels showed that

NDL PCBs increased extracellular Ca^{2+} entry into cells through a number of mechanisms, including activation of L-type voltage-sensitive Ca^{2+} channels and NMDA receptors (Inglefield and Shafer, 2000; Mundy et al., 1999). However, such changes were elicited only at high PCB concentrations (≥10 μM) that were demonstrated to also produce nonspecific changes in membrane fluidity (Kodavanti et al., 1996a). NDL-PCBs were also shown to facilitate the release of Ca^{2+} from intracellular stores through sensitization of two genetically-related Ca^{2+} channels localized to the endoplasmic reticulum (ER): ryanodine receptors (RyR) (Wong and Pessah, 1996; Wong et al., 1997a; Wong et al., 1997b; Wong and Pessah, 1997) and inositol 1,4,5-trisphosphate receptors (IP_3R) (Inglefield et al., 2001). Of these, RyR sensitization is more sensitive to NDL PCBs, and this interaction exhibits a consistent stringent structure–activity relationship, including stereoselectivity, as determined using biochemical, electrophysiological, cellular and in vivo approaches (Pessah et al., 2010; Pessah et al., 2019). Of note, these mechanisms may not be mutually exclusive since RyRs are known to interact with IP_3R, -type voltage-sensitive Ca^{2+} channels and the NMDA receptor, and low μM concentrations of NDL PCBs have been shown to significantly enhance the sensitivity of primary cultured neurons to NMDA- and AMPA-elicited Ca^{2+} signals (Gafni et al., 2004).

This chapter focuses on PCB interactions with the RyR and their contribution to PCB developmental neurotoxicity, but will also discuss how PCB sensitization of RyRs may mediate other biochemical effects implicated in PCB developmental neurotoxicity, specifically, disruption of thyroid hormone (TH) signaling, dopamine depletion and generation of reactive oxygen species (ROS) (Klocke and Lein, 2020; Pessah et al., 2019). In addition, implications of a RyR-based mechanism of PCB developmental neurotoxicity for assessing risks these halogenated organic compounds (HOCs) pose to the developing human brain and critical data gaps are addressed here.

2. PCB–RyR interactions

2.1 RyR structure and function

RyRs are microsomal Ca^{2+} ion channels that are broadly expressed throughout the mammalian central and peripheral nervous systems (Lanner et al., 2010; Pessah et al., 2010). RyRs are the largest known ion channels (>2 MDa) and exist as three mammalian isoforms (RyR 1–3), all of which

are homotetrameric proteins. RyRs associate with cytosolic, endoplasmic reticulum (ER)-anchored and ER luminal proteins to form local Ca^{2+} release units (CRUs) that function to regulate Ca^{2+} release from the ER and to modify gating responses and signal gain of plasma membrane ion channels, notably NMDA receptors and voltage-gated Ca^{2+} channels (Fig. 1). Thus, RyRs function to modulate the amplitude and spatiotemporal fluctuation of intracellular Ca^{2+} during cell activation (Lanner et al., 2010; Pessah et al., 2010). The open probability of the RyR channel is increased by nanomolar concentrations of Ca^{2+} but decreased by millimolar concentrations of Ca^{2+} and Mg^{2+} (Lanner et al., 2010; Pessah et al., 2010). RyRs are

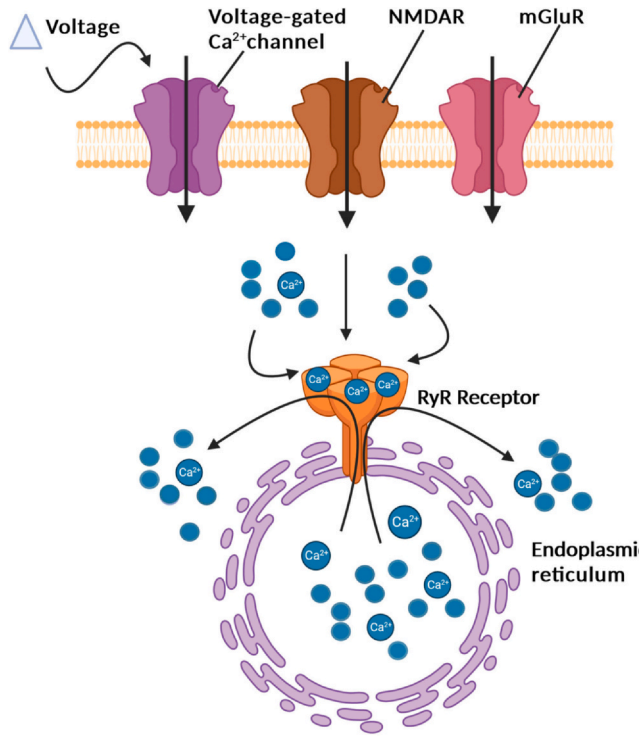

Fig. 1 Schematic of the ryanodine receptor (RyR). The RyR is a large transmembrane Ca^{2+} ion channel embedded in the membrane of the endoplasmic reticulum (ER) responsible for Ca^{2+}-induced Ca^{2+} release (CICR). Binding of Ca^{2+} to the cytoplasmic domains of the RyR stabilizes the ion channel in its open configuration, which increases release of Ca^{2+} from ER stores. In neurons, the RyR mediates CICR in response to Ca^{2+} influx through voltage-gated and ligand-gated Ca^{2+} ion channels embedded in the plasma membrane. NMDAR, *N*-methyl-D-aspartate (NMDA) receptor; mGluR, metabotropic glutamate receptor. *Figure created using* BioRender.com.

also regulated by accessory proteins via phosphorylation, redox modifications, nitrosylation, and/or glutathionylation. Most RyR channel modulators interact with the large cytoplasmic domain while the carboxy-terminal portion of the protein forms the ion-conducting pore (Lanner et al., 2010).

High-affinity tritiated ([^{3}H]Ry) binding sites are expressed in microsomal fractions from rat cerebral cortex, cerebellum, hippocampus and brainstem (Zimanyi and Pessah, 1991) and form Ca^{2+} channels sensitive to the RyR agonist, caffeine (McPherson et al.,1991). While all three RyR isoforms are expressed in the mammalian brain, they are differentially distributed between brain regions, cell types and subcellular localizations, reflecting their participation in specialized functions. Neurons have an extensive ER membrane system that extends deep into the soma to envelope the nucleus and out into the dendrites and axon. Specialized regions of the ER are in close physical approximation to the plasma membrane and are present in more distal aspects of the neuron such as growth cones, axon terminals and dendritic spines. Ca^{2+} release from neuronal ER stores can be evoked by stimulation of RyRs or IP_3Rs, and both receptor types can couple to neurotransmitter-gated receptors and voltage-gated Ca^{2+} channels on the plasma membrane. This organization enables the ER to function not only as a buffer and source of Ca^{2+} in axonal and somatodendritic compartments but also to discriminate between different types of neuronal activity and integrate Ca^{2+} signaling between the plasma membrane, cytosol and nucleus (Bardo et al., 2006; Berridge, 2006). RyR1s anchored to the ER in very close proximity to plasmalemmal L-type voltage-dependent Ca^{2+} channels engage in a form of voltage-induced Ca^{2+} release that is similar to EC coupling in myocytes (De Crescenzo et al., 2006).

RyR channel activity regulates diverse physiological and pathophysiological processes in the nervous system (Berridge, 2006; Lanner et al., 2010; Pessah et al., 2010). RyRs contribute to fundamentally important aspects of neuronal excitability and to both neurochemical and structural aspects of use-dependent synaptic plasticity (Berridge, 1998; Kennedy, 2000; Korkotian and Segal, 1999; Matus, 2000; Segal, 2001). Consistent with the demonstrated role of RyRs in neurotransmission and synaptic plasticity at the cellular level, ligands that directly modulate RyR activity, such as ryanodine, FK506 and rapamycin, alter functional aspects of neuroplasticity in the hippocampus, including long-term potentiation (LTP) (Wang et al., 1996) and long-term depression (LTD) (Li et al., 1998; Wang et al., 1997). Dynamic changes in $[Ca^{2+}]_i$ also play a crucial role in cell

proliferation and differentiation, cell movement and cell death in the developing nervous system (Cline, 2001; Moody and Bosma, 2005; Spitzer et al., 2004; Zheng and Poo, 2007) via regulation of Ca^{2+} signaling pathways that regulate these neurodevelopmental processes (Berridge, 1998; Berridge, 2006; Pessah et al., 2010).

2.2 PCB sensitization of the RyR

NDL PCBs potently and selectively sensitize RyR1 and RyR2 channel activities in sarcoplasmic reticulum membranes isolated from mammalian skeletal and cardiac muscle as determined using radioligand binding studies with [^{3}H]Ry and macroscopic Ca^{2+} flux measurements across isolated SR membrane vesicles (Wong and Pessah, 1996). [^{3}H]Ry binds to only the activated conformation of RyRs with high selectivity and specificity, thereby providing a rapid and quantitative method for screening chemicals that enhance or inhibit channel activity (Pessah et al., 1985; Pessah et al., 1987).

At picomolar to nanomolar concentrations, NDL PCBs interact with RyRs to dramatically increase their sensitivity to activation by nanomolar Ca^{2+} and to attenuate their sensitivity to inhibitory feedback by millimolar Ca^{2+} and Mg^{2+} (Pessah and Wong, 2001), thereby stabilizing the RyR in its full open conformation (Samso et al., 2009). NDL PCBs potently and selectively sensitize both RyR1 and RyR2 channel activities, and PCB-triggered Ca^{2+} release from ER membrane vesicles can be selectively blocked by pretreatment with RyR-selective blockers (FK506 or rapamycin) without inhibiting responses to other RyR channel activators, such as caffeine (Pessah et al., 2010). Rapamycin and FK506 interfere with NDL PCB actions in the same concentration range that promotes the dissociation of the FKBP12/RyR1 complex, suggesting that NDL PCBs interact with a binding site formed at the FKBP12/RyR1 complex interface to enhance channel open probability. Whether RyR-active PCBs influence the phosphorylation, redox state, nitrosylation, and/or glutathionylation state of the receptor complex has not been explored, and an allosteric mechanism has not been ruled out.

A stringent SAR has been identified for PCB sensitization of RyRs, with tetra-ortho PCB 202 being among the most potent and efficacious congeners identified to date, increasing channel open probability at 200 pM (Holland et al., 2017). Two important determinants of the PCB SAR towards RyR1 include: (1) chlorine substitutions at the *ortho*-positions, which restrict the biphenyl rings to non-coplanarity; and (2) *para*-substituted chlorines, which can reduce or eliminate activity (Pessah et al., 2010). With regard to the

former, NDL PCBs possessing 2–3 chlorine *ortho* substitutions are the most potent RyR activators (Pessah et al., 2006), which is consistent with findings from multiple laboratories that NDL, but not DL, PCBs increase intracellular Ca^{2+} in neurons (Kodavanti, 2005). As an example , the 2,3,6-Cl configuration of chlorines on one ring with *ortho*- and *meta*-chloro substitutions on the other ring is optimal for recognizing a binding site within the RyR complex and for sensitizing channel activation. In general, PCBs lacking at least one *ortho*-substitution are inactive toward RyR1 and RyR2, regardless of the degree of chlorination, whereas *para*-chloro substitution lowers the efficacy towards RyR regardless of the presence of one or more *ortho*-substitutions. A similar structure–activity profile has been demonstrated for PCB activation of RyR2 channels isolated from mammalian cardiac muscle (Wong and Pessah, 1996) and fish skeletal muscle (Fritsch and Pessah, 2013).

Since hydroxylated PCBs have been detected in human tissues (Grimm et al., 2015; Li et al., 2022; Zhang et al., 2022), there is currently great interest in the RyR activity of these metabolites relative to the corresponding parent structures. The 4-OH derivative of PCB 30 (4′OH-PCB 30) was found to be significantly more active toward RyR1 than the parent PCB 30 (2,4,6-Cl), suggesting that a *para*-OH group on the phenyl ring that carries no other deactivating substitution confers potency and efficacy towards activating RyR1 (Niknam et al., 2013).

ER preparations isolated from adult rat cerebellum, hippocampus or cortex contain all three RyR isoforms, although RyR1 and RyR2 predominate (Wayman et al., 2012b; Wong et al., 1997a). Of the congeners assayed on ER preparations from rat brain, NDL PCB 95 exhibited the highest potency toward activating high affinity [^{3}H]Ry binding, whereas mono-*ortho* PCB 66 (2,3,4,4′-tetrachlorobiphenyl) and PCB 126 were inactive. Ca^{2+} transport measurements made with cortical ER vesicles revealed that PCB 95 discriminates between inositol 1,4,5-trisphosphate- and ryanodine-sensitive stores, and PCB 95-induced Ca^{2+} release was concentration-dependent and completely inhibited by pharmacological blockade of RyRs (Wong et al., 1997a).

3. RyR-dependent cellular effects of PCBs

Sensitization of RyRs by NDL PCBs has been demonstrated to increase the frequency and amplitude of spontaneous Ca^{2+} oscillations (Wayman et al., 2012a; Yang et al., 2014) and the neuroplasticity of

cultured rat CA1 hippocampal neurons (Wong et al., 1997b). Consistent with this observation, studies in primary rat hippocampal neuron-glia co-cultures showed that picomolar to nanomolar concentrations of PCB 95 activated several Ca^{2+} signaling pathways, including: (1) sequential activation of CaMKK, CaMKIα/γ, MEK/ERK and CREB to increase transcription of Wnt2 (Wayman et al., 2012a); (2) CREB-mediated miR132 upregulation, which suppresses the translation of p250GAP (Lesiak et al., 2014); and (3) mTOR signaling (Keil et al., 2018). These signaling pathways are implicated in transcriptional and translational regulation of dendritic growth and formation of dendritic spines, which are sites of excitatory synaptic contact (Kindler and Kreienkamp, 2012; Konur and Ghosh, 2005; Lohmann and Wong, 2005; Sosanya et al., 2015; Valnegri et al., 2015; Wayman et al., 2008a; Wayman et al., 2008b). Elevated $[Ca^{2+}]_i$ has also been causally linked to the activation of signaling pathways that trigger neuronal apoptosis (Berridge et al., 2000; Ermak and Davies, 2002; Ravagnan et al., 2002; Robertson et al., 2001). Dendritic arborization, spinogenesis and neuronal apoptosis are key determinants of synaptic connectivity and spatiotemporal perturbations in these neurodevelopmental processes are linked to functional deficits (Barone et al., 2000; Pessah et al., 2010; Rice and Barone, 2000). NDL PCBs have been shown to alter the morphogenesis of dendrites, promote dendritic spine maturation, and increase neuronal apoptosis (Klocke and Lein, 2020; Panesar et al., 2022). This section will discuss the contribution of RyR-dependent mechanisms to these outcomes.

3.1 Dendritic arborization and synaptogenesis

Dendritic morphology is a principal determinant of synaptic architecture (Kennedy, 2000; Matus and Shepherd, 2000). The size of the dendritic arbor determines the total synaptic input a neuron can receive, and dendritic branching patterns influence the types and distribution of these inputs (Miller and Jacobs, 1984; Purves, 1975; Purves, 1988; Sejnowski, 1997). Dendritic shape is refined by experience, or activity, and structural plasticity of dendrites is considered a cellular substrate of learning and memory (Leuner and Shors, 2004). Abnormalities in dendritic number, length or branching are thought to underlie the clinical signs of human NDDs (Copf, 2016; Engle, 2010; Garey, 2010; Penzes et al., 2011; Robichaux and Cowan, 2014; Supekar et al., 2013). In experimental animal models, even subtle perturbations of spatial or temporal aspects of dendritic growth are associated with altered neurobehavior. For example, in rodent models, delays

in dendritic maturation of the neocortex as a result of transient depletion of cholinergic input caused persistent learning and memory deficits (Berger-Sweeney and Hohmann, 1997; Hoffman et al., 2002). Increased rates of dendritic growth, as observed in animals exposed to cocaine *in utero*, are also associated with cognitive impairment (Jones et al., 1996; Jones et al., 2000).

In vivo, developmental exposures to Aroclor 1254 have been shown to alter dendritic morphogenesis (Lein et al., 2007; Roegge et al., 2006; Yang et al., 2009). In all three studies, Long-Evans rat dams were exposed to Aroclor 1254 in the diet starting preconception and continuing throughout gestation and lactation, and all three studies tested Aroclor 1254 at 6 mg/kg/day, although one study (Yang et al., 2009) also tested a lower dose of 1 mg/kg/day. All three groups used Golgi staining to quantify dendritic arborization of individual neurons in the offspring's brain. Two of the three studies reported that developmental PCB exposure significantly altered dendritic arborization (Lein et al., 2007; Yang et al., 2009). The first of these two studies (Lein et al., 2007), which quantified dendritic arborization only in male offspring, observed that Aroclor 1254 at 6 mg/kg/day caused a pronounced age-related increase in the rate of dendritic growth in CA1 hippocampal pyramidal neurons and cerebellar Purkinje cells. While dendritic lengths were significantly attenuated in PCB-exposed animals at postnatal day (PND) 22, by PND 60, dendritic growth was comparable to or exceeded that observed in vehicle controls (Lein et al., 2007). The second positive study (Yang et al., 2009), which measured dendritic growth at PND 31 in male offspring, reported that developmental exposure to Aroclor 1254 increased basal dendritic arborization but blocked experience-dependent dendritic growth in cerebellar Purkinje cells and neocortical pyramidal neurons. In the cerebellum, these dendritic effects were only observed in the 1 mg/kg/day exposure group, and in the neocortex, dendritic effects were more pronounced in the 1 mg/kg/day exposure group compared to the 6 mg/kg/day exposure group. Interestingly, the lower, but not the higher, dose of Aroclor 1254 was associated with significant deficits in spatial learning and memory in the Morris water maze (Yang et al., 2009). In contrast, the third study (Roegge et al., 2006), which quantified the dendritic arbor of Purkinje cells at PND 21, reported no changes in primary dendrite length or branching area. This observation is consistent with the second study (Yang et al., 2009) that also observed no effects on dendritic arborization in Purkinje cells in the 6 mg/kg/day Aroclor 1254 exposure group. However, both the second and third studies are at odds with the first study (Lein et al., 2007), which reported that developmental exposure to Aroclor 1254 at 6 mg/kg/day significantly altered the dendritic arborization of Purkinje cells. The reason(s)

for this discrepancy between studies are not known, but since it appears that developmental PCB exposure alters the rate of dendritic growth, perhaps the discrepant findings across studies reflect the fact that dendritic arborization was quantified at different ages.

Studies of Aroclor 1254 do not provide insights as to which PCB congener(s) influence dendritic arborization during development; however, a subsequent study (Wayman et al., 2012b) demonstrated that developmental exposure to PCB 95 phenocopied the dendritic effects of Aroclor 1254. In this study, rats were exposed throughout gestation and lactation to PCB 95 at 0.1, 1, or 6 mg/kg in the maternal diet and the morphology of hippocampal CA1 pyramidal neuronal dendrites was assessed at PND 38 using Golgi staining. Dendritic arborization was significantly increased in pups exposed developmentally to PCB 95 at 0.1 or 1 mg/kg/day. However, dendritic growth in the hippocampus in the 6 mg/kg/day exposure group was not significantly different from that of vehicle controls, recapitulating the non-monotonic dose–response relationship observed earlier for Aroclor 1254 effects on dendritic arborization.

In vitro studies confirmed that PCB 95 (Wayman et al., 2012b), and another NDL RyR-active congener, PCB 136 (Yang et al., 2014), promote dendritic growth. At pM–nM concentrations, PCB 95 (Keil et al., 2018; Wayman et al., 2012a; Wayman et al., 2012b; Yang et al., 2009) and PCB 136 (Yang et al., 2014) increased dendritic arborization in primary rat hippocampal and cortical neuron grown in neuron-glia co-cultures at high cell density. These observations suggest that the effects of PCBs on dendritic growth were autonomous to the brain and occurred independent of systemic effects of PCBs (such as decreased levels of circulating TH). Interestingly, the morphogenic effect of these NDL PCBs was selective to dendritic growth in that axonal growth was not altered in PCB-exposed cultures relative to vehicle controls. Consistent with in vivo observations, the dendritic phenotype exhibited a non-monotonic concentration–response relationship with enhanced dendritic growth observed at nM–pM concentrations but not at low μM concentrations of PCB 95 or 136 (Wayman et al., 2012b; Yang et al., 2014). The biological reason(s) for the lack of response at the higher end of the concentration–response curve remains unknown, but was not due to decreased cell viability (Wayman et al., 2012b; Yang et al., 2014). The dendrite-promoting effects of PCB 95 have also been demonstrated in primary mouse hippocampal and cortical neurons, although in this species, the effect is sex-specific, with the sex-specificity varying between the two brain regions (Keil et al., 2019). The species and regional differences are thought to

reflect species and regional differences in the rates of neuronal maturation (Keil et al., 2019). PCB 95 also promoted the formation of dendritic spines in primary rat hippocampal neurons (Lesiak et al., 2014).

Studies from other laboratories have shown that the hydroxylated metabolites of NDL PCBs can also promote dendritic growth in vitro. The 4-hydroxy metabolites of PCBs 112, 165, and 187 enhanced dendritic arborization in primary mouse cerebellar Purkinje cells (Kimura-Kuroda et al., 2007). Interestingly, the hydroxylated metabolites of NDL PCBs 106, 121, and 159 had no dendrite-promoting activity in this same culture system; however, these compounds inhibited TH-induced dendritic growth (Kimura-Kuroda et al., 2005; Kimura-Kuroda et al., 2007). Whether DL PCBs influence dendritic arborization in the developing brain is not known, but it has been reported that mice born to dams administered TCDD at 0.6 or 3 μg/kg on gestational day 12.5 exhibited increased growth of dendritic branches in both the hippocampus and amygdala at PND 14, and significantly reduced dendritic spine densities at 16 months of age (Kimura et al., 2015). These observations suggest the possibility that DL PCBs may similarly modulate dendritic growth in the developing brain, but this has yet to be directly tested.

RyR activity is implicated in mediating activity- or experience-dependent dendritic growth and spine formation (Adasme et al., 2011; Wayman et al., 2012a). Pulse application of the RyR agonist, caffeine, caused a rapid and significant increase in the size of existing dendritic spines in mature cultures of hippocampal neurons and this effect was blocked by antagonizing concentrations of ryanodine (Korkotian and Segal, 1999), supporting the involvement of RyR in mediating activity-dependent changes in dendritic spine morphology. While pharmacological manipulation of RyRs has been shown to influence activity-dependent dendritic morphogenesis, the specific RyR isoforms associated with any specific effect have yet to be determined.

In vivo observations are consistent with a role for RyR in PCB developmental neurotoxicity. Aroclor 1254 is comprised predominantly of *ortho*-rich PCBs with significant RyR activity (Kostyniak et al., 2005; Wong and Pessah, 1996; Wong et al., 1997a; Yang et al., 2009). In rats, maternal dietary exposure to Aroclor 1254 throughout gestation and lactation altered dendritic growth and plasticity coincident with increased [^{3}H]Ry binding (Yang et al., 2009). Since ryanodine only binds to RyR in its open conformation, increased [^{3}H]Ry binding indicated developmental PCB exposure increased RyR activity in the brain (Pessah et al., 2010).

The dose–response relationship for the effects of Aroclor 1254 on dendritic growth and plasticity was similar to that of Aroclor 1254 effects on RyR expression, but not to PCB effects on TH levels or sex steroid-dependent developmental endpoints (Yang et al., 2009). Increased RyR expression in the brain has also been associated with Aroclor 1254-induced changes in gene expression (Royland and Kodavanti, 2008; Royland et al., 2008) and locomotor activity (Roegge and Schantz, 2006).

In vitro SAR studies confirmed a causal role for RyR sensitization in PCB-induced dendritic growth. PCBs 95 and 136, but not PCB 66, increased dendritic arborization in primary rat hippocampal and cortical neurons (Wayman et al., 2012b; Yang et al., 2009; Yang et al., 2014). PCBs 95 and 136 have potent RyR activity, whereas PCB 66 has negligible RyR activity (Pessah et al., 2006). PCB 136 is a chiral congener that atropselectively sensitizes RyRs (Pessah et al., 2009), and this SAR translated into atropselective effects on dendritic growth. Specifically, the (−)-PCB 136 enantiomer potently sensitized RyR and also enhanced dendritic growth, whereas the (+)-PCB 136 enantiomer lacked RyR activity and had no effect on dendritic growth (Yang et al., 2014). In further support of the hypothesis that RyR-dependent mechanisms underlie PCB effects on dendritic growth, pharmacological blockade of RyRs with FLA365 inhibited the dendrite-promoting activity of PCB 95 and PCB 136 in primary rat hippocampal and cortical cultures (Wayman et al., 2012b; Yang et al., 2009; Yang et al., 2014), and siRNA knockdown of RyR1 or RyR2 inhibited PCB 95-induced dendritic growth in rat dissociated hippocampal cell cultures and hippocampal slice cultures (Wayman et al., 2012b).

Ca^{2+}-dependent translation- and transcription-dependent pathways regulate activity-dependent dendritic growth and spine formation (Schratt et al., 2004; Tsokas et al., 2005; Wayman et al., 2006). PCB 95 promoted dendritic growth by engaging these same signaling pathways (Fig. 2). Ca^{2+} imaging studies of primary rat hippocampal neurons demonstrated that RyR-active congeners, such as PCB 95 and the (−) enantiomer of PCB 136, increased the frequency and amplitude of Ca^{2+} oscillations, whereas congeners that are not RyR-active, such as PCB 66 or the (+) enantiomer of PCB 136, had no discernable effect on neuronal Ca^{2+} oscillations (Wayman et al., 2012a; Yang et al., 2014). FLA365 blocked the intracellular Ca^{2+} oscillations triggered by PCB 95 and (−)-PCB 136, confirming that these PCB effects are RyR-dependent. In primary rat hippocampal neurons, the increase in intracellular Ca^{2+} caused by PCB 95 activated a

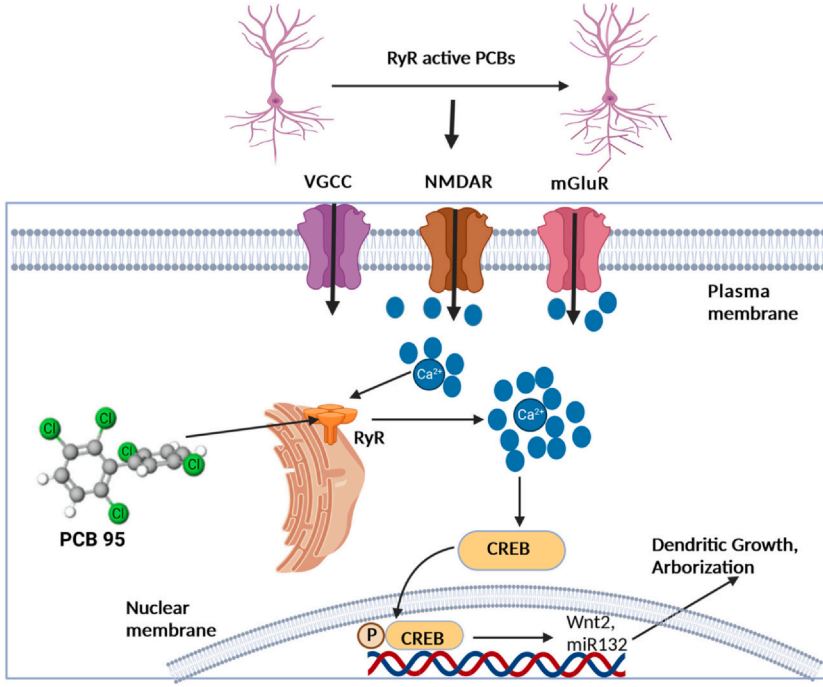

Fig. 2 Ryanodine receptor (RyR)-dependent mechanism of PCB-induced dendritic arborization. Non-dioxin-like (NDL) PCBs with RyR activity, exemplified by PCB 95, promote dendritic growth in pyramidal neurons of the rodent hippocampus and cortex and in Purkinje cells of the rodent cerebellum. Studies in primary hippocampal or cortical neuron-glia co-cultures derived from postnatal day 1 rats have demonstrated that the dendrite-promoting activity of PCBs is mediated by CREB-dependent signaling pathways that are activated by an RyR-dependent increase in intracellular Ca^{2+} concentrations. This same CREB-dependent signaling pathway mediates activity-dependent dendritic growth during normal neurodevelopment mediated by presynaptic release of glutamate or activation of voltage-gated Ca^{2+} ion channels in the plasma membrane. CREB, cAMP response element binding protein; VGCC, voltage-dependent calcium channel; NMDAR, *N*-methyl-D-aspartate (NMDA) receptor; mGluR, metabotropic glutamate receptor. *Figure adapted from Klocke and Lein (2020) and created using BioRender.com.*

Ca^{2+}-dependent translational mechanism involving mechanistic target of rapamycin (mTOR) and pharmacological blockade or siRNA knockdown of mTOR signaling inhibited PCB 95-induced dendritic growth (Keil et al., 2018). In addition, PCB 95 triggered sequential activation of CaMKK, CaMKIα/γ, MEK/ERK and CREB to increase transcription of Wnt2 (Wayman et al., 2012a), which acts in an autocrine fashion to

promote dendritic growth (Wayman et al., 2006). Pharmacological blockade of RyRs inhibited the activation of these signaling molecules, and experimental manipulations to inhibit the signaling molecules in these pathways effectively blocked PCB 95-induced dendritic growth (Keil et al., 2018; Wayman et al., 2012a). Activation of CREB by PCB 95 also upregulated miR132 (Fig. 2), which suppressed the translation of p250GAP to promote synaptogenesis, evidenced by increased dendritic spine density and elevated frequency of miniature excitatory post-synaptic currents (Lesiak et al., 2014). Pharmacological and gene editing approaches that inhibited miR132 were effective in blocking PCB 95-induced dendritic arborization in cultured hippocampal neurons (Lesiak et al., 2014).

3.2 Neuronal apoptosis

Apoptosis is essential for normal brain development (Dikranian et al., 2001; Martin, 2001). Neuronal apoptosis occurs normally in proliferative zones and in postmitotic cells in both the fetal and postnatal brain (White and Barone, 2001). The spatiotemporal pattern of apoptosis in the developing CNS is tightly regulated and disruption of either the timing or the magnitude of apoptosis in a given brain region can alter synaptic connectivity, causing functional deficits even in the absence of obvious pathology (Martin, 2001). Increased Ca^{2+} and ROS are significant triggers of neuronal apoptosis, and RyR activation is a critical component of apoptotic signaling pathways (Berridge et al., 2000; Carmody and Cotter, 2001; Ermak and Davies, 2002; Ravagnan et al., 2002; Robertson et al., 2001). Histologic studies in a rat model of PCB developmental neurotoxicity indicated that exposure to Aroclor 1254 at 0.1 or 1.0 mg/kg/day in the maternal diet throughout gestation and lactation significantly increased apoptosis in the brain of offspring at PND 1, but not PND 21 (Yang and Lein, 2010). Assays of caspase-3 activity and TUNEL staining of the cortex, hippocampus, and cerebellum revealed significantly enhanced apoptosis in all three brain regions, with the most pronounced increase in the cerebellum (Yang and Lein, 2010).

In vitro studies confirmed that Aroclor 1254, and other Aroclor mixtures, induce apoptosis in primary neuronal cell cultures (Howard et al., 2003; Sanchez-Alonso et al., 2004). Studies of individual PCB congeners suggest that the pro-apoptotic activity of these complex PCB mixtures are largely mediated by NDL PCB congeners. An investigation of the apoptotic activity of PCB 52 in human neuronal SK-N-MC cells found that this NDL congener triggered apoptosis via p53-independent mechanism(s)

(Hwang et al., 2001). In a comparative study of PCB 47, a NDL congener, and PCB 77, a DL congener, in primary rat hippocampal neurons, PCB 47, but not PCB 77, was observed to significantly increase caspase-dependent apoptosis (Howard et al., 2003). Neither PCB 47 nor PCB 77 triggered neuronal apoptosis in primary rat cortical neurons established from the same dissection and grown under the same culture conditions. Another comparative study of NDL vs DL PCB congeners using primary cultures of rat cortical neurons indicated that both the NDL congener, PCB 153, and the DL congener, PCB 77, accelerated apoptosis in a time- and concentration-dependent manner (Sanchez-Alonso et al., 2003). The extent of apoptosis was greater in the cultures treated with PCB 77 compared to the cultures treated with PCB 153. The reason(s) for the different outcomes between the two studies with regards to the apoptotic activity of PCB 77 are not known, but may reflect subtle differences in culture systems: primary rat cortical neurons were maintained under serum-free conditions in one study (Howard et al., 2003) but in the presence of serum in the other (Sanchez-Alonso et al., 2003). But a more likely explanation is significant differences in the concentrations of PCB 77 that were tested. In the study in which PCB 77 was not observed to alter neuronal apoptosis, PCB 77 was tested at concentrations $\leq 1.0\,\mu M$ because cytotoxic effects were observed at concentrations $>1.0\,\mu M$ (Howard et al., 2003). In contrast, in the study that observed PCB 77-induced neuronal apoptosis, PCB 77 was tested at 30, 50, and 100 μM, concentrations that caused significant cytotoxicity within 1–3 h after exposure (Sanchez-Alonso et al., 2003).

RyR activity is implicated in the regulation of neuronal apoptosis (Berridge et al., 2000; Ermak and Davies, 2002) and several lines of evidence support a causal relationship between PCB sensitization of RyRs and the pro-apoptotic activity of PCBs. First, PCB 47, but neither PCB 77 nor PCB 104, triggered neuronal apoptosis in primary rat hippocampal neurons (Howard et al., 2003). SAR studies have shown that PCB 47 is a RyR-active congener whereas PCB 77 and PCB 104 have negligible activity at the RyR (Pessah et al., 2006). Furthermore, the pro-apoptotic activity of PCB 47 was inhibited by FLA365, a selective RyR antagonist (Chiesi et al., 1988; Mack et al., 1992), but not by antagonists known to block PCB-mediated Ca^{2+} flux through L-type voltage-sensitive Ca^{2+} channels, NMDA receptors, or IP_3Rs in cultured neurons (Howard et al., 2003) (Fig. 3). The signaling pathway(s) connecting RyR sensitization to neuronal apoptosis are not yet known, but presumably involve caspase-3-dependent (Howard et al., 2003) signaling mechanism(s).

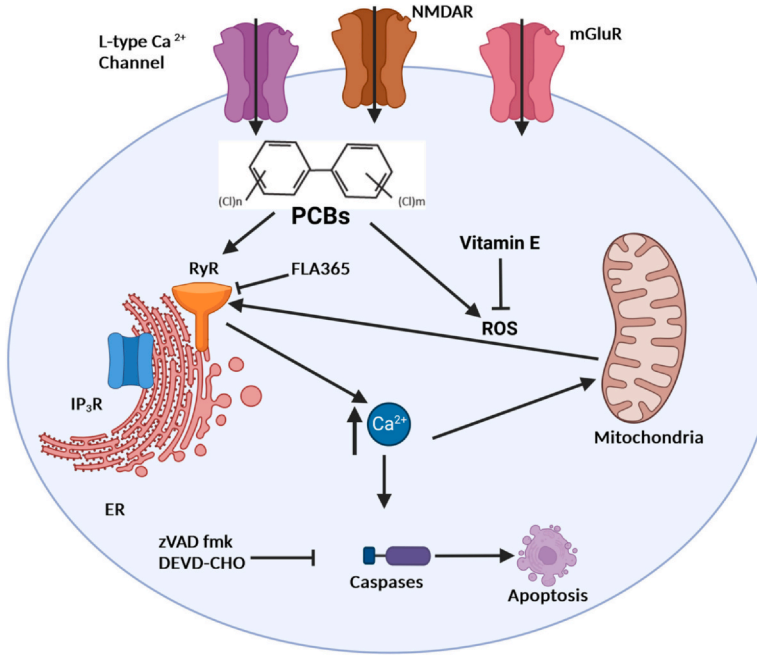

Fig. 3 Proposed RyR-dependent mechanisms of PCB-induced neuronal apoptosis. Non-dioxin-like (NDL) PCBs have been shown to promote apoptosis in primary rat hippocampal neurons and this effect was inhibited by pharmacological block of RyR using FLA365. The pro-apoptotic activity of NDL PCBs was also blocked by pharmacological inhibitors of caspases and by the antioxidant vitamin E. These data suggest a model in which NDL PCBs directly activate the ryanodine receptor (RyR) to increase intracellular Ca^{2+} concentrations ($[Ca_i^{2+}]$) via increased release of Ca^{2+} from endoplasmic reticular (ER) stores. Increased $[Ca^{2+}]_i$ then triggers caspase-dependent apoptosis. Elevated intracellular Ca^{2+} may also increase mitochondrial Ca^{2+} influx, which amplifies mitochondrial generation of reactive oxygen species (ROS) to promote caspase-dependent apoptosis. Alternatively, or in addition, PCBs may generate ROS directly, which then increase intracellular levels of Ca^{2+} via RyR sensitization. Blocking L-type voltage-sensitive Ca^{2+} channels with verapamil or NMDA receptors with APV does not have any effect on PCB-induced apoptosis, suggesting that extracellular calcium is not involved in PCB-induced apoptosis. *Figure adapted from Howard et al., 2003 and created using BioRender.com.*

Neuronal apoptosis induced by Aroclor 1254 or PCB 47 in primary rat hippocampal neurons was also blocked by the antioxidant α-tocopherol (Howard et al., 2003), suggesting that PCBs may trigger neuronal apoptosis not only through RyR-dependent mechanisms, but also ROS-dependent mechanisms (Fig. 3). However, increased ROS could be a consequence of RyR activation. NDL PCBs stabilize RyRs in their open conformation,

which allows release of Ca^{2+} from intracellular stores (Pessah et al., 2010). Increased $[Ca^{2+}]_i$ can initiate apoptosis by directly activating caspases and/or by increasing Ca^{2+} flux into mitochondria, which then triggers production of ROS, resulting in release of cytochrome c from mitochondria with subsequent activation of caspases (Lein et al., 2018). Alternatively, ROS may initiate apoptosis via targeted interactions with the RyR (Carmody and Cotter, 2001; Pessah, 2001). ROS have been shown to interact directly with RyR cysteine residues to heighten the probability of channel opening (Feng et al., 1999; Okabe et al., 2000; Suzuki and Ford, 1999; Xia et al., 2000). Thus, NDL PCBs may sensitize RyRs indirectly via PCB-induced ROS (Howard et al., 2003). As recently noted (Pessah et al., 2019), it may also be that PCBs independently influence RyR activation and ROS generation, and that each of these effects augments the other in a feed-forward mechanism (Fig. 3).

4. Evidence that RyR sensitization mediates the behavioral effects of PCBs

4.1 Experimental animal studies

PCB effects on dendritic growth have been demonstrated to coincide with behavioral phenotypes relevant to behavioral domains altered by PCBs in humans. For example, effects of Aroclor 1254 on dendritic growth in weanling pups exposed during gestation and lactation to this PCB mixture in the maternal diet coincided with deficits in spatial learning and memory (Yang et al., 2009). Developmental exposure to PCB 95 in the maternal diet interfered with the synaptic plasticity and organization of the auditory cortex in weanling rats, causing significant E/I imbalance (Kenet et al., 2007). Developmental exposure to PCB 47 caused deficits in social behavior in mice (Jolous-Jamshidi et al., 2010). However, establishing a causal link between PCB sensitization of RyRs and PCB effects on behavior has proven difficult because pharmacological or genetic deletion of RyR is embryolethal, and in postnatal animals, cardiotoxic. However, a recent study using a larval zebrafish model to compare behavioral phenotypes following developmental exposures to PCBs with varying RyR activity ranging from negligible to very potent demonstrated that the SAR for RyR activity in zebrafish tissues predicted the SAR for PCB effects on behavior (Yaghoobi et al., 2022). This finding, coupled with observations that PCB effects on RyR activity in the brain, dendritic arborization, and

spatial learning and memory exhibited comparable dose–response relationships in rodent models (Yang et al., 2009), provide strong support for a causal relationship between RyR-dependent effects of PCBs on dendritic growth and PCB-induced deficits in learning and memory.

4.2 Human studies

There is some evidence in the human literature to support the hypothesis that RyR-dependent mechanisms contribute to neuropsychiatric outcomes observed in PCB-exposed children. A recent epidemiological study designed to evaluate PCBs as risk factors for autism (Granillo et al., 2019) found that while there were no significant associations between total PCBs and autism, there was an association between RyR-active NDL PCBs and increased risk for autism diagnosis [adjusted OR: 2.63 (95% CI 0.87–7.97)]. The authors concluded "these analyses suggest the need to explore more deeply into subsets of PCBs as risk factors based on their function and structure in larger cohort studies where non-monotonic dose–response patterns can be better evaluated".

5. Implications of RyR-dependent mechanisms of PCB developmental neurotoxicity

5.1 RyR sensitization as a convergent mechanism of PCB developmental neurotoxicity

While there is significant experimental evidence identifying RyRs as critical molecular targets in the developmental neurotoxicity of NDL PCBs, other biological activities have been ascribed to NDL PCBs and implicated in PCB developmental neurotoxicity, including increased intracellular levels of ROS (Fonnum et al., 2006; Mariussen and Fonnum, 2006), disruption of TH signaling (Crofton, 2008; Zoeller, 2007) and dopamine depletion (Mariussen and Fonnum, 2006). There are hints in the literature that RyR sensitization may contribute to these other known biological activities of NDL PCBs. For example, PCB-induced Ca^{2+} release from the ER via RyR sensitization (Wong and Pessah, 1997) may, in turn, increase mitochondrial production of ROS (Ermak and Davies, 2002; Ravagnan et al., 2002) (Fig. 3). This might be a reciprocal interaction in that ROS can directly modulate the channel activity of the RyR (Feng et al., 2000; Pessah, 2001). The thyroid gland is a major target organ of the sympathetic nervous system. Sympathetic neurons express RyRs (Vanterpool et al., 2006) and

neurotransmitter release from sympathetic neurons is regulated by RyR activity (Cong et al., 2004). Conversely, TH regulates RyR expression in at least the heart (Dillmann, 2002; Hudecova et al., 2004; Jiang et al., 2000), suggesting the possibility that PCB effects on TH signaling are mediated in part by changes in RyR expression.

Similarly, emerging evidence implicating RyRs in regulating dopamine homeostasis suggests the possibility that PCB sensitization of RyRs contributes to the effects of PCBs on dopamine. Several mechanisms are currently postulated to contribute to dopamine reductions seen following PCB exposure, including inhibition of two of the enzymes involved in the synthesis of dopamine, specifically, tyrosine hydroxylase and L-aromatic acid decarboxylase (Angus and Contreras, 1996; Angus et al., 1997), as well as decreased striatal levels of the dopamine transporter (DAT) (Caudle et al., 2006) and selective activation of oxidative stress-related pathways in dopaminergic neurons (Lee and Opanashuk, 2004). Ryanodine induces dopamine release from striatal dopaminergic neurons and this effect is significantly attenuated in striatal slices isolated from RyR3 null mice (Wan et al., 1999). It has also been demonstrated that pharmacological manipulations of RyR activity altered somatodendritic dopamine release (Patel et al., 2009), as well as action potential- and NMDA receptor-evoked Ca^{2+} signaling (Cui et al., 2007; Harnett et al., 2009) in midbrain dopaminergic neurons, and that internal Ca^{2+} stores are necessary for the abnormal release of dopamine via reverse transport through the dopamine transporter caused by amphetamine and methamphetamine (Goodwin et al., 2009). Collectively these observations provide biological plausibility to the intriguing speculation that RyR sensitization may be a convergent mechanism of PCB developmental neurotoxicity.

5.2 Assessing PCB risks to the developing human brain

The current approach for assessing PCB risks for the developing brain rely primarily on toxicity related to DL PCBs. Risk assessment platforms often use the toxic equivalence scheme to predict toxicity of complex PCB, dioxin and furan mixtures based on the AhR activity of the individual congeners in the mix. However, this approach fails to account for potential impacts of NDL PCBs on the developing brain (Holland and Pessah, 2021; Pradeep et al., 2019). The SAR data available for RyR activity of PCBs, and the identification of a RyR-based mechanism of PCB developmental neurotoxicity, suggest the utility of developing an alternative toxic equivalence factor (TEF) strategy for NDL PCBs based on their relative

RyR activity (Holland and Pessah, 2021; Pradeep et al., 2019). The feasibility of this approach was recently demonstrated in a study that compared the predicted activity of PCB mixtures as determined using a concentration addition model or a RyR-specific neurotoxic equivalency scheme to the actual RyR activity of the mixture as assessed using [^{3}H]Ry binding assays (Griffin et al., 2023). This strategy of an RyR-based toxic equivalency factor has great appeal for assessing not only the risk of developmental neurotoxicity associated with exposure to mixtures of PCBs but also that of exposures to noncoplanar compounds other than PCBs that also have RyR activity, such as polybrominated diphenyl ethers (PBDEs) and tricolsan (Pessah et al., 2010). RyR-based screening platforms could also be developed to identify novel noncoplanar compounds with RyR activity.

5.3 Identifying gene–environment interactions that influence NDD risk

There has been a significant increase in the incidence of many NDDs, including autism and ADHD, over the past few decades (Lein, 2015). These data together with the tremendous costs that NDDs exact on affected individuals, their families, and society (Hong et al., 2020; Leibson et al., 2020; Schofield et al., 2019), underscore the need to identify factors that confer NDD risk and/or modify symptom severity. While most NDDs have a strong hereditary component, there is growing consensus that the etiopathogenesis of many NDDs involve gene × environment (G × E) interactions (Keil-Stietz and Lein, 2023; Lein, 2015). In contrast to genetic risks, which are currently difficult to reverse, environmental factors are modifiable risk factors. The identification of specific environmental factors that increase NDD risk would enable the primary prevention of NDDs via public health policies aimed at reducing relevant environmental exposures in susceptible populations. Progress has been made in identifying environmental risk factors for NDDs, which include advanced paternal age at conception, complications during pregnancy, maternal diet, and prenatal exposure to psychotropic drugs (Bolte et al., 2019; Carlsson et al., 2020). Environmental chemical contaminants have also been proposed as NDD risk factors (Landrigan, 2010; Landrigan et al., 2012). While experimental animal and predictive toxicology studies have identified environmental chemicals associated with NDD-relevant phenotypes, it has been challenging to demonstrate this association in human studies (Carter and Blizard, 2016; Kalkbrenner et al., 2014; Pelch et al., 2019; Rock and Patisaul, 2018; Rossignol et al., 2014). This is due, in part, to the fact that

there are very few examples of specific gene × environment combinations that have been identified as conferring risk for a given NDD. This likely reflects the fact that the mechanisms by which genetic and environmental factors interact to influence individual NDD risk and/or severity remain speculative. Of the various hypotheses that have been proposed (Keil-Stietz and Lein, 2023; Lein, 2015), one for which there is supporting evidence is the convergence of the genetic and environmental factor on signaling pathways that regulate neurodevelopmental processes altered in NDDs.

Many genetic risk factors for NDDs converge on signaling pathways that critically regulate synaptic connectivity in the developing brain (Delorme et al., 2013; Guang et al., 2018; Landrigan et al., 2012; Masini et al., 2020; Qiu et al., 2012). Neuronal activity plays a significant role in sculpting neural circuits in the developing brain, and Ca^{2+} signaling is the major mechanism linking neuronal activity to dendritogenesis and synaptogenesis in the developing brain (Keil Stietz et al., 2021; Pessah et al., 2010; Wayman et al., 2006). Genes that either control Ca^{2+} signaling or are regulated by Ca^{2+} signaling, are predominant in lists of NDD risk genes (Ansel et al., 2016; Nanou and Catterall, 2018; Nguyen et al., 2018; Pessah et al., 2010). Therefore, Ca^{2+} signaling pathways and the neurodevelopmental processes regulated by them represent molecular and cellular points of convergence for environmental and genetic NDD risk factors (Keil-Stietz and Lein, 2023; Lein, 2015; Stamou et al., 2013).

As discussed in Section 3.1, PCB 95 sensitization of RyRs activated CREB-dependent transcriptional mechanisms (Lesiak et al., 2014; Wayman et al., 2012a) and mTOR-dependent translational mechanisms (Keil et al., 2018) to stimulate dendritic growth and spine formation. CREB dysfunction has been observed in NDDs (Barnby et al., 2005; Ngounou Wetie et al., 2015; Russo, 2017; Todd and Mack, 2001; Zheng et al., 2016), and downstream genes regulated by CREB, such as miR132 and Wnt, have been linked to NDDs or NDD-relevant phenotypes (Bocchi et al., 2017; Kalkman, 2012; Li et al., 2016). TOR signaling is also associated with increased NDD risk (Chaudry and Vasudevan, 2022). Interestingly, human point mutations in *RYR* that increase the sensitivity of these ion channels to PCB 95 in animal models (Kim et al., 2013; Ta and Pessah, 2007) have also been identified in autism candidate gene studies (Lu and Cantor, 2012). Collectively, these observations suggest that RyRs represent a convergent target for NDL PCBs and genes that confer NDD risk (Fig. 4).

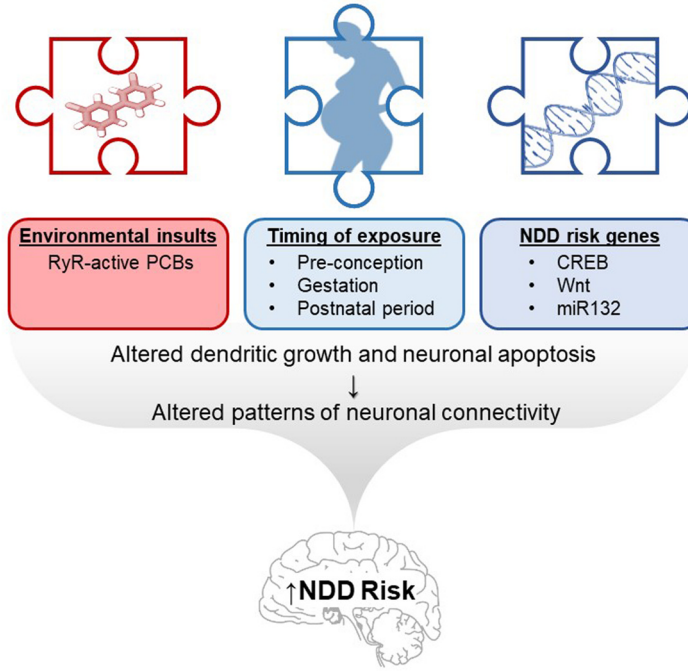

Fig. 4 PCBs may interact with heritable mutations in signaling molecules that regulate dendritic growth and/or neuronal apoptosis to influence NDD risk. *Figure adapted from Klocke and Lein (2020) and created using BioRender.com.*

The hypothesis that NDL PCBs interact with heritable mutations in Ca^{2+} signaling pathways implicated in NDDs to influence neurodevelopmental outcomes was recently tested in experimental animal models (Keil Stietz et al., 2021; Sethi et al., 2021). Mice genetically engineered to express heritable human mutations that alter the fidelity of calcium signaling were leveraged to evaluate whether expression of genetic mutations converging on the same signaling pathways as PCBs increased susceptibility to the neurotoxic effects of PCBs. Mice that expressed a human gain-of-function mutation in *Ryr1*, a CGG repeat expansion in the premutation range in the *Fmr1* gene, or both genetic mutations were exposed throughout gestation and lactation to varying doses of the MARBLES PCB mixture in the maternal diet. The MARBLES mix is comprised of the 12 most abundant PCB congeners identified in the serum of pregnant women living in Northern California who are at increased risk of having their child diagnosed with an NDD (Sethi et al., 2021), and was shown to

have significant RyR activity (Sethi et al., 2019). Morphometric and behavioral analyses of these mice revealed that dendritic morphogenesis and behavior were modulated by complex G × E interactions (Keil Stietz et al., 2021; Sethi et al., 2021). For example, in mouse cortical and hippocampal neurons, genotype influenced the dendritic response to PCBs in a dose-, sex- and brain region-dependent manner. Expression of the RyR mutation or both the RyR and CGG expansion mutations increased dendritic complexity at lower doses of PCBs compared to the dose required to elicit the same dendritic response in wildtype neurons (Keil Stietz et al., 2021). Behavioral studies indicated that developmental exposure of wildtype males to the MARBLES PCB mixture phenocopied social behavior phenotypes observed in mice expressing heritable human mutations in calcium signaling, while expression of these mutations alleviated PCB effects on ultrasonic vocalizations and repetitive behavior, and modified the dose–response relationships and sex-dependent effects of PCBs on social behavior (Sethi et al., 2021). Additional studies using these same mouse models and PCB exposures indicated that G × E interactions influenced the microbiome and intestinal physiology (Rude et al., 2019), as well as serum cytokine and chemokine levels (Matelski et al., 2020) in

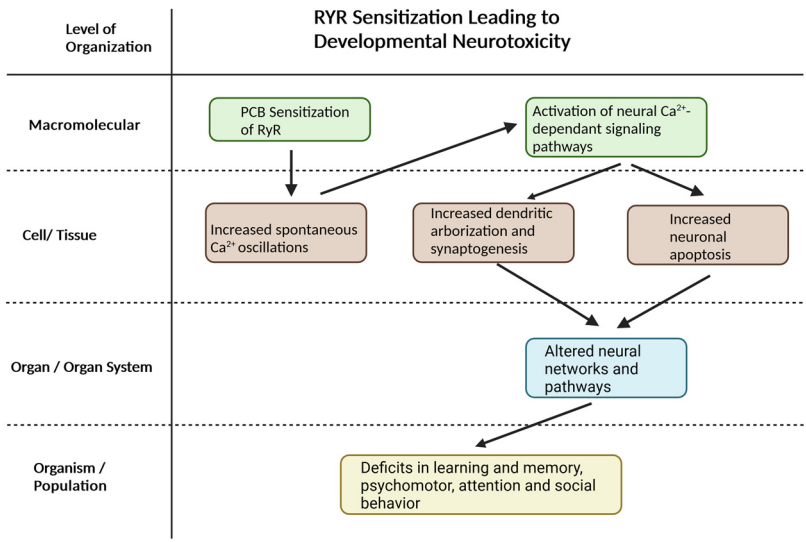

Fig. 5 Proposed adverse outcome pathway (AOP) linking ryanodine receptor (RyR) sensitization to neurodevelopmental outcomes associated with PCB exposures. *Figure adapted from Bal-Price et al. (2015) and created using BioRender.com.*

mouse pups. Collectively, these observations support the hypothesis that heritable deficits in Ca^{2+} signaling pathways also impacted by NDL PCBs modify the neurotoxic response to NDL PCBs, which in turn influence neurodevelopmental outcomes of NDD relevance.

6. Conclusions

Mechanistic studies suggest a model in which NDL PCBs cause neurobehavioral deficits by interfering with neuronal apoptosis and dendritic arborization, and these are mediated, at least in part, by RyR-dependent mechanisms. Based on these data, an adverse outcome pathway (AOP) has been proposed (Bal-Price et al., 2015) that links PCB sensitization of the RyR to behavioral deficits via activation of Ca^{2+} signaling pathways that trigger dendritic growth, spine formation and neuronal apoptosis (Fig. 5). Causal relationships between the key events in this AOP are well-supported by experimental evidence, with the exception of linking PCB sensitization of RyR causally to PCB effects on behavior, which has proven difficult to test experimentally.

A critical data gap in the field, however, is that much of the literature describing mechanisms of PCB developmental neurotoxicity is based on either legacy commercial PCB mixtures or a small subset of individual congeners, leaving significant uncertainty regarding the generalizability of RyR-dependent mechanisms to the broad spectrum of PCBs. In addition to determining congener-specificity, there is a need to identify dose–response relationships of PCB effects on molecular and cellular endpoints, and to establish causal relationships between molecular, cellular and neurobehavioral endpoints with the goal of strengthening adverse outcome pathways linking RyR sensitization to neurotoxic outcomes at the organism and population levels (Bal-Price et al., 2015). The continual evolution of high(er)-throughput methods for analyzing molecular and cellular endpoints will be of great utility in addressing these data gaps.

Additional outstanding questions include: What factor(s) determine the specificity of PCB developmental neurotoxicity and why do NDL PCBs preferentially target the developing nervous system? Neurotoxic outcome is likely influenced by not only the timing of exposure and pharmacokinetic parameters, such as dose, metabolism, and distribution of PCBs within the body, but also by developmentally-regulated expression patterns of RyRs and the complement of accessory proteins that comprise the

calcium release unit, as well as the antioxidant capacity of the cell. Last, there remain significant questions regarding the identity of specific genetic factors that interact with RyR-active NDL PCBs to determine individual risk for neurotoxic outcomes following developmental PCB exposures, and the mechanism(s) by which these G *x* E interactions confer risk.

The identification of RyR-dependent mechanisms of PCB developmental neurotoxicity raises significant concerns given data indicating that RyR-active NDL PCBs are prevalent and readily detected in environmental samples (Pessah et al., 2010) and in human tissues, including the brain of children diagnosed with a NDD (Corrigan et al., 1998; Li et al., 2022; Mitchell et al., 2012; Mouat et al., 2023; Sethi et al., 2019). Recent reports indicating that RyR-dependent mechanisms are not the only mode of action by which NDL PCBs modulate neuronal morphogenesis (Klocke et al., 2019) raise further questions about how RyR-active NDL PCBs interact with NDL PCBs that have negligible RyR activity to influence neurotoxic outcomes. Clearly there is a need for more studies evaluating neurodevelopmental effects of individual PCB congeners and mixtures that better replicate contemporary human exposures than the legacy Aroclor mixtures. This will require a more comprehensive understanding of the congener profile and concentrations of contemporary human maternal and neonatal PCB exposures. To achieve this, it will be necessary to move away from the traditional practice of quantifying legacy indicator PCBs and towards non-biased screening for a broad spectrum of PCBs and their metabolites, including non-legacy PCBs, in relevant human tissue samples.

Evidence supporting RyR-dependent mechanisms of PCB developmental neurotoxicity is also exciting because it suggests scientifically rational, mechanism-based approaches for developing receptor-based screening platforms to identify not only PCB congeners, but also other noncoplanar chemicals, for their potential to cause developmental neurotoxicity. It also supports the development of more rigorous approaches for assessing developmental neurotoxicity risks of complex PCB mixtures by expanding TEF methodology beyond AhR activity to include RyR activity, and thereby capture a broader spectrum of PCBs found in the human chemosphere.

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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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