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Perinatal Exposure to Environmental Polychlorinated Biphenyls Sensitizes Hippocampus to Excitotoxicity *Ex Vivo*

Kyung Ho Kim¹ and Isaac N. Pessah

Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, USA

Summary

Ortho-substituted polychlorinated biphenyls (PCBs) are a concern to human developmental health. Rat dams were exposed to an environmentally relevant mixture of PCBs, Aroclor 1254, or pure congener PCB 95 (6 mg/kg/day) during the perinatal period (GD 5 through PD 21). Hippocampal slices prepared from offspring 1–3 weeks post-weaning were tested for persisting changes in sensitivity to the excitotoxicant picrotoxin. Hippocampal slices were placed on multielectrode arrays. Field excitatory postsynaptic potentials (*f*EPSPs) were recorded from Schaffer Collateral/Commissural fibers in striatum radiatum of the CA1 region in response to single pulse stimuli. After recording baseline excitability, GABA_A receptors were blocked by inclusion of picrotoxin (100 μ M) in the aCSF perfusate. Picrotoxin produced negligible changes in *f*EPSP slope in slices isolated from offspring exposed to vehicle, whereas slices from either PCB test group invariably showed >200% ($p < 0.01$) synaptic facilitation. Picrotoxin produced prominent after-discharges (epileptic wave forms) in the evoked potentials measured from PCB exposed, but not control, hippocampal slices. These results show that developmental exposure to non-coplanar PCBs is sufficient to produce changes in synaptic plasticity that can be unmasked in the presence of GABA_A receptor deficits that persist 1–3 weeks after exposure ceased. Developmental exposure to PCBs may sensitize seizure susceptibility postnatally, especially in susceptible populations with GABA_A receptor signaling deficits.

Keywords

excitotoxicity; non-coplanar PCBs; picrotoxin; hippocampus; hippocampal slice; GABA_A receptor; seizure activity; microelectrode array

Introduction

Recent studies with acutely dissociated rat hippocampal slices showed that *in vitro* exposure to nanomolar concentration of non-coplanar polychlorinated biphenyls (PCBs) of concern to human developmental health can rapidly alter synaptic transmission within CA1 (Kim et al. 2009). Whole-cell voltage clamp recordings obtained from neurons within the primary

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Corresponding author: Isaac N. Pessah, Ph.D., Department of Molecular Biosciences, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, CA 95616, inpessah@ucdavis.edu. Telephone: (530), 752-6696. Fax: (530) 752-4698.

¹Current address: Kyung Ho Kim, D.V.M. Ph.D., Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, 215 Biomedical Science Building, 103 Daehak-Ro, Chongro-Gu, Seoul, Korea, 110-799

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auditory cortex (A1) *in vivo* indicated that rats exposed during gestation and lactation (perinatally) to a non-coplanar PCB found in environmental samples and human tissues -2,2',3,5',6-pentachlorobiphenyl (PCB 95) - exhibited significant alterations in the balance of excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) (Kenet et al. 2007). These changes were associated with abnormal development of receptive fields within A1 (Kenet et al. 2007). Perinatal exposure to a complex PCB mixture, Aroclor 1254, was shown to alter activity dependent dendritic growth in several brain regions and impaired Morris water maze performance in weanling rats (Yang et al. 2009). These results suggested that non-coplanar PCBs are capable of shifting the balance of excitatory and inhibitory neurotransmission as a consequence of developmental exposure. Such effects could have far reaching ramifications on the normal formation and excitability of neural networks. In the pilot study reported here, rats were exposed during the perinatal period *via* maternal dosing of PCB 95, Aroclor 1254, or corn oil vehicle. Hippocampal slices were prepared from the offspring, placed on microelectrode arrays (MEAs), and electrophysiological recordings were performed *ex vivo*. Perinatal exposure to either PCB 95 or the complex mixture Aroclor 1254 significantly enhanced picrotoxin-triggered synaptic facilitation and produced prominent after discharges in *f*EPSP waveforms compared to those prepared from vehicle controls. Developmental exposure to non-coplanar PCBs, either singly as a pure congener or as a complex mixture, persistently enhances susceptibility to a GABA_A receptor signaling deficit.

Material and Methods

Animals and developmental exposure to PCBs

Time-mated rat dams (Sprague Dawley; Charles River Laboratories, Hollister, CA) were randomly assigned to a treatment group. Aroclor 1254 (lot number 124-191-B) and 2,2',3,5',6-pentachlorobiphenyl (PCB 95, >98%) were purchased from AccuStandard (New Haven, CT) and dissolved in 100% corn oil at a final concentration of 24 mg/ml. Dams were offered a cornflake with 0 or 6 mg/kg/day PCB 95 or Aroclor 1254 (75 - 100 μ l corn oil) at 5 pm from gestational day (GD) 5 to postnatal day (PD) 21. The observer documented complete ingestion of each dose. Animals were kept on a 12-hour light/dark cycle throughout the study and had access to food and water *ad libitum*. The day of birth was counted as PD 0. Each litter was kept with their biological mother until weaning (PD 21). After weaning, males from each litter were separated by treatment group and housed thereafter 2-3 animals per cage. All procedures conformed to NIH guidelines and were approved by the University of California Davis Institutional Animal Care and Use Committee.

Hippocampal slice preparation and electrophysiology

The hippocampus was dissected from males (ages 4-6 weeks; PD 28-42) from each treatment group. The method for preparing slices from rat hippocampus and recording field excitatory postsynaptic potential (*f*EPSP) on MEAs was previously described (Kim et al. 2009). Briefly, measurements were made with constant perfusion of artificial cerebral spinal fluid (aCSF; 35 °C) at the slice interface. Single-pulse, biphasic stimuli (10–80 μ A, 0.1 ms) were delivered to Schaffer-Collateral/Commissural pathway within the striatum radiatum in CA1 region at 0.05 Hz (i.e., once every 20 s) (Fig 1A, left panel). The evoked field excitatory postsynaptic potentials (*f*EPSPs) were acquired from recording electrodes chosen based on their proximity to the stimulating electrode. *f*EPSPs were sampled at 20 kHz using a MED 64 multi-channel amplifier, digitized and graphically displayed using Performer® software (Alpha Med Scientific Inc. Osaka, Japan). Baseline *f*EPSPs were experimentally set between 50–60% of maximum of amplitude for each slice. During the stabilization period (at least 30 min), slices that exceeded more than 20% fluctuation in baseline *f*EPSPs were discarded. The *f*EPSP slope was measured using 7 data points between 10% and the

90% of maximum amplitude of the *f*EPSP waveform (Fig. 1A, right panel) using the equation:

$$S_N = \frac{R_{t10\%} - R_{t90\%}}{t_{10\%} - t_{90\%}}$$

Where S_N = slope ($\mu V/msec$), R = response (μV), t = time ($msec$)

Once the slice response stabilized, an additional 10 min of *f*EPSPs slopes were recorded to calculate the mean baseline *f*EPSP slope (baseline period), which was then normalized as 100%. Perfusion of 100 μM picrotoxin (Sigma-Aldrich, St. Louis, MO) dissolved in aCSF was initiated at the completion of baseline period and its influence on *f*EPSP waveform and slope recorded for an additional 30 min.

Statistical Analysis

Results from hippocampal slice experiments were represented as the mean changes in the normalized *f*EPSP slope $\pm$ S.E. ($n = 5-6$ slices per treatment group, each prepared from a different animal). Statistical differences between groups were tested by unpaired Student's-t with Welch's correction (GraphPad Prism, version 5.0). Differences in *f*EPSP slopes before and after addition of picrotoxin were tested by paired Student's-t.

Results and Discussion

Perinatal Aroclor 1254 or PCB 95 enhances picrotoxin excitotoxicity

The major routes of developmental exposure to PCBs in humans are transplacental *in utero* and via breast milk during lactation (Faroon et al., 2001; Pessah et al., 2010). The PCB exposures used in the present study did not influence gestational weight gain, gestational length, litter size, or the mean pup weights (not shown). This exposure protocol with Aroclor 1254 or PCB 95 has been previously shown to influence aspects of learning and behavior in the offspring of exposed rat dams without producing overt toxicity to either dams or pups (Schantz et al. 1997; Crofton et al. 2000; Kenet et al., 2007; Yang et al. 2009). Moreover, the major congeners found in Aroclor 1254, including PCB 95, are prominent in both environmental samples and human tissues (Pessah et al. 2010). Figure 1B and 2B show that control slices subjected to single pulse stimuli showed slight ($108 \pm 11\%$), but statistically non-significant ($p > 0.05$, paired t-test), elevation of *f*EPSP slopes at the end of the 30 min perfusion of aCSF containing picrotoxin. By contrast, hippocampal slices prepared from either Aroclor 1254 or PCB 95 exposed animals showed pronounced elevation of *f*EPSP slopes following exposure to picrotoxin (Figs 1C and 2C), reaching $211 \pm 18\%$ and $218 \pm 22\%$ of baseline, respectively (Figs 1D and 2D; $p < 0.01$). The *f*EPSP waveforms measured after inclusion of picrotoxin in the aCSF invariably (i.e., 100% of the slices tested) exhibited prominent epileptiform after-discharges (epileptic waveforms) in hippocampi prepared from the PCB exposed animals. Epileptic waveforms were not evident in any of the control slices tested (compare Figs 1B and 1C, 2B and 2C).

These data demonstrate that maternal exposure to either an exemplary non-coplanar PCB (PCB 95), or a complex mixture of PCBs predominantly composed of *ortho*-substituted tetra- and penta-chlorinated congeners, is sufficient to persistently enhance the excitotoxic efficacy of GABA_A receptor block with picrotoxin applied to hippocampal slices *ex vivo*.

A current theoretical framework for the etiology of excitotoxicity and seizurogenic activity focuses on the "unbalanced hypothesis" of neurotransmission. This hypothesis proposes

that, in general, seizures are caused by a relative imbalance between excitatory (*e.g.*, glutamate) and inhibitory (*e.g.*, GABA) neurotransmission (Hirose et al. 2000). An imbalance in these major neurotransmitter systems early in development can contribute to abnormal development of local and long-range neural circuits that can contribute to a number of syndromic and idiopathic neurodevelopmental disorders that have high rates of seizure disorders (Belmonte and Bourgeron 2006). Environmental stresses, such as hypoxia, during the neonatal period can produce acute and chronic pathological increases in network excitability in hippocampus CA1, changes that are closely associated with seizure susceptibility (Jensen et al., 1998; Sanchez et al., 2005; Sanchez et al., 2006). For example, significantly decreased amplitudes and frequency of both spontaneous and miniature GABA_A receptor-mediated IPSCs in CA1 pyramidal neurons have been recorded in slices prepared 10 min after hypoxia-induced seizures at PD10 (Sanchez et al 2006).

Perinatal exposure to non-coplanar PCBs *in vivo* enhances sensitivity of hippocampi to picrotoxin *ex vivo*, which blocks GABA_A receptor mediated chloride currents. These results in hippocampus are consistent with previous results indicating a net increase in the ratio of EPSC/IPSC in A1 cortical neurons of rats exposed to PCB 95 during the perinatal period using an identical exposure protocol (Kenet et al., 2007). The balance of excitatory and inhibitory currents in CA1 was not measured in the present pilot study. Never the less, the enhanced efficacy of picrotoxin on enhancing fEPSP slope and the epileptic waveforms that are produced in hippocampal slices from PCB-exposed animals suggest that developmental PCB exposures persistently influence the balance of excitation and inhibition of neural circuits within CA1. These *ex vivo* data would predict that rats exposed perinatally to PCBs via gestational routes and during lactation would exhibit heightened susceptibility to seizurogenic agents administered *in vivo*. Perinatal exposure to PCB 95 (1 mg/kg/day) were shown to reduce latencies to the first myoclonus elicited by flurothyl (bis-2,2,2-trifluorothyl ether) (Lein et al. 2011). Increased susceptibility to seizurogenic agents appears to persist long after PCB exposure terminates. Pentylentetrazole, which kindles seizures within 15-20 subconvulsive doses, enhanced kindled seizure stage severity scores in (1mg/kg/day) PCB 95-compared to vehicle exposed rats when testing was commenced on PD 60 (Lein et al. 2011).

The neurotoxicity of PCB 95 is consistent with those observed with more complex mixtures, including Aroclor 1254 and the environmental mixture known as the Fox River Mixture, both of which are composed primarily of tetra- and penta-substituted non-coplanar PCBs that predominate in both human tissues and environmental matrices (Pessah et al., 2010). Thus developmental exposure to a pure non-coplanar congener (PCB 95) is sufficient to mimic the neurotoxic actions of complex mixtures of current concern to human health. In this regard, congener-specific analyses of brains from weanling rats exposed to A1254 during the perinatal period revealed predominantly *ortho*-substituted congeners at concentrations ranging from 0.5 ng to 3 ng/g wet weight (Yang et al., 2009). Analyses of PCB levels in human brains obtained from the general adult population similarly identified predominantly *ortho*-substituted congeners at concentrations ranging from 0.07 ng to 12 ng/g wet weight (Chu et al., 2003; Covaci et al., 2002; Dewailly et al., 1999).

At least three possible mechanisms could account for the more accentuated response to GABA_A receptor block in hippocampi isolated from PCB exposed offspring *ex vivo* and *in vivo*: (1) altered signaling through ryanodine receptors (Pessah et al. 2010), (2) depression of thyroid hormone (TH) levels (Crofton et al. 2000), and/or (3) allosteric modifications in GABA_A receptor signaling/expression (Fernandes et al. 2010). Although perinatal Aroclor 1254 (6 mg/kg/day) causes mild hypothyroidemia in exposed offspring at PD 31 (Yang et al., 2009), it is unlikely to contribute to the enhanced excitotoxicity of picrotoxin observed in the current communication. Recent evidence indicates that acute application of TH (T3 or

T4) selectively suppresses phasic and tonic GABAergic inhibition (Puia and Losi, 2011). Further, TR α 1^{+/m} mice that have receptor-mediated hypothyroidism and altered development of GABAergic cells (Venero et al., 2005), are in fact seizure resistant (Hadjab-Lallemend et al. 2010). Therefore, hypothyroidemia associated with developmental PCB exposure is unlikely to account for the enhanced sensitivity to picrotoxin observed in the present study. Westerink and colleagues recently reported that non-coplanar PCBs can enhance Cl⁻ currents through GABA_A receptor subunits heterologously expressed in *Xenopus* oocytes, an effect dependent on the pattern of PCB chlorination (Fernandes et al. 2010). However, potentiation of GABA_A receptor signaling is neither consistent with enhanced sensitivity to picrotoxin following developmental exposure to PCB 95 or Aroclor 1254 reported here, nor the enhanced excitation to inhibition currents measured from neurons within the primary auditory cortex of rats developmentally exposed to PCB 95 (Kenet et al. 2007). If PCBs indeed cause persistent potentiation of GABA_A receptor mediated currents in mammalian neurons, such effects would be expected to enhance inhibition and reduce excitotoxicity and seizure susceptibility in adult brain. Never the less, it still remains possible that developmental exposure to PCBs persistently enhances the activity of GABA_A receptors leading to altered expression of GABA_A receptor subunits during the postnatal period. Persistent down regulation of GABA_A receptor subunits could promote an imbalance in inhibitory/excitatory neurotransmission (Olsen and Sieghart 2009), which could be unmasked by exposure to picrotoxin *ex vivo*, as reported here.

Alternatively, the actions of non-coplanar PCBs reported here could result from their ability to alter the spatial and temporal fidelity of Ca²⁺ signals at critical points in development, especially through functional and transcriptional dysregulation of ryanodine receptors (RyRs) (Pessah et al. 2010). In support of this interpretation, perinatal exposure to PCB 95 (Schantz et al., 1997; Lein et al., 2011) and Aroclor 1254 (Yang et al., 2009) were shown to alter the expression and/or function of RyRs in adults. Results from microdialysis studies indicated that local application of ryanodine to rat hippocampus *in situ* altered the balance between glutamatergic and GABAergic transmission (Mori et al. 2005). Results from a study of knock-in mice heterozygous for the R2474S mutation in the ryanodine receptor type 2 (RyR2-R2474S mice) exhibited spontaneous generalized tonic-clonic seizures (Lehnart et al. 2008). One possibility is that PCBs induce a leaky state of RyRs that can increase the sensitivity to pro-convulsants. Perinatal exposure to non-coplanar PCBs may be masked by the influence of normal inhibitory inputs. These results raised the possibility that developmental exposure to RyR active PCBs may significantly enhance seizure susceptibility to chemicals and/or heritable mutations that depress inhibitory neural inputs.

Regardless, if our results showing that developmental exposure to PCB 95 or Aroclor 1254 enhance excitotoxicity to subsequent GABA receptor impairments extend to other non-coplanar chemical of concern to human environmental health, it raises the intriguing question as to whether individuals with heritable deficits in GABAergic signaling might represent especially susceptible populations to PCB exposure. Several neurodevelopmental syndromes with high seizure rates have been hypothesized to possess an increased ratio of excitatory/inhibitory neurotransmission that stems from a complex combination of genetic and environmental variables (Belmonte and Bourgeron 2006; Stafstrom et al. 2011) and could represent particularly susceptible populations to developmental exposures to non-coplanar PCBs and structurally related persistent organic pollutants.

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Figure 1. Developmental exposure to PCB mixture Aroclor 1254 enhances excitotoxicity to picrotoxin *ex vivo*

A. Med-64 multielectrode array (MEA) positioned on a hippocampal slice (left panel) and a depiction of a waveform of a field excitatory postsynaptic potential (fEPSP) indicating the location where slope was measured (right panel). Abbreviation: CA, Cornu Ammon; DG, Dentate Gyrus; S.O., Stratum Oriens; S.T., Stratum Pyramidale; S.R., Stratum Radiatum (the region indicated by the box); S. L.-M.; Stratum Lucidum-Moleculare. Scale: 200 μ V, 10 msec. **B.** Representative fEPSP recorded from a hippocampal slice prepared from a control rat before (at 10 min) and 30 min after the addition of picrotoxin (100 μ M) to the aCSF. **C.** Representative fEPSP recorded from a hippocampal slice prepared from a rat exposed during the perinatal period to Aroclor 1254, before (at 10 min) and after (at 40 min) addition of picrotoxin (100 μ M) in the aCSF. Note the prominent after-discharges observed with all hippocampi prepared from the Aroclor 1254-exposed animals after perfusion of picrotoxin. **D.** Normalized fEPSP slopes recorded from control (n=5) and Aroclor 1254 treated animals (n=6) before and after inclusion of picrotoxin in aCSF perfusate. fEPSP were recorded in response to single biphasic stimuli to Schaffer-Collateral/Commissural pathway of CA1 at 0.05 Hz. Data shown are the means $\pm$ S.E.

Figure 2. Developmental exposure to non-coplanar PCB 95 enhances excitotoxicity to picrotoxin *ex vivo*

A. Structure of PCB 95. **B.** Representative *f*EPSP recorded from a hippocampal slice prepared from a control rat before (at 10 min) and after (at 40 min) the addition of picrotoxin (100μM) to the aCSF. **C.** Representative *f*EPSP recorded from a hippocampal slice prepared from a rat exposed during the perinatal period to PCB 95, before (at 10 min) and after (at 40 min) addition of picrotoxin in (100μM) the aCSF. Note the prominent after-discharges observed with all hippocampi prepared from the PCB 95-exposed animals after perfusion of picrotoxin. **D.** Normalized *f*EPSP slopes recorded from control (n=5) and PCB 95 treated animals (n=5) before and after inclusion of picrotoxin in the aCSF. *f*EPSP were recorded in response to single biphasic stimuli to Schaffer-Collateral/Commissural pathway of CA1 at 0.05 Hz. Data shown are the means±S.E.