Toxicology and Applied Pharmacology 231 (2008) 165–178

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Gene expression profiles in the cerebellum and hippocampus following exposure to a neurotoxicant, Aroclor 1254: Developmental effects ☆

Joyce E. Royland [a], Jinfang Wu [b], Nasser H. Zawia [b], Prasada Rao S. Kodavanti [a,*]

[a] Neurotoxicology Divisions, NHEERL, ORD, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA
[b] Department of Biomedical Sciences and Pharmaceutical, University of Rhode Island, Kingston, RI 02881, USA

ARTICLE INFO

Article history:
Received 29 January 2008
Revised 16 April 2008
Accepted 17 April 2008
Available online 6 May 2008

Keywords:
Polychlorinated biphenyls
Genomics
Nervous system
Development
Aroclor 1254
Learning and memory

ABSTRACT

The developmental consequences of exposure to the polychlorinated biphenyls (PCBs) have been widely studied, making PCBs a unique model to understand issues related to environmental mixture of persistent chemicals. PCB exposure in humans adversely affects neurocognitive development, causes psychomotor difficulties, and contributes to attention deficits in children, all of which seem to be associated with altered patterns of neuronal connectivity. In the present study, we examined gene expression profiles in the rat nervous system following PCB developmental exposure. Pregnant rats (Long-Evans) were dosed perinatally with 0 or 6 mg/kg/day of Aroclor 1254 from gestation day 6 through postnatal day (PND) 21. Gene expression in cerebellum and hippocampus from PND7 and PND14 animals was analyzed with an emphasis on developmental aspects. Changes in gene expression (≥1.5 fold) in control animals identified normal developmental changes. These basal levels of expression were compared to data from Aroclor 1254-treated animals to determine the impact of gestational PCB exposure on developmental parameters. The results indicate that the expression of a number of developmental genes related to cell cycle, synaptic function, cell maintenance, and neurogenesis is significantly altered from PND7 to PND14. Aroclor 1254 treatment appears to dampen the overall growth-related gene expression levels in both regions with the effect being more pronounced in the cerebellum. Functional analysis suggests that Aroclor 1254 delays maturation of the developing nervous system, with the consequences dependent on the ontological state of the brain area and the functional role of the individual gene. Such changes may underlie learning and memory deficits observed in PCB exposed animals and humans.

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Introduction

Polychlorinated biphenyls (PCBs) are a class of persistent organochlorine compounds. Physical and chemical properties that make PCBs industrially valuable also make them resistant to degradation, allow for bioaccumulation in the food-chain, and result in long-lived environmental contamination. Although PCBs were banned in the 1970′s, the contamination continues to be ubiquitous in landfills, sediment, and water (Herrick et al., 2007; ATSDAR, 1999; Wang et al., 2007). Human exposure to PCBs was associated with a number of adverse effects such as skin disorders, cancer, immune dysfunction, neurobehavioral changes, and neuroendocrine disruption (Swanson et al., 1995; Carpenter, 1998). It is of a particular concern that exposure to relatively low concentrations during development may be associated with neurological deficits such as motor dysfunction and impairments in learning and memory (Jacobson and Jacobson, 1996). PCBs are known developmental neurotoxicants in animals and humans (Jacobson and Jacobson, 1996; Jacobson et al., 2002; Kodavanti, 2005) and offer a valuable test chemical to understand the mode of action for developmental neurotoxicity of persistent chemicals. *In utero* and early gestational exposure of rodents, monkeys, and humans to mixtures of polychlorinated biphenyls has been shown to have immediate and long-term consequences (Faroon et al., 2001; Tilson and Kodavanti, 1998; Ribas-Fito et al., 2001). Exposure to PCBs results in adverse neurodevelopmental effects and diminished cognitive function, which may persist into adulthood in humans (Boersma and Lanting, 2000; Lai et al., 2001; Rogan and Gladen, 1992; Vreugdenhil et al., 2004), as well as in rats and monkeys (Hany et al., 1999; Rice, 1999; Rice and Hayward, 1997; Schantz et al., 1989; Widholm et al., 2004).

During the past decades, there has been an attempt to understand the cellular and molecular bases of PCB-induced behavioral and

☆ Preliminary findings were presented at the 44th Annual Meeting of Society of Toxicology, New Orleans, LA (March 6–10, 2006). The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

* Corresponding author. Cellular and Molecular Toxicology Branch, Neurotoxicology Division, B 105-06, NHEERL/ORD, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA. Fax: +1 919 541 0484.
E-mail address: kodavanti.prasada@epa.gov (P.R.S. Kodavanti).

0041-008X/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.taap.2008.04.022

neurological effects in animal models. Both *in vitro* studies using cerebellar granule neuronal cultures and *in vivo* developmental exposure studies indicated that PCBs perturb calcium homeostasis and cause changes in protein kinase C activities in brain (Kodavanti, 2004; 2005; Kodavanti et al., 2000). Other studies indicate that PCBs alter neurotransmitters (Seegal, 1996), decrease circulating thyroxine (T4) levels during developmental exposure (Morse et al., 1996; Crofton et al., 2000), alter the cholinergic system (Juarez de Ku et al., 1994), and cause oxidative stress (Mariussen et al., 2002). While PCBs are known to alter several neurochemical end points (Kodavanti, 2005) and are implicated in the etiology of some neurological diseases (Corrigan et al., 1998), the molecular targets for PCB-induced developmental neurotoxicity are not fully understood.

We recently reported that developmental exposure to PCBs results in alterations in mRNA levels of RC3 neurogranin and spinophilin in rat cortex and/or cerebellum (Lein et al., 2007). These gene changes were partially correlated with changes in dendritic spine density and branching. We now utilize oligonucleotide microarrays to study gene level changes occurring during the brain growth spurt in rat cerebellum and hippocampus following perinatal exposure to either corn oil vehicle or Aroclor 1254, a commercial PCB mixture. We chose cerebellum and hippocampus as brain regions of interest based on the available information on neurochemical changes and adverse effects such as motor activity and cognitive changes associated with PCB exposure (Kodavanti 2005). Microarray analysis is a useful method to obtain a global view of temporal and tissue-specific genomic changes following chemical exposure. To understand the impact of chemicals on brain development, it is necessary to overlay the effects of toxic exposure on normal developmentally-regulated constitutive changes. We chose, for the sake of clarity, to separate this data into two papers, emphasizing developmental changes in gene expression in the first paper and mode of action in the companion paper. In this paper, we report on (a) the developmental changes in gene expression profiles from postnatal day (PND) 7 to PND14 in cerebellum and hippocampus of control rats and (b) the effect of Aroclor 1254 exposure on genomic changes from PND7 to PND14 with emphasis on nervous system development. We have focused mainly on the developmental gene expression profiles in the cerebellum and the hippocampus and how Aroclor 1254 exposure might be altering this developmental gene profile. A separate paper (companion paper, Royland and Kodavanti, 2008) focuses on pathways to understand mode of action and links genomic changes to structural and/or functional correlates of PCB-induced developmental neurotoxicity.

Materials and methods

Chemicals. RNAlater solution for stabilization of tissue RNA, DNA-free kit for removal of contaminating DNA, and the RNA 6000 nanochip kits for checking sample integrity were from Ambion, Inc. (Austin, TX, USA). Trizol reagent from Invitrogen Corp. (Carlsbad, CA, USA) was used for RNA extraction. The RNeasy MinElute Column kit from Qiagen, Inc. (Valencia, CA, USA) was used to further clean-up and concentrate samples. GeneChip Rat Expression Array 230A_2.0 microarrays from Affymetrix (Santa Clara, CA, USA) with 15,923 probe sets were used for gene identification. TaqMan gene expression probe sets for RT-PCR confirmation were obtained from Applied Biosystems (Foster city, CA).

Animals. Long-Evans rats were obtained from Charles River Laboratory (Portage, OR) on gestational day (GD) 3 arrival (the day of insemination was GD 0) and housed in American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved animal facilities. The animals were housed individually in standard plastic hanging cages with sterilized pine shavings as bedding. Food (Purina lab chow) and water were provided *ad libitum*. We have previously reported that this rat chow (4.7 ppb) and water (1.4 ppb) have low levels of PCBs (Kodavanti et al., 1998). Temperature was maintained at 21 ± 2 °C and relative humidity at 50 ± 10% with a 12 h light/dark cycle (6:00–18:00 h). All experiments were approved in advance by the National Health and Environmental Effects Research Laboratory Animal Care Committee of the U.S. Environmental Protection Agency.

Animal dosing. A commercial PCB mixture, Aroclor 1254 (Lot # 124-191; purity > 99%) was purchased from AccuStandard, Inc (New Haven, CT). This lot of Aroclor 1254 has been characterized before with respect to its congener content (Kodavanti et al., 2001). The dosing solutions were prepared in corn oil. In each cohort, at least 15 dams per group were given Aroclor 1254 (0 or 6 mg/kg) in corn oil (2 ml/kg) by oral gavage starting from gestational day (GD) 6 through postnatal day (PND) 21. Dams were left undisturbed on PND1. The rats were weighed and dosed once a day between 8:00 and 10:00 AM. Beginning on GD22, rats were checked twice daily (AM and PM) for births, and the date that birth was first discovered was designated as PND0. All dams (>90% success of pregnancy) gave birth within few hours apart and the litter size varied between 7 and 15 pups. On PND4, litters were culled to 4 each, male and female pups/litter. On PNDs 7 and 14, one male pup from each litter was randomly selected for genomic analysis. The reproductive outcome, general health, and development of rats were similar to those seen in other cohorts and published previously along with neurobehavioral end points (Bushnell et al., 2002; Geller et al., 2001). Brains were quickly dissected on ice, quick-frozen on dry-ice, and stored at −80 °C until RNA extraction and analysis.

RNA extraction. RNA was extracted using an adapted Trizol/RNeasy minicolumn protocol (Invitrogen). Briefly, tissues (cerebellum or hippocampus) were homogenized in 1 ml Trizol, 0.2 ml chloroform added, tubes centrifuged, the aqueous layer removed to a clean, sterile tube, 500 µl ice cold isopropanol added and RNA precipitated overnight at −20 °C. Samples were centrifuged, pellets washed 2 times with 75% ethanol to remove excess salts, dried briefly and RNA re-solubilized in RNase-free H_2O. Contaminating DNA was removed with the Ambion DNA-free kit; RNA content was determined spectrophotometrically on a BioTech plate reader at 230, 260 and 280 λ; and RNA quality was verified by Agilent bioanalyzer analysis using a RNA 6000 nanochip and RNA stored at −80 °C.

Affymetrix chip hybridization. RNA from a single rat pup cerebellum or hippocampus (3 biological replicates/condition/age; 24 arrays total) was hybridized to Affymetrix Rat 230A_2.0 chips by Expression Analysis (Durham, NC) following the basic Affymetrix protocol. That is, 10 µg of total RNA was used to generate cDNA, which in turn was used to make double-stranded cDNA. The cDNA products were incubated with T7 RNA Polymerase and biotinylated ribonucleotides to generate cRNA which was purified and quantified prior to being fragmented, diluted, and denatured in preparation for loading onto the GeneChips. The loaded chips were incubated at 42 °C for ≥16 h, washed with a series of non-stringent (25 °C) and stringent (50 °C) solutions and the fluorescent signal amplified using a biotinylated antibody solution. Fluorescent images were detected in a GeneChip® Scanner 3000 and expression data were extracted using the MicroArray Suite 5.0 software (Affymetrix). Data from these studies will be placed in the NIEHS, Chemical Effects in Biological Systems (CEBS) database (Waters et al., 2003) under accession number 010-00001-0001-000-3.

Real-time PCR protocol. Gene expression levels of selected genes for confirmation of array data were determined using a two-step real-time polymerase chain reaction (RT-PCR) protocol and a comparative C_t experimental design. Genes were selected from the array data that were significantly changed by 1.5 fold, represented a number of expression conditions and were of interest to the experimental question being examined. For instance, Cacnb1 and Gabrb3 correspond to genes of high and low expression, respectively. Gabrb3, Pacsin1, Arnt1, and Cacnb1 were up-regulated and Gpc2 was down-regulated; Cacnb1, Dag1, Gabrb3, Arntl and Pacsin1 displayed tissue- or treatment-specific changes. Sample RNA was first reverse-transcribed to cDNA using an Invitrogen Superscript III first strand synthesis kit following the standard protocol provided by the manufacturer (Invitrogen, CA). Briefly, 2 µg of total RNA was denatured at 65 °C for 5 min in a RNA/primer mixture containing 1 µl of 10 mM dNTP mix and 1 µl of 50 µM oligo dT in a total volume of 10 µl. A cDNA synthesis mixture containing 2 µl of 10× RT buffer (200 mM Tris–HCl, pH 8.4 and 500 mM KCl); 4 µl of 25 mM $MgCl_2$; 2 µl of 0.1 M DTT; 1 µl of RNaseOUT (40 U/µl) recombinant RNase inhibitor; and 1 µl of SuperScript III RT (200 U/µl) per sample was prepared (10 µl total volume). The cDNA synthesis mixture was combined with the RNA/primer mixture (20 µl total volume), cDNA synthesized for 50 min at 50 °C for 50 min, and the reaction terminated with 5 min incubation at 85 °C. Residual RNA was removed by the addition of 1 µl RNase H incubation for 20 min at 37 °C. The final product was stored at −20 °C until used in the RT-PCR reactions. No amplification control (NAC) reactions without reverse transcriptase were carried out under the same conditions.

The RT-PCR was carried out in a 7500 RT-PCR system (Applied Biosystems, CA) as follows: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The RT-PCR mixture (20 µl final volume) consisted of a cDNA generated in the first step reaction, 10 µl of TaqMan Universal PCR Master Mix with ROX as reference dye, 1 µl of TaqMan probe (FAM™/MGB), a probe specific primer mixture (see Table 1 for additional information) and RNase-, DNase- and protease-free water. Utilizing a ubiquitous cDNA generated from a mixture of aliquots from all sample RNAs, a standard curve covering a 3-log dynamic range of cDNA concentrations was generated for the endogenous control and target genes to evaluate PCR efficiency and to determine optimum cDNA concentrations for subsequent analysis. For each target gene, all the samples (12 per tissue) for either the cerebellum or hippocampus were run in triplicate along with the reference gene, beta-actin, and a NAC negative control on the same plate. Variation in beta-actin expression across ages, tissues or treatments varied less than 4%. The reporter dye signal (FAM) was normalized using the passive reference dye, ROX, to eliminate volume-handling error.

Data analysis. Recent evidence suggests that poorly performing perfect match probes on Affymetrix chips can adversely impact data analysis (Mecham et al., 2004a; Mecham et al., 2004b) as expression levels are defined as the mean of all the probe intensities. Therefore,