Polychlorinated Biphenyl–Induced Neurotoxicity in Organotypic Cocultures of Developing Rat Ventral Mesencephalon and Striatum

Gregory D. Lyng,* Abigail Snyder-Keller,* † and Richard F. Seegal* †,1

*School of Public Health, University at Albany, Albany, New York 12222; and †Wadsworth Center, New York State Department of Health, Albany, New York 12201

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Polychlorinated biphenyls (PCBs) are persistent environmental contaminants that are highly toxic to the developing nervous system, particularly via their disruption of dopamine (DA) function. In order to characterize the effects of PCBs on the developing basal ganglia DA system, we utilized an organotypic coculture system of developing rat striatum and ventral mesencephalon (VM). Exposure of the cocultures to an environmentally relevant mixture of PCBs for 1, 3, 7, or 14 days reduced tissue DA concentrations and increased medium levels of DA, homovanillic acid, and 3,4-dihydroxyphenylacetic acid. PCB exposure also increased neuronal cell death in both the VM and the striatum and reduced the number of DA neurons in the VM. Decreases in both tyrosine hydroxylase and DA transporter protein expression were shown by Western blot analysis in PCB-exposed cocultures. There was also an increase in neuronal cell death, identified by Fluoro Jade B, prior to a reduction in the number of VM DA neurons; we hypothesize this increase to be partly due to a loss of gamma-aminobutyric acid (GABA) neurons. Indeed, Western blot analysis revealed up to a 50% reduction in both VM and striatal glutamic acid decarboxylase 65/67. Analysis of tissue PCB levels revealed that concentrations were at or below 10 ppm following all exposure paradigms. This coculture system provides an excellent model to examine the chronology of PCB-induced neurotoxic events in the developing basal ganglia. Our results suggest that PCB-induced neurotoxicity in the developing basal ganglia involves GABAergic neuronal dysfunction, in addition to PCBs’ better-recognized effects on DA function. These findings have important implications for disease states such as Parkinson’s disease and for developmental deficits associated with exposure to PCBs and toxicologically similar environmental contaminants.

Key Words: PCBs; dopamine; GABA; substantia nigra; basal ganglia; development.

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants considered to be a present risk to both wildlife and humans, and they have been banned from production in the United States since 1977. The complex nature of PCB-induced toxicity is due partly to the fact that PCBs are not a single environmental contaminant; instead, there are 209 distinct PCB congeners, including both coplanar and ortho-substituted compounds that differ in their mechanisms of toxicity (ATSDR, 2000). In humans, developmental exposure to PCBs has been linked to cognitive and behavioral deficits in children (Jacobson and Jacobson, 1996; Stewart et al., 2003), while adult exposures have been linked to a higher incidence of Parkinson’s disease mortality (Steenland et al., 2006).

PCB-induced alterations in basal ganglia dopamine (DA) have been observed in a variety of in vitro as well as in vivo systems, including laboratory rodent and nonhuman primate models. Indeed, Bemis and Seegal (1999) have shown, using rat striatal tissue sections, that acute PCB exposure results in decreased levels of tissue DA and increased media DA concentrations. Additional studies using adult rat brain synaptosomes, from either the striatum (Bemis and Seegal 2004) or the whole brain (Mariussen et al., 2001), have shown that PCBs inhibit both the vesicular monoamine transporter (VMAT2) and the dopamine transporter (DAT). PCB-induced inhibition of DA reuptake (DAT) and vesicular packaging (VMAT2) can result in the accumulation of unsequestered DA, which in turn leads to increased production of the DA metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) (Bemis and Seegal 2004, Teng et al., 1997). Additionally, the accumulation of unsequestered DA can result in free-radical formation (Fahn and Cohen, 1992) as well as activation of autoreceptors, with subsequent downregulation of tyrosine hydroxylase (TH) activity (Waggoner et al., 1980), the rate-limiting enzyme in DA synthesis. These changes have been proposed as mechanisms for PCB-induced alterations in DA neurochemistry and subsequent toxicity (Bemis and Seegal, 1999). In fact, Lee and Opanashuk (2004) showed that exposure of the DA MN9D cell line to PCBs produced neuronal death from increased oxidative stress, while Seegal et al. (2004) showed that PCBs induce caspase-mediated neuronal cell death in the N27 midbrain DA cell line.

PCB-induced effects on DA function are also observed in vivo. Seegal et al. (2002) demonstrated, using in vivo microdialysis, that administration of PCBs to adult rats resulted in alterations of striatal extraneuronal DA concentrations, with
noticeable increases in diisylate DA shortly after exposure, followed by decreases in diisylate DA after 3 days or more. Developmental studies of rats exposed to PCBs have shown reductions in frontal cortical and striatal DA concentrations (Seegal et al., 1997). Studies in both adult nonhuman primates and rats exposed to PCBs have shown that chronic PCB exposure significantly reduces the number of DA neurons in the substantia nigra (Seegal, unpublished observations). Additional studies in mice have revealed that a single oral exposure to PCBs results in significant decreases in levels of DAT and VMAT2 protein expression, most likely due to PCB-induced inhibition of these transporters (Richardson and Miller, 2004).

Despite significant evidence that PCBs induce DA dysfunction, both in vitro and in vivo, the mechanisms by which such induction occurs have not been completely elucidated. Although in vivo studies provide evidence of changes in neuronal function, the complexity and lack of experimental accessibility of such studies limit their utility in determining the mechanisms responsible for loss of DA function. On the other hand, in vitro studies can provide insight into mechanisms of action, although in general their design is too simplistic to allow the study of complex systems such as those found in vivo. In order to begin to bridge the results of in vivo and in vitro studies, we utilize an organotypic coculture model based on methods developed by Stoppini et al. (1991) and Snyder-Keller et al. (2001); we have recently further characterized this model in respect to its development of DAergic and GABAergic indices in vitro (Lyng et al., 2007). Organotypic coculture systems permit two distinct regions of brain tissue to be cultured together over an extended time (weeks), while at the same time maintaining the three-dimensional architecture of the tissue. In cocultures of VM and striatum, DA neurons from the VM project to and innervate the striatal tissue in a manner similar to that observed during in vivo development (Snyder-Keller et al., 2001), resulting in increased levels of tissue DA and metabolites, as well as increased protein expression of TH, DAT, and glutamic acid decarboxylase (GAD 65/67) (Lyng et al., 2007). The VM and striatum coculture system not only enables in vivo–like neuron-to-neuron and neuron-to-glial cell interaction but also allows precise control over levels and duration of toxicant exposure; it thereby provides a model in which the chronology of changes in neuronal function can be assessed.

Here, we have used this organotypic coculture model to assess the effects of PCBs on DA function in the developing rat basal ganglia. To test the hypothesis that PCB exposure will result in DAergic dysfunction in organotypic cocultures of VM and striatum, we exposed cultures to an environmentally relevant mixture of PCBs, the Fox River (FR) PCB mixture (Kostyniak et al., 2005), for 1, 3, 7, or 14 days, and we subsequently analyzed the DA neurochemistry, the numbers of DA neurons within the VM, the levels of neuronal cell death, the levels of DAergic and GABAergic proteins, and the congener-specific tissue levels of PCBs.

MATERIALS AND METHODS

Preparation of VM and striatum organotypic cocultures. Cocultures of developing VM and striatum were prepared based on methods described by Lyng et al. (2007) and Snyder-Keller et al. (2001). Under isoflurane anesthesia of the dam, fetuses were removed from timed-pregnant Sprague-Dawley rats (Taconic Farms, Germantown, NY) (the presence of sperm after mating was considered embryonic day [E0]), at E14 (for VM) and E21 (for striatum). Fetal brains were quickly removed and placed in ice-cold Ham’s F-12 medium (Gibco, Carlsbad, CA). The E14 VM was dissected and left intact for subsequent coculturing with an E21 striatum, dissected from 300-μm-thick vibratome sections of the forebrain. VM and striatal tissue pieces were placed 1 mm apart on a Transwell Permeable Support (0.4 μm pore size), in Costar clear six-well culture trays (Corning, Acton, MA), with two cocultures per well. Neurobasal medium (Gibco), supplemented with 20% horse serum, 2% antioxidant-free B-27 supplement (Gibco), penicillin/streptomycin/neomycin (100 μg/ml), bicarbonate (1.2 mg/ml), N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (4.5 mg/ml), and glutamine (2 mM), was used for the first 3 days of culture. After that time, serum-supplemented medium was exchanged for serum-free medium, and cocultures were allowed to incubate, in the presence of vehicle control or PCBs, for an additional 1, 3, 7, or 14 days, at 37°C in a 5% CO2/5% room air-humidified incubator. After these times in culture, the tissue was removed for appropriate analyses. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

PCB selection/preparation of solutions/exposure. We used an environmentally relevant mixture of PCBs, proportional in its congener composition to the PCBs found in fish from the FR in Wisconsin (Kostyniak et al., 2005). We obtained the same FR PCB mixture from the University of Illinois that was characterized by Kostyniak et al. (2005). The FR PCB mixture contains several Aroclors (A) so as to simulate the FR congener profile, specifically, 35% A1242, 35% A1248, 15% A1254, and 15% A1260 (Kostyniak et al., 2005). The FR PCB mixture was dissolved in dimethylformamide (DMF) to create working stock solutions that were further diluted 1000-fold into culture medium. The final medium PCB concentration was either 2 or 8 μM, while the vehicle control solution was 0.1%. PCB- or vehicle-supplemented serum-free medium was added to each coculture after incubation for 3 days in serum-supplemented medium. Cultures were analyzed after 1, 3, 7, or 14 days of exposure to PCBs or vehicle control (corresponding to 4, 6, 10, or 17 total days in vitro).

High-performance liquid chromatography with electrochemical detection. DA levels within the striatum and VM, as well as DA, HVA, and DOPAC levels in the medium, were determined using high-performance liquid chromatography with electrochemical detection (HPLC-ECD), by a protocol based on the methods of Bemis and Seegal (2004). Neurochemical data were corrected for the protein content of the cocultures, using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Briefly, after separation of the VM and striatum, each piece was sonicated in 150 μl of 0.2M HClO4. Medium samples (150 μl) were collected in an additional 150 μl of 0.4M HClO4. Following centrifugation, the supernatants of the tissue samples were used for HPLC-ECD, while the pellet was dissolved in 0.1M NaOH for BCA protein analysis (Pierce). Neurochemical results are expressed as nanograms (ng) of neurotransmitter/metabolite per milligram (mg) of coculture tissue protein for both tissue and medium samples.

TH immunohistochemistry, fluoro jade B labeling, and quantitative analysis. After the above-described PCB or vehicle exposure, cocultures were removed, rinsed in warm phosphate-buffered saline (PBS), and fixed for 48 h at 4°C in 4% paraformaldehyde/4% sucrose. For cryoprotection prior to sectioning, cocultures were placed overnight in 15% sucrose and were then sectioned to 40 μm using a sliding microtome (Leica, Wetzlar, Germany). For TH immunostaining, tissue sections were incubated in 0.2% Triton X-100 in PBS for 15 min, followed by a 2-h incubation in 5% normal goat serum, and 0.2% Triton in PBS. Sections were then incubated for 48 h at 4°C in anti-TH
rabbit primary antibody (1:600; Chemicon, Temecula, CA). Following primary labeling, the tissue sections were rinsed several times in PBS and then incubated for an additional 2 h in goat anti-rabbit Alexa Fluor® 488 secondary antibody (1:200; Molecular Probes, Eugene, OR). After further PBS rinses, samples were imaged using a Zeiss fluorescent microscope with Zeiss Axiosvision® imaging software. Quantitation of TH⁺ neurons was performed using an automated counting/tracing program (Al-Kofahi et al., 2003) that identified TH⁺ neuronal cell bodies using median-based algorithms, thus providing an automated method to quantify the number of TH⁺ neurons in each tissue section. The numbers of TH⁺ neurons in each tissue section was then added together to determine the total number of DA neurons in each cocultured VM. The observer was blinded to the treatment each coculture received.

Neuronal cell death was assessed using the neuron-specific cell death marker, Fluoro Jade B (FJB; Histochem Inc, Jefferson, AR) (Schmued and Hopkins, 2000). PCB-exposed and control organotypic cocultures were sectioned to 40 μm and incubated for 30 min in 0.06% potassium permanganate to limit background staining, followed by a 1-h incubation in 0.001% FJB in 0.1% acetic acid. After labeling with FJB, the entire area of the VM and the striatum were imaged separately, and the fluorescent intensity of the FJB labeling was determined using Image J software (NIH Image). All image collection and analyses were carried out in a blinded manner.

**Western blot protein analysis.** VM and striatal samples were each sonicated in 65 μl of 2% Nonidet P40 with protease inhibitors for Western blot protein analysis. From each sample, 10 μg of protein underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15-lane 4–15% acrylamide separating gels (Pierce), with protein content determined by BCA (Pierce). After electrophoresis, the gels were blot-transferred onto polyvinylidene difluoride membranes as described by Lyng et al. (2007). To limit nonspecific labeling, the membranes were blocked with 5% fish gelatin in PBS with 0.05% Tween-20, overnight at 4°C. Blots were then incubated in primary antibody at 4°C for 24 h with rabbit polyclonal antibody raised against TH or GAD 65/67 (Chemicon, 1:1000) or with a rat monoclonal antibody raised against DAT (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). After rinsing in tris-buffered saline, the blots were incubated for 90 min with biotinylated anti-rabbit (for TH and GAD 65/67) or anti-rat (for DAT) secondary antibody (1:5000; Pierce), followed by incubation with streptavidin-horseradish peroxidase conjugate (1:10,000 in blocking buffer) for 1 h at 4°C. The blots were incubated in SuperSignal/chemiluminescent substrate (Pierce) and developed in a luminescence detection system. Nonspecific labeling, the membranes were stripped with 5% fish gelatin in PBS, incubated for 30 min, and incubated for 5 min in 0.06% potassium permanganate to limit background staining, followed by a 1-h incubation in 0.001% FJB in 0.1% acetic acid. After labeling with FJB, the entire area of the VM and the striatum were imaged separately, and the fluorescent intensity of the FJB labeling was determined using Image J software (NIH Image). All image collection and analyses were carried out in a blinded manner.

**Congener-specific PCB analysis of tissue coculture levels.** Congener-specific PCB analysis of three organotypic cocultures at each PCB exposure level and duration were performed at the Toxicology Research Center, State University of New York at Buffalo, based upon methods described by Kostyniak et al. (2005), Greizerstein et al. (1997), and Howard et al. (2003). Tissue samples were weighed and transferred to a glass vial containing 2.0 ml of n-hexane. PCB congeners #46 and #142 were used as surrogate standards. The mixture was ultrasonicated in a water bath at room temperature for 15 min. One milliliter of the hexane extract was added to a 500-μl Florisil® Sep-Pak® (Waters Corporation, Milford, MA) adsorbent column and eluted with 10.0 ml of n-hexane. The eluant was concentrated to 0.2 ml with a Zymark (Hopkinton, MA) Turbovap® II Concentration Workstation. Congeners #30 and #204 were added as internal standards prior to analysis by capillary column gas chromatography using an Agilent 6890N Gas Chromatograph (Palo Alto, CA) equipped with electron capture detection. Separation of individual PCB congeners was performed on a 60 m × 0.25 mm internal diameter × 0.25-μm film thickness, SPB-5 fused-silica capillary column (Supelco, Bellefonte, PA), with helium carrier gas used at a constant flow rate of 1.5 ml/min. A sample volume of 1.0 μl was injected into the splitless injector, operated in the splitless mode (0.75 min split-less vent time, 100 ml/min) at a temperature of 260°C. The detector temperature was 310°C. The initial oven temperature was 130°C, with programming to 200°C at 4°C/min, then to 210°C at 1.0°C/min, and finally to 280°C at 2.0°C/min. The final temperature was held for 5 min.

**Statistical procedures.** Data were analyzed (SPSS version 12 statistical analysis software) using one-way ANOVA and Bonferroni-corrected post hoc t-test, for determination of the effect of PCB exposure on the numbers of VM DA neurons, DA neurochemistry, FJB fluorescence, DA and GABA protein levels, and PCB levels for the various durations of exposure. Results are based on sample sizes (n) of at least 10 cocultures for each vehicle/PCB dose and duration, except where otherwise indicated. Statistical significance was set at a p value ≤ 0.05. All data are expressed as the mean ± SEM.

**RESULTS**

**PCBs Reduce Striatal and VM DA Concentrations**

Coculture exposure to 8μM FR PCBs significantly reduced both striatal (Fig. 1A) and VM (Fig. 1B) DA concentrations after as little as 1 day, when compared to vehicle controls. Similar to the 1-day exposure results, continued exposure to 8μM FR PCBs for 3, 7, or 14 days resulted in persistent reductions in both VM and striatal DA, with a maximum depletion of tissue DA occurring after 7 days of exposure in the striatum (66% reduction, p < 0.001) and after 14 days of exposure in the VM (60% reduction, p < 0.01) (Fig. 1). Coculture exposure to the lower (2μM) concentration of FR PCBs also resulted in significantly decreased striatal and VM DA levels; however, such a reduction was only apparent after 7 days in the striatum (Fig. 1A) and after 14 days in the VM (Fig. 1B). Vehicle control levels of DA in the striatum and VM closely reflected values previously reported (Lyng et al., 2007), with striatal and VM DA levels reaching a maximum after 7 days of exposure (corresponding to 10 days in vitro), with DA levels of 26.1 ± 3.9 ng/mg protein in the striatum and 38.9 ± 2.6 ng/mg protein in the VM (Fig. 1).

**PCBs Increase Media DA, HVA, and DOPAC Concentrations**

FR PCBs significantly increased levels of DA, DOPAC, and HVA in the medium as early as 1 day after an 8μM FR PCB exposure. Medium DA levels increased to nearly 250% of control levels after 1-day exposure to 8μM FR PCBs (p < 0.01) (Fig. 2A). The medium DA level was also significantly elevated after 7 days of 8μM FR PCB exposure (p < 0.01) and after 14 days of exposure at only the 2μM level (p < 0.05). Fourteen days exposure to 8μM FR PCBs no longer elevated levels of media DA; in fact, DA levels were significantly lower than those of the 2μM exposure (p < 0.05), suggesting advanced neurotoxicity at this longest duration and highest dose.

One-day FR PCB exposure increased medium DOPAC levels (Fig. 2B) to 275% of control levels at the 8μM concentration (p < 0.01), a change similar in magnitude to the increases in medium DA measured at the same time point. While levels of
medium HVA did not increase to the same extent as did medium DA and DOPAC levels in response to FR PCB exposure, HVA did increase nearly twofold following 1-day 8μM FR PCB exposure (p ≤ 0.01). Although there appears to be a trend toward increasing levels of DOPAC and HVA in the medium after 3 or 7 days of PCB exposure, the increases only reattained significance after 14 days of exposure to 8μM PCBs (p ≤ 0.01 and p ≤ 0.05, respectively).

PCBs Reduce the Number of VM DA Neurons

While tissue and medium neurochemical changes were observed as early as 1 day after FR PCB exposure, reductions in the number of VM DA neurons were not found until after 7-day exposure to the 8μM FR PCB concentration (Fig. 3); this...
exposure resulted in a 28% decrease in the number of DA neurons compared to control \( (p \leq 0.05) \). After 14 days of FR PCB exposure, both 2 and 8μM PCBs produced significant reductions in the number of VM DA neurons, with respective 42% \( (p \leq 0.01) \) and 48% \( (p \leq 0.01) \) decreases in neuron number. Vehicle control TH\(^+\) DA neuronal counts after 1-, 3-, 7-, or 14-day exposures were, respectively, 2228 ± 105, 2132 ± 227, 1995 ± 71, and 1426 ± 102 DA neurons. These control counts were similar to counts previously reported in studies conducted without the addition of a DMF vehicle (Lyng et al., 2007).

**PCBs Induce Neuronal Cell Death in Organotypic Cocultures**

In the VM, 8μM FR PCB exposure dose dependently increased FJB-labeled neuronal death after 1, 3, or 7 days of exposure (Fig. 4B). Specifically, 8μM FR PCB exposure increased VM FJB fluorescence to 175% of control after exposure for 1 day \( (p \leq 0.01) \) and to 160% of control after 3 days of exposure \( (p \leq 0.05) \). After 7 days of exposure, 2 and 8μM FR PCBs significantly increased FJB fluorescence to 202% \( (p \leq 0.05) \) and 310% \( (p \leq 0.001) \) of control, respectively. While a 14-day PCB exposure no longer induced significant increases in FJB intensity at the 8μM PCB level, a 2μM exposure still resulted in a significant increase in fluorescence \( (p \leq 0.05) \) (Fig. 4B).

Significant changes in striatal FJB fluorescent intensity occurred less consistently than those in the VM, although an 8μM FR PCB exposure significantly increased FJB fluorescence after both 1-day \( (p \leq 0.05) \) and 7-day PCB exposures \( (p \leq 0.001) \) (Fig. 4A).

**TH Protein Levels**

A 1-day PCB exposure did not produce any significant changes in VM or striatal TH protein expression. Nevertheless, after 3 days of 8μM FR PCB exposure, striatal TH protein expression was decreased nearly 50% from vehicle control levels \( (p \leq 0.05) \) (Fig. 5A), while no significant effect was observed at 2μM in the striatum or at either exposure level in the VM (Fig. 5B). A 7-day FR PCB exposure significantly depleted both striatal (Fig. 5A) and VM (Fig. 5B) TH at the 8μM exposure level, with 49% \( (p \leq 0.01) \) and 45% \( (p \leq 0.05) \) depletions in TH, respectively, while again no effect was observed at the 2μM level. After 14 days, both the 2- and 8μM FR PCB exposures significantly depleted striatal TH protein by 56% \( (p \leq 0.01) \) and 75% \( (p \leq 0.001) \) (Fig. 5A), respectively, and VM TH protein expression was depleted by 34% \( (p \leq 0.05) \) and 52% \( (p \leq 0.01) \) (Fig. 5B), respectively.

**DAT Protein Levels**

Similar to the effects seen for TH protein, a 1-day FR PCB exposure did not significantly change striatal or VM DAT. However, exposure to 2 or 8μM FR PCBs for 3 days resulted in 16% \( (p \leq 0.05) \) and 36% \( (p \leq 0.001) \) reductions in striatal DAT, respectively (Fig. 6A). After 7 days of exposure, striatal DAT was decreased by 39% at the 2μM level \( (p \leq 0.01) \) and by 57% at the 8μM level \( (p \leq 0.001) \) (Fig. 6A), while VM DAT levels decreased by 58% \( (p \leq 0.01) \) and 77% \( (p \leq 0.001) \) after these same exposures (Fig. 6B). Similar to the 7-day exposure results, a 14-day exposure to either the 2 or the 8μM PCBs significantly decreased both striatal and VM DAT, compared to the levels in vehicle control tissue. Specifically, following 14 days of exposure, a 35% reduction in striatal DAT \( (p \leq 0.05) \) and a 33% reduction VM DAT \( (p \leq 0.05) \) were measured at the 2μM FR PCB level, while a 62% reduction in striatal DAT \( (p \leq 0.01) \) and a 64% reduction in VM DAT \( (p \leq 0.01) \) occurred following the 8μM exposure (Fig. 6).

**GAD 65/67 Protein Levels**

Similar to the results for both TH and DAT, levels of GAD 65/67 protein were not significantly altered by a 1-day PCB exposure. However, after a 3-day exposure to 8μM FR PCBs, both striatal and VM GAD 65/67 levels were significantly reduced compared to vehicle control values (by 39%, \( p \leq 0.01 \) and 40%, \( p \leq 0.05 \), respectively) (Fig. 7). After 7 days of PCB exposure, both striatal and VM GAD 65/67 levels were significantly decreased nearly 50% from vehicle control.
exposure, striatal GAD 65/67 was decreased by 52% at 2μM (p ≤ 0.001) and by 76% at 8μM (p ≤ 0.001) (Fig. 7A), while VM GAD 65/67 levels were decreased by 41% (p ≤ 0.01) and 77% (p ≤ 0.001) at 2 and 8μM, respectively (Fig. 7B). A 14-day exposure to either 2 or 8μM PCBs also resulted in significantly decreased striatal GAD 65/67 levels, by 57% (p ≤ 0.01) and 89% (p ≤ 0.001), respectively (Fig. 7A), while only the 8μM level produced changes in VM GAD 65/67 levels (63%...
decrease, \( p \leq 0.01 \) (Fig. 7B). Table 1 summarizes all measures of DA and GABA neuron integrity that were significantly altered in response to PCB exposure, grouped by tissue region (VM and striatum) and duration of exposure.

**Coculture Tissue PCB Concentrations Increase as a Function of Time and Dose**

Levels of PCBs were determined using congener-specific PCB extraction and quantification techniques (Fig. 8). To focus our statistical analysis and focus on relevant PCB congeners, we selected the data only from those individual congeners that have been shown to compose greater than 1% of the total PCBs present in the FR PCB mixture (Kostyniak et al., 2005). This restriction resulted in the analysis of 28 different congener profiles, representing a total of 46 individual PCB congeners, due to coelution; the congener numbers were 4 + 10, 5 + 8, 15 + 17, 16 + 32, 18, 20 + 33 + 53, 22, 28 + 31, 37 + 42, 41 + 64, 44, 47, 49, 52, 56 + 60 + 92, 66 + 95, 70 + 74, 77 + 110, 81 + 87, 97, 99, 101, 105 + 132 + 153, 118, 123 + 149,
138 + 163, and 180 (Fig. 8B). Together, these selected congeners comprise nearly 80% of the total PCBs present in the FR PCB mixture, as well as in our analyzed tissue (see below). PCB concentrations were determined for vehicle, 2, and 8 μM PCB exposures, after 3, 7, or 14 days of exposure. Sums of the above-listed PCB congeners (Fig. 8A) in tissue from FR PCB-exposed organotypic cocultures increased at all exposure durations in a dose-dependent manner. Significant elevations were observed as early as after 3 days of exposure to 8 μM levels of the FR PCB mixture (Fig. 8A). PCB concentrations in exposed cocultures, for 2 and 8 μM exposures, respectively, were determined to be 0.832 ± 0.24 and 2.01 ± 0.40 ng/mg tissue (± SEM) for 3 days, 1.56 ± 0.35 and 3.56 ± 0.58 ng/mg tissue for 7 days, and 2.83 ± 0.41 and 10.01 ± 2.49 ng/mg tissue for 14 days; control levels, in contrast, averaged 0.202 ± 0.023 ng/mg tissue across these exposures.

Figure 8B shows the representative congener composition of the FR PCB mixture (Kostyniak et al., 2005) compared to the average congener composition that we found in PCB-exposed organotypic cocultures. This comparison revealed that certain PCB congeners were more readily retained, while others were more readily excluded than would be predicted from the congener profile of the FR PCB mixture only. In Figure 8B, congeners marked by a ‘‘þ’’ as overrepresented in cocultured tissue exhibited a higher percent composition of PCBs at all exposure levels and durations; these included congeners 66 + 95, 81 + 87, 97, 99, 101, 105 + 132 + 153, 118, 123 + 149, and 138 + 163. Those congeners marked by a ‘‘/’’ as underrepresented in cocultured tissue constituted a lower percent composition than predicted at all exposure levels and durations; the latter included congeners 4 + 10, 5 + 8, 15 + 17, 16 + 32, 18, 20 + 33 + 53, 28 + 31, and 70.

**DISCUSSION**

**PCB-Induced Neurotoxicity**

The dose-dependent decreases in VM and striatal DA concentration and increases in medium DA, DOPAC, and HVA concentrations reported here after as little as 1 day of PCB exposure are similar to effects previously observed in the striatum, both in vivo and in vitro (Bemis and Seegal, 1999; Table 1).

<table>
<thead>
<tr>
<th>Region</th>
<th>PCB dose (μM)</th>
<th>1-day exposure</th>
<th>3-day exposure</th>
<th>7-day exposure</th>
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<td>Striatum</td>
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<td>DA, FJB</td>
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**Note.** #, number of TH+ dopamine neurons; †, significantly increased; ↓, significantly decreased, compared to vehicle control (p ≤ 0.05).
PCB-induced inhibition of DAT and VMAT2 (Mariussen et al., 2001) can result in increased levels of unsequestered extraneuronal and intraneuronal DA, findings supported, respectively, by the elevations in medium DA and its metabolites, HVA and DOPAC, in our study. These transient elevations in unsequestered DA can lead to increased oxidative stress (Fahn and Cohen, 1992; Graham, 1978; Hastings, 1995; Lee and Opanashuk, 2004) as well as the activation of presynaptic DA autoreceptors, which can influence DA synthesis via modulation of TH (Cerrito and Raiteri, 1980; Wolf and Roth, 1990). These changes may account for the persistent depletion of striatal and VM DA content in PCB-exposed cocultures.

We found dose-dependent decreases in the number of VM TH$^+$ DA neurons and the levels of both VM and striatal TH protein. Significant reductions in DA neuron counts were not found until after 7 days of exposure at the 8$\mu$M level, while even greater reductions were determined at both the 2- and the 8$\mu$M levels after 14 days of exposure. These results were supported by a corresponding depletion in VM TH protein. The reduction in the numbers of VM TH$^+$ DA neurons was not unexpected, given that PCBs have been shown to induce neuronal cell death in N27 and MN9D DA cell lines (Lee and Opanashuk, 2004; Seegal et al., 2004) as well as in vivo, where long-term PCB exposure of both nonhuman primates and rats has resulted in decreased numbers of VM DA neurons (Seegal,
unpublished observations). Interestingly, the reductions in the numbers of VM DA neurons and levels of TH protein occur well after the changes in DA neurochemistry, which appear as early as 1 day after PCB exposure. Thus, it appears that DA dysfunction originates at the level of the neuronal terminal, resulting in alterations in DA neurochemistry that precede and possibly lead to the loss of VM TH$^+$ DA neurons.

Increased neuronal cell death, determined by an increase in FJB fluorescence in the VM of cocultures, may explain the reduction in the number of VM TH$^+$ DA neurons, and corresponds with previous findings showing PCB-induced, oxidative stress-mediated, neuronal death in DA cell lines (Lee and Opanashuk, 2004). However, the death of DA neurons cannot fully explain the increased FJB fluorescence in the striatum, for two reasons. First, the majority of striatal neurons are GABAergic, and thus the increase in FJB fluorescence seen here likely reflects a loss of GABAergic neurons. Second, while levels of FJB were substantially elevated in the striatum and VM after 1-day PCB exposure, a decrease in the number of VM TH$^+$ DA neurons was not measured until 7 days of exposure. Since PCB exposure leads to increased oxidative stress (Lee and Opanashuk, 2004), we suggest that GABAergic neurons are more susceptible to oxidative damage than are DA neurons. In fact, Gramsbergen et al. (2002) reported that GABAergic neurons die before DA neurons do, in response to glutathione depletion in an organotypic culture system similar to ours. Thus, we suggest that the PCB-induced increases in FJB fluorescence seen here reflect the death of GABAergic neurons prior to the loss of DA neurons in the VM in response to early alterations in DA neurochemistry and the associated increases in oxidative products.

Indeed, the hypothesized degeneration of GABAergic neurons is supported by our findings of decreased levels of VM and striatal GAD 65/67 protein content, the expression of which has been closely linked to production and release of GABA (Mason et al., 2001; Segovia et al., 1990). Interestingly, it has been shown that the disruption of DA neurons, via neurotoxicant administration, further increases GABA activity; that in turn would increase GAD 65/67 expression (Diaz et al., 2003; Stephenson et al., 2005), an effect not observed in our cocultures. The absence of a compensatory change in GAD expression could be explained by the hypothesized greater susceptibility of GABAergic neurons to PCB exposure in this model of the developing basal ganglia. Since GABA neurons are known to be more susceptible to oxidative stress than are DA neurons (Gramsbergen et al., 2002), we suggest that populations of GABAergic neurons degenerate prior to the loss of DA neurons; accordingly, this results in the absence of an upregulation of GAD 65/67 as would occur in response to a DA neuron–specific neurotoxicant.

Decreased DAT expression in response to PCB exposure, in both the VM and the striatum, is similar to the in vivo findings of Richardson and Miller (2004) and supports the idea of alterations in DA transport and handling in response to PCB exposure in vitro. Striatal DAT protein measures can also be used to assess the extent of DA axonal and terminal innervation of the striatum by the VM DA neurons, given that DAT has been shown to localize to the plasma membranes of DA neurons (Nirenberg et al., 1996) and is considered a surrogate for DA neuronal terminal density. DAT protein results, as well as data on TH protein and DA neurochemistry, support the hypothesis that PCBs do not exert their effect by entirely eliminating the DAergic innervation of the striatum that occurs in the cocultures; instead, these compounds slow the process and/or limit the maximum terminal innervation that can occur.

A loss of synaptic connections between GABA and DA neurons, as suggested by reductions in (1) DAT protein, (2) numbers of DA neurons, and (3) GABA neuron function, as suggested by decreased GAD 65/67 protein and increased striatal FJB fluorescence, may further enhance the neuronal cell death that occurs in response to PCB exposure. DA neurons are thought to receive neurotrophic support through synapses formed in the striatum; disruption of such connections through excitotoxic developmental striatal lesioning (Macaya et al., 1994), 6-hydroxydopamine DA terminal lesioning (Marti et al., 1997), or axotomy of DA neurons (El-Khodor and Burke, 2002) results in increased DA neuronal cell death in the VM. Since striatal neurons project back to VM DA neurons, destruction of both VM-to-striatum and striatum-to-VM synaptic connections, through PCB-induced oxidative damage of neuronal terminals, may enhance neuronal cell death in the two regions, accounting for further increases in FJB fluorescence. Interestingly, FJB fluorescence increased in a dose-dependent manner in the VM, except after the longest duration exposure (14 days) and at the highest level of PCBs (8μM). The reduction in FJB-labeled neuronal death at this time is likely due to the fact that the majority of PCB-susceptible neurons have died and been removed by cellular machinery. FJB only labels neurons that are dead/dying but are still present within the tissue. Once the neurons in the susceptible populations have died and been removed, they can no longer be labeled by FJB.

**Tissue PCB Concentrations**

Tissue PCB concentrations in the VM and striatal organotypic cocultures increased in a dose- and time-dependent fashion when the cocultures were exposed to the FR PCB mixture for 3, 7, or 14 days. An understanding of the tissue levels of PCBs in our exposed cocultures provides valuable insight into the relationship between an in vitro exposure paradigm and in vivo assessments of PCB-induced neurotoxicity. In fact, our highest measured tissue PCB level was only 10 ng/mg tissue (ppm); this maximal level was similar to brain PCB levels determined in vivo after PCB feeding experiments in rodents (Kodavanti et al., 1998), whereas the remaining levels in our study were notably lower than the in vivo results. We did, however, find higher than expected levels of PCBs in our vehicle control tissue (200 ppb), a phenomenon which we suspect is due our
technique of incubating control cocultures in culture wells adjacent to those exposed to PCBs. Nevertheless, we determined that there were no statistical differences between our experimental endpoints in vehicle control tissue cultured adjacent to PCB-exposed samples and those endpoints in vehicle control samples in culture plates where no PCBs were present (data not shown).

Our further analysis, comparing the congener profile of the FR PCB mixture to the congener profile of our PCB-exposed organotypic cocultures, revealed an interesting trend. More highly chlorinated PCB congeners (>1#66) were present at higher-than-anticipated levels, while lightly chlorinated PCB congeners (<1#53), with the exception of congener #70, were present at levels well below the percent composition of the FR PCB mixture. The trend for preferential accumulation of heavy congeners has previously been reported in in vivo rodent studies assessing brain levels of PCBs (Kodavanti et al., 1998). These results indicate that analysis of tissue PCB levels may be as important as is the description of the exposure concentration, if we are to properly interpret in vitro results, and their relationship to effects observed in vivo.

CONCLUSIONS

We have shown that PCBs reduce striatal and VM DA levels while increasing DA and metabolite levels in the medium. PCB-induced reductions in the numbers of TH+ DA neurons in the VM of the organotypic cocultures were supported by decreases in TH and DAT protein. Additionally, PCB-induced increases in both VM and striatal FJB fluorescence, and the timing of reductions in GAD 65/67 protein preceding reductions in TH protein levels or numbers of DA neurons, indicate that GABAergic neurons may in fact be more susceptible to PCB-induced neurotoxicity than are DA neurons. Taken together, these results lead us to hypothesize that an initial loss of DA function occurs via PCB-induced inhibition of DAT and VMAT2; this loss of function causes alterations in DA neurochemistry, downregulation of DAergic proteins, and increased oxidative stress. Oxidative stress likely leads to additional neuronal damage and the eventual loss of both VM and striatal GABA neurons, prior to the death of VM DA neurons.

The proposed mechanisms of PCB-induced alterations in striatal and VM DA remain to be tested in our organotypic coculture system. The hypothesized increases in oxidative damage as well as the hypothesis that PCBs exert their effects by limiting DA terminal innervation of the striatum from the VM will be addressed further in future studies. The measured reductions in VM TH+ DA neurons, combined with the overall DA and GABA dysfunction in this model of the developing basal ganglia, shed further light on the role played by PCBs, and other similar environmental neurotoxicants in the disruption of basal ganglia function during development, as well as in disease states such as Parkinson’s disease.

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REFERENCES


PCB-INDUCED NEUROTOXICITY IN COCULTURES


