

Polychlorinated Biphenyls and Methylmercury Act Synergistically to Reduce Rat Brain Dopamine Content *in Vitro*

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Consumption of contaminated Great Lakes fish by pregnant women is associated with decreased birth weight and deficits in cognitive function in their infants and children. These fish contain many known and suspected anthropogenic neurotoxins, making it difficult to determine which contaminant(s) are responsible for the observed deficits. We have undertaken a series of experiments to determine the relevant toxicants by comparing the neurotoxic effects of two of these contaminants—polychlorinated biphenyls (PCBs) and methylmercury (MeHg)—both of which are recognized neurotoxins. Striatal punches obtained from adult rat brain were exposed to PCBs only, MeHg only, or the two in combination, and tissue and media concentrations of dopamine (DA) and its metabolites were determined by high performance liquid chromatography. Exposure to PCBs only reduced tissue DA and elevated media DA in a dose-dependent fashion. Exposure to MeHg only did not significantly affect either measure. However, when striatal punches were simultaneously exposed to PCBs and MeHg, there were significantly greater decreases in tissue DA concentrations and elevations in media DA than those caused by PCBs only, in the absence of changes in media lactate dehydrogenase concentrations. Elevations in both tissue and media 3,4-dihydroxyphenylacetic acid concentrations were also observed. We suggest that the significant interactions between these two toxicants may be due to a common site of action (i.e., toxicant-induced increases in intracellular calcium and changes in second messenger systems) that influences DA function. The synergism between these contaminants suggests that future revisions of fish-consumption guidelines should consider contaminant interactions. *Key words:* dopamine, methylmercury, polychlorinated biphenyls, rat brain, striatal slice, synergism. *Environ Health Perspect* 107:879–885 (1999). [Online 7 October 1999] <http://ehpnet1.niehs.nih.gov/docs/1999/107p879-885bemis/abstract.html>

An important but daunting task for toxicologists is elucidation of the interactive effects and mechanisms of action of individual toxicants that make up complex mixtures of environmental contaminants. One of the most relevant mixtures of toxicants in the environment is found in contaminated fish, particularly those from the Great Lakes. Epidemiologic studies have demonstrated an association between consumption of contaminated Great Lakes fish by mothers and deficits in growth, development, and cognition in their infants and children (1–3). Because polychlorinated biphenyls (PCBs), at concentrations measured either in breast milk or in fetal cord blood (4), have been associated with these deficits, it has been assumed that PCBs are the contaminant responsible for these deficits. However, Great Lakes fish, including salmon, contain many other known and suspected developmental neurotoxins, including methylmercury (MeHg), chlorinated pesticides, and heavy metals (5,6). Thus, it remains difficult, using only the tools of epidemiology, to determine which contaminant or contaminants are responsible for the observed associations between maternal fish consumption and developmental and cognitive deficits in their infants and children.

To begin to determine which fish-borne contaminants alter central nervous system

function, and whether they interact functionally or synergistically, we examined changes in tissue and media concentrations of dopamine (DA) following *in vitro* exposure of striatal punches from adult rats to PCBs or MeHg alone or in combination. These compounds represent two of the major neurotoxins found in contaminated Great Lakes salmon. We chose to initially look for possible neurotoxic interactions between these two contaminants because of the extensive literature documenting the neurotoxicity of each toxicant (7–9). For this initial study we used striatal tissue punches, rather than cells in culture, because the explanted tissue conserves much of the neuronal complexity and many of the neurotransmitter interactions of the intact brain. We examined changes in DA function because developmental exposure of laboratory rodents to lyophilized Great Lakes fish containing the above contaminants results in significant decreases in regional brain concentrations of DA (10).

Methods

We initially exposed striatal punches to MeHg at media concentrations ranging from 1 to 40 μ M to determine the concentrations of the toxicant that would alter DA function but not induce tissue damage, defined as significant

elevations in media lactate dehydrogenase (LDH) concentrations. Similarly, the PCB doses chosen (10–200 ppm in exposure media), which resulted in altered DA concentrations in the absence of elevated media LDH concentrations, were based on previous striatal slice experiments conducted in our laboratory (11).

After these initial experiments, we characterized the neurochemical effects of combined exposure of striatal slices to PCBs and MeHg by exposing striatal punches from naive adult male rats to a 1:1 Aroclor 1254/1260 mixture at concentrations ranging from 10 to 200 ppm alone or in the presence of different concentrations of MeHg (4, 10, or 14 μ M).

Preparation and incubation of brain tissue. All experiments were performed with the approval of the Wadsworth Center Institutional Animal Care and Use Committee. Naive male Wistar-derived rats, approximately 90 days of age, were decapitated and their brains removed and placed in ice-cold saline. A forebrain block containing the striatum was dissected by hand, mounted on a vibratome, and four 350- μ m thick coronal slices were taken through the striatum. The slices were transferred to a petri dish containing ice-cold HEPES-buffered Hank's saline (HBHS) and two 2-mm diameter punches were obtained from each slice, yielding eight striatal punches from each animal. These punches were distributed across treatment conditions such that tissue from each animal was tested at each exposure condition. Individual punches were placed in 24-well culture plates containing 500 μ L HBHS supplemented with 1% horse serum and 0.2% dimethylsulfoxide (DMSO) (vehicle control) or 0.2% DMSO containing the test compounds of interest. The tissue was incubated for 4 hr in a 37°C shaking water bath under an atmosphere of 95% O₂/5% CO₂. The 4-hr incubation was chosen based on our previous experiments demonstrating

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significant effects of PCB exposure on DA concentrations in striatal slices, without the extreme reductions in dopamine content observed at 6 hr incubation (12). Thus, we were able to examine for potential interactions between PCBs and MeHg.

Preparation of contaminant solutions and selection of doses. The contaminants included PCBs (a 1:1 mixture of Aroclors 1254 and 1260) at concentrations of 10, 20, 40, 100, or 200 ppm and MeHg at concentrations of 1, 4, 10, 14, 20, or 40 μ M. The PCB mixture was chosen because we previously demonstrated homology between the PCB congeners in this mixture and those found in contaminated Great Lakes fish (5). For the PCB/MeHg interaction experiments we chose concentrations of MeHg—4, 10, and 14 μ M—that yielded no effect to minimal effects on our end points without inducing elevations in media LDH concentrations. Stock solutions of each toxicant were dissolved in DMSO and diluted in the exposure medium, resulting in a final DMSO concentration of 0.2%.

HPLC analysis of dopamine and its metabolites. After the 4-hr incubation, a 200- μ L aliquot of media from each well was diluted 1:1 with 0.4 N perchloric acid, and striatal punches were homogenized in 300 μ L 0.2 N perchloric acid. Supernatants from both the tissue homogenates and the perchloric acid-diluted media were analyzed for DA and its metabolites [3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (media samples only)] by HPLC with electrochemical detection as described in Seegal et al. (13).

Measurement of media LDH. In parallel experiments, striatal punches, harvested as described above, were incubated in media containing 0.2% DMSO (control), 4, 10, or 14 μ M MeHg only, 100 or 200 ppm PCBs only, or various combinations of PCBs and MeHg. After incubation, the media were removed and analyzed for LDH content using the Sigma Diagnostics LDH Assay (Sigma, St. Louis, MO) based on the technique of Amador et al. (14).

Statistical analyses. Data are presented from 14 individual experiments that used a range of PCB and MeHg concentrations tested either alone or in combination. The design of each experiment, using 48 tissue punches assigned to treatments in a counter-balanced manner, allowed for either 8 or 12 conditions to be tested with either 6 or 4 punches at each condition, respectively. It was not possible to test all treatment conditions in a single experiment. Because of the variable number of observations and the variance at each test condition, we analyzed the data using the Brown-Forsythe analysis of variance (ANOVA) test, in which the

variances in each experimental treatment are not assumed to be equal (15). This conservative test controls for unequal cell size by weighting the variance according to the number of observations.

Dose-response effects of either PCBs or MeHg alone were analyzed using one-way ANOVA with Bonferroni-corrected post hoc *t*-tests to compare each dose to control. Interactions between varying concentrations of PCBs, in the presence or absence of a constant concentration of MeHg, were analyzed using two-way ANOVA with Bonferroni-corrected post hoc *t*-tests to compare the effects of each combined treatment with the effect induced by the corresponding concentration of PCBs alone. Finally, because of small variations in the control punch DA concentrations between individual experiments, we expressed the data from each experiment as a percentage of its average control value, thus reducing interexperiment variance. The range of absolute values for control tissue and media samples are presented in "Results."

Results

Experiments that examined various exposure times to PCBs, MeHg, and combinations of both revealed no significant effects on the chosen end points before 4 hr (data not shown). Thus, all data represent 4-hr exposure to the previously described treatment conditions. The data presented, expressed as percent of DMSO control values, should be interpreted with regard to the absolute concentrations of tissue and media DA: 372.9 ± 8.9 and 6.2 ± 1.4 ng/mg tissue protein,

respectively. The absolute concentrations of DOPAC in tissue and media exposed to control treatments are 8.9 ± 0.35 and 140.7 ± 7.4 ng/mg tissue protein, respectively. Thus, the apparently large elevations in media DA expressed as a percent of control values actually represent a relatively small change in absolute DA concentrations as compared to the large changes in absolute tissue DA content induced by these toxicants.

Effects of MeHg on dopamine function.

Exposure of striatal punches to MeHg at concentrations ranging from 1 to 40 μ M resulted in a significant dose-dependent decrease in tissue DA concentrations [$F = 85.38$; degrees of freedom (*df*) = 6, 68; $p \leq 0.001$]. Results at concentrations of MeHg at 20 and 40 μ M were significantly different from control values (Table 1).

In contrast, exposure to MeHg resulted in a significant dose-dependent increase in media DA concentrations ($F = 55.98$; *df* = 6, 16; $p \leq 0.001$). Results at 14, 20, and 40 μ M MeHg were significantly different from control (Table 1).

Tissue concentrations of DOPAC were significantly reduced after exposure to 4, 10, 20, and 40 μ M MeHg ($p \leq 0.05$ to $p \leq 0.001$), although media DOPAC concentrations were elevated only at the 20 μ M dose of MeHg (Table 1). The concentrations of HVA in media from punches exposed to all concentrations of MeHg (except 1 μ M) were significantly reduced as compared to control values ($p \leq 0.05$ to $p \leq 0.001$) (data not shown). On the basis of these results, we chose three concentrations of MeHg (4, 10,

Table 1. DA and DOPAC levels^a in tissue and media from striatal punches exposed to MeHg.

| MeHg (μ M) ^b | Tissue DA | Media DA | Tissue DOPAC | Media DOPAC |
|------------------------------|---------------------------------|-----------------------------------|------------------------------|-------------------------------|
| 0 | 100.0 \pm 1.8 ^c | 100.0 \pm 6.3 ^c | 100.0 \pm 3.4 ^c | 100.0 \pm 3.0 ^c |
| 1 | 102.8 \pm 3.1 | 73.3 \pm 10.1 | 95.4 \pm 7.7 | 100.1 \pm 3.7 |
| 4 | 102.2 \pm 2.6 | 116.4 \pm 11.0 | 84.1 \pm 4.0 ^{**} | 94.6 \pm 2.8 |
| 10 | 95.0 \pm 2.6 | 129.7 \pm 11.2 | 83.1 \pm 4.2 ^{**} | 96.5 \pm 3.2 |
| 14 | 83.6 \pm 6.6 | 268.3 \pm 48.2 [*] | 105.0 \pm 10.4 | 103.9 \pm 6.1 |
| 20 | 19.4 \pm 5.8 ^{**} | 1,767.3 \pm 325.6 ^f | 66.2 \pm 6.7 ^f | 181.9 \pm 8.6 ^{**} |
| 40 | 0.687 \pm 0.132 ^{**} | 3,299.6 \pm 235.5 ^{**} | 13.5 \pm 3.3 ^{**} | 81.1 \pm 7.2 |

Abbreviations: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; MeHg, methylmercury.

^aMean \pm standard error, expressed as a percent of the dimethyl sulfoxide control. ^b*n* = 6–84 punches (wells) per dose. ^cAbsolute concentrations of tissue and media DA were 372.9 ± 8.9 and 6.2 ± 1.4 ng/mg tissue protein, respectively, and tissue and media DOPAC were 8.9 ± 0.35 and 140.7 ± 7.4 ng/mg tissue protein, respectively. ^{*} $p \leq 0.1$. ^{**} $p \leq 0.05$. ^f $p \leq 0.01$. ^{**} $p \leq 0.001$.

Table 2. DA and DOPAC levels^a in tissue and media from striatal punches exposed to Aroclor 1254/1260.

| Aroclor 1254/1260 (ppm) ^b | Tissue DA | Media DA | Tissue DOPAC | Media DOPAC |
|--------------------------------------|------------------------------|--------------------------------|------------------------------|------------------------------|
| 0 | 100.0 \pm 1.7 ^c | 100.0 \pm 6.0 ^c | 100.0 \pm 3.4 ^c | 100.0 \pm 3.0 ^c |
| 10 | 89.5 \pm 2.4 ^{**} | 206.3 \pm 27.2 [*] | 120.8 \pm 9.0 [*] | 117.8 \pm 5.1 |
| 20 | 86.5 \pm 2.8 ^f | 233.4 \pm 26.5 ^{**} | 108.5 \pm 3.6 [*] | 119.6 \pm 5.9 |
| 40 | 78.6 \pm 1.7 ^f | 340.6 \pm 29.2 ^f | 114.8 \pm 5.4 ^f | 123.1 \pm 4.0 |
| 100 | 68.4 \pm 2.1 ^f | 438.2 \pm 32.0 ^f | 135.5 \pm 5.7 ^f | 133.7 \pm 4.2 ^f |
| 200 | 58.9 \pm 1.9 ^f | 478.2 \pm 34.0 ^f | 181.7 \pm 7.7 ^f | 153.2 \pm 5.3 ^f |

Abbreviations: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid.

^aMean \pm standard error, expressed as a percent of the dimethyl sulfoxide control. ^b*n* = 23–83 punches (wells) per dose. ^cAbsolute concentrations tissue and media DA were 372.9 ± 8.9 and 6.2 ± 1.4 ng/mg tissue protein, respectively, and tissue and media DOPAC were 8.9 ± 0.35 and 140.7 ± 7.4 ng/mg tissue protein, respectively. ^{*} $p \leq 0.05$. ^{**} $p \leq 0.01$. ^f $p \leq 0.001$.

and 14 μM) to examine for potential interactions with PCBs. These three doses of MeHg spanned the range of doses from no effect at 4 μM MeHg to small nonsignificant effects on DA function, in the absence of gross alterations in DA concentrations or changes in media LDH, at 14 μM .

Effects of PCBs on dopamine function.

Exposure to the Aroclor 1254/1260 mixture at media concentrations ranging from 10 to 200 ppm resulted in a significant dose-dependent decrease in tissue DA concentrations ($F = 63.64$; $df = 5, 212$; $p \leq 0.001$), with all doses significantly different from control at the $p \leq 0.01$ or $p \leq 0.001$ level (Table 2, Figure 1).

Exposure to PCBs also resulted in a significant dose-dependent increase in media DA concentrations ($F = 38.31$; $df = 5, 178$; $p \leq 0.001$) with all doses significantly different from control values at the $p \leq 0.05$ to $p \leq 0.001$ level (Table 2, Figure 2). However, the net effect of PCB exposure was a reduction in total DA concentrations (i.e., punch DA + media DA).

Tissue DOPAC concentrations were significantly elevated after exposure to 100 and 200 ppm PCBs ($p \leq 0.001$) (Table 1). In addition, a significant dose-dependent increase in media DOPAC concentrations ($F = 19.81$; $df = 5, 202$; $p \leq 0.001$) was observed after exposure to PCBs, with all doses significantly different from control at the $p \leq 0.05$ to $p \leq 0.001$ level. Media HVA concentrations were unchanged after exposure to any doses of PCBs (data not shown). These results are comparable to those reported by Chishti et al. (11) using similar concentrations of an identical 1:1 Aroclor 1254/1260 mixture.

Effects of combined PCBs and MeHg on dopamine function.

The effects of coexposure of striatal tissue to MeHg and PCBs on tissue and media concentrations of DA and DOPAC are shown in Figures 1–4. In each panel the indicated p -values represent the significance of the interaction determined using two-way ANOVA with a significant p -value indicating that the slopes of the lines differ and a significant interaction exists when striatal tissue was exposed to both contaminants.

Combined exposure to 4 μM MeHg and 200 ppm PCBs (Figure 1A) resulted in a significant MeHg \times PCB statistical interaction ($F = 3.07$; $df = 5, 78$; $p \leq 0.05$). This treatment resulted in nonsignificant reductions in media DA concentrations at PCB concentrations below 40 ppm and an increase in media DA concentrations above 40 ppm (Figure 2A), resulting in a statistically significant MeHg \times PCB interaction ($F = 2.56$; $df = 5, 51$; $p \leq 0.05$).

Combined exposure to 10 μM MeHg and 20, 40, 100, or 200 ppm PCBs (Figure 1B)

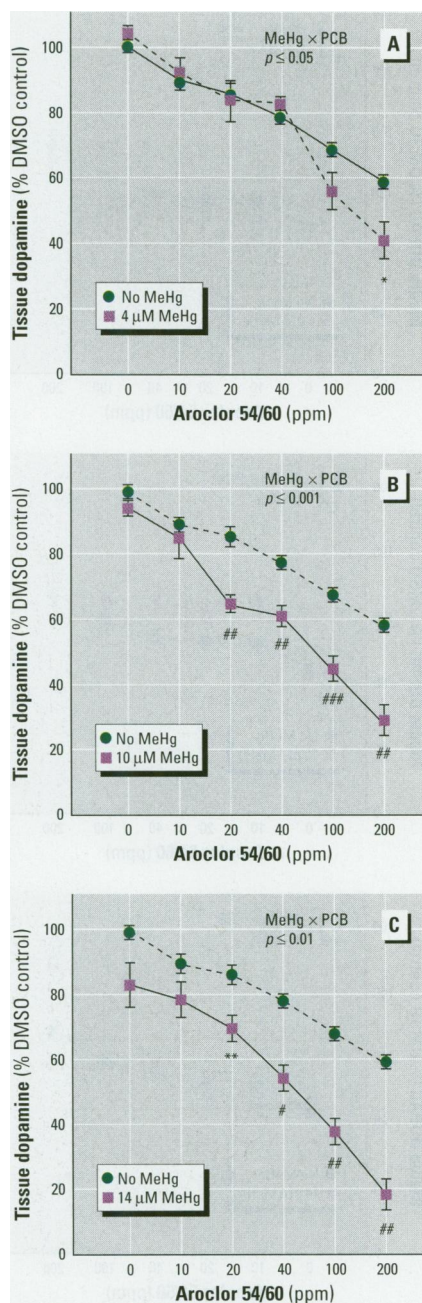


Figure 1. Effect of (A) 4 μM methylmercury, (B) 10 μM MeHg, or (C) 14 μM MeHg alone or in combination with varying concentrations of a 1:1 mixture of 54/60 (10–200 ppm) (PCB) on striatal punch concentrations of dopamine after a 4-hr incubation. Abbreviations: 54/60, Aroclor 1254 + 1260; DMSO, dimethyl sulfoxide; MeHg, methylmercury; PCB, polychlorinated biphenyl. For each concentration, data from 2–14 experiments were combined and are expressed as a percent of the DMSO (vehicle) control. The absolute dopamine concentrations in the control punches were 372.9 ± 8.9 ng/mg tissue protein. The MeHg \times PCB p -value indicates the significance of the MeHg by PCB interaction.

* $p \leq 0.1$, ** $p \leq 0.05$, # $p \leq 0.01$, or ### $p \leq 0.001$ indicates the significance of post hoc t -tests comparing the combined MeHg + PCB treatment with respect to the corresponding dose of PCBs alone.

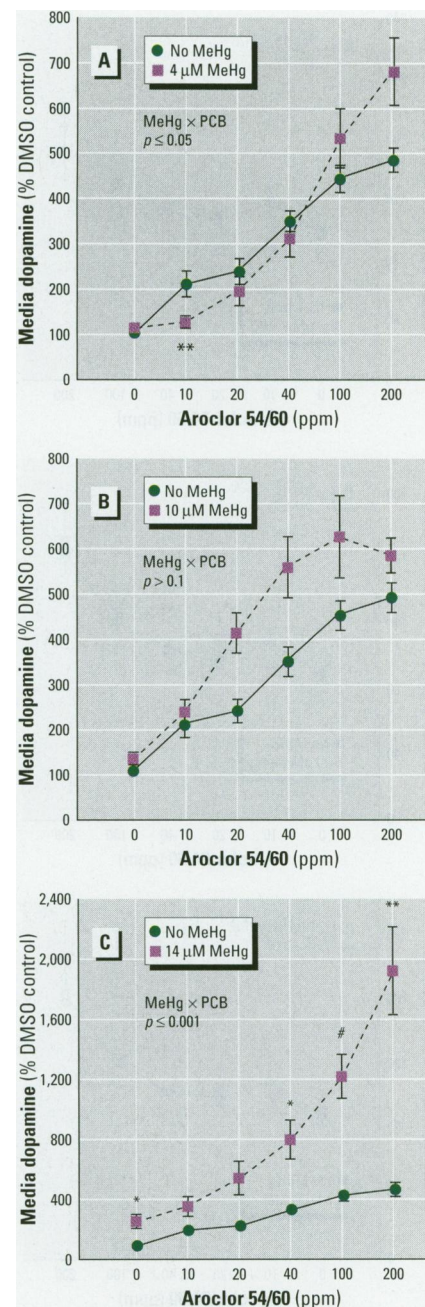


Figure 2. Effect of (A) 4 μM methylmercury, (B) 10 μM MeHg, or (C) 14 μM MeHg alone or in combination with varying concentrations of a 1:1 mixture of 54/60 (10–200 ppm) (PCB) on media concentrations of dopamine after a 4-hr incubation of striatal punches as described in Figure 1. Abbreviations: 54/60, Aroclor 1254 + 1260; DMSO, dimethyl sulfoxide; MeHg, methylmercury; PCB, polychlorinated biphenyl. For each concentration, data from 2–14 experiments were combined and are expressed as a percent of the DMSO (vehicle) control. The absolute dopamine concentrations in media from control punches were 6.2 ± 1.4 ng/mg tissue protein. The MeHg \times PCB p -value indicates the significance of the MeHg by PCB interaction.

* $p \leq 0.1$, ** $p \leq 0.05$, or # $p \leq 0.01$ indicates the significance of post hoc t -tests comparing the combined MeHg + PCB treatment with respect to the corresponding dose of PCBs alone.

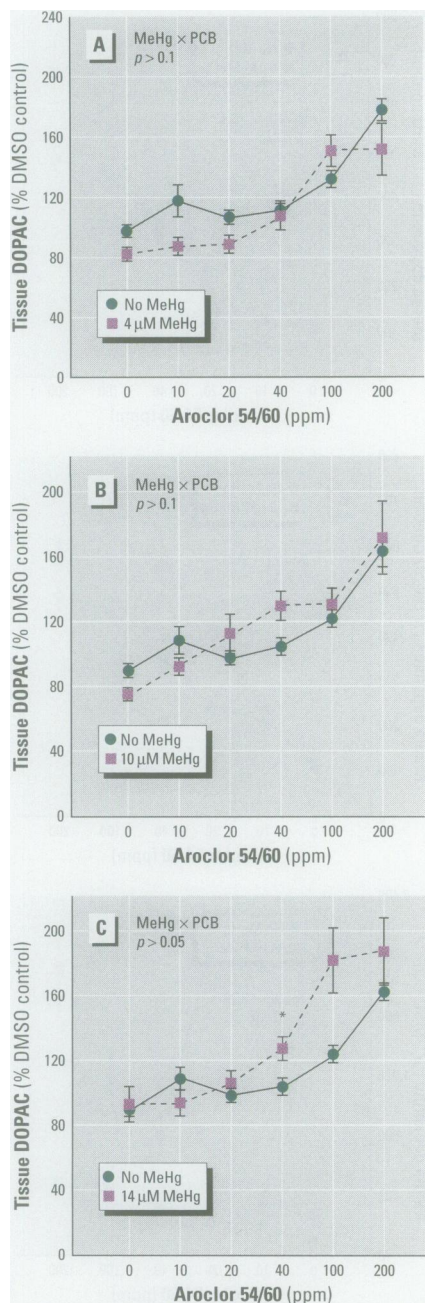


Figure 3. Effect of (A) 4 μM methylmercury, (B) 10 μM MeHg, or (C) 14 μM MeHg alone or in combination with varying concentrations of a 1:1 mixture of 54/60 (10–200 ppm) (PCB) on striatal punch concentrations of DOPAC after a 4-hr incubation. Abbreviations: 54/60, Aroclor 1254 + 1260; DOPAC, 3,4-dihydroxyphenylacetic acid; DMSO, dimethyl sulfoxide; MeHg, methylmercury; PCB, polychlorinated biphenyl. For each concentration, data from 2–14 experiments were combined and are expressed as a percent of the DMSO (vehicle) control. The absolute DOPAC concentrations in the control punches were 8.9 ± 0.35 ng/mg tissue protein. The MeHg \times PCB p -value indicates the significance of the MeHg by PCB interaction.

* $p \leq 0.1$ indicates the significance of post hoc t -tests comparing the combined MeHg + PCB treatment with respect to the corresponding dose of PCBs alone.

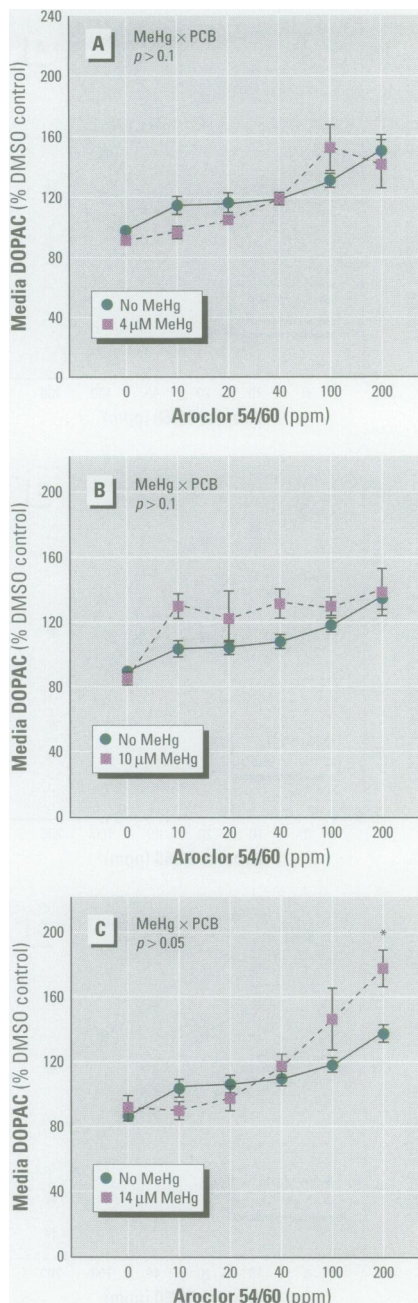


Figure 4. Effect of (A) 4 μM MeHg, (B) 10 μM MeHg, or (C) 14 μM MeHg alone or in combination with varying concentrations of a 1:1 mixture of 54/60 (10–200 ppm) (PCB) on media concentrations of DOPAC following a 4-hr incubation of striatal punches as described in Figure 1. Abbreviations: 54/60, Aroclor 1254 + 1260; DOPAC, 3,4-dihydroxyphenylacetic acid; DMSO, dimethyl sulfoxide; MeHg, methylmercury; PCB, polychlorinated biphenyl. For each concentration, data from 2–14 experiments were combined and are expressed as a percent of the DMSO (vehicle) control. The absolute DOPAC concentrations in media from control punches were 140.7 ± 7.4 ng/mg tissue protein. The MeHg \times PCB p -value indicates the significance of the MeHg by PCB interaction.

* $p \leq 0.05$ indicates the significance of post hoc t -tests comparing the combined MeHg + PCB treatment with respect to the corresponding dose of PCBs alone.

also significantly decreased tissue DA concentrations as compared to exposure to PCBs alone ($p \leq 0.001$), resulting in a significant MeHg \times PCB statistical interaction ($F = 5.09$; $df = 5, 57$; $p \leq 0.001$).

Combined exposure to 14 μM MeHg and varying concentrations of the 1:1 Aroclor 1254/1260 mixture (Figure 1C) also significantly decreased tissue DA concentrations, with combined exposure to all doses of PCB (except 10 ppm PCB) significantly different from exposure to PCBs only at either the $p \leq 0.05$ or $p \leq 0.001$ level, resulting in a significant MeHg \times PCB statistical interaction ($F = 3.45$; $df = 5, 53$; $p \leq 0.01$). This treatment also increased media DA concentrations (Figure 2C) with exposure to 14 μM MeHg only; 14 μM MeHg combined with 40, 100, and 200 ppm PCB was significantly different from exposure to PCBs only at the $p \leq 0.1$ to $p \leq 0.01$ level, which resulted in a MeHg \times PCB statistical interaction ($F = 9.39$; $df = 5, 18$; $p \leq 0.001$). As with PCB-only exposure, the net effect on total DA (punch + media) was a significant reduction.

DOPAC concentrations were elevated in tissue exposed to 14 μM + 40 ppm PCBs only ($p \leq 0.1$) (Figure 3C) and media from tissue exposed to 14 μM MeHg combined with 200 ppm PCB ($p \leq 0.05$) (Figure 4C). Thus, the overall trend in elevations of tissue and media DOPAC concentrations in punches treated with 14 μM MeHg and PCB led to a significant statistical MeHg \times PCB interaction in both tissue ($F = 2.91$; $df = 5, 34$; $p \leq 0.05$) and media ($F = 3.24$; $df = 5, 27$; $p \leq 0.05$) samples. This was the only dose of MeHg (14 μM) at which a statistically significant PCB \times MeHg interaction was observed on tissue or media DOPAC concentrations.

Table 3. Media LDH levels^a from striatal tissue exposed to DMSO control, 54/60, MeHg, or combinations of both.

| Exposure condition ^b | Media LDH |
|---------------------------------------|------------------|
| DMSO control | |
| 0.2% | 60.7 \pm 2.2 |
| 54/60 | |
| 100 ppm | 58.8 \pm 2.4 |
| 200 ppm | 68.4 \pm 6.5 |
| MeHg | |
| 4 μM | 64.0 \pm 1.4 |
| 10 μM | 65.4 \pm 5.0 |
| 14 μM | 66.9 \pm 3.6 |
| 20 μM | 72.1 \pm 4.9** |
| 54/60 + MeHg | |
| 4 μM MEHG + 100 ppm 54/60 | 59.8 \pm 4.3 |
| 10 μM MEHG + 100 ppm 54/60 | 65.6 \pm 7.0 |
| 14 μM MEHG + 100 ppm 54/60 | 70.9 \pm 2.6** |
| 14 μM MEHG + 200 ppm 54/60 | 69.9 \pm 6.8* |

Abbreviations: 54/60, Aroclor 1254/1260; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; MeHg, methylmercury.

^aMean \pm standard error, expressed in international units/liter. ^b $n = 3$ –7 punches (wells) per treatment. * $p \leq 0.1$. ** $p \leq 0.05$.

The addition of 10 or 14 μM MeHg to any of the PCB doses resulted in a simple additive reduction in media HVA concentrations as compared to PCB-only exposed tissue (data not shown). There were no interactive effects observed for HVA after exposure to any of the combinations of PCBs and MeHg.

Effects of toxicant exposure on media concentrations of LDH. To determine if the toxicants alone or in combination induced nonspecific cytotoxic effects, we measured the concentration of LDH released into media, expressed in international units. We chose to use this measure of tissue viability because the measurement of LDH release into media is a commonly used measure of cellular and tissue toxicity (16,17), particularly when applied to organotypic cultures.

Media LDH levels were unaffected after exposure to MeHg at concentrations ≤ 14 μM (Table 3). Media LDH concentrations were not significantly altered after exposure to PCBs at any of the concentrations used in this study [results similar to those reported in Chishti et al. (11)]. However, after combined exposure to 14 μM MeHg and 100 ppm PCBs, a significant (at $p \leq 0.05$) elevation in media LDH was observed, although there was no statistical difference in the combined 14 μM MeHg and 200 ppm PCB exposure condition.

Discussion

This study is part of a larger project to determine the neurologic (i.e., behavioral and neurochemical) effects of developmental exposure of laboratory rodents to the toxicants present in Great Lakes salmon. Although the concentrations of PCBs and MeHg used in these experiments are higher than those seen in Great Lakes fish (PCB content ranged from 0.84 to 1.9 ppm, whereas mercury concentrations were 0.34 ppm) (5,6), the concentrations of these contaminants are similar to, or lower than, those used in other *in vivo* and *in vitro* studies (18–21). Furthermore, some human populations consume contaminated pilot whale meat that contains PCB concentrations as high as 30 ppm and mercury levels in the range of 3.3 ppm (22), well within our range of doses. As presented, these results provide the first evidence of a significant functional interaction between two of the major neurotoxicants present in contaminated Great Lakes salmon and other freshwater and marine species—PCBs and MeHg. To understand the potential mechanisms for the interactions between these contaminants, it is appropriate to first discuss the mechanisms by which the individual contaminants alter DA function.

PCB effects on dopamine function. The data we gathered in intact animals, PC12 cells, and striatal slices exposed to PCBs

suggest two distinct but interdependent mechanisms that may be responsible for the decreases in DA concentrations observed after exposure to PCBs.

First, PCBs reduce tissue DA concentrations in the above test systems (10,11,23), most likely due to the inhibition of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis. Inhibition of TH by PCBs has been demonstrated in striatal slices exposed to NSD-1015, which inhibits aromatic amino acid decarboxylase (AADC). AADC is the enzyme that converts 3,4-dihydroxyphenylalanine (L-DOPA), an intermediate product formed by the TH catalyzed conversion of tyrosine, to DA (24). Thus, PCBs reduce L-DOPA content in striatal slices after exposure to NSD-1015 + PCBs, demonstrating an inhibition of TH activity (12). In addition, Choksi et al. (25) reported reductions in TH activity in rat brain striatal minces exposed to specific *ortho*-substituted PCB congeners.

Second, in both striatal slices (11) and punches (data presented here) we noted significant elevations in media concentrations of DA, which we believe is a reasonable surrogate for changes that occur in the extracellular space of the tissue punch or the brain. These elevations may be due to PCB-enhanced neurotransmitter release and/or inhibition of DA uptake at either the dopamine transporter (DAT) or the vesicular monoamine transporter (VMAT). Thus, Messeri et al. (26) reported elevations in spontaneous DA release from bovine chromaffin cells exposed to PCBs—results similar to those obtained in our experiments. Furthermore, Mariussen et al. (27) showed that *ortho*-substituted PCBs inhibit DA uptake by VMAT in vesicles isolated from rat striatal synaptosomes, resulting in elevated media levels of DA. The reported elevations of both tissue and media DOPAC concentrations further support the proposed increases in intracellular DA levels because DOPAC is formed from free intracellular DA via the action of intracellular monoamine oxidase (MAO) (28). Thus, increased tissue and extracellular DOPAC concentrations may reflect increased free intracellular DA concentrations elicited by exposure to PCBs. In addition, elevations in free intracellular DA concentrations result in end-product inhibition of TH activity (29,30) and elevations in extracellular DA by a carrier-mediated process that moves DA out of the terminal via DAT (31). Thus, elevations in extracellular DA or exposure to DA agonists result in activation of presynaptic DA autoreceptors and inhibition of DA synthesis at the level of TH (32,33).

HVA is formed from extracellular DA by the catabolic activity of catechol-*O*-methyltransferase (COMT) and MAO (28). The

reported elevations in media DA elicited by exposure to PCBs or MeHg would be expected to result in increased media HVA concentrations. However, the lack of effect on media HVA reported here has previously been observed in our *in vitro* preparation after exposure to PCBs (11) and most likely represents the effects of relatively short incubation times and the relatively small elevations in absolute media DA concentrations as compared to the large overall reduction in tissue DA content.

In summary, these data provide evidence that PCBs inhibit the synthetic activity of TH in several model systems. We suggest that the elevations in media DA and, by inference, extracellular DA concentrations represent an initial event induced by PCB exposure which, by the mechanisms described above, contributes to the PCB-induced decrease in DA synthesis.

MeHg effects on dopamine. Both *in vivo* and *in vitro* exposure to MeHg alters DA function. Using a mouse striatal slice preparation similar to the one used here, MeHg exposure, at concentrations considerably higher than we used (i.e., 50 μM), resulted in significantly elevated media DA concentrations (20). Similarly, when adult rats were exposed intraperitoneally to MeHg at concentrations ranging from 0.1 to 2 mg/day (for a total dose of 4 or 6 mg), DA concentrations in striatal dialysates obtained by *in vivo* microdialysis were significantly elevated (21).

Both groups of investigators concluded that the MeHg-induced increases in extracellular DA concentrations may be due to enhanced DA release. These results are similar to those obtained by Komulainen (34), who exposed striatal synaptosomes to MeHg and reported dose-dependent inhibition of uptake of DA and enhanced release of DA from synaptosomes.

Combined effects of PCBs and MeHg on dopamine function. The major effects of combined exposure of striatal punches to PCBs and MeHg are a reduction in punch DA concentrations and an increase in media DA concentrations beyond that seen after exposure to PCBs or MeHg separately. There are several possible explanations for this synergism, which suggests a similar site of action.

Both PCBs and MeHg elevate intracellular Ca^{2+} in *in vitro* neuronal cell preparations, and elevations in intracellular Ca^{2+} alter DA function. Kodavanti et al. (19) exposed cerebellar granule cells to *ortho*-substituted PCB congeners and demonstrated a significant increase in intracellular calcium concentrations. Similarly, Pessah (35) demonstrated elevations in intracellular calcium in PC12 cells after exposure to *ortho*-substituted PCB congeners. These elevations

are due to PCB-induced activation of intracellular ryanodine channels that act as release sites for calcium sequestration stores in endoplasmic reticulum and mitochondria. MeHg exposure also results in elevations in intracellular calcium concentrations in both continuous cell lines and primary cells. Using the calcium-sensitive dye fura 2, Marty and Atchison (36) demonstrated a two-component elevation in intracellular calcium in cerebellar granule cells exposed to MeHg. They concluded that the smaller, more rapid onset increase in intracellular calcium involved MeHg-induced liberation of calcium from inositol 1,4,5-triphosphate (IP3)-sensitive intracellular stores.

The second and larger elevation in intracellular calcium involved the influx of extracellular calcium due to MeHg acting on multiple sites of entry of extracellular calcium into granule cells. Furthermore, Minnema et al. (37) showed that exposure of mitochondria isolated from whole rat brain to MeHg results in significant efflux of labeled calcium from these calcium storage organelles. These effects again complement the action of PCBs on the disruption of intracellular calcium homeostasis via sequestration of calcium in mitochondria. Finally, in cultured cerebellar granule cells exposed to MeHg, Sarafian (38) observed increased intracellular calcium as well as IP3 levels. He demonstrated that MeHg did not directly activate protein kinase C (PKC), but most likely functions through one or both of the reported elevations in the second messengers, calcium and IP3. Verity (39) published a more complete review of MeHg effects on calcium.

The similar actions of these two environmental toxicants on regulation of intracellular calcium concentrations suggest a potential mechanism that might account for their significant interactive effects on the release of DA into media and on DA synthesis. Hanneman et al. (40) reported that elevations in intracellular calcium in rat hippocampal slices, induced by exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, increased the activity of PKC. In turn, activation of PKC in both mouse and rat striatal synaptosomes led to phosphorylation of DAT (41,42), resulting in increased extracellular DA concentrations. Furthermore, an increase in intracellular calcium can also initiate the release of DA from synaptic vesicles docked at the plasma membrane (43). Thus, toxicant-induced elevations in intracellular calcium may lead to both inhibition of DA uptake, mediated by DAT, and enhanced release of intracellular DA—events that ultimately result in decreased DA synthesis (32,33).

It is also conceivable that the observed interactive effects on punch DA concentrations are toxicokinetic in nature (i.e., either

MeHg increases the intracellular concentrations of PCBs or PCBs increase intracellular concentrations of MeHg). However, we believe that elevated toxicant levels were not responsible for the observed interactions, particularly in punches exposed to either 4 or 10 μ M MeHg plus PCBs, for the following reasons. First, in preliminary studies (data not shown) there were no differences in PCB content between PCB- and PCB + MeHg-exposed punches. Second, there were no detectable differences in media LDH concentrations, except for the 14 μ M + PCB-exposed punches. These data not only suggest that the preparations were viable, but also suggest that MeHg exposure, at least at the 4 and 10 μ M MeHg concentrations used in these experiments, did not significantly alter membrane permeability and, by inference, increase intracellular concentrations of the toxicants.

In summary, we have shown that exposure of rat striatal punches to PCBs and MeHg decreases punch DA content and elevates media DA levels to a greater extent than exposure to the single toxicants alone in the absence of significant changes in media LDH concentrations, which provides a recognized measure of both the integrity of the plasma membrane and the viability of the striatal punch. The magnitude of the decrease in punch DA content after combined exposure to MeHg and PCBs (a 20–50% decrease depending on the level of the contaminants studied) is not as great as that seen in rat brain after developmental exposure to the complex mixture of contaminants found in Great Lakes salmon (10). Although there are methodologic differences between these studies (e.g., *in vivo* vs. *in vitro*), we suggest that a major difference is related to the greater number of putative neurotoxicants present in the contaminated fish as compared to the two neurotoxicants studied here. Examination of binary pairs of contaminants (e.g., MeHg + PCBs) is but a preliminary step in the investigation of the neurotoxic potential of complex contaminant mixtures. Thus, we have begun to examine functional interactions between PCBs and other fish-borne contaminants, including the chlorinated pesticide chlordane, and we demonstrated similar changes in striatal punch DA function. Examination of the neurotoxic actions of tertiary and higher order combinations of toxicants provides a logical next step toward understanding the actions of complex mixtures of environmentally relevant toxicants on nervous system function. Thus, our results warrant examination of the impact of other environmental mixtures on human health, suggest that future fish-consumption advisories consider the likelihood that environmental contaminants may act synergistically, and provide a strong rationale

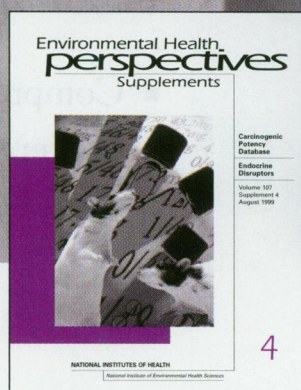
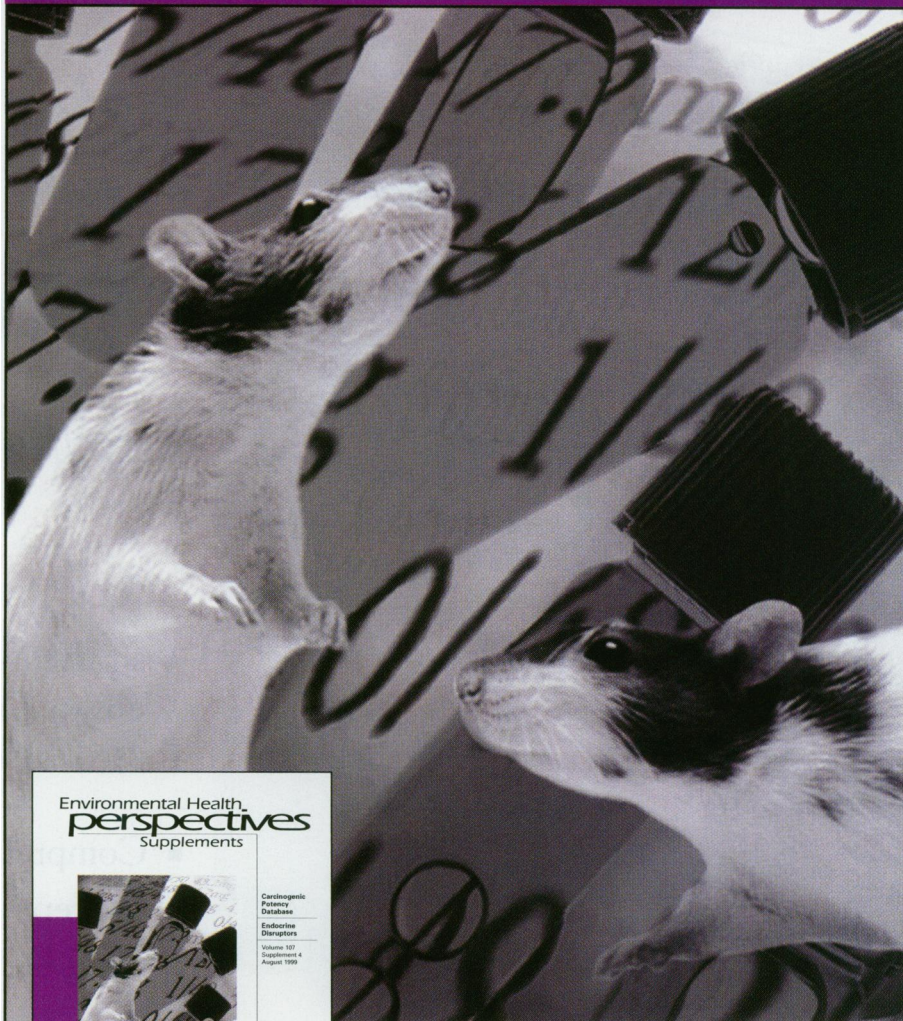
for examining for possible interaction effects of this and other contaminant mixtures in the intact developing animal.

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