RODENT NEUROTOXICITY BIOASSAYS FOR SCREENING CONTAMINATED GREAT LAKES FISH

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Abstract—Standard laboratory rat neurotoxicity protocols were used to study the consequences resulting from the consumption of walleye (Stizostedion vitreum), whitefish (Coregonus clupeaformis), and lake trout (Salvelinus namaycush) from Lake Superior (LS) and the consumption of carp (Cyprinus carpio) from Little Lake Butte des Morts (LLBM) near Oshkosh, Wisconsin, USA. Two 90-d subchronic studies are described, including a 45-d exposure to fish diets using male Sprague-Dawley hooded rats, and a 90-d exposure to fish diets using female rats of the same species. Behavioral alterations were tested using a battery of behavioral tests. In addition, pharmacologic challenges using apomorphine and d-amphetamine were administered to the rats to reveal latent neurotoxic effects. Cumulative fish consumption data were recorded daily, weight gain recorded weekly, and behavioral data collected prior to exposure, and on days 7, 14, 55 ± 2, 85 ± 2. Motor activity data were collected on days 30 ± 2, 60 ± 2, and 90 ± 2 of the feeding protocols. Brain tissue from rodents fed these fish were subsequently analyzed for either mercury (Hg) or polychlorinated biphenyls (PCB). Mercury concentrations were increased in the brains of the walleye-fed rats, and PCB concentrations ranged from 0.5 nl/L to 10 nl/L in the brains of rats fed carp from LLBM, a Lake Michigan tributary. Adult male rats fed LLBM carp for 45 d exhibited the greatest behavioral responses to the dopaminergic agonist apomorphine on the accelerating rotarod, although these differences were not significant. The 90-d exposure of LS walleye or Hg-spiked LS walleye resulted in behavioral alterations on tactile startle response and second footplay. d-Amphetamine challenge caused changes in tactile startle response, second footplay, and accelerating rotarod performance after consuming walleye diets. Rats fed LLBM carp had altered behavioral responses to apomorphine on the accelerating rotarod.

Keywords—Rats Neurotoxicity PCB Methylmercury Great Lakes fish

INTRODUCTION

The present study examines the consequences resulting from the consumption of Lake Superior (LS) fish (walleye [Stizostedion vitreum], lake trout [Salvelinus namaycush], and whitefish [Coregonus clupeaformis]) and the consumption of carp (Cyprinus carpio) from a Lake Michigan tributary (Little Lake Butte des Morts, LLBM).

Polychlorinated biphenyls (PCB) and methylmercury (MeHg) are common, persistent environmental contaminants in the Great Lakes and related tributaries. Persistent environmental contaminants are cause for human health advisories regarding the consumption of contaminated fish. The consumption of large sport fish by eagles may be responsible for reproductive failures [1].

Both PCB and MeHg are known to cause neurobehavioral toxicity in laboratory animals and humans [2,3]. Neurobehavioral effects have been observed in rodents fed a diet of Lake Ontario salmon contaminated with PCB [3]. Effects included reduced exploratory activity and decreased rearing and nose-poke behavior in comparison with controls. In the presence of a negative event, such as a mild shock, rats fed salmon from Lake Ontario showed greater reactions to events than controls [4]. When exposed to MeHg [5], rats have demonstrated changes in open field activity and learned behavior.

Although the effects of MeHg and PCB have been documented in both human studies and animal models [5,6], the effects from chronic environmental exposure in humans are not well defined. Neurobehavioral testing for assessing human health risks associated with environmental contaminants is a relatively new field. However, a large quantity of rodent neurotoxicity tests has been proposed and validated for evaluating the toxicity of new pesticides and other commonly manufactured chemicals. Tests have been published by the U.S. Environmental Protection Agency (EPA) in the Federal Register (Reference 40 CFR 798.6050 and 6200) [7] for screening chemicals for neurotoxicity under the Toxic Substances Control Act (TSCA) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Additional tests (e.g., accelerating rotarod [8], sophisticated startle response measurements [9], and the use of pharmacologic challenges) are not required for neurotoxicity screening under the EPA guidelines; however, they often add considerable sensitivity and specificity to testing protocols.

When animal models are used for human toxicology studies, pharmacologic challenges may provide comparable data between species to illustrate any additional latent neurotoxic effects [10]. For example, apomorphine is a dopaminergic agonist that may be used to reveal PCB-related neurochemical alterations, and d-amphetamine is a nonspecific stimulant that reveals MeHg-induced changes in brain morphology [11]. Pharmacologic challenges can be a useful tool that adds considerable power when making interspecies extrapolations [10].

Rodent feeding groups for this study were based upon the reported fish consumption habits of the Red Cliff Band of LS Chipewa. Many North American peoples, including natives such as the Red Cliff Chippewa (Ojibway), depend upon the Great Lakes fishery for both cultural and economic reasons.
These subchronic rodent studies are intended as neurotoxicity bioassays for human health risks associated with consuming contaminated Great Lakes fish.

Contaminant burdens of Great Lakes fish selected for this study are listed in Table 1. Laboratory determinations are for total Hg, however, in fish 90 to 95% of Hg occurs in the methylated form (MeHg) [12]. Therefore, the results are reported as total Hg, but in actuality are comprised of mainly MeHg. With regard to brain tissue, MeHg more readily crosses the blood–brain barrier when compared to other forms of Hg [13].

MATERIALS AND METHODS

Fish collection

Walleye, whitefish, and trout were collected in the Apostle Islands region of LS (Wisconsin management unit #2) and prepared by Red Cliff fishermen. Walleye and trout were prepared with the skin attached, and whitefish were scaled and filleted with the skin attached. Pacific salmon were purchased through local vendors and filleted with the skin attached and used as a negative control for the feeding studies. Carp were obtained from the Wisconsin Department of Natural Resources by electrofishing in LLBM near Oshkosh, Wisconsin, USA, and filleted with skin removed. Carp were used as a positive control for PCB contamination. A potato diet was included to represent the practice of eating potatoes as a replacement for fish, a reported PCB contamination. A potato diet was included to represent the practice of eating potatoes as a replacement for fish, a reported PCB contamination. A potato diet was included to represent the practice of eating potatoes as a replacement for fish, a reported PCB contamination.

Diets were provided using ceramic food jars with a stainless steel food follower and nose-hole lid. Food jars were weighed before feeding and upon removal to determine the amount of food consumed.

Neurobehavioral tests

Behavioral measures included a modified Functional Observational Battery similar to Moser et al. [14], motor activity using figure 8 mazes (F8M), accelerating rotarod (AR) performance, startle responses, grip strength, and landing foot splay. All behavioral tests except motor activity were conducted prior to dosing in both exposure studies, and on days 1, 7, 14, 55 + 2, and 85 + 2 of dosing. Motor activity was assessed prior to dosing, and on days 30 + 2, 60 + 2, and 90 + 2 in both studies. Startle responses (San Diego Instruments, San Diego, CA, USA) are a stereotypic motor response to a sudden intense audio or tactile stimulation. When stimuli are elicited repetitively, habituation occurs [9]. Rats were placed in a polypropylene tube with air holes inside the startle response chamber. Each rat was allowed to acclimate in the chamber for 1 min with a background noise of 56 dB. The response to three types of stimuli were recorded (audio, tactile, and inhibitory prepulse). The audio stimuli was a single 118-dB tone having a duration of 300 ms. The tactile stimuli was an air puff to the back of the neck of the rat for 20 ms at 15 psi. The inhibitory prepulse was a single tone of 102 dB for 5 ms followed by an air puff of 15 psi for 20 ms. After the acclimation period, the three stimuli were administered in random order six times, with the time interval between stimuli randomly varied between 5 and 9 s. All responses were analyzed for changes in amplitude as converted by a piezo disk located under the platform holding the tube.

Grip strength (designed by Dr. J. Mattson, Dow Chemical, Midland, MI, USA), was tested by placing the rats fore- or hindlimbs on a wire mesh attached to a dynamometer. The rat was pulled backward until it released the mesh. The resistance to the pull was measured in kilograms and the average of three trials used for analyses.

Foot splay was recorded by painting the rats’ rear foot pads with tempera paint. The animals were held parallel to the landing surface at a height of 30 cm and released onto a sheet of paper. The initial foot placement was circled and the distance between pad marks was measured for two trials.

The AR (Omni-Rotor Treadmill Model RR, Omnitech Electronics, Columbus, OH, USA) is a rotating rod apparatus used to test locomotor performance of rats. Each compartment has a digital timer with infrared detectors that monitor the length of time an animal remains on the rod. An electric grid provides a mild scrambled shock that serves as a negative reinforcement for stepping off the rod. Prior to the feeding study, all rats were trained on the AR apparatus over approximately a 2-week period using an eight-phase training schedule with a final goal of maintaining 20 rpm for 2 min [8]. Upon completion of the training, the rats were ranked according to rotarod performance and distributed between the feeding groups. The eight rats with the lowest scores were dropped from the study. During the testing
phase, the acceleration of the rotarod was 1 rpm/s and the negative stimulus turned off. The rats were left on the beam until they stepped off, and the maximum time on the rod (in seconds) was recorded.

Motor activity was determined using the F8M (San Diego Instruments), which consists of a plexiglas maze with a wire mesh floor. Eight infrared beams are positioned around the maze and function to record rat movement by beam breaks that are counted and summed every 5 min for a period of 60 min.

Pharmacologic challenge

After both exposure studies the rats were given a pharmacologic challenge. Apomorphine injections (0.15 mg/kg, subcutaneously [s.c.]) were given to the 32 male rats after the 45-d exposure. The MeHg-contaminated group (walleye, spoked-walleye, and whitefish) of the 90-d exposure received d-amphetamine (2.5 mg/kg, s.c.) while the PCB-contaminated group (LLBM carp, trout, and Pacific salmon) received apomorphine (0.15 mg/kg, s.c.). Rats were then tested on the behavioral measures as stated previously.

Tissue collection

Tissues were collected within 2 weeks of completion of the 90-d subchronic studies using the following procedures. Rats were decapitated and blood was collected in heparinized vacutainer tubes with aluminum foil-covered septa. The brains were quickly removed and dissected on a glass petri dish on ice. The brain was halved along the corpus callosum. One half was weighed and placed in a polypropylene capped centrifuge tube for PCB analysis and the other half for Hg analysis.

Blood and brain tissues were collected for specific PCB congener analyses from all rats in the first subchronic study and from those rats fed diets of trout, Pacific salmon, and LLBM carp in the second study. Brain and blood tissues were collected from rats in the second study fed walleye or whitefish and were analyzed for total Hg.

Hg and PCB analyses

Mercury analyses were performed using the atomic absorption technique involving a Hg cold vapor analyzer [15]. Approximately 1.0 to 1.1 g of brain tissue or 0.1 to 0.2 mL of rat blood was placed into an acid washed biochemical oxygen demand (BOD) bottle. Mercury standards, blanks, and spikes were prepared with each set of tissue samples. Approximately 1.0 ml of concentrated nitric acid and 4.0 ml of concentrated sulfuric acid was added to each sample. The mixture was heated in a water bath for 1 h or until the samples were dissolved. After cooling, 20.0 ml of 5% potassium permanganate solution (15.0 ml for blood) and 8.0 ml of 5% potassium persulfate solution was added. The samples sat overnight allowing the organic forms of Hg to oxidize into inorganic forms. Approximately 10.0 ml of 10% hydroxylamine hydrochloride–10% sodium chloride solution was added to each sample and swirled until no purple coloration of potassium permanganate remained. Just prior to analysis, 5.0 ml of 10% stannous chloride solution was added to the BOD bottle; immediately following, the BOD bottle was attached to the aeration system and the maximum absorbance of each sample was recorded.

Concentrations of specific PCB congeners in the blood and brain tissues were determined at the University of Illinois Department of Veterinary Biosciences. The PCB were acquired from Ultra Scientific and used as standards for the subchronic rodent feeding studies (Appendix 1). Approximately 0.5 g of brain tissue was weighed out and placed in a glass beaker. Each sample was spiked with PCB 14 and 166 at known amounts to determine recovery values. Sodium sulfate and hexane were added to the tissue and ground with a glass rod until homogeneous. The sample was then sonicated and quantitatively transferred onto a silica gel column. The silica gel column separated the sample into three fractions, isolating the PCB into the second fraction (SG-2). The SG-2 fraction was dried down to less than 1 ml under nitrogen gas, and internal standards PCB congener 30 and octochloronaphthalene (OCN) were added. The sample was adjusted to 1 ml and placed into an autoinjection vial. Sample analysis was performed using a Hewlett Packard model 5790A series gas chromatograph equipped with a DB-5 60-m column and an electron capture detector. [16,17,18].

Statistical analyses

A multivariate analysis of variance (MANOVA, SAS Institute version 6.03) [19] was used to test all behavioral dependent variables (footprint, grip strength, accelerod, audio startle, tactile startle, and inhibitory prepulse) looking for diet × day interactions as the primary fish feeding group effect. Body weight was used as a covariate. A univariate analysis of variance (ANOVA) model was examined for each dependent variable using the general linear model type III tests, when appropriate, to control for a weight covariate. Dunnett’s contrast between whitefish and the MeHg groups or the Pacific salmon and the PCB groups was calculated for each dependent variable across all days.

Brain/liver weights were tested using ANOVA procedures and Dunnett’s contrast as noted above. The F8M data were analyzed using a repeated-measures ANOVA model, which tested for within-subject and between-subject effects. The day × time × diet three-way interaction was used as the primary significance test for fish feeding group effect.

For the pharmacologic data, a repeated-measures ANOVA (SAS Institute version 6.03) [19] using all behavioral dependent variables plus the weight covariate was used to examine the diet × day and/or the drug × diet interactions. The repeated measures analysis permitted an examination of the within-subject and between-subject diet effects.

RESULTS

The 90-d subchronic with 45-d feeding exposure

Minimal behavioral effects were seen after 45 d of feeding the fish diets. None of the behavioral test measures showed significant alterations in responses or activity. The rats readily ate their diets and had consistent weight gains, except for the rats fed the potato diet. The high starch and low protein content of the potato diet appeared to have significantly restricted growth.

Congener-specific PCB analysis of brain data was performed on the rats fed LLBM carp from the 45-d exposure. Following the IUPAC nomenclature for PCB congener identification, the highest brain PCB concentrations were of congeners 47, 70, 48, 28, and 74, in descending order, which were found in the carp fed group (Table 2). No PCB were detected in the brains of rats fed Pacific salmon, potatoes, or rat chow after 45 d of fish consumption. These data are consistent with the food stock analysis (Table 1).

The 90-d subchronic with 90-d feeding exposure

Female rats readily ate their diets and had consistent weight gains. Increased Hg levels were found in the brains of rats fed
Table 2. Comparison of mean brain values of rodents fed a diet of LLBM carp

<table>
<thead>
<tr>
<th>PCB congener</th>
<th>45-d exposure</th>
<th>Range (ng/ml)</th>
<th>90-d exposure</th>
<th>Range (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>3.14</td>
<td>1.78–5.04</td>
<td>0.65</td>
<td>LOQ—3.19</td>
</tr>
<tr>
<td>47 + 48</td>
<td>13.36</td>
<td>3.29–11.34</td>
<td>18.47</td>
<td>LOQ—19.04</td>
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<tr>
<td>49</td>
<td>LOQ&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<tr>
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<td>4.87</td>
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<td>LOQ—4.08</td>
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<tr>
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</tr>
<tr>
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<td>LOQ</td>
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</tr>
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<td>0.74–1.19</td>
<td>LOQ</td>
<td>LOQ</td>
</tr>
<tr>
<td>153</td>
<td>1.36</td>
<td>1.02–1.70</td>
<td>6.32</td>
<td>LOQ—10.54</td>
</tr>
</tbody>
</table>

<sup>a</sup> LOQ = below limit of quantitation of 0.5 ng/ml for individual congeners.

walleye (0.067 µg/ml) and spiked walleye (1.571 µg/ml) compared to whitefish (0.022 µg/ml). Brain weights in rats fed walleye (1.855 g) were significantly (<i>p</i> < 0.05) greater than those fed whitefish (1.749 g). Liver weights appeared to be increased compared to salmon in all fish groups except LS whitefish (Table 3), although not statistically significant, and no gross pathological signs were noted in any feeding group.

Of those rats fed MeHg-contaminated diets, significant behavioral effects (<i>p</i> < 0.05) were seen during the 90-d feeding study and during the D-amphetamine challenge on the AR (Fig. 1), second footsplay (Fig. 2), and tactile startle response (Fig. 3). No behavioral effects were seen on grip strength, F8M, or audio or inhibitory prepulse after 90 d of exposure.

Rats fed the PCB-contaminated diets had no significant behavioral effects on any of the behavioral measures during the 90-d feeding study. After the apomorphine challenge was administered, significant behavior effects (<i>p</i> < 0.05) were seen only on the AR in the groups fed LLBM carp (Fig. 4).

Several potentially neurotoxic PCB congeners were found in the brains of rats fed LLBM carp. Congener-specific PCB analyses were also completed on the brain tissues from the second study (90-d exposure). The six highest congeners detected in the LLBM carp fed group were 48 + 47, 153, 52, 74, and 49, respectively (Table 2). Low concentrations of PCB congeners 52 and 49 were detected in the Pacific salmon and trout groups; the other congeners were not consistently found in rats from those two feeding groups.

The adult male rats from the first subchronic study displayed
can alter dopaminergic activity. Although no direct behavioral activity [20]. Therefore, it is possible that decreased dopaminergic activity or had no effect on dopamine tested against control. * Statistically significant.

few direct behavioral effects. The LLBM carp-fed group showed the greatest response to the dopaminergic agonist apomorphine for accelerated performance, although this response difference was not statistically significant.

**DISCUSSION**

Pharmacological agents are used to address several types of mechanistic questions, including the effects a neurotoxicant has on a specific neurochemical or neuroanatomical parameter [11]. The coadministration of apomorphine, a known dopaminergic agonist, is an attempt to better define the mechanism of action of these environmental toxicants by agonizing or antagonizing the effects of an unknown compound. Because certain PCB detected in the present study have been shown to deplete dopamine concentrations [20], a dopaminergic agonist was administered at the end of an exposure. Although no direct behavioral alterations could be attributed to PCB burdens, it is possible that behavioral alterations are related to dopamine concentrations. The carp-fed group, which was highest in PCB, showed statistically significant alterations after the dopaminergic challenge, while other groups showed no significant difference. It should be noted that no organochlorine compounds other than PCB could be detected in the carp tissue.

All PCB congeners found in the brain tissue of rats contained ortho substitutions. In addition, the congeners that occurred in the highest concentration contained at least one substitution in the para position. These findings are consistent with the findings of other studies [20] (R. Seegal, personal communication), which have shown that PCB congeners with substitutions in the ortho position occur at the highest concentrations in the brain tissue. In similar studies, chlorination in the para position reduces dopamine, while chlorination in the meta position either decreased dopaminergic activity or had no effect on dopamine activity [20]. Therefore, it is possible that ortho-substituted PCB can alter dopaminergic activity. Although no direct behavioral changes were detected in our study, simultaneous administration of the diet and a dopaminergic agonist did cause alteration in the AR performance.

Distribution of total PCB body burdens are influenced by the resultant metabolites with considerable differences between PCB congeners and animal species as a result of differential basal activities and substrate specificities of P450 enzymes. Hepatic microsomal enzyme induction by PCB involves cytochrome P450 types IA1, IA2, IIB1, and IIB2 and has been directly correlated to an increase in liver weight of animals when compared to controls [21,22]. Liver weights were increased in all rodents fed diets of PCB-contaminated fish for 90 d (Table 3). Brain concentrations of PCB 70 and 28 were higher in the 45-d exposure (first subchronic) than in the 90-d exposure (second subchronic). Because liver weights were increased, one possible explanation for their concentration difference is the induction of hepatic microsomal enzymes. In the second subchronic study PCB congeners 47, 48, 49, and 153 increased in concentration as exposure lengthened. Adjacent unsubstituted carbon atoms in the meta and para positions are preferentially subjected to metabolism, which may proceed via an arene oxide intermediate [23]. Because PCB 153 has chlorine substitutions in the meta and para positions, the increased concentration corresponding to exposure period would be expected. Alternately, the residue concentrations of PCB 47, 48, and 49 may have been altered due to differences between the sexes. Female rats can have different basal activities of enzymes than males and in some cases have shown lengthened pharmacological activity and increased toxicity [24].

Because of the levels of Hg in the brains of rats fed walleye, MeHg contamination may be responsible for the behavioral decrements seen in the rats during the 90-d feeding and during the d-amphetamine challenge. The observed behavioral difference occurred subsequent to mild, negative stimuli associated with learned behavior. Specifically, the second landing foot splay occurs immediately following the first trial, and rats receive a mild foot shock if they are unable to stay on the rotating rod. Deficits in learning a water-escape T-maze [25] have been observed in rat pups exposed to MeHg postweaning. Disruption of the morphological and biochemical development of the nervous system also occurred with a direct exposure of MeHg in young pups [25]. These apparent overreactions to negative stimuli are similar to the observations made by Daly [4] of rats fed Lake Ontario fish.

Future dose response feeding studies are essential to deter-
mine a cause effect relationship between the fish diets in these experiments and resultant behavioral alterations. No adverse effects can be attributed to any specific contaminant, although PCB and MeHg were the contaminants examined in this study. The interactions between these and other environmental contaminants need to be assessed further to determine their effects.

Acknowledgement—This research was funded in part by a grant from the Great Lakes Protection Fund, Chicago, Illinois, USA, with additional collaborations with the University of Minnesota in Duluth, and the Department of Veterinary Biosciences, University of Illinois in Champaign–Urbana. Shawn Gerstenberger will include the data reported herein as partial fulfillment of his Ph.D. dissertation. The Wisconsin Department of Natural Resources, under the direction of M. Meyer and T. Doelger, provided the LLBM carp. All use of animals was reviewed and conducted in compliance with the National Institute of Health’s guidelines for the care and use of laboratory animals. Publication 75 of the Lake Superior Research Institute series.

REFERENCES

APPENDIX 1
Polychlorinated biphenyl congeners and their chlorine substitution patterns used as standards for the rodent tissue analysis

<table>
<thead>
<tr>
<th>Congener (IUPAC no.)</th>
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