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Neuromotor deficits and mercury concentrations in rats exposed to methyl mercury and fish oil

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Abstract

It has been suggested that docosahexaenoic acid (DHA) or other $n-3$ polyunsaturated fatty acids (PUFAs) may prevent or ameliorate methyl mercury's neurotoxicity. To examine interactions between PUFAs and methyl mercury exposure, sixty-six female Long–Evans rats were exposed to methyl mercury continuously via drinking water from fifteen weeks of age. Water included methyl mercury concentrations of 0, 0.5, and 5.0 ppm, creating estimated intakes of about 0, 40, and 400 μg/kg/day across exposure groups. An additional fifty-eight female offspring were exposed to methyl mercury only during gestation. Rats consumed one of two diets, each based on AIN-93 formulation, providing a 2 (generation) X 2 (diet) X 3 (methyl mercury exposure) factorial experimental design. A "coconut oil" diet (1/3 of fats were provided by coconut oil) was marginally adequate in $n-3$ PUFAs and contained no DHA. A "fish oil" diet was rich in $n-3$ fatty acids, including DHA. The diets were approximately equal in $n-6$ fatty acids. Forelimb grip strength declined with age for all groups, but the decline was greatest for those exposed chronically to 400 μg/kg/day of methyl mercury. This high-dose group also displayed hind limb crossing, gait disorders, and diminished running wheel activity. Dietary $n-3$ fatty acids did not influence these effects. Chronic exposure to 400 μg/kg/day of methyl mercury resulted in blood and brain concentrations of about 70 and 10 ppm, respectively, approximately 50-fold higher than concentrations seen in rats exposed to 40 μg/kg/day. Rats that became ill and died before the experiment ended had higher concentrations of mercury than their cohorts who survived to the end. Organic mercury was highly correlated with total mercury in these rats but inorganic mercury remained approximately constant. Some deaths were due to urolithiasis (kidney or bladder stones) associated with a dietary contaminant and that was eventually fatal to 22% of the females in the colony. Neurobehavioral effects are reported on rats that did not become ill.

Keywords: Methyl mercury; Chronic exposure; Developmental exposure; Motor deficits; Docosahexaenoic acid; Polyunsaturated fatty acids; Brain mercury; Blood mercury; Aging

1. Introduction

Methyl mercury (MeHg) is a known environmental neurotoxicant that, when ingested, results in sensory and motor deficits. The pernicious effects of high level MeHg exposure are well documented by human epidemiological studies that followed poisonings in Minimata Bay (Japan) and Iraq. Signs and symptoms of chronic exposure include paresthesia, ataxia, constriction of the visual field, tremor, mental deterioration, and dysarthria [3,2,15]. Studies using laboratory animals have also reported ataxia, tremor, paralysis, hind limb spasticity, and decreased muscle tone after adult-onset exposures to MeHg [6,16,22]. One of the central characteristics of environmental MeHg toxicity is a latent period between ingestion and behavioral symptoms [58]. Following the aforementioned Japanese study, some participants displayed no impairments until well after the peak exposure period, when difficulties in fine motor control during daily activities (such as dressing and eating) appeared [17,51].

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The consequences of developmental methyl mercury exposure have generated widespread concerns. MeHg can cause severe developmental damage even in the absence of overt maternal symptoms. Fetal, infantile, and childhood intoxication could also occur at much lower exposure levels than seen in adults [15]. Such toxic effects may remain "silent" until unveiled by secondary stressors such as aging. In one investigation of delayed neurotoxicity of methyl mercury, developing Macaca monkeys received 50 μg/kg/day of MeHg until 6.5–7 years of age [42]. Observations at 13 years of age revealed clumsiness, slowness, and other fine motor deficits in exposed monkeys. Another study exposed pregnant rats to 0, 0.5, and 6.4 ppm of MeHg via drinking water, and later tested female offspring in a procedure that specifically targeted high-rate behavior. Lever-pressing was reinforced on a multiple DRH 9:4 extinction schedule and this behavior was followed through the aging process. Although no signs of neurotoxicity were present in developing and middle-aged rats, declines in reinforcer rates were noted in geriatric rats exposed prenatally to methyl mercury [35].

Human exposure to MeHg occurs primarily through the consumption of large predatory fish, leading to suggestions that people limit their intake of certain fish species known to have high concentrations of MeHg. Fish also have many nutritional benefits, however. Docosahexaenoic Acid (DHA) and other $n-3$ polyunsaturated fatty acids (PUFAs) found in fish are critical to neural development and function [8,32,53,54]. Interestingly, the functional domains impaired by methyl mercury generally overlap with those also impaired by $n-3$ PUFA deficiency. Thus, visual, motor, and reinforcement systems are all affected by methyl mercury exposure [34,45,43] and $n-3$ PUFA deficiency [10,41,53]. It is no surprise, therefore, that $n-3$ PUFAs in fish have been hypothesized to ameliorate MeHg's neurotoxic effects and decrease the risks associated with eating MeHg contaminated foods [25,31].

A previous investigation [11] studied a MeHg-exposed population in the Seychelles, in which fish was the major dietary component. Increased MeHg exposure was not associated with any deficits as measured by an extensive behavioral and cognitive test battery. Conversely, another report noted subtle cognitive deficits in children exposed to MeHg through ingestion of whale meat and fish in the Faeroe Islands [14,13]. This divergent evidence may reflect the presence of essential fatty acids in fish, or may result from additional toxicants (e.g., polychlorinated biphenyls) specific to the whale population in the Faeroe study [4,13].

Although epidemiological results are valuable, they generally lack the ability to report a lucid exposure history [15]. In animal studies, the diet and exposure history can be controlled with precision. The present experiment was designed to determine blood and brain concentrations associated with chronic exposure, identify motor deficits that develop following low-level methyl mercury exposure, and investigate the role that $n-3$ PUFAs (specifically DHA) might play in moderating these effects. Motor function of aging offspring exposed during gestation only was also evaluated for comparison.

Female rats received drinking water containing 0, 0.5, or 5 ppm of methyl mercury beginning as young adults. Rats also received one of two diets according to a 2 (generation) $\times$ 2 (diet) $\times$ 3 (methyl mercury) experimental design. One diet was rich in DHA and other $n-3$ polyunsaturated fatty acids due to the presence of fish oil. The other diet included coconut oil instead of fish oil. The coconut oil diet had no DHA and only marginal levels of its important precursor, α linoleic acid (ALA). Two noteworthy considerations entered the design of the nutritional component of this study. First, the concentration of $n-3$ fatty acids in the diet was neither extremely deficient nor was it excessive. Instead, the two diets are best described as marginal and sufficient [53,55,57]. Second, the diets contained approximately the same concentrations of $n-6$ PUFAs, so differing levels of these fatty acids were not a concern when interpreting these experiments. It might be noted that diets are a mixture and it is impossible to control for everything, so the $n-3$ deficient diet had more saturated fats than the $n-3$ sufficient diet, a difference that models human nutrition.

During a portion, perhaps up to three months, of this lifespan study, shipments of the AIN-93 diet to many labs in the U.S. and Canada contained racemic, rather than levo, bitartaric acid to stabilize choline [19]. This supposedly inert ingredient caused urolithiasis, kidney and bladder stones, that was fatal for 22% of the females in the present study over the course of the entire study [37]. The cause of the urolithiasis was not ascertained until almost 1 1/2 years into the study. Neurobehavioral evaluations are reported only on rats that did not show signs of illness at or within months of when the evaluations were conducted, but brain and blood mercury concentrations are reported separately on animals that became ill. Since urolithiasis is related to renal chemistry [26] and the neurobehavioral evaluations reflect neural function, these results are not believed to be biased by using surviving rats, but that possibility should be acknowledged.

The mercury concentrations used here produce approximately 0, 40, and 400 μg/kg/day consumption in rats [36], intakes that are higher than those used the primate studies referred to above. A higher intake is required to overcome the mercury binding capacity of rat blood, which has approximately ten times as much hemoglobin as mouse, monkey, or human blood [24] and which binds mercury readily. Neonatal brain mercury, a better biomarker of exposure for comparisons across species [7], observed after 40 and 400 μg/kg/day of maternal consumption is in the low to moderate range [36]. While the relevant biomarkers have been reported for developmental exposure to this regimen, neither the consequences of chronic, adult-onset exposure nor the effects of dietary PUFAs are known.

2. Methods

2.1. Subjects and breeding

Seventy-two female Long-Evans rats (designated the F0 generation) were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and arrived at 80 days of age. Mating occurred between 17 and 25 weeks of age, when females were paired with unexposed adult males overnight. From the initial colony, sixty-six dams were used for strength and motor testing after their offspring were weaned. Forty-nine offspring from the original round of breeding were used in the present study.

Due to initial difficulties in breeding with some mercury-exposed rats on the coconut oil diet, a second cohort of offspring was bred to generate adequate sample sizes in two experimental groups (400 μg/kg/day MeHg dose group and corresponding controls, described below). The result was the addition of nine offspring that were ~20 weeks younger than the previous cohort, but were otherwise identical. In total, fifty-eight female offspring (termed the F1 generation) underwent testing in this study. Every rat was implanted subcutaneously with an electronic chip (Bio Medic Data Systems Inc., Seaford, DE) containing its identification number.

2.2. Diet and exposure

2.2.1. Diet for both generations

Within a week after arriving at the colony, F0 rats were assigned to one of two isocaloric diet groups (termed coconut oil and fish oil) such that the mean body weight of each group was the same. Diets were based on the AIN-93 formulation for semipurified diets but they differed in fat mixtures. The semipurified diet was used because it offers more precise control of mineral and fatty acid concentrations than chow diets [39,40]. The fat mixture of both diets consisted of 42.8% palm oil, 9.2% safflower oil, and 15.0% soybean oil. The coconut oil diet also contained 33% coconut oil, whereas the fish oil diet contained 33% blend of fish, especially menhaden, oils. The fish oil used was the EPAX mixture (Pronova Biocare, Lysaker, Norway). For the fish oil diet, the $n-3$ fatty acids α linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid comprised 1.6, 5.4, and 11.2% of total fatty acids, respectively. For the coconut oil diet, these comprised 1.0–1.6, 0, and 0% of total fatty acids. The fish oil and coconut oil diets contained 23.2 and 18.1% of total fatty acids as $n-6$ PUFAs. The ratios of $n-6$ to $n-3$ fatty acids in the fish oil and coconut oil diets were 2.1 and 16.5, respectively. The diets were purchased from either Dyets, Inc. (Bethlehem, PA) or Research Diets, Inc. (New Brunswick, NJ). The breeder (F0 generation) rats began this diet shortly after arriving at the colony and remained on it for life. The F1 generation began this diet at weaning and remained on it for life.

Urolithiasis resulted in the death of 22% of the female breeders and offspring from the first cohort of rats [37]. This problem was eventually traced, with great difficulty, to the inadvertent presence of racemic, rather than levo tartaric acid used to stabilize choline; choline chloride replaced choline bitartaric acid once this problem was discovered [19]. The second cohort was less affected because it was exposed to racemic bitartaric acid for less time. The diet was used across all three mercury groups and both diet groups. There was an elevated prevalence of urolithiasis in the F0 rats (breeders) exposed chronically to 5 ppm of mercury in their drinking water as compared with all other groups [37]. Only animals with no clinical signs of urolithiasis were used for the behavioral or neurological evaluations reported here. Blood and brain concentrations of mercury were also evaluated in animals that were euthanized due to illness.

2.2.2. Chronic MeHg exposure in the F0 generation

Table 1 contains the sample sizes for each experimental group across age categories (for both F0 and F1 generations). Fig. 1 illustrates the time course of methyl mercury exposure for both generations.

After three weeks of ad libitum diet consumption, F0 rats were weighed and pseudo-randomly assigned to one of three methyl mercury exposure groups such that mean body weights were approximately equal in all groups. Mercury was delivered as methyl mercuric chloride (Alfa Aesar, Ward Hill, MA) via the sole source of drinking water in one of three concentrations: 0, 0.5, and 5 ppm. It is estimated that this produces methyl mercury intakes of about 0 μg/kg/day (control), 40 μg/kg/day, and 400 μg/kg/day [36]. Fluid consumption was checked periodically to ensure that it approximated values reported in the earlier study.

After their offspring were weaned, F0 generation animals were weighed at least once per week and maintained at a bodyweight of about 270 g. Maternal exposure was temporarily terminated for five days prior to weaning (PN days 16–21) to prevent pups from obtaining methyl mercury drinking water.

2.2.3. Developmental MeHg exposure in F1 generation

F1 rats experienced developmental methyl mercury exposure exclusively via maternal contact. As previously reported for rats [36] and confirmed by others using mice [48], this regimen produces gestational exposure only, as exposure during lactation is negligible. Offspring were weaned at PN day 21, when they were moved to separate cages and began the semipurified diet. Body weights were recorded weekly when rats were idle, and daily during participation in other behavioral experiments. Offspring were fed ad libitum until they reached 240 g, when feed was restricted.

2.3. Housing

During breeding, F0 rats were singly housed in $22 \times 20 \times 45$ cm shoebox cages with wire tops. At all other

Table 1
Motor, strength, and observational testing across aging

MeHg dose (μg/kg/day)	F0 Generation (breeders)							F1 Generation (offspring)				
	Age in months	Forelimb grip strength		Running wheel performance		Hind limb crossing		Age in months	Forelimb grip strength		Running wheel performance	
		N	Mean±SEM	*N*	Mean±SEM	*N*	*p*		*N*	Mean±SEM	*N*	Mean±SEM
0	7	22	494±39.3	–	–	–	–	8±2	–	–	16	136±13.9
	11	21	310±24.3	15	95.6±9.34	21	0	9±1	18	36±26.0	–	–
	14	21	223±17.8	–	–	21	0	14±1	–	–	14	144±14.9
	18	13	219±27.1	–	–	13	0	20±1	–	–	13	124±15.4
	30	–	–	8	52.6±6.19	9	0	27±1	13	240±30.4	–	–
40	7	22	415±33.0	–	–	–	–	8±2	–	–	16	142±14.4
	11	21	325±22.7	14	91.8±18.1	21	0	9±1	17	345±19.8	–	–
	14	18	247±15.1	–	–	18	0	14±1	–	–	15	134±10.6
	18	13	248±25.2	–	–	13	0	20±1	–	–	13	147±25.0
	30	–	–	8	54.5±11.6	9	0	27±1	12	247±24.9	–	–
400	7	22	452±31.6	–	–	–	–	8±2	–	–	16	126±12.8
	11	21	222±24.0	18	36.6±6.35	21	0.38	9±1	14	369±25.6	–	–
	14	19	164±18.9	–	–	20	0.65	14±1	–	–	15	117±9.24
	18	13	85.7±9.44	–	–	14	0.64	20±1	–	–	12	112±12.1
	30	–	–	3	6.78±1.83	3	1.00	27±1	9	263±23.1	–	–

times, rats (both F0 and F1) shared a cage with a same-sex member of the matching experimental group. A clear Plexiglas divider placed diagonally in the cage separated the rats and allowed for individual control of food and fluid intake. The sole source of water intake was a plastic bottle capped with a rubber stopper. Water bottles and stoppers were assigned to exposure groups in order to prevent cross contamination. Aspen chip bedding was provided and changed weekly. All animals were housed in rooms with a 12-h light–dark cycle, with lights on at 0600 hours. Temperature was maintained at 24 °C. The Laboratory Animal Health facilities are AAALAC accredited. All experiments were approved by the Auburn University Animal Care and Use Committee.

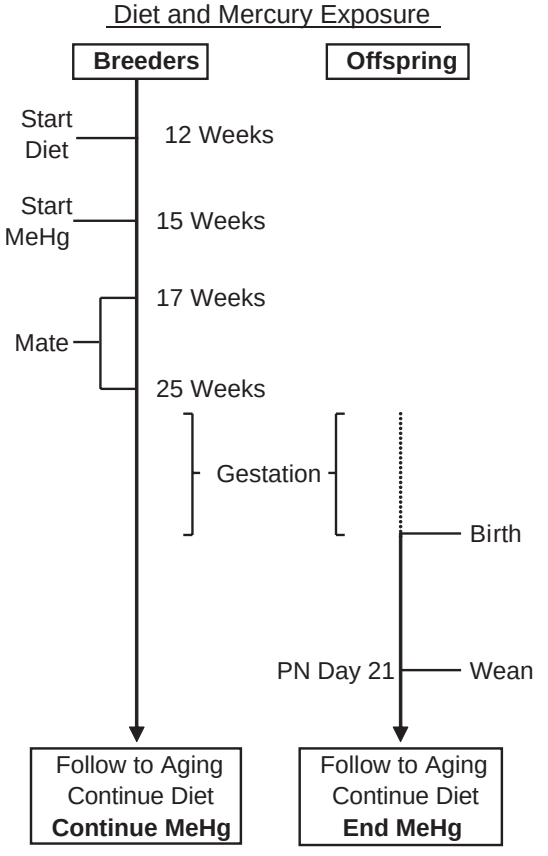

Fig. 1. Time course of diet and MeHg exposure for Breeder (F0) and Offspring (F1) generations.

2.4. Apparatus

Forelimb grip strength was determined using an Imada DPS digital force gauge (Imada, Inc., Northbrook, IL). A local machine shop fabricated an 18×8 cm grid out of 2 mm diameter copper wire. Eighteen 2×2.5 cm rectangle openings provided a reliable surface for forelimb grip. A threaded tip on the grid connected it to the input of the force gauge. A vise secured the gauge to a table when in use.

Running wheel tests employed seven Lafayette galvanized steel activity wheels (Lafayette Instrument Co., Lafayette, IN). Prior to conducting this experiment each unit was cleaned, primed, and painted with a black enamel finish. About 40–60 grams of mass was required to initiate movement of the wheel. Each wheel was 36 cm in diameter and 14 cm in width. An $11 \times 11 \times 22$ cm stationary chamber was accessible from the wheel during the experiment, and included a small ceramic bowl containing fresh tap water. A serial counter attached to the wheel recorded total revolutions.

2.5. Procedure

2.5.1. F0 generation

Functional testing of F0 rats included forelimb grip strength, running wheel activity, and gait and hind limb cross observations. Forelimb grip strength assessments in F0

animals occurred at 7, 11, 14, and 18 months of age. Each animal was weighed prior to testing and had received no food for ~20 h. The experimenter held the rat by the torso and tail and placed its forelimbs on the mesh grid attached to the force gauge. Then, in a quick smooth, rapid movement, the rat was pulled away from the grid by the base of the tail. Movement was parallel to the floor. The peak force (in grams) was recorded. On each testing date, rats underwent three trials that were separated by about 5 s. The mean and maximum scores from the three trials were computed for statistical analysis. Only means are reported here. The same person conducted all grip strength tests.

At 11–12 and 30 months of age, free access to a running wheel was provided for 12 h overnight, beginning at ~2100 hours. Total revolutions were calculated following the 12 h period. Seven rats ran concurrently at each testing date. Wheel position and chronological order were counterbalanced between experimental groups. A one-way ANOVA using post hoc analysis revealed no effect of running wheel on performance, indicating that the wheels performed similarly. All rats were weighed prior to testing and had received no food for about 7 h.

Hind limb cross in F0 rats was evaluated at 11, 14, 18, and 30 months of age. An experimenter held a rat by the base of the tail for 2–5 s (perpendicular to the floor) and recorded the position of the hind limbs [18]. Gait in an open arena was also observed at 14, 18, and 30 months of age.

2.5.2. F1 generation

Offspring underwent both running wheel and forelimb grip strength testing. These rats were not tested for hind limb cross and gait at specifically defined ages as were the F0 rats. They were, however, handled daily during other behavioral tests and gait and the position of the hind limbs were observed routinely. F1 rats were grouped into age categories for activity wheel testing to increase group population sizes.

The combination of testing dates from the first and second cohorts (described above) resulted in testing age categories of 8 (±2) months, 14 (±1) months, and 20 (±1) months. At these dates, rats were provided free access to a running wheel for 16.5 h beginning at ~1600 h. Again, bodyweights were recorded before testing. Rats had received no food for ~2 h. All offspring were tested twice during the eight and fourteen month categories, and the average of these two scores was recorded for analysis. Grip strength testing of offspring rats (all from cohort 1) occurred at 9 (±1) and 27 (±1) months of age, and used methods identical to those described above.

2.6. Brain tissue and blood methyl mercury levels following chronic exposure

For blood and tissue collection, animals were anesthetized with pentobarbital and euthanized with either cardiac puncture or decapitation. Blood was collected and stored in EDTA-containing tubes. The brain was removed, cut in half longitudinally, and frozen. Total and inorganic mercury in the whole blood and brain were analyzed by the Institute for Toxicology/INSPQ (Sainte-Foy, QC, Canada) using cold vapor atomic absorption spectrophotometry. Organic mercury was determined by subtracting inorganic mercury from total mercury. Most animals were euthanized after 85 weeks of exposure (over 125 weeks of age). Some animals were euthanized at a younger age because they became moribund before the experiment ended. Tissue analyses were conducted separately for these cases.

2.7. Data analysis

Analyses of forelimb grip strength were based on mean resistance in grams. Running wheel activity was measured in total revolutions and revolutions per hour; using a rate measure minimizes any potential influence of the different session lengths experienced by the F0 and F1 rats. A three-way (MeHg × Diet × Age) repeated measures ANOVA (multivariate design) was used for grip strength data from F0 and F1 rats, and with running wheel data from F1 rats. This required that a specific case have a data entry under each test date, otherwise it was excluded from analysis. Separate two-way ANOVAs (MeHg × Diet) were used to analyze grip strength and running wheel data at each testing age. Statistical significance was assessed using an alpha level of 0.05. The litter served as the experimental unit for all statistical analyses of F1 rats. SYSTAT and Sigma Plot software (SYSTAT Software, Inc., Richmond, CA) were used for all statistical and graphical analyses.

Results from the hind limb cross and gait tests were coded with a "0" (normal) or a "1" (impaired). A log-linear model was then employed to analyze the presence of a hind limb cross and gait disorders in F0 animals. The likelihood-ratio (LR) Chi-square value derived from this model was used to evaluate the impact of methyl mercury and PUFA exposure on the observed frequency of hind limb crossing.

3. Results

3.1. Forelimb grip strength

3.1.1. F0 generation (Chronic exposure)

Box plots in Fig. 2 display the median, 10th, 25th, 75th, and 90th percentile mean grip strength for each F0 group at the first four testing ages. Points lying outside the 10th and 90th percentiles are plotted separately. The bar graph shows the mean and standard error of the mean as a function of age and methyl mercury group. A main effect of methyl mercury exposure was found at 11 months [$F(2,57)=6.14$, $p=.004$], 14 months [$F(2, 52)=6.29$, $p=.004$] and 18 months [$F(2, 33)=16.28$, $p<.001$] of age. No effects of MeHg were detected at 7 months [$F(2, 60)=1.34$, $p=.27$]. Post hoc tests confirmed what is evident in the figure; only the highest exposure level produced a significant reduction in forelimb grip strength. There was no effect of diet and no interaction

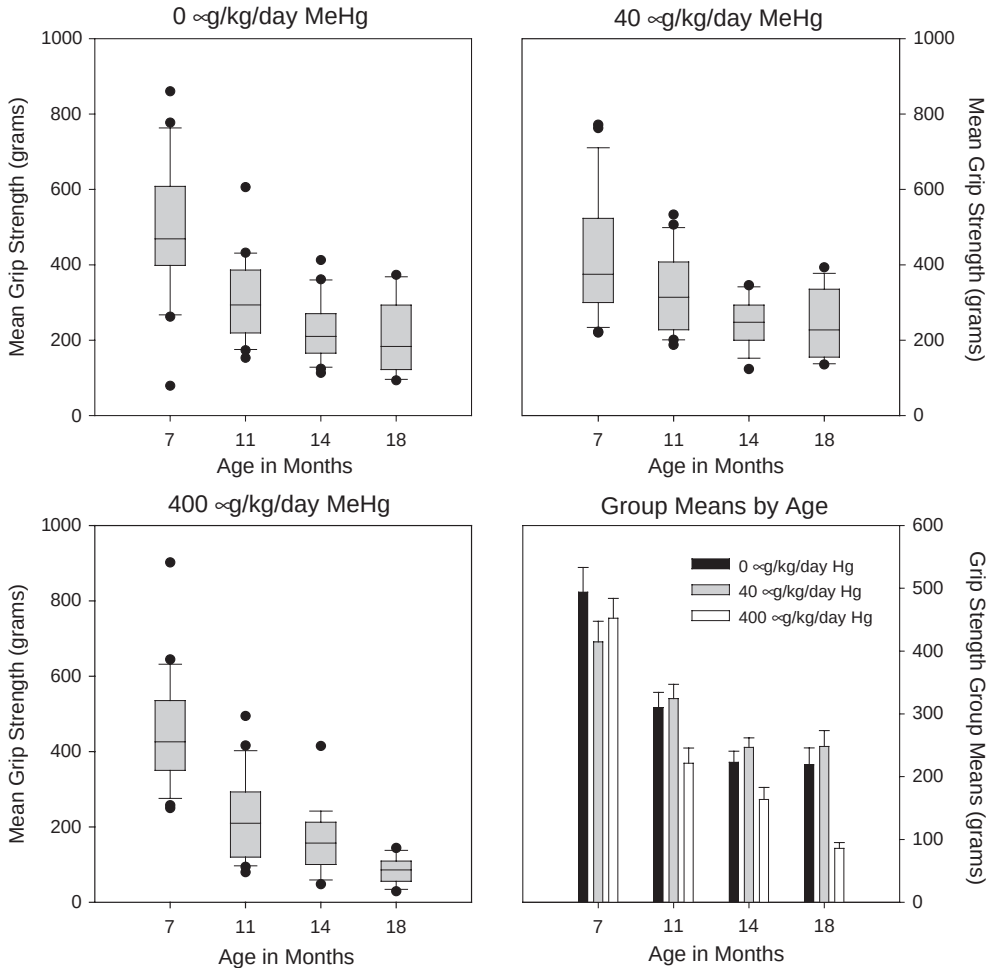

Fig. 2. Box plots marking the 10th, 25th, median, 75th, and 90th percentile mean grip strength scores for each MeHg dose. Testing age is represented on the *x*-axis and mean grip strength on the *y*-axis. Outliers are plotted separately. The bottom right figure displays the mean grip strength for each MeHg group across aging. This analysis revealed a primary effect of MeHg dose, which increased with age and continued exposure. Diet had no significant effect; both groups are combined.

between diet and methyl mercury (all p's >0.1), so the two diet groups are combined for graphical presentation.

Interactions with age were analyzed using a RMANOVA on rats surviving to 18 months. Age was treated as a repeated measure and methyl mercury dose and diet as between-group measures. This analysis revealed a main effect of age [$F(3, 99)=59.04$, $p<.001$] as well as an interaction between age and methyl mercury dose [$F(6, 99)=3.18$, $p=0.007$]. An interaction between age and diet was also identified [$F(3, 99)=4.72$, $p=.004$]. At 7 months of age, the fish oil group recorded slightly higher grip strength scores, yet at 11 months the scores were slightly lower. There were no effects of diet at 14 and 18 months. Because this effect was not systematic and appeared only on this variable, it is not displayed graphically. All group scores are presented in Table 1.

3.1.2. F1 generation (Gestational exposure)

Forelimb grip strength was unaffected by prenatal exposure to methyl mercury or chronic diet maintenance (all p's >0.35). Box plots in Fig. 3 display the 10th, 25th, 75th, and 90th percentiles as well as the median for mean grip strength scores. A RMANOVA (MeHg × Diet × Age) using the litter as the statistical unit and age in months as the repeated measure revealed a main effect of age [$F(1, 26)=14.83$, $p=.001$], but no other significant effects or interactions. Thus, apparent declines in grip strength across aging seemed to be unrelated to experimental factors.

3.2. Running wheel performance

3.2.1. F0 generation (Chronic exposure)

Fig. 4 shows the median, 10th, 25th, 75th, and 90th percentile running rates (revolutions per hour) for the F0 generation at the two testing periods. Outliers are plotted separately. Testing at 30 months of age suffered from low sample sizes (reported in Table 1), thus the 10th and 90th percentile values were not calculated. A main effect of methyl mercury was noticed at both 11–12 [$F(2, 41)=8.32$, $p<.001$] and 30 months [$F(2, 13)=5.75$, $p=.016$]. No effect of diet and no interaction between methyl mercury and diet were detected.

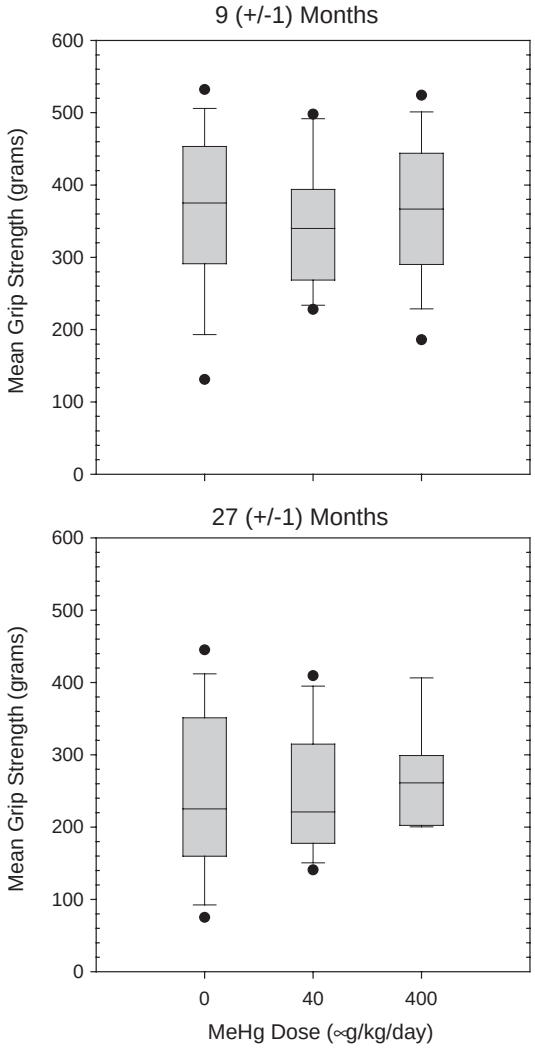

Fig. 3. Mean forelimb grip strength as a function of gestational MeHg exposure, presented as the 10th, 25th, median, 75th, and 90th percentile scores. Although performance decreased with age, the decrease was unrelated to MeHg or PUFA exposure. Diet groups are combined.

3.2.2. F1 generation (Gestational exposure)

Running wheel activity in F1 animals was not significantly affected by methyl mercury or $n-3$ PUFA exposure at any age (all p's >0.4). A three-way (MeHg × Diet × Age) RMANOVA was conducted using wheel revolutions per hour as the dependent measure. Performance was not affected by age [$F(2, 64)=0.24$, $p=.786$], and there was no interaction between age and methyl mercury dose [$F(4, 64)=0.39$, $p=.819$] or age and diet [$F(2, 64)=1.88$, $p=.161$].

3.3. Hind limb cross and gait: F0 generation

Only animals exposed to a chronic, high dose of methyl mercury displayed crossed hind limbs (Table 1). Significant effects of methyl mercury on the presence of a hind limb cross were found at 11 months ($X^2=59.71$, $p<.001$), 14 months ($X^2=56.41$, $p<.001$), and 18 months ($X^2=37.44$, $p<.001$) of age. Graphical and post hoc statistical comparisons revealed that the effect was due to the high-dose rats. Fig. 5 shows the proportion of hind limb crossing as a function of experimental group and age. As exposure duration increased, high-dose animals became more likely to exhibit a hind limb cross or gait deficits. Diet did not affect the prevalence of hind limb crossing. Gait impairments were largely correlated with the presence of a hind limb cross (simple matching dichotomy coefficient $>.9$ at all ages), and included ataxia, impaired coordination, excessively splayed or adducted hind limbs, and dragging of hind limbs.

3.4. Brain and blood levels resulting from chronic exposure

Fig. 6 shows blood and brain mercury concentration after more than 96 weeks (672 days) of exposure. There was a

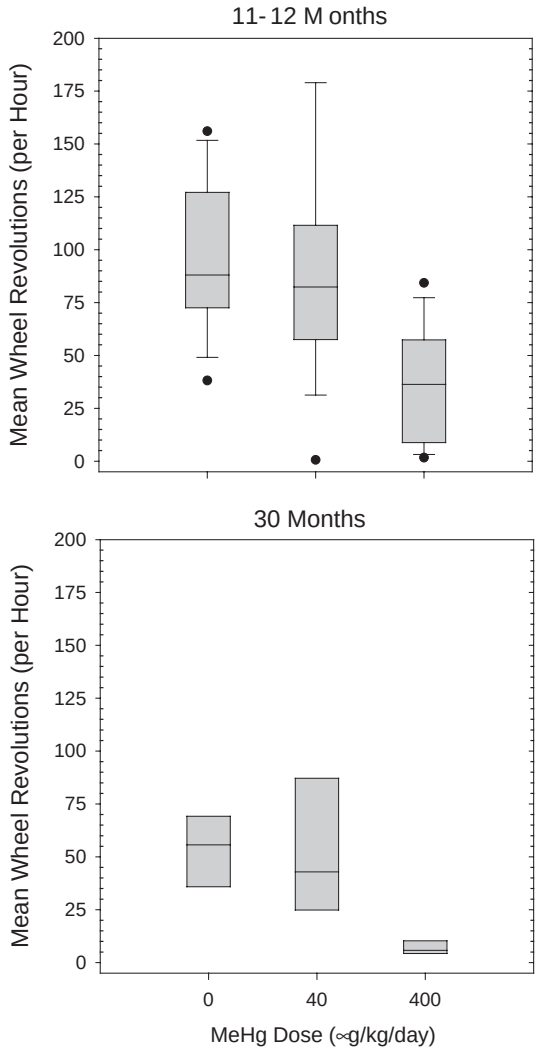

Fig. 4. Running wheel revolutions per hour at 11–12 months and 30 months, represented as the 10th, 25th, median, 75th, and 90th percentile scores. Points lying outside these percentiles are plotted separately. Running wheel performance was severely impaired by a 400 μg/kg/day MeHg dose at both ages. Again, diet groups are combined. Rats were exposed chronically to methylmercury.

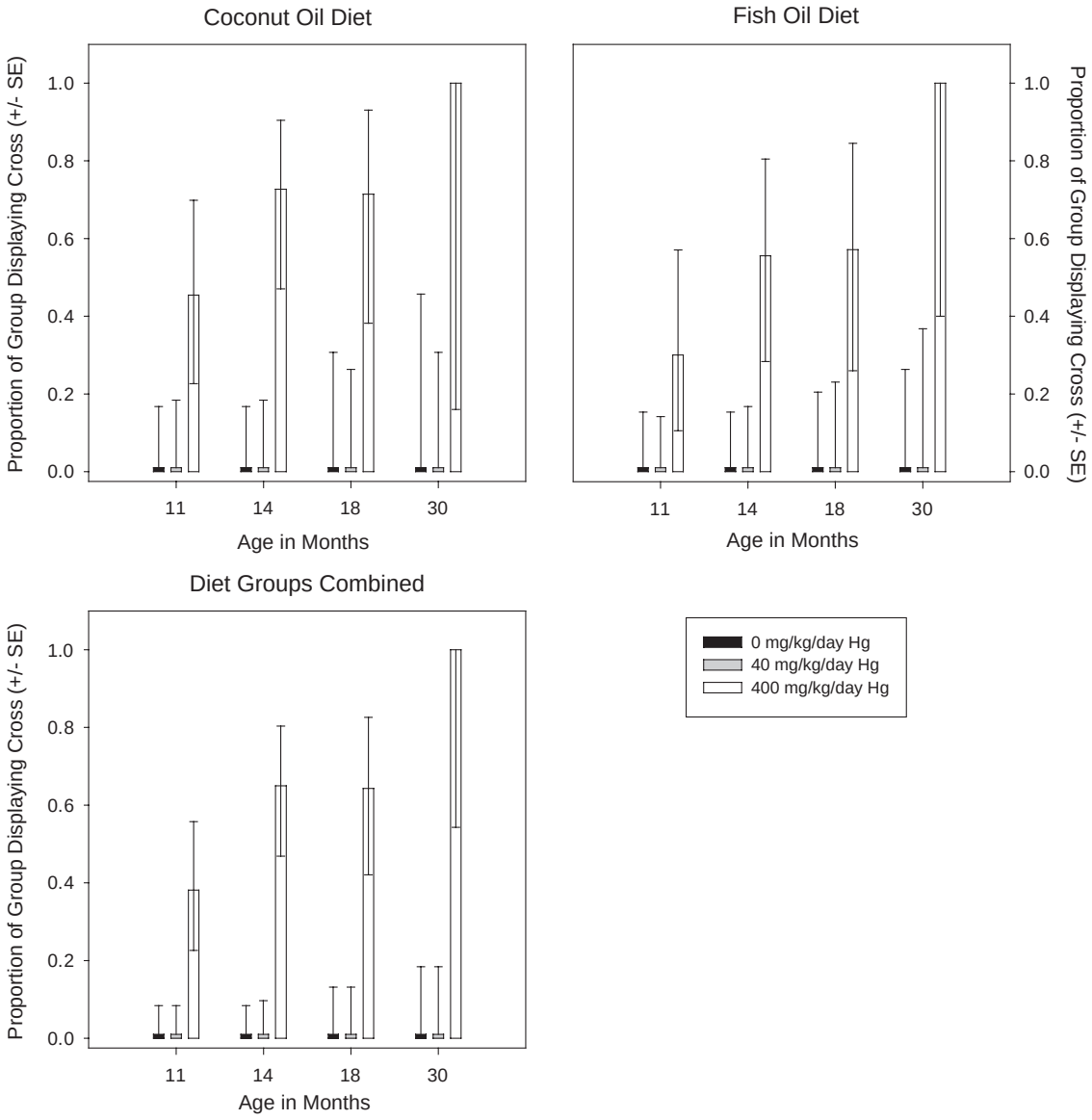

Fig. 5. Proportion of rats displaying a hind limb cross at sequential testing ages stratified by MeHg dose and separated by diet (top panels) and for both diet groups combined (bottom left). The standard error of the proportion was determined using the binomial distribution. Rats were held by the base of the tail for 2–5 seconds and the position of the hind limbs was recorded. The high-dose animals tended to display crossed hind limbs and flexion, whereas the control animals exhibited exemplary postural command.

main effect of mercury exposure on brain mercury concentration [$F(1, 19)=221$, $p<.000$], but no effect of diet and no interaction with diet (p's>0.1). Note that the 10-fold increase in mercury concentration in drinking water resulted in a greater than 50-fold increase in brain concentration.

Blood concentration also increased with mercury dose but, as with brain concentration, there was no effect of diet and no interaction between diet and drinking water concentration. Rats consuming tap water without mercury had blood mercury concentrations of about 0.0025 ppm (not shown in the figure). A brain:blood ratio of about 0.125 appeared regardless of mercury or dietary exposures. This is evident in the parallelism of the lines in Fig. 6.

Brain and blood concentrations of mercury were also determined in rats that were euthanized during the course of the study. Most of these deaths were due to urolithiasis caused by racemic bitartaric acid improperly included in the AIN-93 diets [37]. Total mercury in the brains of rats exposed chronically to 5 ppm of MeHg was higher in rats that died at a younger age (Fig. 7, top panel). The regression line describing this relationship has a slope of -0.032 ppm/day ($p<0.01$) or -0.97 ppm/month. There were too few fatalities among the rats exposed to 0.5 ppm to support this analysis in that group. The brain:blood ratio of mercury did not change but stayed at a value of 0.17. The cause of death had no discernable influence on these measures.

The proportion of brain mercury that was in an inorganic form increased about three-fold over the course of about 500 days of exposure (Fig. 7, middle panel). The slope

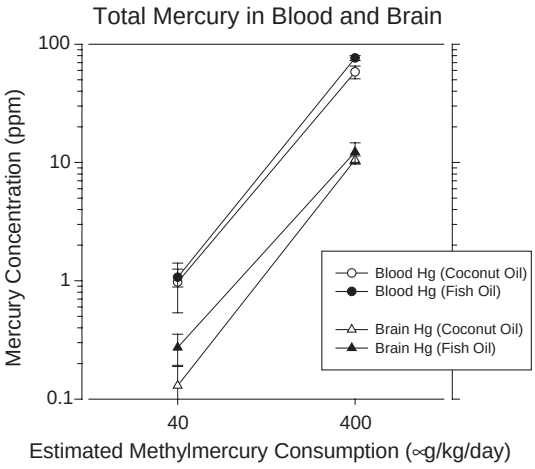

Fig. 6. Brain and blood concentrations for rats with 0.5 or 5 ppm of mercury in their drinking water, which led to consumption levels of approximately 40 and 400 μg/kg/day. Data from rats on Coconut and fish oil diets are shown separately.

describing this increase was 0.00019/day or about 0.0057/month (different from zero, $p<0.01$). Inspection of this middle panel reveals a cluster of rats in which a proportion of about 0.05 appears regardless of age of death. These cases were reviewed in an effort to identify some unifying characteristic that might explain why inorganic mercury did not increase with age. The rats forming this cluster could not be distinguished from other rats on any variable examined, including cause of death, blood mercury concentration, blood:brain mercury ratio, or brain mercury concentration.

Although the proportion of mercury that was inorganic increased, the absolute value remained at a constant concentration regardless of the duration of exposure. A regression analysis failed to reveal a statistically significant relationship between the concentration of inorganic mercury in the brain and the duration of exposure. To examine how the percent of mercury that was inorganic could increase even as total inorganic mercury remained constant, the relationship between organic and inorganic mercury was regressed against total mercury in the brain. The results are presented in the bottom panel of Fig. 7. Initially, the regression was conducted after forcing an intercept of zero, on the assumption that the absence of brain mercury implies the absence of both organic and inorganic mercury. Organic mercury increased with total mercury (slope = 0.94, different from zero with $p<0.000001$). Inorganic mercury in the brain increased slightly with total mercury, a shallow slope of 0.06 describes this relationship, (different from 0 with $p<0.00001$) but there was a non-random pattern to the residuals, indicative of a poor fit. Specifically, the regression line underestimated inorganic mercury at low concentrations of total mercury and overestimated it at higher concentrations. Including the intercept as a free parameter had little effect on the regression of organic mercury against total mercury, but it did make the slope describing inorganic

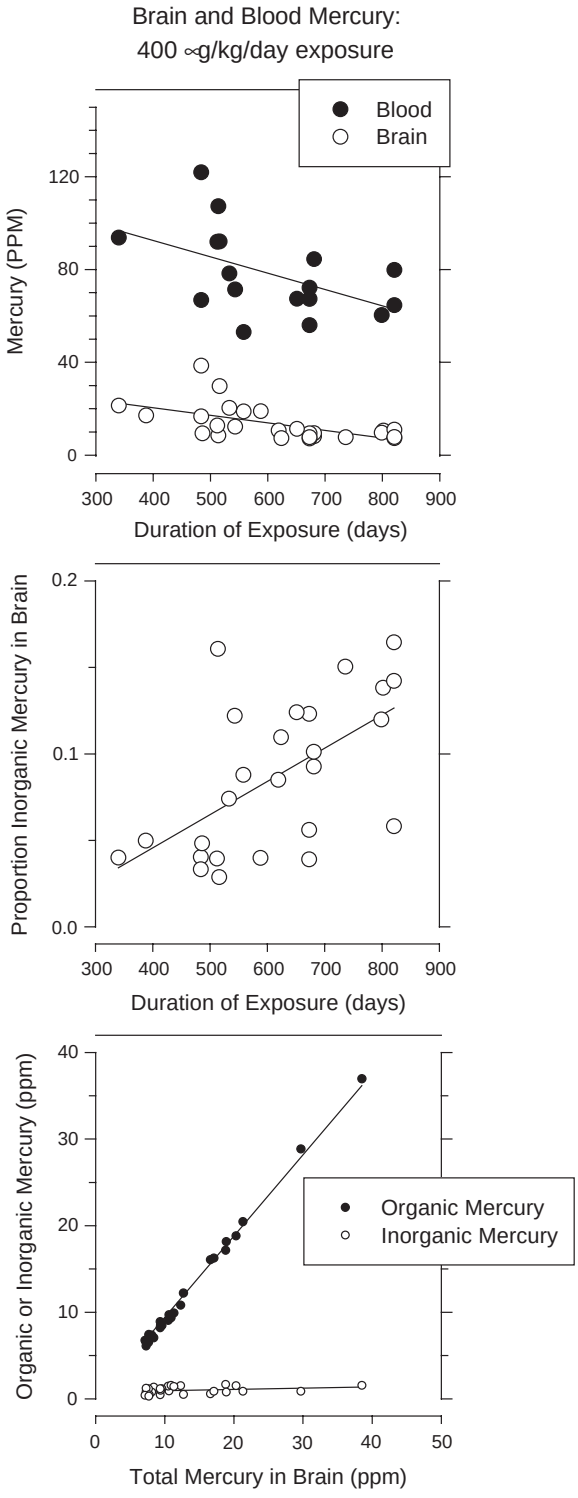

Fig. 7. Top panel: Concentration of mercury in the brain and blood of rats that survived to the end of the experiment. The middle panel shows proportion of brain mercury that was inorganic mercury for all animals consuming about 400 μg/kg/day of mercury chronically, including those that became ill and died. The bottom panel relates organic and inorganic mercury to total mercury for rats consuming 400 μg/kg/day. See text for details.

mercury indistinguishable from zero, consistent with a constant, unchanging value over the course of the assessment period. Relaxing the requirement that the line passes through the origin improved the residuals and resulted in a slope that was indistinguishable from zero and an intercept of 0.8±0.16 ppm ($p<0.01$).

4. Discussion

4.1. Neuromotor effects

Daily exposure to an estimated 400 μg/kg of methyl mercury resulted in neurological disorders including hind limb crossing, gait disturbances, and decreased forelimb grip strength. Daily exposure to a dose that was 10-fold lower had no such effects, despite the fact that cumulative mercury exposure in this group became quite high. The presence or absence of DHA in the diet had no influence on the development of neurological signs.

The first indications of motor debilitation occurred after approximately 7 1/2 months of chronic MeHg exposure and at eleven months of age, regardless of measure. Forelimb grip strength and running wheel activity were significantly impaired by the higher MeHg dose at each subsequent age category. An age-related decline in grip strength was also observed in all groups, yet high-dose rats were impaired at an earlier age and to a greater degree. Thus, methyl mercury exposure may have amplified weakness associated with aging. Alternatively, aging may have compounded the toxic motor consequences of MeHg exposure. The present experiment directly confounds exposure duration with age so the effect of either cannot be examined separately. However, the trend observed here is consistent with previous reports of delayed or latent neurotoxic injury attributable to prior or chronic MeHg exposure. Taken together, these and past findings signify that compensatory mechanisms are compromised when dually burdened by methylmercury and aging processes.

The prevalence of hind limb crossing and gait disorders also increased with continued exposure and age. These deficits were never observed in controls, in animals exposed to 40 μg/kg/day, or in animals exposed developmentally. Therefore, the neurological signs reported here are uniquely associated with adult-onset methyl mercury exposure. In a direct comparison of developmental and adult-onset exposures, the administration of 10 mg/kg/day (10,000 μg/kg/day) of methyl mercury beginning at different stages of development produced hind limb crossing and other motor deficits only when exposure began in adulthood [56].

Hind limb crossing and other debilitating motor effects have also been reported with adult-onset MeHg exposures produced by the addition of 5 ppm of mercury to the diet. That regimen resulted in intakes of approximately 230 μg/kg/day for adult rats, assuming 16 gm/day of food and 350 gm rats, as reported [46]. Oral intubation of 10 mg/kg/day (10,000 μg/kg/day) for several weeks also resulted in similar signs [49,56]. In combination, the data from these studies and the present one suggest that overt neurological signs can be associated with exposure regimens that result in intakes of at least 230 μg/kg/day but apparently not intakes of 40 μg/kg/day. The latency to the appearance of signs is directly related to the daily dose, with higher exposure rates producing them more quickly.

4.2. Blood and brain mercury

A ten-fold increase in daily MeHg exposure resulted in greater than a 50-fold increase in brain concentration of methyl mercury. Brain mercury also increases more rapidly than exposure levels in mice [48] and neonatal rats [36]. The proportion of total mercury that was inorganic mercury increased with exposure duration, as also reported with nonhuman primates [52]. In the present experiment, however, for which inorganic mercury could be determined for the high-exposure group only, changes in this proportion were due to changes in organic mercury. Regression analyses indicated that inorganic mercury levels remained constant at a value of about 0.8 ppm throughout the experiment, even in rats that died before the study ended and even as organic mercury varied over an eight-fold range. Apparently this concentration was established before the first rat died. In nonhuman primates the concentration of inorganic mercury in the brain did not appear to increase with exposure duration over 12 months but did show some increase after 18 months of exposure [52]. Inorganic mercury is uncorrelated with total mercury in humans [23].

This constant value of inorganic mercury cannot represent a general background level since inorganic mercury was undetectable, and therefore below this value, in the animals exposed to lower daily intakes and in controls. Instead, this value seems to represent an upper limit on the concentration of inorganic mercury in whole brain. Therefore, the increase in the proportion of mercury that is inorganic indicated in the middle panel of Fig. 7 is not due to increased inorganic mercury after prolonged exposure but rather to high organic mercury concentrations in the animals that died early. This is so even with the protracted exposure duration in the present study. The exposure rates of 40 and 400 μg/kg/day used in the present study appear to span a threshold above which the toxicity of methyl mercury differs qualitatively from that seen at lower daily intakes.

Mercury concentrations were lower in both the brains and blood of rats that survived until the end than in those that died during the course of the present study. In healthy rats, brain mercury stabilizes and blood mercury rises with continued exposure [60]. The actual time course with which stabilization occurs in the brain is not known at present, but it appears to be within about six months to a year at estimated daily intakes of about 250 μg/kg/day in rats [60].

Many early deaths in the present experiment were due to urolithiasis and rats chronically exposed to 400 μg/kg/day

were more vulnerable than others [37]. The cause for the correlation between early death and elevated mercury levels cannot be determined at present. Individual differences in tissue concentrations of mercury can be accounted for only partly by differences in the uptake and elimination of mercury or by differences in fluid consumption [36]. It is possible that the disease process that lead to death significantly impaired the ability to eliminate mercury. Alternatively, rats that retained more mercury became more vulnerable to disease. In considering the deaths associated with urolithiasis, the first possibility seems more likely. Urolithiasis was linked to severe kidney disease, and the kidney is involved in mercury excretion. The elevated mercury could be secondary to urinary tract disease in those cases, a suggestion that is consistent with observations that brain mercury stabilizes within about six months in healthy rats [60]. Some rats died of other causes, so the reason for their elevated mercury must also remain unknown at present. Whatever the specific relationship between early fatality and mercury concentration, it appears to be related to organic mercury (at least in blood and brain) since inorganic mercury did not change. It should be noted that these very high concentrations are unrelated to performance on the neurobehavioral tests, since the illness that lead to the rats' death removed them from testing.

4.3. Potential mechanisms

Forelimb grip strength, running wheel activity, hind limb crossing, and gait analysis are sensitive to the motor and sensory effects of many neurotoxicants [28,30,61]. Deficits in forelimb grip strength have been observed following damage to spinal motor neurons, and decreased running wheel revolutions appear when sensorimotor (particularly cerebellar) pathways have been affected [38,61]. Hind limb crossing and gait abnormalities may indicate several modes of motor insult [33,49]. In conjunction with other neurobehavioral methods, these tests can reliably identify certain motor deficits.

Although the present investigation was not designed to identify the neural mechanisms of methyl mercury toxicity, our behavioral results are consistent with cellular and neural outcomes observed in other studies. Other investigators have reported severe mercury-induced damage to granule cells in the cerebellum [1,9,20,21]. Given that the cerebellum is instrumental in motor execution, feedback, and modulation, damage to this area could produce the gait impairments or inferior motor performance detected by the running wheel task. However, striatal and motor cortical injuries have also been linked to both chronic and acute methyl mercury exposure [12,27,50], and their potential contribution to the present results should not be dismissed.

Flexion and hind limb crossing are separable signs of methyl mercury-exposure and the appearance of flexion may emerge earlier [18]. Flaccidity and impaired coordination appeared in the hind limbs of rats delivered 10,000 μg/kg/day MeHg for 10 consecutive days [49]. Later pathological analyses revealed cytoplasmic vacuolation selectively in large motor neurons of the spinal anterior horn. Such lesions result in excessive activity of flexor muscles and consequent flexion, especially if they involve the descending motor pathways. In the present experiments, flexion was roughly coincident with hind limb crossing, but was noted only after the systemic observations had begun, so a relative time course for this sign cannot be ascertained here.

Dorsal root ganglion lesions have been linked to hind limb crossing associated with adult-onset methyl mercury exposure [56]. Interestingly, hind limb crossing occurred even before cerebellar damage was detected in that report, suggestive of a spinal mechanism for this sign. Su and colleagues [49] did not report on the dorsal root ganglion, also a target of methyl mercury exposure, so it is not possible to rule out peripheral sensory function as a contributor to hind limb crossing.

In vivo, in vitro, and pathological investigations of MeHg toxicity have implicated altered cerebellar, brain stem, spinal, and striatal functioning in the etiology of adult MeHg neurotoxicity [21,29], but there may be qualitative differences between low and high concentrations of methyl mercury. For example, cerebellar granule cell cultures are profoundly affected by both lower (1 μM) and higher (5–10 μM) acute doses of MeHg [9]. However, a 1 μM (0.2 ppm) concentration resulted in delayed cerebellar granule cell apoptosis, while the 5 and 10 μM (1 and 2 ppm) concentrations led to cellular necrosis. Dose-dependent effects on apoptosis in dorsal root ganglia have also been described [59], and abnormal cytoskeletal development and morphology [18,20] appear in the 0.3–0.1 μM range. By comparison, whole-brain mercury concentrations produced in the present study averaged about 0.2 ppm in the 40 μg/kg/day group and about 10 ppm (range about 8 to 40 ppm) in the 400 μg/kg/day exposure group. The high tissue levels associated with overt neurological signs in the present study are consistent with the high micromolar concentrations that produce cell death in vitro. The more subtle effects associated with lower concentrations studied in vitro appear not to produce overt signs visible with the sorts of observations reported here, but these lower concentrations are consistent with effects reported with operant behavior [34,35,44].

4.4. Dietary interactions

Fish are the most common source of methyl mercury and are also a rich source of $n-3$ fatty acids, especially docosahexaenoic acid (DHA). The present study was designed to examine whether the presence or absence of $n-3$ polyunsaturated fatty acids in fish influences blood or brain concentrations of mercury; $n-6$ polyunsaturated fatty acids and total fat content were held approximately constant. No influence of $n-3$ fatty acids was seen on any measure of blood or brain mercury levels, regardless of the health of

the animals in question. Total mercury, inorganic mercury, blood:brain ratios, and inorganic:total ratios were not affected by diet and the presence of $n-3$ fatty acids in the diet did not influence the appearance of neurological signs.

There was an unanticipated dietary interaction, urolithiasis, that has been discussed elsewhere. It is unlikely that urolithiasis affected the other conclusions reached in the present study. Urolithiasis does not produce any of the neurological signs or symptoms associated with methyl mercury exposure [5,26,47]. Because running wheel activity could be affected by end-stage urolithiasis, animals that were ill or that became ill shortly after the test were not included in the analyses reported here. Moreover, urolithiasis appeared in all exposure groups and there was no evidence that it affected the performance of control animals, of rats exposed during gestation, or of rats exposed to the lower concentration of methyl mercury. Other signs, such as gait disturbances related to hind-limb dragging and the presence of hind-limb cross, are associated with methyl mercury but not urolithiasis. Perhaps most important, the effects reported here were linked to methyl mercury dose and exposure regimen and bore no relationship to urolithiasis, which appeared across all exposure groups.

It might be argued that the rats studied here represent a biased sample because they were not affected by urolithiasis when they were being tested, i.e., that some unknown metabolic difference between rats that were and were not affected by urolithiasis skewed the present results. Urine chemistry influences the appearance of urolithiasis and the important factor in urine chemistry has been identified as racemic bitartaric acid [19,26,37]. Apart from the high mercury concentrations found in rats that became ill, which certainly could be important, it seems unlikely that urinary tract chemistry would alter the conclusions drawn here regarding the appearance of neurobehavioral effects of methyl mercury exposure, markers of exposure, or the absence of an interaction with dietary PUFAs.

4.5. Summary

Gait, running wheel activity, forelimb grip strength, and hind limb crossing were all associated with adult-onset exposure to 400 μg/kg/day of methyl mercury, and did not appear with any of the other exposure regimens. These signs were associated with brain and blood concentrations of mercury of approximately 10 and 70 ppm, respectively, but were not associated with a low or high ratio of $n-3$ to $n-6$ polyunsaturated fatty acids. The absence of effects in the lower exposure level or with developmental exposure does not indicate that these exposures are without motor effects, only that more refined techniques, such as high-rate operant behavior, may be necessary to detect deficits [33,35].

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