# Deficits in the Brain Growth in Rats Induced by Methylmercury Treatment during the Brain Growth Spurt

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This paper describes the deficits in brain regional growth of rats treated with methylmercury (MeHg) among the postnatal developing phases. Rats were orally administered 10 mg/kg/day of methylmercury chloride (MMC) for 10 consecutive days from postnatal days 1 (PD-1), -14 and -35, which corresponded to the early-, late- and post-brain growth spurt, respectively. Weight-matched control rats were periodically isolated from their mother or diet and placed in an incubator for intervals of 4 to 10 hr in order to adjust the body weight to MMC-treated rats. The earlier the postnatal phase the higher the resistance to body weight loss induced by MMC. The rats were dissected on the day after final MMC treatment and the weight of organs and their mercury (Hg) concentrations were measured. Hg accumulation in the brain on the day after final treatment with 10 mg/kg/day of MMC was highest in the rats treated during the late-brain growth spurt. On the other hand, Hg accumulations in the liver and kidney increased rapidly with development of postnatal phases. Then, the brain/kidney and brain/liver ratio of Hg concentration were much higher in early postnatal rats than in later one. The weight of brain regions in MMC-treated rats was compared with those in weight-matched control rats. The significantly lower weight of the cerebrum, cerebellum and midbrain + diencephalon were confirmed in rats treated with MMC during the early-brain growth spurt. The significantly lower cerebellum weight was confirmed in rats treated with MMC during the late-brain growth spurt. The Significant differences were not observed in the brain regions in rats treated during post-brain growth spurt. In the case of human, a similar reduction of the brain weight occurred in the fetal and non-fetal infantile Minamata disease patients. The experiment using postnatal rats succeeded to reproduce the deficit in the brain growth during the early- and late brain growth spurt by MMC treatment.

**Key words** —— methylmercury, brain growth spurt, cerebellum, neonatal rat, neurological disorder

## INTRODUCTION

MeHg is known as a highly toxic environmental pollutant. Many infants were congenitally affected by MeHg in the epidemics in Minamata and Niigata, Japan, and Iraq.[1] It was reported the Minamata infants had severe cerebral palsy, whereas their mothers had mild or no manifestations of poisoning.[2–5] Data from animal studies indicated that the mercury concentration in fetus brain was 2–4 fold higher than in the mother's,[6–8] and thus the fetus may be more susceptible to its toxic effects. Further, Takeuchi[4] suggested that MeHg affected the nerve cells after considerable differentiation had occurred during the intermediate or later period of fetal life. Therefore, it is important that animal studies also focus on the effect of MeHg on the brain at the rapid growth period that corresponds which occurs during the late gestation period in humans.[2]

Rapid brain growth occurs primarily during the third trimester in humans, whereas in rats it occurs after parturition.[4,9–13] Therefore, we hypothesized that the effects of MeHg on the postnatal development of the rat brain may help in understanding the neurotoxicity to the human fetal brain at late gestation. In the previous experiment, we succeeded in producing widespread neuronal degeneration in the brain and spinal sensory ganglia in rats following oral administration of MeHg during the postnatal

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development phase.[14] The extensive distribution of the lesions was quite similar to human fetal cases of MeHg intoxication in Minamata, Japan,[3,5,14,15] which can not be seen in exposure during the prenatal neonatal periods in rats.[16–18] Further, recent studies using rats[15] indicated that Hg concentrations of the brain and blood decreased rapidly during the suckling period, suggesting that an experiment using suckling offspring maintained by mothers might not be suitable to extrapolate the effects during the late gestation period in human. For these two reasons, we used neonatal rats with artificial MeHg administration to evaluate the effects on the late gestation period in humans.

In the case of human, obvious reductions of the brain weight were reported in the severe cases of fetal and non-fetal infantile Minamata disease patients.[4,5] The present study investigated the effects of MeHg on the brain weight growth in rats treated during the early-, late- and post-brain growth spurts.

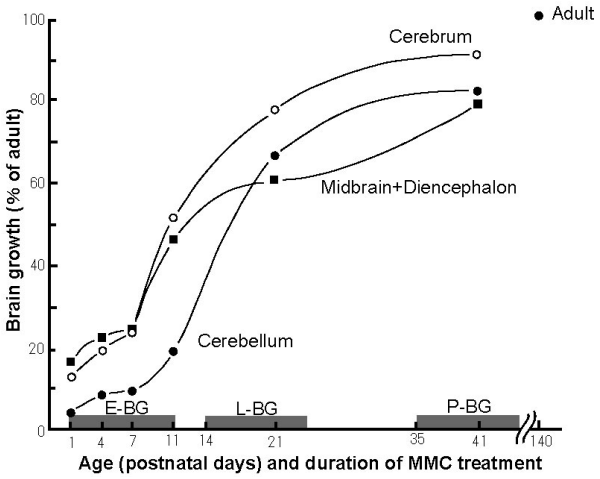


**Fig. 1.** Comparative Weights of the Brain Regions (Erebral Cortex, Cerebellum and Midbrain + Diencephalon) in Developing Rats and the Duration of Methylmercury Chloride (Methylmercury) Treatment

The weights have been calculated as a percentage of adult (PD-140) value. Each point represents a mean of four rats and smooth lines drawn by eye through the points. From the growth curve, we set the duration of methylmercury treatment as follows: during PD-1 to -10 corresponded to the early-brain growth spurt (E-BG), days 14 to 23 to the late-brain growth spurt (L-BG) and days 35 to 44 to the post-brain growth spurt (P-BG).

## MATERIALS AND METHODS

**Animals** —— Adult male and female Wistar rats supplied by CLEA Japan (Tokyo, Japan) were mated, and females were maintained on a 12-hr light/12-hr dark cycle at 23°C. within 24 hr of birth, a litter was randomly reduced to six male neonates, which were then maintained by a dam until weaning on postnatal day 21 (PD-21). One hundred mg methylmercury chloride (MMC) (98% of purity; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), 48.2 mg L-cysteine (SIGMA, St. Louis, U.S.A.): each combination corresponding in a molecular ratio 1 : 1, were dissolved in 6 ml of distilled water and 4 ml of condensed milk. Neonatal rats were orally administered each of 0 (control and weight-matched) and 10 mg/kg/day MMC for 10 consecutive days, with an aid of using a microman-pipette (Gilson, Villiers le Bel, France) for the suckling rats according to the method Sakamoto *et al.*[14,19,20] and a stainless catheter for the postnatal rats.

**Duration of MeHg Treatment** —— The weight of brain regions (cerebral cortex, cerebellum and midbrain + diencephalons) on PD-1, -4, -7, -11, -21, -41, and -140 of four rats were measured to assess their growth. The mean weight of the brain regions at each postnatal day was calculated as a percentage of adult rat (PD-140) and show in Fig. 1. From the growth curve, the significant neonatal brain growth (brain growth spurt) in the rat seems to occur during the first three weeks after birth. Therefore, we set the duration of MeHg treatment as follows: during postnatal days 1 to 10 covered one week of the early-brain growth (E-BG), days 14 to 23 covered one week of the late-brain growth spurt (L-BG) and days 35 to 44 corresponded to the post-brain growth spurt (P-BG).

**Brain Weight Evaluation** —— Twenty four male rats on PD-1, -14 and -35 were randomly divided into three sub-groups (MeHg-treated rats, weight-matched controls and normal controls). MeHg-treated rats were orally administered with 10 mg/kg/day of MeHg for 10 consecutive days. Normal and weight-matched controls were orally administered an equivalent volume of same ingredient without MeHg. Weight-matched controls were periodically isolated from their mother or diet and placed in an incubator (37°C) for intervals of 4 to 10 hr. Using this procedure the body weights of weight-matched controls were maintained within ± 5% of MeHg-treated rats. Six of the rats eliminating the largest and the smallest in each group were selected and dissected under pentobarbital anesthesia on the day after the final treatment. To remove blood from the tissues, each rat was perfused *via* the heart with physiological saline. The brain was carefully re-

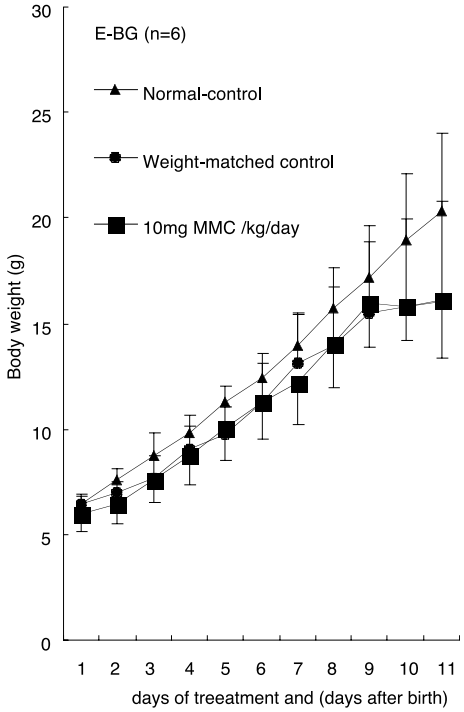


**Fig. 2-1.** Changes in Body Weight of Rats Treated with Methylmercury Chloride during E-BG, Weight-Matched and Normal Controls

Rats were administered 10 mg/kg/day of methylmercury for 10 consecutive days from PD-1. Each point represents a mean of eight rats.

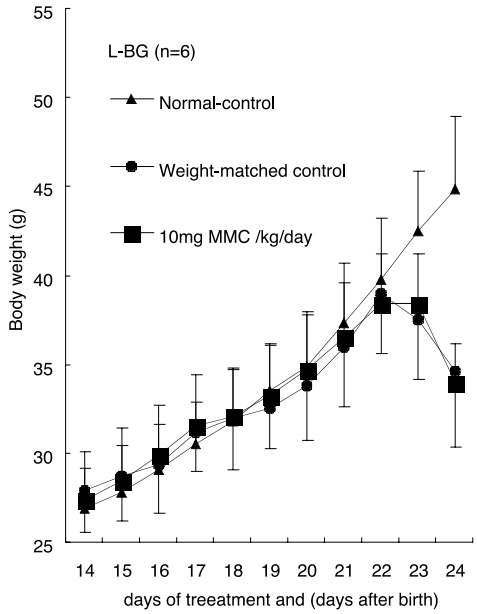


**Fig. 2-2.** Changes in Body Weight of Rats Treated with Methylmercury Chloride during L-BG, Weight-Matched and Normal Controls

Rats were administered 10 mg/kg/day of methylmercury for 10 consecutive days from PD-14. Each point represents a mean of eight rats. Asterrisk indicated the onset of hindlimb-crossing.

moved and separated from the spinal cord at the decussation of pyramids, and the liver and kidney were also removed and weighted. Brain regions were obtained by dissecting into cerebral cortex, cerebellum and midbrain + diencephalon, and weights were determined for each region.

**Mercury Determination** —— Total Hg determinations in the brain, liver and kidney were performed according to the oxygen combustion-gold amalgamation method with a Sugiyamagen mercury Analyzer, following procedures described by Jacobs.[21)]

**Statistics** —— Statistics significance was tested by Student's *t*-test or analysis of variance (ANOVA) tests. The level of significance was put at $p < 0.05$ and $p < 0.01$.

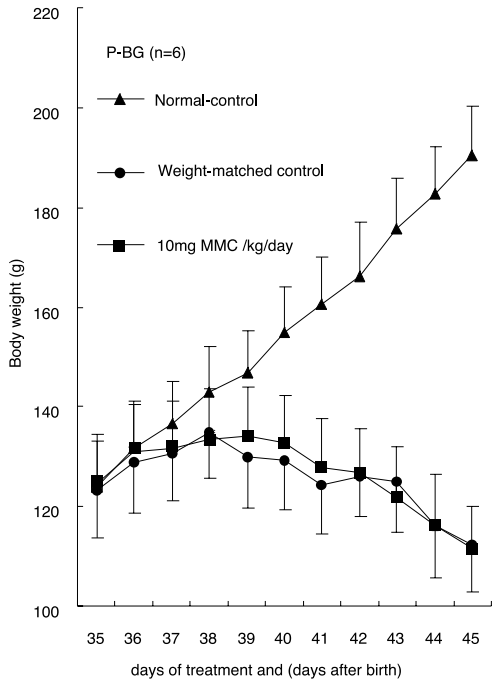


**Fig. 2-3.** Changes in Body Weight of Rats Treated with Methylmercury Chloride during P-BG, Weight-Matched and Normal Controls

Rats were administered 10 mg/kg/day of methylmercury for 10 consecutive days from PD-35. Each point represents a mean of eight rats.

## RESULTS

### Body Weight Growth and Neurological Symptoms

Figures 2-1, -2, and -3 show the changes in mean body weight of MeHg-treated rats (10 mg/kg/day of MeHg for 10 consecutive days from PD-1, -14 and -35), weight-matched and normal controls. The

**Table 1.** Percentage of Total Mercury dose and Mercury Concentration in the Brain, Liver and Kidney of Rats Treated with 10 mg/kg/day for 10 Consecutive Days from PD-1, PD-14 and PD-35, Respective

| | | % dose | | | Total mercury concentration ($\mu$g/g) | | |
|---|---|---|---|---|---|---|---|
| | (n) | Brain | Liver | Kidney | Brain | Liver | Kidney |
| PD-1 | 6 | 1.66 | 3.23 | 1.22 | 20.3 ± 2.2* | 46.7 ± 7.2** | 46.7 ± 3.4** |
| PD-14 | 6 | 1.58 | 3.85 | 1.83 | 30.4 ± 1.8** | 76.1 ± 13.0** | 73.0 ± 5.2** |
| PD-35 | 6 | 0.33 | 3.73 | 1.67 | 24.3 ± 2.3 | 103 ± 13.8 | 143.2 ± 13.7 |

Significant differences from PD-35 rats are show by $*p < 0.05$; $**p < 0.01$ by Student's $t$-test.

weight gain was lowered by MeHg treatment in E-BG rats, but no weight loss was observed. The weight loss of L-BG rats began suddenly on day 10 and severe hindlimb-crossing appeared in all of them on day 11. Three of them showed backward bending of their hind-limbs, and none could walk on the floor. The severe myoclonic jerking of the hindlimb began suddenly in two of them, and they died after fluttering on the floor for a while. The weight loss began on day 5 in PD-35 rats. The hindlimb-crossing did not appear during the period of this experiment. During the experiment, significant differences were not found in the body weights between MeHg-treated rats and weight-matched controls for E-BG, L-BG and P-BG rats.

**Mercury Distribution**

Table 1 shows the Hg concentrations in the brain, liver and kidney of E-BG, L-BG and P-BG rats on the day after the final treatment with 10 mg/kg/day of MeHg for 10 consecutive days. There were significant differences of Hg concentrations in the brain, liver and kidney among E-BG, L-BG and P-BG rats by ANOVA. The mean Hg concentrations in the brain were the highest in L-BG rats, followed by E-BG rats and P-BG rats, The mean Hg concentrations in the liver and kidney (46.7 and 24.3 $\mu$g/g) in E-BG rats and increased markedly with development in postnatal phase.

**Weight of Body, Brain Regions, Liver and Kidney**

Table 2 shows the weights of body, brain regions, liver and kidneys in MeHg-treated rats (10 mg/kg/day of MeHg for 10 consecutive days from PD-1, -14 and -35, respectively), weight-matched controls and normal controls. On the day after the final MeHg treatment, the average body weight of MeHg-treated rats was 25, 26 and 41% less than normal controls in E-BG, L-BG and P-BG rats, respectively. The average whole brain weight of MeHg-treated rats was significantly less than weight-matched controls in E-BG rats, but not in L-BG and P-BG rats. The whole brain weight of MeHg-treated rats was 15% less than normal controls and 14% less than weight-matched controls in E-BG rats. A significant weight loss was observed in all of the brain regions, particularly in the cerebellum (19% less than weight-matched controls) in E-BG rats. In L-BG rats, the weight loss was significant and predominant in cerebellum (11% less than weight-matched controls). No significant decline in the cerebellar weight was observed in P-BG rats. The liver weights in MMC-treated rats were significantly lower than that of weight-matched controls in E-BG and P-BG rats but not in L-BG rats. The kidney weights of MMC-treated rats were significantly higher than that of weight-matched controls in E-BG, L-BG and P-BG rats, respectively. The weight-matched controls/MMC-treated rats ratio of kidney weight was highest in P-BG rats (1.46), followed by L-BG and E-BG rats (1.37 and 1.16, respectively).

## DISCUSSION

Walker *et al.*[22] recommended the relative cerebellar weight as a indicator of developmental neurotoxicity. Also, in the case of human, a similar restriction in the brain weight occurred in the fetal and non-fetal infantile Minamata disease patients, not in the adult ones.[4,5] Our previous experiment indicated that the Hg concentrations in the blood and brain of pups maintained by MeHg administered mothers rapidly decreased during the suckling after the birth suggesting MeHg hardly transferred to pups through milk.[15] This fact suggests that an experiment using suckling pups maintained by mothers is not suitable to examine the effects of MeHg exposure on the postnatal developing brain in rats. On the other hand, we succeeded in causing widespread neuronal degeneration in the brain and spinal sensory ganalia in rats following oral administration of MeHg during the postnatal developing phase.[14] The extensive dis-

**Table 2.** Organ and Body Weights[a)] of MMC-Treated (10 mg/kg/day for 10 days from PD-1, -14 and -35), Weight Matched and Normal Controls

| | E-BG[b)] | | | |
|---|---|---|---|---|
| ($n$ = 6) | N-Cont | WM-Cont | MMC (% of WM-Cont) | |
| Brain (mg) | 994 ± 47 | 986 ± 43 | 846 ± 32 | (85.8)* |
| Cerebrum | 628 ± 28 | 626 ± 29 | 531 ± 25 | (84.8)* |
| Cerebellum | 94 ± 10 | 89 ± 8 | 72 ± 6 | (80.8)** |
| Midbrain + Diencephalon | 272 ± 11 | 271 ± 12 | 246 ± 10 | (90.7)** |
| Liver (mg) | 638 ± 134 | 437 ± 39 | 517 ± 56 | (118.3)** |
| Kidney (mg) | 244 ± 51 | 186 ± 14 | 217 ± 11 | (116.6)** |
| Body weight (g) | 20.4 ± 3.6 | 15.8 ± 1.1 | 15.6 ± 1.7 | (97.4) |
| | L-BG[c)] | | | |
| ($n$ = 6) | N-Cont | WM-Cont | MMC (% of WM-Cont) | |
| Brain (mg) | 1436 ± 37 | 1423 ± 37 | 1385 ± 38 | (97.3) |
| Cerebrum | 875 ± 25 | 857 ± 26 | 854 ± 32 | (99.6) |
| Cerebellum | 183 ± 4 | 188 ± 10 | 168 ± 3 | (89.3)* |
| Midbrain + Diencephalon | 378 ± 16 | 378 ± 12 | 363 ± 13 | (96.0) |
| Liver (mg) | 1602 ± 180 | 1495 ± 159 | 1195 ± 79 | (79.9)* |
| Kidney (mg) | 504 ± 45 | 456 ± 57 | 602 ± 59 | (132.0)* |
| Body weight (g) | 45.3 ± 4.0 | 35.9 ± 2.7 | 33.7 ± 2.1 | (95.2) |
| | P-BG[d)] | | | |
| ($n$ = 6) | N-Cont | WM-Cont | MMC (% of WM-Cont) | |
| Brain (mg) | 1716 ± 74 | 1704 ± 56 | 1666 ± 57 | (97.4) |
| Cerebrum | 1007 ± 42 | 1003 ± 39 | 984 ± 38 | (98.1) |
| Cerebellum | 242 ± 11 | 244 ± 9 | 235 ± 9 | (96.3) |
| Midbrain + Diencephalon | 467 ± 23 | 457 ± 16 | 447 ± 14 | (97.8) |
| Liver (mg) | 9439 ± 469 | 3835 ± 274 | 4501 ± 533 | (117.3)** |
| Kidney (mg) | 1822 ± 284 | 1150 ± 98 | 1683 ± 216 | (146.3)* |
| Body weight (g) | 190.3 ± 9.9 | 114.5 ± 6.3 | 111.8 ± 8.3 | (97.6) |

*a*) Values are mean ± S.D. for 6 rats per group. Significant levels (between MMC): $*p < 0.01$, $**p < 0.05$. *b*) Rats treated with MMC during early-brain growth spurt (from postnatal days 1 to 10). *c*) Rats treated with MMC during late-brain growth spurt (from postnatal days 14 to 23). *d*) Rats treated with MMC during post-brain growth spurt (from postnatal days 35 to 44).

tribution of the lesions was quite similar in fetal cases of MeHg intoxication in Minamata, Japan,[3,4)] which can not be seen in exposure during the neonatal periods in rats.[17,18)] Thus the effects of MeHg in humans exposed prenatally during third trimester need to be studied using postnatal developing rats. The present study investigated the effects of high dose of MeHg on the growth of brain regions in rats treated during the early-, late- and post-brain growth spurts (E-BG, L-BG, and P-BG). Body weight loss at a toxic MeHg level is a typical phenomenon in adult rats and mice, and is caused by anorexia.[1)] The E-BG and L-BG rats seemed to be more resistant than P-BG rats to the anorexic effect of MeHg and no body weight decline was induced in E-BG rats. However, significant difference in body weight were observed between control and MMC-treated groups in all aged groups. Therefore, we made a weight-matched control group to evaluate not the anorexia but MeHg itself to the growth of the brain.

The growth period is characterized by function immaturity of organs and the brain regions, which could be the main reason for the differences in Hg accumulation among organs and the brain regions. For example, the glomerular filtration rate is low in immature kidneys.[23)] The slow blood flow into the renal tissue may cause less accumulation of MeHg in the kidney of neonatal rats. Renal glutamyltraspeptidase plays an important role in renal intake of MeHg.[24)] Therefore, the low activity of the enzyme in the neonatal period[25)] may be another reason for low Hg accumulation in the kidneys during this pe-

riod. Inouye *et al.*[26] reported that Hg accumulation in the kidney of the mouse fetus was considerably lower than with the dam. The liver weight loss in MeHg treated rats was much large in the later postnatal rats than in the earlier ones. This may indicate that the anorexic effects of MeHg was stronger in the later postnatal rats. The lower kidney weight in weight-matched controls compared to the normal controls may be caused by the poor nutrition owing to the artificial anorexia in the weight-matched controls. On the other hand, the MeHg-treated rats/weight-matched controls' ratio of kidney weight increased with development in the postnatal phase. The mechanism is unclear, but it was reported that MeHg treatment caused renal enlargement in rats.[24] The increased in Hg concentration in the kidney with the development in postnatal phase (Table 1) may explain the age-related enlargement of the kidney caused by MeHg treatment.

All the brain regions, especially cerebellar growth was restricted by MeHg treatment in E-BG rats, whereas only cerebellar growth was restricted in L-BG rats. The deficit of the cerebellar growth was not observed in P-BG rats. The deficit of the brain regions did not have any direct relationships between Hg concentrations among the growth stages. The brain growth spurt is a period of maximal brain vulnerability.[27] Dobbing and Sands[13] mentioned it seemed reasonable that if vulnerability was related to the rate of growth, the part of the brain growing fastest would presumable show the greatest effects of growth restriction. In this point of view, the cerebellum may be the predominant region in the brain for the growth deficit induced by MeHg treatment, because the fastest growth occurred during the neonatal period as shown Fig. 1. However, in previous animal studies,[28] few if any very small deficits in the brain weight were found in neonatal rats after MeHg treatment. Differences in the MeHg dosage and/or in the selection of brain regions may account for the differences in outcomes between the abovementioned studies and ours. An important feature of our experiment is that we succeeded in administering a considerably large amount of MeHg continuously without wounding the neonatal rats. The epidermis of neonatal rats is too weak and narrow to inject MeHg several times, and the wound from the injection may cause misleading results in the subsequent behavioral test. The growth of the brain is accompanied by an increase in dendritic differentiation and branching of the axons.[27] MeHg treatment during the brain growth spurt may affect their structural growth especially in cerebellum. Golgi preparations of cerebellum of MeHg-treated neonatal rats showed a significant reduction in dendritic arborization of Purkinje cells.[29]

In summary, the deficit in the brain growth was confirmed in rats treated with MeHg using postnatal developing rats suggesting that the experiment using postnatal developing rats will contribute to understands the effects on the prenatal developing brain in human.

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