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Increased Body:Brain Weight Ratio in Developing Rats after Low Exposure to Organic Lead

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Tetramethyl lead in an oily vehicle was administered to rats at weekly intervals during gestation and early postnatal life, raising the total lead concentration in the brain to about 1 μg/g. Birth weight was unaffected, but postnatal body growth was stimulated more than brain growth, resulting in a higher body:brain weight ratio. Histological measures of brain myelination, dendritic growth, granule cell production, and retinal receptor development showed no deficit. We conclude that the body:brain weight ratio is the most sensitive of the parameters measured for detecting the effect on development of exposure to a low concentration of tetramethyl lead. The latter is neurotoxic at higher concentrations, and the stimulating effect on body growth of a low concentration is an example of "hormesis," a phenomenon which has been noted with other toxins.

INTRODUCTION

The organic lead compounds that are burned in petroleum in thousands of tons each year are largely converted to inorganic forms before reaching most humans. However, 5 to 10% of airborne lead in cities consists of organic lead compounds (8). Tetra- and trialkyl lead compounds have lipid solubilities that enable them to cross the blood-brain barrier and enter the brain whereas inorganic forms are largely excluded. Organic lead has been measured at as much as 0.05 μg/g in the brains of some inhabitants of Copenhagen (13). To our knowledge, the chronic effect of low concentrations of organic lead compounds on developing brains has not been studied. However, neuronal damage has been reported after large acute doses (14,

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15), and in tissue culture chick embryo brain cells have been found to grow fewer nerve processes when exposed to triethyl lead chloride at 0.57 mg/liter or 0.36 μg/g as lead (1).

Triethyl lead chloride given to young rats at doses of 5 + 3 μg/g as lead reduced tissue oxidation and hence protein synthesis and the deposition of myelin in the developing brain (10). With incubated slices of brain, the same compound at 0.62 μg/g as lead reduced the incorporation of leucine into myelin proteins (11). In adult mice, a single dose of the same compound at 5 μg/g as lead caused a reversible reduction in the number of glial cells in the anterior commissure (19).

Earlier studies of human and animal neuropathology associated with organic lead were reviewed by Grandjean and Nielsen (7). We do not know which is the most sensitive target within the developing nervous system for the assessment of the toxicity of organic lead compounds. We studied the growth of the retinal rod receptors because this occurs rapidly and is suitable for accurate measurement. The rod receptors contribute a component to the electroretinogram that is sensitive to heavy metal ions, including lead at 1 μg/g (6).

Because of the biochemical indication of an interference with myelin formation (10), we counted the number of optic nerve fibers that had acquired myelin sheaths 13 days after birth. The area of the corpus callosum at the midline in sagittal section was also measured to reflect the progress of myelination. The growth of dendrites can be measured conveniently by light microscopy in the hippocampus, and this growth has been shown to be sensitive to inorganic lead (4). Accordingly, we measured the thickness of the stratum radiatum containing the apical dendrites of the pyramidal cells, and the thickness of the infrapyramidal stratum moleculare in the dentate gyrus, containing the dendrites of the granule cells. In the cerebellum, the external germinal layer becomes thinner as granule cell production approaches completion in the young rat, so we measured the mean thickness of this layer, looking for a retardation in granule cell production, as occurs in hypothyroidism.

Tetramethyl lead (TML) was available to us, but there is little guide to the minimal chronic dose that might cause structural changes in the developing nervous sytem. At 6 μg/kg/day for 6 months, TML did not cause clinical signs of toxicity in monkeys (9). Male rats initially 22 days old that received TML 1 mg/kg daily for 100 doses during 21 weeks gained 14% less body weight than controls, but similarly treated female rats gained 9% more (16). A single lethal dose was 108 mg/kg. We decided to administer TML at 22 mg/kg at weekly intervals throughout gestation and early postnatal life, and to use an oily vehicle to yield a slow and sustained release of the toxin.

MATERIALS AND METHODS

Long–Evans rats were mated, and the day on which vaginal sperm were found counted as E_0. The pregnant rats were injected subcutaneously on E_7, E_{14}, and E_{21} with a volume of about 0.1 ml olive oil that contained TML with toluene, or toluene alone (controls). Litters were born on E_{22} and all reduced to eight pups which were injected subcutaneously 7 and 14 days after the last prenatal injection with the same injectate in a volume of 5 to 15 μl. The injectate contained 300 mg TML (Associated Octel Co., Ltd.) and 300 mg toluene in 5 ml olive oil, or 300 mg of toluene alone, and was injected at 0.37 ml/kg body weight to provide a dose of 22 mg TML/kg body weight. Rats were fed a commercial cubed rat food and water *ad libitum*, housed in an air-conditioned room at 22°C, and exposed to 12 h of artificial light per day.

The control and experimental rats were killed either on postnatal day 13 or on day 16 (35 and 38 days after conception). The control and experimental groups were each drawn from four litters at 13 days and from three litters at 16 days, and contained the same numbers of pups at all times. The rat pups were anesthetized with chloroform and perfused through the left cardiac ventricle with 0.1 *M* phosphate buffer, *p*H 7.3, containing 4% formaldehyde and 1% glutaraldehyde. The brains were removed and weighed, and the eyes opened to allow fixative to penetrate the retinas. After secondary fixation in osmium tetroxide for 24 h, the retinas were embedded in Araldite and sectioned tangentially, at 0.5 μm, through the head of the optic nerve. Sections were stained with 1% toluidine blue in 1% potassium metaborate, and coded.

An oil immersion 100× objective lens was used with a Leitz microscope drawing tube to trace the outer and inner segments of the rod receptors in 10 to 15 sectors across a complete section of retina. The area and length of each sector were measured on a Zeiss MOP image analyzer, and the average width of the segments was obtained by dividing the area of each sector by its length. The mean width of the outer and inner segments in each retina was obtained from the 10 to 15 sectors measured. Cross sections of the optic nerve were cut at 0.5 μm in Araldite, and axons that had acquired a myelin sheath were counted under the same lens in 10 squares (each 21 × 21 μm defined by an eye-piece graticule) for each nerve.

Sagittal sections of the brain of 25 μm thickness were cut on a freezing microtome at the midline for the corpus callosum and cerebellar vermis, and 2 mm to the right of the midline for the hippocampus. These sections were stained overnight in 0.001% toluidine blue with 0.001% thionin in phosphate buffer at *p*H 7.3. Sections were then mounted from buffer and dried on the slide, dipped in xylol, and coverslipped with DPX. The

structures to be measured were viewed at appropriate magnification and drawn accurately with the drawing tube attached to the Leitz microscope. Areas and lengths were measured with the Zeiss MOP, and divided to obtain an average measure of thickness. The code written on the slides was broken after the measurements were complete. For electron microscopy, the Araldite-embedded blocks were cut at 60 nm, stained with lead citrate, and viewed in a JEOL 100S microscope. The numbers of rats used are stated in the footnotes of the tables, which show means and standard error of the mean and probabilities calculated by Student's *t* test.

Brain lead was measured in litters of rats injected with toluene or TML as described above and killed with chloroform on postnatal days 13 or 28. Whole brains from lead-injected rats or vehicle-injected controls were pooled and homogenized in an all-glass apparatus. Aliquots of the homogenates were digested with nitric and perchloric acids at 60°C under reflux and analyzed by background-corrected furnace atomic absorption spectroscopy. Standard additions of TML were made to the digests for calibration. The analysis was done by Mr. B. James, Analytical Reference Laboratory, Melbourne, and measured total lead in organic and inorganic forms.

RESULTS

Brain Lead. The mean total concentration of organic plus inorganic lead was 0.08 μg/g fresh brain in the vehicle-injected control rats, 1.15 μg/g in the TML-treated rats at 13 days, and 0.98 μg/g at 28 days.

Brain and Body Weights. As shown in Tables 1 and 2, the body and brain weights of the pups treated with TML were slightly greater than those of the controls for both male and female pups at both 13 and 16 days after birth. Only three of these eight comparisons showed statistically significant differences, as indicated by the asterisks. The body weight and body:brain weight ratio was significantly increased by TML except in males at 13 days. The coefficient of variation (not shown) was not increased by TML.

The question of whether or not any effect on body weight was present at birth was examined in 14 control litters and 15 litters treated with TML, some litters being part of a parallel study at the same dosage (5). As shown in Table 3, the birth weights and numbers were lower in the litters treated with TML, but none of the differences was statistically significant.

Retinal Development. The total length of the combined inner and outer segments of the photoreceptors was about 22 μm at 13 days, 35 μm at 16 days, and 60 μm in an adult rat. Those times of biopsy were chosen to measure the receptors at a period of rapid and early development. No differences were found between the male and female rats, whose brain weights did not differ significantly, and the retinal measurements were

TABLE 1

Effect of Tetramethyl Lead (TML) on Body, Brain, and Retinal Development of Rats at Day 13[a]

	Control	TML	Percentage difference
Body wt (g)			
Male	22.8 ± 0.9	23.6 ± 0.7	+3.4
Female	21.2 ± 0.3	24.1 ± 0.2	+14*
Body:brain wt (g)			
Male	21.9 ± 1.1	21.9 ± 1.4	−0.3
Female	19.7 ± 0.5	22.4 ± 0.7	+14*
Brain wt (g)			
Male	1.04 ± .01	1.10 ± .03	+4.9
Female	1.05 ± .02	1.08 ± .04	+2.8
Retinal receptor length (μm)	22.2 ± 0.8	22.5 ± 1.4	+1.6
Optic nerve myelinated axons per 100 μm^2	10.0 ± 0.4	11.3 ± 0.7	+13
Corpus callosum area (mm^2)	1.65 ± 0.3	1.63 ± .08	−1.3
Dentate moleculare thickness (μm)	95.9 ± 4.1	122.0 ± 3.9	+27*
Stratum radiatum thickness (μm)	357 ± 3	411 ± 16	+15*
Stratum pyramidale thickness (μm)	64.6 ± 1.9	67.0 ± 6.1	+3.7
Cerebellum EGL[b] thickness (μm)	57.3 ± 3.4	52.9 ± 1.7	−7.7

[a] Ten male and eight female rats treated with TML were compared with 9 male and 11 female control rats in gross and microscopic measures of body and retinal development. Control and experimental groups were each drawn from four litters. The measures of brain development came from a single litter of two male and three female rats treated with TML and a single litter of four male and five female control rats. Values are $\bar{x} \pm$ SEM.

[b] External germinal layer.

* $P < 0.05$.

pooled. At 13 days, the total receptor length is shown (Table 1) because the boundary between the inner and outer segments was ill-defined by light microscopy. Electron microscopy showed the junction to differ in position considerably from one photoreceptor to the next. At 16 days, however, the junction was distinct and separate measurements of the inner and outer segments are shown in Table 2. No significant differences were found in photoreceptor development in the TML-treated rats at either 13 or 16 days. Electron microscopy found two small hemorrhages and one dead bipolar cell in the TML-treated retinas at 13 days, but no other structural changes were apparent.

Cross sections of the optic nerve showed the myelin sheaths to be in an early stage of development at 13 days, and the number counted in a fixed

TABLE 2

Effect of Tetramethyl Lead (TML) on Body, Brain, and Retinal Development of Rats at Day 16[a]

	Control	TML	Percentage difference
Body wt (g)			
Male	32.4 ± 0.7	35.4 ± 0.8	+9.5*
Female	29.2 ± 0.7	33.8 ± 0.7	+15.6*
Body:brain wt (g)			
Male	28.2 ± 0.4	29.9 ± 0.5	+6.0*
Female	26.5 ± 0.7	29.4 ± 0.7	+10.9*
Brain wt (g)			
Male	1.15 ± .002	1.19 ± .02	+3.2
Female	1.10 ± .03	1.15 ± .01	+4.2
Retinal receptor length (μm)			
Outer segment	20.8 ± 0.6	21.2 ± 0.9	+1.8
Inner segment	13.2 ± 0.7	14.1 ± 0.5	+7.1
Total	34.0 ± 1.1	35.3 ± 1.3	+3.9

[a] Six male and six female rats treated with TML were compared with five male and five female control rats. Values are $\bar{x}$ ± SEM.

* $P < 0.05$.

area of graticule was 13% higher in the TML-treated nerves (Table 1). This difference was not statistically significant when the mean counts for six TML treated nerves were compared with the mean counts for six control nerves.

TABLE 3

Birth Weights and Numbers of Pups in 14 Control Litters and 15 Litters Treated with Tetramethyl Lead[a]

	Control	TML	Difference (%)
Number of pups born per litter	9.36 ± 0.61	9.07 ± 0.69	−3.1
Total weight of pups per litter (g)	58.4 ± 3.0	54.5 ± 3.3	−6.7
Average weight of one pup (g)	6.37 ± 0.25	6.15 ± 0.16	−3.5

[a] Values are $\bar{x}$ ± SEM.

Brain Development. At 13 days, the cross-sectional area of the corpus callosum at the midline was well defined, and yielded area measurements of small variance, but there was no significant difference between TML and control brains (Table 1). However, in the hippocampus 2 mm from the midline, the stratum radiatum, occupied by the apical dendrites of the pyramidal cells, was significantly thicker in the TML-treated rats, although the thickness of the cell body layer (stratum pyramidale) was unaffected. In the dentate gyrus the mean thickness of the stratum moleculare occupied by granule cell dendrites was significantly increased after TML treatment. After decoding the slides, the latter difference of 27% was qualitatively apparent on inspecting the sections. However, these brain measures were made on only one litter of TML-treated rats and one control litter. In the cerebellum, there was no significant difference in the thickness of the external germinal layer, which was still producing granule cells by mitosis.

DISCUSSION

Tetramethyl lead increased the body weights and body:brain weight ratios of female rats at 13 days and of both males and females at 16 days. In the parallel study using the same dosage of TML, Ferris and Cragg (5) found the body:brain weight ratio increased at 18 and at 28 days in both males and females. Differences of body weight were not significant at birth. This stimulation of postnatal body growth by TML does not seem to have been described in studies with inorganic lead, but has been found with niobium (17). Stimulation of growth in other organisms at low concentrations of a toxin has been seen with petroleum hydrocarbons (12) and with copper and other toxins (18) and has been named "hormesis." The mechanism is not known, but it is possible that the extra metabolism required for detoxification acts as a stimulus to body growth.

Brain weight was not significantly affected at 13 or 16 days in the present study, but was reduced significantly at 18 days in male rats and at 28 days in both male and female rats (5). The occurrence of these opposite effects on brain and body weights makes the body:brain weight ratio a sensitive measure for detecting the effect of a minimal toxic dose of TML. Moreover, for a given amount of labor, far more weights can be measured than histological variables, and an increased number of observations allows a smaller difference due to the toxin to attain statistical significance.

The biochemically measured deficits in myelination produced by triethyl lead (10) were not manifested in our study by a reduction in area of the corpus callosum or in the number of axons that had acquired myelin sheaths in the optic nerve. Growth of the retinal receptors was unaffected at 13 and 16 days after birth. The parameters of brain development

measured at 13 days did not show a deficit after TML treatment, and the same was true at 18 and 28 days (5). The dendritic layers of the hippocampus were thicker at 13 days in one litter of rats treated with TML. There was no deficit in brain weight at 13 or 16 days after birth, or at 18 days in female rats, but in males at 18 days and in males and females at 28 days a significant reduction in brain weight was apparent (5). Thus we have not identified any specific structure that is sensitive to a low exposure to TML during development. However, the significant increase in body:brain weight ratio in both male and female rats at all four ages studied (except males at 13 days) is due to an increase in body weight without a corresponding increase in brain weight, there being a significant decrease in brain weight at 28 days. This action of TML is compatible with its neurotoxic effects at higher dosages, but it is still not known how it limits brain development. We have not followed the rats beyond 28 days, and because brain weight continues to increase slowly during the 1st year of life it is possible that greater relative deficits might appear in the TML-treated rats.

The significant differences in body:brain weight ratio that we measured were due to a lead concentration of about 1 μg/g in brain tissue, lower than that in most studies of inorganic lead that have reported significant effects. Our rats received a total dose of 88 μg/g TML (4 $\times$ 22 mg/kg), or 68 μg/g as lead, and the small amount of lead retained may be related to the slow release of TML from the oily vehicle. The total lead content of our rat brains, 1 μg/g, was considerably greater than the 0.09 μg/g reported in brains of people not exposed to lead by their occupation (2) though values as high as 0.18 μg/g have also been reported (13). The content of organic lead in the brains of children is still unknown.

Although we report significant effects on body:brain weight ratio in Long–Evans rats, this may not be the best species for testing lead toxicity. It has been found that triethyl lead binds to hemoglobin in rat blood but not in human blood (3) and this substantially affects the pharmacokinetics. In unpublished work, we have found that the growth of 1-day-old chickens is more sensitive to TML than is that of rats.

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