ed under stressful situations and are involved in mediating the effects of stress.

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Developmental exposure to organic lead causes permanent hippocampal damage in Fischer-344 rats

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Summary. The long-term consequences of neonatal exposure to triethyl lead, the putative neurotoxic metabolite of the anti-knock gasoline additive tetraethyl lead, were examined with respect to central nervous system (CNS) development. We presently report a series of studies in which exposure of neonatal rats to organic lead produces profound CNS damage in adulthood as indicated by dose-dependent, persistent behavioral hyperreactivity as well as dose-dependent, preferential, and permanent damage to the hippocampus. General morphological parameters of brain development were not altered. Pharmacological probes of neurotransmitter system integrity suggested a functional and dose-dependent relationship between this behavioral hyperreactivity and hippocampal damage via cholinergic, but not dopaminergic, pathways. Furthermore, these alterations were not accompanied by long-term alterations in motor activity and were not attributable to the presence of lead in adult neural tissue. Finally, these behavioral, anatomical, and pharmacological indices of developmental exposure to organic lead were dissociable from any effects of early undernutrition. These data collectively indicate that organolead compounds may pose a potent neurotoxic threat to the developing CNS.
Key words. Triethyl lead; development; central nervous system; neurotoxicity; hyperreactivity; hippocampus; cholinergic; rat.

Organolead compounds were synthesized in the 1920's for use as gasoline anti-knock additives[1], and constitute the major sources of human lead exposure and environmental lead contamination[2]. Despite the recent decline in use of leaded gasoline additives in the U.S. (from 73% of total gasoline consumption in 1977 to 18% in 1988)[3], world-wide mine production of lead remains higher in the current decade than at any time in history, with the exception of the 1970's[4]. Of particular concern is the possibility that lead may be biotransformed into the more toxic organolead compounds[5,6]. Organic lead gasoline additives are highly lipid soluble, rapidly metabolized, and readily cross the blood-brain barrier[7,8]. These physiochemical properties make the central nervous system (CNS) a critical target organ for organolead toxicity.

With respect to potential health effects, it has been estimated that up to 20% of lead in the brains of urban dwellers is of the organic form[9]. Unlike the case with inorganic lead toxicity, however, routine blood and urine monitoring are of questionable values because of a relatively short residence time of organic lead in blood and a lack of correlation of organic lead levels in urine with CNS effects[10]. A lack of accumulation of organic lead in bone also renders this prognostic aid ineffective[10]. Therapeutic treatment of organolead poisoning, such as by chelating agents, is also problematic. Based on the fact that EDTA binds Pb^{2+} firmly, chelation therapy is often recommended; however, as it does not bind Et_3Pb^+ or Et_2Pb^{2+} it may not be very useful[11].

Adverse health effects of organolead toxicity may be further exacerbated for specific subpopulations. Indeed,

a 'fetal gasoline syndrome' has been reported to accompany maternal gasoline sniffing[12]. Although experimental studies have yet to detect any teratogenic abnormalities[13], the protracted development of the mammalian CNS places the immature organism at high risk for organolead toxicity. Also at particular risk to high-dose organolead exposure are juveniles involved in the recreational sniffing of leaded gasoline. Such individuals experience a range of neurological abnormalities which may affect attentional and cognitive abilities[14]. Clinical autopsy findings of recreational gasoline sniffers[15,16] have noted cell necrosis in hippocampal and cerebellar brain regions. These data suggest that despite a lack of overt teratogenesis, neurotoxic consequences of early organolead exposure are quite likely. No studies have, to our knowledge, provided a comprehensive, multidisciplinary examination of the neurotoxic potential of organic lead in developing mammals.

The offspring of Fischer-344 dams (Harlan Laboratories, Indianapolis, IN) bred at the NIH/NIEHS animal facilities were culled to eight pups per litter (4 male, 4 female) on postnatal day 3 (birth = day 0 ± 12 h). Litters were maintained under standard colony conditions with continuous access to food and water. Recognizing that organic lead compounds are rapidly absorbed regardless of the route of exposure, we took advantage of the precision and safety of subcutaneous exposure[10]. Triethyl lead (TEL), the putative neurotoxic metabolite of the gasoline additive tetraethyl lead[17,18], was investigated. At 5 days of age, one pup of each sex per litter ($N_{litter} = 16$) was administered a single subcutaneous injection of either the vehicle (deionized water), 4.5-, or 9.0-mg/kg of TEL chloride. Analysis of total levels was performed by nitric acid digestion and graphite furnace atomic absorption techniques[19]. These doses of TEL elevated blood and brain lead levels at weaning (day 22) [blood: controls = 7; 4.5 mg/kg TEL = 15; 9.0 mg/kg TEL = 35 µg/dl and whole brain: controls = 0.2; 4.5 mg/kg TEL = 1.5; 9.0 mg/kg TEL = 3.0 µg/g dry weight]. Lead was not detectable (< 2 ng/ml) in the brains of TEL-treated animals at 140 days of age. The fourth pup of each sex was undernourished by periodic placement with a nipple-ligated surrogate dam[20]. This procedure controlled for the mild (12%–19%) preweaning body weight loss from TEL treatment. Growth curves for the undernourished animals were matched to the animals administered 9.0 mg/kg of TEL through the first two weeks of life. Undernourished control animals provided an appropriate baseline for our behavioral, anatomical, and pharmacological studies. Planned orthogonal contrasts were used to test specific relationships among the group means[21]. The first set of contrasts directly evaluated TEL-effects relative to the treatment control group. The second orthogonal set described dose-dependent linear or quadratic functions. And finally, the effect of TEL was compared to the undernutrition control group.

In our first longitudinal observations we examined several measures of gross neurological function-exploratory behavior, motor control, and reflex responsiveness (table 1). No TEL-induced body weight deficits were present. The behavior of TEL-treated animals appeared quite normal; we observed no treatment-induced deficits in exploratory behavior or gross motor activity, and no body tremor, when testing began in adulthood (day 90). Within-session habituation (not shown) was comparable across treatment groups. However, control animals displayed significant habituation to the motor activity test

Table 1. Effects of neonatal triethyl lead exposure (TEL) on physical growth, behavioral indices of neurological function, and brain morphology upon maturation to adulthood

Parameter	Vehicle control	4.5 mg/kg TEL	9.0 mg/kg TEL	Undernourished control
Physical growth (Mean D91-D120 weight; grams)				
Males	336 ± 4	345 ± 4	334 ± 6	327 ± 5
Females	200 ± 3	204 ± 2	201 ± 3	195 ± 2
Behavioral indices				
Exploratory motor activity (9 min totals)	678 ± 25	682 ± 25	659 ± 29	637 ± 21
Spectral analysis of movement (Hz & Power)	13.7 ± 0.2	13.6 ± 0.1	13.8 ± 0.3	13.7 ± 0.2
	78.4 ± 0.6	77.7 ± 0.6	78.0 ± 0.6	77.8 ± 0.6
Indices of brain development				
Cortical width	1414 ± 18	1442 ± 24	1410 ± 24	1409 ± 27
Corpus callosum width	282 ± 12	270 ± 7	288 ± 10	301 ± 13
Hippocampal width[a,b]	2026 ± 27	2027 ± 24	1950 ± 23	2019 ± 21

[a] Significant linear dose-dependent effect of TEL. [b] Effect of TEL (9.0 mg/kg) significantly different from nutritional controls (see text for details and exact statistical values). Statistical evaluations failed to confirm any long-term impairment in young adult body weight following TEL exposure. No significant TEL-induced alterations in undifferentiated motor activity were detectable. Although an early gross motor tremor had been noted as an acute effect (postnatal days 10 and 14) there was no evidence of any persistent fine motor tremor as a consequence of neonatal TEL. Morphometric measures of corpus callosum and cortical widths failed to detect any significant TEL-induced decrements in general neural indices of development, but the hippocampus was selectively damaged by exposure to TEL.

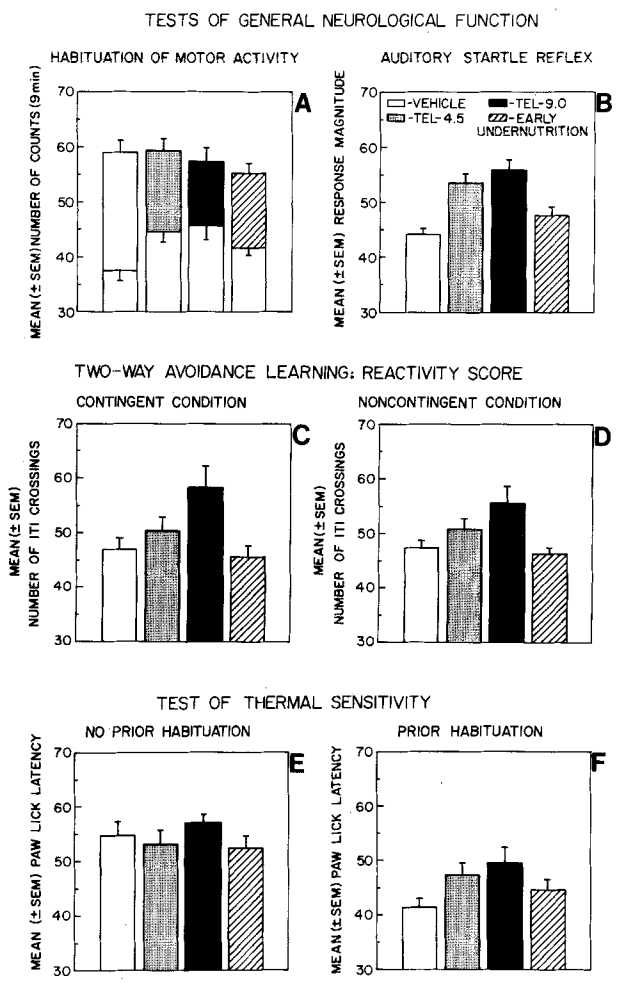

Figure 1. Results of the neurological tests administered to adult rats that were exposed to TEL during development. *A* Between session habituation of motor activity was attenuated by early TEL-exposure. *B* Reactivity of animals to a suprathreshold stimulus was increased by TEL-exposure. *C* and *D* Successful avoidance responding was not altered by neonatal TEL-exposure; however, marked changes in responding during the intertrial interval (ITI) were observed in both the contingent and noncontingent conditions. *E* and *F* Mean paw lick latency indicated that TEL-exposure did not alter sensitivity to thermal stimuli. TEL animals did not habituate to the testing situation. The results of each task are depicted as normalized *Z*-scores; this facilitates direct comparison of the relative changes across all test data without concern for different response units.

environment by a second test, while, surprisingly, TEL-treated animals remained highly active (fig. 1A). Between-session habituation of motor activity was attenuated as a linear dose-response function of neonatal TEL [$F(1,119) = 11.7$, $p < 0.001$]. Early undernutrition per se does not account for the TEL-exposed animals' relative failure to habituate to the test environment [$F(1,119) = 6.8$, $p < 0.01$].

Auditory startle evaluations demonstrated a linear TEL dose-dependent increase in reactivity to an 8 kHz tone (fig. 1B). Reactivity of the animals to a suprathreshold auditory stimulus (8 kHz, 110 dB) varies as a linear dose-response function of TEL [$F(1,119) = 31.7$, $p < 0.001$]. This increased reactivity was not due to potential alterations in early nutritional status [$F(1,119) = 15.1$, $p < 0.001$]. These latter observations suggest that early TEL exposure induces a persistent, generalized hyperreactivity to environmental stimuli.

Subsequent assessment of the integrity of CNS function was provided by examination of cognitive and sensory processes in an attempt to define a locus of neurological dysfunction. We investigated the effects of neonatal TEL on acquisition of a two-way conditioned avoidance response, a classic index of cognitive function. Because of a potential disruption in performance resulting from generalized hyperreactivity, rather than from a cognitive impairment, pseudoconditioning controls were also included. All animals received 120 aquisition trials (day 107). For the conventionally trained group, a trial began with the initiation of the conditional (warning) stimulus, a nondirectional tone and a directional light cue, followed 5 seconds later by the onset of an unconditioned footshock stimulus. Responding prior to, or after, the onset of the unconditioned stimulus was scored as an avoidance or escape response, respectively. Animals in the pseudoconditioning group received noncontingent 'training', that is, presentation of the conditional stimulus was temporally uncorrelated with the unconditional stimulus[22]. All sessions contained 120 trials and began with a 2-min adaptation period. For the learning-memory condition, the intertrial interval was a variable 60-s schedule (35 to 85 s); for the random control condition, either a conditional or unconditional stimulus occurred, on average, every 30 (5 to 55) s. This random procedure was conservative in retaining an avoidance contingency which, if met, precluded delivery of the next scheduled shock.

No significant alteration in number of successful avoidance responses was observed as a function of TEL exposure. However, under both contingency conditions, TEL-exposed animals exhibited significantly enhanced intertrial interval responding (fig. 1C, D). As linear dose-dependent TEL effects were present for both the contingently trained [$F(1,115) = 10.5$, $p < 0.001$] and noncontingent control animals [$F(1,115) = 5.7$, $p < 0.001$], this increased responding did not reflect an alteration in avoidance learning, but reflected an increase in reactivity. This increased reactivity was not attributable to early nutritional status for either the contingent [$F(1,115) = 9.7$, $p < 0.002$] or noncontingent groups [$F(1,115) = 4.6$, $p < 0.03$]. Subsequent data indicated no detectable differences in flinch or jump thresholds suggesting the difference in intertrial interval responding was not due to altered sensory function. This differential response pattern suggested, once again, a predominant dose-dependent increase in the reactivity of TEL-treated rats to their environment.

One possible explanation for the hyperreactivity in the learning task would be an alteration in sensory function. Accordingly, we first examined tactile sensory detection thresholds using psychophysical techniques (day 114). Mean flinch thresholds of 0.076, 0.076, 0.078, and

0.074 mA and mean jump thresholds of 0.39, 0.38, 0.38, and 0.39 mA were noted for the vehicle, 4.5-TEL, 9.0-TEL, and undernourished controls, respectively. Second, we evaluated the animals' sensitivity to thermal stimulation using a hotplate 'analgesia' test (52.8 °C ± 0.2 °C). For six days prior to the test trial, one-half of the animals of each group were habituated to an ambient temperature apparatus. It was hypothesized that if both TEL-habituated and TEL-nonhabituated groups displayed altered hotplate response latencies, then potential alterations in thermal sensitivity existed. However, if alterations were observed only in the nonhabituated condition, then alterations in reactivity to the test stimuli would again be suspected. A dose-dependent increase in paw lick latency was observed (fig. 1E, F), but only for the habituated animals (day 136). Mean paw lick response latency (interrater reliability of > 0.95) indicated that TEL did not alter sensitivity to thermal stimuli. However, TEL-treated animals previously exposed to a nonfunctional hotplate over six consecutive days were markedly different from controls on the subsequent test day. These data again suggested that early TEL-exposed animals failed to show comparable habituation to controls when repeatedly exposed to the nonfunctional hotplate. This alteration was confirmed as a significant linear dose-response function for early TEL [$F(1,117) = 9.3$, $p < 0.003$] which was independent of early nutritional status [$F(1,117) = 5.6$, $p < 0.02$].

Collectively, these findings suggested that neonatal TEL-exposure produces long-term alterations in behavioral reactivity which are independent of sensory modality as well as early nutritional status. The unifying construct of generalized hyperreactivity suggested a marked similarity to those behavioral effects that often accompany septal-hippocampal lesions in adult animals [23]. The possibility of insult to this region was thus followed by a direct examination of CNS tissue. Thus, one-half of the animals from the behavioral study ($N_{subjects} = 64$) were randomly selected and sacrificed for analytical studies (day 140). Regional brain dissections [24] and subsequent analyses of lead levels were performed. Of the eight brain regions examined, only hippocampal wet weight was depressed in a linear dose-dependent manner in both sexes of TEL-exposed animals. We were unable to detect lead in any brain region of adult rats (< 2 ng/ml) [19]. The remaining animals from the behavioral study ($N_{subjects} = 64$) were sacrificed for quantitative neuromorphometric analyses (day 141). The brains were Paraffin-processed, sectioned (10 microns), and stained with Luxol Fast Blue/Cresyl Echt Violet. Morphometric measurements were made bilaterally, on coded and carefully matched slides for each of the 64 animals. For quantitative purposes, coronal sections were matched using several conspicuous subcortical landmarks, and measurements were obtained using an microscope eyepiece scale and stage micrometer. Regional measurements were made to determine the extent of general neural deficits [cortical thickness, corpus callosum width, and hippocampal width]. Neuropathological examination of serial sections through the entire brain of four TEL-treated animals found no gross pathology, and cell loss only in the hippocampal formation (R. M. Booze and M. K. Krigman, unpublished observations). Accordingly, the hippocampus was examined in detail with multiple intrahippocampal measurements (pyramidal cell field width and cell density in CA1, CA3a, and CA3c, dentate granule cell layer width and density, molecular layer width, and the intrahilar width).

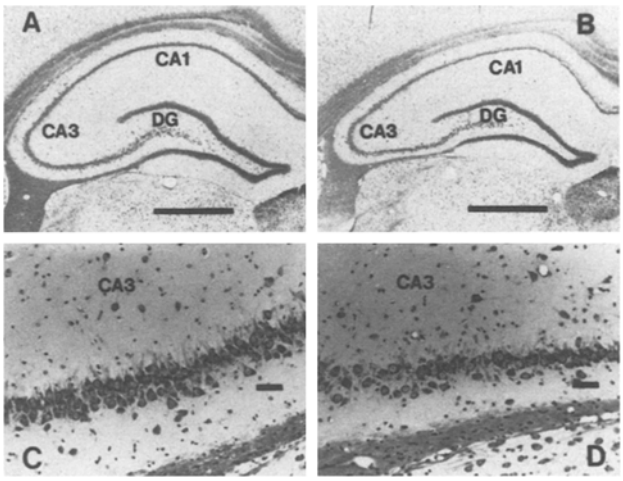

Figure 2. The whole hippocampal formation and close-up of area CA3c are illustrated for control (*A* and *C*) and 9.0 mg/kg TEL-exposed animals (*B* and *D*). A general thinning of the pyramidal cell layer and a corresponding reduction in pyramidal cell number is apparent. *A* and *B*, calibration bar = 1.0 mm. *C* and *D*, calibration bar = 0.05 mm.

The neonatal TEL treatment produced no decrements in general developmental parameters, i.e., corpus callosum or cortical widths (table 1). In contrast, despite the general anatomical intactness of the hippocampus (fig. 2B), significant reductions in hippocampal width were induced by TEL in a linear dose-dependent manner [$F(1,56) = 4.9$, $p < 0.03$] and were independent of early nutritional status [$F(1,56) = 6.5$, $p < 0.02$]. Intrahippocampal measurements (table 2) indicated significant linear dose-dependent TEL alterations for width of pyramidal cells fields CA1, CA3a, and CA3c [$Fs(1,56) > 24.0$, $ps < 0.001$] which were independent of nutritional status [$Fs(1,56) > 18.0$, $ps < 0.001$]. Significant linear dose-dependent TEL decreases in density estimates were also observed in pyramidal cell fields CA3a and CA3c [$Fs(1,56) > 41.0$, $ps < 0.001$] and were not due to early undernutrition [$Fs(1,56) > 34.0$, $ps < 0.001$]. A linear dose-dependent TEL-induced decrease in density of pyramidal cell field CA1 was also suggested [$F(1,56) = 6.4$, $p < 0.01$] and confirmed to be independent of early nutritional effects [$F(1,56) = 13.2$, $p < 0.001$]. The intrahilar width of the dentate gyrus and the width of the molecular layer were similarly affected; linear dose-dependent effects of TEL were noted [$Fs(1,56) > 34.0$, $ps < 0.001$] which were independent of

Table 2. Effects of neonatal triethyl lead exposure (TEL) on intrahippocampal indices of brain development upon maturation to adulthood

Parameter	Vehicle control	4.5 mg/kg TEL	9.0 mg/kg TEL	Undernourished control
CA1				
Width[a,b]	52 ± 1	58 ± 2	64 ± 2	51 ± 1
Density[a,b]	3.11 ± 0.06	3.25 ± 0.09	2.86 ± 0.05	3.04 ± 0.07
CA3a				
Width[a,b]	57 ± 2	49 ± 2	36 ± 2	52 ± 3
Density[a,b]	2.32 ± 0.08	1.97 ± 0.10	1.44 ± 0.07	2.13 ± 0.11
CA3c				
Width[a,b]	60 ± 2	47 ± 2	26 ± 1	54 ± 2
Density[a,b]	2.44 ± 0.08	1.82 ± 0.09	1.15 ± 0.04	2.14 ± 0.09
Dentate hilar				
Width[a,b]	523 ± 6	517 ± 6	444 ± 10	518 ± 10
Molecular layer				
Width[a,b]	289 ± 4	287 ± 4	259 ± 3	288 ± 3
Granular layer				
Width	78 ± 1	78 ± 1	78 ± 1	79 ± 1
Density	6.04 ± 0.11	6.11 ± 0.11	5.94 ± 0.08	6.06 ± 0.10

[a] Significant linear dose-dependent effect of TEL. [b] Effect of TEL (9.0 mg/kg) significantly different from nutritional controls. (See text for details and exact statistical values). Intrahippocampal measurements indicated that exposure to TEL during early development permanently compromised the integrity of the hippocampus. TEL produced substantial dose-dependent damage to pyramidal cell field CA3 and milder damage to pyramidal cell field CA1, but no damage to the granule cells. Width measurements represent the average of three adjacent, but not overlapping fields. Density was estimated by the number of cells subtended per measurement.

nutritional effects [$Fs(1,56) > 41.0$, $ps < 0.001$]. No alterations in width or density of the granule cells were detectable as a function of neonatal TEL [$Fs(1,56) < 1.0$]. Intrahippocampal morphometric measurements indicated preferential cell pathology-pyramidal cells were damaged, whereas granule cells appeared intact. Furthermore, these quantitative techniques confirmed that neonatal exposure to TEL resulted in a permanent, dose-dependent, loss of pyramidal cells in area CA3 of the hippocampus with a milder loss and spreading of CA1 pyramidal cells. The CA3 pyramidal cells form a major link in the excitatory pathway for the passing of information through the hippocampus with projections to CA1 pyramidal cells, the septum, and the contralateral hippocampal CA1 and CA3 pyramidal cells. The spreading of area CA1 pyramidal cells may be hypothesized to reflect a compensatory reaction to loss of the CA3 Schaffer collaterals that may normally provide an organizing influence on CA1. Recognizing the current views of hippocampal function as that of a 'supramodal' association area involved in the processing of information from all sensory modalities[25], the TEL-induced damage to hippocampal CA3 cell populations could reasonably account for the demonstrated compromises in the animal's behavior to many varied stimuli.

Hippocampal damage has been found to produce secondary damage in several interconnected brain regions, particularly in the dopaminergic basal ganglia systems[26] and the cholinergic projections within the septal-hippocampal pathway[27]. As neonatal exposure to TEL was found to produce preferential hippocampal damage, another series of studies examined the functional integrity of dopaminergic and cholinergic systems. Apomorphine (a centrally-acting dopaminergic receptor agonist) and scopolamine (a muscarinic cholinergic antagonist) were used as pharmacological probes of nervous system function. Animals were dosed with TEL as in the previous studies ($N_{subjects} = 104$), and allowed to mature to adulthood. The dopaminergic study involved two tests separated by a 7-day interval. Within each test day, a predrug-postdrug determination of motor activity was performed with predrug values obtained 15 min after saline injection and postdrug values obtained 15 min after apomorphine injection. In their first test the animals received either 0.1 or 1.0 mg/kg of apomorphine while in the second test the treatments were reversed. Although apomorphine (0, 0.1, 1.0 mg/kg) produced a dose-dependent increase in stereotypic behavior, there were no TEL-induced alterations in pharmacological drug sensitivity. A parallel study was conducted for the cholinergic receptor antagonists, scopolamine and methylscopolamine (peripherally-acting). Two weeks after apomorphine testing, the animals' response to scopolamine (1.0 mg/kg) and the quaternary ammonium derivative, methylscopolamine (1.0 mg/kg), was determined. Neonatal TEL-exposed animals were increased in sensitivity to scopolamine as indicated by their attenuated within-session habituation (fig. 3). An overall analysis of variance indicated a significant cholinergic drug by linear time interaction [$F(1,96) = 7.7$, $p < 0.007$]. Tests of simple main effects indicated this decreased rate of habituation following scopolamine varied as a linear function [$F(1,96) = 5.8$, $p < 0.02$] and was not accountable to either baseline differences or to the peripherally mediated cholinergic action of methylscopolamine. This increased sensitivity to scopolamine was also dissociable from any effect of neonatal TEL on early nutritional status [$F(1,96) > 6.4$, $p < 0.01$]. Thus, the long-term TEL-in-

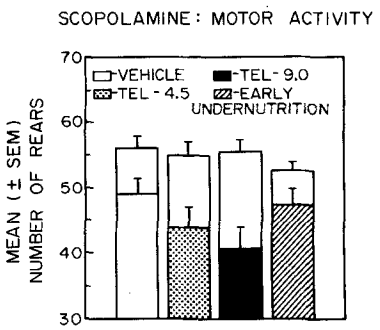

Figure 3. Mean rearing activity during the initial 5 min (upper bars) and mean decrease in rearing during the subsequent 10 min (lower bars) are shown under the influence of 1.0 mg/kg scopolamine. TEL-exposed animals were increased in sensitivity to scopolamine relative to controls as evidenced by a significantly greater increase in drug-induced motor activity. This facilitation was independent of early undernutrition effects, peripherally-mediated effects (methylscopolamine), and baseline differences.

duced alterations in behavioral reactivity appear to be mediated by central cholinergic, but not dopaminergic, systems.

In summary, we have demonstrated that an organolead compound preferentially damages a select area of the developing rat CNS: the hippocampus. Since no known replacement of neurons occurs in the mature CNS, the TEL-induced damage constitutes an irreversible injury. The mechanism by which TEL-induced hippocampal damage occurs remains to be investigated. We speculate that the TEL-induced damage is an excitotoxic mediated event, in which the hippocampal mossy fiber pathway plays a major role. Future studies can be directed at this hypothesis using more specific stains (i.e., Timm's stain) or focusing on the excitatory and inhibitory neurochemical systems within the hippocampus during early development.

The effects and extent of low-level organolead exposure on human brain development are at present largely unexplored. However, it has been estimated that organolead compounds account for at least 10% of the lead in the urban environment[28,29], and a significant fraction of lead in human brain tissue[11]. The present data also bear resemblance to the hippocampal damage induced by inorganic lead exposure, albeit this latter morphological damage occurs only after much higher doses of lead[30,31]. Although our experimental results do not permit direct extrapolation to chronic low-level human organolead exposures, they do indicate that organic lead gasoline additives pose a potent neurotoxic threat to the normal development and functioning of the CNS. The replication and extension of our results in other rat strains and additional species will provide important data for increasing the accuracy of risk estimation for human exposures.

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