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Effects of Triethyl Lead on Hot-Plate Responsiveness and Biochemical Properties of Hippocampus

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BONDY, S. C., J. S. HONG, H. A. TILSON AND T. J. WALSH. *Effects of triethyl lead on hot-plate responsiveness and biochemical properties of the hippocampus.* PHARMACOL BIOCHEM BEHAV **22**(6) 1007–1011, 1985.—Rats treated with a single dose of triethyl lead chloride (TEL) by subcutaneous injection (7.9 mg/kg) showed a transient increase in latencies to lick the hind paw during hot-plate testing. The time course of triethyl lead-induced antinociception was temporally associated with depressed binding capacity of benzodiazepine receptor sites and reduced levels of Substance P. Both of these changes appeared to be confined to the hippocampus and were not apparent in the cortex or striatum of treated rats. Met-enkephalin levels were not altered in any region studied at any time during the 21-day postdosing period. Lead levels within the brain were higher than blood levels 1 week after triethyl lead injection. Although changes in more than one factor may account for the antinociceptive effect of triethyl lead, the hippocampus seems especially vulnerable to this amphiphilic organometal.

Triethyl lead Antinociception Benzodiazepine receptor binding Hippocampus

TETRAETHYL and tetramethyl lead derivatives are commonly manufactured industrial materials. In addition to their widespread use as antiknock additives in gasoline, they are also used as wood preservatives, catalysts and stabilizers [6]. The major toxic metabolic product of tetraethyl lead is triethyl lead (TEL) which is formed by hepatic enzymes [4]. TEL is very toxic, the estimated lethal dose in humans being 0.25 g, and has a specifically neurotoxic effect [6]. Humans exposed to tetraethyl lead displayed several signs of neurotoxicity involving motor (tremor, weakness), sensory, emotional (irritability, depression) and cognitive (memory impairment) processess [6]. Although recovery of function has been reported in some cases, Razsudov [13] noted long-term alterations in intellectual capacity and decreased working ability in some patients previously exposed to tetraethyl lead.

Tilson *et al.* [18] reported that short-term exposure to TEL produced dose- and time-dependent alterations in the behavior of rats. One consistent observation was that TEL elevated latencies to respond to an aversive thermal stimulus (hot-plate, tail flick procedures) during the first two weeks after dosing. However, shock thresholds as determined by nose-poke operant titration procedure or the flinch-jump method were not affected. In a subsequent paper [20], several organometal compounds (trimethyl lead, triethyl and trimethyl tin), as well as TEL, were found to elevate latencies to respond to a noxious thermal stimulus. In an attempt to elucidate the possible neurochemical substrate for the neurobehavioral effects of TEL, Hong *et al.* [9] found time-related decreases in Met-enkephalin levels in the septum of TEL-exposed rats exhibiting antinociception. Subsequent experiments indicated that the antinociception produced by TEL could be attenuated by pretreatment with chlordiazepoxide and a relatively high dose (10 mg/kg) of naloxone. Because chlordiazepoxide alters reactivity to stressful conditions, one interpretation of the findings [9] is that TEL-induced alterations in responding to the hot-plate may represent an alteration in emotionality or reactivity to noxious stimuli; attenuation by naloxone also suggests a possible opiate involvement.

That chlordiazepoxide altered the responsiveness of TEL-exposed animals to the hot-plate, suggests that this neurotoxicant may have altered the function of benzodiazepine receptors. The behavioral and clinical potency of benzodiazepines correlate well with their affinity for the benzodiazepine receptor [1,2]. Moreover, it has been reported that intense short-term stress produced a biphasic alteration in ^{3}H-flunitrazepam binding in the hippocampus [11]. The purpose of this study was to compare the antinociception induced by TEL to various neurochemical measures that might be associated with responsiveness to noxious stimulation. In addition to benzodiazepine receptors, levels of the peptides Met-enkephalin and Substance P were determined. The hippocampus was emphasized in this study due to the susceptibility of this area to the neurotoxic effects of some alkyl lead compounds [15].

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METHOD

Male Fischer 344 rats, obtained from Harlan Industries (Indianapolis, IN), were used. Animals were housed under constant temperature (20±2°C) and humidity (50±10%). The colony was maintained on a 12-hr light/dark cycle, with lights on at 0700. Both laboratory chow (NIH Diet No. 31) and tap water were freely available. All animals were 90 to 120 days of age and weighed between 213 and 294 g at the beginning of the study.

Rats were injected subcutaneously with 75% of the LD_{50} of TEL; 7.9 mg/kg body weight [18] while controls received an equal volume of distilled water (1 ml/kg body weight). TEL chloride was obtained from Ventron Co., Danvers, MA. The purity, immediately prior to use, was 97–98% as judged by dithizone colorimetric analysis [5] (P. Chen, personal communication), the major contaminant appearing to be inorganic lead.

Behavioral Testing

A hot-plate apparatus was used for testing. The surface of the hot-plate was maintained at 57.5±0.2°C. Each rat was picked up by the tail and placed onto the surface of the apparatus which was enclosed within a small Plexiglas chamber in order to prevent escape. The time it took to lick one of the hindpaws was recorded to an arbitrary maximum of 45 sec. To minimize potential circadian variations in responsiveness, animals were always tested between 1300 and 1530 hr.

Neuropeptide Assay

The tissue levels of Met-enkephalin and Substance P were determined by radioimmunoassay (RIA) methods as described previously [7]. Briefly, tissue was homogenized in 2 N acetic acid and immersed in boiling water for 5 min. After centrifugation at 25,000 g for 20 min the supernatant fluid was lyophilized. The residue was then reconstituted in distilled water and aliquots were used for RIA. The specificity of antisera against Met-enkephalin and Substance P has been described in previous reports [7]. Fractionation of brain extract by column chromatography, followed by RIA with antiserum against Met-enkephalin, revealed that over 90% of the immunoreactivity appeared in the fraction where authentic Met-enkephalin was eluted [8,21]. This indicates that this antiserum does not cross-react with the enkephalin precursors or related peptides unless these larger molecules are trypsinized [21]. Using the same method, we found that over 90% of Substance P immunoreactivity of brain tissue extract represented authentic Substance P. The recovery calculated by adding a known amount of peptide into brain extract was over 90%.

Benzodiazepine Receptors

A crude membrane fraction was prepared from brain regions by homogenization of tissue in 19 vol of 0.32 M sucrose followed by centrifugation (50,000 g, 10 min). The precipitate from this step was then homogenized in distilled water, pH 7.4, and recentrifuged. The final pellet was suspended in 40 mM Tris-HCl buffer, pH 7.4, at a concentration representing 50 mg original tissue/ml. Binding incubations were carried out in triplicate in a final volume of 1 ml containing 40 mM Tris-HCl, pH 7.4, containing 10^{-9} M[^{3}H-methyl] flunitrazepam (77 Ci/mM). The amount of tissue used per tube corresponded to 5–10 mg original wet weight and contained 300–400 μg membrane protein as determined by Lowry *et al.* [10]. At the end of a 15-min incubation at 0°C samples were filtered on glass fiber disks (25-mm diameter, 0.3 μm pore size, Gelman Inc., Ann Arbor, MI) and washed twice rapidly with 5 ml Tris buffer. Filter disks were then dried and counted in 5 ml of a scintillation mixture using a Packard Tri-Carb 2660 scintillation counter at an efficiency of 38–43% in order to determine membrane bound radioactivity. Control incubations were carried out simultaneously with the experimental series containing unlabeled competing ligand in order to determine the extent of nonspecific binding. The final concentration of benzodiazepine in control incubations was 10^{-6} M. Specific binding was taken to be that binding that was displaced in the presence of this large excess of the competing compound.

TABLE 1

BENZODIAZEPINE RECEPTORS IN RAT BRAIN REGIONS AT VARIOUS TIMES AFTER SUBCUTANEOUS INJECTION OF TRIETHYL LEAD

Time after dosing		Hippocampus	Frontal cortex	Striatum
		(pmoles ^{3}H-flunitrazepam bound per g protein)		
1 day	Control	338 ± 34	432 ± 25	764 ± 75
	TEL	289 ± 22*	460 ± 36	759 ± 25
21 days	Control	346 ± 36	435 ± 25	761 ± 48
	TEL	359 ± 39	424 ± 34	706 ± 37

Values represent means from 6 animals/group ± SE. TEL dose was 7.9 mg/kg body weight.

*$p<0.05$ that value differs from corresponding control (Fisher's least significant difference test following two-way ANOVA for overall significance).

Lead Analysis

Lead concentrations in blood and brain were carried out by Environmental Science Associates, Bedford, MA, using anodic stripping voltametry methods. Samples were randomized and coded.

Statistical Analysis

Differences between groups were assessed using Fisher's least significant difference test after a two-way analysis of variance [12]. The accepted level of significance in all cases was $p<0.05$ using a two-tailed distribution. Each data point represents values derived from six to eight individual animals. In the case of the lead levels in blood and brain, overall significance was determined using the Kruskal-Wallis one-way analysis of variance [16]; differences between means were determined using Mann-Whitney U-tests.

RESULTS

The binding of ^{3}H-flunitrazepam to membranes of various brain regions was assayed in rats 1 and 21 days after a single injection of 7.9 mg TEL chloride (Table 1). A significant depression of the amount of label bound was apparent in hippocampal membranes 1 day after dosing. This was regionally specific in that no parallel change was found in

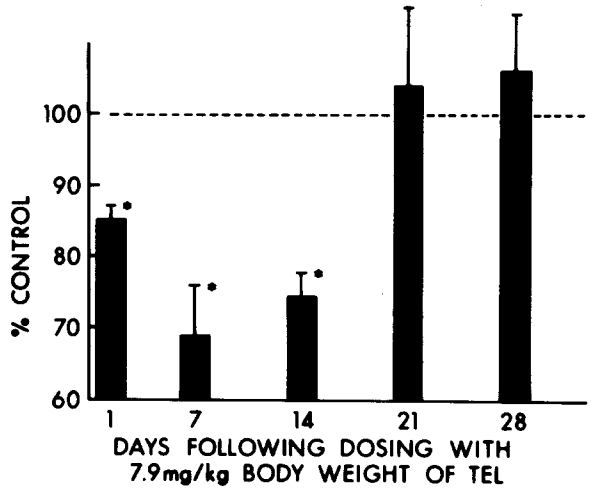

FIG. 1. Binding of flunitrazepam to hippocampal membranes of triethyl-lead-treated rats. Animals received a dose of 75% of the LD_{50} of TEL by subcutaneous injection. Data are presented as a percentage of saline injected controls for each test day. Control values ranged between 96 and 128 pmoles flunitrazepam bound/g protein. *$p<0.05$ that value differs from the corresponding control.

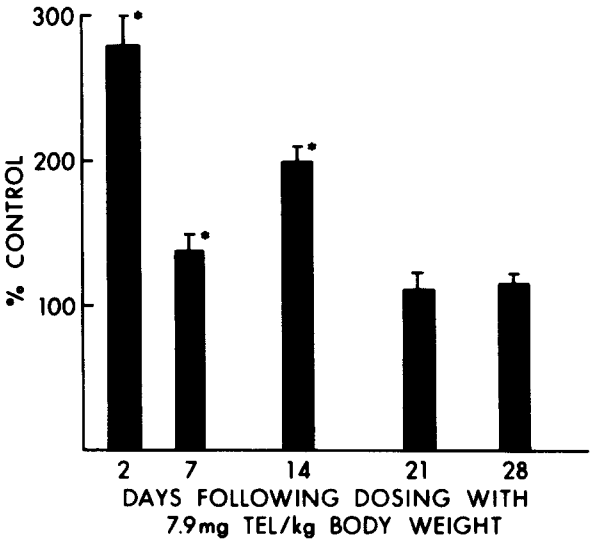

FIG. 2. Hot-plate latencies of triethyl-lead-treated rats. Animals received 7.9 mg/kg TEL by subcutaneous injection. Data are presented as a percentage of saline-injected controls for each test day. Control values ranged between 9.5 and 10.9 sec. Bars represent standard errors. *$p<0.05$ that value differs from the corresponding control.

striatum or frontal cortex. By 21 days after injection, the binding intensity in TEL-treated rats did not differ significantly from control values in any of the three regions studied. A more detailed time course showed hippocampal benzodiazepine receptor binding to be depressed at 2, 7 and 14 days postdosing after which time values returned to the control range (Fig. 1). The latencies to respond on the hot-plate were significantly increased in those animals having decreased benzodiazepine binding in the hippocampus (Fig. 2). Responsiveness to the hot plate was the same as controls when receptor binding had returned to control levels at 21 and 28 days postdosing.

Neuropeptide assays revealed a significant depression in hippocampal Substance P content at 7, 14 and 21 days after TEL dosing while frontal cortical levels of Substance P were unchanged by treatment at all time points studied (Table 2). Corresponding cortical and hippocampal levels of Met-enkephalin were also unchanged.

Blood and brain levels of lead were determined 1 week after dosing with TEL (7.9 mg/kg body weight). For comparative purposes, such levels were also measured after a subcutaneous injection of lead nitrate at a much higher dose (100 mg/kg). After TEL injection, blood levels of TEL were very high and brain levels were around 27% of the blood values (Table 3). Lead nitrate administration resulted in a 100% increment of blood lead while brain levels were approximately 20% of blood levels.

DISCUSSION

A single dose of triethyl lead (TEL) given subcutaneously to rats decreased benzodiazepine receptor binding in the hippocampus, but not frontal cortex or striatum, 24 hours postdosing. Subsequent experiments demonstrated that hip-

TABLE 2

PEPTIDE LEVELS IN HIPPOCAMPUS AND FRONTAL CORTEX AFTER A SINGLE INJECTION OF TRIETHYL LEAD CHLORIDE (7.9 mg/kg BODY WEIGHT)

Region	Time after TEL-dosing (days) 7	14	21
Hippocampus			
Met-enkephalin			
Control	1.35 ± 0.07	1.35 ± 0.09	1.46 ± 0.08
TEL	1.46 ± 0.08	1.36 ± 0.08	1.34 ± 0.04
Substance P			
Control	0.36 ± 0.01	0.38 ± 0.01	0.42 ± 0.03
TEL	0.32 ± 0.01*	0.34 ± 0.01*	0.36 ± 0.01*
Frontal Cortex			
Met-enkephalin			
Control	1.09 ± 0.04	1.14 ± 0.06	1.22 ± 0.05
TEL	1.11 ± 0.06	1.06 ± 0.02	1.18 ± 0.03
Substance P			
Control	0.31 ± 0.01	0.32 ± 0.01	0.29 ± 0.03
TEL	0.28 ± 0.01	0.31 ± 0.08	0.37 ± 0.04

Data are presented as the mean from 6 animals/group ± SE. Values are ng peptide/10 mg wet tissue.

*$p<0.05$ that value differs from corresponding control (Fisher's least significant difference test following two-way ANOVA for overall significance).

TABLE 3

BLOOD AND BRAIN LEAD LEVELS, 1 WEEK AFTER SUBCUTANEOUS INJECTION OF A LEAD COMPOUND

	Blood	Brain
	(μg/100 g tissue)	
Controls (saline injected)	5.2 ± 1.5	2.5 ± 0.8
Lead nitrate (100 mg/kg body weight)	27.2 ± 3.0*	5.4 ± 0.5*
Triethyl lead (7.9 mg/kg body weight)	775.0 ± 131.0*†	210.0 ± 12.3*†

Each value is the mean from 5 animals ± SE.
*Differs from saline injected controls ($p<0.05$).
†Differs from corresponding lead nitrate value ($p<0.05$).

pocampal benzodiazepine receptors were decreased 2, 7 and 14 days postdosing and were at control levels at 21 and 28 days after dosing. These decreases in receptor binding were associated temporally with increases in latencies to respond on a hot-plate (i.e., antinociception).

The results obtained in the present experiment are in accord with work in which it was suggested that TEL increased latencies to respond to the hot-plate possibly by disrupting the normal function of limbic forebrain areas involved in the control or modulation of emotional reactivity [9]. This previous research found that short-term repeated administration of TEL decreased septal Met-enkephalin in animals having increased hot plate latencies. Furthermore, it was shown that the behavioral effect produced by TEL could be attenuated by a relatively high dose of naloxone (10 mg/kg) or pretreatment with chlordiazepoxide, a benzodiazepine anxiolytic. However, we were not able to reverse the analgesia caused by a single TEL dose (7.9 mg/kg) with chlordiazepoxide (data not shown).

One interpretation of the results of the present data is that TEL-induced increases in hot-plate latencies are associated with alterations in benzodiazepine receptors in the limbic forebrain. Studies using ^{3}H-flunitrazepam have indicated that benzodiazepine receptors are relatively more concentrated in brain areas thought to participate in the sensory and emotional processing of environmental events, including the modulation of behavioral reactivity to those stimuli [19]. Medina *et al.* [11] reported that acutely stressed rats showed a time-dependent decrease followed by an increase in benzodiazepine binding in the hippocampus; results from our laboratory have also shown that RO15,1788, a selective benzodiazepine antagonist, increases hot plate latencies in the absence of effects on neuromotor function (Walsh *et al.*, submitted). Moreover, acute or repeated stress has been shown to produce antinociception by nonopiate, as well as opiate mechanisms [3].

Acute exposure to TEL also decreased Substance P in the hippocampus at 7, 14 and 21 days postdosing; Substance P in the frontal cortex was not affected at any time after dosing. Changes in hippocampal Substance P were generally associated in time with decreased reactivity to the hot-plate. These data are in accord with those reported by Hong *et al.* [9] who found that Substance P was decreased in the hippocampus in rats 8 days after receiving 5 daily injections of 1 mg/kg of TEL; effects on Substance P were not observed in the striatum, frontal cortex, septum, hypothalamus or pituitary. As in the case of repeated dosing with TEL [9], alterations in Met-enkephalin in the hippocampus and frontal cortex were not seen following acute exposure.

The data with Substance P are interesting in view of the fact that this peptide is not found in intrinsic neurons of the hippocampus, but only in afferents terminating in the pyramidal layer of CA2 and 3 regions [14]. Thus, TEL-induced effects on hippocampal Substance P suggest impairment of normal inputs to the hippocampus. The present results with hippocampal Substance P are also of interest in view of previous data showing that Substance P in the spinal cord was not altered at a time when TEL-exposed rats exhibited elevated latencies to respond on the hot plate [9]. It has been proposed that Substance P is involved in the transmission of impulses from nociceptive afferents in the dorsal horn of the spinal cord [17]. Although evidence is rapidly accumulating that Substance P can modulate neural activity in the central nervous system, its function is not fully understood. Our data showing that hippocampal Substance P is altered at a time when animals have elevated latencies to respond to a noxious thermal stimulus suggest that Substance P may modulate or relay information concerning noxious stimulation at the central level as well as in the spinal cord. Additional work needs to be done to clarify this issue.

In summary, the acute administration of TEL produced time-dependent alterations in hippocampal benzodiazepine receptor binding intensity and levels of Substance P; these biochemical changes were generally associated in time with increased latencies to respond to a noxious thermal stimulus. These data and those from Hong *et al.* [9] suggest that TEL alters emotional reactivity to aversive environmental stimuli possibly by altering systems in the limbic forebrain. The antinociception produced in experimental animals following treatment with TEL cannot clearly be attributed to a single biochemical parameter but seems to involve several loci.

The penetrance of TEL into the bloodstream from the subcutaneous injection site was high, while a much larger dose of inorganic lead had much less of an effect on circulating levels of lead. This large difference is probably due to the tendency of inorganic lead to form insoluble salts and remain at the injection site while the amphiphilic TEL may be able to pass more rapidly across membranes. It is probably this latter characteristic that allows TEL to pass more readily than inorganic lead through the bloodbrain barrier. Brain lead levels increased to 27% of the blood values in the case of TEL, while the increased level of inorganic lead in brain was only around 11% of the increase of blood lead over background.

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