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Intrahippocampal Administration of Lead (Pb) Impairs Performance of Rats in the Morris Water Maze

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JETT, D. A., A. C. KUHLMANN AND T. R. GUILARTE. *Intrahippocampal administration of lead (Pb) impairs performance of rats in the Morris water maze.* PHARMACOL BIOCHEM BEHAV **57**(1/2) 263–269, 1997.—We examined spatial learning in the Morris water maze after daily acute bilateral micro-injection of 13.9 ng sodium acetate (NaAc) or 37.9 ng lead acetate (PbAc) in 1 μl volumes into the dorsal hippocampus of normal adult rats. After six days of injections and water maze training, rats injected with NaAc were able to find a hidden platform in 8.3 s, and those injected with PbAc were significantly slower (15.2 s; $p < 0.02$). In a second experiment, rats were trained to find a hidden platform before injections began and then tested in order to determine if intrahippocampal injections of Pb affected the recall of a previously learned task. The escape latency on the first day after injections began was increased slightly when compared to the last day of training before injections, however the NaAc and PbAc groups were not significantly different over three days of injections. Both treatment groups performed as well as they did before injections began by the second day of injections. These results suggest that the direct injection of Pb into the hippocampus impairs the acquisition, but not the recall of the spatial learning task in the Morris water maze. © 1997 Elsevier Science Inc.

Lead Hippocampus Micro-injection Rats Morris water maze Learning

THERE has been renewed interest in lead (Pb) neurotoxicity because of an urgent need to delineate the underlying mechanisms of cognitive deficits observed in children with blood Pb levels as low as 10 μg/dL, a level much lower than what was previously thought to be toxic (30). It has been suggested that there may not be a threshold for the neurotoxic effects of this ubiquitous toxicant (28). Chronic low-level exposure to Pb during development results in learning impairments in both children (8) and experimental animals (5). Furthermore, it appears that children are more sensitive to Pb intoxication than adults (24). There is considerable evidence to suggest that many of the neurochemical (14), neuroanatomical (1), and neurophysiological (4,16) sites that are sensitive to Pb also play crucial roles in spatial learning and memory in the Morris water maze (18,21). For example, Pb inhibits *N*-Methyl-d-Aspartate (NMDA) receptor function (2,10,11, 13), and the Morris swim task appears to be extremely sensitive to pharmacological manipulation of this receptor-ion channel complex (18).

It has been proposed that there are two distinct mechanisms of Pb neurotoxicity (29). First, Pb interferes with the development and differentiation of the central nervous system, and second, Pb impairs cognitive processes by direct pharmacological interaction with specific sites that are important in neurotransmission. We have previously shown that rats which were exposed to Pb during development were significantly impaired in the Morris swim task (15). The impairment in this behavioral task is presumably due in part to the known disruptive effects that Pb has on the developing hippocampus (1,14), a structure known to be essential for spatial learning (22).

It is not known whether the deficits in spatial learning can be observed in normal adult rats exposed acutely to Pb. Furthermore, it is not known if direct hippocampal administration of Pb alters behaviors known to be associated with this brain structure. In the present study, we tested these hypotheses by administering Pb to normal adult rats by bilateral microinjection into the dorsal hippocampus, and examining spatial learning and memory in the Morris water maze.

METHOD

Subjects and Pb Exposure

Adult male Long–Evans rats (58–60 days old) with cannulae surgically implanted bilaterally into the dorsal hippocam-

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TABLE 1

[^{3}H]-PK11195 AUTORADIOGRAPHY IN NaAc AND PbAc-INJECTED RATS USED IN THE MORRIS SWIM TASK

	Experiment 1		Experiment 2	
	fmol/mg tissue	*N*	fmol/mg tissue	*N*
Na Acetate	91.8 ± 7.4	7	93.9 ± 10.4	7
Pb Acetate	103.5 ± 7.3	9	89.2 ± 8.0	8

Given are the mean ± SEM specific binding measured bilaterally in the hippocampus of rats in the two treatment groups.

pus were purchased from a commercial laboratory (Zivic-Miller, Pittsburgh, PA). These rats were housed individually and maintained at 23°C on a 12/12 h light/dark cycle. Food and water were allowed ad lib. Stereotaxic coordinates of the cannula were 3.8 mm posterior to bregma, 2.2 mm lateral to the midline, and 3.2 mm ventral to the skull surface of bregma, according to Paxinos and Watson (27). Rats were allowed to recover for seven days after surgery before injections and behavioral testing began. Sodium acetate (NaAc) or Pb acetate (PbAc) were dissolved in saline to make equimolar (100 μM) solutions at pH 6.9. Solutions were made daily and 1 μl containing 13.9 ng NaAc or 37.9 ng PbAc was injected bilaterally into each hippocampus of unrestrained rats at a flow rate of 0.5 μl/min using a CMA/120 micro-injection system for freely-moving animals (Bioanalytical Systems, Inc., West Lafayette, IN). The initial side of injection was alternated daily for each rat. One minute was allowed for diffusion of the solution before removing the cannula, and a 20 min post-injection period occurred before beginning behavioral testing. At the end of the study, the rats were euthanized by decapitation and the brain removed for histological examination with cresyl violet staining and [^{3}H]-PK11195 autoradiography.

Experiment 1

Spatial learning and memory was assessed using the Morris water maze (22). A pool 1.8 m in diameter and 0.7 m deep was situated in a room containing several pictures and objects around the periphery to serve as extramaze cues. The pool was filled with water made opaque with non-toxic white paint (Van Aken Int., Cucamonga, CA). Water temperature was maintained at 20 ± 2°C with aquarium heaters. Rats were acclimated to the room and the testing procedure on the day before testing by allowing them to swim for 60 s in the pool. On the first day of testing, NaAc and PbAc injections were made according to the above procedures and the behavioral session began by allowing the rats to orient to visual cues around the test room while resting for 20 s on the hidden platform positioned 15 cm from the edge of the pool in one of the quadrants. The platform remained in the same position for the entire study. This is typically referred to as a test for reference or long-term memory. The rat was then placed in the pool facing the wall in one of four quadrants and allowed to swim to the platform. A maximum trial length of 60 s was used, i.e., if the platform was not found within the allotted time, the rat was manually placed onto the platform. This procedure was repeated with each rat from starting positions in all four quadrants in random order alternating between NaAc (N = 7) and PbAc (N = 9) treated rats. The rat was allowed a 20 s rest period (inter-trial interval) between each trial and at the end of a session. Bilateral injections and water maze testing were conducted daily for the duration of the study. A session of four trials was conducted each day from 8:00–11:00 A.M. for each rat, and rats were tested on consecutive days until the study was completed.

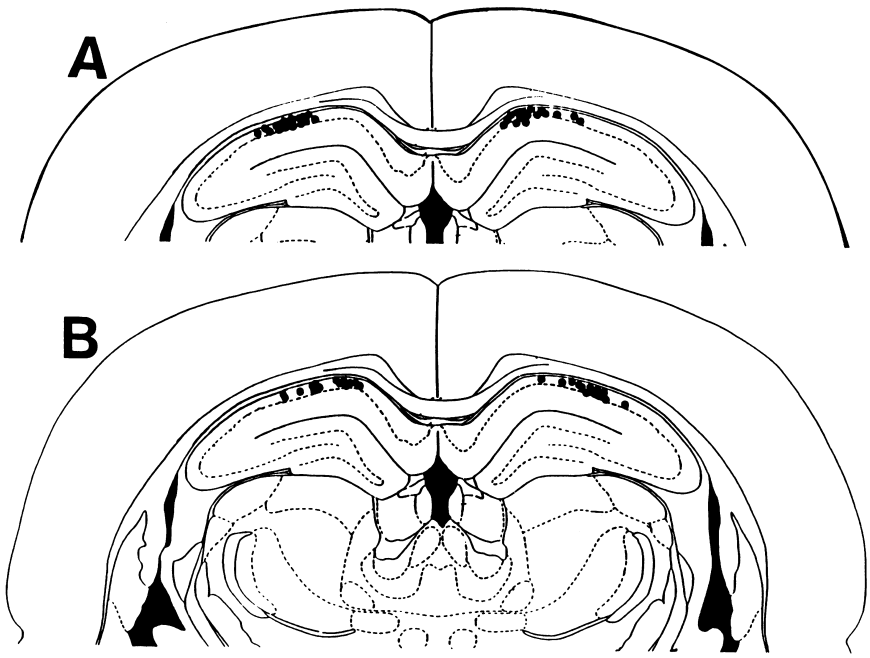

FIG. 1. Diagram of the cannula placements for rats used in swim task experiments 1 (A) and 2 (B). Each filled circle represents an area 1 mm below the tip of the cannula guide. This is the estimated location of the injection site.

Experiment 2

The procedures in this experiment followed those in experiment 1 except that the NaAc and PbAc injections were not made until after the criterion of finding the platform in ≤10 s was achieved. This occurred on the sixth day of the swim task training, and intrahippocampal injections began on day 7. Rats were randomly assigned to two groups, one that would receive NaAc, and one to receive PbAc after training. Rats in both groups (see Table 1 for sample sizes) were then injected and tested in the swim task daily, with the platform remaining in the same position as during the training period. In both experiments, the escape latency (time to the hidden platform) and pathlength (distance to the hidden platform) were recorded using a Videomex-V Image Analyzer (Columbus Instruments, Columbus, OH).

Histology and Autoradiography

At the end of both sets of behavioral experiments, the rats were euthanized by decapitation and the brains frozen at −70°C. Coronal 20 μm brain sections were obtained with a cryostat microtome from all of the rats and stained in 0.5% cresyl violet. These sections were subjected to gross and microscopic histological examination, noting any evidence of cell damage or loss. Sections were also used for [^{3}H]-PK11195 autoradiography. This ligand binds to the peripheral benzodiazepine receptor, a sensitive indicator of reactive gliosis resulting from chemical or physical brain cell damage (9,19). The autoradiography method has been previously described (9). Briefly, brain sections were incubated in 50 mM Tris-HCL buffer, pH 7.4 containing 1 nM [^{3}H]-PK11195 (85.5 Ci/mmole, NEN) for 30 min at 25°C. Non-specific binding was determined with 10 μM PK11195 in adjacent brain sections. After rinsing with ice cold Tris-HCL (2 × 5 s) and deionized water (2 × 2 s), sections were dried under cool air, desiccated overnight, and apposed to [^{3}H]-Hyperfilm (Amersham) along with [^{3}H]-Microscale (Amersham) standards of known radioactivity. The resulting autoradiograms were digitized using a MCID Image Analysis System and quantitated using the NIH Image 1.55 analysis computer program.

Direct measurement of the extent of diffusion by Pb injected into the hippocampus was not possible due to the difficulty and expense in obtaining radioactive Pb in order to generate autoradiographic images of the diffusion of the Pb solution after injection. However, the extent of the diffusion was estimated by less direct means. For example, two rats that

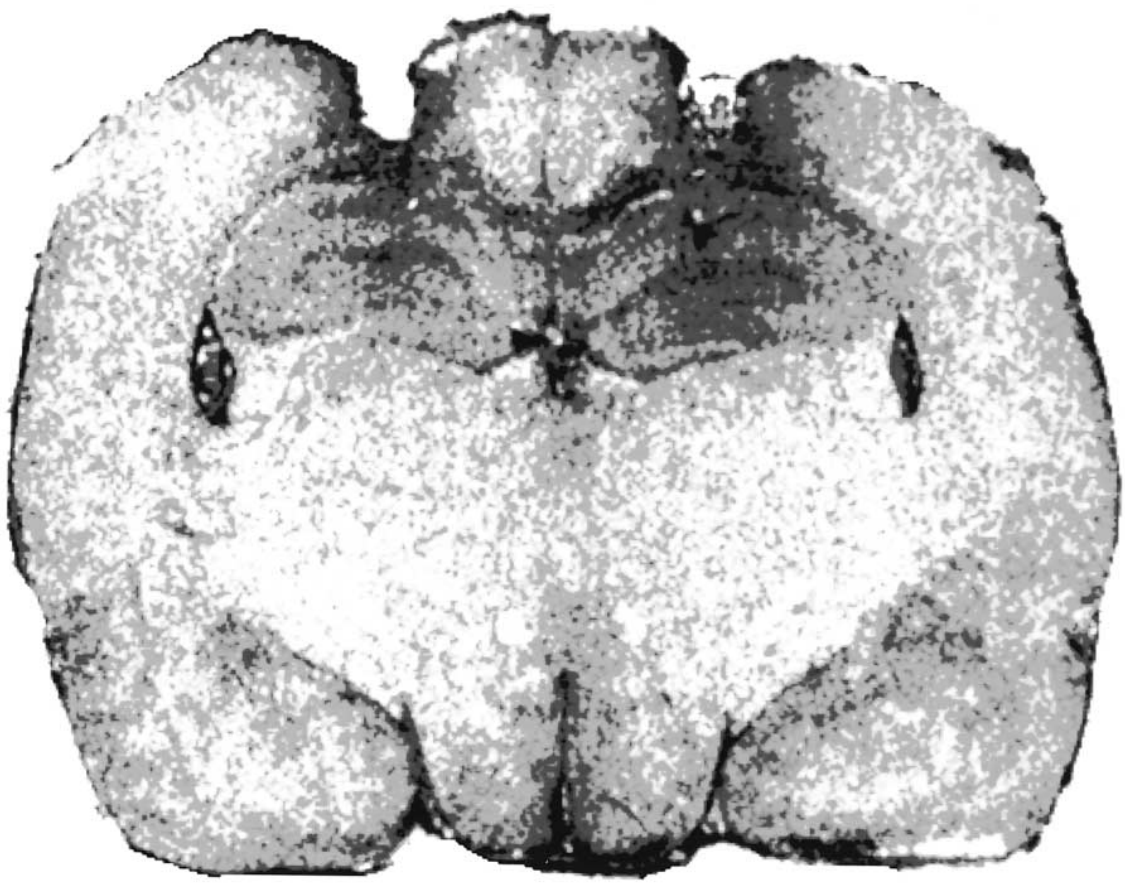

FIG. 2. Representative autoradiogram of [^{3}H]-PK11195 binding to 20 μm coronal brain section taken from a rat used in the Morris swim task that was injected with PbAc. The darker red areas in the hippocampus and around the areas of cannula guide insertion represent increased levels of [^{3}H]-PK11195 binding. Cannula guides extended from the surface of the brain to the dorsal hippocampus. Yellow areas within the brain neuropil represent low levels of binding. High levels of [^{3}H]-PK11195 binding are normally present in the choroid plexus and in the ventricles.

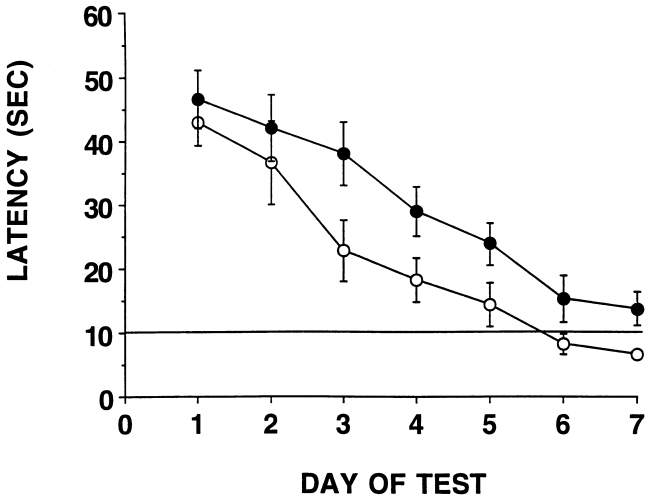

FIG. 3. Escape latency of rats used in experiment 1 that were injected bilaterally with NaAc (n = 7; open circle) or PbAc (n = 9; closed circle). Rats were tested in four trials daily, 20 minutes after intrahippocampal injections for seven consecutive days. Each point represents mean ± SEM of nine rats. The solid line indicates the escape latency criteria of ≤ 10 s.

were subjected to the same daily PbAc injections in experiment 1 (7 days), were injected with cresyl violet to determine the area of diffusion at the end of the experiment (day 7). The injection volume and flow rate was identical as with PbAc, and the rats were euthanized 20 min after injections. After obtaining 20 μm coronal sections of the brains from these rats, they were stained briefly with cresyl violet to outline structural landmarks. The extent of diffusion was determined by examining the much darker stained injected dye. Also, the extent of diffusion was estimated by the cannula placement determinations and the extent of the area in the hippocampus with increased [^{3}H]-PK11195 binding as determined from the autoradiographic images (see Fig. 2).

Statistical Analysis

The average escape latency of four daily trials was used as a measure of performance in the swim task for each rat on a given day. Latency data were analyzed using one-way repeated measures analysis of variance (RANOVA), with Treatment as the main effect, and Day as the repeated measure. Probe, visual cue, and swim speed data were analyzed by a one-way ANOVA, as were body and brain to body weight ratios. Student's *t*-tests were used for comparison of individual means.

RESULTS

There were no significant effects of Pb injections on body weight: (day 0: NaAc = 273.4 ± 7.2 gm, PbAc = 271.0 ± 2.9 gm; day 8: NaAc = 283.9 ± 9.0, PbAc = 278.5 ± 4.1 gm), or on the whole brain wet weight to body weight ratio at the time of euthanasia (NaAc = 0.73 ± 0.02, PbAc = 0.73 ± 0.02). The cannula placements were determined for each animal by examining sections stained for histopathology. The placement of the cannula tip was localized to the CA1 subfield of the dorsal hippocampus for all rats used in experiments 1 and 2 (Fig. 1). Histological microscopic examination of the injected rats indicated that in both PbAc and NaAc-injected rats, there

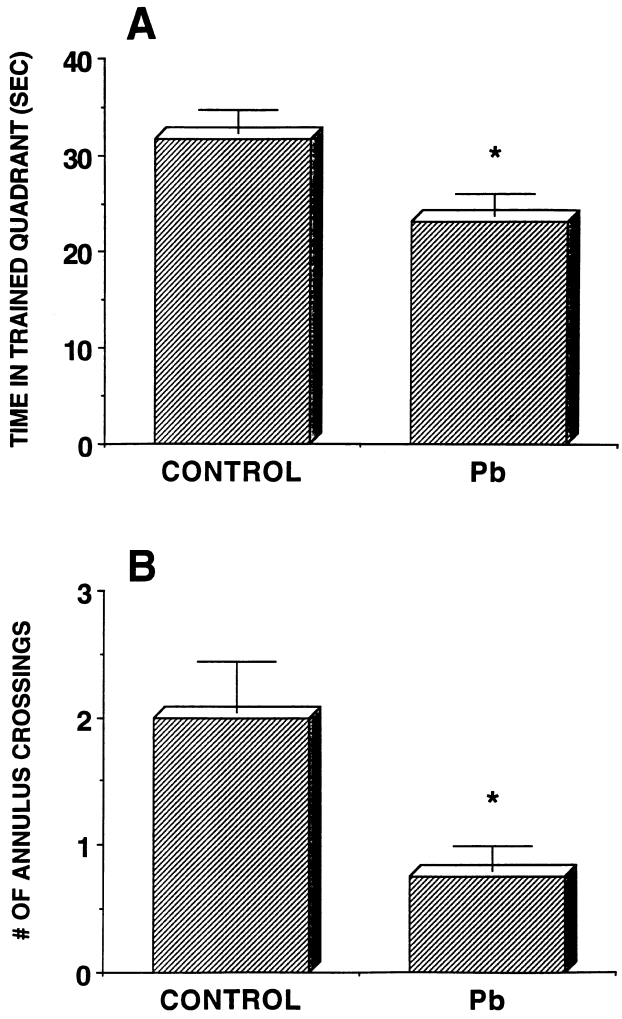

FIG. 4. Time (sec) spent in the quadrant of the pool in which rats injected with NaAc (n = 7) or PbAc (n = 9) were trained to find a hidden platform (A), and the number of times rats crossed the former position of the platform (annulus) (B), during probe tests (see text for procedure). Each bar represents mean ± SEM. *$p < 0.05$.

was damage localized to the site of cannula insertion, but there were no histopathological changes present that could be associated with the Pb exposure only. As expected severe damage was associated with the physical disturbance of the cannula guide.

Quantitative autoradiography of [^{3}H]-PK11195 binding to peripheral benzodiazepine receptors was used as a biomarker of reactive gliosis (9, 19) and the findings supported the histopathological results. The pattern of [^{3}H]-PK11195 binding indicated that neuronal damage-induced reactive gliosis was limited almost entirely to the hippocampal formation, with increased binding nearer to the site of the cannula tip (Fig. 2). The average level of specific [^{3}H]-PK11195 binding from bilateral measurements taken from the hippocampus were essentially the same for both NaAc and PbAc-injected rats in experiments 1 and 2 (Table 1). The area of diffusion was estimated from dye-injected rats to extend into the CA1 oriens and radiatum layers, however the [^{3}H]-PK11195 autoradiograms indicate that repeated injections of either NaAc or

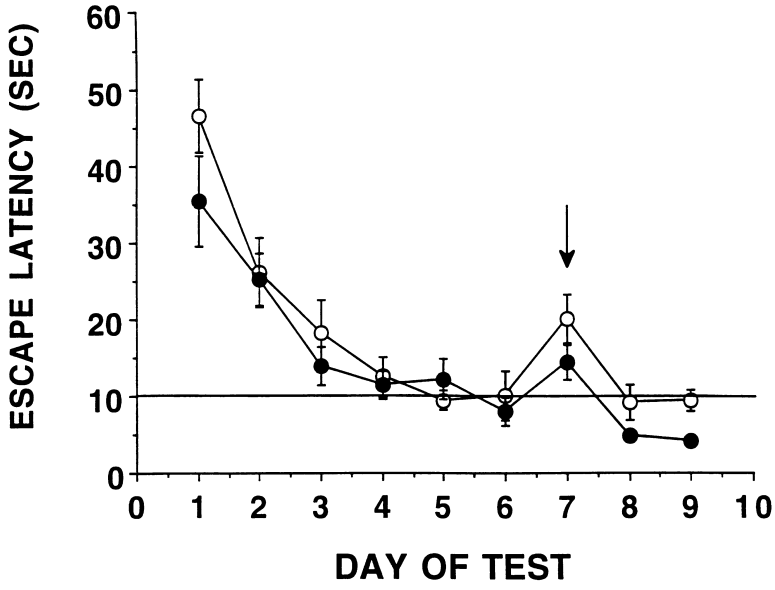

FIG. 5. Escape latency of rats used in experiment 2 that were injected bilaterally with NaAc (n = 7; open circle) or PbAc (n = 8; closed circle). Rats were tested in four trials daily. Injections began on day 7 (indicated by arrow). Each point represents mean ± SEM. The solid line indicates the escape latency criteria of ⩽10 s.

PbAc may have resulted in more extensive diffusion, but still limited to the hippocampal formation (Fig. 2).

Experiment 1: Acquisition

Preliminary water maze studies were conducted to determine the criterion to be used for the Morris swim task, i.e. the average minimum time to find the platform by normal rats. This criterion was determined to be ⩽10 s, and was achieved in 5–7 days (data not shown). Rats injected with NaAc (control group) were able to reach criterion in six days, whereas those injected with PbAc were slower (Fig. 3). Repeated measures analysis of variance (RANOVA) indicated that the latency of rats in the PbAc-injected group was on average 8.4 s greater than those injected with NaAc [$F(1, 14) = 7.1$; $p < 0.02$].

These results were supported by probe tests in which rats were allowed to swim for 60 s in the pool with the platform removed and the time and distance in each quadrant were recorded automatically. The rats were tested in the same randomized order as with the acquisition phase. The probe test was conducted after the experiment was terminated (day 8). Rats in the control group spent 9.8 s longer than Pb-injected rats in the quadrant in which training occurred (one-way ANOVA, $F(1, 14) = 8.5$; $p < 0.02$] (Fig. 4A). Additionally, the exact location of the platform was delineated digitally (annulus) during these probe tests. Significantly fewer platform annulus crossings during probe tests were observed in Pb-injected rats [one-way ANOVA, $F(1, 14) = 5.1$; $p < 0.04$] (Fig. 4B).

In order to test for potential differences in visual, motor, or motivational function resulting from the intrahippocampal injections, we conducted visual cue tests in which a black flag was extended above the platform to serve as a clear visual marker of its position. Rats were then placed in the opposite quadrant (relative to the platform) and released into the pool. This procedure was repeated in all four quadrants. Rats immediately swam to the platform, and latencies of NaAc (control) (7.0 ± 1.0 s) and PbAc-treated (9.1 ± 1.6 s) rats were not significantly different. Swimming speed during the probe test was calculated from recorded distances and the treatment groups were not significantly different by one-way ANOVA (NaAc = 21.9 ± 0.7 cm/s; PbAc = 20.4 ± 0.6 cm/s). Swimming speed for the two groups was also determined during the acclimation period before testing in the water maze began (day 0), and NaAc (23.2 ± 0.8 cm/s) and PbAc-injected (21.4 ± 1.0 cm/s) were not significantly different. These data indicate that the deficits in the Morris swim task could not be accounted for by differences in swimming speed, motivation to complete the task, or by visual impairment.

Experiment 2: Recall

In a second experiment, we tested whether intrahippocampal PbAc injections could affect the recall of a previously learned platform position in the water maze. Rats were injected as in the first experiment except that injections began after six days of training when the ⩽10 s criterion was met by both treatment groups (Fig. 5). On the first day of injections (day 7), both NaAc and PbAc-injected rats had similar escape latencies, but took several seconds longer to find the platform than during testing on the previous day (Fig. 5). This effect was observed equally in both treatment groups, and was not present on the following two days (days 8–9) when both groups were well under the ⩽10 s criterion (Fig. 5). Thus, the study was terminated since the criterion was met after 2–3 days of intrahippocampal injections.

DISCUSSION

These studies indicate that: (i) micro-injection of Pb into the hippocampus of normal adult rats caused significant impairment of performance in the Morris water maze, and (ii) the effects of Pb appear to be limited to acquisition of the

task, rather than recall of a previously learned task. These learning deficits observed after direct intrahippocampal microinjections of Pb suggest that the acute behavioral toxicity of Pb may be due to its direct interference with hippocampal function in adult rats, perhaps by its demonstrated antagonist action at NMDA receptors (2,10). We chose the dose of Pb used in this study as a starting point for developing the microinjection procedure for use with the Morris swim task. The amount of Pb injected bilaterally was similar to the amounts of NMDA and muscarinic receptor antagonists used in other intrahippocampal injection studies (26). We plan to conduct future studies to determine dose-response relationships for the effects of Pb on swim task performance.

Our observations with intrahippocampal injections of Pb are in agreement with previous studies showing that the NMDA antagonists MK-801 and AP5 impair acquisition, but not the recall of spatial learning in the Morris swim task (12, 17). AP5-induced deficits in the Morris swim task have also been linked quantitatively to the inhibition of long-term potentiation (LTP), a synaptic model for learning and memory (7,21). Similarly, Pb has been shown to inhibit LTP in both in vitro (3) and in vivo (16) models. Morris et al. (20) have shown that intrahippocampal injection of NMDA antagonists impair spatial learning in the water maze. Other studies utilizing intrahippocampal administration of drugs demonstrate the importance of NMDA and muscarinic receptors within this structure in spatial learning behavior (25,31). Thus, it appears that our behavioral data are consistent with the hypothesis that NMDA and muscarinic receptors are involved in the neurotoxicity of Pb.

Our results identify the hippocampus as an anatomical site for the behavioral toxicity of Pb. This is important in light of the fact that the hippocampus and related limbic structures play key roles in spatial learning in the water maze (22), and hippocampal lesions produce similar behavioral effects to those observed in rats chronically exposed to Pb (6,23). The histopathological results and [^{3}H]-PK11195 autoradiography obtained from the rats used in the present study indicate that the effect of the PbAc injections were mediated through changes within the hippocampus, rather than a global effect throughout the brain. The failure to detect a differential effect of the PbAc and NaAc injections on cell loss or reactive gliosis indicates that the behavioral effects produced by intrahippocampal injections of PbAc were not simply due to Pb-induced damage, but to the direct pharmacological action of Pb on neuronal targets such as NMDA receptors within the hippocampus. In summary, this micro-injection technique may be a valuable tool for further investigations on the behavioral toxicity of Pb.

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