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Evidence for Hippocampal Role in Place Avoidance Other Than Merely Memory Storage

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Summary

Spatial navigation is used as a popular animal model of higher cognitive functions in people. The data suggest that the hippocampus is important for both storing spatial memories and for performing spatial computations necessary for navigation. Animals use multiple behavioral strategies to solve spatial tasks often using multiple memory systems. We investigated how inactivation of the rat hippocampus affects performance in a place avoidance task to determine if the role of the hippocampus in this task could be attributed to memory storage/retrieval or to the computations needed for navigation. Injecting tetrodotoxin (TTX) into both hippocampi impaired conditioned place avoidance, but after injecting only one hippocampus, the rats learned the place avoidance as well as without any injections. Retention of the place avoidance learned with one hippocampus was not impaired when the injection was switched to the hippocampus that had not been injected during learning. The result suggests that during learning, the hippocampus did not store the place avoidance memory.

Key words

Navigation • Multiple memories • Temporary inactivation • Tetrodotoxin • Rat

Introduction

The participation of hippocampus in human memory is suggested by extensive retrograde amnesia following bilateral hippocampal damage (Scoville and Milner 1957). Additional evidence from experimental studies suggests that the hippocampus is involved in rapid encoding of information (Morris and Frey 1997, Nakazawa *et al.* 2004), indexing of memory storage sites in the neocortex (Tyler and DiScenna 1986), and inhibition of inappropriate behavioral responses (McNaughton 1997). The role of hippocampus in rodent spatial memory and navigation has been studied in a variety of water mazes (Morris 1981) and dry maze tasks (Olton *et al.* 1979, Lanke *et al.* 1993, Cimadevilla *et al.* 2000a). When assessing the role of the hippocampus in memory, it is important to keep in mind that multiple memory systems and multiple navigational strategies (Bureš and Fenton 2000) are available to animals (Packard and McGaugh 1996, Stolberg 2005). Hippocampus-dependent locale strategies (O'Keefe and Nadel 1978) for navigation in water mazes are preferred in some experimental conditions (Morris *et al.* 1982) but not in others (Ramos 2001, 2002, Pouzet *et al.* 2002).

These and other studies have demonstrated that both hippocampus-dependent and hippocampus-independent behavioral strategies are used for water maze navigation (Eichenbaum *et al.* 1990, Whishaw *et al.* 1995). In navigation tasks on dry open fields, it was quickly determined that even when rats use locale strategies (Bureš *et al.* 1997, Fenton *et al.* 1998), they also simultaneously learn and use associations between self-motion stimuli and stationary local stimuli (Stuchlík *et al.* 2001, Stuchlík and Bureš 2002). Hippocampal dysfunction also impaired tasks requiring navigation in dry spaces (Olton *et al.* 1979, McDonald and White 1995, Cimadevilla *et al.* 2000a, 2001, Wesierska *et al.* 2005). There is therefore little doubt that the hippocampus participates in these tasks, but it is not clear whether the role of the hippocampus is important in memory storage and retrieval or in the computations that manipulate memories stored in extrahippocampal sites.

The present study investigated how hippocampal dysfunction affected the performance of a simple conditioned place avoidance task in a dry open field. Experiment 1 tested if injections of tetrodotoxin (TTX) into both hippocampi impair a familiar conditioned place avoidance. Experiment 2 tested whether or not the TTX-induced deficit observed in Experiment 1 could be attributed to a frank impairment of memory storage or retrieval.

Methods

Animals

Twenty-four adult (300-500 g) male Long-Evans rats from the Institute's breeding colony were used. They were housed three per cage within an air-conditioned vivarium in 25x25x50cm transparent plastic cages. All manipulations were carried out during the light phase of a 12 h light: 12 h dark cycle. The animals were food-deprived before the start of the place avoidance training (see below - Pretraining). Access to water was always *ad libitum*. All experiments were carried out in accordance with the Animal Protection Act of the Czech Republic, NIH guidelines and the directive of the European Communities Council (86/609/EEC).

Surgery

The animals were implanted with guide cannulae aimed at the dorsal hippocampi, which allowed infusions of TTX. Rats were anesthetized with thiopental (50 mg/kg i.p., Spofa, Prague, Czech Republic) and the skull was exposed. Two holes were drilled to aim two 10 mm-long stainless steel guide cannulae (22 ga, OD=0.70 mm, ID=0.40 mm) bilaterally above the dorsal hippocampus (AP 3.5; L 2.6; H 2.0). Two anchoring bolts were implanted into slits drilled into the skull, then cemented to the bone and the cannulae with dental acrylic. Lubricated stainless steel stylets were inserted into the cannulae to prevent occlusion. Each rat was also implanted with a subcutaneous low-impedance electrode. The electrode was made by bending a hypodermic needle (25 ga, OD=0.50 mm, ID=0.25 mm) into a U-shaped pin, piercing the skin between the shoulders, and soldering a screw to the sharp end. The screw prevented the electrode from slipping out, and provided purchase for an alligator clip that could connect the electrode to a wire for delivering electric current. The rats had at least one week to recover from the surgery. The method was previously described in detail (Fenton and Bureš 1993, Fenton *et al.* 1995, Cimadevilla *et al.* 2000a,b, Cimadevilla *et al.* 2001, Klement *et al.* 2005).

Intrahippocampal injection

Tygon tubing (Small Parts, Miami Lakes, FL, USA) connected an injection needle (30 ga, OD=0.30 mm, ID=0.15 mm) to a 10 µl Hamilton syringe. The rat was restrained by hand and the stylets were removed. The injection needle was inserted through one of the guide cannulae until a stopper positioned the needle tip 3.5 mm ventral to bregma. One µl of TTX solution (5 ng/µl saline; Sigma, St. Louis, MO) was continuously injected over a minute. The injection needle was left in place for another minute and then slowly retracted. The rats received the injection one hour before training. One bilateral injection of TTX was administered before the behavioral training started in order to habituate the rats to the injection procedure and to the TTX-induced state before training. If unilateral injections were given, then they were left/right counter-balanced across animals.

Injection site verification

After completion of the experiments, the rats were anesthetized with an overdose (100 mg/kg) of thiopental (Spofa, Czech Republic) and transcardially perfused with saline and then 4 % formaldehyde. The brains were removed, sectioned and stained with cresyl violet and the injection sites were verified to be in the dorsal hippocampus within 0.5 mm of the target. Typically we observed a cortical lesion made by the implanted cannula and a more subtle lesion produced by

the injection needle that extended into the hippocampus. Animals with lesions extending beyond the hippocampus and the overlying cortex were excluded from the study (n=4). A detailed description of the TTX injection, evaluation of the possible extent of the TTX diffusion and its electrophysiological effects were recently published (Klement *et al.* 2005).

Apparatus

The place avoidance system (Bio-Signal Group, DE, USA) was used. The system was adapted from that used in previous studies (Bures *et al.* 1997, Fenton *et al.* 1998, Cimadevilla *et al.* 2000a, 2001). The arena was a uniform metal circle, 82 cm in diameter with a 5 cm high wall. It was elevated 76 cm above the floor. The arena was centered in a room with multiple distal landmarks. The rat wore a latex harness that held infrared light-emitting diodes (LEDs) between the shoulders. The LEDs were powered through a cable. The cable also carried the shock current which was connected to the implanted electrode by an alligator clip. Shock current (50 Hz, 0.5 s, 0.5-0.7 mA) from a constant current source was delivered through the implanted low impedance (of the order of 100 Ω) shock electrode. Since the impedance of the contact between the rat's paws and the grounded arena was three orders of magnitude higher, the major voltage drop was across the paws. The shock current was monitored by the experimenter. A PC-based tracking system (iTrack; Bio-Signal Group, DE, USA) analyzed the signal from an overhead television camera and recorded the position of the LEDs on the rat 25 or 50 times per second. The position time series was stored for off-line analyses (TrackAnalysis; Bio-Signal Group, DE, USA).

Pretraining

The rats were food deprived to 85 % of their free feeding weight and trained in the experimental arena for 7-10 days until they continuously foraged for food before place avoidance training started. The food pellets were dropped into the arena from an overhead feeder every 20 s. There were no shocks. Each rat received one bilateral injection of TTX into both hippocampi to habituate them to the injection procedure and to the TTX-induced state before the place avoidance training started.

Place avoidance paradigm

The rats were conditioned to avoid a 60 ° sector of the space while foraging for scattered food pellets on a dry arena (Bureš *et al.* 1997). The rat was placed on the arena across from the to-be-avoided sector facing away from the arena center. Whenever the rat entered the to-be avoided sector for more than 0.5 s, the tracking system delivered a mild constant current foot-shock and counted an entrance. The current amplitude for each rat was adjusted in the first session to be the minimum necessary to elicit flinch and escape responses (Cimadevilla *et al.* 2000) and was not changed afterwards. If the rat did not leave the sector, additional shocks were given every 1.5 s until the rat left the sector, but no more entrances were counted until the rat left the sector for more than 0.5 s. Each session lasted 20 min. In order to account for potential TTX-induced differences in locomotion, to measure place avoidance memory, we used the distance the animal had walked before it entered the to-be-avoided sector for the first time in a session (D1). The total number of entrances (E) was used to measure avoidance and the total distance (TD) walked during the session was used to measure the overall activity. Linearity (L) of the path was calculated to further characterize the animals' locomotion (Wesierska *et al.* 2005). Linearity was calculated as the average ratio of the linear distance in each two-second episode divided by the sum of the distances along the path determined each 40 ms of the episode.

Experimental design

Experiment 1 tested whether place avoidance is sensitive to bilateral hippocampal dysfunction. Ten rats were trained for 5 days in the place avoidance task with no injections. On day 6, they were injected with TTX in both hippocampi and one hour later, they were trained in the same place avoidance task.

Experiment 2 tested if the hippocampus stored the place avoidance memory. After place avoidance had been learned with only one functional hippocampus, we tested if inactivating the other hippocampus impaired retrieval of the avoidance that was learned with the now inactive hippocampus. A different group of 10 rats was trained for four days in the place avoidance task. They were injected with TTX into one hippocampus one hour before the training. After a three day break, the training was resumed for 5 more days to ensure avoidance was stable with daily TTX injections in the same hippocampus. The next day, the rats were divided into two groups and injected with TTX in the contralateral (n=5) or the ipsilateral (n=5) hippocampus one hour before testing retention of place avoidance in a session

with no shocks.

Data analyses

Intact place avoidance learning during 5 days was characterized by one-way ANOVA with repeated measures on individual days. The effect of bilateral TTX injection was analyzed by comparing the performance on days 5 and 6 by paired t-tests. This within-subject design provided control for the individual differences in assessing the effect of TTX. The effect of unilateral TTX injection was assessed by comparing performance on the first four training days in the injected animals with performance of the animals in Experiment 1 using two-way ANOVA with repeated measurement on separate days. T-tests on the last day of training and on the retention test compared the performance of animals that on the retention test were injected in the same or opposite hippocampus as during training. Newman-Keuls *post-hoc* comparisons were performed when appropriate. Significance was accepted at $p<0.05$. Means $\pm$ S.E.M. are reported.

Results

Experiment 1

The uninjected rats rapidly learned to avoid a place during 5 days. They reduced the number of entrances (E) and increased the distance they walked before entering the shock zone for the first time (D1). Presumably because they had learned the shock was applied only in a particular part of the arena, they also increased the total distance (TD) they walked as training continued. There was a significant effect of days on all measures (D1: $F_{4,36}=9.77$, $p<10^{-4}$; E: $F_{4,36}=2.88$, $p<0.05$; TD: $F_{4,36}=11.6$, $p<10^{-5}$). *Post-hoc* tests indicated the rats walked longer before making the first entrance on day 4 than on day 1 and on day 5 they walked longer than on the other days. The rats made significantly fewer entrances on day 4 than on day 1.

The bilateral injection of TTX impaired avoidance (Fig. 1). The distance to the first entrance decreased (D1: $t_9=3.66$, $p<0.01$) and the number of entrances (E: $t_9=2.71$, $p<0.05$) increased, but the total distance did not change (TD: $t_9=1.78$, $p>0.1$).

Experiment 2

The place avoidance of rats with unilateral TTX injection was equivalent to that of uninjected rats over the four days. Animals in both groups avoided for a similar

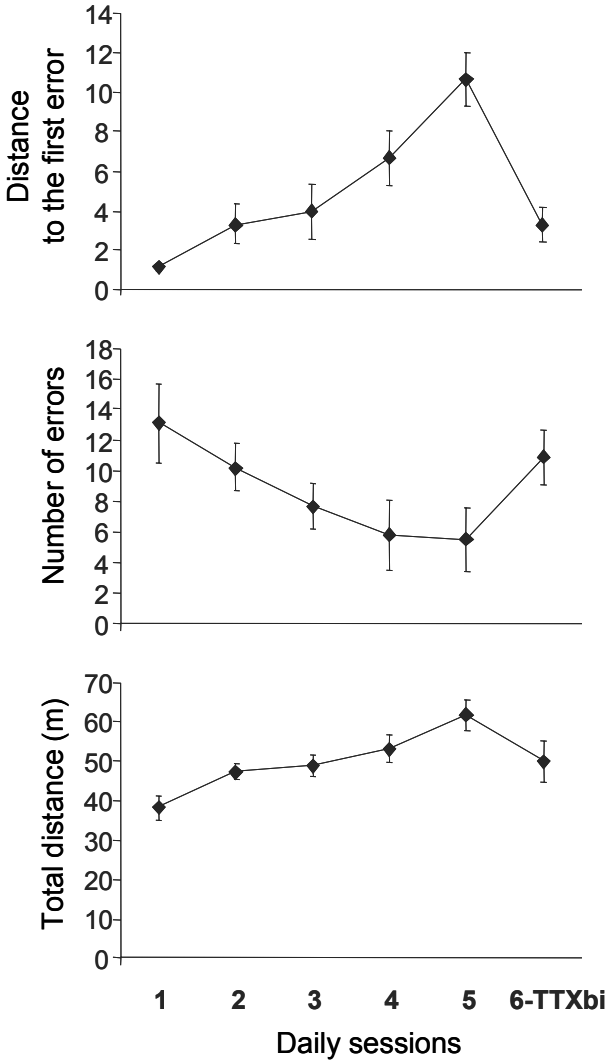

Fig. 1. Experiment 1: The uninjected rats (n=10) learned the place avoidance in 5 days. The bilateral TTX injection on day 6, disturbed the place avoidance but not locomotion.

distance before making the first entrance and made a similar number of entrances. There were significant effects of days (D1: $F_{3,54}=14.0$, $p<10^{-5}$; E: $F_{3,54}=14.3$, $p<10^{-5}$) but not groups (D1: $F_{1,18}=0.75$, $p>0.3$; E: $F_{1,18}=0.19$, $p>0.6$). There was a significant group by day interaction on the number of entrances (D1: $F_{3,54}=0.34$, $p>0.7$; E: $F_{3,54}=4.66$, $p<0.01$). The *post hoc* tests confirmed that the animals entered the to-be avoided sector earlier on day 1 and later on day 4 than on other days and that the rats with unilateral TTX injection made more entrances on the first day (Fig. 2).

In contrast, there was a significant effect of groups ($F_{1,18}=6.88$, $p<0.02$) and the interaction ($F_{3,54}=16.3$, $p<10^{-6}$), but no effect of days on the total distance ($F_{3,54}=1.38$, $p>0.2$). The *post hoc* tests indicated that while the controls moved the least on day 1 and

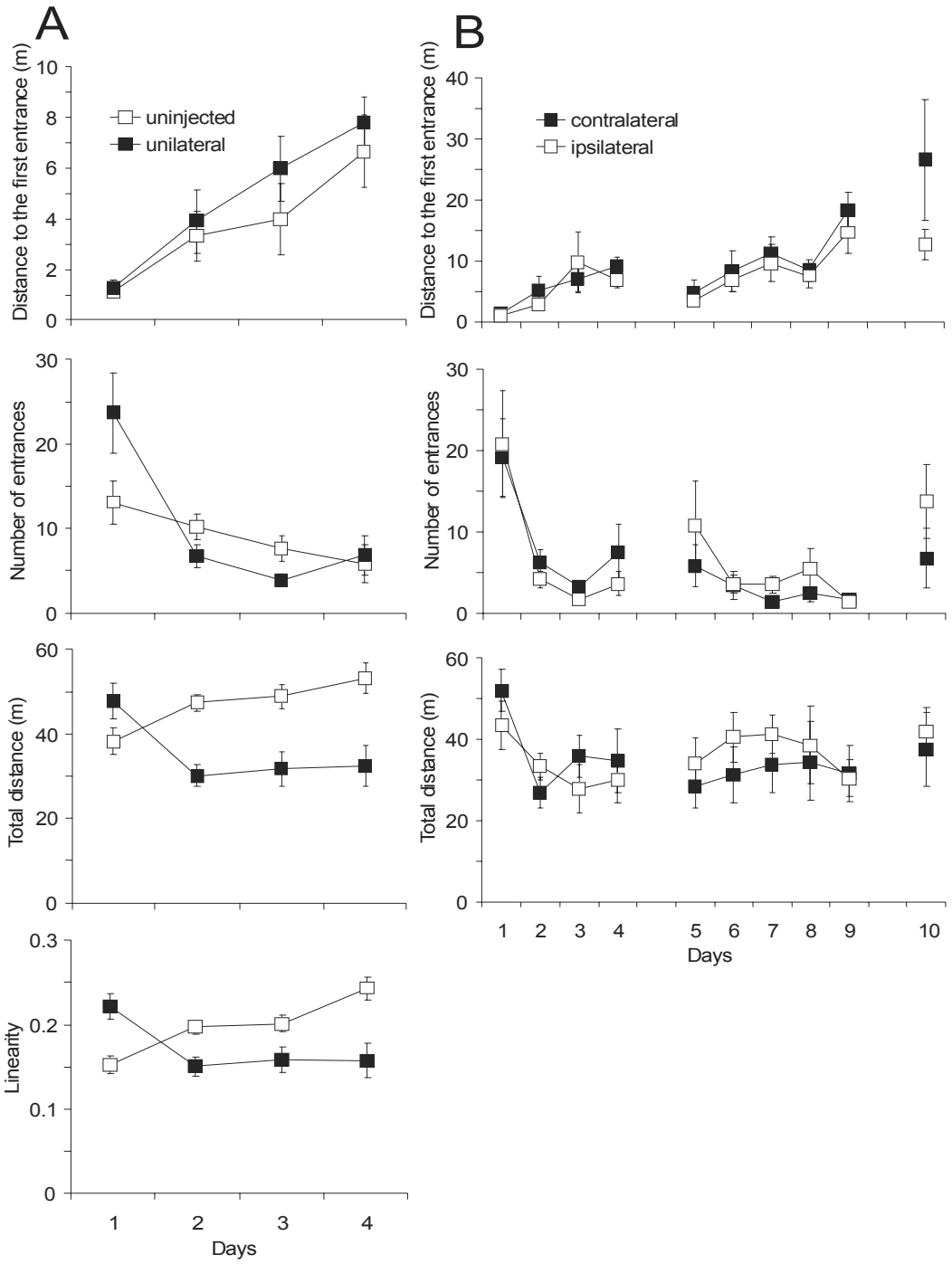

Fig. 2. Experiment 2: A) Unilateral injections of TTX had no effect on the number of entrances and the distance to the first entrance during the first 4 days of training. However, the rats with the TTX injection moved less and their paths were less linear from day 2. B) After 5 more days of training with daily TTX injections, changing the side of the injection (contralateral, n=5) did not disturb retrieval of the avoidance (day 10) compared to rats injected in the same hippocampus as during previous training (ipsilateral, n=5).

moved more on subsequent days, the TTX-injected rats moved the most on day 1 and decreased walking on the following days. Analysis of linearity (L) of the animals' trajectories revealed a similar pattern. There was no effect of groups ($F_{1,18}$=2.55, $p>0.1$), but a significant effect of days ($F_{3,54}$=3.13, $p<0.05$) and the interaction ($F_{3,54}$=26.7, $p<10^{-6}$). The *post-hoc* tests revealed that the linearity increased from day 1 to day 4 in the control group, but it decreased from day 1 to day 2 and thereafter remained lower in the unilaterally injected group. To summarize, the data indicated the rats in the unilateral group learned to avoid the to-be avoided area as well as the uninjected rats, but their movements were different.

Changing the side of the TTX injection did not

disturb place avoidance (Fig. 2). The groups differed neither on the last training trial (D1: t_8=0.72, p>0.4; E: t_8=0.52, p>0.6; TD: t_8=0.13, p>0.8) nor on the retention test (D1: t_8=1.22, p>0.2; E: t_8=1.07, p>0.3; TD: t_8=0.34, p>0.7). This result does not support the hypothesis that the place avoidance memory was stored in the hippocampus that was active during training.

Discussion

The temporary inactivation of hippocampus by injections of TTX was used and described previously (Fenton and Bureš 1993, Cimadevilla *et al.* 2001, Klement *et al.* 2005). The 5 ng dose of TTX was determined to block neural transmission throughout the hippocampus (Klement *et al.* 2005) for about 5 h (Zhuravin and Bureš 1991). No signs of ataxia were observed prior to training, indicating the TTX did not diffuse to the thalamus.

Experiment 1 demonstrated that hippocampus participates in normal performance of place avoidance. In accordance with Wesierska *et al.* (2005), we showed that unlike in active distal cue-based place avoidance on a rotating arena (Cimadevilla *et al.* 2001), rats can learn the place avoidance on a stable arena with one hippocampus injected with TTX. We also found that the unilateral TTX injection decreased the locomotion and made the paths less linear. This contrasts with the uninjected rats, which increased the distance they walked with training. These effects on locomotion are not likely due to a general inhibitory effect of the injection because it only appeared after the second day of training, suggesting the inhibition of locomotion was acquired. Perhaps in response to the TTX injection the rats may have adopted a different strategy for avoiding than the uninjected rats (Bureš and Lánský 2004).

The impairment of place avoidance by the TTX injections provided an opportunity to determine if the functional hippocampus had stored the place-shock association. If the role of the hippocampus was to store the place avoidance memory, then injecting the other hippocampus should impair retention of place avoidance as was shown in a water maze task (Fenton and Bureš 1993). Alternatively, if the memory was stored in extra-hippocampal sites, then changing which hippocampus was injected should not be impairing. Experiment 2 showed that switching the side of the TTX injection did not impair place avoidance suggesting the role of hippocampus in place avoidance is not to store the place-shock association. The possibility that the memory was transferred to the untrained hippocampus is unlikely because memory for water maze escape lateralized to one hippocampus did not spontaneously transfer to the untrained hippocampus without returning the rats to the training environment with both hippocampi functional (Fenton and Bures 1993, Fenton *et al.* 1995).

These data, however, do not distinguish between competing theories of hippocampal function that do not posit it has an essential role in memory storage. The data are essentially compatible with the idea that multiple traces of experience are acquired and stored in neocortex and that the hippocampus indexes and cross-associates subsequently acquired traces (Nadel and Moscovitch 1997, 2001). According to this multiple memory trace hypothesis, the hippocampus only temporarily stores memory and references to extrahippocampal sites, which provide the actual memory storage for future retrieval.

The behavioral inhibition account of hippocampal function posits that the hippocampus acts to solve behavioral conflicts by inhibiting inappropriate learned (or inherent) responses (McNaughton 1997, McNaughton and Wickens 2003). The hypolocomotion induced by unilateral TTX injection is compatible with the rats learning to inhibit moving in order to avoid shock, which may be an alternative to using a locale strategy for mapping the to-be-avoided area.

The conclusion that the role of hippocampus was not in memory storage is based on the assumption that if place avoidance memories were stored in the active hippocampus during training then those memories would remain lateralized to that hippocampus. It was demonstrated in water maze tasks that indeed, after unilateral injections of TTX (Fenton and Bureš 1993) or lidocaine (Fenton *et al.* 1995) the place response remained lateralized to one hippocampus. Taken together with the water maze results, the present findings suggest that place avoidance can be acquired using a different strategy than is used in the water maze. This strategy does not seem to require memory storage in the hippocampus.

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