Long-lasting training in the Barnes maze prompts hippocampal spinogenesis and habituation in rats

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There is a constant need to assess spatial memory in small rodents to elucidate the basics of cognition in neuroscience experiments. Thus, the significance of the Barnes maze in the biology of hippocampal and cortical neural function cannot be overemphasized. Despite the wide use of the Barnes maze, the effect of maze task training on the structure of hippocampal neurons is yet to be elucidated. Adult Sprague-Dawley rats were subjected to intense training on the Barnes maze (3 months). Subsequently, the hippocampus (cornus ammonis and dentate gyrus) of separate sets of rats was processed for Golgi Colonnier techniques (silver impregnation) and adenoviral-green fluorescent protein labeling (immunohistochemistry). Our results showed that training the animals on the Barne maze increased spinogenesis significantly in the cornus ammonis and dentate gyrus neurons. In addition, we identified a critical time point at which the rats habituated to the trial without escaping box (the probe trial) and could not be tested further in the maze. Taken together, we deduced that a prolonged test on the dry land maze facilitated habituation and caused an increase in hippocampal dendritic spine count. As such, the dry land maze is a suitable paradigm for assessing spatial memory in rats. However, precautions should be taken in selecting suitable experimental controls on the basis of the duration of a study. ***NeuroReport*** **00:000–000** **Copyright © 2017 Wolters Kluwer Health, Inc. All rights reserved.**

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Introduction

Episodic memory is a high-order cognitive process that is conserved in mammals. Despite the series of studies carried out in this area, the basis of episodic memory is only partially understood. Memory is an outcome of cognitive function. As such, understanding memory formation, consolidation, and retrieval in small rodents often elucidates broad aspects of cognitive processing that could have important translational significance.

The spatial memory is a subtype of episodic memory, which has been shown to involve, mostly, the hippocampal neural system [1]. Several studies have shown the effect of an enriched environment on neural plasticity [2]. Furthermore, in small rodents, an enriched environment promoted hippocampal neurogenesis in the dentate gyrus (DG) [3] and increased dendritic spine density in the occipital cortex [4]. In another study, an enriched environment rescued cornus ammonis (CA1) dendritic spine density in mice injected with streptozotozin [5]. Although the dry land maze differs from an enriched environment, we hypothesized that a long-lasting training would have a similar effect on hippocampal neuron spinogenesis.

Since its invention [6], the dry land maze has provided a straightforward approach for experimental assessment of spatial memory in small rodents. Structurally, the dry land maze is a circular platform with 20 holes, under one of which is an escape box. The task on a dry land maze involves placing a rat on the platform; then, the animal is trained to find the hole associated with the escape box to escape aversive stimuli such as bright light and high-pitch noise.

After several training sessions (acquisition trials), a probe trial without an escape box is conducted. This is aimed at determining the extent to which the animal remembers the location of the escape hole. Notably, besides the basic rules of the paradigm, we and other groups have adapted the protocol of the maze in accordance to the specific requirements of the experimental question. Some of these modifications include a probe trial conducted 1–5 days after the acquisition phase. In a previous experiment of our group [7], on comparing young (4–6 months old), old (26 months old), and senile (29–32 months old) Sprague-Dawley female rats, we found differences in the long-term probe trial between the young and old rats. Also, we found prominent differences in acquisition trial performance between the old and the senile rats. In another study [8], using a modified version of the dry land maze, we showed that insulin-like growth factor-1 gene therapy improved spatial memory in senile rats by assessing two probe trials: first, before gene therapy, and subsequently 17 days after gene therapy had

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been implemented. However, it remained unclear whether the experimental rats can both learn and habituate to the maze task in a probe trial.

In this study, we show that rats can habituate to the probe trials in the dry land maze. Using the Golgi–Colonnier technique or green fluorescent protein (*GFP*) labeling, we compared the morphology and density of dendritic spines for untrained control rats and rats trained on the maze. Ultimately, we showed that a prolonged training on the maze increased dendritic spine density and was associated with habituation to the maze tasks.

Animals and methods

Animals

All animals were housed in a temperature-controlled room (22 ± 3°C) on a 12 : 12 h light/dark cycle, with food and water available *ad libitum*. All experiments with animals were conducted following the Animal Welfare Guidelines of National Institute of Health (INIBIOLP's Animal Welfare Assurance No A5647-01). The ethical acceptability of the animal protocols used in this study was approved by our institutional IACUC (Protocol # T09-01-2013).

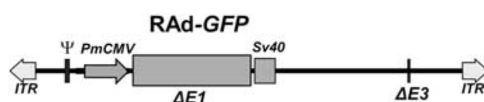
Twelve 3-month-old female Sprague-Dawley rats weighting 199 ± 1 g were used for this study. A group of four rats was not trained (naive), whereas a group of eight rats was trained in the dry land maze for 3 months. Subsequently, a total of three animals per group were used for the Golgi–Colonnier technique to outline the morphology of dendritic spines. In addition, in one of the naive rats, we injected a recombinant adenovirus coding for the *GFP* (RAd-*GFP*) into the hippocampus to outline the morphology of CA1 and granular cell layer (GCL) neurons of the DG (Fig. 1).

Dry land maze

For this study, we adopted a modified version of the procedure described previously by our group [7,8]. At day 0, the animals were habituated to the starting chamber and escape box by placing them inside each one for 180 s. The task was organized into three separate sessions at 1-month intervals. Every season lasted 9 days and consisted of four acquisition trials per day. As the first session was the original encounter of the animals with the maze, they were trained thoroughly to ensure that the task was learnt correctly. At the end of session 1, a probe trial was conducted to assess spatial memory as a preference for the goal hole (hole 0). For the second and third sessions, the rats were subjected to a probe trial every 3 days (Fig. 2a). In sum, the animals were subjected to a standard intensive training at the beginning of the protocol and were subsequently subjected to several probe trials to determine whether habituation to this test phase could take place.

The behavioral performances were recorded using a computer-linked video camera mounted 110 cm above the platform as described previously [8]. The performance of the subjects was determined using the Kinovea v0.7.6 software (*http://www.kinovea.org*). For statistical analysis, latency to escape box and errors were averaged for the four trials for each experimental day.

Fig. 1



Schematic illustration of the Rad-*GFP* adenovector. ψ, Packaging signal; *ΔE1* and *ΔE3*, deletions of genes *E1* and *E3* in the Ad 5 genome; *ITR*, inverted terminal repeats; *PmCMV*, murine cytomegalovirus promoter; Rad-*GFP*, a recombinant adenovirus coding for the green fluorescent protein; *SV40*, Simian virus 40 polyadenylation signal.

Adenoviral injection and green fluorescent protein labeling

To observe the morphology of CA1 and GCL neurons, we administered an intrahippocampal injection of a RAd vector harboring the gene for *GFP* (RAd-*GFP*). This vector was constructed in-house using a procedure described previously by our group [9]. The cDNA of *GFP* was placed under the control of the mouse cytomegalovirus (*mCMV*) promoter. The new adenovector was rescued from HEK293 cell lysates and plaque purified. In subsequent processing, the virus was purified from the lysate by ultracentrifugation in a CsCl gradient. The final adenoviral stock was titrated using a serial dilution plaque assay [9]. Adenoviral plaque forming units (10^9) prepared in a 2 μl solution were injected bilaterally into the hippocampus using the following coordinates relative to the Bregma: anteroposterior: − 3.8; lateral: ± 2.7; dorsoventral: − 2.7 and − 3.5 for the CA1 and GCL, respectively [10]. Two days after the RAd-*GFP* injection, the animal was deeply anesthesized and transcardially perfused, following which the brain was processed for immunohistochemistry.

Immunohistochemistry

Vibratome (Leica, Wetzlar, Germany)-sliced coronal brain sections were used for this procedure (60 μm thick). After blocking in serum, free-floating sections were incubated overnight at 4°C with the anti-GFP antibody (1 : 10 000, #A6455; Invitrogen). Subsequently, sections were incubated with a biotinylated anti-rabbit antibody (1 : 300, BA-1000) for 2 h and the avidin–biotin–peroxidase complex (1 : 500, PK-6100, Vector ABC Elite Kit, Vector Labs, Inc., Burlingame, California, USA) for 90 min. Sites of immunoreaction were developed using 3, 3′-diamino benzidinetetrahydro-chloride.

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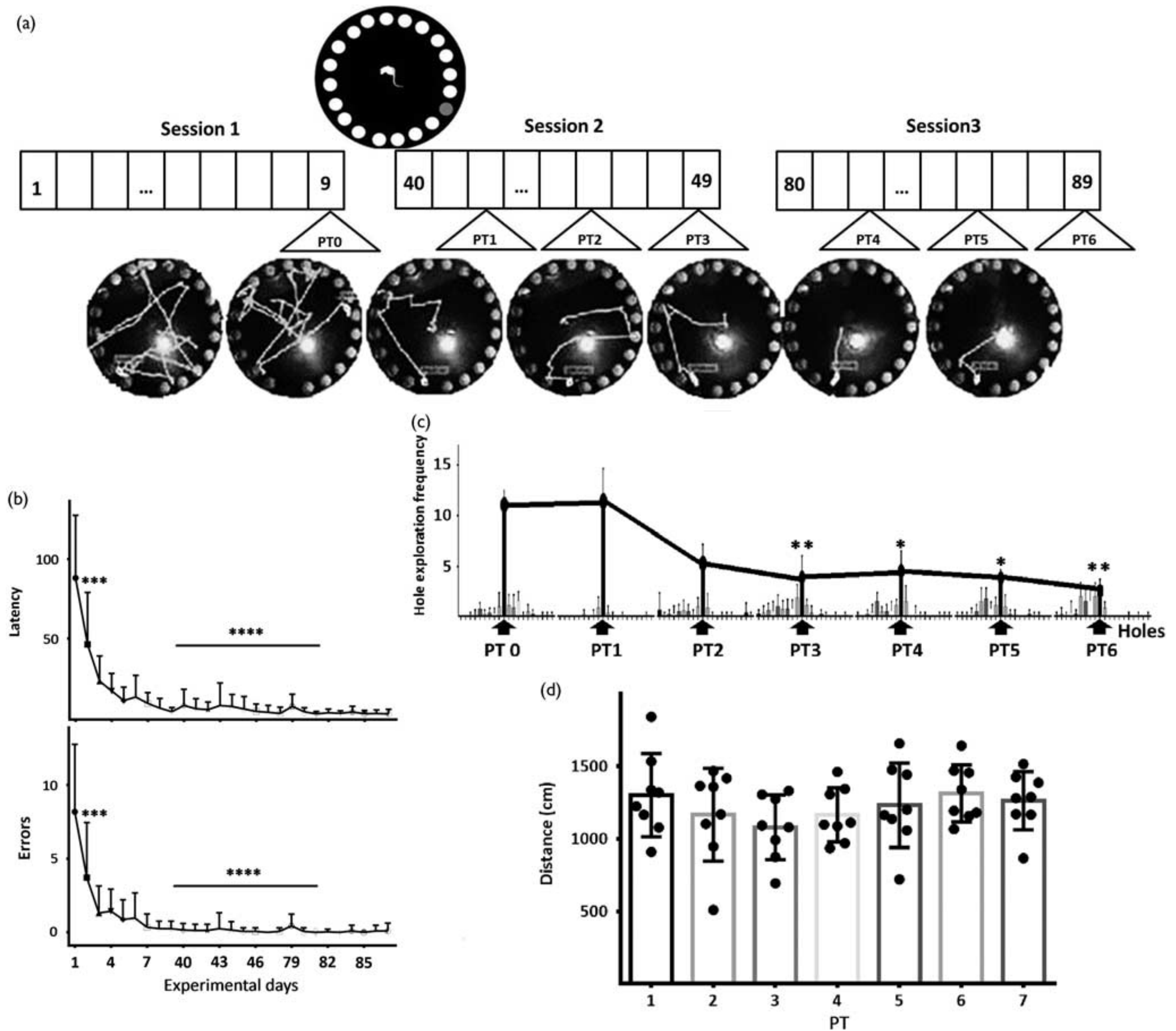
Fig. 2



(a) Schematic illustration of the behavioral task design. A total of three separate sessions, at 1-month intervals, were conducted. For each session, a total of nine experimental days, each with four acquisition trials, were conducted. Lower panel: Representative behavioral patterns showing the trace of area covered by the animal, in the maze, during the course of the trials. Toward the end of the trials, the animals followed a rectilinear path to the goal hole. (b) Graphs showing the time-dependent statistical change in the latency and errors of behavioral parameters for the duration of the experiment. (c) Representative histogram showing the hole exploration frequency for probe trials (PT) 0–6. (d) Bar chart showing the statistical relationship for the distance covered by the animals for probe trials 0–6. The data were presented as mean (bars) ± SEM (error bars). *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Golgi Colonnier staining

Animals were transcardially perfused with phosphate-buffered formaldehyde (4% formaldehyde in PBS). The brain was dissected and fixed in 4% phosphate-buffered formaldehyde for 24 h. Subsequently, the sections were processed using a modified version of the Golgi–Colonnier technique [11]. Postfixed brains were sectioned to obtain 5 mm thick coronal slices exposing the hippocampus. Subsequently, the tissue was incubated in a dark cupboard at room temperature in the following solutions diluted in distilled water: 2.5% potassium dicromate, 5% glutaraldehyde for 5 days; 3.5% potassium dicromate for 1 day; 2% silver nitrate for 1 day; 3.5% potassium dicromate for 3 days; 2% silver nitrate for 1 day; and finally in 30% sucrose for 1 day.

To highlight the microscopic morphology of the hippocampus, the 5 mm thick brain slices were further trimmed to obtain 200 μm thick sections. Individual sections

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were gradually dehydrated as follows: water, ethanol 70%, ethanol 100%, and xylene. Ultimately, the sections were mounted on gelatinized slides and covered with Canadian Balsam.

Image acquisition and data analysis

RAd-*GFP* expression images were acquired using an Olympus BX-51 microscope attached to an Olympus DP70 CCD video camera (Olympus, Tokyo, Japan). Golgi–Colonnier images were acquired using Hamatsu Nanozoomer (Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan). Calibrated images of dendrites were acquired at ×100 magnification, dendritic spines were zoomed and identified in the Image Pro Plus v5.1 software (Media Cybernetics Inc., Silver Spring, Maryland, USA), and manually counted by a scientist who was unaware of the sampling procedure. For this study, we assessed dendritic spine density for apical dendrites facing the stratum radiatum of CA1 pyramidal neurons and for GCL neurons situated in the DG. We have focused specifically on these regions as they are anatomically clearly defined and particularly vulnerable to degeneration in patients with Alzheimer's disease [12].

Dendritic spine density was determined using previously published criteria [5,13]. Briefly, for the CA1 neuron apical dendrites, 10 neurons were identified per animal and dendritic segments at least 20 μm long of apical dendrites facing the stratum radiatum were mapped along the entire slice thickness to acquire images of dendritic spines at all focal planes. In the case of GCL neurons, spine count was assessed on the middle third of the molecular layer. Thus, 10 neurons were considered for every animal and spine density was calculated in dendritic segments at least 40 μm long. Finally, dendritic spine count per 10 μm (dendritic spine density) was calculated.

Statistical analysis

Behavioral parameters were analyzed by repeated measures (RM) one-way analysis of variance (ANOVA), with the Greenhouser–Geisser correction and Dunnet's post-hoc test for multiple comparisons versus a control. Mean comparisons between dendritic spine densities were performed using the unpaired *t*-test. All statistical analyses and plots were carried out in GraphPad v6 Software, Inc., San Diego, CA, USA. Data are shown as mean ± SEM.

Results

The rats learnt the task, albeit habituation to the probe trials was observed

To study habituation to the probe trial and the impact of the dry land maze on the hippocampal (CA1 and GCL) spinogenesis, we implemented a long-lasting behavioral testing procedure (Fig. 2a). The outcome of this study showed that the latency to escape box was markedly reduced after the second experimental day. Furthermore, a more significant decrease was recorded continuously on subsequent experimental days (RM one-way ANOVA; day factor $F=76.85$, $d.f.=26$, $P<0.0001$; Dunnet's post-hoc test day 2 vs. 1 (control) $P<0.001$; subsequent days vs. 1 $P<0.0001$). In addition, a similar pattern was observed for the magnitude of errors made (RM one-way ANOVA; day factor $F=1.997$, $d.f.=26$, $P<0.0001$; Dunnet's post-hoc test day 2 vs. 1 (control) $P<0.001$; subsequent days vs. 1 $P<0.0001$) (Fig. 2b).

Goal hole exploration decreased significantly from probe trial 0 to 6 [RM one-way ANOVA probe trial factor $F=1.178$, $d.f.=6$, $P<0.0001$; Dunnet's post-hoc test $P<0.05$ from probe trial 3 onwards vs. probe trial 0 (control); Fig. 2c]. As shown in Fig. 2c, the animals lost interest (habituated) in exploring the goal hole after the third probe trial. However, they sought an escape from the maze by exploring other holes. This showed that the animals did not lose motivation. In support of this outcome, the animals showed no significant change in the distance covered among the probe trials (Fig. 2d).

Long-term training in the dry land maze enhances hippocampal spinogenesis in cornus ammonis and granular cell layer neurons

The morphology and density (count) of dendritic spines was highlighted using a Golgi–Colonnier staining (Fig. 3a–d). As this histological procedure is susceptible to artifacts, we validated it by comparing the morphology of Golgi-stained cells with immunostained RAd-*GFP*-labeled neurons within the CA1 and DG regions of the hippocampus. The rationale for the use of *GFP*-labeled neurons was to create a positive control for whole-neuron impregnation as the *GFP* shows a cytoplasmic localization. As expected, we observed that the Golgi stain labeled both the soma and the neuron processes, similar to the observation for *GFP* staining in the same region of the hippocampus. Interestingly, the Golgi-stained neurons showed highly discernible dendritic spines, a morphological feature that cannot be distinguished by means of *GFP* immunohistochemistry at light microscopy (Fig. 3e and f).

To determine the effect of training in the dry land maze on spinogenesis, we assessed dendritic spine density for CA1 pyramidal neurons apical dendrites and GCL neuron dendrites. Thus, we found that dendritic spine density increased in both regions studied, compared with the control rats, which had not been subjected to the behavioral task (unpaired *t*-test, $P=0.0043$ and 0.0347 for CA1 and GCL, respectively) (Fig. 3g and h).

Discussion

Taken together, the outcome of this study shows that a long-term training in the dry land maze has a significant impact on the behavior and dendritic morphology of the animals. Our results showed that the animals learnt the

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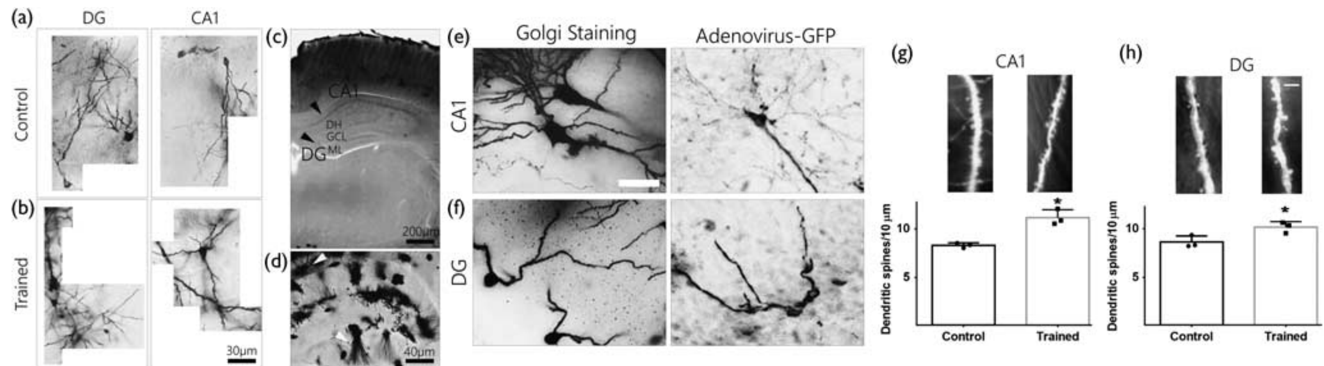
Fig. 3



(a–d) Representative images showing CA1 neurons labeled with silver stain in the hippocampus of control and trained rats (scale bar = 30, 40, 200 μm). (e and f) Representative images of stained neurons in the dentate gyrus and CA1 of the hippocampus. Note that both Golgi and *GFP* staining showed a similar outline to the neuronal morphology (scale bar = 25 μm). (g and h) CA1 and DG neuronal dendritic spine count increased in trained rats compared with the controls. The data were presented as mean (bars) ± SEM (error bars). $P < 0.05$; scale bar for g and h, 2 μm. CA1, cornus ammonis; DG, dentate gyrus; *GFP*, green fluorescent protein.

task rapidly and were characterized by a significant goal hole-seeking behavior during the acquisition trials. This continued until the very end of our study in the last acquisition trial (day 89; Fig. 2a). Strikingly, when the rats were subjected to the fourth probe trial, we noted that they lost ~ 60% of their original interest in exploring the goal hole, a trend that continued until the last probe trial was evaluated (probe trial 6 at day 89; Figs. 2a and c). After this point, it was challenging to test the spatial memory in the rats with this maze. As shown in Fig. 2c, the animals did not lose motivation to escape from the maze. In addition, we observed that the animals covered a similar distance in all the probe trials, which indicated that loss of interest in goal hole exploration was not because of decreased motility (Fig. 2d). Interestingly, some tested animals jumped out of the maze in the probe trial 5 and probe trial 6 (data not shown), which also suggests that they were motivated to escape. In previous studies, we have made use of two probe trials to test memory at two different time points [7,8]. Although these protocols were adapted to specific scientific aims, and yielded consistent results, we consider the present data very valuable for determining and optimizing test durations for future studies.

Previous studies have shown that switching from a standard to an enriched cage (enriched environment) has a significant impact on hippocampal neurons, learning, and memory [2,3]. On the basis of these propositions, we asked whether retaining a standard cage while subjecting the animal to a rigorous learning task may produce a similar effect on learning ability and hippocampal spinogenesis. Evidently, an increase in the tendency to escape from the dry land maze was associated with an increase in hippocampal spinogenesis, being a trait exclusively dependent on the training in this paradigm. We confirmed this by assessing dendritic spine density in two hippocampal neuronal populations associated with the spatial memory – the CA1 pyramidal neurons and GCL neurons [14].

For this experiment, we used the Golgi staining method to outline the morphology of hippocampal dendritic spines. This provided a detailed staining of entire neurons by the stochastic deposition of silver salts onto their membranes. Even though the most widely used protocol in the last 15 years is the Golgi–Cox method [15], for this work, we used the cost-effective Golgi–Colonnier technique [11]. As a validation for this method, the morphology of the neurons was compared with *GFP*-expressing neurons located in the CA1 and GCL, which showed an expected similarity.

This report aimed at guiding the choice of duration on future studies involving the use of the dry land maze. We strongly recommend that a maximum of three probe trials be run. As the training caused changes in dendritic spine density, our study shows that rats already tested on the Barnes maze cannot be used as naive or intact experimental controls for subsequent experiments.

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Conflicts of interest

There are no conflicts of interest.

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