

Video Article

# The Double-H Maze: A Robust Behavioral Test for Learning and Memory in Rodents

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## Abstract

Spatial cognition research in rodents typically employs the use of maze tasks, whose attributes vary from one maze to the next. These tasks vary by their behavioral flexibility and required memory duration, the number of goals and pathways, and also the overall task complexity. A confounding feature in many of these tasks is the lack of control over the strategy employed by the rodents to reach the goal, e.g., allocentric (declarative-like) or egocentric (procedural) based strategies. The double-H maze is a novel water-escape memory task that addresses this issue, by allowing the experimenter to direct the type of strategy learned during the training period. The double-H maze is a transparent device, which consists of a central alleyway with three arms protruding on both sides, along with an escape platform submerged at the extremity of one of these arms.

Rats can be trained using an allocentric strategy by alternating the start position in the maze in an unpredictable manner (see protocol 1; §4.7), thus requiring them to learn the location of the platform based on the available allothetic cues. Alternatively, an egocentric learning strategy (protocol 2; §4.8) can be employed by releasing the rats from the same position during each trial, until they learn the procedural pattern required to reach the goal. This task has been proven to allow for the formation of stable memory traces.

Memory can be probed following the training period in a misleading probe trial, in which the starting position for the rats alternates. Following an egocentric learning paradigm, rats typically resort to an allocentric-based strategy, but only when their initial view on the extra-maze cues differs markedly from their original position. This task is ideally suited to explore the effects of drugs/perturbations on allocentric/egocentric memory performance, as well as the interactions between these two memory systems.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/52667/>

## Introduction

In animals, learning is principally mediated by the hippocampal- and striatal-based memory systems<sup>1,2</sup>, which play central roles regarding place- and procedural-memory, respectively. The relationship between these two systems is complex, and they are known to interact with each other in cooperative or competitive manners<sup>1,3</sup>. In addition, studies have shown that the influence of either of these memory systems on animal behavior can increase following the absence or damage of the other system<sup>4-7</sup>. Both of these systems are connected to the prefrontal cortex via the thalamus.

Numerous neurological disorders and neurodegenerative diseases can affect spatial cognition in humans, which rely on the interplay between procedural and declarative memory systems. Examples include Parkinson's disease (PD), Huntington's disease (HD)<sup>8-10</sup>, Alzheimer's disease (AD)<sup>11-14</sup>, as well as amyotrophic lateral sclerosis (ALS)<sup>15</sup>. Animal models, which are relevant to these disorders can be induced through various drug treatments which block certain receptors<sup>16</sup>, as well as through targeted lesions. When such animals are used with spatial memory tasks, a valuable insight can be gained into the underlying mechanisms related to these disorders, as well as to various treatment options.

There are many different types of spatial memory tasks in rodents, which collectively are designed to assess specific aspects of learning and memory, as well as the effects of potential treatments for various disorders<sup>17,18</sup>. These tasks can be distinguished by the number of goals and pathways, the degree of behavioral flexibility in solving the task, the memory duration or delay, as well as the choice of strategy used in solving the task. A good performance may be acquired based on external cues or landmarks which are used to orientate the animal towards the goal (an allocentric or place strategy). Alternatively, a rodent may develop a strategy which is based on bodily direction and cues with regards to the direction to move in (an egocentric or procedural strategy), e.g., if a rat knows that the goal is one left turn followed by one right turn, then there is little need for an allocentric or place strategy. Maze tasks often differ based on the degree of flexibility offered to the rodent in solving them. For

instance, in the Morris Water Maze, a dry version of the latter (e.g.,<sup>19</sup>) or the Barnes maze (e.g.,<sup>20</sup>), there are potentially infinite routes the rat can take to reach the goal. In the Morris Water Maze, for example, the location of the goal may be learned based on external landmarks or cues (allocentric strategy), or by simply swimming in circles towards the center until the platform is found (egocentric strategy)<sup>21</sup>. Certain tasks have multiple goals and a high degree of flexibility, such as the cone-field task<sup>22</sup> or Olton's radial maze<sup>23</sup>. At the other end of the scale are tasks, which offer limited flexibility in reaching the goal, e.g., the Stone maze, or the alternating version of the T-maze. These tasks provide only one correct way of reaching the goal and facilitate the emergence of cognitive routines that are principally governed by the striatal-based procedural memory system.

The double-H maze is a novel spatial memory testing device, which was designed to allow the experimenter to direct the type of strategy that is learned by rodents in solving the task<sup>24</sup>. Consisting of three parallel run arms intersected by a perpendicular central alleyway, the double-H maze is a water-escape task in which rodents learn to reach an escape platform that is immersed in one of the maze locations. During training, a procedural strategy can be developed by maintaining the same start and goal locations throughout. Alternatively, an allocentric strategy may be developed by alternating the starting location in a random order, thus requiring the rat to learn the location of the hidden platform based on environmental cues as it has to do in a water maze. This overcomes an obstacle present in many different maze tasks, in which the experimenter otherwise has little control over the type of strategy that rodents utilize. This is important when considering that the effects of certain cognition-enhancing drug candidates rely on the hippocampal-based place-memory system, thus the emergence of cognitive routines or procedures may confound the interpretation of the behavioral observations when animals, for example switch from allocentric to procedural memory during the course of training. Similarly, it may be desirable to assess the effects of drugs and treatments on procedural memory, without the influence of allocentric place-based memory. Finally, this device can be utilized to study the cooperative or competitive interactions between these memory systems, and the conditions under which rodents may switch from one system to another.

## Protocol

### 1. General Considerations

This protocol is approved by the Animal Care and Use Committee of University Hospital Freiburg (same for Strasbourg). Visual acuity is necessary for performance in tests of spatial learning. Rodents with impaired visual systems are thus not suitable. Also, lighting must be sufficient in order for the rats to see the different cues located on the surrounding walls. It is useful to utilize basic-shaped (square, circle, triangle) but well-contrasted cues (e.g., black-painted cues on a white-painted background). Likewise, severe motoric deficits are exclusion criteria because swimming is required for this test and drowning may occur. Finally, hyper-anxious rodents can display a strongly biased search behavior, which impacts on performance.

### 2. Apparatus Set Up

1. Construct a double-H maze consisting of a 160 cm central alleyway, which is intersected at both ends and in the center by three 160 cm parallel run arms (see **Figure 1**). Ensure that the central alleyway and its perpendicular arms are 20 cm wide, and are surrounded by 35 cm high transparent Plexiglas walls. Thickness of the Plexiglas is 6 mm for all parts.
2. Secure the walls in place using glue and screws, and waterproof the maze using silicone joints at all internal angles. These joints can be replaced easily should they lose their waterproofness. Place a drain outlet at the extremity of one of the corner arms (or in the middle of the maze) for emptying.
3. Place the maze on an 80 cm high table, with adequate space around it for a) walking around the maze, and b) placement of well-contrasted cues. Designate each arm in the maze by its position, i.e., northwest (NW), north (N), northeast (NE), southwest (SW), south (S), and southeast (SE).
4. Place a ceiling-mounted camera above the maze for post-test analysis of animal behavior, using either manual or automatic (video-tracking) methods (see §5).

### 3. General Comments

1. Prior to use, fill the maze with water to an approximate height of 18 cm (200 L). This is high enough to prevent rats from touching the bottom of the maze with their feet, but shallow enough to prevent escape.
2. After pre-training, render the water opaque by mixing 250 g skim milk powder. Change the water on a daily basis to prevent the milk-water from becoming rancid. Maintain the water temperature between 21 - 23 °C to provide incentive for the rats to seek the escape platform.  
NOTE: As rats become familiar with the position of the platform during pre-training, its position is moved to a different arm during training.
3. Prior to use, immerse a 17 cm high, 10 cm diameter platform at the extremity of one of the corner arms (NE, NW, SE or SW). Ensure the height to be submerged is 1 cm below the surface of the water surface. Train the rats to reach the target platform using either an allocentric or egocentric learning strategy, which is dependent on the type of paradigm used (see §4).

### 4. Basic Training Protocols

NOTE: Rats are typically provided with an initial day of pre-training, which allows them to become familiar with the maze.

1. For pre-training release the rat from the extremity of one of the center arms (e.g., S arm) and place the goal platform at the extremity of one of the corner arms (e.g., NE), then give the rats 4 consecutive 60 sec trials in which to reach the target platform.
2. Upon reaching the escape platform, allow the rat to wait there for 15 sec, so that they can rest and observe their surroundings. Regardless of the start position, always block the opposite arm with a transparent guillotine door, which prevents entry.  
NOTE: During pre-training, leave the water transparent, and adjust the platform height such that it protrudes 1 cm above the water's surface, thus making it visible to the rat.

3. Perform daily training session consisting of up to 4 consecutive trials, separated by a 10 sec gap at least (discrete training, *i.e.*, with intervals of several minutes between trials, is an alternative).
4. For training, relocate the platform from its pre-training position to the chosen arm (*e.g.*, NW), and submerge it at its extremity 1 cm below the water surface. Now render the water opaque by addition of milk powder and perform the following training (section 4).
5. For rats that do not reach the target platform within 60 sec, return them to the starting position, and gently guide them to the platform by the experimenter.
6. Measure several variables during the training and probe sessions, such as, distance swam, latency to goal arm / platform, time spent in each arm, as well as the number of initial/repetitive errors (see **Figure 2**). Keep in mind that latencies may be influenced by motor difficulties. Should the case arise, distance and errors appear as more reliable variables regarding cognitive performance.
7. Allocentric Strategy Training:
  1. Day 1 - Pre-Training:
    1. Do not add milk powder for this step. Place escape platform protruding 1 cm above the water's surface in a fixed location. Train rats in 4 consecutive trials to reach the platform.
  2. Days 2 - 5 - Training:
    1. Add 250 g of skim milk powder to the water to render it opaque. Move the platform to a different arm (*e.g.*, from NE to NW) and add water so that the platform is 1 cm below the water surface. Release rats from either the N or S arm in unpredictable sequences for each session, such that both arms are used twice as trial start in a single session (4 trials/day, *e.g.*, SNNS, NSNS; see **Figure 3**).
  3. Day 6 - Probe Session:
    1. Remove platform for the probe trial. Release rats from a different arm to those used during training (*e.g.*, SW), and allow them to swim for 60 sec. See **Figure 4** for representative swim tracks. Analysis of the time spent in the target arm (former location of the platform) gives an indication about whether rats use a spatial strategy, another type of strategy, a sequential combination of different strategies (see below), or a disorganized search pattern.
8. Egocentric Strategy Training:
  1. Day 1 - Pre-Training:
    1. This is the same as first step in the allocentric strategy training (step 4.1). Do not add milk powder for this step. Place escape platform protruding 1 cm above the water's surface in a fixed location. Train rats in 4 consecutive trials to reach the platform.
  2. Days 2 - 5 - Training:
    1. Add 250 g of skim milk powder to the water to render it opaque. Move the platform to a different arm and add water so that the platform is 1 cm below the water surface. Release rats from the same start arm (S or N; see **Figure 5**) for every trial (4 trials/day).
  3. Day 6 - Probe Session:
    1. Remove platform for the probe trial. Rats are released from an arm different from where they were released during the training. Allow the rats to swim for 60 sec. Block the opposite arm with a transparent guillotine door.  
NOTE: Modify the above training strategies for according to the requirements of the particular experiment, *e.g.*, Testing Drug Effects *etc.*
9. Dry the rat off with absorbent towels after each session in the water.

## 5. Analysis

1. Perform measurements of latencies, initial and repetitive errors, first choices and response type manually by recording these variables from the videos taken from the overhead camera.
2. Alternatively if available, utilize commercially available video-tracking software and configured to record these variables automatically.
3. Statistical Analysis:  
NOTE: The specific implementation of statistical analysis depends on the study, which is taking place.
  1. Perform a one, two or three-way ANOVAs regarding the initial/repetitive errors, latency to goal arm/platform, and time spent in the target arm; with factors that include the test day, and treatment group(s).
  2. Where necessary, follow these ANOVAs using post-hoc Newman-Keuls multiple comparisons tests. To compare performance, use a reference value (*e.g.*, the time spent in the target arm during a probe trial vs. the chance level), and perform t-test.
  3. If required, use non parametric statistics in addition (*e.g.*,  $\chi^2$ , see below) or instead when conditions for parametric ones are not fulfilled.

## Representative Results

### Egocentric Learning Strategy

A study was carried out to determine whether the chosen memory strategy in rats changes based on alterations of their perspective of external environmental cues, following an egocentric-learning paradigm<sup>25</sup>. Rats were trained over 4 days (4 trials/day) to reach a goal arm located at NE, and were subsequently tested on the fifth day using a misleading probe trial, in which the start arm was either moved 60 cm to the left (*i.e.*, NE start for animals released from the N arm during training; SW start for animals trained from the S arm), or rotated 180°. During the probe trial, rats were shown to develop a preference for procedural-memory responses when the platform was shifted 60 cm to the left of its normal position (at 92.3% using 26 rats; see **Figure 6**). This was evident for rats that were trained in both the N and S arms (and subsequently released from the

NE and SW arms during the probe-trial, respectively). By contrast, only 8 out of 25 rats (32%) that were released from the opposite arm from that which they had been trained in (*i.e.*, a change from N to S, and vice-versa) had displayed a procedural response, with a higher presence of place responses. A  $\chi^2$  analysis showed that the difference in this distribution was highly significant ( $\chi^2 = 19.80$ ,  $p < 0.001$ ). In the rotation group, 5 out of the 13 rats, which were released from the N arm during training, had displayed a behavior that didn't account for either a place or procedural response (designated as "OTHER" in **Figure 6**), which was significant ( $\chi^2 = 5.77$ ,  $p < 0.05$ ). To summarize, rats that had been released in the probe trial with a similar environmental perspective to that observed during training (60 cm lateral shift) had predominantly utilized an egocentric strategy, whereas rats with large changes in their environmental perspective (via 180° rotation) had displayed a higher incidence of place or "OTHER" responses.

### Deep-Brain Stimulation in the Dorsal-Striatum - Implications for Procedural Learning

The procedural-learning paradigm has been utilized in rats, which underwent deep-brain stimulation (DBS) in the dorsal striatum<sup>26</sup>, in an effort to understand how it affects the animals' acquisition, and choice of strategy in a misleading probe trial. Rats were split into three groups (stimulation, sham-stimulation and control) and were trained for three consecutive days (4 trials/day) to swim from the start position in the S arm, to an escape platform hidden in the NE arm. Immediately following each training session rats were administered DBS over 4 hr in alternating 20 min periods. Rats were subsequently given a 20 sec probe trial 24 hr after the last training day in which the platform was removed, and the start location was shifted 60 cm to the left (SW arm). Whilst this study had reported no significant differences between the stimulation (STIM) and sham-stimulation (SHAM) groups during acquisition performance, they had displayed an altered behavior during the probe trial. Performance of each of the groups of rats during the probe trial is shown in **Figure 7**. Although each of the three groups of rats had demonstrated an above-average time spent in the target arm ( $p < 0.01$ ; at 2.9 sec chance level), there were no significant differences between the groups ( $F(2,42) = 1.4$ ), as analyzed using a one-way ANOVA (Group). Chance level was determined in this case by dividing the goal arm surface area over the total maze area, and multiplying by the probe-trial time (20 sec). Furthermore, no significant differences were observed between the groups regarding the latency to reach the former platform location ( $F(2,24) = 0.5$ ). Generally speaking each of the groups of rats required between 8 and 11 sec to reach the arm where the platform had been located during the training period, with between 6 and 8 sec spent in the target arm (~35% of probe-trial duration). This was significantly better than the chance level (at 2.9 sec;  $p < 0.01$ ). First-arm choice was different between the STIM and CONT rats also, with 42% of the STIM rats choosing the N arm (indicating a procedural memory response), 42% choosing NW (other response) as their first choice, and the remaining 14% choosing the NE arm (place response). By contrast, 65% of the CONT rats swam directly to the N arm, with only 7% choosing the NW one. The differences between these groups were significant, as assessed using a  $\chi^2$  statistic ( $\chi^2 = 4.09$ ,  $p < 0.05$ ). Taken together, the observations made during the probe trial indicate a modification of processes underlying procedural memory in the rats that received deep-brain stimulation.

### Procedural Memory Testing in Rat Models of Neurological Disorders

#### Schizophrenia

Procedural learning and memory in rats has been probed using animal models of relevance to schizophrenia, using an egocentric-learning strategy in the double-H maze. Lecourtier and colleagues<sup>27</sup> studied how a neonatal ventral-hippocampal lesion (NVHL) affects procedural memory in rats, at postnatal days 84 - 87. After a day of pre-training, rats were trained over 3 consecutive days (4 trials/day) in which they were released from the S arm (with the N arm blocked by a transparent guillotine door), and the platform was located at the extremity of the NE arm. On the next consecutive day following the training period, a misleading probe trial took place in which the platform was removed, and the rats were released from the SW arm (with the S arm blocked), and were allowed to swim for 60 sec.

Results obtained during the 3-day training show the mean latency to reach the platform (**Figure 8A**, top), and the mean number of errors made (**Figure 8A**, bottom). Over the 3-day training period, there was a significant effect of Training Day on platform latency ( $F(2,38) = 46.67$ ,  $p = 0.0000$ ) and the number of errors made ( $F(2,38) = 7.06$ ,  $p = 0.002$ ), which was reflected by a reduced platform latency and number of errors (respectively), throughout the course of training by both groups of rats. When comparing the performance of both groups of rats, there were significant effects of Lesion on both the platform latency ( $F(1,19) = 25.81$ ,  $p = 0.00006$ ), and also on the number of errors made ( $F(1,19) = 14.92$ ,  $p = 0.001$ ), in which case the NVHL rats were shown to perform worse. No significant interactions between Training Day and Lesion were observed regarding the latency to reach the target platform ( $F(2,38) = 0.34$ ,  $p = 0.72$ ) or the number of errors ( $F(2,38) = 0.18$ ,  $p = 0.18$ ), which can be explained by the presence of a similar trend of improvement in performance throughout the training period, for both groups of rats. Typical swim tracks as recorded during the start of the training, and the probe-trial are depicted in **Figure 9**. The proportion of first-choices that rats used during the probe-trial was analyzed using a  $\chi^2$  test, in which case comparisons were made based on the proportion of choices that corresponded to a procedural learning-based strategy. Whilst 8/8 rats from the sham group (100%) had displayed a procedural learning-based choice during the probe session, only 1/13 rats from the NVHL group (7.7%) had displayed this behavior, with the remaining choices being in an arm that is not reflected by either a place- or procedural-strategy (**Figure 8B**, bottom). These differences were statistically significant ( $\chi^2 = 17.23$ ,  $p = 0.0000$ ). Generally speaking, these findings highlight a deficit in the ability of NVHL rats to a) acquire the task, and b) acquire a procedural memory-based response, as compared to the sham group. This latter finding reflects a spatial disorientation in the NVHL rats.

#### Huntington's Disease

Procedural learning and memory have also been probed in a transgenic rat model of Huntington's disease (tgHD), using a similar paradigm as that previously described<sup>28</sup>. 40 tgHD rats (25 males; 5 homozygote, 15 heterozygote, 5 wild-type, and 15 females; 4 homozygote, 5 heterozygote, 5 wild-type) were tested in the double-H maze using an egocentric-learning strategy at 13 months. Animals were trained over 4 days with 4 trials per day (days 1 - 4; **Figure 10A**) followed by a single probe trial (Probe 1, day 5; **Figure 10B**). On Day 6, animals underwent an additional 4 trials of training, which was then followed by a second probe trial on Day 7 (Probe 2; **Figure 10C**). Throughout the training period all three groups of rats had displayed a progressive improvement in acquisition performance, although there were no statistically significant differences between these groups (**Figure 10D**; Genotype,  $F(2,43) = 2.9$ ,  $p = 0.07$ ). In spite of this, there is a clear tendency for the homozygote rats to not perform as well as the heterozygote and wild-type rats during the first trial of each training or probe session, which is perhaps indicative of an information recall deficit (**Figure 10D**, Genotype  $\times$  Trials,  $F(38,646) = 2.6$ ,  $p < 0.001$ ).

During both the first and second probe-trials there were no significant differences between the groups of rats regarding the latency to reach the platform location (**Figure 10E and F**, Genotype,  $F(2,34) = 1.6$  and  $0.1$ , both n.s.), or the time spent in the target arm (**Figure 10G and H**, Genotype,  $F(2,34) = 2.7$  and  $1.3$ , both n.s.). However, when the amount of initial and repetitive errors between the groups were assessed, homozygote rats were shown to have a significantly increased number of initial errors (**Figure 10I**: Genotype,  $F(2,34) = 11.1$ ,  $p < 0.001$ ; post hoc Newman–Keuls test, homozygote tgHD > wild-type, heterozygote tgHD,  $p < 0.05$ ), and repetitive errors (**Figure 10J**: Genotype,  $F(2,34) = 12.1$ ,  $p < 0.001$ ; post hoc Newman–Keuls test, homozygote tgHD > wild-type, heterozygote tgHD,  $p < 0.05$ ). Taken together, this data is suggestive of a distinct memory deficit in the tgHD rats.

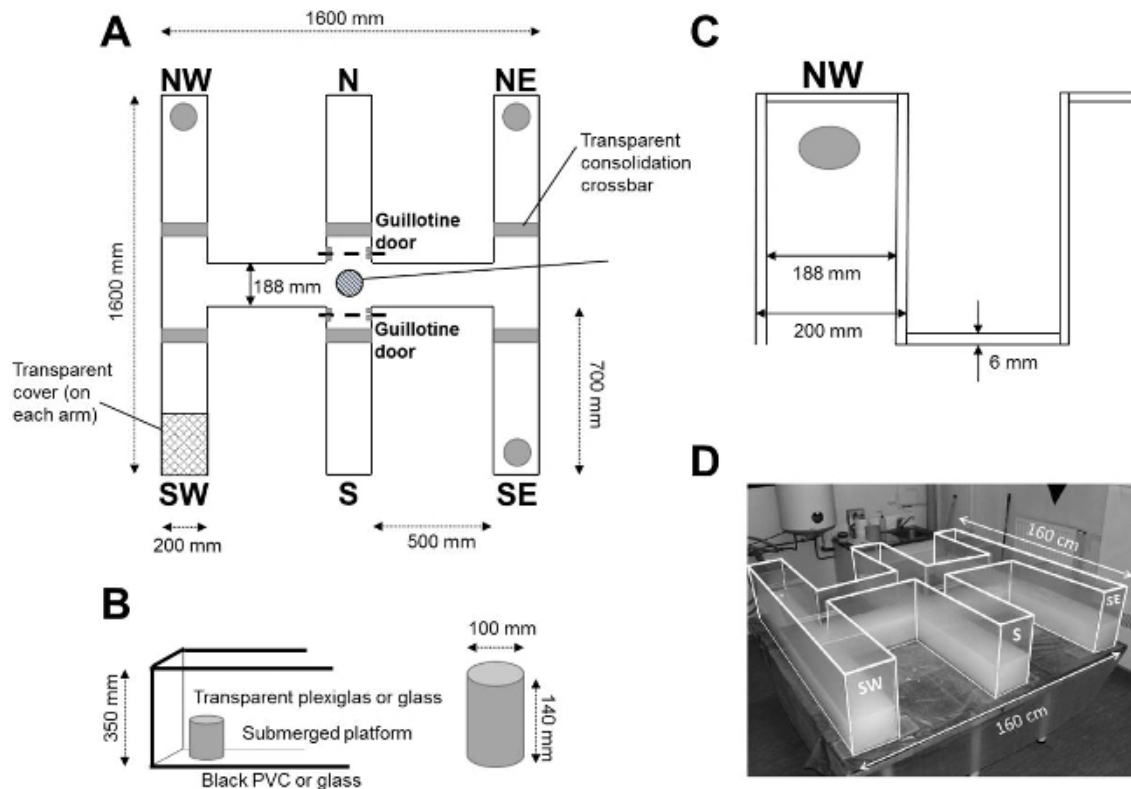
#### Allocentric Learning and Memory Performance following a Bilateral Muscimol Inactivation of the mPFC or dHip

The role of the reuniens and rhomboid (ReRh) thalamic nuclei regarding the regulation of cortico-hippocampal interactions was studied in an allocentric-learning paradigm using the double-H maze and a bilateral muscimol (MSCI) inactivation of these brain regions<sup>29</sup>. One experiment in this study is described here, in which rats were trained over 4 days (4 trials/day), with the escape platform was located at the extremity of the NE arm. They were released from either N or S arm in a randomized sequence (e.g., S, N, N, S; see §4.7 for explanation). Two probe trials were given (1) 24 hr after the first 2 days of training and (2) 24 hr after 2 additional days of training. Thus animals were trained on days 1, 2, 4 and 5; with probe trials taking place on days 3 and 6. 30 min prior to each probe trial, rats were infused with 0.26 or 0.70 nmol in 1  $\mu$ l of muscimol (MSCI) in the medial prefrontal cortex (mPFC) or dorsal hippocampus (dHip). The remaining animals were infused with an equivalent volume of phosphate-buffered saline (PBS) as a control.

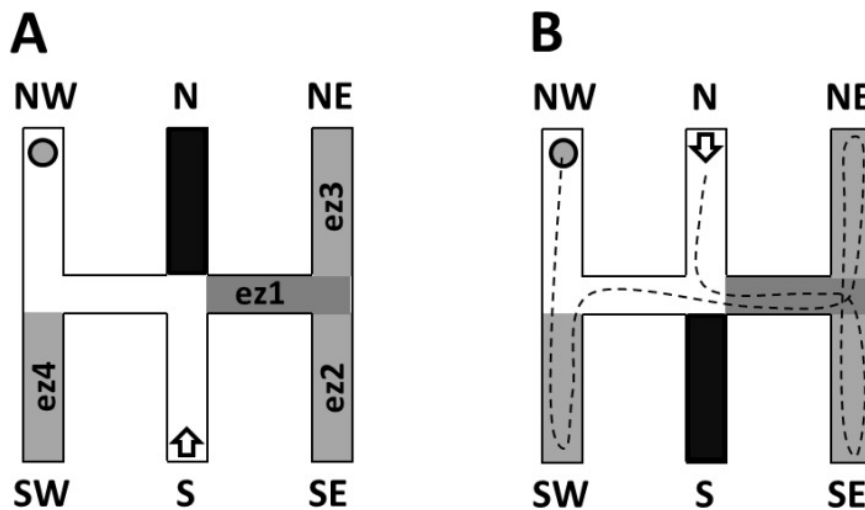
During the training sessions (Days 1 - 2, and 4 - 5), all groups of rats had shown a comparable performance regarding the overall distance swum in the maze (**Figure 11A-B**). Whilst the distance swum had shown to shorten over days ( $F(3,159) = 59.3$ ,  $p < 0.0001$ ), there was no significant effect of brain region ( $F(1,153) = 0.2$ , n.s.), inactivation ( $F(3,53) = 0.3$ , n.s.), and no significant interaction between structure and inactivation ( $F(2,53)=0.8$ , n.s.). Taken together, this shows a comparable pattern of learning between all of the groups of rats over the 4 training sessions, with no significant differences between them.

Representative swim paths for the probe and training sessions are shown in **Figure 11E**. Regarding the time spent in the place-arm by each of the groups of rats (**Figure 11C-D**), the performance was shown to be significantly above chance level for the mPFC and dHip groups pre-treated with PBS on the second probe trial ( $p < 0.05$ ), and above chance level for the mPFC-PBS group during the first probe trial ( $p < 0.05$ ). When MSCI was introduced to the mPFC, the time spent by rats in the place-arm did not differ from chance on either probe trial, for either the 0.26 or 0.70 nmol concentration. By contrast, the dHip-MSCI group had spent significantly less time in the procedural-response arm (than chance) for both concentrations during the first probe-trial, but their performance did not differ from chance during the second probe trial (both concentrations, again). In the second probe trial, a  $2 \times 2 \times 2$  ANOVA of the time spent in the place arm (NE) showed significant structure ( $F(1,53)=11.2$ ,  $p < 0.01$ ), inactivation ( $F(2,53)=17.4$ ,  $p < 0.001$ ), and probe trial ( $F(1,53)=9.0$ ,  $p < 0.01$ ) effects. What this shows is that the ability of the control rats to find the platform arm improved markedly after 4 days of training (as opposed to 2 days) – an effect, which was disrupted in both the mPFC and dHip inactivation groups. Generally speaking, these disruptions were more pronounced in the dHip groups, particularly given that performance deficits were observed for both the 0.26 and the 0.70 nmol concentrations of MSCI. By contrast the mPFC-inactivation deficits were more pronounced at the higher concentration of MSCI.

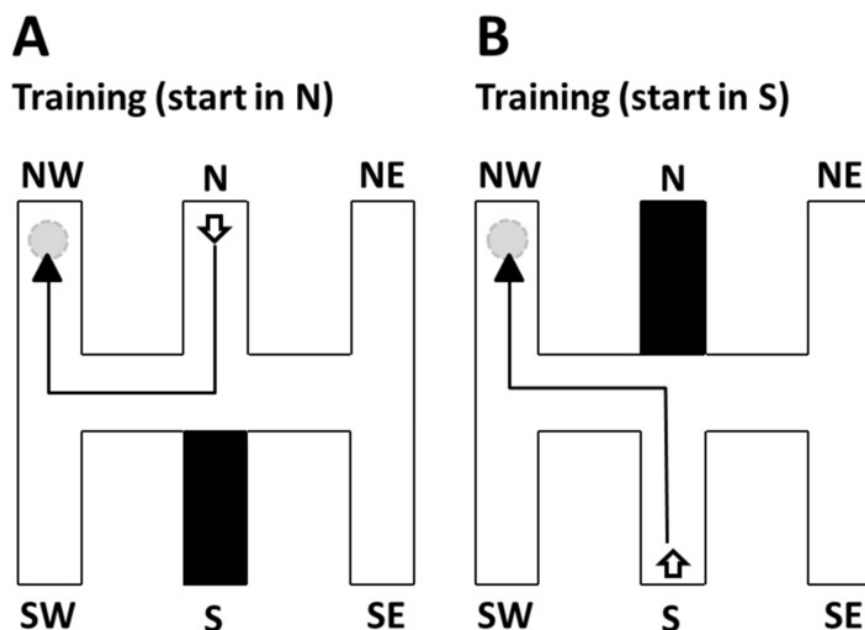




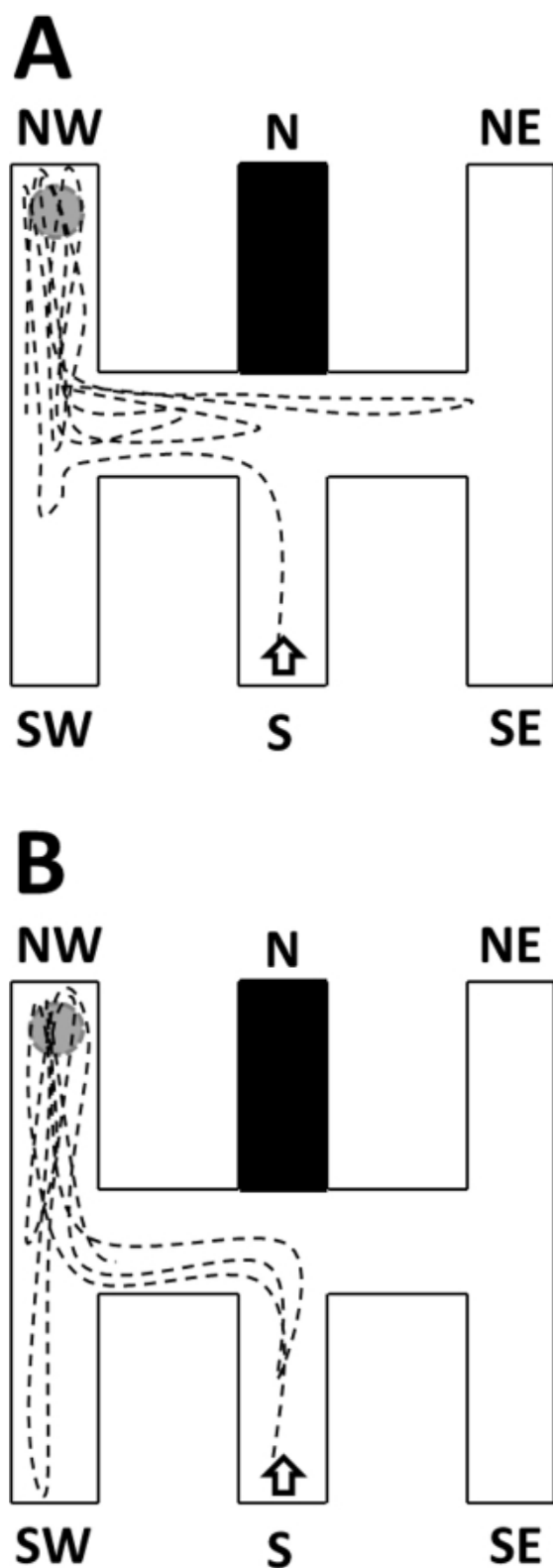
**Figure 1. Double-H maze schematic.** The Double-H Maze occupies a 1,600 x 1,600 mm square with an internal surface area of approximately 1.084 m<sup>2</sup>, and consists of wall panels, guillotine doors, crossbars, and transparent covers at the end of each arm (A). A 100 mm diameter, 140 mm high cylinder is placed at the end of one of the goal arms to serve as an escape platform (B). The thickness of the wall panels throughout the maze is 6 mm. A photograph of the Double-H Maze is shown (D; reprinted from<sup>24</sup>), with the maze edges highlighted for clarity. For further information on the maze construction, blueprints of the maze are available upon request. [Please click here to view a larger version of this figure.](#)



**Figure 2. Layout of zones for error analysis.** Bird's-eye view of the double-H maze, showing the error zones (ez1 - ez4) for 2 different starting positions (South - A; North - B). The error zones are highlighted in grey, and the closed arm (via guillotine door) is highlighted in black. Error zones are determined based on any possible deviations the rat could make from the shortest path between the start and the goal arms. Thus upon being released from the S arm (A), an initial right turn would result in the rat entering error zone 1 (ez1), which constitutes an initial error. Repetitive errors are counted when this happens more than once, for each zone. The hatched line in B highlights an example swim path, which places the rat into all 4 error zones prior to reaching the goal in NW, thus constituting 4 initial errors (ez1, ez3, ez2, and ez4). Reprinted from<sup>24</sup>. [Please click here to view a larger version of this figure.](#)



**Figure 3. Protocol used in allocentric strategy training.** An allocentric training paradigm takes place over 3 - 4 days, and rats are given up to 4 trials per day. Rats are released from the N (**A**) and the S (**B**) arm in a randomized sequence (e.g., NSSN, NSNS, SSNN, etc.), with the opposite arm blocked by a transparent guillotine door. The escape platform is fixed in the same location (NW in this case). For the probe trial, the platform was removed and the rats are released from the S arm, and given 60 sec to swim inside the maze. Reprinted from <sup>24</sup>. [Please click here to view a larger version of this figure.](#)

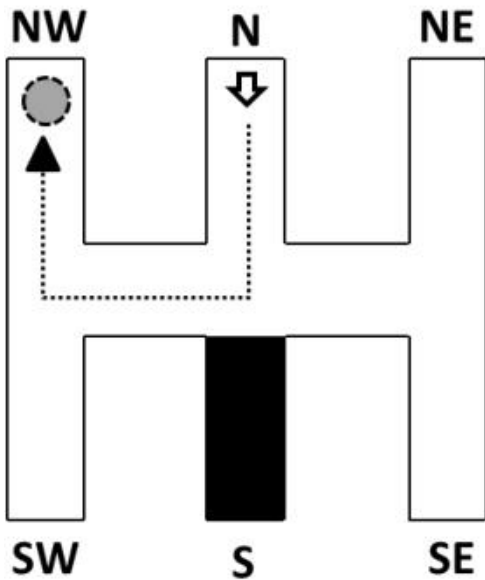


**Figure 4. Examples of swim tracks from allocentric strategy training.** Swim track examples are depicted as hatched lines during a probe trial, which was given after a 1 day delay following 4 training days (at 4 trials/day). Rats were released from the S arm and the platform was removed from its NW (training) position. In **A**, the track corresponds to a latency to reach the former platform location of 6.62 sec, and the time spent in this arm was 25.16 sec (chance at 8.2 sec). In **B**, the track corresponds to a latency of 14.02 sec, with a time spent inside the arm at 26.45 sec. Reprinted from <sup>24</sup>. [Please click here to view a larger version of this figure.](#)



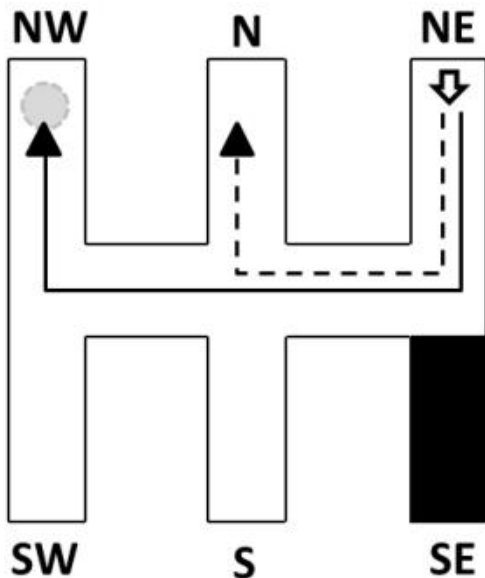
**A**

**Training (all days, all trials)**



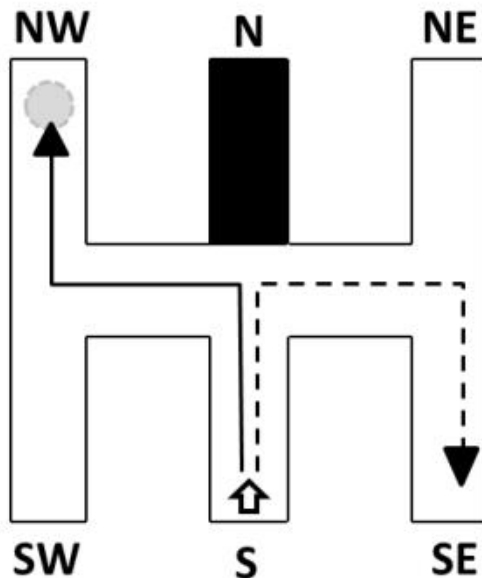
**B**

**Probe trial, start in NE**

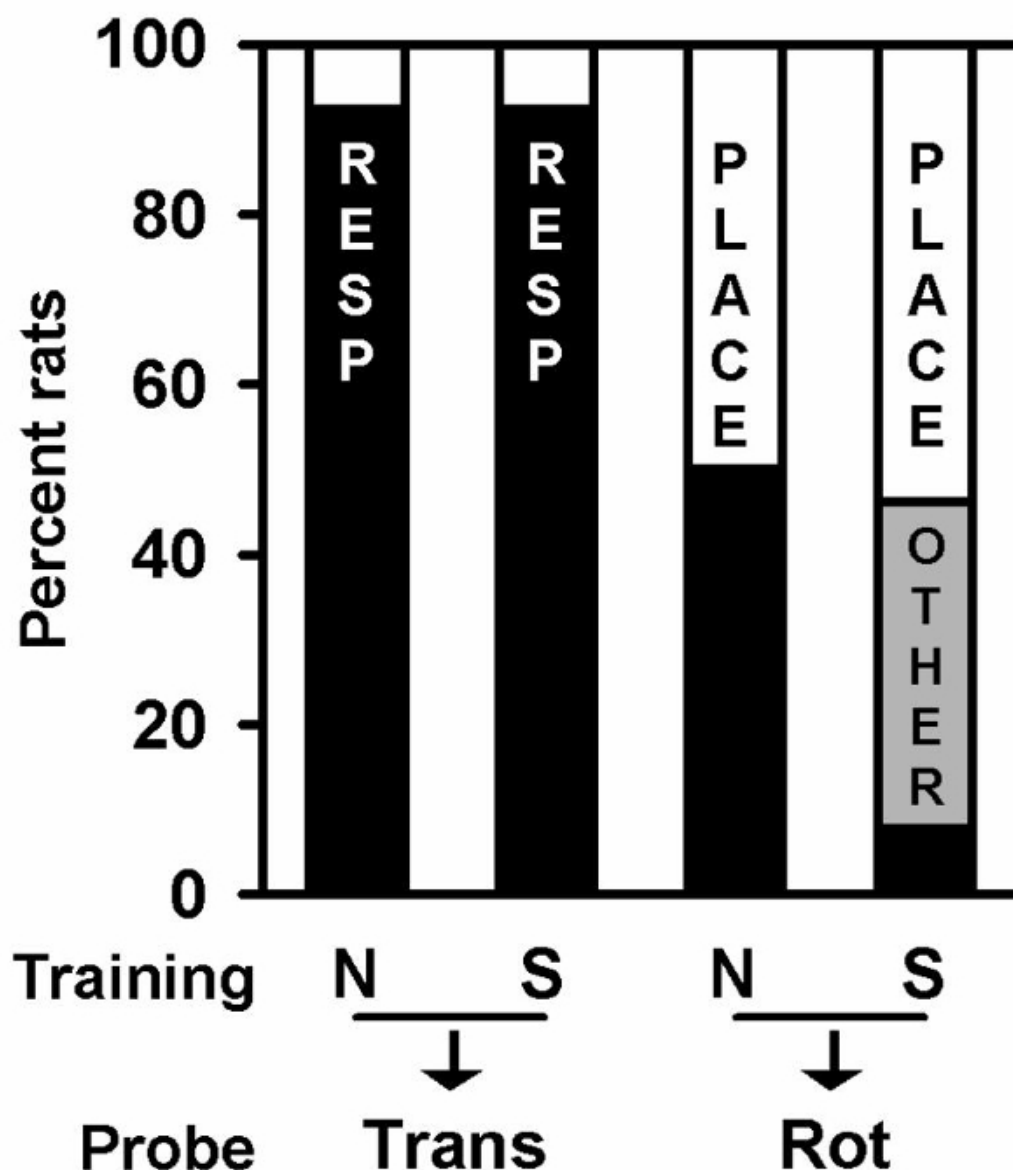


**C**

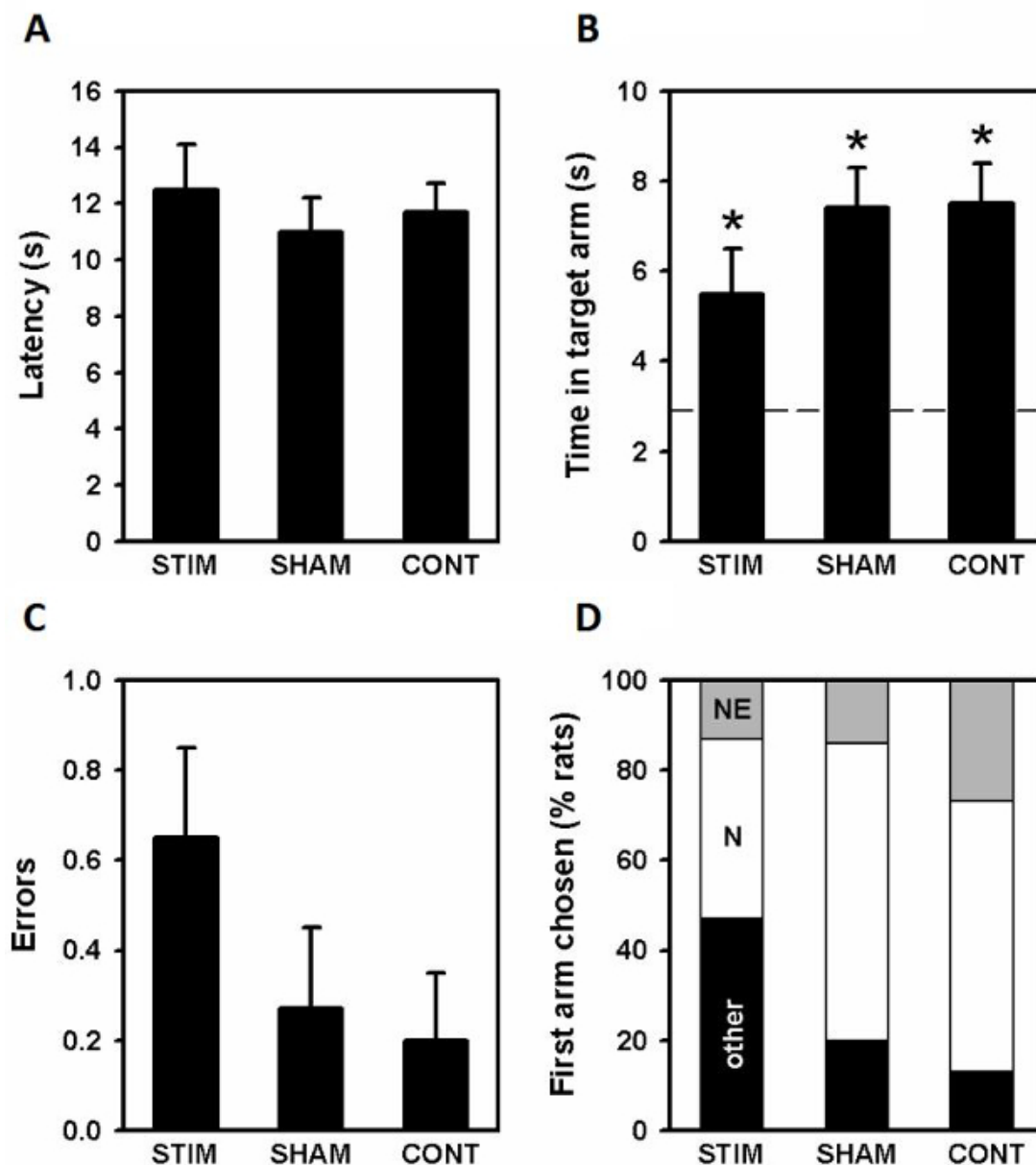
**Probe trial, start in S**



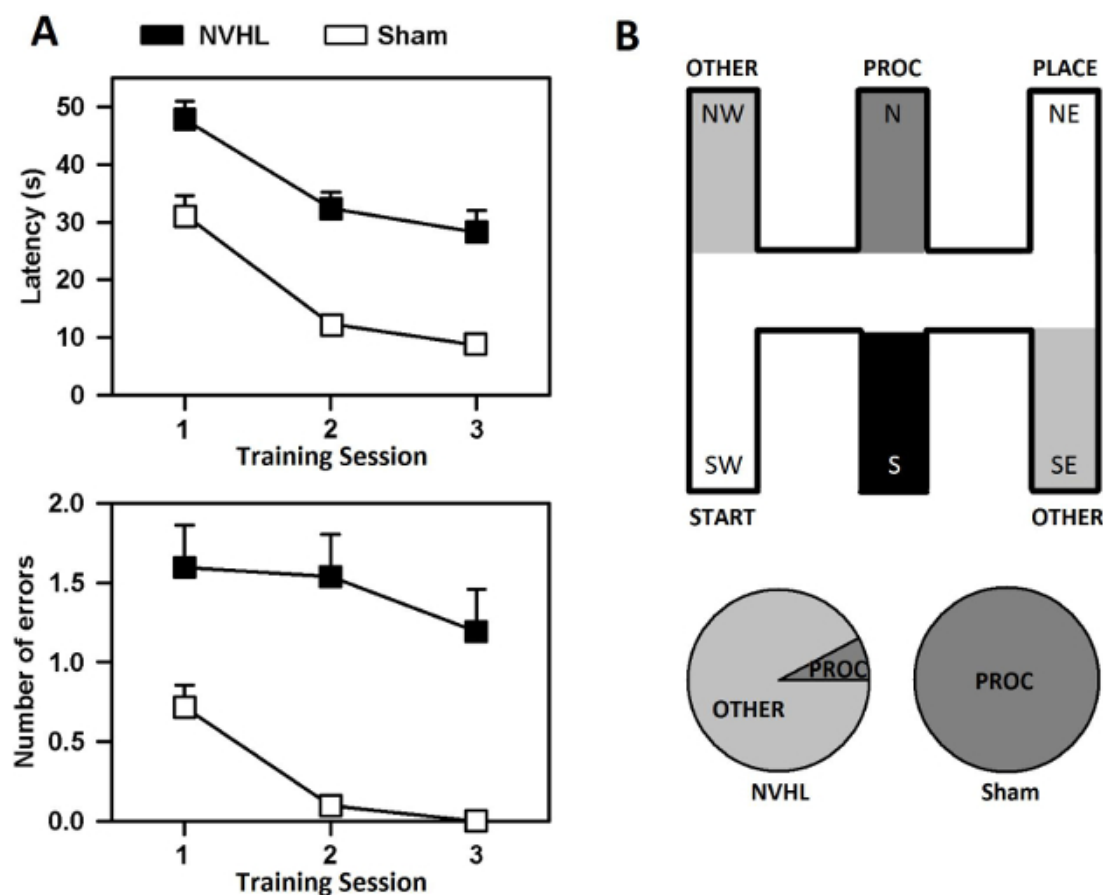
**Figure 5. Protocol Used in Egocentric Strategy Training.** During training (A), rats were released from the N arm of the double-H maze (white arrow), with the S arm closed off by a transparent guillotine door. The escape platform was located in the NW arm, and rats were trained to locate this arm over 4 consecutive days (at 4 trials/day). During the probe trial, the escape platform was removed, and the rats were released from either the NE (B, white arrow), or the S arm (C, white arrow), with the SE or N arms closed off, respectively. The probe trial lasted 60 sec and rats would make either an egocentric response using procedural memory (swim to N, for NE start; swim to SE, for S start), a place response using place memory (swim to NW), or neither. [Please click here to view a larger version of this figure.](#)



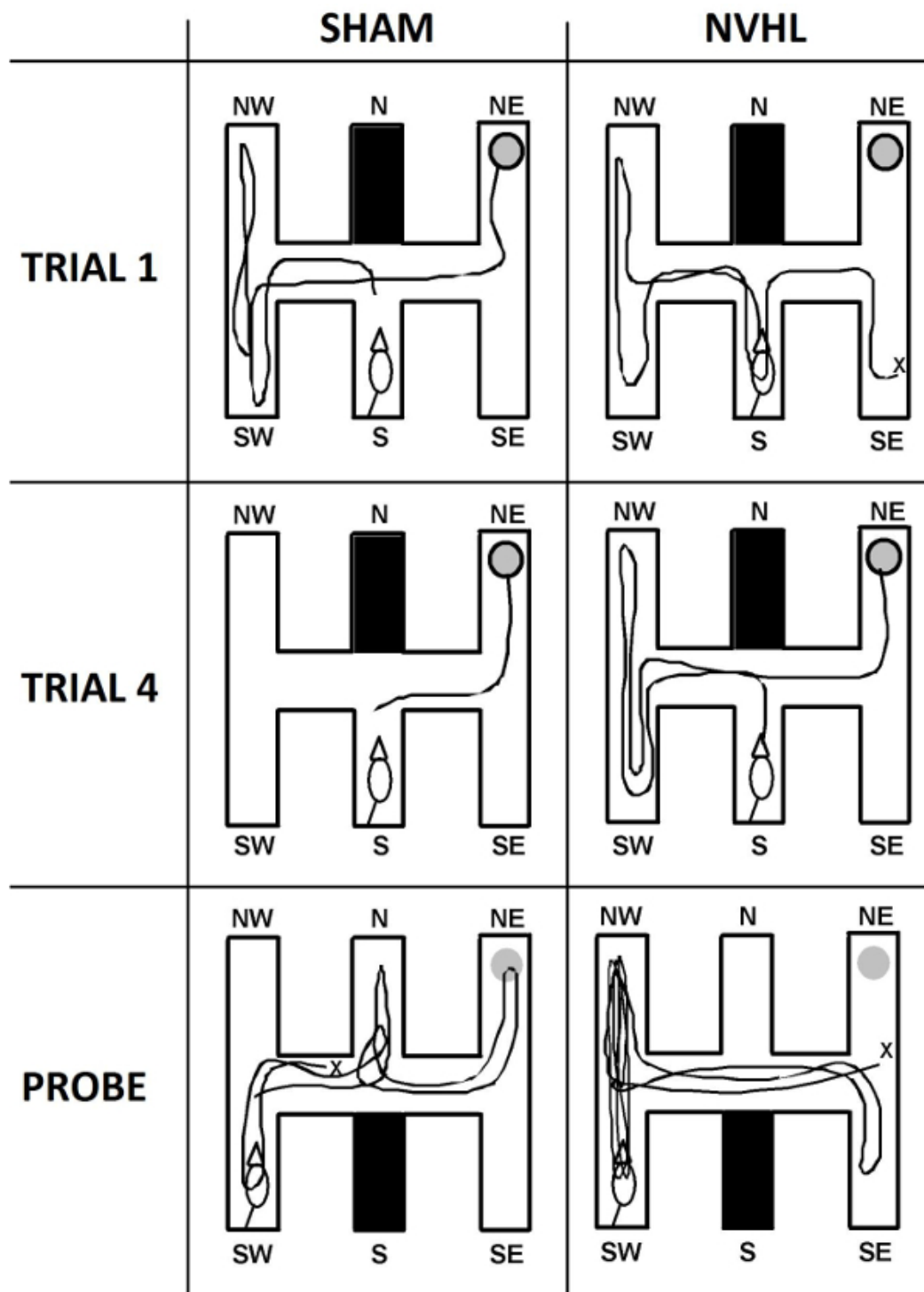
**Figure 6. Performance during the Probe Trial.** Percentage of responses made by each of the 4 groups of rats are shown for the probe trial. Regardless of rats being trained from either the N or S arm, a shift of the start arm by 60 cm to the left (Trans) resulted predominantly in procedural responses (RESP; 92.3%). However the two groups of rats that had their start arm rotated 180° (Rot) displayed a higher amount of place responses (PLACE). This indicates that subtle shifts in the environmental view (in the Trans groups) resulted in more rats switching to place-memory to find the platform location. Responses that were neither place nor procedural responses were designated as OTHER. Reprinted from <sup>25</sup>. [Please click here to view a larger version of this figure.](#)



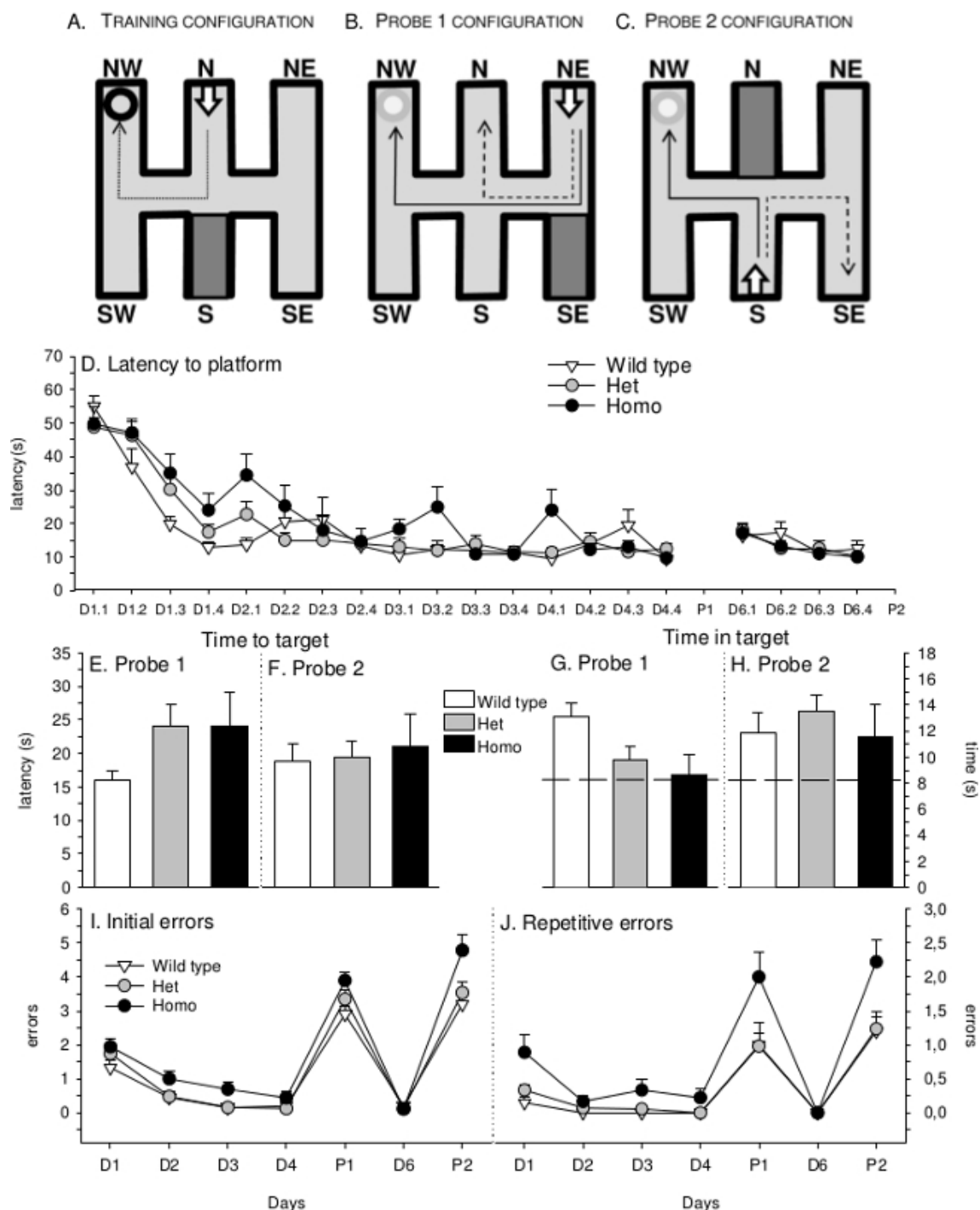
**Figure 7. Effect of HFS on memory performance in the double-H maze.** Rats underwent a 20 sec probe session following 3 days of training in an egocentric paradigm (4 trials/day). Data are shown for the probe session that took place on the next consecutive day, with the stimulation (STIM), sham-stimulation (SHAM) and control (CONT) groups of rats. There were no significant differences observed between the three groups of rats regarding the mean latency to reach the training platform location (**A**), and in the mean time spent in the target arm (**B**). In **B**, \* represents a significant difference in the time spent in the target arm from chance level (as indicated by a hatched line;  $p < 0.05$ ). Regarding the mean number of errors made by each group during the first 20 sec, a significant difference was observed between the STIM and CONT groups ( $p < 0.05$ ). The percentage of procedural, place and "OTHER" responses for each group is shown in (**D**), as white, grey and black bars, respectively. The difference between STIM and CONT rats regarding the N and NW responses was significant ( $\chi^2 = 4.09$ ,  $p < 0.05$ ), which perhaps highlights an altered use of procedural strategy in the STIM during the probe session. Reprinted from <sup>26</sup>. [Please click here to view a larger version of this figure.](#)



**Figure 8. NVHL-Induced deficits of procedural memory.** During each of the 3 training days, rats with neonatal ventral hippocampal lesions (NVHL) spent a significantly greater amount of time reaching the target platform than sham-operated animals (**A**; top). Also, the NVHL group made more errors prior to locating the target platform (**A**; bottom). The maze layout during the probe trial is shown in **B** (top), with the start arm located in SW, and each of the accessible arms highlighted based on the rats chosen strategy when being released into the maze: place response (PLACE; NE), procedural memory (PROC; N) and neither (OTHER; NW and SE). The S arm was closed off with a guillotine door. During the probe trial NVHL rats displayed a predominance of OTHER responses that accounted for neither place nor procedural memory, in contrast to the sham group which had all displayed procedural responses (**B**; bottom). Overall the impaired training performance and the lack of clear strategy during the probe session in the NVHL rats demonstrate learning deficits and spatial disorientation, respectively. Reprinted from <sup>27</sup>. [Please click here to view a larger version of this figure.](#)

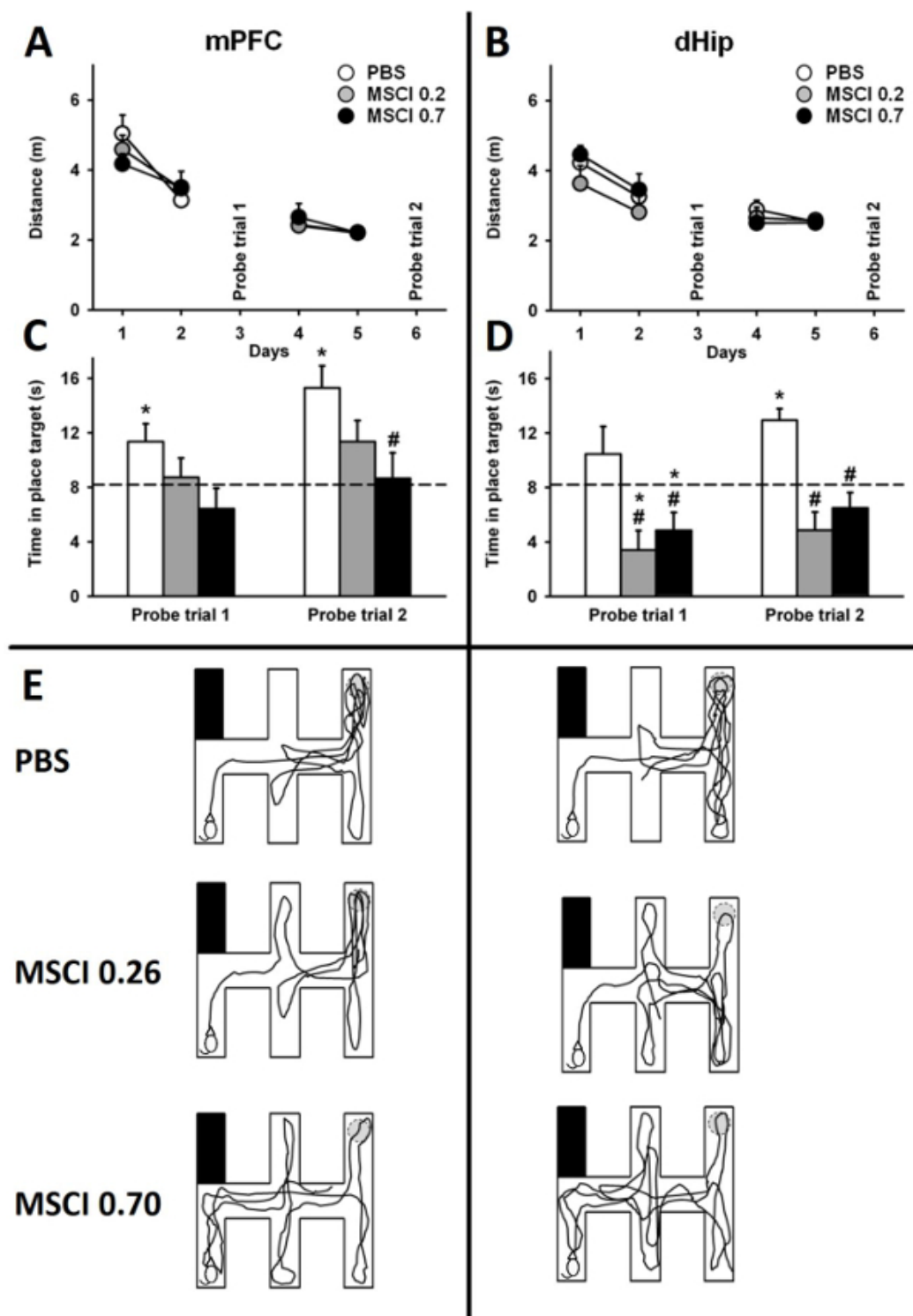


**Figure 9. Typical swim tracks during training and retention.** Typical swim tracks are shown for both the sham-operated (SHAM) and neonatal ventral hippocampal lesion (NVHL) groups, for the first and fourth trials during the first test day (top and middle), and during the probe trial (bottom). During the training phase the N arm was closed off with a transparent guillotine door, and rats were released from the S arm. During the probe session the start arm that was used during training (S) was closed off with a transparent guillotine door, and rats were released from the SW arm. An "x" denotes the location where rats were taken out of the maze after the 60 sec trial period had elapsed. A procedural-memory response was counted when the rat had moved first into the N arm. Reprinted from <sup>27</sup>. [Please click here to view a larger version of this figure.](#)



**Figure 10. Training and retention performance in rat models of Huntington's Disease.** The training (A) and probe (B-C) configurations of the double-H maze at 13 months are shown, including the start points (identified by a white arrow) and the closed-off arm (highlighted in grey). During the training phase, rats swam from the N arm to a platform, which was located at the extremity of the NW arm. During the probe sessions the platform was removed, and rats were released from either the NE arm (Probe 1; B) or the S arm (Probe 2; C). In both cases a hatched line indicates a procedural-memory based response, whereas a solid line indicates a place-memory response. Latency to reach the platform during training (D), Probe 1 (E) and Probe 2 (F) sessions are shown for the three groups of rats. The time that rats spent in the target arm (previous platform location) during the first (G) and second (H) probe sessions are also shown, with the hatched line representing the chance-level (8.25 sec). The initial (I) and repetitive (J) errors during each of the training and probe sessions are shown for each group. All data is shown as mean  $\pm$  SEM. Reprinted from <sup>28</sup>. [Please click here to view a larger version of this figure.](#)





**Figure 11. Training and retention performance in rats with dHip or mPFC inactivation.** All groups of rats were trained on days 1 - 2 and 4 - 5 using an allocentric-learning paradigm (4 trials/day), with probe sessions taking place on days 3 and 6. During the probe session rats were released from the SW arm, with the NE arm closed by a transparent guillotine door (E). Rats received a bilateral infusion of either 0.26 or 0.70 nmol of MSCI, or PBS, into either the medial prefrontal cortex (mPFC) or dorsal hippocampus (dHip) prior to each probe session. The average distance swum for mPFC (A) and dHip (B) rats prior to reaching the escape platform are shown for each of the training days, with no significant difference among the groups. During the probe trial, the time spent in the target place-response arm (NE) during the 60 sec period are shown (C-D). Typical swim paths are shown for each of the groups of rats during the probe session (E). All data is shown as mean  $\pm$  SEM, with # indicating a significant difference compared with PBS ( $p < 0.05$ ), and \* indicating a significant level shown as a hatched line). [Please click here to view a larger version of this figure.](#)

## Discussion

### Comments on Study Design and Analysis

Since its conception, the double-H maze has been utilized in a number of behavioral experiments in rats, which collectively were designed to study egocentric and/or allocentric responses in rats under normal<sup>24,25</sup> and altered<sup>26-29</sup> brain states. The latter studies include striatal deep-brain stimulation (DBS)<sup>26</sup>, animal models of neurological disorders<sup>27,28</sup>, as well as bilateral deactivations of various cortico-hippocampal brain regions using muscimol<sup>29</sup>. Many of the studies described utilized either an allocentric- or egocentric-learning strategy, as summarized in §4.7 and §4.8, respectively; although subtle variations to the core paradigm (e.g., a 180° rotation in the start arm during a misleading probe trial<sup>25</sup>) are made where appropriate to the particular study. The specific nature of the training paradigm depends on the study, which is taking place (e.g.,<sup>24-29</sup>). An initial error is counted whenever the rat deviates from the correct goal path back into either the start arm or into a new maze segment, for the first time. A repetitive error is counted whenever this happens more than once. The correct goal path is defined as the shortest route between the starting position and platform / goal arm. For example if the starting position is in the S arm, and the escape platform is in the NW arm, the resultant 4 error zones (ez1 – ez4) are located in the SW, NE and SE arms, as well as the east portion of the central alleyway (**Figure 2A**). The example swim trace in **Figure 2B** represents 4 initial errors, as the rat had visited each of the 4 error zones once. Rats are only considered to have entered an arm/maze segment when all 4 paws are inside it.

### Statistical Analysis

Given the tight correlation between the latency (between start position and platform) and the distance swum, the latter variable is often omitted. However, in rats displaying obvious motor problems, the distance or the number of errors seem a more reliable variable. For rats, which have been trained on an egocentric paradigm, their first swim choice inside the double-H maze during the probe trial constitutes either an egocentric, allocentric or "OTHER" response, depending on where they swim to first. This is analyzed with a  $\chi^2$  statistic. Regarding the time that rats spent in the target arm during the probe trials, the chance-level was calculated based on the proportion of surface area in this arm, relative to the rest of the maze (13.76%). Thus 13.76% x 60 sec = 8.25 sec.

### Modification of Training Strategies for e.g., Testing Drug Effects etc.

Numerous adaptations to the standard allocentric and egocentric learning protocols have been utilized across various studies, which have tested rats in the double-H maze. The frequency and duration of training sessions may be configured according to a particular protocol. For example, Pol-Bodetto and colleagues vary the number of test days (2, 4 or 6, as well as the number of trials per day (3 or 4)<sup>24</sup>. Furthermore, whilst the majority of studies utilizes a 60 sec probe trial (e.g.,<sup>24,25,27-29</sup>), Schumacher and colleagues used 20 sec<sup>26</sup>. Animals may be treated with drugs either before training<sup>24</sup> or before the probe session<sup>29</sup>. Finally, the location of the starting position during the probe session may be varied to give the animals a similar (e.g., Schumacher *et al.*<sup>26</sup>) or markedly altered (e.g.,<sup>24,25</sup>) initial view of the extra-maze cues.

### General Considerations

The double-H maze task has proven itself to be a simple memory task, which can be rapidly acquired by rodents, and requires no prior reinforcement techniques such as food restriction. Its basic principle involves rats swimming to reach a hidden escape platform, and subsequently learning its position, much like the Morris Water Maze. Rats can be trained to a stable performance in as little as 2 days (6 trials/day), giving this task a considerable advantage with regards to time-conscious studies such as pre-clinical applications and drug screening. The training protocol can be tailored to suit an allocentric or egocentric learning paradigm, and probe trials taken place throughout can provide an insight into the dynamic balance between the two memory systems that govern these behaviors, including the ability of rats to switch from one system to the other. Finally, numerous adaptations to the standard protocol (e.g., a lateral displacement of the start arm, vs. a rotation) can be carried out to reinforce the use of a particular strategy in a misleading probe trial<sup>25</sup>.

### Acquisition and Trace Duration

In numerous studies using the double-H maze, both the allocentric<sup>24,29</sup> and egocentric<sup>24-28</sup> training paradigms have led to stable memory traces, even in the presence of amnesic drugs, lesions and high-frequency stimulation (see below). Notably in an allocentric protocol (see §4.7) rats are not able to develop a routine for solving the task, because the starting position is randomized across the trials (mainly N or S; more can be used to increase the spatial demand of the task). Performance throughout the task has been measured by counting the initial and repetitive errors (deviations away from the task goal), as well as measuring the latency to reach the goal arm/platform. Additional measures made during the misleading probe trials include the time spent in the place-response arm, as well as the initial choice rats made upon being released into the maze. Consistently, in the studies that have taken place so far, these variables have been seen to gradually change over the relatively short training period in a stable manner, thus reflecting a performance improvement. For example in a 6 day allocentric-learning protocol, rats were observed to reduce the number of initial errors (from ~2 to almost 0) and repetitive errors (from ~0.5 to 0), as well as their latency to reach the escape platform (from ~35 sec to ~10 sec)<sup>24</sup>. During this particular study it was evident in the probe trials that rats had learned and could retrieve the location of the escape platform, as revealed by the mean latency to enter the target arm being below 8 sec (as opposed to the average first trial latency of 42.8 sec). Furthermore rats have been shown to retain the location of the escape platform even at the longest post-acquisition delay (18 days) between the last training day and the probe session<sup>24</sup>. In addition, no statistical evidence for performance degradation was found at shorter delays (1 and 5 days).

### Switch from Place to Procedural Memory

Previously, Packard and McGaugh<sup>7</sup> had demonstrated the tendency for rats to switch from a place to a procedural response, once they had acquired a repetitive task inside a cross maze. In this experiment rats were consistently released from the same start point in order to reach a food reward at another fixed point. Saline-treated rats had demonstrated a response strategy accounting for place memory after 8 days of training, and procedural memory after 16 days of training; in misleading probe trials whereby the start arm was moved to a different location. This phenomenon has been subsequently demonstrated following extended procedural learning in the double-H maze<sup>24,25</sup>, in which rats were

consistently released from the same starting arm (using an egocentric strategy; see §4.8). In one of the first studies, which utilized the double-H maze, it was found that subtle alterations in the environmental perspective in a misleading probe trial (a 60 cm lateral shift in the start position), led to mainly procedural responses in rats, as opposed to rats which had undergone a 180° shift in the start location and had subsequently displayed a much higher incidence of place responses<sup>24</sup>. This effect was attributed to rats utilizing a procedural-based strategy when their environmental perspective was similar enough to that seen during the training sessions. This was further explored in a later study which took into account the starting position (rats split into N and S arm starting position), in which groups of rats trained from either the N or S arm experienced either a lateral or rotational shift, the latter of which markedly altered the initial environmental perspective on the maze<sup>25</sup>. They found not only similar results regarding the choice of strategy based on the initial environmental perspective, but also this choice was relatively invariant of the position in which rats were initially trained in the maze. Taken together, these observations demonstrate that adjustments of the starting position in the double-H maze (during the misleading probe trials) can facilitate either a procedural or place response, depending on how markedly different the rats initial perspective on environmental cues are. For large differences, as seen in rats that start from the opposite arm from which they have been trained, they resort mainly to place memory. This is interesting because it demonstrates an ability for rats to switch to a previously learned place-memory on demand, when the situation calls for it, and also further reinforces earlier findings which suggest that rats learn a procedural response after acquiring the task using place memory<sup>7</sup>. This may be of benefit in future studies in which the experimenter may wish to know whether a rat can switch from procedural to place memory following a particular treatment.

## Performance Deficits in Animal Models of Memory Impairment

Since its conception, the double-H maze has been utilized in several studies which assessed the effects of memory impairment in rodents, through selective hippocampal lesions<sup>27</sup>, inactivation of cortico-hippocampal brain regions<sup>29</sup>, striatal deep-brain stimulation (DBS)<sup>26</sup>, as well as through the use of various systemically-administered drugs<sup>24</sup>. Whilst all of these examples had displayed performance differences in the post-training probe trials, the performance of treated and control rats had normalized towards the end of the training sessions. This highlights the simplicity of the task in that it can be rapidly acquired even in the presence of drugs affecting cholinergic or glutamatergic neurotransmission<sup>24</sup>, for instance. Various alterations in performance had been noted in the probe sessions. DBS of the dorsal striatum had revealed a significantly altered distribution of place/procedural/other responses in rats (vs. control rats) that undertook a misleading probe-trial following, an egocentric learning paradigm<sup>26</sup>. This was proposed to result from a modification of the neural processes that underlie procedural memory, in the rats that had received DBS. Bilateral muscimol inactivation of cortico-hippocampal brain regions (at 0.70 and 0.26 nmol) was found to induce major deficits in strategy shifting when rats were tested on a misleading probe trial<sup>29</sup>. In this study it was noticed that these deficits were also present through bilateral inactivation of the reuniens and rhomboid (ReRh) thalamic nuclei, which subsequently highlighted its possible role in strategy shifting in tasks that require both cortical and hippocampal information exchanges. The memory deficits in animal models of neurological diseases have also been assessed in the double-H maze<sup>27,28</sup>. Rats with neonatal ventral hippocampal lesions (NVHL) had displayed a significantly longer latency to reach the target platform ( $p = 0.00006$ ) as well as an increased number of errors made ( $p = 0.001$ ), as compared to control rats<sup>29</sup>. Furthermore, NVHL rats had displayed primarily responses during the probe trial that had accounted for neither place nor procedural memory (as opposed to the control rats which had all displayed procedural responses), which highlighted a spatial disorganization. Finally, transgenic rat models of Huntington's disease had shown clear memory deficits during the probe trials, following a procedural learning paradigm<sup>28</sup>. Taken together, this evidence highlights an intriguing role for the double-H maze to be utilized in studies which assess neurological impairments (and potential treatment options) in animals, since it allows for a fine control over the type of learning and strategy that is required for the task completion.

## Benefits and Drawbacks

To summarize previous discussion, the double-H maze is a simple and rapidly acquired task, which can lead to stable memory traces. Furthermore, it can enable the experimenter to direct the type of learning that is involved, namely allocentric or egocentric learning. The dynamic interactions between these two memory systems can be observed in a probe trial, and assessed under a variety of conditions (e.g., lesions, drugs, DBS), as previously described. Given that the double-H maze is a water maze, one of its primary drawbacks involves acute stress for the animals when placed inside the water, as with any water maze (e.g., <sup>30</sup>). Furthermore, many of the dependent variables in the task (e.g., latency to target platform) necessitate normal motor performance in rats. This is important because rats with motor impairments (e.g., models of Parkinson's disease) are known to swim differently to control animals, thus longer latencies in the task may be incorrectly interpreted as memory impairments. In such case, distance can be used as a last resort. Given that the water must be rendered opaque using skim milk powder, the maze must be emptied and refilled every day in order to prevent the water from becoming rancid, which itself is a time-consuming task, depending on the laboratory setup. Milk powder, however, could be replaced by a synthetic dye. Finally, unlike other maze tasks, the use of water prevents the use of simultaneous EEG recording (using either a cable tether or wireless recording system), unless a) an implantable system is used (which seldom have the transmission range to cover the double-H maze), or b) effort is undertaken to waterproof the system.

## Future Enhancements to this Method

Since its conception, the double-H maze has been involved in several studies, which have each made subtle adaptations to the standard protocol, as described in the representative results. Numerous other variables may be recorded that are relevant to the future studies that will take place, such as the second choice rats make following a procedural response during a probe session. For example rats may switch to place memory immediately after discovering that the escape platform is missing (negative feedback). Other behavioral measures can include an analysis of the velocity of swimming, as well as the characteristics of swim paths taken by the rat. As observed during in-house studies, rats that swim along a particular side of a double-H arm have a tendency to turn in that particular direction once they reach the following junction. Such behaviors may be recorded using commercially available video-tracking software. Finally, regarding the current allocentric-learning protocol, it cannot be excluded that this procedure might lead rats to the development of two routines. Furthermore the starting point in the probe trial could be the same as one of the training starting points. Thus a more appropriate protocol to train an allocentric strategy might consist of using 4 starting arms/day from where the rat is released in an unpredictable sequence (e.g., N, NW, SE, S; S, N, NW, SE; etc.). One of the remaining arms would be where the escape platform is located during training sessions (e.g., NE), the other one being closed during training but used as the starting arm for the probe trial (e.g., SW). While there are no published results with this protocol, ongoing experiments have shown that this protocol works perfectly with much less doubts about the spatial nature of the learning.

## Conclusion

The double-H is an efficient and reliable maze test, which can be used to assess place and procedural learning in rats. Its relatively short acquisition times make it ideal for rapid preclinical evaluations of drug candidates, and the lack of performance degradation over almost 3 post-acquisition weeks highlights a role in the identification of the spatiotemporal dynamics of remote memories at the system level. Furthermore, it allows for studies based on the dynamic interplay between procedural and declarative memory systems.

## Disclosures

The authors have nothing to disclose.

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