

Where to place the rewards? Exploration bias in mice influences performance in the classic hole-board spatial memory test.

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Abstract

The classic hole-board paradigm (a square arena with 16 holes arranged equidistantly in a 4x4 pattern) assesses both exploration and spatial memory in rodents. For spatial memory training, food rewards are hidden in a fixed set of holes. The animal must not visit (i.e. nose-poke) the holes that are never baited (reference memory; RM) nor re-visit a baited hole within a session (working memory; WM). However, previous exploratory bias may affect performance during reward searching. During habituation sessions with either all holes rewarded or all holes empty, mice intrinsically preferred poking peripheral holes (especially those located in the maze's corners) over centre holes. During spatial memory training, mice progressively shifted their hole pokes and staying time to the central area that contained hidden rewards, while mice exposed to the empty apparatus still preferred the periphery. A group of pseudotrained mice, for whom rewards were located randomly throughout the maze, also increased their central preference. Furthermore, reward location influenced memory measures. Most repeated pokes (WM-errors) were scored in the locations that were most intrinsically appealing to mice (i.e. the corner and wall baited holes), supporting a strong influence of previous exploratory bias. Regarding RM, finding rewards located in the centre holes, which were initially less preferred, entailed more difficulty and required more trials to learn. This outcome was confirmed by a second experiment that varied the pattern of rewarded holes, as well as the starting positions. Therefore, reward location is a relevant aspect to consider when designing a hole-board memory task.

Keywords

Habituation, periphery, anxiety, corner, reference memory, working memory

1. Introduction

The hole-board task has been widely used to study both exploration and cognition in rodents (van der Staay et al. 2012). The animal must learn to find the holes baited with a reward (frequently four holes out of 16), usually guided by extra-maze cues that allow for spatial orientation. However, as occurs with many pre-clinical models for memory assessment, a caveat of the hole-board paradigm is that cognitive performance is inferred from exploratory activity (i.e. hole exploration; van der Staay et al. 2012). Therefore, it is important to consider that rodents show strong intrinsic exploratory bias when they are tested in numerous experimental settings. For example, regarding a stimulus's attributes, objects that are novel or spherical are usually more explored than familiar or cylindrical objects (Antunes and Biala 2012; Winne et al., 2015). Maze corners, in particular, seem to provide a salient recognisable stimulus configuration that makes them attractive for rodents to explore and stay (Yaski and Eilam 2007). Moreover, either in water or dry mazes of a variety of shapes (e.g. circular, square, plus-shaped), there is a clear intrinsic preference for exploring the 'safer' areas, which are usually those that are least illuminated or closest to walls, such as the maze periphery (Bourin and Hascoet 2003; Eilam and Golani 1989; Pellow et al. 1985; Simon et al. 1994; Yaski and Eilam 2007). Rodents can even select a particular zone in a maze, preferably near a shelter or a salient landmark, to use as a 'home base' from which to start exploring the environment (Eilam and Golani 1989; Yaski and Eilam 2007).

Nevertheless, there is no consensus with respect to where to place the rewards in a hole-board arena. This aspect has been little addressed, to the point that the locations of rewards are frequently omitted from the methods description of hole-board manuscripts (e.g. Castilla-Ortega et al. 2010; van der Staay et al. 1990; Woldeit and Korz 2010). In this study, we report that intrinsic exploratory bias and reward location influence memory-related measures in mice assessed in a classic 16-hole hole-board apparatus (van der Staay et al. 2012).

2. Methods

2.1. *Animals*

Male c57BL/6J mice were acquired from Janvier (Le Genest-Saint-Isle, France) and were 8–12 weeks old at the beginning of behavioural testing. A total of 50 mice were used for Experiment I, whereas 16 mice were used for Experiment II. From one week before starting the experiments, mice were single-housed in cages containing nesting material, kept in a 12 h light-dark cycle (lights on at 8:00 a.m.) at standard humidity and temperature conditions. Procedures were in accordance with the European (Directive 2010/63/UE) and Spanish regulations (Real Decreto 53/20130 and Ley 32/2007) for animal research.

2.2. *Food Deprivation*

As described previously (Sampedro-Piquero et al. 2018), for three days before starting the hole-board testing, mice were submitted to mild food deprivation (~3 g/day of their habitual pellet diet; A04, SAFE DIETS, Paris, France) and received two pieces (~0.06 g each) of sweet chocolate cereal (Choco Krispies®, Kellogg) in their home cage per day,

to become habituated to this new flavour. Once behavioural training started, the home-cage diet consisted of ~3 g of pellet food/day.

2.3. Hole-board Apparatus and Data Collection

We used an automatic version of a ‘classic’ hole-board maze (CIBERTEC, Madrid, Spain). A square white arena (45 x 45 cm) was surrounded with clear Plexiglas walls (25 cm high) and contained 16 equidistant holes (5.5 cm apart, 2.5 cm diameter, 3 cm depth) in its central zone. The automatic hole-board was equipped with infrared beams that horizontally crossed the holes at a depth of 1 cm. The hole-board was connected to custom computer software that allowed for task design and data acquisition. A false bottom was filled daily with cereal to prevent discrimination of the baited holes based on olfaction (Sampedro-Piquero et al. 2018).

Holes were numbered as depicted in **Figure 1b** and were classified within three different hole type categories, according to their location: ‘corner’ holes (hole numbers: 1, 4, 13 and 16), ‘wall’ holes (hole numbers: 2, 3, 5, 9, 8, 12, 14, 15) or ‘centre’ holes (hole numbers: 6, 7, 10, 11) (**Figure 1b**). A hole nose-poking was automatically scored by the hole-board software each time the mouse introduced its nose into a hole, deep enough to interrupt the infrared beams. The software also provided the frequency of poking for each specific hole, the latency to finish the trial and memory-relevant parameters (i.e. pokes to baited or unbaited holes).

Bias for different arena regions of the maze were also studied. Video recordings of the sessions were analysed with the software Ethovision XT 12 (Noldus, Wageningen, The Netherlands). This software tracks the centre body point of a mouse and measures the time this point is registered inside selected zones of the apparatus. Thirteen imaginary zones within the maze arena were considered to be of interest (**Figure 1e**). Each of these regions was an equal size (7x7 cm) and was classified within three different zone types: ‘corner’ zones, ‘wall’ zones or ‘centre’ zones, as depicted in **Figure 1e**. While having its centre body point inside one zone of interest, the mouse is only able to poke holes that are classified as the same type as the zone (e.g. from the centre zone, the mouse is only able to reach centre holes). The rest of the maze arena was not considered for zone analysis, since it entailed intermediate regions that could provide confounding data (e.g. while having its centre body point in an intermediate region, the mouse could reach to poke different hole types). Nevertheless, it should be stressed that the time-in-zone measure is independent of hole poking, as mice could also stay in any selected zone of the apparatus without poking into any hole.

2.4. Behavioural Procedure

Behavioural assessment was carried out between 8:30 a.m. and 2:00 p.m. The testing room was 5-sided, in the shape of a rectangle with a corner cut off. The holeboard was placed asymmetrically towards the narrow end of the room. The testing room was illuminated to 46 lux and black cardboard in different geometric shapes (i.e cross, square and four vertical bars) was placed on the walls as cues to facilitate spatial orientation (**Supplementary Figure S1**). Sessions were recorded in zenith view by a digital camera hanging from the ceiling. The hole-board apparatus was rotated 90° daily to prevent

orientation using intra-maze cues, and it was cleaned with 30% ethanol after each session to remove odour cues.

2.4.1. Hole-board training for Experiment I

For the first experiment, hole-board training was performed as described previously (Castilla-Ortega et al. 2010; Sampedro-Piquero et al. 2018). During the first two testing days, mice underwent a 6 min habituation session per day, during which they freely explored the entire apparatus. For 33 mice, all holes were baited (rewarded) with a piece of cereal (~0.03 g), so in these ‘shaping’ sessions, mice learned to eat from the holes. To study whether the addition of rewards influenced exploratory behaviour, another group of 17 mice performed the habituation sessions with empty holes and received the cereal in their home cage (‘Empty’ group; **Figure 1a**).

The following day, mice that received habituation sessions with all holes baited were assigned either to the ‘Learning’ (n = 17) or the pseudotrained (‘Pseudo’; n = 16) treatments (**Figure 1a**). The Learning mice underwent spatial memory training over four consecutive days. Each training day consisted of two session blocks separated by a 90 min interval; each session block included two consecutive training sessions separated by 1 min (i.e. a total of four training sessions per day). For spatial learning, a fixed pattern of four holes was baited with cereal, while the non-baited holes were always empty (**Figure 1a**). Importantly, the baited holes comprised one hole placed in a corner zone, one hole placed in the wall zone, and two holes placed in the centre zone, as depicted in **Figures 1a** and **2a**. Mice began each training session from one of the four corner zones (**Figure 2a**). For half of the mice, all four possible starting locations were used within a training day in random order. The rest of the mice started the session from the opposite corner to avoid any bias attributed to the starting corner zone. Sessions lasted until all four rewards were found or a maximum time of 4 min had passed.

The Pseudo mice underwent the same training protocol as the Learning mice, but the pattern of four baited holes varied unpredictably from trial to trial (**Figure 1a**). Thus, Pseudo mice were searching for rewards through the maze, but they could not find them in any particular location of the apparatus, so the long-term memory component of the task was prevented.

Finally, to further study hole-board exploration without motivation by food rewards, the mice that performed the habituation sessions with empty holes were subsequently exposed to the hole-board apparatus (‘Empty’ group, n = 17; **Figure 1a**) for a number and duration of training sessions that were comparable to those of the Learning and Pseudo mice, but no holes were ever baited as they received the cereal in their home-cage.

2.4.2. Hole-board training for Experiment II

Since Experiment I suggested an exploration bias for the corner and wall holes, a second experiment was carried out to confirm this outcome, while ruling out possible confounding factors. Hence, a group of 16 mice underwent two habituation sessions (with all holes baited) and four spatial training days, as described for the Learning mice in Experiment I. However, only three holes (one corner hole, one wall hole and one centre hole) were baited during training, so mice would have a comparable number of rewards

per hole type. Additionally, the pattern of baited holes was rotated every four mice, so any potential bias for any specific side of the apparatus would be controlled for (i.e. the four possible patterns of baited holes were: holes 6, 12 and 13 as in **Figure 3e**; holes 1, 7 and 14; holes 4, 5 and 11; and holes 3, 10 and 16). The reward pattern was assigned to each mouse so the corner hole rewarded during learning did not correspond to the corner hole that the mouse explored the most during habituation. Furthermore, mice in this experiment were never released from corners at the beginning of the sessions, to rule out that this aspect of the protocol could explain a bias for the corner zones or holes. In this regard, during habituation sessions, mice were released from the centre arena zone (**Figure 3a**) so they could reach the central holes without crossing the periphery. During spatial learning sessions, mice were released either from the centre of the arena (in approximately 1/3 of the sessions) or from any of the four wall zones (in approximately 2/3 of the sessions), but never from a corner zone (**Figure 3e**).

2.5. Data Processing and Statistical Analyses

Both hole poking (i.e. number of nose-pokes to each hole) and time in zone (i.e. duration, in seconds, of the mouse's central body point registered inside the zone of interest) were recorded in every session and an average per minute was calculated, because mice could take different times to complete a training session. To control for different hole poking frequencies between mice (i.e. a different intensity of exploratory activity), the number of nose-pokes/minute to each hole was divided by the total number of hole nose-pokes/minute performed by the animal in that session. This yielded an 'adjusted hole poking' frequency for each hole.

Subsequently, bias for each hole type (i.e. corner, wall or centre holes) was studied for habituation and training. The 'adjusted hole poking' measures for holes of a similar type were summed and corrected by the total number of holes of that type that were present in the apparatus (i.e. divided by 4 for the corner and centre holes, or by 8 for the wall holes), in order to accommodate that there were twice as many wall zones as corner or centre holes. This resulted in an adjusted *hole poking rate per hole type*. Similarly, the seconds in a zone/minute were summed between zones of a similar type (corner, wall or centre zones) and averaged by the total number of zones of that type (i.e. divided by 4 for corner zones, 1 for the centre zone, 8 for wall zones). All the exploration measures were averaged across sessions to obtain a unique measure for the habituation and training phases.

Memory measures during spatial training were calculated per session and averaged every two sessions (i.e. per session block). As in the previous literature (Castilla-Ortega et al. 2010; Douma et al. 1998; van der Staay et al. 1990), working memory was studied considering the set of baited holes only, in order to minimise the potential influence of reference memory performance on the working memory measure (e.g. in the case of non-baited holes, once the animal learns that a hole is never baited, the hole is unlikely to be poked, which prevents the mouse from performing both reference and working memory errors). Both working memory (WM) errors (i.e. pokes into a baited hole that was already poked during the session) and reference memory (RM) errors (i.e. every poke into a non-baited hole) were scored separately for each of the baited holes. For RM, the \sum RM error was calculated as the sum of the RM errors committed across the session until that specific

baited hole was poked for the first time (**Supplementary Figure S2**). In this way, rewards placed in baited holes with a high Σ RM error were found later in the mouse's path than rewards placed in baited holes with a low Σ RM error, which were found earlier.

Variables were analysed using a mixed repeated measures ANOVA followed by *post hoc* Fisher's least significant difference (LSD) test. The effect size was calculated by partial eta-squared (η_p^2). Significant ANOVA effects were often associated with a 'large' effect size (≥ 0.14 ; **Table 1**; Richardson 2011). Pearson's correlation coefficients were calculated. Significance was considered at $p \leq 0.05$. Both significant and non-significant ANOVA effects are reported.

3. Results

3.1. Experiment I: An exploratory bias for the 'corner' holes and zone was attenuated by spatial learning or pseudotraining, but persisted in mice without food rewards

In Experiment I, the analysis of the habituation sessions revealed that, irrespectively of the addition of food rewards, mice showed a preference to poke the corner holes over any other hole type (*post hoc* $**p < 0.001$ and $##p < 0.001$; **Figure 1c**), while the wall holes were also more preferred than the holes located in the centre (ANOVA [1] in **Table 1**; *post hoc* $##p < 0.001$; $\#p < 0.05$ **Figure 1c**). Furthermore, during habituation, both groups of mice notably preferred to stay in a corner arena zone compared to either a wall or a centre zone (ANOVA [2] in **Table 1**; *post hoc* $**p < 0.001$ and $##p < 0.001$; **Figure 1f,h**). This suggests that, in either the absence or the presence of food, mice had a clear tendency to set a 'home base' (i.e. the location where they spent the most time) in one or several of the hole-board corners, from where they took roundtrips for exploration or foraging (Dorfman et al. 2016; Weiss et al. 2017). The addition of food rewards during the habituation period did not affect the total frequency of hole pokes (**Supplementary Figure S3**).

Through the training phase, both hole and zone exploration patterns described during habituation persisted in the mice repeatedly exposed to the empty hole-board, who still preferred poking peripheral holes and staying in corner zones despite becoming familiarised with the maze during training. However, initial exploratory bias was notably modified for mice submitted to spatial learning or pseudotraining (**Figure 1d, 1g, 1i**). Specifically, both the Learning and Pseudo mice reduced their preference for poking corner holes in favour of increasing centre hole poking, compared to the Empty mice (ANOVA [3] in **Table 1**; *post hoc* $\$p < 0.05$, $\$\$p < 0.001$; **Figure 1d**). Concomitantly, their preference for the corner zone was reduced, while their time in the centre zone increased (ANOVA [4] in **Table 1**; *post hoc* $\$p < 0.05$, $\$\$p < 0.001$; **Figure 1g, 1i**). This suggests that an intrinsic exploratory bias for corners was overcome by motivation for finding the rewards sparsely distributed through the maze, either in the learning or in the pseudotraining treatment. Interestingly, the fact that the 'Learning' mice always had two centre holes baited probably explained why they showed even more preference for the centre holes and zone than the Pseudo group (*post hoc* $\&\&p < 0.001$; **Figure 1d, 1g**).

It is also worth mentioning that the Empty mice gradually habituated to the apparatus as they reduced their total hole poking rate across the training phase, but the Learning mice

maintained a higher hole poking rate, probably due to increased motivation as result of food searching. The Pseudo mice greatly increased their frequency of hole pokes compared to the other two groups (**Supplementary Figure S3**). This evidence, combined with their aforementioned increased preference for the central elements of the arena (compared to the Empty mice), suggests that the Pseudo mice developed a food searching strategy that achieved a fast coverage of the whole apparatus, in order to find the randomly hidden rewards.

3.2. Experiment I: Location of the baited hole influenced both working and reference memory errors

Working and reference memory errors were scored for the Learning mice during the training phase. The analysis per session block showed that both error types progressively decreased across training (**Supplementary Figures S4a and S5a**), but differently depending on the baited hole in question (ANOVAs [5] and [6] in **Table 1**; **Figure 2b,d**). For both memory measures, the most notable differences were found in the first half of training, especially at the first session block, in which the baited corner hole showed more repeated pokes (highest WM error) and it was poked faster (i.e. poked for the first time; lowest \sum RM error) than every other baited hole (**Supplementary Figures S4b, S4c, S5b, S5c**). Overall, the baited corner hole resulted in more working memory errors during training than the wall baited hole (*post hoc* $*p < 0.05$), while the centre holes were the least repeatedly poked (*post hoc* $##p < 0.001$; one way ANOVA: ‘hole’: $F_{3,64} = 31.355$, $p < 0.000$; $\eta_p^2 = 0.595$; **Figure 2c**). For reference memory, the baited corner hole was overall more easily found than the wall baited hole (*post hoc* $*p < 0.05$), while rewards placed in centre holes were the hardest to locate (*post hoc* $##p < 0.001$; one way ANOVA: ‘hole’: $F_{3,64} = 19.977$, $p < 0.000$; $\eta_p^2 = 0.484$; **Figure 2e**). Additional data of repeated poking in the non-baited holes has been included for the interested readers (**Supplementary Figure S6**).

Finally, the Pseudo mice also reduced their overall amount of WM errors progressively across training (i.e from 4.09 ± 0.64 errors in the first session block to 2.28 ± 0.40 errors in the eighth session block; data not shown), as previously reported (Sampedro-Piquero et al. 2018; one way ANOVA: ‘block’: $F_{7,120} = 4.155$, $p < 0.000$; $\eta_p^2 = 0.195$). Interestingly, this improvement in WM occurred in the absence of RM, as for each session, the four rewards could be hidden in random holes of any hole type.

3.3. Experiment II: Additional data confirmed a preference for corner holes in a hole-board setting

Because mice in Experiment I were always released from maze corners, and the pattern of baited holes was the same for every mice, it was possible that these particular baited holes, located in a fixed side of the apparatus, were preferred or avoided for reasons other than their hole type (e.g. illumination, noise); an additional experiment (Experiment II) was carried out to confirm the results, while controlling for the potential influence of these procedural factors.

While the training phase protocol differed in the two experiments, mice in Experiment II performed the habituation sessions identically to mice in Experiment I, with 16 holes

baited; the only difference was that they started the session from the centre maze zone instead of from a corner zone (**Figure 3a**). Thus, it was possible to directly compare these two groups of mice during habituation, in order to study whether the starting zone influenced intrinsic exploratory bias. Results showed that releasing mice from the centre of the apparatus did not diminish their intrinsic initial preference for the peripheral holes, nor did it prevent mice from establishing the corner zones as their home base (ANOVAs [7] and [8] in Table 1; **Figure 3a, 3b, 3c**).

During spatial training, mice progressively reduced both working and reference memory errors [**Supplementary Figures S7a, S8a; Figure 3f, 3h**]. Furthermore, working memory performance confirmed that most hole re-visits were attributed to the baited corner hole followed by the wall corner hole (*post hoc* $*p < 0.05$; **Figure 3g**); the centre baited hole was the least re-visited (*post hoc* $\#p < 0.05$, $\#\#p < 0.001$; **Figure 3g**; one way ANOVA: ‘hole’: $F_{2,45} = 18.774$, $p < 0.000$; $\eta_p^2 = 0.455$; **Figure 3g, Supplementary Figure S7b, S7c**). Finally, Σ RM errors were increased for the baited centre hole (*post hoc* $\#p < 0.05$, $\#\#p < 0.001$; **Figure 3i**), confirming that this reward was the hardest to find (one way ANOVA: ‘hole’: $F_{2,45} = 10.958$, $p < 0.000$; $\eta_p^2 = 0.328$; **Figure 3i, Supplementary Figure S8b, S8c**). Differing from Experiment I, rewards placed in the wall and corner holes were found at a similar rate (**Figure 3i, Supplementary Figure S8c**). The increased chance of finding the baited corner hole sooner than the wall baited hole reported for Experiment I (**Figure 2d, 2e**) was likely attenuated in Experiment II because mice never started a training session from a corner zone.

3.4. Working and reference memory measures were directly correlated across training

An overview of the memory results in both experiments suggested that the tendency to repeatedly poke the peripheral holes (i.e. committing more WM errors) was concomitant to the tendency to avoid poking the central baited holes (i.e. committing more RM errors until the central rewards were finally found; **Figures 2 and 3**). To further investigate this relationship, the correlation between WM and RM errors was tested for each session block and for the whole training; there was a strong tendency for these measures to be directly correlated (**Table 2**).

4. Discussion

These results showed an inherent exploratory bias in mice tested in a classic hole-board paradigm, which affected hole pokes, exploration of the arena and memory-related measures. Intrinsically, mice clearly preferred exploring the hole-board periphery over the centre (in regard to both holes and arena zones), which may be attributed to the natural tendency of rodents to choose ‘protected’ (i.e. surrounded by walls) over unprotected spaces to optimise safety and decrease anxiety (Simon et al. 1994; Whishaw et al. 2006). Interestingly, we noted that such preference for the periphery was mostly explained by a bias for the corner holes and zones, which has been reported in C57BL/6J mice and other mouse strains (Moy et al. 2008; Wernecke and Fendt 2015). The inherent bias for corners was maintained even when mice were initially placed in the centre zone of the apparatus. While the preference for maze corners likely contributed to the mice’s emotional state (i.e. the corners may be perceived as the ‘most protected’ area), another possible explanation is that corners may also present a salient stimulus configuration that is more

attractive for mice to explore (Yaski and Eilam 2007). In any case, it is important that this exploratory bias was not invariable, since mice trained for searching rewards through the maze (placed either in fixed or in random locations) progressively increased the relative exploration of the central maze elements, as well as their total frequency of hole explorations. This change in exploratory patterns was attributed to food searching and not to a mere influence of an increased familiarity or sense of safety by virtue of experience, because mice similarly exposed to the apparatus, but in the absence of rewards, extensively maintained their preference for the corners and periphery, and progressively reduced (habituated) their hole poking rate. In other words, inherent hole-board exploratory patterns may be flexibly adapted given that there is enough motivation to do so, such as when appetitive or novel stimuli (e.g. food, odours) are placed in the less-visited holes (Moy et al. 2008; Wernecke and Fendt 2015). Inability to modify hole exploratory behaviour in response to stimuli has been interpreted as a symptom of autism-like resistance to change (Moy et al. 2008).

Since mice show a strong bias for re-visiting certain hole locations, it is worth questioning to what extent the re-visits to baited holes during spatial training (which are typically scored as WM errors) are indeed influenced by a short-term WM memory component (i.e. the mouse re-visits a baited hole because it has *forgotten* its previous visit) or by the mice's innate exploratory tendency (i.e. the mouse re-visits a baited hole because it is *intrinsically motivated* to perform a hole re-visit). Importantly, the fact that WM errors were mainly scored in the most intrinsically appealing holes — primarily in the baited corner hole and secondarily in the baited wall hole — suggests an influence of previous exploratory bias on the WM measure. However, this was apparent mainly in the initial sessions of spatial training when mice were still unaware of the rewards' positions. As spatial training progressed, a gradual reduction in WM errors was observed (Castilla-Ortega et al. 2010; Kuc et al. 2006; Sampedro-Piquero et al. 2018), which was directly correlated with improvement in RM performance. In other words, less RM errors were associated with less WM errors. This occurred even when the WM and RM measures used in this study were calculated independently from the other (contrary to commonly published RM ratios, which count re-visits to baited holes — WM errors — as RM correct responses; Castilla-Ortega et al. 2010; Douma et al. 1998).

Findings showing a progressive WM improvement somewhat contradict the nature of working memory, which is supposed to represent a limited short-term capacity that may not be augmented by training and, moreover, should be independent from long-term memory storage (Cowan 2008). A possible explanation for the association of WM and RM performance in the hole-board is that mice progressively learn a task rule (i.e. 'there is only one reward per hole for every session and thus hole re-visits are never rewarded'), which may involve a long-term memory component that leads the animal to progressively avoid the unnecessary hole re-visits (Castilla-Ortega et al. 2010), since mice will prioritise finding food over their exploratory bias. Similarly, as mice learn that central holes are (or *may be*) baited, they will shift the exploration to the central maze area, thus being driven away from the peripheral holes that are more likely to be re-visited for WM errors. It should be noted that, even in the absence of RM for specific hole locations, the aforementioned rules or food-searching strategies may also be acquired by pseudotrained mice. Pseudotrained mice learn that rewards are hidden *somewhere* across the arena, so

an effective maze exploration strategy — one that avoids hole re-visits and covers the central maze area — is also in demand. While it has been demonstrated that learning such procedural rules involves less cognitive effort and triggers less hippocampal neuroplasticity than proper RM for fixed reward location (Sampedro-Piquero et al. 2018), pseudotrained mice would still increase the exploration of the central maze zones and progressively reduce their WM errors across training (Sampedro-Piquero et al. 2018).

Considering all this, especially at the initial stages of spatial training, WM measures may be partially influenced by intrinsic exploratory bias and improved by the gradual learning of procedural rules, food searching strategies and/or reference memory for the rewards' location. Genuine WM capacity may be better studied by examining WM errors during the late phases of hole-board training, when procedural rules have been learned, initial exploratory bias has been inhibited, and RM has reached a performance plateau. On the other hand, mice's inherent exploratory bias also influenced RM performance. In this study, the peripheral baited holes — especially the corner ones — were readily explored from the first sessions, when the animal was still naïve. Thus, rewards hidden in holes with intrinsic appeal are more likely to be found by chance. Because of the higher initial probability of exploring a baited corner or a wall hole, RM learning across training was mainly evidenced by improvement in finding the centre baited hole(s), which were initially avoided. Importantly, the influence of intrinsic exploratory bias does not rule out the possibility that the centre baited locations may also be more difficult to learn as they are completely surrounded by other holes, probably entailing a less salient and less discriminative stimulus configuration that requires additional cognitive efforts for spatial pattern separation (Creer et al. 2010).

In conclusion, this study supports that performance during spatial training in a hole-board paradigm may be initially influenced by inherent exploratory bias and, as the animal progressively learns, it becomes driven by motivation for food rewards. In other words, until the animal has grasped the aim of the hole-board task, it may have no reason to inhibit its natural tendency to perseverate in visiting (or re-visiting) the intrinsically preferred holes or zones. This may obscure measurement of both RM and WM during the earlier phases of training. In any case, the location of rewards in relation to animals' exploratory preferences is a relevant aspect that should be acknowledged. Considering that exploratory preferences may be modulated by the particular experimental settings (i.e. room illumination, ventilation), researchers may be interested in analysing the exploratory pattern of their animals during initial maze exposures prior to learning, in order to decide where to locate rewards depending on their experimental aims. Rewards placed in the inherently most-preferred holes may be scarcely modulated by RM, but they will be more reflective of intrinsic exploratory activity, including hole perseverations, and they may help to incentivise animals for the task by providing a reward that is easy to obtain. On the contrary, rewards placed in the less preferred holes are more diagnostic of rodent spatial RM (as well as of its flexibility to adapt its previous exploratory pattern), and they may involve different levels of 'difficulty' depending on the number of holes surrounding them or whether they are placed closer to the periphery.

There are other hole-board maze variants, such as the modified hole-board, in which two rows of six holes each are displayed in the middle of a squared board, thus avoiding the

existence of central vs. peripheral holes (Labots et al. 2015); another variant is the circular hole-board, in which holes are disposed in circle, avoiding corners, periphery or centre holes. These other hole-board variants may also be checked for previous spatial preferences, since these exist in mazes of a wide variety of shapes (Eilam and Golani 1989). For example, in arenas that do not have walls, the edge has an effect that is reminiscent of walls (Clark et al. 2006). In addition, in circular arenas where a cardinal physical structure such as the corners is eliminated, rodents would still establish a home base and display a preference for certain arena sectors (Clark et al. 2006). Therefore, the testing of memory in circular mazes may also be biased by spatial preferences.

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Conflict of interest statement

The authors do not have any conflicts of interest.

Figure Legends

Fig. 1. Hole and zone exploratory preferences of Experiment I mice in the hole-board. (A) Experimental protocol used for mice in Experiment I. (B) Classification of the 16 holes into three different hole types: corner (Co), wall (W) or centre (CE) holes. (C) Exploration rate per hole type during the habituation phase, with or without rewards. This measure is based on the frequency of nose-pokes in each hole type, after controlling for the different number of holes in each hole type category, session duration and hole exploration frequency. (D) Exploration rate per hole type during training in mice submitted to either learning, pseudotraining or empty maze treatments. (E) 13 similar zones of interest were imaginarily drawn over the maze to analyse zone exploration (i.e. when the mouse's centre body point was registered inside the zone, controlled for session duration). (F) Zone exploration during habituation or (G) training phases, which is also represented by heatmaps (H, I). *Post hoc* LSD: within group comparisons: $**p < 0.001$ vs. wall hole or zone; $##p < 0.001$ vs. centre hole or zone; between group comparisons: $\$p < 0.05$; $\$\$p < 0.001$ vs. the 'Empty' group; $\&p < 0.05$; $\&\&p < 0.001$ vs. the 'Pseudo' group. Data are expressed as mean \pm S.E.M. W = wall; CE = centre; Co = corner.

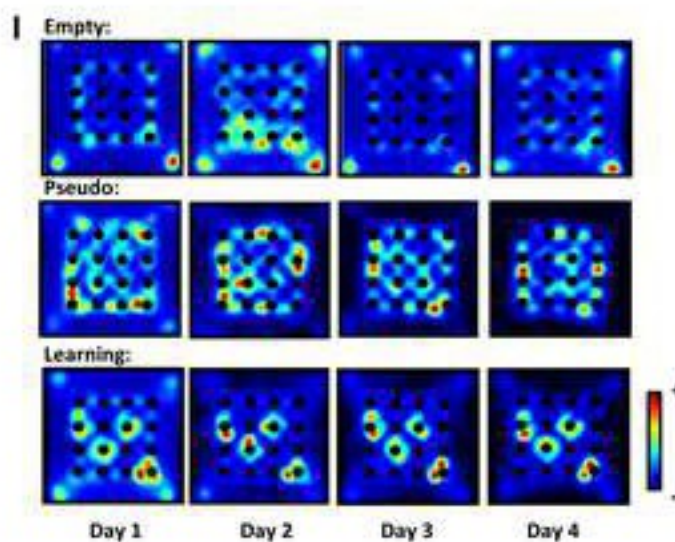
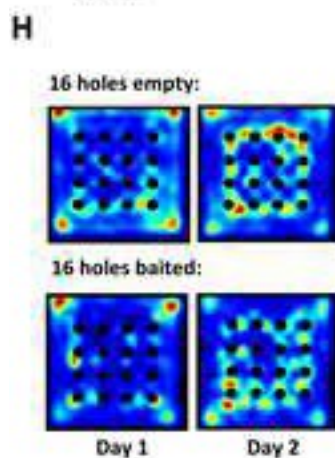
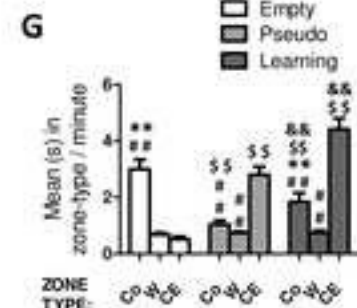
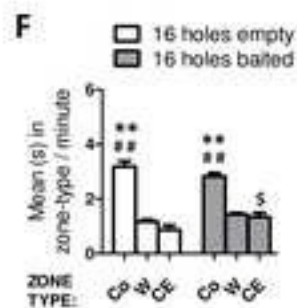
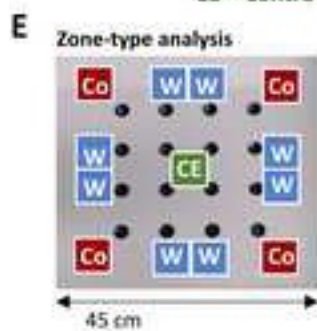
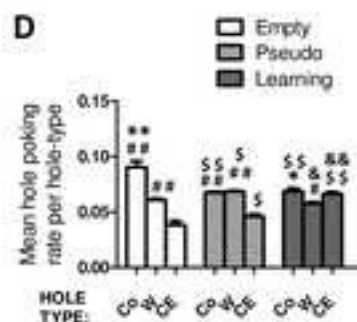
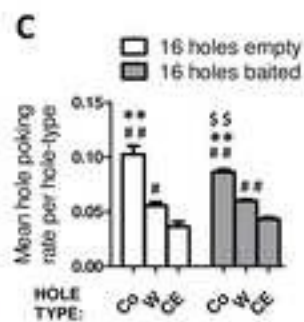
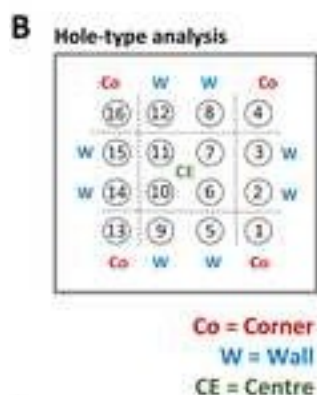
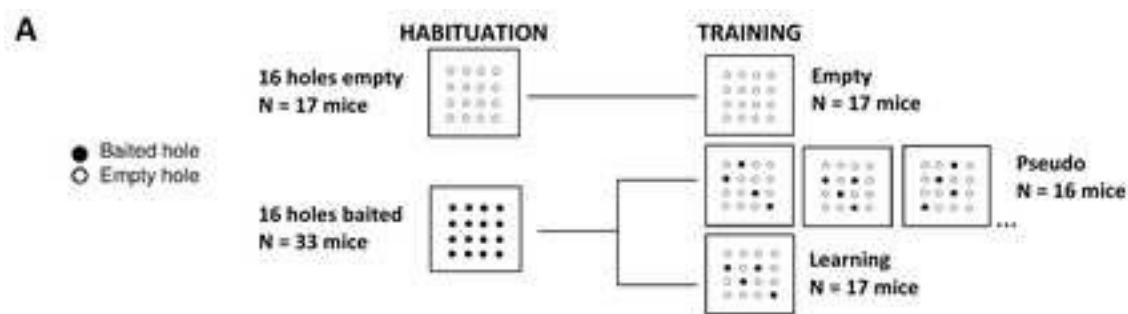
Fig. 2. Memory performance in the 'Learning' mice from Experiment I. (A) Pattern of baited holes. Mice started each session from one of the maze corners. (B, C) Working memory errors committed in each baited hole, analysed per session block (B) or per training (C). (D, E) Σ Reference memory errors (calculated as detailed in the Supplementary Figure S2) committed before finding each baited hole, analysed per session block (D) or per training (E). *Post hoc* LSD for B and D is shown in Supplementary Figures S3 and S4. *Post hoc* LSD for C and E: $##p < 0.001$ vs. any of the baited centre holes; $*p < 0.05$ vs. the baited wall zone. Data are expressed as mean \pm S.E.M. W = wall; CE = centre; Co = corner.

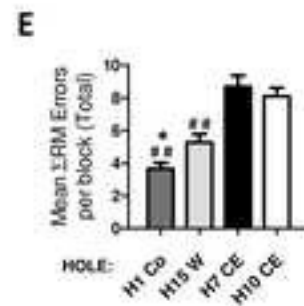
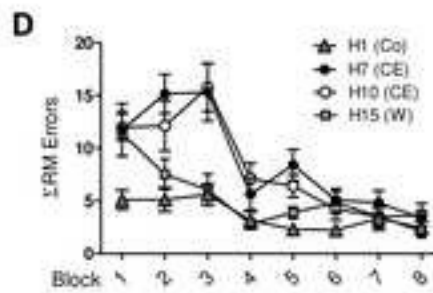
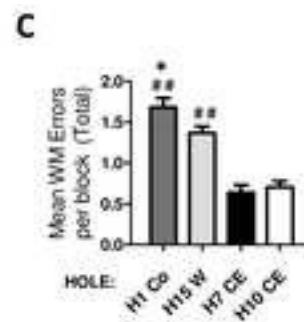
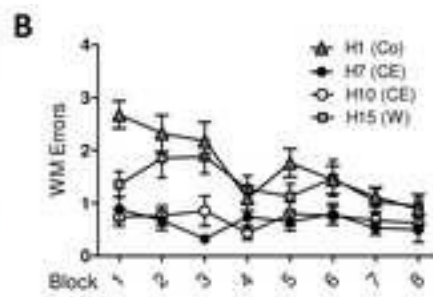
Fig. 3. Results from Experiment II. (A) For the habituation sessions, mice from Experiment II ('Exp. II'; $n = 16$ mice) were released into the apparatus from the centre zone, whereas mice from Experiment I ('Exp. I') were released from a corner zone. (B, C) Comparison of mice from Experiment II and Experiment I ('16 holes baited' group) in terms of both hole (B) and zone (C) exploration during the habituation sessions. (E) Pattern of baited holes during learning. The pattern of holes was different (rotated) for each group of four mice. Mice started each training session from the centre zone or a wall zone. (F, G) Working memory errors committed in each baited hole, analysed per session block (F) or per training (G). (H, I) Σ Reference memory errors (calculated as detailed in Supplementary Figure S2) committed until finding each baited hole, analysed per session block (H) or per training (I). *Post hoc* LSD for F and G is shown in Supplementary Figures S5 and S5. *Post hoc* LSD for C and E: $\#p < 0.05$; $##p < 0.001$ vs. any of the baited centre holes; $*p < 0.05$ vs. the baited wall zone. Data are expressed as mean \pm S.E.M. W = wall; CE = centre; Co = corner.

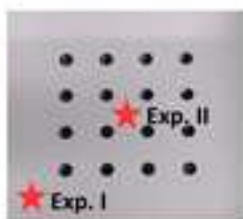
References

- Antunes M, Biala G (2012) The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn Process* 13:93-110. Bourin M, Hascoet M (2003) The mouse light/dark box test. *Eur J Pharmacol* 463:55-65. [https://doi.org/10.1016/S0014-2999\(03\)01274-3](https://doi.org/10.1016/S0014-2999(03)01274-3)
- Castilla-Ortega E, Sanchez-Lopez J, Hoyo-Becerra C, Matas-Rico E, Zambrana-Infantes E, Chun J, De Fonseca FR, Pedraza C, Estivill-Torrus G, Santin LJ (2010) Exploratory, anxiety and spatial memory impairments are dissociated in mice lacking the LPA1 receptor. *Neurobiol Learn Mem* 94:73-82. <https://doi.org/10.1016/j.nlm.2010.04.003>
- Clark BJ, Hamilton DA, Wishaw IQ (2006) Motor activity (exploration) and formation of home bases in mice (C57BL/6) influenced by visual and tactile cues: modification of movement distribution, distance, location, and speed. *Physiol Behav* 87:805-816 <https://doi.org/10.1016/j.physbeh.2006.01.026>
- Cowan N (2008) What are the differences between long-term, short-term, and working memory? *Prog Brain Res* 169:323-338. [https://doi.org/10.1016/S0079-6123\(07\)00020-9](https://doi.org/10.1016/S0079-6123(07)00020-9)
- Creer DJ, Romberg C, Saksida LM, van Praag H, Bussey TJ (2010) Running enhances spatial pattern separation in mice. *Proc Natl Acad Sci U S A* 107:2367-2372. <https://doi.org/10.1073/pnas.0911725107>
- Dorfman A, Nielbo KL, Eilam D (2016) Traveling companions add complexity and hinder performance in the spatial behavior of rats. *PLoS One* 11:e0146137. <https://doi.org/10.1371/journal.pone.0146137>
- Douma BR, Korte SM, Buwalda B, la Fleur SE, Bohus B, Luiten PG (1998) Repeated blockade of mineralocorticoid receptors, but not of glucocorticoid receptors impairs food rewarded spatial learning. *Psychoneuroendocrinology* 23:33-44. [https://doi.org/10.1016/S0306-4530\(97\)00091-7](https://doi.org/10.1016/S0306-4530(97)00091-7)
- Eilam D, Golani I (1989) Home base behavior of rats (*Rattus norvegicus*) exploring a novel environment. *Behav Brain Res* 39:199-211. [https://doi.org/10.1016/S0166-4328\(89\)80102-0](https://doi.org/10.1016/S0166-4328(89)80102-0)
- Kuc KA, Gregersen BM, Gannon KS, Dodart JC (2006) Holeboard discrimination learning in mice. *Genes Brain Behav* 5:355-363. <https://doi.org/10.1111/j.1601-183X.2005.00168.x>
- Labots M, Van Lith HA, Ohl F, Arndt SS (2015) The modified hole board-measuring behavior, cognition and social interaction in mice and rats. *J Vis Exp* 8:52529. <https://doi.org/10.3791/52529>
- Moy SS, Nadler JJ, Poe MD, Nonneman RJ, Young NB, Koller BH, Crawley JN, Duncan GE, Bodfish JW (2008) Development of a mouse test for repetitive, restricted behaviors: relevance to autism. *Behav Brain Res* 188:178-194. <https://doi.org/10.1016/j.bbr.2007.10.029>
- Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 14:149-167. [https://doi.org/10.1016/0165-0270\(85\)90031-7](https://doi.org/10.1016/0165-0270(85)90031-7)
- Richardson JTE (2011) Eta squared and partial eta squared as measures of effect size in educational research. *Educ Res Rev* 6:135-147. <https://doi.org/10.1016/j.edurev.2010.12.001>
- Sampedro-Piquero P, Moreno-Fernandez RD, Mañas-Padilla MC, Gil-Rodriguez S, Gavito AL, Pavon FJ, Pedraza C, Garcia-Fernandez M, Ladron de Guevara-Miranda D, Santin LJ, Castilla-Ortega E (2018) Training memory without aversion: Appetitive hole-board spatial learning increases adult hippocampal neurogenesis. *Neurobiol Learn Mem* 151:35-42. <https://doi.org/10.1016/j.nlm.2018.03.023>

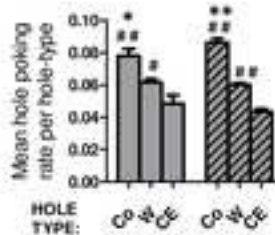
- Simon P, Dupuis R, Costentin J (1994) Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. *Behav Brain Res* 61:59-64. [https://doi.org/10.1016/0166-4328\(94\)90008-6](https://doi.org/10.1016/0166-4328(94)90008-6)
- van der Staay FJ, Gieling ET, Pinzon NE, Nordquist RE, Ohl F (2012) The appetitively motivated "cognitive" holeboard: a family of complex spatial discrimination tasks for assessing learning and memory. *Neurosci Biobehav Rev* 36: 379-403. <https://doi.org/10.1016/j.neubiorev.2011.07.008>
- van der Staay FJ, van Nies J, Raaijmakers W (1990) The effects of aging in rats on working and reference memory performance in a spatial holeboard discrimination task. *Behav Neural Biol* 53: 356-370. [https://doi.org/10.1016/0163-1047\(90\)90226-V](https://doi.org/10.1016/0163-1047(90)90226-V)
- Weiss O, Dorfman A, Ram T, Zadicario P, Eilam D (2017) Rats do not eat alone in public: Food-deprived rats socialize rather than competing for baits. *PLoS One* 12: e0173302. <https://doi.org/10.1371/journal.pone.0173302>
- Wernecke KE, Fendt M (2015) The olfactory hole-board test in rats: a new paradigm to study aversion and preferences to odors. *Front Behav Neurosci* 9: 223. <https://doi.org/10.3389/fnbeh.2015.00223>
- Whishaw IQ, Gharbawie OA, Clark BJ, Lehmann H (2006) The exploratory behavior of rats in an open environment optimizes security. *Behav Brain Res* 171: 230-239. <https://doi.org/10.1016/j.bbr.2006.03.037>
- Winne J, Teixeira L, de Andrade Pessoa J, Gavioli EC, Soares-Rachetti V, Andre E, Lobao-Soares B (2015) There is more to the picture than meets the rat: a study on rodent geometric shape and proportion preferences. *Behav Brain Res* 284: 187-195. <https://doi.org/10.1016/j.bbr.2015.02.018>
- Woldeit ML, Korz V (2010) Theta oscillations during holeboard training in rats: different learning strategies entail different context-dependent modulations in the hippocampus. *Neuroscience* 165: 642-653. <https://doi.org/10.1016/j.neuroscience.2009.11.002>
- Yaski O, Eilam D (2007) The impact of landmark properties in shaping exploration and navigation. *Anim Cogn* 10: 415-428. <https://doi.org/10.1007/s10071-007-0073-8>



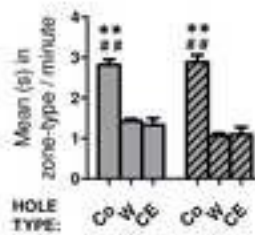


A**B**

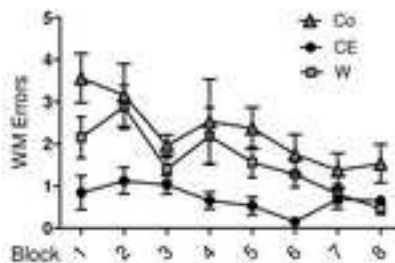
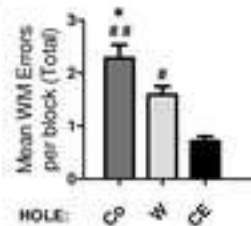
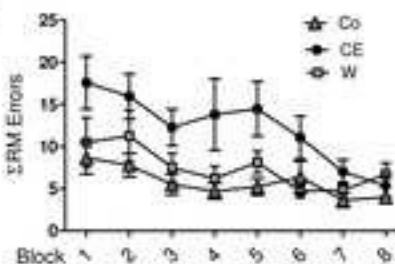
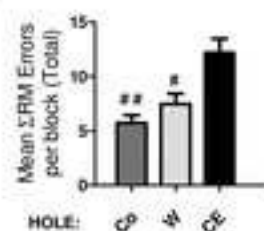
Experiment I (corner)
Experiment II (center)

**C**

Experiment I (corner)
Experiment II (center)

**E**

★ = Start position
○ = Baited hole

F**G****H****I**

Analysis number	ANOVA effects and effect size (η_p^2)
Experiment I (section 3.1)	
[1] ANOVA (treatment –IV- x hole-type –rDV-) –habituation-	‘treatment’: $F(1, 48) = 2.917, p = 0.094; \eta_p^2 = 0.057$ ‘hole-type’: $F(2, 96) = 97.642, p < 0.000^{**}; \eta_p^2 = 0.670^{++}$ ‘treatment x hole-type’: $F(2, 96) = 5.180, p = 0.007^*; \eta_p^2 = 0.097^+$
[2] ANOVA (treatment –IV- x zone-type –rDV-) –habituation-	‘treatment’: $F(1, 48) = 1.849, p = 0.180; \eta_p^2 = 0.037$ ‘zone-type’: $F(2, 96) = 76.790, p < 0.000^{**}; \eta_p^2 = 0.615^{++}$ ‘treatment x zone-type’: $F(2, 96) = 3.252, p = 0.043^*; \eta_p^2 = 0.063^+$
[3] ANOVA (treatment –IV- x hole-type –rDV-) –training-	‘treatment’: $F(2, 47) = 20.741, p < 0.000^{**}; \eta_p^2 = 0.469^{++}$ ‘hole-type’: $F(2, 94) = 45.180, p < 0.000^{**}; \eta_p^2 = 0.490^{++}$ ‘treatment x hole-type’: $F(4, 94) = 18.896, p < 0.000^{**}; \eta_p^2 = 0.446^{++}$
[4] ANOVA (treatment –IV- x zone-type –rDV-) –training-	‘treatment’: $F(2, 47) = 13.253, p < 0.000^{**}; \eta_p^2 = 0.360^{++}$ ‘zone-type’: $F(2, 94) = 48.639, p < 0.000^{**}; \eta_p^2 = 0.589^{++}$ ‘treatment x zone-type’: $F(4, 94) = 37.180, p < 0.000^{**}; \eta_p^2 = 0.613^{++}$
Experiment I (section 3.2)	
[5] ANOVA (baited hole –IV- x session block –rDV-) for working memory	‘hole’: $F(3, 64) = 31.355, p < 0.000^{**}; \eta_p^2 = 0.600^{++}$ ‘block’: $F(7, 448) = 4.907, p < 0.000^{**}; \eta_p^2 = 0.071^+$ ‘hole x block’: $F(4, 141) = 27.716, p < 0.000^{**}; \eta_p^2 = 0.084^+$
[6] ANOVA (baited hole –IV- x session block –rDV-) for reference memory	‘hole’: $F(3, 64) = 19.977, p < 0.000^{**}; \eta_p^2 = 0.484^{++}$ ‘block’: $F(7, 448) = 22.624, p < 0.000^{**}; \eta_p^2 = 0.261^{++}$ ‘hole x block’: $F(21, 448) = 2.468, p < 0.000^{**}; \eta_p^2 = 0.104^+$
Experiment II (section 3.3)	
[7] ANOVA (experiment –IV- x hole-type –rDV-) –habituation-	‘experiment’: $F(1, 47) = 0.498, p = 0.483; \eta_p^2 = 0.010$ ‘hole-type’: $F(2, 94) = 59.012, p < 0.000^{**}; \eta_p^2 = 0.557^{++}$ ‘experiment x hole-type’: $F(2, 94) = 2.120, p = 0.125; \eta_p^2 = 0.043^+$
[8] ANOVA (experiment –IV- x zone-type –rDV-) –training-	‘experiment’: $F(1, 47) = 3.419, p = 0.071; \eta_p^2 = 0.068$ ‘zone-type’: $F(2, 94) = 61.476, p < 0.000^{**}; \eta_p^2 = 0.567^{++}$ ‘experiment x zone-type’: $F(2, 94) = 0.868, p = 0.423; \eta_p^2 = 0.018$
[9] ANOVA (baited hole –IV- x session block –rDV-) for working memory	‘hole’: $F(2, 45) = 18.774, p < 0.000^{**}; \eta_p^2 = 0.455^{++}$ ‘block’: $F(7, 315) = 5.203, p < 0.000^{**}; \eta_p^2 = 0.104^+$ ‘hole x block’: $F(14, 315) = 1.027, p = 0.044^*; \eta_p^2 = 0.084^+$
[10] ANOVA (baited hole –IV- x session block –rDV-) for reference memory	‘hole’: $F(2, 45) = 18.774, p < 0.000^{**}; \eta_p^2 = 0.328^{++}$ ‘block’: $F(7, 315) = 5.427, p < 0.000^{**}; \eta_p^2 = 0.108^+$ ‘hole x block’: $F(14, 315) = 0.909, p = 0.549; \eta_p^2 = 0.107^+$

ANOVA effect. * $p < 0.05$; ** $p < 0.001$

Partial eta-squared is interpreted based on Cohen’s rule: $\eta_p^2 < 0.009$ small; $\eta_p^2 > 0.0588$ medium (+); $\eta_p^2 > 0.1379$ large (++) (Richardson, 2011).

IV: Independent variable (between subjects); rDV: Within-subjects dependent variable with repeated measures

Table 1. Statistical results for the mixed ANOVAs with repeated measures.

Pearson's correlations among WM and RM errors across training									
Session block	1	2	3	4	5	6	7	8	Mean total
<i>Experiment I</i>									
<i>r</i> =	0.239	0.693*	0.804**	0.729**	0.652*	0.734*	0.583*	0.436	0.515*
<i>Experiment II</i>									
<i>r</i> =	0.831**	0.593*	-0.057	0.914**	0.712*	0.994**	0.635*	0.726*	0.850**

Table 2. Relationship among memory-related measures. Pearson's correlations between total WM and total RM errors across training sessions (* $p < 0.05$; ** $p < 0.001$). The total number of errors is shown in **Supplementary Figures S3a and S4a** (Experiment I) and in **Supplementary Figures S5a and S6a** (Experiment II)

Supplementary material

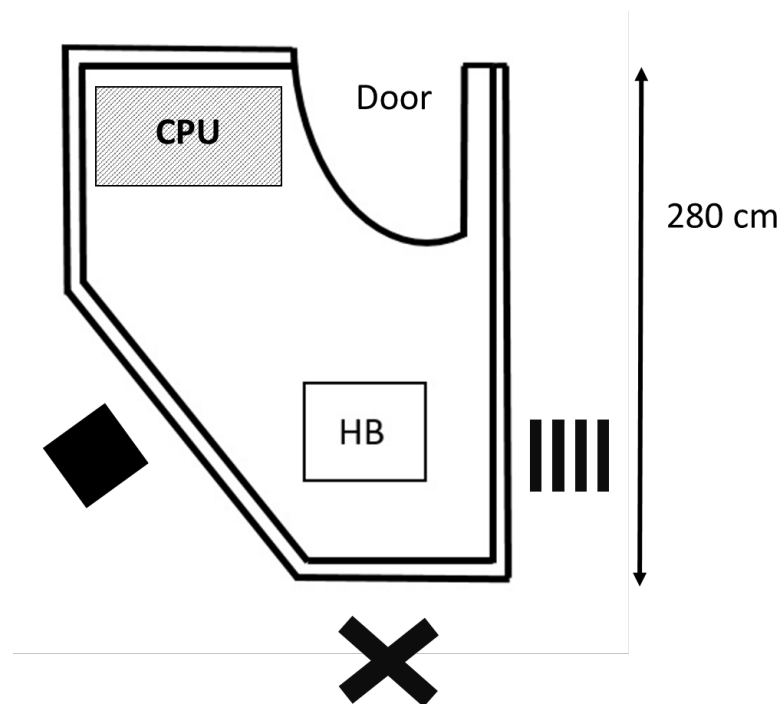


Figure S1. Diagram of the experimental room. Black cardboards representing different geometric shapes were hung on the wall as extra-maze spatial cues. HB: Hole-board; CPU: Computer.

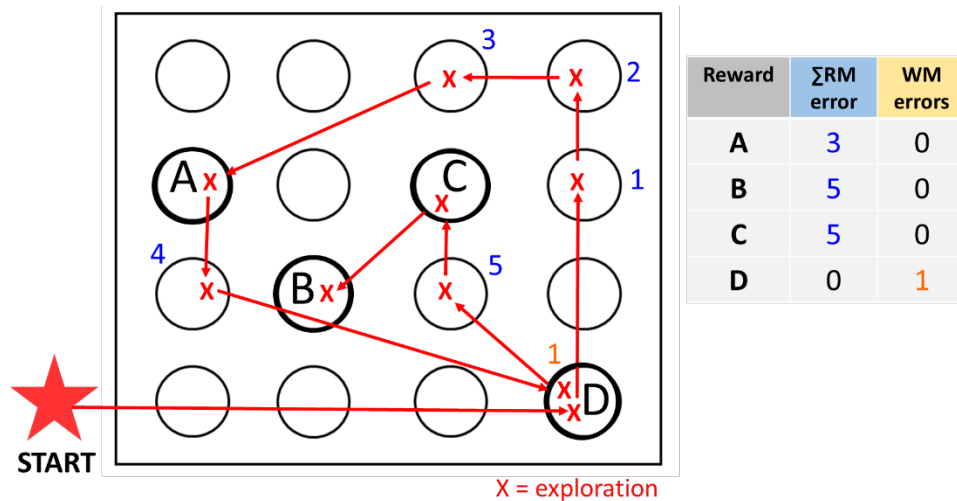


Figure S2. Example of analysis of a training session (four holes baited: A, B, C, D). Arrows point out the path of the animal while hole explorations are indicated by ‘X’. The session is finished after finding the last reward. As observed, the ‘ Σ RM error’ for each reward refers to the sum (i.e. the total number) of RM errors (i.e. explorations to un-baited holes) committed until such reward is found. In other words, it measures how fast or easily that reward is located by the animal. If two rewards are found consecutively (e.g. rewards C and B), their Σ RM error is the same. Working memory errors are re-visits to a baited hole which has already been explored within that session. Reference memory (RM) errors are written in blue color, while working memory (WM) errors are written in orange.

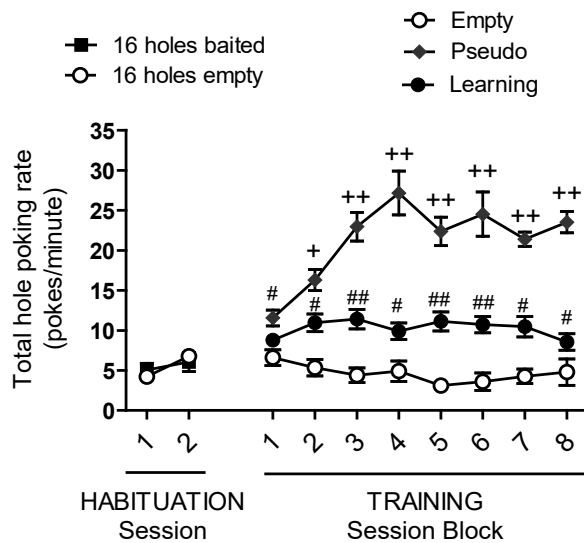


Figure S3. Hole exploration frequencies (hole pokes per minute) in mice from Experiment I. (A) During the two habituation sessions, the addition of food rewards in all holes did not influence the frequency of hole pokes. However, the treatment that mice received during training sessions notably influenced their hole exploratory activity [repeated measures ANOVA during training phase: ‘treatment’: $F(2, 47) = 100.04$, $p < 0.000$; $\eta_p^2 = 0.810$; ‘block’: $F(7, 329) = 5.237$, $p < 0.000$; $\eta_p^2 = 0.100$; ‘treatment x block’: $F(14, 329) = 7.455$, $p < 0.000$; $\eta_p^2 = 0.241$].

Mice that were repeatedly exposed to the empty hole board gradually habituated and lost interest in poking holes compared with the Learning mice that had four food rewards hidden in a fixed set of holes. The Pseudo mice, which had the rewards hidden randomly in each session, greatly increased their hole poking rate, probably as a food searching strategy that allowed them to cover the whole maze in a reduced amount of time. Post hoc LSD: between group comparisons: $\#p < 0.05$; $\#\#p < 0.001$ vs the Empty group; $+p < 0.05$; $++p < 0.001$ vs both the Empty and the Learning groups.

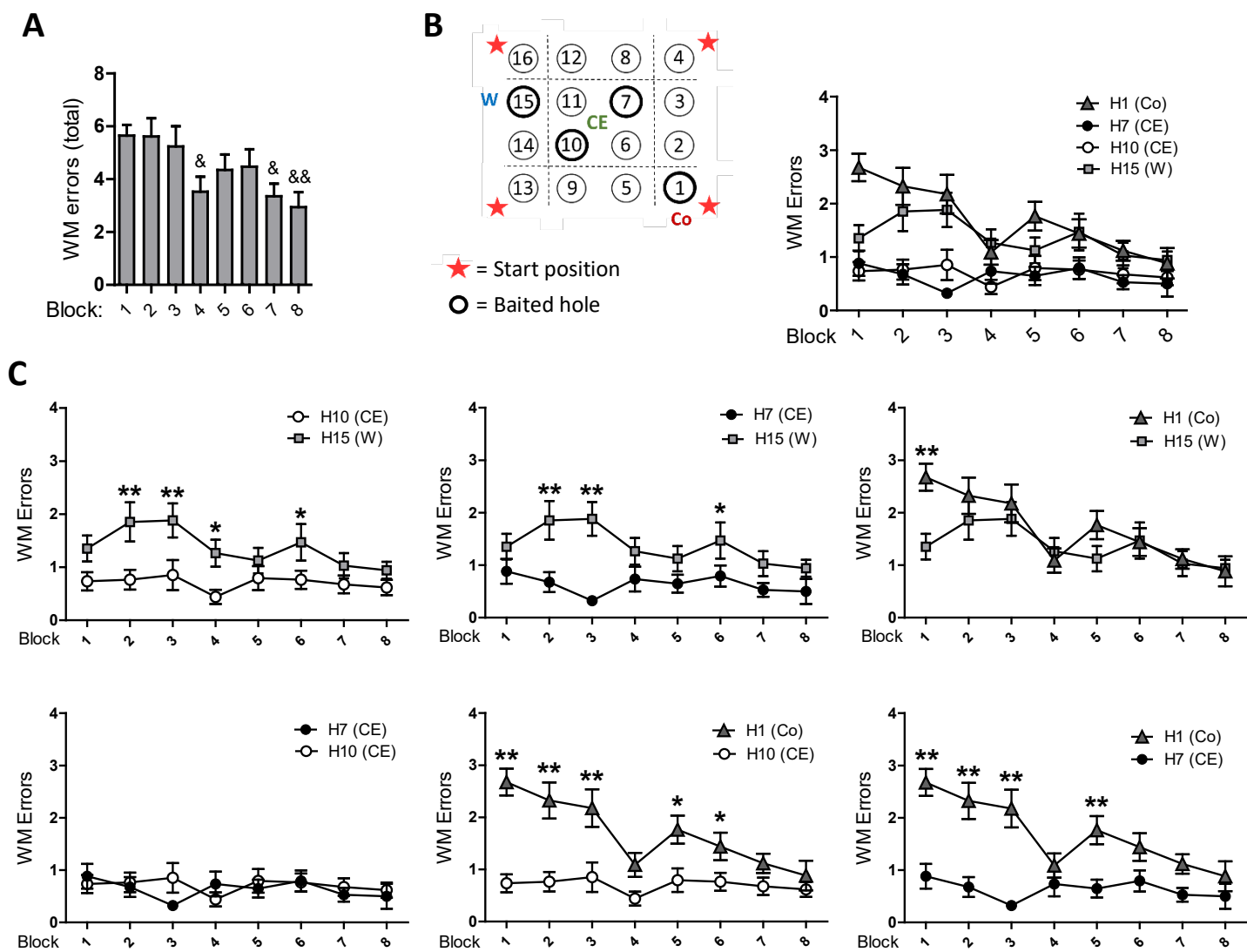


Figure S4. Working memory errors in mice from Experiment I. (A) Total number of WM errors decreased across training [one way ANOVA per session block: $F(7, 128) = 3.069$, $p = 0.005$; $\eta_p^2 = 0.144$]. (B) Working memory errors per baited hole. Post hoc comparisons between holes are detailed in (C). The WM errors were mainly attributed to re-visits to the baited corner hole and, secondly, to the baited wall hole, while the baited centre holes rarely scored any WM errors. The main effects of the ANOVA are shown in the manuscript. Post hoc LSD: within group comparisons: (A); $&p < 0.05$; $&&p < 0.001$ vs session blocks 1-3 (C); between groups comparisons: $*p < 0.05$; $**p < 0.001$ vs the other reward location. Data are expressed as mean \pm S.E.M. Co: corner; CE: center; W: wall

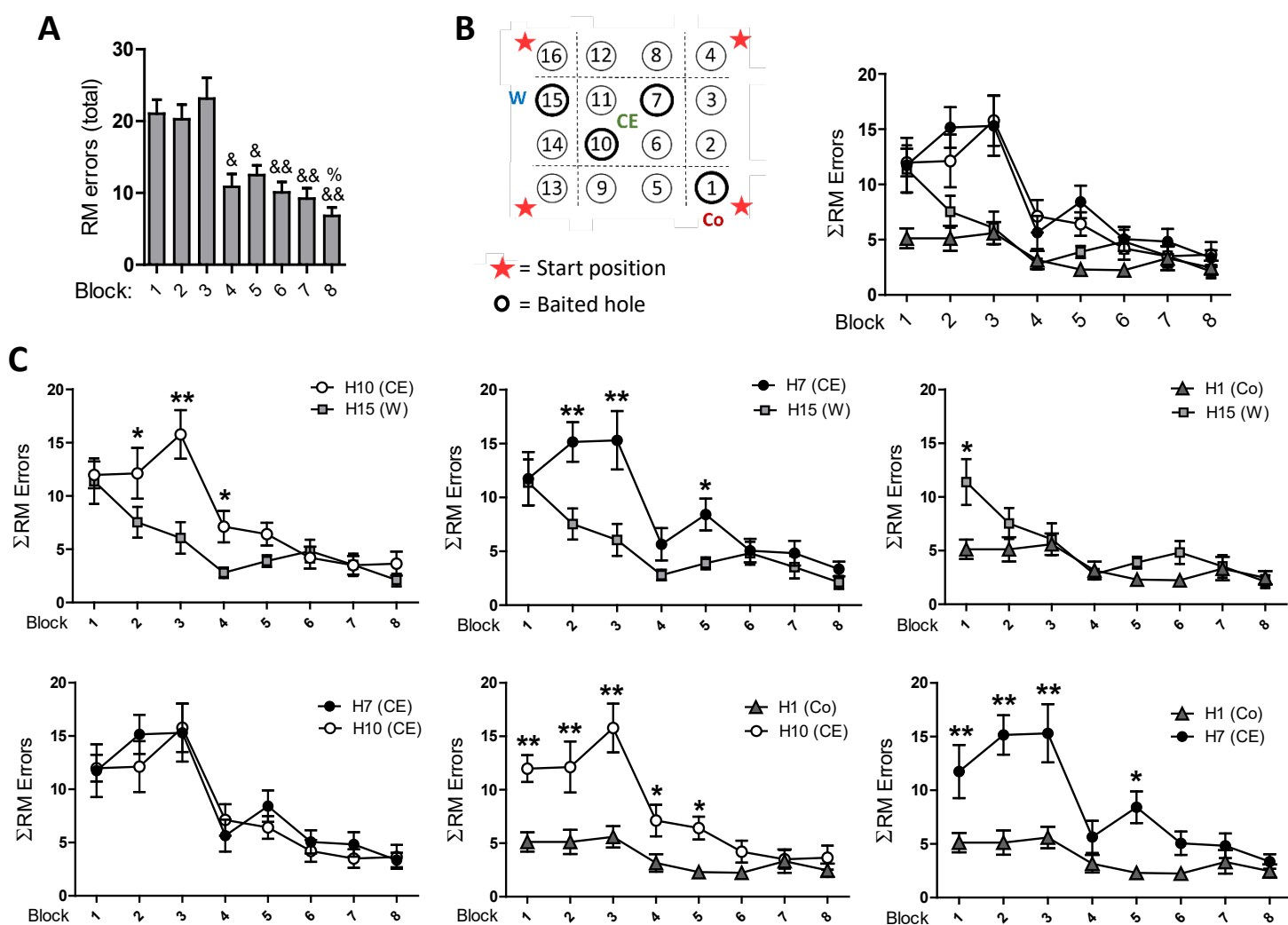


Figure S5. Reference memory errors in mice from Experiment I. (A) Total number of RM errors decreased across training [one way ANOVA per session block: $F(7, 128) = 11.660$, $p < 0.000$; $\eta_p^2 = 0.389$]. (B) Σ RM memory errors per baited hole. Post hoc comparisons between rewards are detailed in (C). The baited corner hole was rapidly found (i.e. per chance) as indicated by a low Σ RM error from the first session block, so subsequent performance was scarcely modified by learning. In contrast, the Σ RM error was initially higher in the baited wall and center holes, which were more efficiently located as learning advanced. In the late training sessions (6-8), the mouse would find any of the four rewards at a similar chance, committing a low number of RM errors through its path. Post hoc LSD: within group comparisons: (A); $\&p < 0.05$; $\&\&p < 0.001$ vs session blocks 1-3; $\%p < 0.05$ vs session block 5 (C); between groups comparisons: $*p < 0.05$; $**p < 0.001$ vs the other reward location. Data are expressed as mean \pm S.E.M. Co: corner; CE: center; W: wall

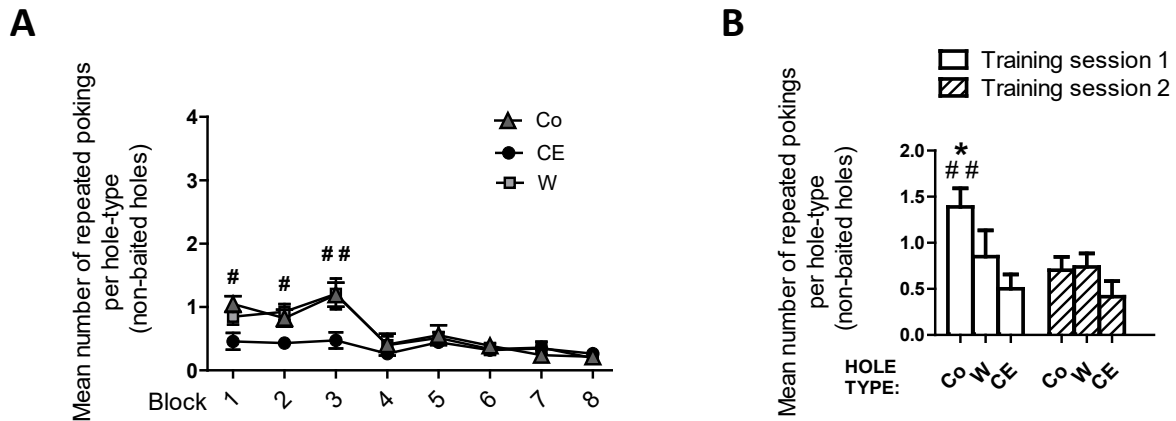


Figure S6. Repeated pokings in the non-baited holes for mice in Experiment I. (A) This measure was calculated as the number of repeated pokes into a non-baited hole that was already poked during the session, and then divided by the number of non-baited holes of each hole type (as shown in **Figure 2a** in the manuscript: 3 non-baited corner holes –holes: 4, 13 and 16–; 7 non-baited wall holes–holes: 2, 3,5, 9, 8, 12, 14; and 2 non-baited center holes –holes: 6 and 11–). Results were averaged in session blocks of two session each as in the main manuscript. Interestingly, data (A) showed a similar tendency than the WM errors in the baited holes, since mice would be more likely to re-visit the peripheral holes (corner and wall zones) than the centre holes during the initial training phase [repeated measures ANOVA (‘hole type’ x ‘session block’): ‘hole type’: $F(2, 51) = 5.113, p = 0.009; \eta^2 = 0.167$; ‘session block’: $F(7, 357) = 15.811, p = 0.000; \eta^2 = 0.237$; ‘hole type x session block’: $F(14, 357) = 2.282, p = 0.005; \eta^2 = 0.082$]. Nevertheless, contrary to the WM errors in baited holes, in this case there were no differences between the corner hole type and the wall hole type.

Because of this, we examined the repeated pokings in non-baited holes that were performed in the first training session where mice were still completely naïve to the rewards’ location (B). Results showed that a preference for the corner hole over the wall hole was indeed present in the first training session, but this was rapidly habituated from this session onwards [one way ANOVA per ‘hole type’ on the first training session: $F(2, 51) = 7.265, p = 0.002; \eta^2 = 0.221$]. This probably reflects that mice rapidly learned which corner holes — that may be more salient and easier to discriminate than the wall or center locations—were unbaited; therefore they tended to ignore them from the second training session. Considering this, repeated poking in non-baited holes may be strongly influenced by reference memory.

Post hoc LSD: between groups comparisons: $\#p < 0.05$; $\#\#p < 0.001$ vs the center holes; $*p < 0.05$ vs the wall holes. Data are expressed as mean \pm S.E.M. Co: corner; CE: center; W: wall

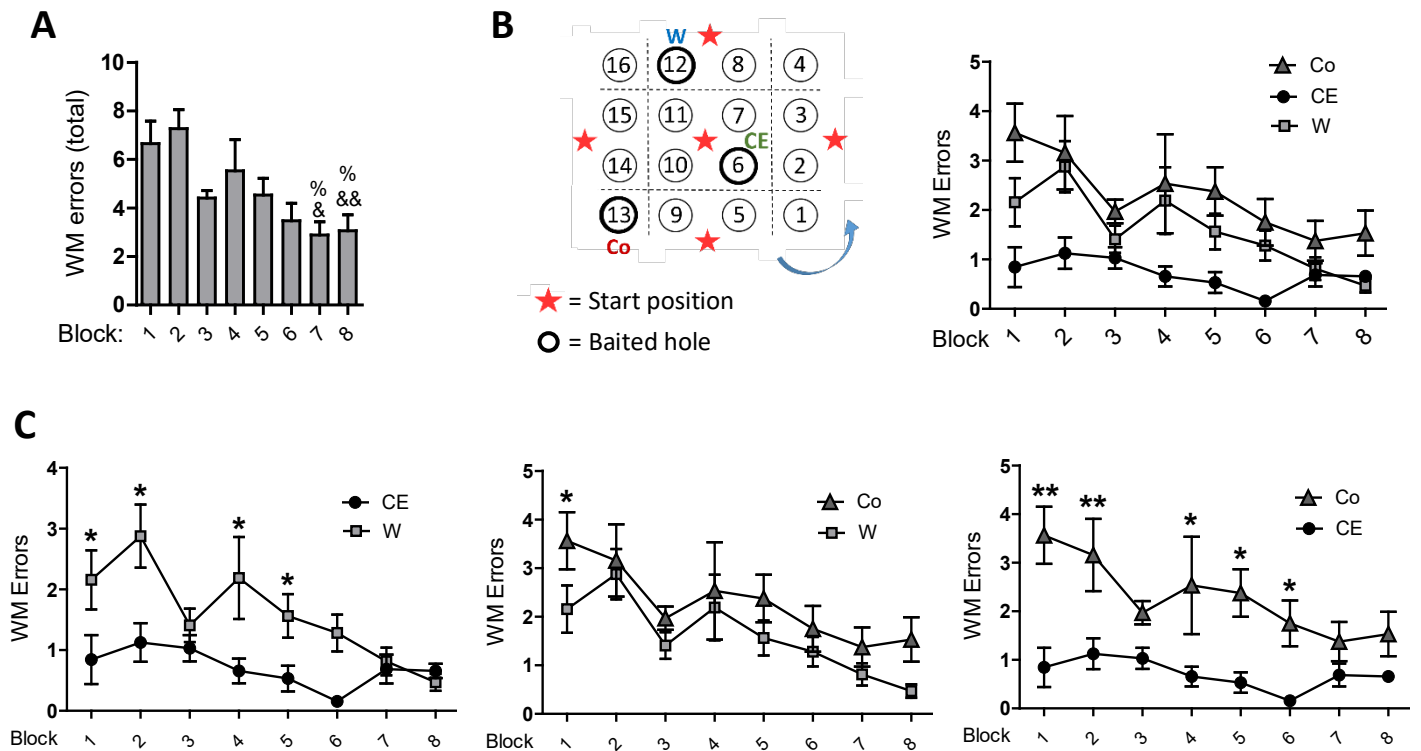


Figure S7. Working memory errors in mice from Experiment II. (A) Total number of WM errors decreased across training [one way ANOVA per session block: $F(7, 120) = 4.253, p < 0.000; \eta_p^2 = 0.199$]. (B) Working memory errors per baited hole. Post hoc comparisons between holes are detailed in (C). Similarly to Experiment I, the WM errors were mainly attributed to re-visits to the baited corner hole and, secondly, to the baited wall hole. The main effects of the ANOVA are shown in the manuscript. Post hoc LSD: within group comparisons: (A); $\&p < 0.05$; $\&\&p < 0.001$ vs session blocks 1-2; $\%p < 0.05$ vs session block 4 (C); between groups comparisons: $*p < 0.05$; $**p < 0.001$ vs the other reward location. Data are expressed as mean \pm S.E.M. Co: corner; CE: center; W: wall

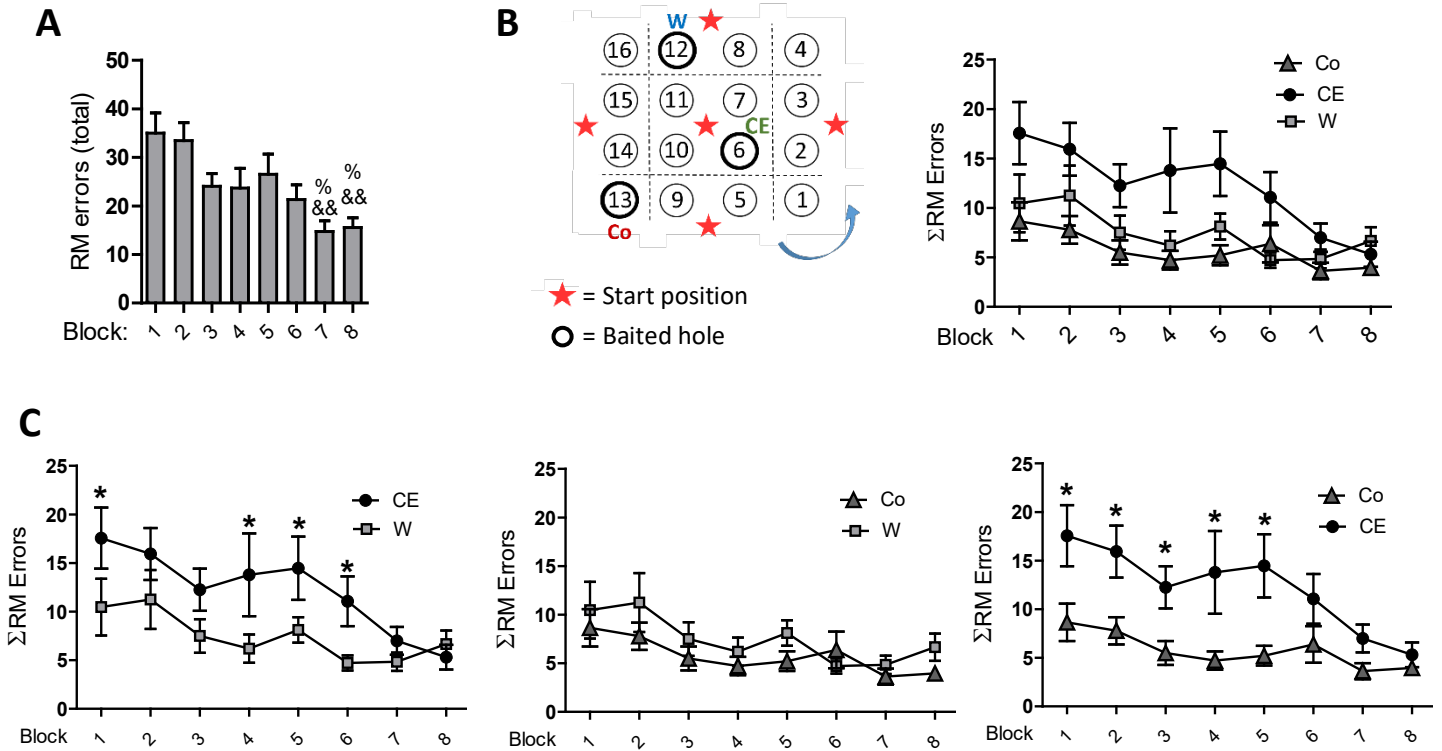


Figure S8. Reference memory errors in mice from Experiment II. (A) Total number of RM errors decreased across training [$F(7, 120) = 5.354, p < 0.000; \eta_p^2 = 0.238$]. (B) Σ RM memory errors per baited hole. Post hoc comparisons between holes are detailed in (C). This experiment was similar to Experiment I in that the baited centre hole was more difficult to find than the baited corner and wall holes. The main effects of the ANOVA are shown in the manuscript. Post hoc LSD: within group comparisons: (A); $\&p < 0.05$; $\&\&p < 0.001$ vs session blocks 1-2; $\%p < 0.05$ vs session block 5 (C); between groups comparisons: $*p < 0.05$; $**p < 0.001$ vs the other reward location. Data are expressed as mean \pm S.E.M. Co: corner; CE: center; W: wall