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Search strategy selection in the Morris water maze indicates allocentric map formation during learning that underpins spatial memory formation

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ABSTRACT

Using a Matlab classification algorithm, we demonstrate that a highly salient distal cue array is required for significantly increased likelihoods of spatial search strategy selection during Morris water maze spatial learning. We hypothesized that increased spatial search strategy selection during spatial learning would be the key measure demonstrating the formation of an allocentric map to the escape location. Spatial memory, as indicated by quadrant preference for the area of the pool formally containing the hidden platform, was assessed as the main measure that this allocentric map had formed during spatial learning. Our C57BL/6J wild-type (WT) mice exhibit quadrant preference in the highly salient cue paradigm but not the low, corresponding with a 120% increase in the odds of a spatial search strategy selection during learning. In contrast, quadrant preference remains absent in serotonin 1A receptor (5-HT_{1A}R) knockout (KO) mice, who exhibit impaired search strategy selection during spatial learning. Additionally, we also aimed to assess the impact of the quality of the distal cue array on the spatial learning curves of both latency to platform and path length using mixed-effect regression models and found no significant associations or interactions. In contrast, we demonstrated that the spatial learning curve for search strategy selection was absent during training in the low saliency paradigm. Therefore, we propose that allocentric search strategy selection during spatial learning is the learning parameter in mice that robustly indicates the formation of a cognitive map for the escape goal location. These results also suggest that both latency to platform and path length spatial learning curves do not discriminate between allocentric and egocentric spatial learning and do not reliably predict spatial memory formation. We also show that spatial memory, as indicated by the absolute time in the quadrant formerly containing the hidden platform alone (without reference to the other areas of the pool), was not sensitive to cue saliency or impaired in 5-HT_{1A}R KO mice. Importantly, in the absence of a search strategy analysis, this suggests that to establish that the Morris water maze has worked (i.e. control mice have formed an allocentric map to the escape goal location), a measure of quadrant preference needs to be reported to establish spatial memory formation. This has implications for studies that claim hippocampal functioning is impaired using latency to platform or path length differences within the existing Morris water maze literature.

1. Introduction

Tolman first proposed that a cognitive map is created during goal-driven maze behaviour (Tolman, 1948). His idea was the first to suggest that during learning rodents spatial coding systems can be viewpoint variant (egocentric person-centred view) or viewpoint invariant (allocentric world-centred view). Tolman hypothesized that during maze learning rodents sample their environments prior to making responses through vicarious trial and error behaviours that develop an overall representation of the maze based on partial experience. This sampling is then incorporated into a cognitive map that enables the animal to dynamically learn the allocentric location of a reward and solve the maze. The Morris water maze is one of the most widely cited behavioural tasks in neuroscience. It was elegantly designed to test the

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hypothesis that the hippocampus creates this viewpoint invariant cognitive map of space (O'Keefe and Nadel, 1978). Although rodents are natural swimmers, the stressful nature of that experience supplies the motivation to find an escape that is the goal of the water maze. To prevent egocentric learning to solve the maze, local cues to guide escape behaviour during navigation are eliminated by using a circular pool that is uniform throughout its circumference with a submerged platform as a hidden escape goal. Surrounding the pool are placed a configuration of salient distal cues that rodents are meant to vicariously sample during spatial learning to form an allocentric reference map. Learning, in the words of Morris, is "acquiring the entire array of cues" (Morris, 1984). In his seminal water maze paper, Morris notes in principle that rats can escape through egocentric random or unsystematic search paths but that in reality rats quickly learn to escape by swimming to the hidden platform location regardless of start location (Morris, 1984). He thus proposed that rats escape by allocentrically learning the spatial position of the platform relative to the distal cue array. The quality of this cognitive map is then tested in a retention probe where the spatial memory is assessed by preference of the rodent for the platform area when the platform is absent.

However, despite its intelligent design, the Morris water maze does not completely eliminate the problem of local cues being present. Spatial learning in mice is most often reported using latency to platform or path length to platform, highly correlated escape parameters that are easily drawn from tracking software (Vorhees & Williams, 2006). Morris introduced latency to platform by noting that the curve steepness interpreted as learning is due to both the egocentric and allocentric spatial learning about the hidden platform location (Morris, 1984). Furthermore, it was subsequently demonstrated that hippocampal lesioned rats learn the escape location with similar latencies to controls (DiMattia & Kesner, 1988). Analysis of their swim paths and path directionality showed they were adopting strategies independent of the distal cues by using a wide arc from the perimeter wall, a strategy now known in mice as chaining (Wolfer & Lipp, 2000). The ratio between the search area in the pool and the platform size is a critical and often overlooked variable that can determine the degree of reliance on non-distal cue strategies like chaining (Vorhees & Williams, 2006). If this ratio is too small the animal will learn the task without acquiring the distal array of cues while to the experimenter this difference is undetectable during spatial learning using latency to platform. Presumably, if the distal array of cues is not salient enough one would expect a similar effect.

Furthermore, an early report in rats demonstrated that latency or path length to platform spatial learning curves do not predict spatial memory formation because key factors that influence probe performance are not captured (Gallagher, Burwell, & Burchinal, 1993). However, the first bearing of an animal does not correlate strongly with any of these measures and is related to the path directionality measure used in Morris's early work (Morris, 1984). Path directionality is the deviation of the first bearing of an animal from the correct angle heading towards the platform, which they demonstrate narrows as spatial learning progresses. Path directionality measures are inherent to the strategy classification hierarchy for mice introduced by Wolfer and Lipp using time-tagged xy coordinates from spatial learning trials (Wolfer & Lipp, 2000). This classification captures the egocentric learning Morris describes (Morris, 1984) (progressing from thigmotaxis to random searching and scanning), notes the chaining response described by DiMattia and Kesner (1988), and demarcates the beginning of allocentric learning when focused searching using the distal cue array becomes the defining feature of navigation. As the cognitive map is strengthened, this search strategy narrows in precision to direct swims to the escape location, where mice focally search in that location if their first bearing is slightly askew. They propose that this transition predicts retention probe performance (Wolfer & Lipp, 2000). Using a Matlab algorithm, this assessment can now be performed in a reasonable time frame (Garthe, Behr, & Kempermann, 2009).

As described above, it has been clearly established that an allocentric map to the hidden platform requires a salient distal cue array and that this cognitive map underlies the exhibition of spatial memory on the retention probe. The objective of this study was to utilize the Matlab classification algorithm to assess the requirement of salient cues for increased spatial search selection during Morris water maze spatial learning. We hypothesized that the likelihood of spatial search strategy selection is the key learning measure that reflects the formation of an allocentric map to the escape location during spatial learning. To test that hypothesis, we trained animals with a high saliency or low saliency distal cue array and assessed measures related to spatial learning and performance on the retention probe in both paradigms. We used quadrant preference on the retention probe as the main outcome measure to establish that a cognitive map to the escape goal had formed during spatial learning. In a similar design to Gallagher et al., we included animals with a spatial memory impairment, serotonin 1A receptor (5-HT_{1A}R) knockout (KO) mice, as a spatial learning and memory impairment control to strengthen our conclusions (Gallagher et al., 1993). We also assessed the effect of cue saliency on spatial learning curves for both latency to platform and path length, as well as their relationship to spatial search strategy selection. Through this approach, we aimed to definitively determine if those spatial learning measures are also appropriate indicators of allocentric learning and spatial memory formation in mice.

2. Methods

2.1. Animals and housing

Serotonin 1A receptor (5-HT_{1A}R) knockout (KO) mice and wild-type (WT) littermates on a C57BL/6J background were obtained by heterozygous breeding at the Florey Institute of Neuroscience and Mental Health (van den Buuse, Ruimschotel, Martin, Risbrough, & Halberstadt, 2011). After weaning, female and male mice were separately group-housed (3–5 per cage) by genotype in open-top standard-housed cages (34 cm × 16 cm × 16 cm) with *ab libitum* access to water and food on a 12 h light/ 12 h dark cycle (lights on at 7 am). Male and female animals were used in this study and two separate cohorts of mice were trained on the Morris water maze (MWM) at around 12 weeks of age. The first cohort obtained was trained on the MWM using the low cue saliency configuration while the second cohort was trained using the high cue saliency configuration. Separate cohorts were used to eliminate the potential confounds of mice having already acquired a distal cue array and having had previous task training from our conclusions. All experiments were performed blind to genotype in accordance with the guidelines of the Florey Institute's Animal Ethics Committee and the National Health and Medical Research Council (NHMRC).

2.2. Behavioural testing

2.2.1. Spatial learning assessment

The Morris water maze protocol used was designed according to Vorhees & Williams, 2006 (Vorhees & Williams, 2006). A circular 1.2 m diameter pool with a height of 0.5 m was used with an uniform interior and unobtrusive seams to diminish the presence of proximal cues during experimentation. The pool was placed on the floor and filled with water to a height of approximately

30 cm so that the 10 cm diameter circular platform was placed 0.5 cm below the water surface. Thus, the key measure of the task difficulty, the search area to target area ratio, was 143:1. It was specifically chosen as it is the appropriate difficulty for the C57BL/6J mice used in our study (Vorhees & Williams, 2006). Additionally, smaller search area to target area ratios than 143:1 would have increased the likelihood of the distance of the platform from the pool wall becoming a proximal cue. On the other hand, larger ratios would have increased the task difficulty to a point where significantly longer training (either through more trials per day or an extended period of days) would be required for mice to acquire the escape goal. The water was made opaque with nontoxic white paint to hide the platform and kept at a temperature of 22 ± 2 °C to provide escape motivation. The pool was divided into cardinal points and the hidden platform location remained constant throughout experimentation in the middle of the south-western (SW) quadrant.

During spatial learning, mice were trained for four trials per day for five consecutive days. To promote invariant cue representations forming during training, distinct start locations were used for each trial: north-western (NW), northern (N), eastern (E), or south-eastern (SE). These distal positions relative to the hidden platform were chosen to minimise mice randomly achieving the escape goal at the beginning of navigation. The start location trial assignments were randomised across the five days of training. During each trial, mice were allowed to search for the escape location for a maximum of 60 s. If they did not find the hidden platform location during this period they were gently guided to it. After finding the platform, mice were left on it for 30 s. If mice jumped from the escape location during this period, they were gently guided back to the goal location. These measures were strictly adhered to as spatial mapping of the hidden platform location is known to take place during both navigation and exploratory rearing once the escape has been found (Harvey et al., 2008). The experimenter was hidden behind a curtain during each trial; if mice were required to be gently navigated to the goal location the experimenter was hidden during the 30 s opportunity for exploratory rearing. Mice were then removed and placed in holding containers underneath heating lamps with 50 W infrared bulbs that were turned on during experimentation to warm the mice for the approximately 20 min before their next trial. Mice were trained sequentially in blocks of 10–13 mice for each trial and the inter-trial interval was the time taken for all the other mice to perform their trials, a suggested strategy to prevent hypothermia-related confounds during training (Iivonen, Nurminen, Harri, Tanila, & Puoliväli, 2003).

To indicate whether spatial learning occurred during the training described above, we used three outcome measures that had different natures (i.e. time to event, continuous, or binary). The first, latency to platform, described the time to an escape and could have been censored (e.g. when an animal did not find the hidden platform location during the 60 s trial and thus had to be guided to the escape location), so was thus a time to event outcome. The second, path length, was the distance traversed by a mouse during each trial, so was a continuous outcome. The last, the search strategy, was a measure of how mice found/attempted to find the hidden platform during each trial. The search strategy data was dichotomized as non-spatial vs spatial (described in great detail below), so was thus a binary outcome measure.

2.2.2. Design of low and high saliency distal cue arrays

A preliminary study in our lab revealed that training mice with the distal cue array described below as the low cue saliency condition resulted in mice not exhibiting spatial memory. Thus we took an approach to increase the saliency in the room. We added larger posters, increased the abundance of smaller posters and placed them higher up the walls, exposed one side of the room by removing a curtain, and moved 3D objects to more distal locations from the pool (Fig. 1, Supplementary Fig. 1). In a second preliminary study, we confirmed these changes resulted in mice exhibiting spatial memory. From these observations, we defined the salience by the quantity, the quality (i.e. size), the visibility, and the distance from the pool edge for 3D objects. The low saliency condition was defined by two small posters and two 3D objects proximal to the pool and the high saliency was defined by an abundance of small posters, large posters, and 3D objects distal to the pool as described below.

2.2.3. Low cue saliency paradigm

In addition to the existing structures within the room, mice were provided a distal cue array containing two 2D and two 3D objects (Fig. 1A, Supplementary Fig. 1A). Printed on A4 paper (30 × 42 cm) a large black X and a set of grated bars were placed in the middle of the west and south wall respectively. In the north, a 1.3 m metal stand with a yellow bucket (15 cm diameter; 30 cm height) glued together 40 cm from the top was placed 16 cm from the pool. In the east, a metallic black lamp (1.5 m) was placed 50 cm from the edge of the pool. Two days of 'cued learning' was used to control for the ability of mice in each genotype to learn to swim to a visible goal. In this paradigm, a red funnel 10 cm tall was inserted in the middle of the platform and all other visual cues in the room were hidden beneath white sheets. Mice were trained from four novel start positions to find four randomised platform locations. All other elements of the training occurred as described above. No effect of genotype was observed during cued learning (data not shown).

2.2.4. High cue saliency paradigm

In this case, mice were provided with a distal cue array that had an increased number of high-contrast 2D cues printed on A4 paper. We also raised the location of the 2D cues on the walls to allow increased visibility during navigation. Additionally, we removed the curtain that previously hid other potential 3D cues including the heating lamps with their visibly lit infrared bulbs, the holding containers and the corridor leading to the room door. We moved the yellow bucket stand described above to the south-east corner of the room and removed the black lamp from the room. We also included larger 2D objects, a large black poster (85 × 62 cm) was placed in the south-west corner and an English flag (150 × 85 cm) was placed in the north on the remaining curtain that was left in place to continue hiding the experimenter and recording equipment (Fig. 1B, Supplementary Fig. 1B). No cued learning was performed prior to the experimentation.

2.2.5. Spatial memory assessment

To assess long-term spatial memory a retention probe with the hidden platform removed from the pool was performed 24 h after the last day of training in either paradigm. The trial lasted 1 min and was started when mice were placed in the pool at a novel distal start position (north-east) to test the quality of the allocentric map formed during training. The mouse behaviour was analysed for only the first 30 s as the absence of the hidden platform leads mice to extinguish the representation of the goal location with prolonged exposure. Preference for the target quadrant was the main measure we used to establish if mice had acquired a viewpoint invariant representation of the former goal location. We also analysed differences in the absolute time target quadrant without reference to chance or time in the other three quadrants as this is a commonly used measure of spatial memory strength. A multitude of platform related measures, which are often used as additional outcome measures of spatial memory formation, were also recorded or derived (Vorhees & Williams, 2006). Annulus crossings

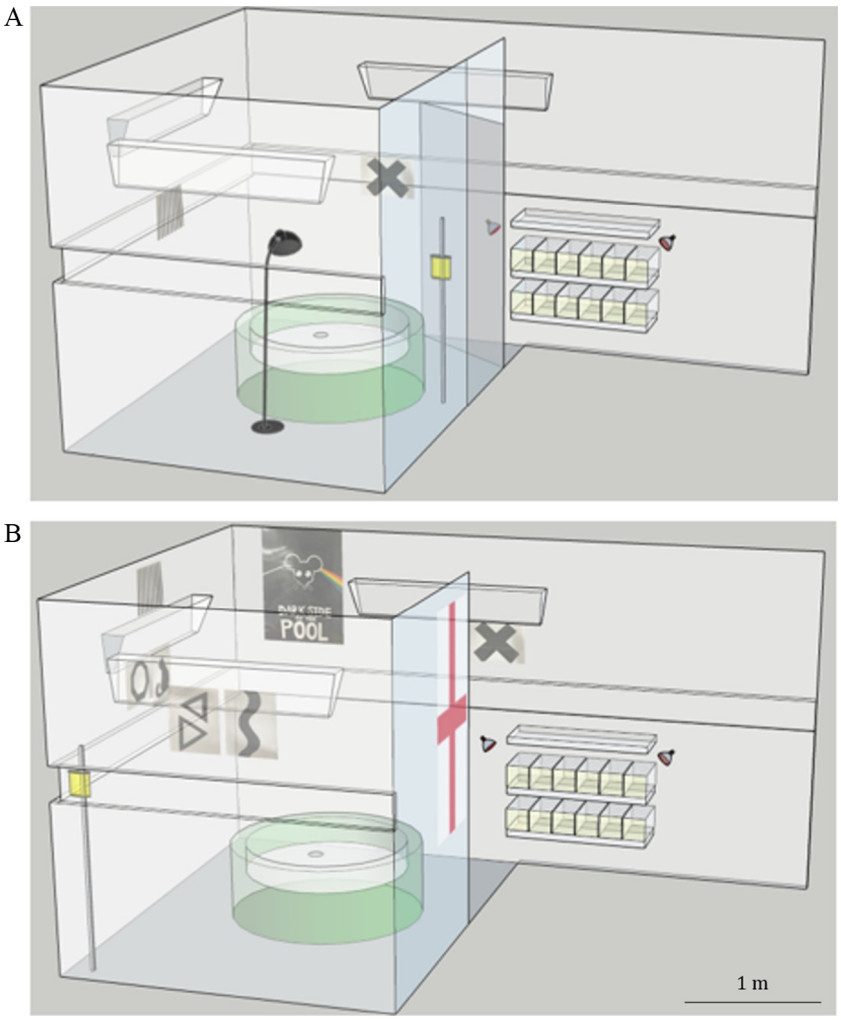

Fig. 1. Morris water maze experimentation room drawn to scale for each cue saliency paradigm. The hidden platform position is visible in the SW quadrant of the pool, from which the four distal start locations (NW, N, E, and SE) can be extrapolated. The computer and the experimenter (not illustrated) were hidden behind a large curtain in the NE corner of the room during training in both paradigms. (A) The low cue saliency paradigm, with the exact locations of the two 2D cues and 3D cues surrounding the pool. Curtains in the NW corner of the room block the view of the heating lamps and holding containers. (B) The high saliency cue paradigm, characterised by a significant increase in both the number and the size of 2D and 3D cues. This included revealing the heating lamps, holding containers and corridor to the room door by removing the curtain obstructing that view. Cues were placed higher up on the walls and further from the pool than in the low saliency configuration. N: north; E: east; NW: north-west; SE: south-east; SW: south-west.

were recorded as bouts in the exact former location of the hidden platform, determined by the centre of the mouse entering that location during the probe trial. Proximity to the platform was recorded by assessing the average distance of mice from the centre of the former goal location during the probe. To quantify accuracy for the former platform location, the annulus crossing index (ACI) was calculated by subtracting the annulus crossings (defined as above) of the equivalent platform position in each of the other quadrants from the annulus crossings of the actual escape location (Janus, 2004). We also used the same logic to derive a novel ACI based on proximity but in this case used the proximity to the platform from the same locations described above. For both the ACI (bouts) and the ACI (proximity), the accuracy for the former goal position is thus expressed by positive ACI values.

2.2.6. Morris water maze learning analysis

The learning parameters latency to platform and path length were derived from Topscan tracking software (Clever Sys, Restin, Virginia). We also performed a search strategy analysis using time-tagged xy-coordinates derived from the same software and an algorithm in Matlab (Mathworks, Natick, Massachusetts) where the script was coded identically to previously published work (Garthe et al., 2009). The seven search strategies were distinguished from each other by their essential attributes (in order of their precision) as follows: direct swim - a maintained heading in the exact direction of the platform; focal search - a localized search near the platform; directed search - a preference for the goal-directed corridor from the start location toward the escape location; chaining - searching in the annulus zone defined by the radial distance of the platform from the pool wall; scanning - preference for the centre zone of the pool area where the distal cue array is maximally visible; thigmotaxis - preference for the wall zone of the pool and random search - searching indiscriminately throughout all pool areas (Fig. 2). In order to distinguish the strategies from each other, we first extracted the arena boundary values for the pool and the platform coordinates within it. These values from the tracking software were then normalised and inputted into the Matlab algorithm. Through this process, the algorithm then generated a set of zones in the pool (wall, annulus and centre) and an ellipsoid around the platform location required for the essential attributes of the strategies to be referenced against (Fig. 2A). On a trial by trial basis across all training days, xy coordi-

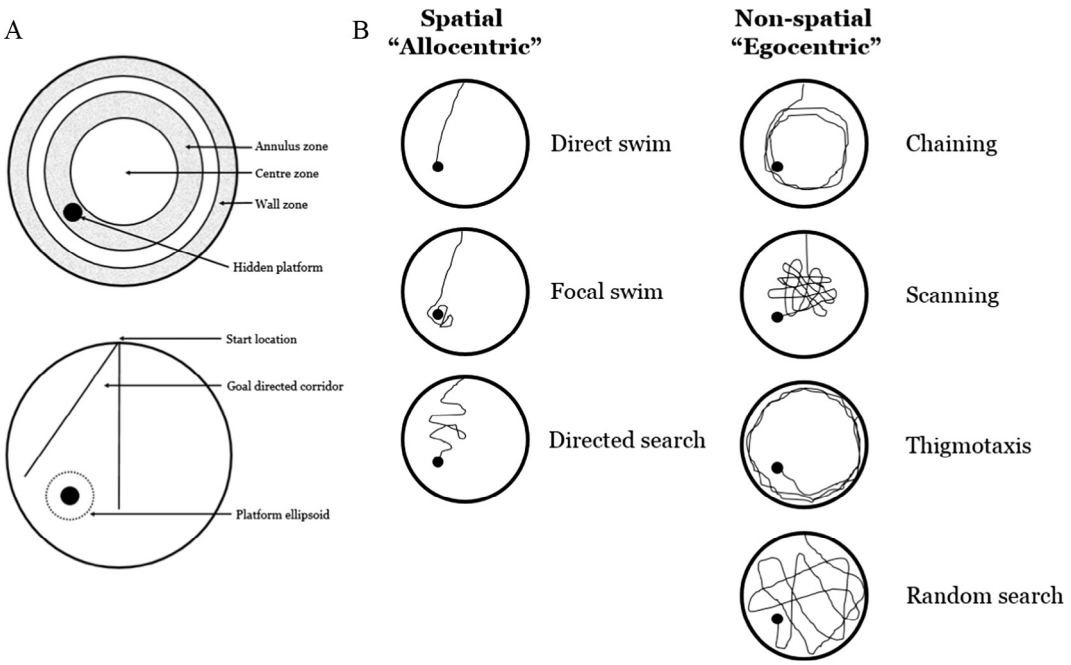

Fig. 2. The objective determination of the search strategy employed during spatial learning in the Morris water maze on a trial by trial basis using the Matlab algorithm (A) In order to objectively identify the various qualitative aspects of a trial's swim path needed to distinguish the strategies from each other, the algorithm first determines the hidden platform location, creates an ellipsoid around it, and generates a set of three zones in the pool (wall, annulus and centre). Then, using the time tagged xy coordinates, the Matlab script establishes the goal-directed corridor and determines a set of numerical parameters (the absolute heading error, path efficiency index, surface coverage, and average distances) required for the essential attributes of the strategies to then be referenced against. (B) The various strategies (listed in both columns from most to least precise) are grouped by the type of learning taking place, with more precise strategies being spatial or allocentric (direct swim, focal search and directed search) and the least precise strategies being non-spatial or egocentric (chaining, scanning, thigmotaxis, random search). A set of criteria representing the abstract properties specific to each strategy is defined within the algorithm. To avoid misclassification due to shared features among those definitions, the Matlab script sorts the more precise swim paths (which are enclosed by the swim paths of other strategies) as special cases before sorting the less defined ones. The objective swim path attributes determined for each trial are then serially matched against the criteria of the strategies from those most precisely defined to those least precisely defined. The first strategy criteria that does not exclude these swim path attributes is the strategy outputted by the algorithm for that trial.

nates for each mouse were then inputted into the algorithm. The Matlab script first determined a set of numerical parameters (the surface coverage, absolute heading error, path efficiency index, goal-directed corridor and average distance to points of interest) and then objectively identified the various qualitative aspects of the trial's swim path. Each strategy was defined in the algorithm by a set of criteria representing the abstract properties specific to that strategy (see (Garthe et al., 2009)). To avoid misclassification due to shared features among the seven strategy definitions, the Matlab script was written to first sort the more precise swim paths (which are enclosed by the swim paths of other strategies) as special cases before sorting the less defined ones. Thus, the objective swim path attributes determined for that trial are then serially matched against the criteria of the strategies from those most precisely defined to those least precisely defined in the order of precision listed above. Finally, the first strategy criteria that did not exclude the swim path attributes determined the strategy employed during that particular trial. Notably, since the algorithm output was on a trial by trial basis, it was possible for an animal to adopt a range of strategies across training days. The various strategies can be classified according to the type of learning taking place (Fig. 2B), with the least precise strategies being non-spatial or egocentric (chaining, scanning, thigmotaxis, random search) and the more precise strategies being spatial or allocentric (direct swim, focal search and directed search) (Wolfer & Lipp, 2000). Thus, on the basis of this clear division in precision, the strategy data were dichotomised as non-spatial (egocentric) or spatial (allocentric). Ultimately, this was used as input for statistical analysis in Stata (Survey Design and Analysis Services Pty Ltd, ACT, Australia). The % spatial search strategy was used as an indicator of allocentric spatial learning.

2.3. Statistics

2.3.1. Spatial learning

Due to the repeated measures nature of the data where individual test outcomes are nested within each individual animal, we utilized mixed-effect regression models to investigate the effect of cue saliency on all spatial learning outcome measures of interest. Additionally, search strategy (binary) and latency to platform (time to event) were not continuous outcomes, so this statistical approach was required to analyse the effect of cue saliency in those cases. The advantages of this modelling approach are two-fold: it accounts for potential missing data in a more robust way as well as presenting cue saliency effect estimates and their precision in addition to the p-values. Individual animals were treated as random effects for all regression models. Significance threshold was set at $p < 0.05$ for all analysis. For each regression analysis, we first assessed the overall effect of cue saliency on the dependent variable (search strategy, latency to platform, or path length) and used day (learning), genotype (learning impairment), start location, and strategy selection (when not the dependent variable) as covariates for adjustment purposes. The effect of cue saliency on the association between a given outcome measure and an independent variable (day, search strategy, start location) was examined by introducing an appropriate interaction term into the regression model. If the interaction was statistically significant, we then undertook further subgroup analyses in high vs. low cue saliency.

The type of regression model used and type of effect size reported was determined by the nature of the outcome measure. For the search strategy analysis (binary outcome), a random-effects logistic regression model was used. Effect sizes

were estimated as odds ratios (OR) with corresponding 95% confidence intervals (95% CIs) to quantify the precision of the estimated effects. In the case of latency to platform (time to event outcome), survival analysis was appropriate for analysis of those repeated-measures data. In survival analyses data can be censored for many reasons, including when the critical event (i.e. the mouse finding the escape goal) has not yet occurred at the end of the time of analysis (i.e. the allotted 60 s for each trial). Censorship avoids introducing bias in the modelling by omitting 60 s trials where no escape occurred (i.e. by treating these cases as if they are missing data). For the data to be censored in this fashion, latency to platform was inputted as a combination of two variables for each trial, the time to the escape goal, and whether or not the mouse escaped. We employed a shared frailty Cox regression model- a well-established model that is used with survival data to specifically model within-mouse correlation of repeated measures and may be thought of as a random-effects model for survival data (Cleves, 2008; Gutierrez, 2002). It was also used to explore the potential relationship between latency to platform and search strategy. Effect sizes were estimated as hazard ratios (HR) of the escape occurring at any time over the 60 s trial period with corresponding 95% CIs to quantify the precision of the estimated effects. The HR is thus a measure of the relative risk of reaching the platform at a given point in time. HRs greater than 1 corresponded to an increased likelihood of a beneficial outcome for the mouse (despite the counterintuitive effect size name). For the path length data (continuous outcome), a clustered median regression model was used. It was also used to explore the potential relationship between path length and search strategy. Effect sizes were reported as median differences (in cm) with corresponding 95% CIs to quantify the precision of the estimated effects. All mixed-effect regression analysis performed has been provided in Supplementary materials (Supplementary Tables 1–3). For investigating the effect of cue saliency on spatial learning outcomes, a repeated measures analysis of variance (RM-ANOVA) could only have been applied to the path length data because it was the sole continuous outcome. However, we would have been unable to adjust for important factors, such as search strategy or start location, we wanted to include in our analyses.

2.3.2. Spatial memory formation

For the determination of long-term spatial memory formation, we assessed whether mice had quadrant preference for the target quadrant. Quadrant preference has been historically defined as the time spent in the target quadrant being significantly different to time spent in each of the other quadrants through RM-ANOVA analysis (Vorhees & Williams, 2006). However, a RM-ANOVA was not appropriate as knowledge of time spent in three of the quadrants automatically determines the time in the final quadrant, thus violating the independence of observations assumption required to perform it. Instead we assessed quadrant preference for each group using point estimates of the time in target quadrant mean with the precision of that estimate (the 95% CI) to test the null hypothesis that the time spent in the target quadrant was not different to chance (i.e. 25% of time during probe). A group was determined to possess quadrant preference only if the 95% CI of the time in target quadrant mean did not overlap with chance. For all additional outcome measures of spatial memory we investigated, data were analysed by two-way ANOVA with genotype and cue saliency as between-group factors. No sex differences were found for any parameter measured, therefore we did not adjust for gender in our regression analyses and used pooled data from both sexes for assessing quadrant preference and for all ANOVA analyses.

3. Results

3.1. The effect of cue saliency on spatial learning

3.1.1. Search strategy selection

The first main objective of this study was to assess the requirement of salient cues for increased spatial search selection during Morris water maze spatial learning. We demonstrate a striking effect of the distal cue array saliency on spatial search strategy selection (Fig. 3A). Mice were 120% more likely to adopt a spatial search strategy to find the hidden platform if the saliency was high [cue: OR 2.21, 95% CI (1.20; 4.04), p = 0.01]. Our analysis of search strategies also indicated that spatial learning was taking place in all mice. There was a clear learning effect, as the adjusted odds of adopting an allocentric strategy increased by approximately 30% per day [day: OR 1.27, 95% CI (1.12; 1.45), p < 0.001]. This analysis also identified significant differences between WT and serotonin 1A receptor (5-HT_{1A}R) knockout (KO) mice. Assuming similar cue, start location and day, the 5-HT_{1A}R KO mice were over 50% less likely to choose an allocentric strategy [genotype: OR 0.46, 95% CI (0.25; 0.86), p = 0.014]. There was a significant interaction with the effect of cue saliency on the association between search strategy selection and day (p = 0.03). The subsequent subgroup analysis within each cue paradigm revealed that the spatial learning curve was present only when mice were trained with a highly salient cue array. There was a significant increase in the odds to adopt an allocentric strategy over training in the high saliency paradigm [day: OR 1.33, 95% CI (1.12; 1.57), p = 0.001] but not if the saliency was low [day: OR 1.21, 95% CI (0.97; 1.50), p = 0.086].

The immediate viewpoints of the distal cue array were distinct for mice placed in the pool at each of the four start locations using both saliency paradigms (see Fig. 1). We hypothesized that these differential vantage points of the distal cue array would influence the likelihood that a spatial search strategy would occur so we also investigated start location as a covariate in the analyses. Assuming similar cue, genotype, and day no statistically significant changes were observed in the adjusted odds of a spatial search strategy due to start location. However, a significant interaction with the effect of cue saliency was identified between search strategy selection and start location (p < 0.001). The subsequent subgroup analysis within each cue paradigm revealed that the spatial search selection likelihood was not equivalent across the four distinct start locations we employed during spatial learning trials (Fig. 3B). When restricting analysis to the high saliency paradigm, the adjusted likelihood of a spatial search strategy occurring was significantly different when comparing the individual start locations. The eastern [OR 3.16; 95% CI (1.58; 6.34), p = 0.001] and south-eastern [OR 2.30, 95% CI (1.13; 4.68), p = 0.022] but not the north-western start location (OR 1.81, 95% CI (0.88; 3.74), p = 0.11) had significantly increased likelihoods for mice to select a spatial search strategy compared to the northern start position. In contrast, when restricting analysis to the low saliency paradigm, the same hierarchy existed in terms of adjusted likelihood, but in this case it was the complete reverse. In this case, assuming similar cue, day, and genotype, the eastern [OR 0.26, 95%CI (0.11; 0.64), p = 0.004] and south-eastern [OR 0.26, 95% CI (0.11; 0.64), p = 0.003] but not the north-western location [OR 0.66, 95% CI (0.32; 1.38), p = 0.27] had significantly decreased likelihoods for mice to select a spatial search strategy compared to the northern start position.

3.1.2. Latency to platform

A secondary objective of our spatial learning analysis was to determine the requirement of salient cues for a latency to platform learning curve to exist during spatial learning. We also aimed to

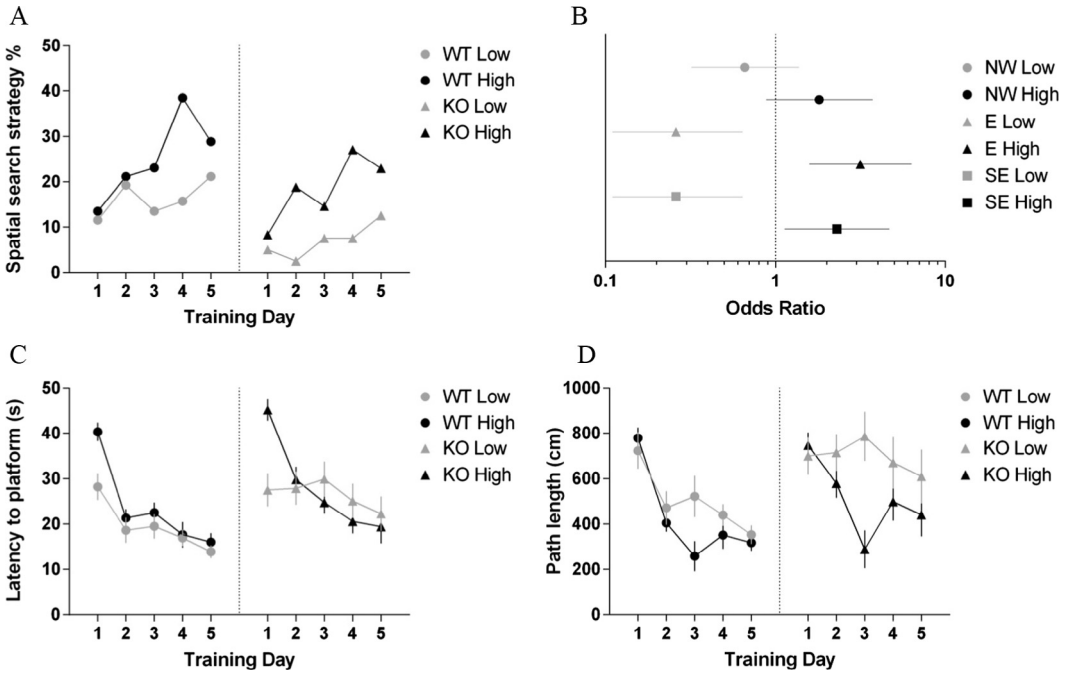

Fig. 3. During spatial learning search strategy selection but not latency or path length is significantly altered by the saliency of the distal cue array. (A) Cue saliency increases the likelihood that allocentric search strategy is selected during spatial learning [OR 2.21, 95% CI (1.20; 4.06), p = 0.01]. Furthermore, as evidenced by the likelihood of allocentric search strategy selection increasing across training days, spatial learning takes place under the high [OR 1.33, 95% CI (1.12; 1.57), p < 0.001] but not the low [OR 1.21, 95% CI 0.97–1.50, p = 0.086] saliency configuration. (B) A significant interaction between cue saliency and start location was identified for search strategy selection. In the high paradigm, the odds ratios for search strategy likelihoods at the eastern start positions but not the north-western are significantly increased compared to the northern location (i.e. the 95% confidence interval does not overlap with 1). In contrast, in the low paradigm, the odds ratios the eastern start positions but not the north-western are significantly decreased compared to the northern location. The effect size in both cases are approximately equal in magnitude. C) No significant effect of the distal cue array saliency was identified for latency to platform during training [HR 0.84, 95% CI (0.68; 1.05), p = 0.12]. However, there is a clear effect of learning as mice have shorter latencies as training progresses [HR 1.15, 95% CI (1.07; 1.23); p < 0.001]. D) No significant effect of cue saliency for the path length chosen during training was observed [cue: −59.76, 95% CI (−133.99; 14.46), p = 0.11]. This is despite a clear effect of learning as mice have shorter path lengths as training progresses [day: −32.26, 95% CI (−58.25; −6.28), p = 0.015]. 5-HT_{1A}R KO animal performance indicates an impairment using all three measures. Search strategy data are expressed as a percentage of spatial strategies selected within all trials on a given day; start locations are expressed as odds ratios with 95% CIs; latency and path length data is expressed as mean ± SEM; n = 10–13; E: east; NW: north-west; SE: south-east.

establish the adjusted association between latency to platform and spatial search strategy selection. The time taken for mice to reach the hidden platform location across training was assessed for each saliency condition (Fig. 3C). Our shared frailty Cox regression analysis (see Methods) of latency to platform identified that there was no significant change in the adjusted risk of mice reaching the platform due to cue saliency [cue: HR 0.84, 95% CI (0.68; 1.05), p = 0.12]. It also revealed, assuming similar cue, day and genotype, that at any point of time mice were 115% more likely to find the hidden platform location if they selected a spatial search strategy to find it [strategy: HR 2.15, 95% CI (1.74; 2.66), p < 0.001]. We also showed an overall learning effect as mice were 15% more likely to find the platform at any given point in time with each training day [day: HR 1.15, 95% CI (1.07; 1.23), p < 0.001]. A learning impairment in 5-HT_{1A}R KO mice was also found, as they are approximately 30% less likely to find the platform [genotype: HR 0.71, 95% CI (0.58; 0.89), p = 0.002]. There was a significant interaction with the effect of cue saliency on the association between latency to platform and search strategy selection (p < 0.001) but not latency to platform and day (p = 0.70). This was reflected by differences in the influence of a spatial search strategy selection on the adjusted likelihood of mice reaching the platform under the two saliency paradigms, from being approximately 1.5 times more likely if testing with a high saliency [strategy: HR 1.49, 95% CI (1.15; 1.93), p = 0.003] compared to approximately 5.5 times more likely with low saliency [strategy: HR 5.52, 95% CI (3.72; 8.17), p < 0.001]. No statistically significant differences or interactions with cue saliency were observed in the investigation into whether the different start locations changed the likelihood for mice to find the hidden platform.

3.1.3. Path length to platform

To complete the secondary objective of our spatial learning analysis we then assessed the requirement of salient cues for a path length to platform learning curve to exist and established the adjusted association between path length and spatial search strategy selection. The spatial learning curves for the length of the path taken for mice to reach the hidden platform were derived in each cue saliency paradigm (Fig. 3D). No significant effects of cue paradigm on path length [median difference in path length by cue: −59.76, 95% CI (−133.99 cm; 14.46), p = 0.11] were identified using our median clustered regression analysis (see Methods). However, assuming similar cue, day and genotype, a spatial search strategy selection significantly reduced the path length [strategy: −135.20, 95% CI (−227.08 cm; −43.33), p = 0.004]. There was also a clear learning effect as adjusted path lengths were shortened across days [day: −32.26, 95% CI (−58.25 cm; −6.28), p = 0.015]. Additionally, a learning impairment in 5-HT_{1A}R KO animals was demonstrated as the same analysis revealed that they took longer adjusted path lengths [genotype: 109.08 cm, 95% CI (10.42; 207.74), p = 0.03]. There was a significant interaction with the effect of cue saliency on the association between path length and search strategy selection (p = 0.001) but not path length and day (p = 0.34). This was reflected by differences in the influence of a spatial search strategy selection on the adjusted path length, from significantly associated with decreased path lengths if testing with

a low saliency paradigm [strategy: −285.11, 95%CI (−385.53 cm; −184.69), p < 0.001] compared to no significant association given a high saliency cue paradigm [strategy: −25.48 cm, 95% CI (−170.81; 119.85), p = 0.73]. No significant differences or interactions with cue saliency were observed during our analyses into whether the different start locations influenced the path length mice employed to find the hidden platform.

3.2. The effect of cue saliency on spatial memory

3.2.1. Quadrant preference

The second main objective of this study was to assess the requirement of a salient distal cue array for spatial memory formation (i.e. the ability to demonstrate that a cognitive map to the escape goal location had formed during spatial learning on a retention probe). We used preference for the target quadrant (see methods) as the main outcome measure that spatial memory had formed. Our analysis of quadrant preference showed that only WT animals trained with the high saliency paradigm demonstrated spatial memory on the retention probe (Fig. 4). In the low saliency cue paradigm, both WT [8.94 s, 95% CI (6.42; 11.47)] and 5-HT_{1A}R KO animals (8.82 s, 95% CI (6.28; 11.37)) did not demonstrate quadrant preference, as their 95% CIs overlap with the chance performance (25% - 7.5 s). This comparison within the high saliency paradigm identified intact quadrant preference in WT animals [12.42 s, 95% CI (9.23; 15.61)] with a 95% CI that overlapped with 50% of time in target quadrant. 5-HT_{1A}R KO mice, included as a positive control for spatial memory impairment, exhibited no quadrant preference [9.00 s, 95% CI (7.43–10.56)] and thus had impaired spatial memory despite the increase in cue saliency.

3.2.2. Additional retention probe outcome measures

A secondary objective of our spatial memory analysis was to determine the requirement of salient cues for spatial memory formation using additional spatial memory retention probe outcome measures routinely employed in Morris water maze studies. 5-HT_{1A}R KO mice were again used as a positive control for spatial memory impairment. Thus, we also assessed the effect of cue saliency, genotype and potential interactions between both factors on a broad sample of these outcome measures during the retention probe (Fig. 5). The absolute time in target quadrant (Fig. 5A) is used as the key measure of spatial memory strength, but we found no significant effects of cue saliency ($F_{1,43}$ = 0.88, p = 0.35) or genotype ($F_{1,43}$ = 2.17, p = 0.15) and no cue × genotype interaction ($F_{1,43}$ = 1.87, p = 0.18). In contrast, the latency for the mouse to visit the exact location where the platform resided during training (Fig. 5B), was sensitive to cue saliency ($F_{1,43}$ = 10.76, p = 0.002) but not genotype ($F_{1,43}$ = 1.37, p = 0.25). Additionally, no significant interaction was identified between those factors ($F_{1,43}$ = 0.21, p = 0.65) for latency to platform on the retention probe. Similarly, the number of times a mouse crosses the former platform location (Fig. 5C) was also sensitive to cue saliency ($F_{1,43}$ = 4.49, p = 0.04) but no significant effect of genotype ($F_{1,43}$ = 1.46, p = 0.23) or a significant cue × genotype interaction ($F_{1,43}$ = 0.040, p = 0.84) were observed. However, no significant effects of cue saliency ($F_{1,43}$ = 3.30, p = 0.076) and genotype ($F_{1,43}$ = 3.65, p = 0.063) or significant interaction between those factors ($F_{1,43}$ = 0.16, p = 0.7) were observed using an annulus crossing index (ACI) derived from crossings in the four equivalent platform locations as an outcome measure (Fig. 5D), Analysing the average proximity of the mouse from the former platform location during training (Fig. 5E), we also found a significant effect of cue saliency ($F_{1,43}$ = 5.29, p = 0.026) but no significant effect of genotype ($F_{1,43}$ = 2.45, p = 0.13) or a significant cue × genotype interaction ($F_{1,43}$ = 0.74, p = 0.4) were identified. Additionally, the version of the ACI derived using the average proximity from each equivalent platform location (Fig. 5F) showed again a significant effect of cue saliency ($F_{1,43}$ = 10.92, p = 0.002). In a similar fashion to the above results, no statistically significant effect of genotype ($F_{1,43}$ = 1.69, p = 0.2) or a significant interaction between cue and genotype ($F_{1,43}$ = 1.16, p = 0.29) were observed.

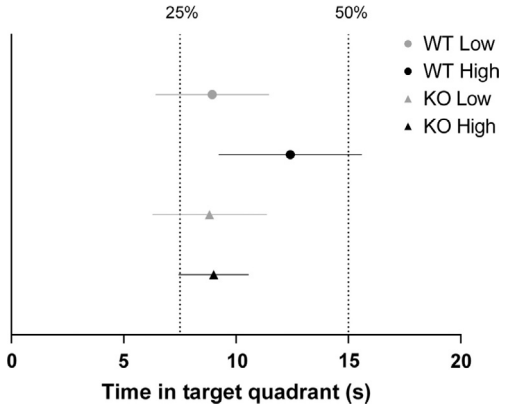

Fig. 4. Spatial memory formation is demonstrated on the retention probe only in wild-type (WT) mice trained with a highly salient distal cue array. WT mice exhibit quadrant preference in the high saliency paradigm (i.e. the 95% CI does not overlap with 25% of retention probe time, the chance performance) and thus demonstrate spatial memory for the escape goal location. However, WT mice trained in the low saliency paradigm do not display quadrant preference (i.e. the 95% CI overlaps with chance performance) and thus do not demonstrate spatial memory. As expected, 5-HT_{1A}R KO animals display no quadrant preference when trained in both paradigms and thus have impaired spatial memory in either case. Data is expressed as mean ± 95% CI; n = 10–13.

4. Discussion

The main objectives of this study were to determine if search strategy selection during spatial learning and spatial memory demonstration on the retention probe were dependent on the quality of the distal cue array. We established this dependency in both cases. We also wanted to test our hypothesis that search strategy selection is the key spatial learning measure that predicts the formation of an allocentric map to the escape location (and thus predicts intact spatial memory). Several lines of evidence in our study support accepting this hypothesis. Firstly, we found that spatial memory was exhibited only by WT mice trained in the high but not the low saliency paradigm. Secondly, the odds of a spatial search strategy increased by 120% in the high compared to the low saliency condition and a spatial learning curve during training was present on this measure only in the high saliency paradigm. Finally, spatial learning curves for both latency to platform and path length had no significant effect of, or interaction with, cue saliency despite the differences in spatial memory in each paradigm. Overall, these lines of reasoning all strongly support accepting our original hypothesis and are explored in greater detail below.

4.1. The effect of cue saliency on search strategy selection and spatial memory

Morris defined spatial learning as "acquiring the entire array of cues" (Morris, 1984) so in this study we hypothesized that search strategy selection is the spatial learning outcome measure that demonstrates this acquisition is taking place. Three key pieces of evidence from our investigation into the effect of cue saliency on search strategy support accepting this hypothesis. Firstly, the adjusted odds of a spatial search strategy occurring increased by 120% if a salient distal cue array was provided. This demonstrated

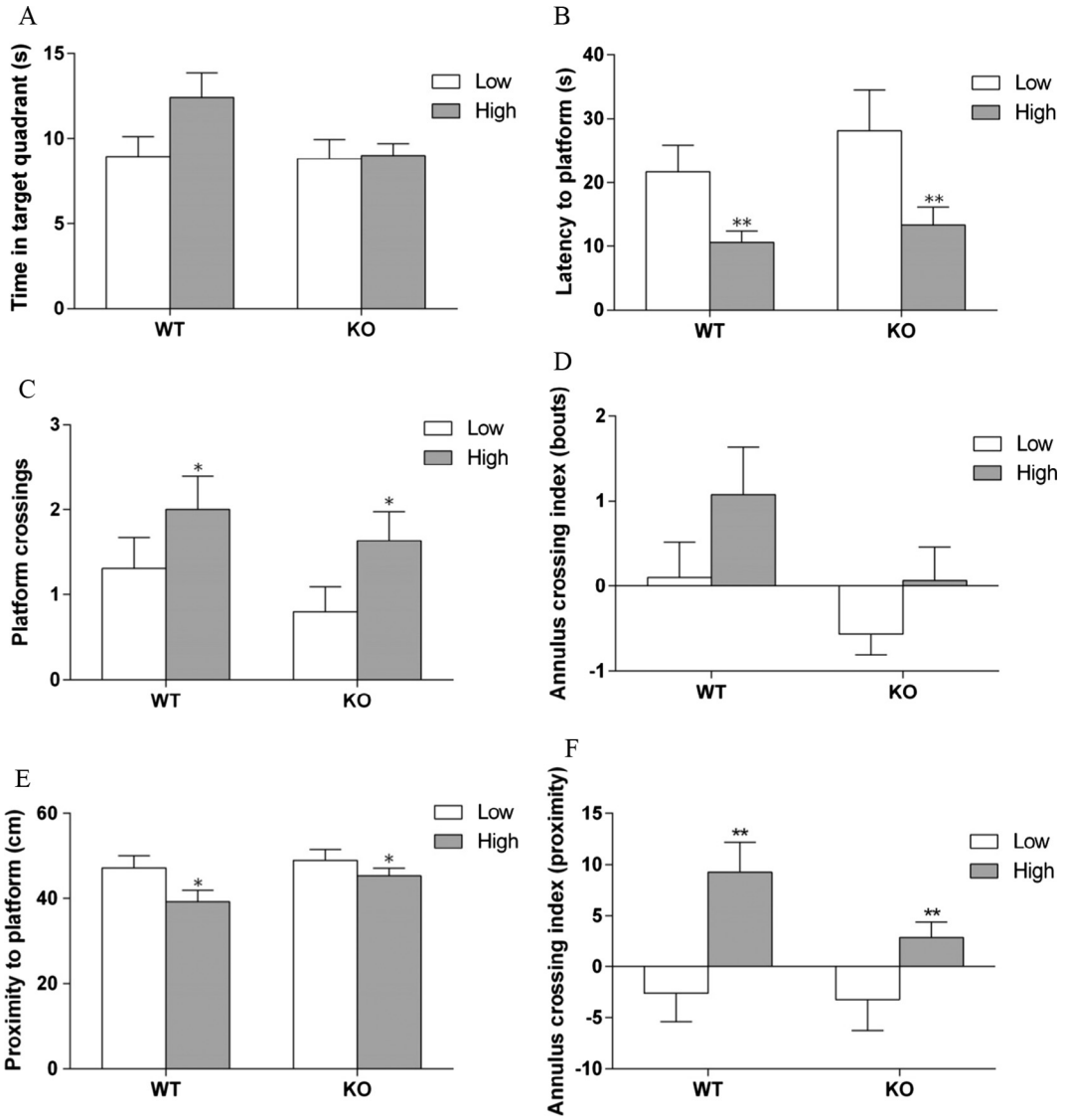

Fig. 5. Additional outcome measures indicating spatial memory formation do not indicate impaired memory in 5-HT_{1A}R KO mice despite several being sensitive to cue saliency. (A) No significant effect of cue saliency was identified using the absolute time in target quadrant as an outcome measure for spatial memory. (B) There is a significant effect of cue saliency on latency to platform during the retention probe. Under the high saliency condition, mice have shorter latencies to the former platform location (C) The number of times mice visited the former platform location is significantly increased if high saliency cues are available for navigation during training. (D) No significant effects of cue saliency were identified on the annulus crossing index (ACI). This ACI was derived using the number of times mice frequented the equivalent location of the hidden platform in the other three quadrants. (E) The high saliency configuration significantly decreases the average proximity to the centre of the former platform location during the probe trial. (F) A novel ACI derived using the average distance to the centre of all possible platform locations is significantly increased under the high saliency condition. No statistically significant differences were identified in 5-HT_{1A}R KO animal performance using any of the above measures. Data is expressed as mean ± SEM; n = 10–13; main effect of saliency: $*p < 0.05$; $**p < 0.01$.

that spatial search strategy selection was dependent upon the distal cue array. Secondly, a search strategy spatial learning curve was present in the high saliency paradigm but absent in the low. This result established that mice were transitioning from egocentric navigation to utilizing allocentric routes to the escape location only when trained with a salient distal cue array. It is worth noting that the % spatial search strategy was less than 50% during training for WT mice in the highly salient condition, indicating that on a trial by trial basis mice were still adopting multiple different strategies on any given day. It is possible that mice could adopt a spatial strategy and, after unsuccessfully finding the escape goal, revert back to a non-spatial strategy within the same trial. In this case, the algorithm would have most likely ranked the trial as non-spatial, which is a limitation to its sensitivity we must acknowledge. However, from our subjective rankings in those circumstances, mice tended to transition between the allocentric strategies or egocentric strategies within the same trial. Notably, this behaviour is accounted for by the dichotomization we employed. Finally, the likelihood of a spatial search strategy selection from the eastern start positions was increased using the high saliency paradigm but decreased using the low. This further substantiates the evidence described above that allocentric learning requires salient distal cues. It also suggests that mice were not uniformly acquiring the distal array of cues as they were attempting to form an allocentric reference map to the escape goal in either paradigm, but were instead heavily reliant on certain cues. Variable start locations have been proposed to force mice to acquire the entire array of distal cues (Eichenbaum, Stewart, & Morris, 1990). The idea was that mice assimilate the varying initial visual perspectives posed from the unique start locations for

navigation to be successful. However, our results are more consistent with a recent report dissociating cues processed for navigation from those for associative properties within a distal cue array (Scaplen, Gulati, Heimer McGinn, & Burwell, 2014). In that study, the cues processed for navigation were strongly related to size. Therefore, the cues most critical for navigation were most likely the exposed holding containers and hallway in the north-west corner of the room in the high saliency paradigm and the black lamp in the east in the low (see Fig. 1). Supporting this idea, in both paradigms these cues were hidden from the initial visual perspective of the mice in the start locations with the lowered likelihood of spatial search strategy occurring. From these three lines of evidence in our study, we conclude that search strategy selection is a spatial learning outcome that measures the acquisition of the distal cue array.

The second main objective of this study was to assess the requirement of a salient distal cue array for spatial memory formation. For mice to display spatial memory on the retention probe they must demonstrate that an allocentric cognitive map to the escape goal location had formed during spatial learning. Outcome measures establishing the presence of spatial memory must therefore capture the animal's preference for the area of the pool where the escape was expected to be found (i.e. to test the null hypothesis that mice were not significantly preferring any area of the pool). Thus, in our study, we used preference for the target quadrant as our main spatial memory outcome measure. This is because we were interested in establishing whether spatial memory had formed but not in assessing the spatial memory strength in our various groups. Using this approach, we established that WT animals display intact spatial memory in the high but not the low saliency paradigm. Additionally, 5-HT1AR KO mice, included as a positive control for spatial memory impairment, did not display intact spatial memory in the high saliency paradigm. Thus, our quadrant preference analysis indicates that a salient distal cue array is required for spatial memory formation and that mice with impaired hippocampal functioning fail to adequately acquire it. Furthermore, we showed that additional indicators of spatial memory formation, outcome measures relating to the former platform location, were also sensitive to cue saliency during spatial learning. If significant interactions were found during these ANOVA analyses we would have performed subgroup analysis within the saliency paradigms but no such interactions were identified/observed. However, if we did restrict our analysis to the high or low saliency paradigm for each of these measures, no statistically significant differences were observed between WT and 5-HT_{1A}R KO mice (analyses not shown). Thus, these additional outcome measures of spatial memory formation did not reveal the spatial memory impairment in 5-HT_{1A}R KO mice so were not in complete agreement with the quadrant preference results previously discussed. Furthermore, no statistically significant effects of cue were observed in the analysis of the absolute time in target quadrant, implying this measure may not reflect the formation of an allocentric map to the escape location. This has implications for the current literature as the absolute time in target quadrant is often used as an indicator of the presence or absence of spatial memory on the retention probe (Vorhees & Williams, 2006). Our results suggest that a measure of quadrant preference should be routinely included to first establish intact spatial memory. Once intact spatial memory is established for each group, absolute time in target can then be used to draw biologically meaningful inferences about the spatial memory strength in group comparisons. In summary, all of this evidence supports the conclusion that a salient distal cue array is required for spatial memory formation.

Having established that both search strategy selection during spatial learning and spatial memory exhibition during the retention probe require a salient distal cue array, we will now explore whether the conclusion that spatial search strategy selection predicts spatial memory is valid. In this study, we found that spatial memory formation exists in our WT animals only if a spatial learning curve existed in spatial search strategy selection (in the high saliency paradigm). This finding suggests that spatial search strategy learning curves can be used to predict the formation of the allocentric map to the escape location (and thus spatial memory demonstration on the retention probe). A previous study that interrogated the relationship between acquiring the array of distal cues during spatial learning and spatial memory formation revealed key insights (Harvey et al., 2008). It established that distal cue associations form both during swimming itself and exploratory rearing once the mouse has found the escape on the hidden platform. That study elegantly showed a requirement for the necessity of the distal cue array to be present during both behaviours for intact spatial memory to occur on the retention probe. Furthermore, they found that if you rotate the distal cue arrangement by 180 degrees after spatial learning has finished before the memory probe is conducted, mice will exhibit quadrant preference in the opposite quadrant to where the escape was located during training. This nicely established that the allocentric map formed during training guides the behaviour on the retention probe. These observations were in alignment with early observations that habituation of exploratory rearing takes place during spatial learning, and is disinhibited if the platform location is altered during training (Sutherland, Whishaw, & Kolb, 1983). We extend those findings in our present study by demonstrating that distal cue associations formed to create the allocentric map are reflected by spatial search strategy selection increases during spatial learning. Thus we conclude that search strategy selection can be used to predict spatial memory on the retention probe.

The decision to accept the hypothesis that spatial search strategy selection during spatial learning predicts spatial memory formation is strongly supported by the existing literature using this measure. The hierarchical classification of search strategy has been in the literature for over 15 years, but the use of an objective Matlab algorithm was first pioneered in 2009 (Garthe et al., 2009; Wolfer & Lipp, 2000). Several studies using an earlier form of search strategy analysis found that mutant animals with a reduced incidence of spatial strategy selection during spatial learning have impaired spatial memory (Brody & Holtzman, 2006; Janus, 2004; Lo, Callaerts-Vegh, Nunes, Rodrigues, & D'Hooge, 2013). Studies using the Matlab classification approach adopted in our study are in agreement, having demonstrated that greater use of spatial search strategy selection corresponds with increased spatial memory strength (Gil-Mohapel et al., 2013; Stone et al., 2011). The most comparable study is the latest from the researchers who initially developed the algorithm (Garthe, Roeder, & Kempermann, 2016). They were the first to adopt the regression analysis used here to empirically interrogate the effects of various treatments on search strategy selection. For example, using this approach they found that a significant 30% reduction in spatial search strategy in mice with suppressed adult neurogenesis corresponded to a lack of quadrant preference on the spatial memory retention probe. This is an equivalent effect size to our previous results using the 5-HT_{1A}R KO, where we demonstrated that a similar significant reduction in spatial search strategy selection coincided with a lack of quadrant preference (Rogers et al., 2016). In the present study, the 120% increase in the odds of a spatial search strategy selection corresponded with the restoration of quadrant preference in our WT animals in the high saliency paradigm. As expected the 5-HT_{1A}R KO did not have intact spatial memory in either case due to the reduction in spatial search strategy likelihood we reported during spatial learning. In those studies described above that employed a search strategy analysis, mice were trained on average for 5 days with 5 trials per day, yielding an average of 25

trials being administered during spatial learning. These numbers are close to the training mice underwent in this study (5 training days; 4 trials per day; 20 total trials), which were chosen to avoid overtraining the animals. However, the results in our study may have been more optimal if the mice were trained for an extra day. Additionally, similar to our approach, all of these studies used a measure of quadrant preference to establish the presence of spatial memory formation. The time in target quadrant was compared to chance (Brody & Holtzman, 2006; Gil-Mohapel et al., 2013; Lo, Callaerts-Vegh, Nunes, Rodrigues, & D'Hooge, 2013; Rogers et al., 2016), maps of average position was compared to chance (Garthe et al., 2016; Janus, 2004), or the time in target quadrant was compared to time in the other three quadrants or their average (Garthe et al., 2009; Stone et al., 2011). Taken together, these findings strongly support the major conclusion of our study that spatial search selection learning curves during spatial learning reliably predict spatial memory demonstration on the retention probe.

4.2. The effect of cue saliency on latency to platform and path length

The secondary objective of our spatial learning analysis was to determine the requirement of salient cues for latency to platform or path length to platform learning curves to exist during spatial learning. Using our mixed-effect regression approach, no significant effects of, or interactions with, the quality of the distal cue array were identified for latency to platform and path length. This was despite the fact that a spatial learning curve during training was found in the analysis of both latency to platform and path length when examining the effect of cue saliency. This suggests that conclusions drawn from spatial learning curves using those measures are confounded by elements of the task that are not associated with the acquisition of the distal cues. This is supported by the fact that before the beginning of spatial learning mice in the low cue paradigm underwent cued learning with curtains surrounding the pool to eliminate all distal cues mice could use for navigation. Cued learning taught mice in the low paradigm the basic task requirements that the hidden platform was the escape goal, that escape was found by searching, and that escape occurred away from the pool wall. Having mice acquire these procedural aspects is proposed to be advantageous for beginning spatial learning (Vorhees & Williams, 2006). This experience lowered the average latency to platform in the low saliency paradigm in both genotypes on the first day of training, an effect independent of acquiring the distal cue array. Notably, it did not also result in differences in the percentage of strategies that were spatial. We defined spatial learning as the acquisition of the distal cue array so we are confident that this training only moderately reduces the statistical validity of our comparisons between high versus low saliency spatial learning curves. Furthermore, no statistically significant differences or interactions were identified for both latency to platform or path length with start location. This provides further evidence suggesting that these measures are not reliant on the distal cue array. Taken together, these results suggest that spatial learning curves for latency to platform or path length do not solely measure the acquisition of the distal cue array. Thus these spatial learning curves do not differentiate allocentric from egocentric learning or predict spatial memory formation.

The advantages of the non-traditional statistical analysis we conducted in this study strengthen the validity of these conclusions regarding latency to platform/path length spatial learning curves. Firstly, the mixed modelling approach we employed allows the analysis of outcome measures that are non-continuous (such as search strategy or latency to platform). For instance, being able to analyse latency to platform as a time to event outcome accounts for the situation in which mice do not find the escape instead of entering a blanket value of 60 s in those cases. This strengthens our conclusions as it reduces bias within our modelling in a fashion not available with a traditional RM-ANOVA analysis. Secondly, mixed modelling takes into account multiple potential sources of error inherent to individual responses by including them as random effects. The advantage here is that it can thus account for factors that vary randomly within and/or between individual mice. In complex phenomena like mouse behaviour on the Morris water maze, a large number of important factors may be influencing the outcome measure of interest. A third strength of the mixed modelling approach is that it allows the experimenter to adjust for multiple important factors in one analysis. We can illustrate how advantageous this can be using our path length dataset. Path length is a continuous variable so a traditional RM-ANOVA analysis would have been appropriate. However, choosing this approach we could not have adjusted for how start location and search strategy were influencing the effect of cue on path length. This RM-ANOVA analysis includes day (within-subject) and cue/genotype (between-subject) as factors while the equivalent median clustered regression analysis adjusts for day, cue, and genotype as covariates. A significant effect of cue on path length was identified using both of these equivalent analyses (analyses not shown). Thus, for our path length analysis adjusting for start location and search strategy as additional important factors account for the increased p-value we reported for cue as a covariate. These differences reveal that the significant effect of cue on path length in the RM-ANOVA result can be accounted for by a combination of the effect of start location and search strategy on path length. Thus, for path length as an outcome measure, the mixed modelling can better capture complexity of the mouse behaviour by incorporating these additional important factors into one analysis. All these advantages described above add validity to our conclusions that spatial learning curves for these measures do not dissociate egocentric learning from the allocentric learning the task was designed to measure. An excellent overview describing why this is the case can be found elsewhere (Gallagher et al., 1993). Therefore, we advocate that analysis of the search strategy is the most appropriate way of detecting allocentric learning as Morris originally defined it (Morris, 1984).

Finally, using our regression approach we also aimed to establish the adjusted association between search strategy selection and both latency to platform and path length during spatial learning. In the case of latency to platform, we showed that mice were 115% more likely to escape at any given time if adopting a spatial search strategy across both our saliency conditions. Additionally, we demonstrated that spatial search strategies corresponded with reduced path length, confirming the intuitive conclusion one would draw from the representative search strategy classifications (see Fig. 2). Furthermore, the significant interactions with cue we revealed for both datasets are informative. In the low saliency condition the likelihood of escape with a spatial search strategy was nearly five-fold larger and the relationship between reduced path length and spatial search strategy was present (but not under the high saliency paradigm). These results reflect the relative rarity of spatial search strategies in the low saliency paradigm compared to the high. Taken together, these results illustrate that, if available to an animal based on previous experience, spatial search strategies are reinforced for future trials as mice are less likely to spend superfluous time navigating in the pool to find the escape goal.

5. Conclusions

The Morris water maze was designed to test the hypothesis that the hippocampus creates an allocentric cognitive map. The distal cue array provides the means within which the escape goal location can be mapped as a viewpoint invariant representation within

the hippocampus. The quality of this map is subsequently tested on a retention probe with the escape location removed. Our objective in this study was to determine if spatial search strategy analysis was the most appropriate measure of the formation of this cognitive map by manipulating the quality of the distal cue array. We found that significant increases in spatial search strategy selection occurred only if a highly salient cue array was used and that this predicted spatial memory for the escape location. The differences we uncovered in the likelihoods of search strategy selection amongst our various start locations support these conclusions. In contrast, we demonstrated that spatial learning curves during training existed using latency to platform or path length that were not significantly influenced by the quality of the distal cue array or the start location, and also did not reliably predict spatial memory formation. Furthermore, we also showed that spatial memory, as indicated by absolute time in the target quadrant, was not sensitive to cue saliency or impaired in 5-HT_{1A}R KO mice. Importantly, this suggests that, in the absence of a search strategy analysis, a measure of quadrant preference should be included to establish that control mice have formed an allocentric map to the escape goal location before performing group comparisons of spatial memory strength using absolute time in target quadrant as an outcome measure.

There is a need for increased translational validity of conclusions drawn from preclinical research (Freedman, Cockburn, & Simcoe, 2015). This challenge requires increased sophistication of analysis in order to avoid animal waste and economic expense (Check Hayden, 2014; Perrin, 2014). The utility of adopting this philosophy was illustrated by the mixed-effect regression approach we employed for our spatial learning analysis, that allowed us to adjust for additional factors including start location and search strategy, and thus revealed novel insights. Furthermore, there are currently no mandatory guidelines for reporting either the design or analysis of the Morris water maze. We suggest the exact nature of the distal cue array, the search to target ratio and a measure of quadrant preference must be reported as these are crucial factors determining whether the paradigm is assessing the formation of an allocentric map. From our findings, we strongly advocate that analysis of latency to platform or path length is inappropriate for drawing conclusions of impaired hippocampal functioning without a measure of quadrant preference establishing spatial memory exists. For example, recent datasets that claim impaired hippocampal functioning with evidence of latency to platform spatial learning curve differences without retention probe results (Park et al., 2016) or, more ominously, with absolute time in target quadrant on the retention probe used to establish intact spatial memory (when a measure of quadrant preference would not establish it) (Cha et al., 2016; Meng et al., 2015), should be treated as incomplete and potentially misleading. Finally, the search strategy analysis employed here can be performed in a reasonable time frame with minimal expertise. As we provide strong evidence it crucially indicates whether true hippocampal spatial learning is taking place, it should become a staple part of Morris water maze analyses.

Conflict of interest

The authors have no conflict of interest to report.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2016.12.007.

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