Subject Section

Unbiased classification of spatial strategies in the Barnes maze

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Abstract

Motivation: Spatial learning is one of the most widely studied cognitive domains in neuroscience. The Morris water maze and the Barnes maze are the most commonly used techniques to assess spatial learning and memory in rodents. Despite the fact that these tasks are well-validated paradigms for testing spatial learning abilities, manual categorization of performance into behavioral strategies is subject to individual interpretation, and thus to bias. We have previously described an unbiased machine-learning algorithm to classify spatial strategies in the Morris water maze.
Results: Here we offer a support vector machine (SVM) – based, automated, Barnes-maze unbiased strategy (BUNS) classification algorithm, as well as a cognitive score scale that can be used for memory acquisition, reversal training and probe trials. The BUNS algorithm can greatly benefit Barnes maze users as it provides a standardized method of strategy classification and cognitive scoring scale, which cannot be derived from typical Barnes maze data analysis.
Availability: Freely available on the web at http://okunlab.wix.com/okunlab as a MATLAB application.
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Supplementary information: Supplementary data are available at *Bioinformatics* online.

1 Introduction

While the molecular and genetic tools applied in neuroscience are becoming ever more sophisticated, the most robust tasks that assess cognitive spatial memory have been largely unchanged since the early 1980's. The Morris water maze (MWM), described by Richard Morris (Morris, 1984), and the Barnes maze, described by Carol Barnes (Barnes, 1979), are the most widely used paradigms for assessing spatial learning in rodents (Vorhees and Williams, 2006). We have recently described the MUST-C (Morris water maze Unbiased Strategy Classification) algorithm, which rapidly classifies spatial swim strategies in the MWM and provides an unbiased assessment of the cognitive capacity of rodents (Illouz, et al., 2015). Similar to the MWM, the convenient and robust nature of the Barnes maze has facilitated the investigation of spatial learning in both control and disease states. For example, assessing spatial

learning using the Barnes maze has important implications for many pathologies in which learning is impaired such as aging (Krause, et al., 2008), Alzheimer's disease (AD) (Puzzo, et al., 2014), neurodevelopmental disorders, mental retardation, pharmacological assays to assess drug efficiency and more.

The Barnes maze also has advantages in assessing improvements in spatial capacity, such as following enriched environment (Bonaccorsi, et al., 2013), caloric restriction (Ma, et al., 2014), or exercise (Inoue, et al., 2015). In contrast to the MWM paradigm, the Barnes maze is a dry maze, reducing water-related stress that enables the use of in-vivo devices such as optogenetics. However, assessment of performance in the Barnes maze is susceptible to experimenter bias, calling for a more objective method of data analysis.

The difference between the MWM and Barnes maze tasks, often results in different sets of navigation strategies utilized by rodents (Illouz,

et al., 2015). As a consequence, these differences call for a novel classifier dedicated to the Barnes maze.

In the Barnes maze, rodents are trained to find an escape box hidden under the surface of a circular table by relying on extra-maze cues (O'Leary and Brown, 2012). The high illumination used in this task (1350 lux), causes anxiety in the mice, providing the motivation to find the hidden chamber. Performance in this task relies on hippocampal place cells, validating its use as a task for spatial learning (Bimonte-Nelson, 2015). The efficiency in which mice perform this task is then quantified using specific parameters such as the latency to reach the escape chamber, total distance traveled, mean moving speed, and path efficiency (calculated as the distance between the first and last locations divided by the total distance). Additional attributes are typically used to control for the animal's behavior, such as immobility and mean speed. Both cognitive and non-cognitive traits are evident during spatial navigation, including changes in mean speed, path efficiency, duration, and body rotations. Mice typically exhibit specific navigation strategies when tested in the Barnes maze, reflecting the extent of their spatial acquisition of the environment and thus provide information about the integrity of hippocampus-dependent functions and cognitive state. Based on narrower previous strategy definitions (direct, serial and mixed) (Harrison, et al., 2006) as well as our empirical data, we have defined 6 main navigation strategies for the Barnes maze. This broader range of spatial strategies is described from highly spatial to non-spatial strategies: (a) *direct*, reflects the optimal acquisition of the target's location, as the animal efficiently identifies its location and the target's location and thus uses the shortest trajectory possible (b) *corrected,* reflects a slightly lower efficiency, as the animal makes a minor correction in the trajectory towards the target, and often follows re-angualtion (c) *long-correction* consist of a major correction in the trajectory, as the animal navigates to a distal region of the environment, re-angulates and make a direct movement towards the target (d) *focused search* reflects a localized scanning of the region surrounding the target (e) *serial search* reflects a systematic non-spatial method of enhancing the probability of finding the target, as the animal travels from one hole to the adjacent hole in a serial manner (f) *random search* reflects no acquisition of the target location, as the animal utilizes a non-systematic method of enhancing the probability of finding the target by traveling randomly in the environment. The specific combination of strategies employed by mice in the Barnes maze are considered representative of the level of cognitive resources being used for the task (Bimonte-Nelson, 2015). However, despite thorough assessment of trajectory parameters, non-spatial navigation strategies can increase in efficiency over time, resulting in a decreased latency to reach the escape box. Moreover, simple quantification of latency, mean speed, distance and other parameters describing performance in the Barnes maze often result in only a partial understanding of mouse behavior and cognitive capacity. In order to avoid such erroneous or incomplete interpretation of behavioral data in the Barnes maze, manual assessments of mouse behavior are often required. Manual assessments of spatial strategies are time-consuming, and different experimental conditions alter searching attributes used in the apparatus, hindering cross-experiment analysis of mouse behavior. Moreover, manual assessments are prone to experimenter bias during analysis. Thus, manual assessment fails to reliably detect important aspects of cognitive spatial behavior in the Barnes maze, due to reduced sensitivity to nuances, bias, and environmental factors.

For these reasons, we constructed the Barnes maze unbiased strategy (BUNS) classification tool, an automatic classification algorithm, freely available for the analysis of mouse behavior. This tool uses generic features of the mouse path to enable unbiased analysis of spatial learning strategies, and presents a cognitive scoring scale based on the spatial learning strategy used by the mice. We have compared performance of mice in the Barnes maze in order to validate and assess the sensitivity of our algorithm. . Our data demonstrate that the BUNS algorithm provides unbiased analysis of Barnes maze behavioral data and extends the capacity of researchers to understand the mechanisms of spatial learning and memory in rodents.

2 Methods

Animals. Congenic 8 week old *male* mice (*n*=15) from a C57bl/6 genetic background were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a reversed 12:12hr cycle to enable cognitive assessment during the animals' activity period. Animal care and experimental procedures followed Bar Ilan University guidelines and were approved by the Bar Ilan University Animal Care and Use Committee.

Barnes maze. A circular table 105 cm high with a diameter of 92 cm was used. Eighteen holes are located at the perimeter of the table at equal distances, each with a diameter of 5 cm. One hole only (the target hole) leads to an escape chamber in which the animal can hide. Illumination was measured at the center of the table and maintained at 1350 lux in order to encourage the animal's motivation to search for the target hole. During the habituation phase, which lasted one day, the animal was placed in a cylinder at the center of the maze. Five seconds later, the cylinder was removed and the mouse was allowed to explore the environment for 120 seconds. Mice that found the target hole were able to enter the escape chamber; mice that did not find it within this period of time were placed back in the cylinder, now located above the target hole. In this phase mice were given one trial only.

Four visual cues were presented on the walls surrounding the Barnes table (Supplementary Figure 1). In the spatial acquisition phase, mice were given 120 seconds per trial to find the target hole. Mice that did not find it were placed at the target hole area until they located it. Mice were divided into groups of 4. Each animal was given 2, 3 or 4 trials (separate experiments) with an inter-trial interval of 10 minutes. Thus, all four mice completed a trial before the next trial began. This procedure was repeated daily until no significant improvement in performance was identified. Following spatial acquisition, a probe test was conducted with closed holes and no escape chamber. Animals were given a single 60s trial to explore the environment. Following the probe test, the target hole and escape chamber were moved 180° from the original target location. Similar to the spatial acquisition phase, mice were given 4 non-sequential trials, 120s each, to find the new escape chamber. This procedure was repeated daily until no significant improvement in latency was identified. Latency to reach the escape chamber, total distance travelled and mean speed were automatically calculated by the video tracking system (ANY-maze, Stoelting Co, Wood Dale, IL, USA).

Algorithm

Support vector machine. Support vector machine (SVM) (Boser, et al., 1992; Vapnik, 1998) is a supervised learning classification technique. It has a strong theoretical basis and has been proven to be successful in numerous classification tasks from handwritten digit recognition to bioinformatics (Osuna, et al., 1997; Tong and Koller, 2002). Linear SVM separates a binary-labeled training data set by a hyper plane (decision boundary) that maximizes the margin between the two classes. A test set point is labeled according to its location in the feature space, relative to

the decision boundary. The kernel function used in this study is the radial basis function. We used the MATLAB SVM function (The MathWorks, Inc., Natick, Massachusetts, United States) in this classifier.

Our Training set consists of over 600 labeled trials, which include six possible strategies. Input is generated by video tracking software (ANY-maze, Stoelting Co, Wood Dale, IL, USA) and contains a computer-graphic X, Y coordinate of the animal's center-point or head location according to the user's preference. Animal coordinates are converted into Cartesian coordinates to extract a set of 15 factors characterizing different aspects of the animal trajectory (Supp. Table 1), such as trial duration, total distance, absolute angles and path efficiency. Variables were chosen to characterize the physical properties of the different strategies (time, distance and speed) as well as the spatial performance of the animal (path efficiency, number of circles, distance from perimeter). All data were normalized using the Z-score method (see Supplementary Methods).

Prediction accuracy. Prediction accuracy of the BUNS algorithm was compared to 962 trials that had been manually classified by researchers using confusion matrices.

Multi-class classification. In order to categorize data into multiple classes, we performed a set of hierarchically ordered binary decisions to separate the data into one of several subclasses. To build the suitable tree we first performed an unsupervised binary *k-mean* clustering. This analysis was conducted with 1-5 hierarchical layers (2-16 clusters, Figure 2F). A separation index was calculated for each branch as the percentage of trials belonging to a single strategy divided by the sum of percentages of all other strategies in this branch. The strategy's location was determined according to the maximal separation index obtained (Figure 2F). Based on this unsupervised dendrogram we have designed a similar tree for the SVM-analysis (Figure 2G). As demonstrated, related strategies are neighboring branches, for example, direct and corrected are both highly cognitive strategies and therefore located at the same branch. However, they are also based on the strategy's actual features. For example, although *long correction* is theoretically related to *correction*, it was located near *focused search* due to its high distance. At each decision junction, the relevant features were selected in the same manner. In the first layers, more general features, such as *trial length,* were used to separate the data into *short* or *long* trials. At deeper layers, each decision was made by a different set of finer features relevant to the theoretical basis and actual characteristics of the junction, such as *number of sequential hole visitations* to separate *serial* from *random*.

Cognitive score. We provide a quantification method for performance in the Barnes maze, as each training trial was scored with a numerical value in a non-arbitrary manner. To do so we have applied the well-known unsupervised machine learning method of *k-means* clustering with possible 1-5 layers. The optimal tree was then chosen according to the highest homogeneity of its branches. Next we scaled each strategy according to its location in the dendrogram, that is, the number of junctions from the optimal *direct* strategy (scored 1, Figure 2G): random = 0, serial = 0.25, focused search = 0.5, long correction = 0.5, corrected = 0.75, and direct = 1.

Statistical Analysis. All statistical methods can be found in supplementary information.

3 Results

Spatial learning in the Barnes maze. We developed an SVM-based Barnes maze unbiased strategy classifier (BUNS) algorithm for the classification of spatial strategies in the Barnes maze. In order to assess whether the algorithm detects changes in the navigation strategy of mice, we used BUNS to analyze raw data from three Barnes maze experiments conducted with 2, 3, or 4 non-consecutive trials per day ($n = 15$ per group). Mice tested with 3 and 4 trials per day exhibited a significant reduction in latency to reach the escape box ranging from 72.5±4.1s and 78.3±6.8s respectively on the first day, to 18.5±1.2s, 21.4±2.1s respectively on the last day ($P<0.0001$, Figure 1A). Mice trained with only 2 trials per day exhibited a milder reduction in latency ranging from 66.62±8s to 56.8±7.5s at day 9 ($P<0.0001$, Figure 1A). The distance traveled was also reduced in mice receiving 3 or 4 trials, ranging from 2.95±0.23m and 3.6±0.4m covered to 1.15±0.07m, 1.04±0.1m on the last trial, respectively ($P<0.0001$, Figure 1B). In contrast, mice trained with only 2 trials per day did not exhibit a reduction in the distance traveled (2.74±0.4 m to 2.6±0.38 m, $P<0.0001$, Figure 1B). Path efficiency on the last day of training was higher in mice that received 3 or 4 trials: 0.48±0.03 and 0.54±0.03 compared with 2 trials, 0.32±0.04 on the last day of training ($P<0.001$, Figure 1C). Moreover, mice tested with 3 and 4 trials exhibited a significant enhancement in path efficiency, whereas mice tested with 2 trials did not ($P<0.001$, $P<0.0001$, $P=0.97$, respectively, Figure 1C). Mean speed increased in mice trained with 3 and 4 trials per day, from 0.039±0.002 m/sec, 0.042±0.002 m/sec to 0.049±0.002 m/sec, 0.052±0.002 m/sec on the last day of training ($P<0.05$, Figure 1D). However, mean speed did not differ between groups in mice trained with only 2 trials per day ($P=0.95$, Figure 1D). Thus, 3 trials per day is the minimum number required for successful spatial learning by the mice in the Barnes maze.

Although the conventional measures of latency, distance, path efficiency and mean speed provide a general quantification of the spatial learning process, they are not discriminative enough between all navigation strategies. For example, latency and distance distinguish between direct and random (Figures 2B, C), however they do not sufficiently discriminate between finer differences such as between direct and corrected search, or long correction and focused search, since their distributions overlap in different strategies (Figures 2B, C). Path efficiency provides a better measure of spatial acquisition, as it reflects the efficacy of the animal's navigation strategy based on its trajectory. However, this variable also fails to provide a fine discrimination between direct and corrected strategies, as their distributions partially overlap in different strategies (Figure 2D). Mean speed does not provide any discriminating information, as there is no sufficient variation between strategies (Figure 2E). Nevertheless, the animal's speed does affect the latency to reach the target, and thus provides information about the motor performance of the tested strain. Because the parameters used in the Barnes maze have substantial overlap, manual assessment is often required to accurately differentiate between individual strategies, introducing potential bias in analysis. The BUNS algorithm provides fine discrimination of learning strategies and sets a cognitive scale to enable the researcher to better understand the cognitive state of the subjects.

Spatial strategy classification using the SVM. The BUNS algorithm we employed (Figure 2F) was composed of five binary decision junctions (Figure 2G). This architecture was based on an unsupervised tree obtained by performing *k-means* binary clustering analysis with 1-5

layers of separation (2-16 clusters, Figure 2F). The optimal tree was determined according to the maximal separation indices (Figure 2F). X and Y coordinates were used to generate 15 parameters (Supp. table 1), which were then normalized to Z-score values (see Supplementary algorithm). These factors were fed to the SVM algorithm, which was previously trained using labeled trials (see Figure 2A, Algorithm). The prediction accuracy of the BUNS algorithm for experiments using 2, 3 and 4 trials per day was 93%, 90% and 89%, respectively (Figure 3A). These prediction rates are represented in their respective confusion matrices (Figures 3B-D). In order to assess the classification bias of human judges and the BUNS algorithm, we have compared the classification results of three independent judges and the BUNS algorithm with previously validated human labels. The bias of the human classification is significantly higher than the machine bias (0.5±0.005, 0.2, respectively, $P<0.05$, Supp. Figure 3,4) and the classification standard deviation was 9-times the magnitude of the bias. In addition, the human classification suffers from between-judges differences, as the between-subject standard deviation was 0.53±0.39, the stable machine classification suffers from no such variance ($P<0.0001$, Supp. Figures 3,4).

Using this algorithm, we found that mice tested with 2 trials per day shifted from mostly random search on the first day (69.3±8.4%) to mostly serial search (36±8.7%) on the last day (Figure 4A, Supp. Table 2). Mice tested with 3 trials per day shifted from mostly random (27.2±6.6%) and serial search on day 1 (29.8±6.8%, Figure 4B, Supp. Table 2), to mostly corrected search on day 6 (33.3±7%, Figure 4A, Supp. Table 2). Mice tested with 4 trials per day shifted from mostly random search on day 1 (45±7.4%), to mostly direct (23.3±6.3%) and corrected (38.3±7.2%) search on the last day (Figure 4C, Supp. Table 2). The cognitive level of a particular strategy used by mice can be quantified in order to provide a definitive score for spatial cognitive capacity. In order to accomplish this, we attributed a scoring scale for the different navigation strategies based on their relevance to spatial learning (see the Algorithm section, distance between strategies in Figure 2F), that is, the scaled distance of each strategy in the hierarchical tree from direct swim (Figure 2G), quantified as number of junctions between them. This analysis indicates that mice tested with 2 trials per day obtained significantly lower cognitive scores compared to mice receiving 4 trials ($P<0.05$, Figure 4D). In addition, mice tested with 3 trials per day obtained significantly lower cognitive scores compared to those with 4 trials ($P<0.05$, Figure 4D). This observation is further strengthened when measuring the latency distribution of the mice. While mice trained with 3 and 4 trials per day exhibited a shift in the latency distribution throughout training ($P=2.5*10^{-19}$ and $1.31*10^{-12}$, Supplementary Figure 2A-F), mice trained with 2 trials per day exhibited equal distribution between days ($P=0.53$, Supplementary Figure 2A-I).

To confirm that mice relied on spatial navigation to reach the hidden platform, mice trained with 4 trials per day ($n = 15$) were given a probe trial twenty-four hours following the last training day. All mice exhibited a high preference towards the escape box ($P<0.01$, Figure 5A). Reference memory errors were defined as the first entry into a non-target hole. Additional entries to non-target holes were considered working memory errors. During the probe trial, mice exhibited a higher rate of working memory errors than reference memory errors ($P<0.0001$, Figure 5B). Since reference memory errors were less than the potential maximal value (17 non-target holes) the high number of working memory errors reflects the animal's tendency to repeatedly visit holes near the target, rather than search in more distant holes. Thus, these data confirm the process of spatial navigation in this task.

Similar to the acquisition stage, we again employed the SVM algorithm in analysis of navigation strategies in probe trials. In this SVM variant, we included a binary algorithm with two decision junctions (Figure 6A). As before, X and Y coordinates were used to generate 15 parameters (see Supp. table 1). These parameters were normalized to Z-score values, and then fed to a new SVM algorithm (with the same kernel), which was trained separately with over 120 relevant pre-labeled trials (Figure 6B). The prediction accuracy of the BUNS algorithm in the probe trial analysis was 97.7% (Figure 6C). This prediction accuracy is also represented in a confusion matrix (Figure 6D). Using this algorithm, we found that during probe trials, the navigation strategy of the mice mostly consisted of focused search (57±12%), serial search (14±8.9%) and serial-random search (29±11%) (Figure 6E, Supp. Table 3). To quantify the cognitive capacity of mouse performance, we again assigned a scoring scale for search strategies unique to the probe trials based on their relevance to spatial learning (see the Algorithm section). Mice tested with 4 trials per day obtained a score of 0.68±0.1 (Figure 6F).

If spatial information is not reinforced, spatial memory extinction can result (Rossato et al., 2006). To further demonstrate that the SVM algorithm is capable of detecting spatial memory extinction, we conducted an additional reversal task only in mice tested with 4 trials per day ($n = 15$). Mice exhibited a significant reduction in latency to reach the escape box from 70.1 ±6.4s to 17.7±1.9s ($P<0.0001$, Figure 7A). This was accompanied by a reduction in the distance travelled from 3.1±0.28m to 1.05±0.15m ($P<0.0001$, Figure 7B). Similarly, path efficiency also improved from 0.19±0.02 to 0.54±0.03 ($P<0.0001$, Figure 7C). The mean speed of the mice was significantly lower on the first day compared to subsequent days ($P<0.05$, Figure 7D).

Using the data from the reversal task, we next employed the BUNS analysis to reveal navigation strategies used by the mice during memory extinction of the previous location of the escape box and memory acquisition of the new location of the escape box. Mice shifted from mostly serial (40±6.3%) and random (45±6.4%) search on the first day to mostly direct (20±5.1%) and corrected (31.6±6%) on the last day (Figure 7E, Supp. Table 4). The cognitive score increased significantly from 0.19±0.03 on the first day to 0.6±0.03 on the last day ($P<0.0001$, Figure 7F).

4 Discussion

The BUNS algorithm offers an accurate and unbiased determination of the spatial strategies utilized by mice in the Barnes maze, a widely used task for the assessment of cognitive learning in rodents. Our data confirm that the BUNS algorithm can be effectively used to analyze Barnes Maze data in a reduced-bias and stable fashion. Further, we have created a cognitive score scale that assesses memory acquisition, reversal training and probe trials.

While performance in spatial learning tasks such as the Barnes maze is typically quantified using factors such as latency and path efficiency, these parameters are limited in their ability to illustrate the complexity of the animal's spatial cognitive capacity. In addition, since researchers in different laboratories use different experimental setups and animals from different backgrounds, the ability to quantify the various spatial strategies exhibited by the mice enables investigators to directly compare phenotypic data obtained in different apparatuses. Notably, the BUNS tool enables this analysis to be performed in different variants of the Barnes maze, such as during acquisition, probe trials, reversal, and

memory extinction, which enables one to obtain a complete picture of the spatial cognition phenotype of the mice.

The BUNS tool exhibits sensitivity that exceeds the discrimination obtained by typical parameters such as latency, distance and path efficiency. In fact, when comparing the performance of the mice, which were given 3 trials per day vs. 4 trials per day, no significant difference was observed in either latency, distance or path efficiency. However, the cognitive score derived from the spatial strategy assessment did reveal a higher cognitive score obtained by mice trained with 4 trials per day compared with mice trained with 3 trials per day. Moreover, our findings reveal a limitation in the spatial acquisition of the target hole when animals were given 2 trials per day. Thus, the BUNS algorithm was able to tease out slight differences in navigation strategies that enabled a differential classification and separation of the mice.

Current methodologies and analysis of the Barnes maze, while effective, still neglect to discriminate between important differences in navigation strategy and cognitive ability in mice. The BUNS tool enables efficient, accurate and objective comparison between mouse strains and experimental conditions that was previously unavailable. Overall, the BUNS algorithm offers the research community a powerful tool in assessing spatial learning in rodents.
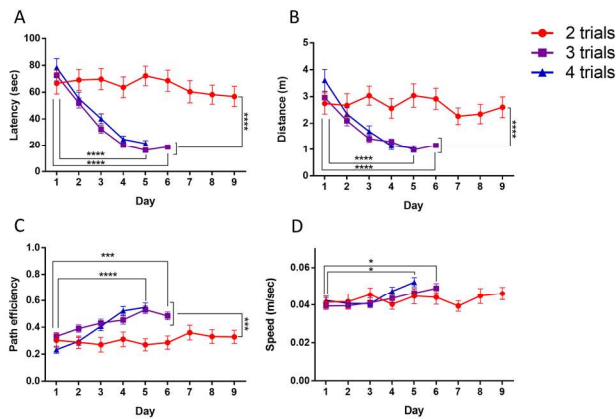
Acknowledgements

Figure 1. Target acquisition in the Barnes maze is dependent on the number of trials. Mice were trained in the Barnes maze until optimal performance was maintained for two consecutive days ($n = 15$ per group), measured by latency to reach the target hole. Mice trained with 2 trials per day exhibited a slower learning curve compared to mice tested with 3 or 4 trials per day in the following parameters: (**A**) latency to reach the target hole, $P<0.0001$ and a time effect for 3,4 trials-per-day $P<0.0001$ (**B**) total distance traveled, $P<0.0001$, time effect for 3,4 trials-per-day $P<0.0001$ and (**C**) path efficiency,

$P<0.001$, time effect for 3,4 trials-per-day $P<0.001$ and $P<0.0001$ accordingly. (**D**) Mean speed increased significantly over time in mice trained with 3 or 4 trials per day ($P<0.05$, Figure 1D) but not with 2 trials per day ($P=0.95$). Two-way RM ANOVA. **** $P<0.0001$, *** $P<0.001$, * $P<0.05$.
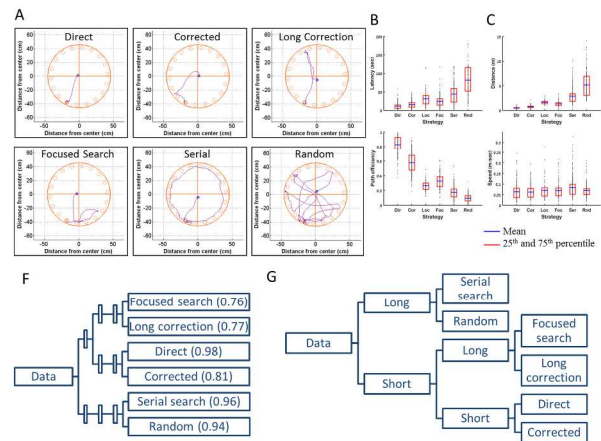

Figure 2. Spatial strategy classification by the BUNS algorithm during task learning. (A) Examples of search strategies employed by mice in the Barnes maze, sorted from highly spatial (top-left) to non-spatial (bottom-right)**:** Direct, Corrected, Long correction, Focused search, Serial search and Random Search. **(B-E)** Typical Barnes maze variables are insufficient to assess mouse performance. Distributions of **(B)** latency, **(C)** distance, **(D)** path efficiency, and **(E)** mean speed by search strategy reveal that these variables are insufficient for a manual linear classification. A vast overlap between strategies is observed, even if only the middle 50% of each variable (in red) is under consideration. **(F)** Binary hierarchical tree derived from an unsupervised analysis (*k-Means*, see the Algorithm section) with optimal separation indices (indicated in parentheses, only relevant branches are presented). (G) Binary choice tree describing decisions taken by the BUNS algorithm, based on the unsupervised tree.
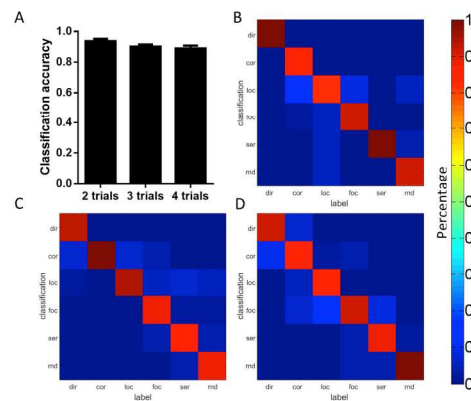

Figure 3. Prediction accuracy of the BUNS algorithm. (A) Percentage of success in spatial strategy classification in Barnes maze experiments conducted with 2, 3, and 4 trials per day. Confusion matrices expand the BUNS algorithm prediction accuracy in Barnes maze experiments conducted with **(B)** 2, **(C)** 3, or **(D)** 4 trials per day.
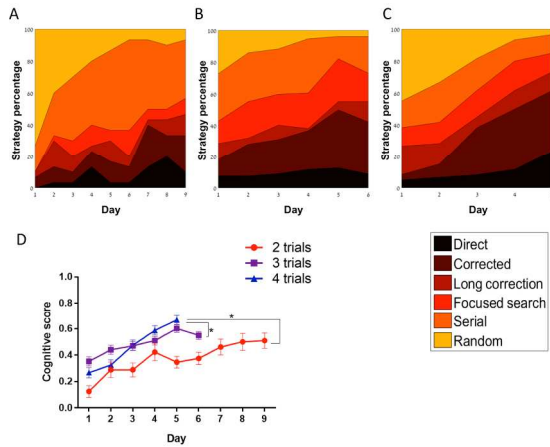
Figure 4. Navigation strategies utilized by mice trained with 2, 3, or 4 trials per day in the Barnes maze. Classification of the spatial strategies was conducted using the BUNS algorithm. The percentage of strategy usage per day (*n* = 15 per group) is shown for (**A**) 2, (**B**) 3 or (**C**) 4 trials per day. Strategies ranged from non-spatial (yellow, random search) to highly spatial (black, direct). (**D**) Cognitive scores for mice tested with 2 trials per day are significantly lower than mice trained with 4 trials per day, *P*<0.05. Cognitive scores for mice tested with 3 trials per day are lower than mice trained with 4 trials per day, *P*<0.05. Two-way ANOVA repeated measures. * *P*<0.05.
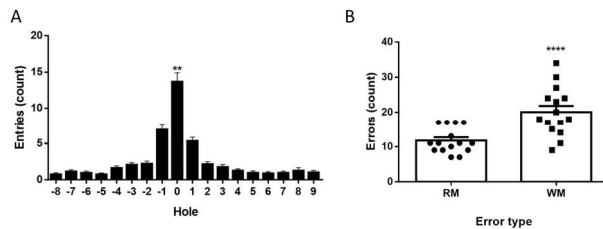

Figure 5. Performance in the probe test reflects memory retention of the target location. Following optimal acquisition of the target location, a probe test was conducted (*n* = 15). Animals were given 60 seconds to explore the environment in the absence of the escape chamber. (**A**) A Gaussian-shaped distribution of entries into the zone surrounding the target hole (marked as zero) implies a strong reference memory of the escape chamber location. (**B**) Working memory errors were higher than reference memory errors which did not reach its maximal value (17, the maximal number of holes). **P<0.01, ****P<0.0001.
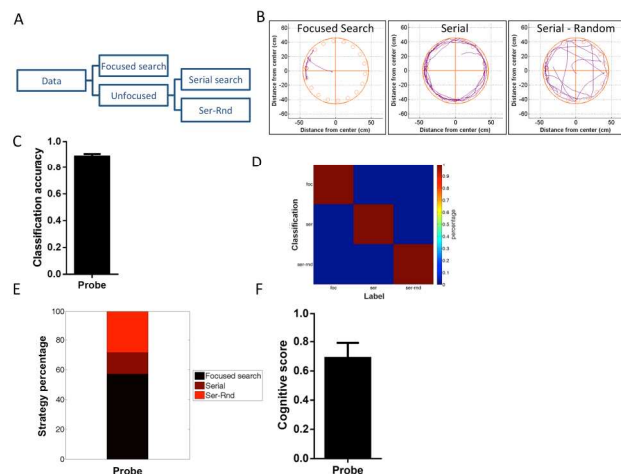

Figure 6. Classification of spatial strategies utilized during the Probe test. (**A**) Binary choice tree denoting binary decisions taken by the BUNS algorithm. (**B**) Examples of navigation strategies employed by mice (*n* = 15) in the probe test of the Barnes maze, sorted from highly spatial (left) to non-spatial (right): Focused search, Serial, and Serial-Random. (**C**) Percentage of success in spatial strategy classification in the probe test. (**D**) Confusion matrices illustrate the BUNS algorithm prediction accuracy in the Barnes maze probe test. (**E**) Percentage of strategy usage in the probe test. Strategies ranged from non-spatial (red, serial-random) to highly spatial (black, focused search). (**F**) Cognitive scores of mice tested in the probe test quantify the different strategies used. One-way ANOVA repeated measures. ** *P*<0.01, **** *P*<0.0001.
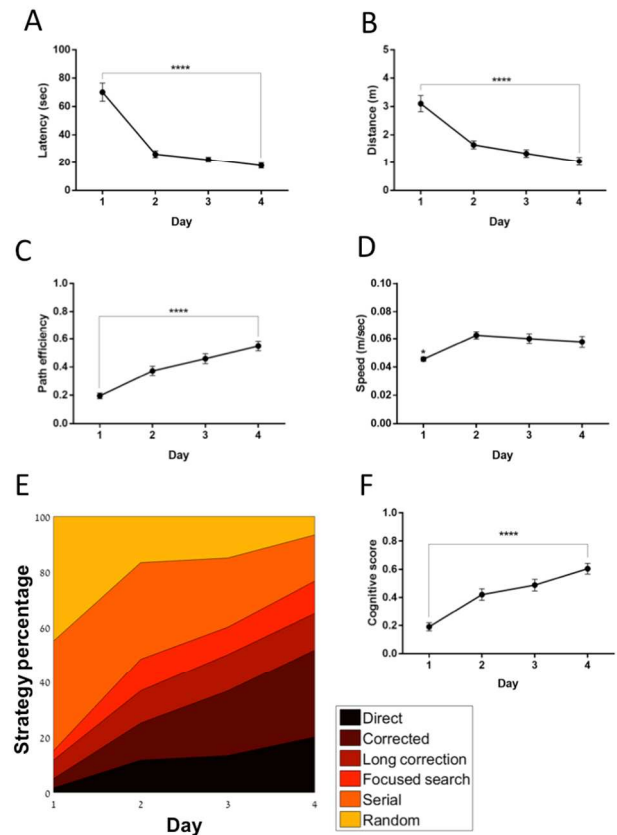

Figure 7. Spatial strategy classification by the BUNS algorithm during reversal acquisition in mice tested with 4 trials per day. Following the acquisition and probe test phases, the target hole was relocated 180 degrees from the original target. Mice (*n* = 15) were trained on the task until optimal performance was maintained for two consecutive days, measured by latency to reach the target hole. The following parameters were measured: (**A**) latency to reach the target hole, *P*<0.0001, (**B**) total distance traveled, *P*<0.0001 (**C**) path efficiency, *P*<0.0001, and (**D**) mean speed *P*<0.05. Classification of the spatial strategies was conducted using the BUNS algorithm. (**E**) Percentage of strategy used each day of the test. The utility of highly spatial strategies increased gradually throughout the course of the reversal task (**F**) Cognitive scores increased significantly throughout the task. Two-way ANOVA repeated measures. * *P*<0.05, **** *P*<0.0001.

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