Development and Validation of a Novel Framework to Study the Neural Basis of Spatial Learning and Navigation in Rodents

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Abstract

The ability of an organism to navigate towards remembered locations requires an internal representation of space. This representation can be referenced against either external cues (landmark-based navigation) or internal vestibular and proprioceptive cues (path integration). While it has been shown that the hippocampus is essential for spatial cognition, the precise neurobiological underpinnings of our ability to navigate to locations stored in spatial memory is not well understood. Recent electrophysiological and behavioural evidence suggests hilar mossy cells may play a functionally significant role in spatial cognition. However, spatial mazes previously described in the literature are confounded by stress or otherwise incompatible with capturing mossy cell involvement in spatial learning. Here I developed the Hidden Food Maze (HFM), in which mice learn to find a food reward in a large circular arena that requires no experimenter handling. I show that the HFM produces reliable learning curves in C57Bl/6 mice and that a robust learning criterion can be established. Notably, I found that mice rely predominantly on path integration when only distal visual cues are available in the HFM task. Preliminary results suggest that intra-maze cues are available, mice use them for their initial orientation and then appear to use path integration after cue identification. For my Master's Thesis, I will describe results from only experiments with distal visual cues. I conclude that the Hidden Food Maze provides a naturalistic assessment of spatial learning in mice, in a format that is amenable for in vivo electrophysiological and imaging approaches from awake and behaving animals and thus provides a powerful and flexible means to investigate the neural bases of navigation.

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List of Abbreviations

CA1	Cornu Ammonis region 1
CA3	Cornu Ammonis region 3
DDmg	magnocellular dorsal pallium
DG	Dentate gyrus
EC	Entorhinal cortex
GCL	Granule cell layer
HFM	Hidden Food Maze
HPC	Hippocampus
IEG	Immediate early gene
IHC	Immunohistochemistry
IN	Interneuron
MC	Mossy cell
MEC	Medial entorhinal cortex
MWM	Morris Water Maze
LEC	Lateral entorhinal cortex
PI	Path-integration
PVC	Polyvinyl chloride (plastic)
REL	Rotationally equivalent location

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1. Introduction

1.1 The Behaviour of Memory & Navigation

Navigation is the ability to maintain a course reliably and flexibly from one known location to another. It is essential to everyday function and may require a mental representation of the spatial relations between identified environmental features. Many mobile organisms possess some degree of navigational skill. Beyond the obvious importance for everyday function, navigational ability is also an important symptom marker as it is known to deteriorate in Alzheimer's disease and dementias (Hort et al., 2007). The importance of navigation in daily life and its clinical significance motivates research on its neural bases and, in particular, how the brain knits together myriad of external and idiothetic (internal) sensory cues to enable coherent navigation.

Navigational tasks can be investigated with respect to orientation mechanisms or spatial cognition. Studying orientation mechanisms is investigating on how an organism finds its directional bearings in an environment utilizing landmarks, vestibular cues, or even geomagnetic fields. The latter line of inquiry asks how these spatial orientations are mentally represented and processed for goal-directed navigation. Behavioural experiments can allow us to discriminate what orientation mechanisms are used, allowing us to investigate the cognitive basis for navigation.

The goal of my thesis is to develop and validate a behavioural platform that tests navigational ability in order to investigate the neural basis of navigation and spatial memory in rodents.

1.1.1 Path Integration vs. Landmark-Based Navigation

Navigation orientation mechanisms can be broadly separated into two categories: path integration and landmark-based navigation, which utilize internal and external cues respectively.

The *Cataglyphis fortis* worker ant travels thousands of times its own body length across the desert each day to search for food. Its path is meandering, but when it reaches a food location, the ant can turn back home following the most direct path. How does the ant know which direction is home? Wehner and Flatt (1972) demonstrated that the ants were not finding their nest based on external landmarks. The ants could only find their way back if they had travelled out themselves, but not when they were

manually moved by the researchers. When the ants were captured and moved away from the nest, the worker ants behaved as though they were lost. Wehner & Flatt concluded that the ants were keeping track of their positions using interoceptive information, or information based on the body's own movements. This is a navigational strategy called path-integration, so-called because it relies on the animal constantly integrating their own movements along their path to keep track of their position.

Path integration, also called dead reckoning, is a system that uses idiothetic (internal) cues as an orientation mechanism, as opposed to external cues in the environment. Cognitively, the animals are continuously storing in memory all their intermediate displacements and rotations with respect to their point of departure (Alyan, 1996).

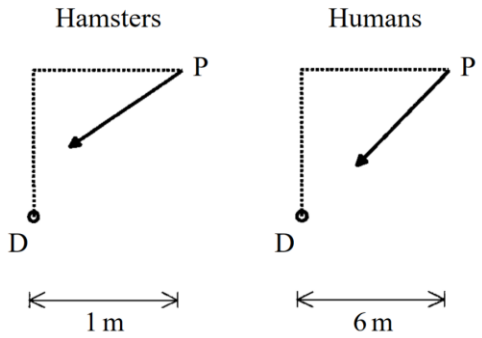


Figure 1. Path Integration. When blindfolded and led on a 2-armed journey from point D to point P (dotted line), both hamsters and humans can turn approximately back to point D (solid line) without external sensory information. Fujita et al., 1993.

An animal relying solely on path integration should be able to navigate in darkness, in the absence of external landmarks, and not require an exact re-tracing of steps (Figure 1). However, animals using path integration should not be able to adapt to changes in their environment if they are not accounted for by the animal's own movements, such as when animals are moved by external forces, or when the external environment shifts. A fundamental feature of path integration is that it is prone to cumulative errors. Self-movement and rotations are not integrated perfectly, so longer paths will be less accurate compared to shorter ones (Etienne et al., 1996).

In contrast to path integration, navigational strategies that do rely on external (allothetic) cues are broadly called **landmark-based navigation**. These landmark cues can be proximal, distal, singular, or multiple.

In landmark-based navigation, the animal's orientation mechanism relies on external landmarks to find its bearings. The landmark might be acting as a beacon the animal can move towards directly, or it may act as a relative reference point in which the animal can compute its position relative to a hidden target in another location. A hallmark feature of landmark-based navigation is disorientation following a disappearance or change of the relevant landmarks' location. Unlike path integration, the accuracy of a navigational route using landmarks should not be affected by the distance travelled. The animals should be able to navigate correctly towards their goal location regardless of their starting point.

It is important to note that path integration and landmark navigation are not opposing systems. The two systems are different but complementary. There is evidence that animals routinely update their path integration according to local landmarks, and the two systems mostly work cooperatively during navigation (Etienne et al., 2004). Some reports suggest idiothetic and allothetic information is integrated to form an allocentric map within the MEC (Wang et al., 2020). Although the two systems are undoubtedly intertwined, it is not well understood exactly where and how they interact and update one another and to what degree.

1.1.2 Interaction of Navigation Strategies

Many species show some ability to utilize both path integration and landmark navigation strategies.

Although predominantly a path-integrator, the *Cataglyphis* desert ant can follow visual guides when its path integration has brought it near the zero position, e.g. when it is very close to home (Wehner, 2003). In this case, the ant can learn to associate particular visual images with motor responses that lead back to the nest opening. Although the exact biological underpinnings of navigation in the desert ant is unknown, the ant does not appear to construct a unified cartographic map using these landmarks in combination with path integration. Instead, it only uses one or the other, switching to path-integration when far from the nest and visual cues when it has arrived close to the nest entrance. In the ant's case, path integration and landmarks are used distinctly and sequentially.

Studies have shown that there is large variation in how landmarks are treated between different species (Rodrigo, 2002). Many vertebrates show a greater degree of interaction between the two strategies. Alyan (1996) showed that landmarks can be used to orient path integration in mice when they are setting out from home. If the mice are passively rotated by 90° while they are in their nesting box, the

mice will not be misdirected. The mice can use landmarks to correctly orient themselves. However, if the mice are rotated at a target location that is away from their nest, they will be misdirected in a way that is consistent with pure path integration. This study suggests that the mice are using local guiding stimuli when initially setting out from a home location but are relying solely on path integration when traveling outside their residence.

Other research shows that path integration in rodents can be reset if the animal is presented with conflicting visual cues even outside the nest. Etienne *et al.* (2004) showed that when presented with visual cues that conflict with their path integration, golden hamsters will favour following visual cues on an outward foraging route, leading them to search for their home in a different location than where they started from. In this case, visual cues dominate over path integration when there is an information mismatch. The hamster's reorientation could be explained by the fact that visual cues in this experiment were made more noticeable due to a flash of light that illuminated them. Hamsters are also more diurnal creatures than mice, and thus visual information might be more relevant in their sensory world (Gattermann et al., 2008). This suggest the reliance on path integration versus landmark cues for navigation is a function of their differing levels of saliency in the particular sensory system and species under study.

Studies show that landmark utilization is influenced by factors such as whether the landmarks are local, distal, geometric, or non-geometric. Mammals tend to rely first on the geometry of an environment (e.g. the shape and three-dimensional boundaries of a space) to re-orient themselves before learning to use other environmental features. Rats and young humans (<2yrs old) use only geometric information when re-orienting themselves (Hermer & Spelke, 1994; Margules & Gallistel, 1988). Rats persistently rely on environment shape to discern their position, even when the location of food reward followed salient non-geometric cues. Further studies suggest this is affected by whether or not the task is appetitive or aversive, and the size of the room, with appetitive tasks and small rooms leading to increased reliance on geometry (Twyman & Newcombe, 2010; Vallortigara et al., 2005).

A review of navigation literature by Knierim & Hamilton (2011) presented evidence that most behavioural results in rats point toward the local frame dominating over distal cues. They suggest that distal cues provide broad directional orientation information while local cues allow for the recording of precise spatial locations. Hamilton *et al.* showed that rats are quicker to treat distal cues as a relative

reference of direction rather than as an absolute cue for location. In further studies with humans navigating in virtual reality, they showed that the ability to directly navigate towards a platform based on distal cues worsened if the boundaries of the local environment were not visible (Hamilton et al., 2009). They proposed that distal cues contributed to initial orientation while proximal contribute to subsequent navigation towards a precise location. It is unclear from these experiments how path integration plays a role in conjunction with distal or local landmarks. Indeed, it seems that the particularities of behavioural setups can interact with an animal's navigational process in diverse ways, influencing how the animal chooses its navigation strategy.

1.2 Hippocampus – The physiological basis for navigation

Tolman (1948) hypothesized that idiothetic and allothetic sensory cues come together to form a coherent navigational representation of space through the means of a cognitive map. A cognitive map includes a representation of the spatial relations of features in the organism's environment independent of its own location. According to cognitive map theory, organisms form a spatial framework in which elements such as stable objects and the content and time of occurrence of discrete events can be associated to create a coherent internal representation of external reality. This representation can then be used to plan future actions, including navigating with respect to expected rewards or threats. This theory is supported by experiments that showed that rats, when given the opportunity, will take novel shortcuts rather than following an established trained path (Hsiao, 1929). This theory also counters hypotheses that animal navigation is solely stimulus-response learning.

The discovery of spatially tuned neurons in the hippocampus gave strong support to the theory of a cognitive map. These neurons, so-called "place cells", have spatially-selective firing properties that increased firing frequency when the animal enters a particular location (O'Keefe & Dostrovsky, 1971). The hippocampus is the primary brain region associated with formation and storage of new memories, as well as the control of flexible planning in goal-oriented navigation. Ablation of the hippocampus in rodents leads to chance performance in navigational tasks while heightened activity in hippocampal granule cells is associated with improved performance in the Morris Water Maze and other spatial navigation tasks (Carter et al., 2015; de Bruin et al., 2001). There is therefore strong causal evidence that the hippocampus is essential for carrying out navigation.

Beyond place cells, there are a myriad of other spatially sensitive cell types that are implicated in spatial cognition. Grid cells were discovered in the medial entorhinal cortex (MEC) that generate a spatial signal for place cells within the hippocampus (Hafting et al., 2005). Much like place cells, grid cells also fire in specific spatial fields of an environment, but unlike place cells, grid cells have multiple fields of sensitivity that are arranged in a hexagonal grid-like pattern.

Within the entorhinal cortex, a host of other spatially tuned cell types continue to be discovered. Boundary cells were found in the MEC. These cells fired when the animal was near the perimeter of an environment (Solstad et al., 2008). Object cells were found in the lateral entorhinal cortex (LEC) and fire if an object is present in specific spatial locations (Tsao et al., 2013). The MEC and the LEC represent the where/what pathway that dissociate context information from content information. It has been hypothesized that MEC is responsible for path integration and landmarks, while LEC is responsible for the content of experiences (Knierim & Hamilton, 2011; Wang et al., 2020). Distinctive features of space are encoded by different cell types, and the properties of how these cell types respond dynamically to environmental changes can reveal much about how the hippocampus computes spatial signals.

1.2.1 Gymnotiform fish experiments suggest mossy cells are involved in navigation

Previous research in Dr. Leonard Maler's lab studied a brain region called the magnocellular component of the dorsal pallium (DDmg) in electric gymnotiform fish and presented evidence that it is homologous to the hilar mossy cells in mammal hippocampus (Figure 2). Their research has demonstrated that electric Gymnotiform fish are capable of learning a spatial navigation task that requires them to remember to location of a food reward in a large circular pool. They show the fish learn to rely on local landmarks placed within the pool, with search performance decreasing if the landmarks are removed (Jun et al., 2016).

The study shows neurons in the fish DDmg connect to putative homologs of CA3 hippocampal field and form a recurrent loop with the dentate gyrus (DG) (Elliott et al., 2017). DDmg appears to become active when electric fish are presented with spatial and social novelty in a behavioural learning task, but activity is greatly reduced when fish habituate to the stimuli. Specifically, immediate early gene (IEG) activity increased in the DDmg when landmark cues are moved following training. Similar results have been reported for mossy cells (Bernstein et al., 2019). From this research, Elliot *et al*. hypothesized that

mossy cells also function in a similar way in mammals, and implement pattern separation of objects, including landmarks used for allocentric spatial learning.

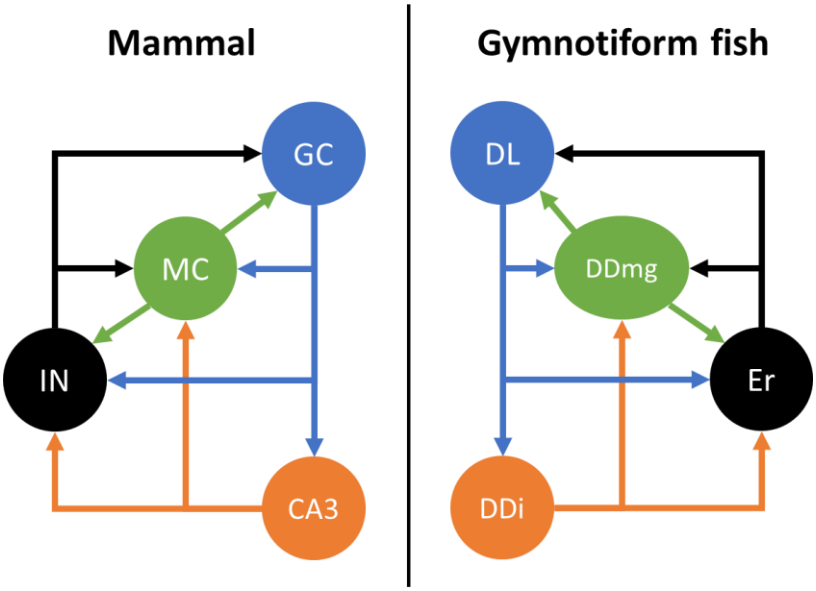


Figure 2. Hypothesized homologous fish and mammal hippocampal circuits. The mammalian mossy cells (MC) are hypothesized to be homologous to the fish DDmg. Both cells share similar circuit connections and they are hypothesized to share similar functions in pattern separation.

1.2.2 Mossy Cells

Hippocampal mossy cells are neurons located in the hilus of the dentate gyrus. They are glutamatergic, receive input from DG granule cells and then feedback to the DG inner molecular layer where they contact granule cells and interneurons (Sun et al., 2017). Mossy cells have large and complex proximal spines (thorny excrescences), and possess several defining electrophysiological characteristics that distinguish them from neighbouring GABA interneurons and CA3 pyramidal cells (Scharfman & Myers, 2013).

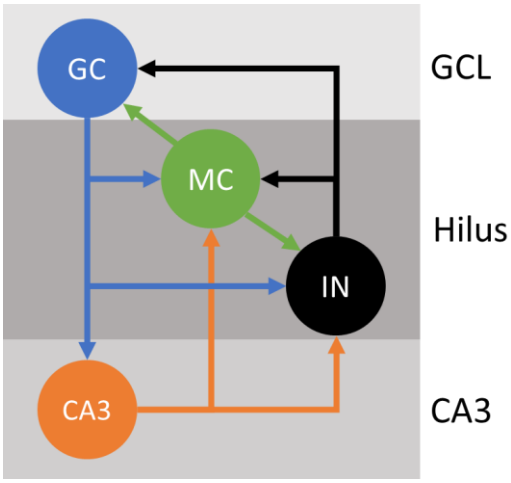


Figure 3. Mossy Cell Circuitry. Mossy cells (MC) receive inputs from granule cells (GC), CA3, and hilus interneurons (IN). MCs output back to interneurons and GC, creating a feedback loop.

Mossy cells have a low firing threshold, high spontaneous activity, and are vulnerable to excitotoxicity. Their loss is implicated in temporal lobe epilepsy and degradation of memory function (Magloczky & Freund, 1993; Scharfman & Myers, 2013). The function of mossy cells is not clear and there is ongoing investigation into whether mossy cells contribute to spatial learning and pattern separation/completion.

In vivo recordings have shown that mossy cells have robust place cell properties that are not simply inherited from the upstream granule cells (Danielson et al., 2017). Unlike granule cells in the dentate gyrus which generate place fields sparingly in a given enclosure, each mossy cell often has multiple place fields in the same enclosure (GoodSmith et al., 2019). Mossy cells remap easier than granule cells in response to changes in local cues (Senzai & Buzsáki, 2017). The computations performed by the promiscuous remapping properties and multiple place fields of mossy cells combined with the sparse, steady place fields of granule cells are completely unknown.

Recent electrophysiological experiments in freely moving rats show that mossy cells are sensitive to local landmark locations. MC place fields respond maximally near local cues and the fields remap to follow local cues rotations (GoodSmith et al., 2019; Jung et al., 2019). Due to this compelling electrophysiological evidence of spatial coding in mossy cells, they are likely contributing to spatial memory and perhaps to the pattern separation computations required for remembering individual landmarks(GoodSmith et al., 2019; Jinde et al., 2012). This raises a critical question: what is the activity of mossy cells as an animal learns to navigate in a new environment, and how is their activity related to the allothetic and idiothetic aspects of successful navigation?

1.3 Laboratory Tests of Navigation

In order to study the contribution of mossy cells to spatial memory and navigation, a behavioural task is needed that can target and quantify navigational behaviour.

There are many pre-existing spatial tasks. The radial arm maze is one of the earliest tasks. It is an 8-armed apparatus that has food baited in some arms. The location of the food remained in a fixed spatial relationship relative to some distal cues. However, this task runs into a problem of being solvable using working memory rather than spatial reference memory by the animal remembering which arms it has previously visited (Olton & Samuelson, 1976).

An answer to the radial arm maze is the Morris Water Maze, which eliminates the working memory component. The MWM is probably the most famous spatial memory task. In this task, rodents are trained to find a hidden platform submerged in a circular pool of opaque water. The platform remains in a fixed position relative to distal cues outside the pool. There are no cues within the pool that offer any orientation information. General consensus among researchers has been that the rodents learn to navigate in this task based on the relationship of distal cues to the escape platform (Rogers et al., 2017). It is noteworthy that the MWM relies on escaping from an aversive situation – the mouse is forced to continue swimming until it finds the hidden platform.

However, there are several problems with the MWM relating to the study of navigation and these are connected to the aversive stimuli associated with immersion in water.
There is evidence that the MWM causes stress, exhaustion, hypothermia, and floating behaviour (Sankowski et al., 2019). The task was originally designed for rats and later adapted for mice. Compared to rats, mice show more variability in performance. There is also large variability in performance between different strains of mice. For the highly excitable mossy cells, stress in the MWM presents a serious obstacle. Although, some argue that the MWM is not significantly more stressful than food deprivation (Vorhees & Williams, 2014). However, different sex responses to stress has been seen affecting performance in the MWM. The MWM usually shows male rodents performing better than females. However, less stressful variations of the task producing differing results, suggesting it may be a factor of stress rather than sex (Sankowski et al., 2019).

Although it was long thought that animals were solving the MWM based on learning their target's position relative to distal cues, modifications of the task show that rats learn to navigate to locations based on the local apparatus reference frame, with the distal cues only providing directional information and not information for specific locations. This was demonstrated after rodents had learned to find a target based on a landmark, the entire maze was moved closer to the landmark. Instead of shifting their search location based on the closer landmark, the animals continued searching in the same relative location, showing they only registered directional information from the landmark rather than specific distance information (Hamilton et al., 2008).

Most obviously, a critical fundamental limitation of the MWM is the aquatic nature of the task which makes compatibility with electrophysiology equipment difficult. A similar dry task is the Barnes Maze, where the mice is placed in a circular open field and they must find the location of an escape hole. The escape hole maintains a fixed relationship with distal cues in the room. Like the MWM, the Barnes Maze is motivated by aversion to escape aversive stimuli in the arena – water and open spaces respectively. The Barnes Maze is a dry task, which is more naturalistic compared with the MWM. However, a problem with the Barnes Maze is that it can be solved by serial search. Solutions are always on the perimeter of the arena, so the task cannot distinguish between whether the mice are deriving specific location information from distal landmarks or merely compass direction. It is technically not a test of cognitive mapping, rather it can be solved purely by directional association.

Ideally, a navigation task that test the cognitive map theory should require the animal to locate a specific place in the arena rather than a direction. There are some land-based foraging tasks that exist, namely the oasis maze, cheese board task, and variations of the buried food task (Clark et al., 2005; Ariane S. Etienne et al., 2004; Kesner et al., 1991). They are not nearly as standardized as the above-mentioned MWM and Barnes Maze and often are highly customized to address particular experimental questions. Other navigation tasks that allow for electrophysiological recording often require the animal to be head-fixed, which interferes with the natural contribution of vestibular cues (Chen et al., 2018; Jung et al., 2019; Richardson et al., 2011). For that purpose, we have decided to create our own foraging task that is inspired by elements of existing tasks but is adapted for mice and minimize stress.

An ideal spatial learning and navigation task has several required design parameters:

1) The maze must be a test of navigational ability that is hippocampal-dependent. This means the test should not be able to be solved through merely following odour trails or using stimulus-response learning.
2) The task should be compatible with mice that have head recording devices for electrophysiology or imaging experiments.
3) The experimental conditions of the task should resemble the earlier fish experiments by Jun et al (2016) and Fotowat et al (2019) in order to better draw comparisons as to the operation of similar neural circuits (Fotowat et al., 2013, 2019; Jun et al., 2016).
4) The task should minimize stress, such as experimenter handling and aversive stimuli, in order to maximise the signal to noise ratio of the highly excitable mossy cells. The task should use a rewarding stimulus to identify an otherwise hidden location - reward based learning should minimize stress.

2. Objective

The objective of my thesis is to develop a behavioural task that can study the neural basis of learning and navigation. I hypothesize that mossy cells play a behavioural role in navigation and, specifically, in pattern separation of landmarks and spatial memory.

The goals of my MSc project include:

1) Designing a navigational task that is minimally stressful and relies on spatial cognition
2) Validating that the behavioural task can be learned by mice
3) Investigating what orientation mechanisms control task performance and which navigation strategies are employed to solve the spatial task

3. Methods

3.1 Animals

All animals were housed in the University of Ottawa Animal Care and Veterinary Services facility. C57Bl/6 wild-type male mice were ordered from Charles River, arriving at 8-9 weeks old. Mice were individually housed in 12h light/12h dark cycles (lights on at 2:00PM EST, lights off at 2:00AM EST). Animals had had food and water available *ad libitum*. The temperature of the room was kept at 22.5°C

and the humidity was 40%. Mice were habituated in ACVS facilities for 1 week and began testing when they were 10-11 weeks old. Testing of each cohort took place over the course of approximately 23 days, 1-2 hours after the lights turned off (3-4PM).

All animal procedures were conducted with the approval of the University of Ottawa's Animal Care Committee and in accordance with guidelines set out by the Canadian Council of Animal Care.

3.2 Apparatus: The Hidden Food Maze

3.2.1 Maze Design

The Hidden Food Maze (HFM) is a framework that trains mice to search for a food reward hidden in an open, circular arena. The protocol for the task was inspired by the Cheese Board spatial task in which rats are placed inside an arena with many holes in the ground, one of which contains a food reward (Kesner et al., 1991). Unlike the task by Kesner *et al*. (1991), the HFM does not require the animal to be handled, and the pattern of holes is arbitrary rather than grid-like. This task design was also chosen because it mirrors the setup of the Morris Water Maze (MWM), which can test allocentric navigation separate from response learning. Like the MWM, mice are searching for a target location in a circular environment based on distal cues from one of four starting locations. In contrast to the MWM, this new task is a dry maze that is food motivated, which is more naturalistic and less stressful. The following describes how the maze design addresses each of the required parameters.

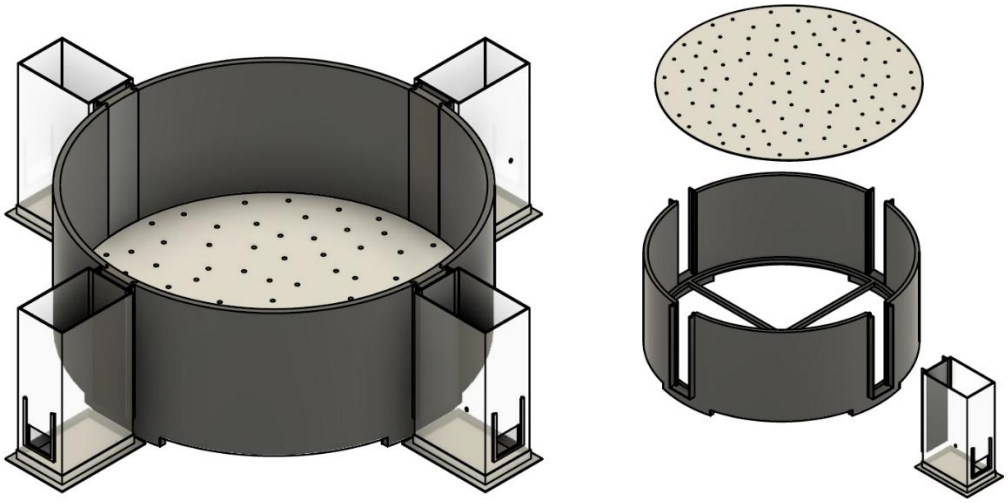


Figure 4. Hidden Food Maze Design. Fully assembled (left) and separated into components (right). The maze includes the main black ring, the removable floor, and 4 removable home cages that are separated from the arena with a sliding door. The floor of the maze is 120cm in diameter, and walls are 45cm tall. The home cage has a 10.5 x 6.5 cm floor area.

Parameter 1: The task is a hippocampal-dependent test of spatial navigation

The maze has a removable floor that can be washed & rotated between trials to eliminate odor trails that mice from previous trials might have left behind. The pattern of the holes is rotationally symmetrical, so the pattern looks the same regardless if the floor is rotated 90°, 180°, or 270° with respect to the mouse's entrance. This ensures that the mouse will have the same initial view regardless of which entrance it starts from and how the floor is rotated. It also eliminates any potential direction cues it may derive from the floor such as odours or scratches. The holes in the floor are encircled by a downward facing rib so it cannot be easily seen whether the hole is open, or has been closed by a food containing insert, unless the mouse stood directly over the hole. Thus, the mice will not be able to discern the contents of the hole just by looking across the floor from their home, but instead will need to approach the hole and look inside.

The design of the maze itself is completely round and uniform from the inside so as not to provide any directional cues. The floor of the arena is encircled by tall black walls. The walls are symmetrical and designed to eliminate geometric cues that would give away directional information from asymmetries in the environment shape. The arena is resting on a subfloor that contains food dust. The holes of the maze are open to the bottom, allowing food powder to be distributed beneath the maze to saturate the maze with food odours. This reduces the odor cue from the food in the target location, so mice cannot rely on odour as a search strategy.

In order to eliminate the home entrance as a geographic cue, hallway blinders can be installed in the passageway so the mouse cannot see which entrance is open when inside the arena. These hallway blinders are two walls that form an S-shaped hallway, so the mice can still freely travel between the arena and the home cage, but the opening of the home cage is obscured. These hallway blinders were not equipped during the experiments described in this thesis.

Only distal landmarks are available - these are visual cues that are affixed to the walls, encouraging the mice to pay attention to landmarks and perform allocentric navigation. These design features carefully control for directional information so the mouse can rely only on the cues the experimenter provide.

Parameter 2: The task should be compatible with electrophysiology equipment

Implanted recording electrodes often require a wired connection on the top of the mouse's head. All components of this task are open at the top, including the hallway blinders, which allows it to be compatible with head wires. The walls tall enough so that no mice can jump high enough to escape, eliminating the necessity for a ceiling. The junction between the walls and the door have been smoothed to not catch and tangle any wires.

The open ceiling also conveniently eliminates the need for a ventilation device for the home cages.

Parameter 3: The task should resemble earlier fish experiments

Like the fish behavioural experiments by Jun et al. (2016), this task takes place in a round arena where the subject is housed in behind one of the entrances. Both tasks are appetitive tasks that has the animal search for a hidden food reward in a circular arena using spatial memory.

In previous experiments, fish were allowed to remain in the tank following the task in order to investigate IEG activity through immunohistochemistry staining. Allowing them to remain undisturbed minimized noise in the cell staining. Similarly, because the main testing arena is connected to the home cage during the trial, the mice can remain unperturbed after the trial ends for an indefinite amount of time because it has free access to the home cage. This allows for IEG expression with low background noise, allowing us to investigate cellular activity using IHC staining if we choose, allowing us to compare results.

Parameter 4: The task should minimize stress

The task is food motivated. As opposed to the Morris Water Maze or the Barnes Maze where the motivation is to escape aversive stimuli – swimming & open space respectively – the hidden food maze is motivated by the positive stimuli of a food reward.

The mice are food restricted, which may function as a stressor, however the willingness of mice to explore open space suggests the mice are not stressed. Time spent in open space, as well as rearing behaviour are indicative of decreased anxiety (Sturman et al., 2018).

Mice can perform the entirety of the trial without experimenter handling. The maze doors are designed to slide upwards and provide access to the main arena. When the trial is finished, the experimenter can slide the doors back into place and the mouse is confined to its home cage once again.

When moving mice to a different starting quadrant, the home cages are designed to slide out from the maze and into a new entrance so the mice do not need to be picked up. The home cages are designed to include their own doors, separate from the doors that provide entry into the maze, so the home cage can be freely moved and the mice are still securely confined. The detachable home cages allows the mice to be moved to different starting locations, allowing us to control against navigational strategies that rely on response learning or path-integration.

3.2.2 Experimental Setup

Behaviour Apparatus Details:

The arena's circular floor is 120cm in diameter and made of white PVC plastic. The floor has 100 holes (1.2cm diameter) arbitrarily dispersed throughout the surface, with 25 holes in each quadrant. The size of the hole was determined experimentally to be smaller than an adult mouse can fit through. Each hole is encircled by a 1cm plastic rib, sticking downwards, which can be covered with a cap in order to hold food but remains invisible from the surface. The surface of the floor is sanded to be matte to avoid generating reflections from the lights which might interfere with the cameras or distract the mice.

The walls are made of solid black PVC plastic which forms a cylinder around the maze and is open at the top. The walls are 1cm thick and 45cm tall, which is tall enough so the mice cannot jump out. There are crossbeams and a ridge that runs around the interior of the cylinder which the floor rests on, holding the floor above the subfloor.

The home cages are 27cm x 16.5cm x 45cm and they are open at the top. The home cages contain a food hopper and a water bottle feeder. The dimensions of the home cage are based on commercial mouse cages and comply with the Canadian Council on Animal Care mouse housing standards.

The maze and home cages are raised 7cm from the ground, resting on a wooden pallet that has been painted black. The black colour was chosen to maximally eliminate all visual cues just in case the mouse could look down underneath the floor through the holes.

The maze was designed using Autodesk Fusion 360, a 3D modeling program. The physical maze was built by Canus Plastics Inc. from our designs.

Lighting:

Four LED flashlights were aimed at a white ceiling in order to create dim, diffuse lighting throughout the maze. The illuminance is measured to be 50 lux at the surface of the arena.

Video Recording:

The recording camera is an ELP 2MP USB webcam with infrared lighting. Videos were recorded at 1920 x 1080 resolution and analyzed in Ethovision. Videos were recorded on AMCap webcam recording software. Tracking was done with Ethovision XT12 (Noldus) based on contrast detection. Mice were tracked according to 3 body points at 30 frames per second.

3.2.3 Behavioural Protocol

Pre-training Habituation:

5 days before training, mice are transferred from standard animal facility cages to the experimental home cages attached to the Hidden Food Task. Mice arrive to the facility singly housed and continue to be individually housed throughout.

Animals are food restricted, with 10% of their body weight in food given back each day and which they could consume *ad libitum*. Water is freely available throughout the experiment. Based on experimentation, animals are maintained at 80% of their original body weight.

On the first two days, mice are allowed to habituate to their new home cages and food restriction is started. On day 3, the door dividing the home cage and the arena is removed and each mouse is given free access to explore the arena for 15 minutes. 50% of the holes in the arena are filled with food treats (a piece of Cheerio), and each mouse is given 10 minutes to forage for food. This is repeated on day 4, where 25% of the arena's holes are filled with food treats. On day 5, only 4 holes contain treats, 1 in each quadrant. Mice pass the pre-training stage when they successfully find treats from all four locations. Training in the spatial learning task then commences.

Spatial Training:

One of the holes in the area is capped from the bottom with a food-containing insert that is not visible from the surface. Extra-maze landmarks (described in the Results section) are placed on the walls of the maze to serve as location cues. The HFM is compatible with intra-maze cues that can be slotted into the

holes of the arena, but those experiments will not be described in this thesis. At the start of the trial, the door is removed, and the mice are given a maximum of 15 minutes to find the target hole. The mice have free access to return to their home cage during the trial. The door is re-inserted after the mouse has found the food reward and returned home. If the mouse has not returned home by itself after 1 minute of finishing the food reward, it is gently guided back by the experimenter. The mice are guided back by holding the removable doors behind the mice, which prompt them to run back to their home cage. If it did not find the food after 15 minutes, the mouse is guided towards the food location and allowed to return to its home cage. There is an inter-trial interval of approximately 20 minutes. The trial setup differed between experiments, as described below.

Experiment 1: Changing Entrances

In the first experiment, the protocol mimics classic Morris Water Maze setups to test allocentric landmark-based learning. The mice are trained to locate a food reward that has a fixed relationship with 4 visual cues on the walls (Figure 5). Each mouse enters the arena from one of four entrances (randomly chosen). The entrance changes every day.

There are 4 mice per cohort. All of them are living individually in the home cages and have followed the habituation protocol described in the methods above.

During the spatial training phase, mice are given 2 trials per day. The location of the target hole containing the food is the same for every mouse. The floor is washed and rotated between each trial. The home cage location does not change between trials within a day. At the end of the day, the home cage is arbitrarily moved to a new entrance location. This allows the mice to habituate to the new location overnight.

If the mouse can learn to use the visual cues, it is expected that the mice will get faster at finding the food over time, showing a learning curve that gets faster as the number of trials increase. We would also expect a place preference for the target quadrant during probe trials. If the mouse cannot use visual cues but can use path integration, we would expect to see an improvement on the second trial of each day, when their entrance to the arena did not change. However, we would not see an overall decrease in the learning curve across days. If the mouse is unable to learn the spatial location using either visual cues or path integration, we would expect to not see any improvement in performance over trials.

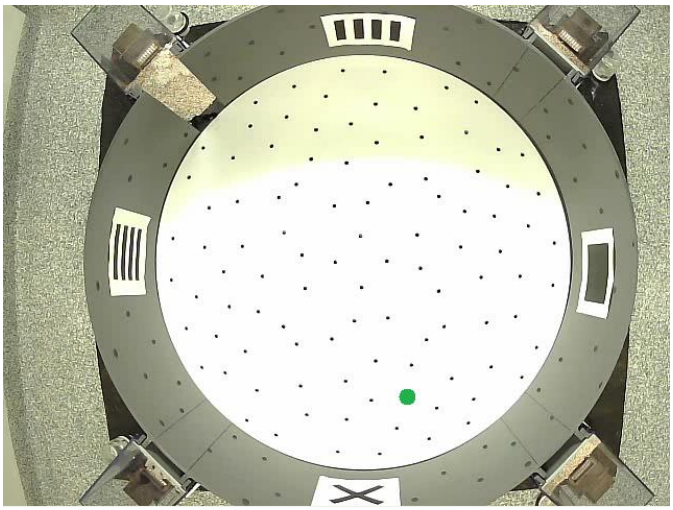


Figure 5. Visual Landmarks. Four large black shapes on a white background (cross, square, vertical lines, and horizontal lines) are taped on the walls. The green dot indicates the location of the target hole.

Experiment 2: Same Entrances

This experiment tests the ability of mice to navigate using visual cues in cooperation with path integration. In this experiment, the protocol is the same as Experiment 1 except the mice are not moved to different starting locations between trials. The static entrances allow the mice to potentially rely on both landmarks and path integration to remember the location of the food reward. This protocol mimics earlier fish experiments.

The same 4 visual cues as described in Experiment 1 were fixed to the walls. This experiment was carried out with a new cohort of 4 mice. In some iterations of this experiment, the location of the food reward was different for different mice in order to ensure the training was not due to imperceptible cues left behind by a previous mouse. Nevertheless, the location of food does not change between trials for the same mouse.

If path integration is contributing to navigation, we would expect to see a difference between the learning curve of Experiment 1 and Experiment 2.

Experiment 3: Rotations

In this condition, after mice have been trained to remember the target location from the Experiment 2 protocol, their home cages were rotated to another quadrant and tested on how well they found the food from a new starting location. The first rotation was 180° from the initial position, followed by a 90°

rotation and another 180° rotation so the mouse ends up at each of the 4 entrances. The home cages were rotated to a new quadrant every 3 days. Mice were given 2 trials a day, with 6 trials at each new entrance location.

The results of this experiment should reflect whether the mice learned to find the target hole according to its relationship with visual cues or whether path integration dominates. If visual cues dominate, mice should correctly find the target following a rotation because the relationship between the target hole and the visual cues does not change. If mice are relying on path integration to find the target hole, the mice should not compensate for the rotation and search in the rotationally equivalent hole from the target.

In a separate cohort, a probe trial was given after 2 rotations in order to investigate whether mice had formed a quadrant preference despite the rotations.

Experiment 4: No Landmarks

This experiment tests whether the distal visual cues make a difference in the learning curve of mice during rotations. In this experiment, all visual cues are removed from the walls during rotation and subsequent training. The setup is identical to the protocol in Experiment 3.

If the mice were relying on the provided visual cues, we would expect that results would differ in the learning curve performance between cohorts with landmarks (Experiment 3) and without landmarks (Experiment 4).

Experiment 5: Dark Trials

How well do mice perform this task in complete darkness, in the absence of all visual cues? This experiment is meant to control for the effects of other visual cues besides the 4 cues on the wall.
In this condition, the mice go through standard training as per Experiment 2, then the lights are turned off prior to the doors being opened and the mice perform trials in the dark. The lights are opened in between trials so the mice do not acclimatize to the darkness. The camera tracks the mice using infrared lighting. The following protocols were tested: darkness during a regular trial with food present in the arena, darkness following a rotation of the home entrance, and darkness in a probe trial where there is

no food present in the arena. This condition is meant to help identify the contribution of vision to mice navigation in this task.

3.3 Data Analysis and Statistical Tests

Path tracking analysis was conducted within the 120cm diameter circle of the arena. This zone excludes the walls of the arena from analysis.
Latency to target was calculated from when the nose point of the mouse enters the arena until the nose of the mouse enters the target hole.
Number of holes searched was counted based on when the nose point of the mouse poked into a hole and the mouse either paused or changed direction immediately afterwards. This is done to minimize false positives of the mouse merely running past a hole without searching it.
Distance, time, and number of holes counted were pooled for the entire cohort (N=4), unless otherwise noted. Speed and distance were measured from the center point of the mouse body.

It was necessary to exclude certain outlier trials. If a mouse became sick or exhibited unusual stress behaviour, it was eliminated from the statistical calculation. A mouse was deemed unusually stressed if there are more than 3 fecal boluses and multiple urine puddles found in the arena following exploration, or there is unusual exploration behaviour such as the mouse not searching for food. In total, one mouse was not included in Experiment 5's results.

Significance testing was conducted in a manner that was appropriate for each dataset. Paired comparisons utilized the t-test for parametric data and the Wilcoxon signed-rank test for non-parametric data. The significance cut-off was set at 0.05. All statistics were calculated using R: A language and environment for statistical computing. URL http://www.R-project.org/.

4. Results

Experiment 1: Changing entrances

During training when entrances were changed every day, mice did not show a clear learning curve in terms of latency to target. Average time to reach the target for the first 10 trials = 115.47 seconds (SD=135.99) and the average time in the last 10 trials = 105.14 seconds (SD=114.74). The average

latency to target across all the trials is 135.40s (SD=156.78). There is not a significant difference in performance between the first 10 and the last 10 trials (*N* = 40 trials, *P* = .847, *P* value not significant, Wilcoxon Signed-Rank Test). The number of holes searched significantly decreases when comparing the first 10 trials (M=41.4 holes, SD=46.7) and the last 10 trials (M=10.3 holes, SD=11.1) (*N* = 40 trials, *P* < .001, Wilcoxon Signed-Rank Test).

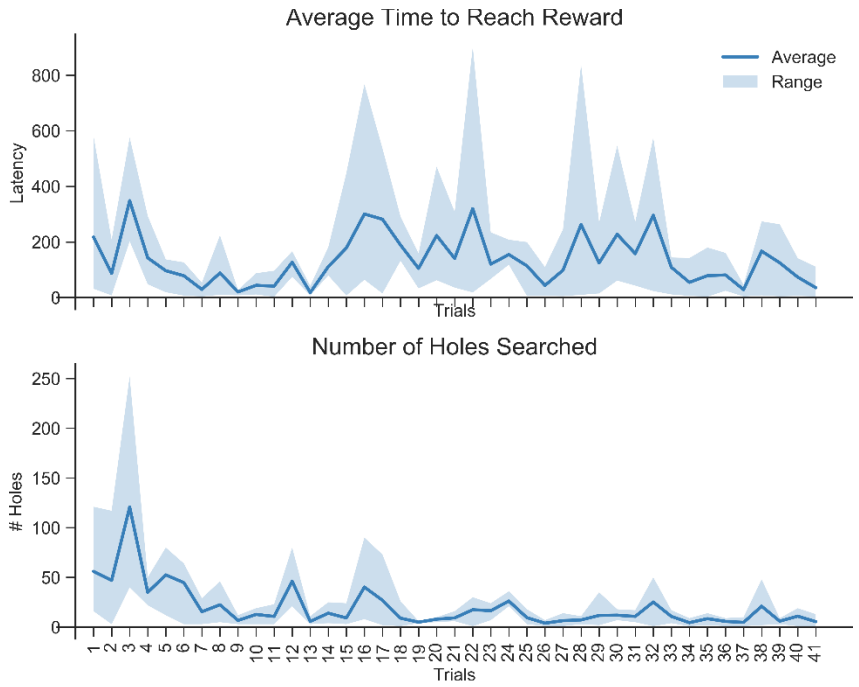


Figure 6. Experiment 1: Changing entrance learning curves. Graphs show the latency to reach food reward (top) and number of holes searched before correct hole (bottom). N = 4. Blue line is the mean, light blue area represents the data range. Mice do not appear to exhibit an improvement trend in terms of latency.

The distance mice traveled before reaching the target closely corresponds to latency (Figure 7 A). Comparison between distance traveled during the first 3 trials (M=1039.7cm, SD=1030.73) and the last 3 trials (M=554.7cm, SD=652.83) show there is no significant difference (*N* = 12 trials, *P* = .339, *P* value not significant, Wilcoxon Signed-Rank Test). Similarly, the speed in which the mice traveled in the arena did not significantly differ between the first 3 trials (M = 8.93 cm/s, SD = 2.57) and the last 3 trials (M = 9.50 cm/s, SD = 4.49) (*N* = 12 trials, *P* = .569, *P* value not significant, Wilcoxon Signed-Rank Test).

Despite the decrease in latency, mice did not appear to exhibit spatial search strategies, suggesting they decreased for other reasons such as decreased novelty or increased search efficiency (Figure 7 B).

Search trajectories did not concentrate in the target quadrant vs the other quadrants, suggesting mice searched less holes per distance travelled but were not better at searching closer to the food. Based on this pilot experiment, the data suggests 3 possibilities: mice were not using landmarks to find food, mice were unable to distinguish between landmarks used, or mice are unable to learn to task because it is too difficult.

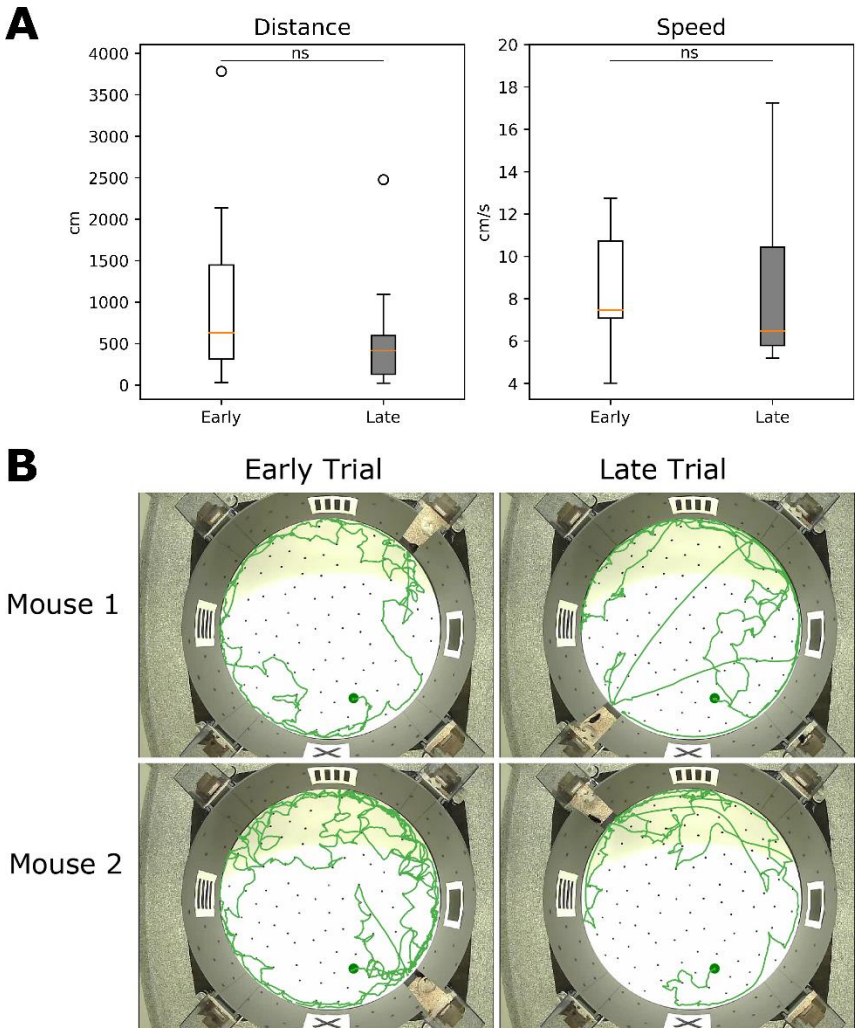


Figure 7. (A) Comparison of distance and speed traveled in early trials (first 3 trials) and late trials (last 3 trials). N = 12. (B) Example path traces comparing early and late trials. First column shows Trial 3, last column shows Trial 38. Search paths do not show evidence of spatial search strategy. Green dot indicates the target hole. Green line is the path trace. N = 2.

To analyze whether mice can learn the task if they could employ path integration in conjunction with landmarks, comparisons were made between the 1st trial of the day and the 2nd trial of the day. Since mice were not rotated between the first and second trials of the day, a difference in performance on the second trial would suggest the contribution of path integration on learning. Comparison of latency and #

of holes searched in both the early trials and late trials showed a highly significant improvement on the 2nd trial of the day (Figure 8). First 10 trials: Mean of 1st Trial = 163.94s, SD = 178.38. Mean of 2nd Trial = 105.13, SD = 132.23. *P* = .0024. The result is significant at *P* < .05, one tailed Wilcoxon Signed-Rank Test. Last 10 trials: Mean of 1st Trial = 168.33s, SD = 206.58. Mean of 2nd Trial = 99.45s, SD = 84.41. *P* = .03288. The result is significant at *P* < .05, one tailed Wilcoxon Signed-Rank Test. This improvement suggests that mice can use path integration as a spatial strategy to learn the target location.

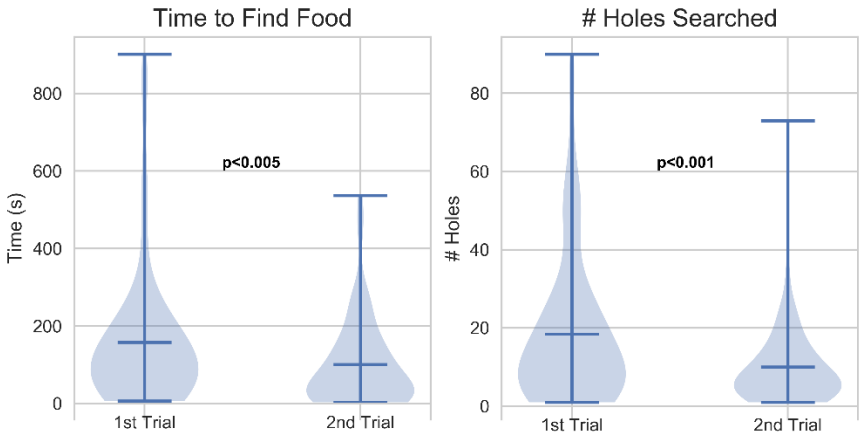
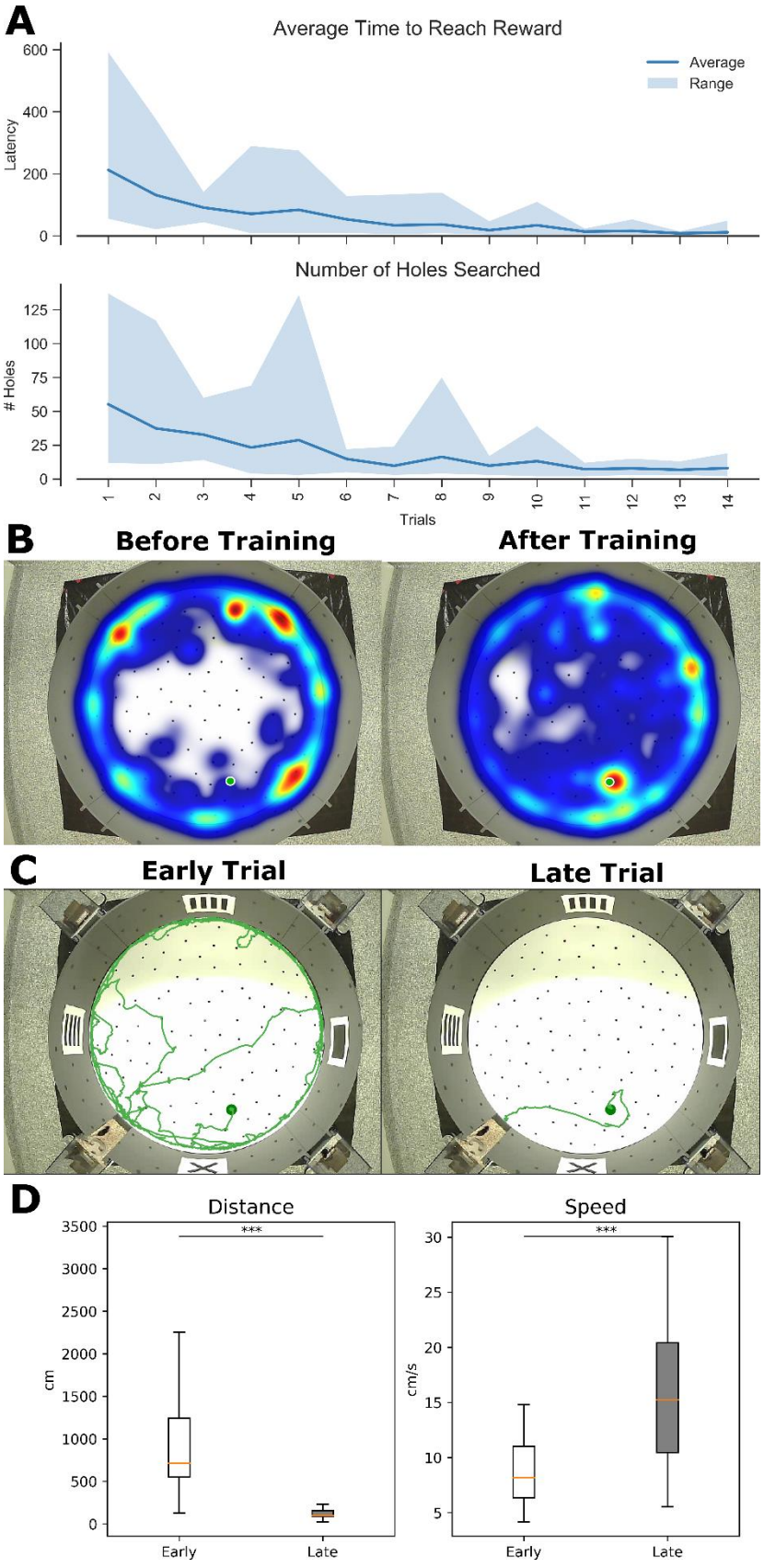


Figure 8. Comparison of 1st trial of the day and the 2nd trial of the day in the metrics of latency and number of holes searched. N = 144 trials, 36 trials per mouse. Mice perform significantly better in both measures on the second trial of the day. Middle line is the mean.

Lighting causes mice to be sleepy and remain in their cages. Mice are nocturnal and thus, to test the mice when they were more active, the trials were conducted during the dark cycle in subsequent experiments. After the change, mice were much faster to rouse and begin exploring after the home door was opened.

Experiment 2: Static Entrances


A
Average Time to Reach Reward
Average
Range
Latency
600
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Number of Holes Searched
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125
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Trials
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Before Training
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Early
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30
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15
10
5
Early
Late

Figure 9. Experiment 2: Unchanging entrances. (A) Average latency to target (top) and average number of holes searched before the correct target (bottom). N = 12 mice. Blue line is the mean, light blue area represents the data range. (B) Heatmaps of probe trials pre-training (left) and post-training (right). N = 4. Target is indicated on the figure with a green circle with a white outline. (C) Example path traces of a mouse in an early trial (Trial 3) and a late trial (Trial 14). Target marked with green circle. (D) Comparison of distance and speed of first 3 trials (Early) and last 3 trials (Late). N = 12 trials

The findings from Experiment 1 (Trial#1 vs Trial #2 stats), provide evidence that mice could learn the task if allowed to use path integration. Experiment 2 tested this hypothesis by allowing mice to enter from the same entrance every trial.

Mice show a strong improvement in their performance over time (Figure 9 A). Comparison between first and last 3 trials show a highly significant improvement in terms of latency ($P < .001$) and number of holes searched ($P < .001$, $N = 36$ trials, 3 trials per mouse, Wilcoxon Signed-Rank Test). Mean latency of the first 3 trials = 145.34s (SD=114.90), mean latency of the last 3 trials = 11.98s (SD=11.94). Mean # holes in the first 3 trials = 41.89 (SD=30.13), mean # holes in the last 3 trials = 7.56 (SD=3.91).

The decrease in latency is reflected in a corresponding significant decrease in distance traveled between early and late trials ($N = 35$, Z = -5.1594, $P < .001$, Wilcoxon signed-rank test). The speed in which the mouse travels significantly increases between the early trials ($M = 8.71$, $SD = 2.68$) and the late trials ($M = 15.61$, $SD = 6.08$, $t(34) = 7.83$, $P < .001$, t-test). The increase in speed, as well as the shorter distances travelled, suggest the mice have become more certain about the location of the target as the training progresses.

Comparison of the first trials of the day in the rotation experiment (Experiment 1) and the static experiment (Experiment 2) show there is no significant difference between their performance early in the training, but the difference becomes significant during late training. Mean of latency for first 6 trials of the first trials of the day in the rotation experiment = 149.28s (SD=148.16), mean latency for the first 6 trials of the first trials of the day in the static experiment = 121.03s (SD=118.03). $N = 28$ trials, 6 trials per mouse, $P = .702$, P value is not significant, Mann-Whitney U Test. Mean latency of 6 late trials of the first trials of the day in the rotation experiment = 165.12s (SD=159.90), mean latency of 6 late trials of the first trials of the day in the static experiment = 13.86 (SD=14.40). $N = 28$ trials, 6 trials per mouse, $P < .0001$, The P value is significant, Mann-Whitney U Test. All visual landmarks remained the same between the two experiments, suggesting the improvement in performance is due to the availability of path-integration as a strategy.

Mice reach a learning plateau in approximately 7 trials (Figure 9 A). Number of holes searched and latency to target are highly correlated, making the two measures rough estimates for one another.

A probe trial was given after trial 14 where mice could freely explore in the absence of food. Path heatmaps show mice spend a greater portion of time searching in the trained target hole compared with exploration before training (Figure 9 B). On average, mice spent 0.74s (SD=1.3) within 5cm radius of the target hole in the first 5 minutes of the trial before training. After training, they spent 28.61s (SD=21.50) around the target hole during the probe trial. The increase in time is significant (N = 12 mice, P = .00222, P is significant at < .05, Wilcoxon Signed-Rank Test). The increase in time spent around the target location suggest mice have acquired a spatial strategy to find the food location. Although the mice in Experiment 1 were given far more trials, they never acquired a spatial search strategy as seen in Experiment 2. Since performance in this experiment with static entrances (Experiment 2) is a dramatic improvement compared to performance with changing entrances (Experiment 1), it suggests path integration plays a large role in spatial learning in mice. However, it is unclear from these experiments alone whether mice are using visual landmarks at all in conjunction to path integration.

Experiment 3: Rotations

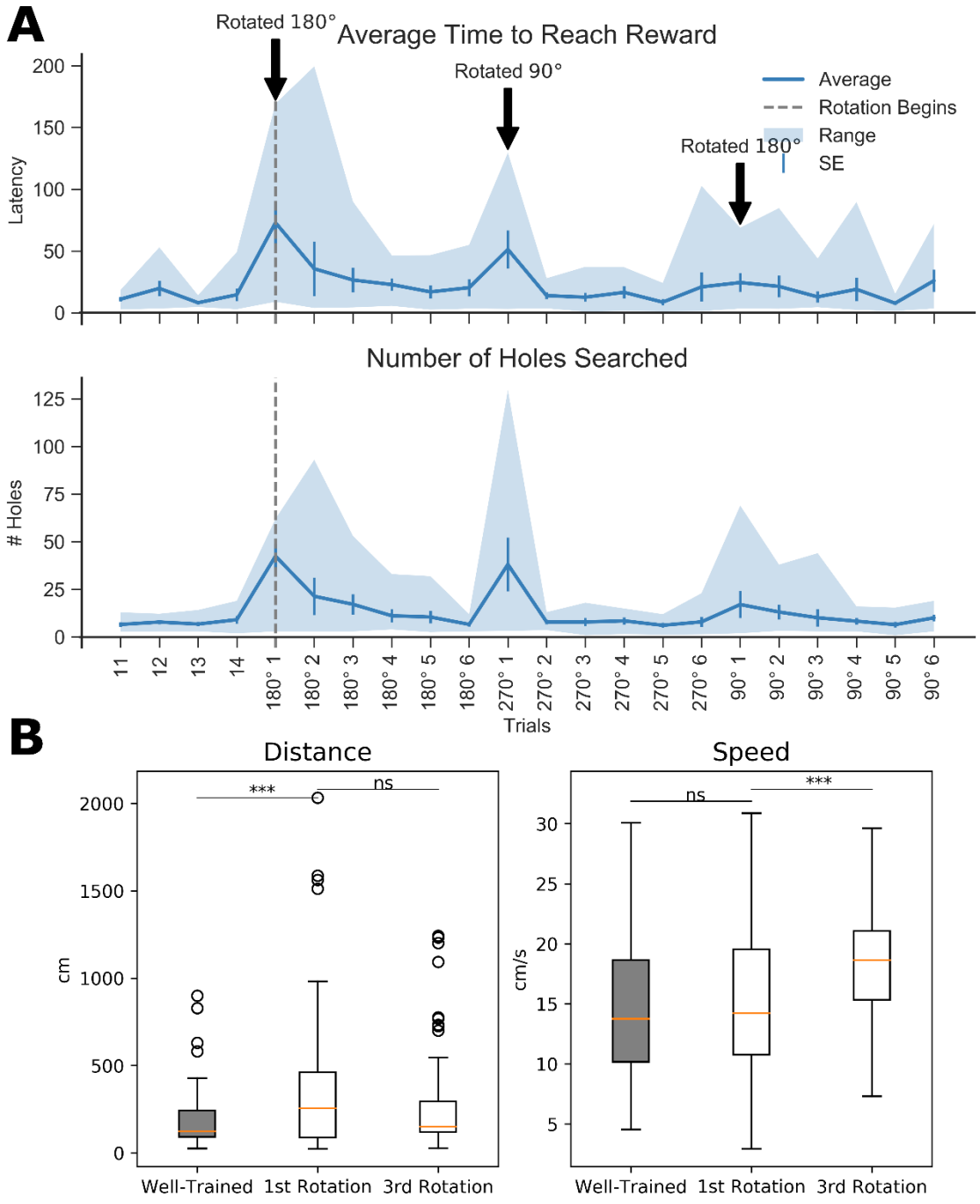

Figure 10. Rotation Experiment: (A) Learning curves of well-trained mice are subject to home cage rotations. Rotations occur before the 1st trial, the 7th trial, and the 13th trial. N = 8. Blue line is the mean, light blue area represents the data range. Trials before the dashed line is the baseline performance before rotation (N = 8 mice), representing the baseline performance. Vertical lines are standard error. (B) Comparison of the mice's distance and speed before and during rotations. Well-trained (grey box) represents the baseline performance of mice during the last 6 trials of training. 1st Rotation shows the distribution of distance and speed traveled during the 6 trials of the first 180° rotation. 3rd rotation represents the performance during the 6 trials of the last 180° rotation. N =12 trials.

Based on Experiment 2, mice were clearly able to learn the task, but it is uncertain whether their performance was purely attributed to path integration or if they also used visual landmarks. In order to test whether visual landmarks contributed to navigation, well-trained mice following the protocol from Experiment 2 were subject to a series of home cage rotations.

Mice perform worse following each rotation in terms of both increased latency and increased number of holes searched (Figure 10). Performance returns to levels near baseline within 6 trials in the new location but decreases again when the next rotation occurs. The average well-trained latency = 14.64s (SD=15.07), the average latency following the 1st Rotation = 73.02s (SD=50.72), average latency following 2nd rotation = 51.41 (SD=46.11), average latency following 3rd rotation = 24.55 (SD=22.79). There is a significant difference between group means of the latency during baseline and each of the 3 rotations (*N* = 8 mice, *P* = .0155, *P* is significant at > 0.05, one-way ANOVA test).

Mice travel significantly longer distances in the rotation conditions compared to their baseline performance when they were well-trained on static entrances. Comparison between the 1st rotation and the 3rd rotation show that there is not a significant difference in distance traveled, although there is a slight downward trend (N = 12 mice, 70 trials, *Z* = -1.741, *P* = .08186, Wilcoxon Signed-Rank Test).

On the other hand, mice do not significantly increase their speed between their baseline performance and the 1st rotation. However, mice show a significant increase in speed between the 1st and 3rd rotations. This suggests that mice are increasing their speed as their trials increase, which is an explanation of why they decrease latency during subsequent rotations. However, because distance does not correspondingly decrease, it suggests that the performance is not due to spatial learning.

The decrease in performance appears to diminish with successive rotations. The increased latency and # of holes searched following the 3rd rotation is less than the increase initially seen following the 1st rotation. The average latency of the last 2 well-trained trials is 11.43s (SD=10.72). The average latency of the 2 trials following the 1st rotation = 54.36s (SD=58.41). The average latency of the 2 trials following the 3rd rotation = 22.99s (SD=23.25). The difference between the well-trained condition and the 1st rotation is significant (N = 16 trials, P = .0114, Wilcoxon Signed-Rank Test). The difference between the well-trained condition and the 3rd rotation is not significant (P = .1096, P is not significant at <.05,

Wilcoxon Signed-Rank Test). This pattern raises the question whether mice can learn to use visual landmarks to improve to their performance in successive rotations.

Path trajectories suggest that mice revisit the rotationally equivalent location (REL) following a rotation of their home cage, strongly suggesting that mice rely on path integration to find the food reward (Figure 11).

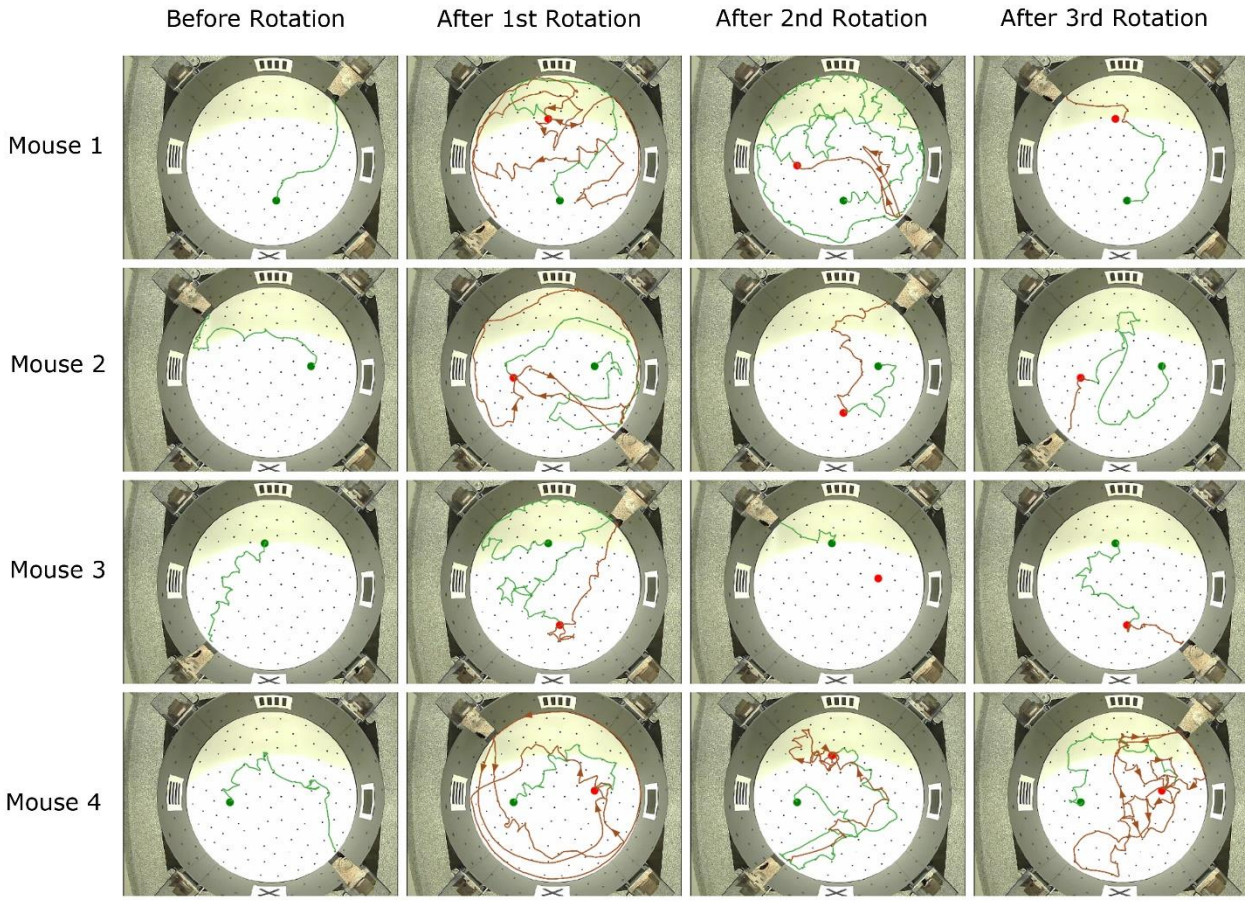

Figure 11. Path traces of mice during rotations. N = 4. Green dot represents the actual target. Red dot is the rotationally equivalent target. Dark red path is the initial trajectory of the mouse as it heads towards the REL. Small arrows indicate trajectory. If the mouse does not visit the REL, the red path is not shown. The green line is the path the mouse takes towards the correct food location. This path starts after the mouse last visits the REL (if applicable) or from the home cage.

Path tracking shows that mice consistently re-visit the REL, even after the 3rd rotation. The mice revisit the REL following the 1st rotation 7 out of 8 trials. Mice revisit the REL following then 2nd rotation 7 out of 8 times. Mice revisit the REL following the 3rd rotation 7 out of 8 times. Total revisits following a

rotation is 87.5% (21/24) (N = 8 mice, 3 rotation trials per mouse, 24 trials total). In 47.6% (10/21) of those visits, mice searched the REL more than once. This result strongly supports that mice are committing a perseverative error by relying predominantly on path integration over visual landmarks. It also demonstrates that mice are not using chemical cues such as odor to find the food reward because they will search empty holes at or near the REL.

In some cases, it did appear as if the mice did know how to go from the REL to the true food location. In Figure 11, Mouse 1 after the 3rd rotation appears to take a somewhat direct path to the correct target after visiting the REL. Similar cases are seen with Mouse 2 after the 2nd rotation and Mouse 3 after the 3rd rotation. It is possible that the mice stumbled upon the correct target by chance, but there is the possibility the mice have learned to use visual landmarks to re-orient after path integration has failed. By the 6th trial following rotation, most mice stop revisiting the previous REL. Removal of landmarks would help clarify whether visual cues have any consistent impact.

Experiment 4: No Landmarks

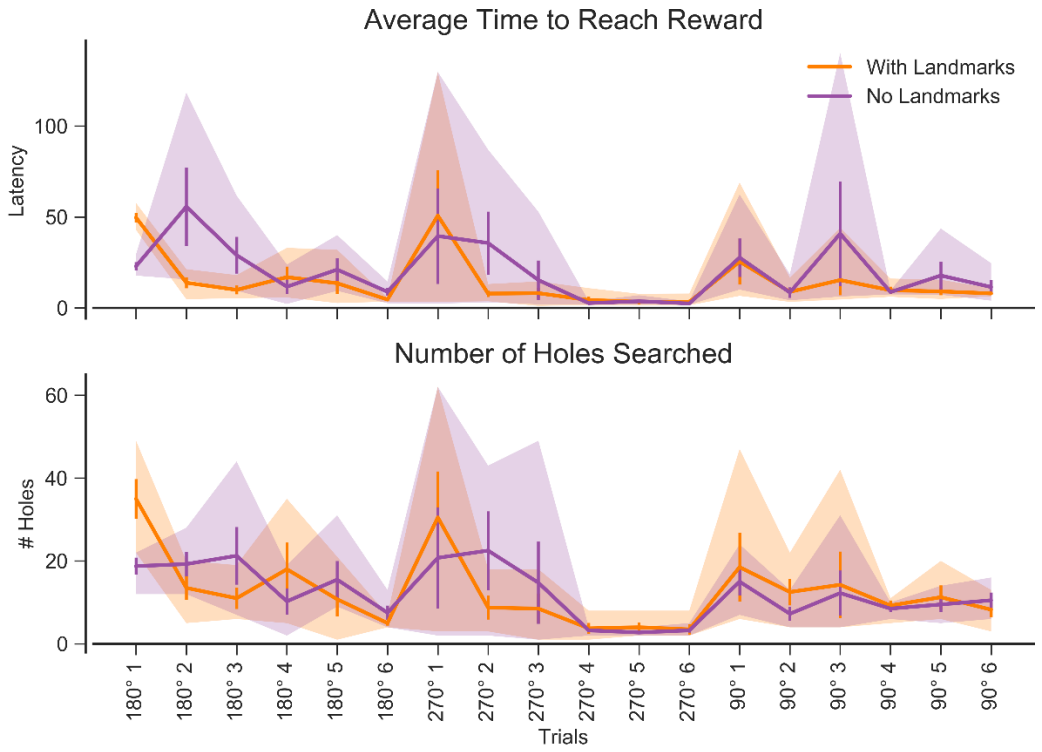

Figure 12. Rotation learning curves with landmarks and no landmarks. Orange line = visual landmarks present. Purple line = landmarks absent. Error bars = SE. Shaded region = data range. N = 4 mice per experimental group.

In Experiment 4, well trained mice were given 3 rotations (as per Experiment 3) but the visual cues on the wall were removed prior to the rotations. The performance curves of the mice closely mirror the mice in Experiment 3 (Figure 12). Analysis of the path tracking show that mice will revisit the rotationally equivalent location regardless of whether landmarks are present.

The difference in speed between the two cohorts was not significant (*N* = 8 mice, 18 trials each, *P* = .27400, paired t-test). The similarity of the results support that the mice were not relying on the visual cues present on the walls to improve their performance. Even when landmarks were removed, there is a downward trend of performance improving upon successive rotations, suggesting the mice is learning something unrelated to landmarks in order to improve search efficiency.

Comparing the mice's traveling speed over the course of the rotations reveals no apparent difference between landmark and no landmark conditions (Figure 13), although there is a slight increasing speed trend overall (Best fit line adjusted R-squared = 0.17450), but it is not significant. This trend does not appear to be affected by rotations, suggesting that speed increases with increased exposure to the arena independent of the mouse's distance traveled. The increase in speed may be a possible explanation why the mice have slightly improved search latency in later trials.

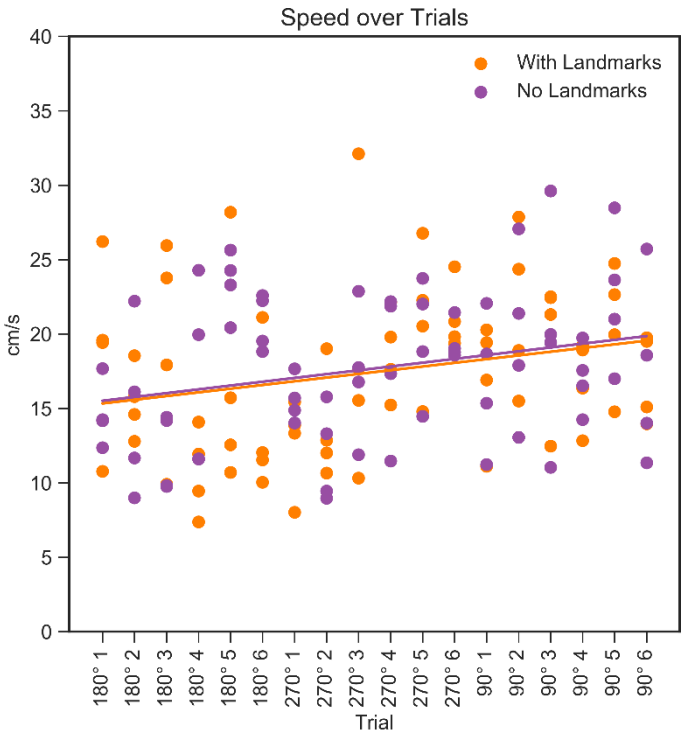

Figure 13. Scatterplot of speed over trials comparing Landmark and No Landmark conditions. N = 8 mice. Orange dots represent mice with visual landmarks during rotation. Purple dots represent mice without visual landmarks. Colours correspond to the best fit line of the respective conditions. With Landmarks R^2 = 0.17450. No Landmarks R^2 = 0.18219

It is possible the visual landmarks that were removed were not the visual cues mice were relying on. To address this possibility, mice were made to perform the trial in complete darkness.

Experiment 5: Dark Trials

Mice were trained normally as per Experiment 2 and given a probe trial. They were given two additional trials and then given another probe trial in complete darkness. Mice were then rotated 180° and 90° and trained at the new locations (9 trials each) and given a final probe trial. One mouse fell ill during training and was excluded from the dataset.

As expected, mice showed a strong preference to the target quadrant during the regular probe trial. The preference remains in the dark probe trial, albeit to a lesser degree. The preference is abolished in the probe trial following rotations (Figure 14 A). Because the mice performed better in the dark probe than the rotation probe, the findings support that mice are able to remember the food location through path

integration alone and that the ability is abolished if they are moved so PI becomes ineffective. Notably, in the Rotation probe trial's heatmap, one can see that the mouse spends time in each of the 4 rotationally equivalent target holes (Figure 14 B), suggesting that the mouse has retained PI information about the location of the target within a quadrant, but is confused about the overall orientation of the maze. However, their performance in the dark is not as precise as their performance in the light, suggesting that vision still plays a role in increasing the precision of the mouse's search strategy. Alternatively, perhaps the unfamiliarity of the dark arena contributed to the decreased in search precision.

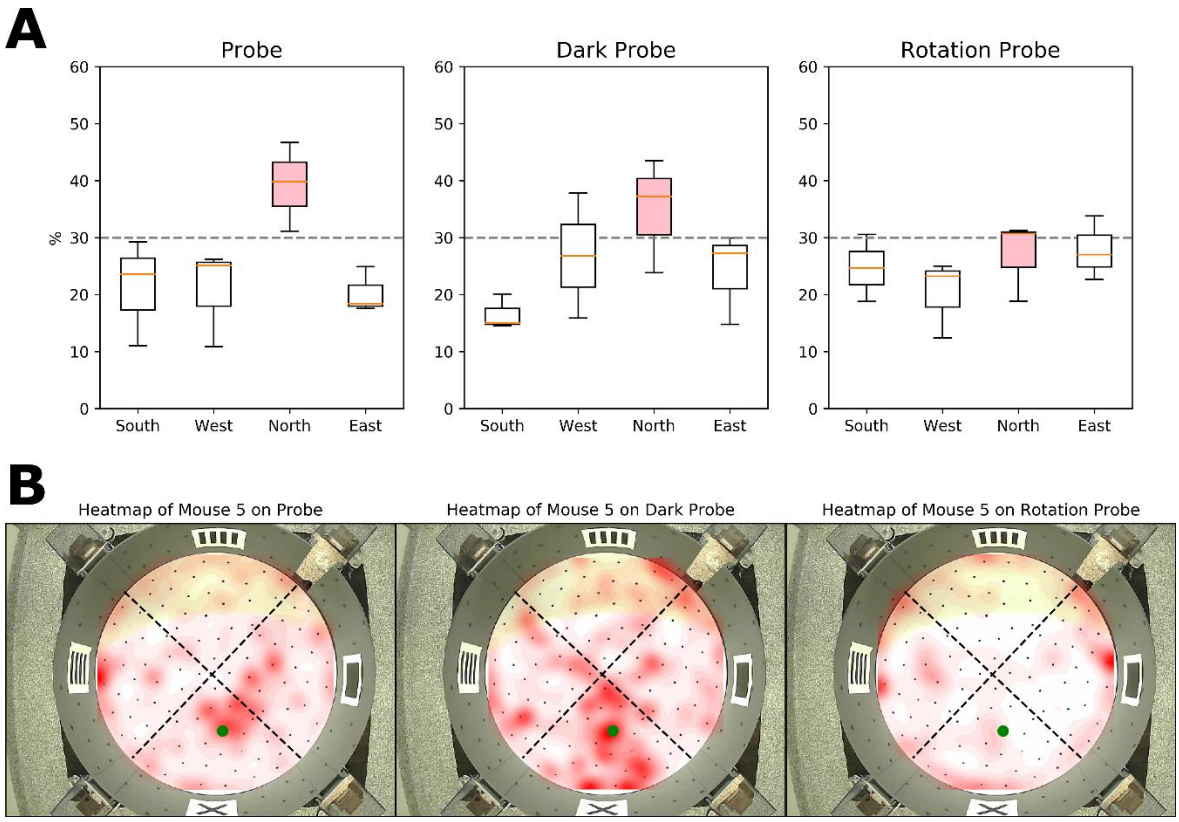

Figure 14. (A) Quadrant preference in 3 probe trials. Data represents percentage of time spent in each quadrant. Shaded box indicates target quadrant (North). N = 3. Dotted line indicates 30% threshold.(B) Heatmap example of one mouse during each of the 3 probes. In these images, the bottom of the image is considered "North" as it corresponds to the boxplots in 14A. Opacity of red indicates time spent in a location. Quadrants are delineated with dotted lines. Target is annotated by a green circle. 5 minutes are represented.

5. Discussion

Experiments on the Hidden Food Maze have demonstrated that mice use predominantly, perhaps solely, path integration as a navigation strategy and do not rely on visual landmarks located outside their immediate navigation environment. Mice can successfully learn the HFM when path-integration strategies are available and fail to learn the task when path integration strategy is robustly degraded through home cage rotations. When faced with contrasting cues between visual cues and path integration, mice showed a dominance of path integration as their navigational strategy.

5.1 Mice do not learn to use visual distal cues in the Hidden Food Maze

Our behavioural results in the Hidden Food Maze provide strong evidence that mice do not use large visual cues on the walls to orient themselves. During Experiment 1, the mice failed to improve performance when entrances were changed every trial and only visual landmarks could be used to predict the target location. Evidence for the lack of spatial learning is suggested by both the lack of significant improvement in latency and number of holes searched, but most evidently seen in the lack of spatial search patterns that concentrated in the target location.

The failure of the mice to learn the spatial location of the food could alternatively be explained by the task being too hard to learn. However, 1st Trial vs. 2nd Trial of the day comparison revealed that mice perform significantly better on the 2nd trial of the day when there was no entrance rotation. This improved performance suggests that mice can effectively orient themselves in this arena and that the rotation is the cause of the disorientation.

5.2 Mice can successfully learn the HFM when entrances are static

Significant and consistent performance improvement of mice during the Static Entrances experiment (Experiment 2) demonstrate that mice can successfully learn to solve the Hidden Food Maze when the entrances did not move between trials. Significant decreases in latency, number of holes searched, and distance traveled before reaching the correct target all point to successful acquisition of task knowledge.

Probe trials reveal evidence that mice learn to solve the task using spatial cognition. Heatmap visualizations of probe trials show a strong preference towards the exact target hole in the absence of food stimuli, providing strong evidence that the mice have learned the location of the target through

spatial memory. During probe trials, any non-spatial cues which could serve as beacons were eliminated: food was removed to eliminate any odor or visual cue at the target, the surface of the floor was washed with cleaning solution to remove any odor trails of previous mice, and the floor itself was rotated so any small surface scratches would not serve as a cue. Regardless, mice robustly showed a strong location preference towards the target hole. Notably, unlike other experiments such as the Barnes Maze where target location can be solved only in terms of remembering the correct orientation, the Hidden Food Maze requires mice to travel to the exact location of the target, keeping track of both the correct orientation and the correct displacement. The ability of mice to correctly locate the target hole in the absence of other non-spatial cues supports that mice learned to navigate towards the target based on spatial strategies that can keep track of both orientation and displacement.

5.3 Mice are using path integration to solve the Hidden Food Maze

The Hidden Food Maze demonstrates that mice show a strong preference for path-integration over landmark-based cue navigation. Mice were only able to successfully learn the task when path integration was possible: when the entrances were held static so there was a consistent spatial relationship between the entrance and the target (Experiment 2). When mice had become well trained, the same mice would follow different trajectories to reach the target during different trials, suggesting the presence of an internal map that can track the mouse's position in space. This strongly suggests mice are using path integration as opposed to non-spatial strategies such as rote memorization of the distance and angle needed to reach the target.

Furthermore, search paths of mice during rotation experiments (Experiment 3) revealed that mice on most trials did not compensate for a rotation but instead searched in the rotationally equivalent location of the target. If the mice were using the fixed visual cues, they should have been able to compensate for the rotation by navigating using these cues. They showed a memory of the target based on its spatial relationship to the entrance as opposed to the fixed visual cues on the wall. The inability to take allocentric information into account is a characteristic of path integration.

5.4 When path integration and visual cues conflict, path integration will dominate

Rotation experiments (Experiment 3) which brought path integration and visual cues into conflict revealed that mice will travel to the location predicted by path integration as opposed to the location

predicted by visual landmarks. The mice's failure to compensate for the rotation using landmarks provides strong evidence that PI dominates over visual cues as an orientation strategy.

However, mice did appear to be quicker at finding the correct target upon subsequent rotations, leaving open the possibility that the visual cues might provide a supplementary or delayed assistance in orientation in addition to path integration. Yet, when subject to additional subsequent rotations, the mice did not appear to demonstrate the ability to switch away from PI to a landmark-based navigation strategy.

5.5 Mice can perform path integration in the absence of visual landmarks

The final evidence that mice do not use visual cues is observed during the Landmarks/No Landmarks experiment (Experiment 4), in which the mice's performance appears to not be significantly altered by the presence or absence of visual cues on the wall. The absence of landmarks did not change the learning curve during entrance rotations, suggesting that any changes in behaviour of the mice were not due to the presence of visual cues. One explanation why the mice get quicker at finding the target during subsequent rotations is that that the mice have learned to narrow their search within a certain radius from the wall, since all targets were necessarily in rotationally symmetrical locations. This is supported by observing probe trial data following rotations, where a preference for searching in each of the 4 rotationally symmetrical targets in each quadrant can be seen in some mice. Another explanation is that mice walk faster as they become more comfortable in the arena, improving their latency performance despite not adopting a visual cue-based strategy.

Another hallmark feature of path-integration is the ability to navigate using idiothetic cues alone, without the use of any external cues. To test whether the mice were truly path integrating and not using an unknown visual cue, well-trained mice were tested in total darkness in a probe trial. During the dark trials (Experiment 5), mice displayed a preference for searching in the correct target quadrant, albeit the preferences was less pronounced than in the light condition. In comparison, when mice were subjected to a few rotations, the preference was abolished. This suggests the mice can remember the target location using only idiothetic cues, although their precision is increased by vision.

5.6 The Hidden Food Maze provides a behavioural framework for future hippocampal recording experiments

Our HFM behavioural results are consistent with previous studies that show rodents prefer to use path integration when navigating outside their home. Although previous research suggest mice can orient using visual cues when setting out from a starting location, it is not clear what types of cues they used. Our experiments show that 2-dimentional shapes on the wall are not used by mice for orientation in the HFM. Instead, their travel strategy appears to be based on path integration and perhaps orientation due to the 3-dimentional geometry of the entrance and arena.

Why do the mice not use the 2D visual cues? It is known from previous research that mice can differentiate between the types of shapes we used in this task (Horner et al., 2013; Prusky & Douglas, 2004). However, they are simply not using them for navigation towards a hidden food source. Perhaps the cues were not salient enough to be noticed. The geometry of the cue might also be important for orientation. Mice are nocturnal animals and may rely more on touch than vision for navigation and 3-dimentional cues might be more effectively used for navigation (Diamond et al., 2008). Furthermore, cognitive performance between mice and rats is known to be highly strain dependent (Kim et al., 2015; Kumar et al., 2015).

We are furthering our investigation on whether mice can use 3D intra-maze cues for orientation. I have gathered preliminary data (not shown and not included), that suggest that mice can use intra maze cues for learning the hidden food location. The intra maze cues may act as a relative reference point in which the animal performs an initial orientation, followed by using path integration to travel to the target. These results suggest that the mode of navigation used by mice is not fixed but may flexibly adapt in response to specific environmental constraints.

The results of these experiments have potential implications on hilar mossy cell research. If we expect mossy cell activity to encode navigational information, it implies that the features encoded will be predictive of navigational behaviour. Our research suggest mossy cells will likely not be as responsive to 2D visual cues compared to other cues. This behavioural setup provides a unique opportunity to investigate how spatial properties are encoded under a dominant path integration strategy as well as a combined PI + landmark strategy.

Overall, the work presented herein validates the use of the Hidden Food Maze as a robust, flexible, and versatile test to probe the neural bases of rodent spatial learning. Its minimally stressful design, low requirements for experimenter handling, and compatibility with electrophysiology equipment poises it to be used to investigate how spatial cells in the hippocampus contribute to navigational behaviour.

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