Chapter 2

The Mazes

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Abstract

How does learning and remembering happen? The answer to this question gives us a lot to talk about. Indeed, we are now in an exciting time of science when technological advances in the neuroscience field are meeting the demands and eagerness of scientists who wish to study relationships between the brain and cognition. Learning and memory experimenters have worked with great resolve to answer the mystery of these processes, and research in rats and mice has been especially prolific. Data from rodents have pioneered profound discoveries unlocking many mysteries of how learning and remembering occur. This chapter provides the background information necessary to understand this prior research, and also to perform a sound maze learning and memory rodent experiment. Learning and memory processing is multidimensional and complex, and rodent mazes can tap the different stages and depths of this processing by varying apparatus types and protocols. When studying cognition in rodents, it is necessary to acknowledge the multitude of factors involved in the process of quantifying maze scores in order to properly interpret data in terms of performance. In this chapter, critical terms are operationally defined, including memory types tested by various protocols applied to different apparatuses. Also reviewed are optimizing experimental designs, as well as the most frequently used rodent mazes in terms of setting up the apparatus, deciding the protocol to use in the chosen apparatus, actual testing procedures, behavior quantification, and data interpretation. Caveats, control procedures, and cautionary tales are discussed in detail. All of this is considered within the perspective that scientists must be clear about what is being evaluated; for maze studies, this means first broadly defining learning and memory, and then more specifically operationally defining the variables used to quantify types of measurements. Moreover, care is taken to reflect on how there are ample opportunities for unanticipated interactions to arise in behavioral research, with specific examples and respective solutions noted. Some of these interactive factors causing variability that could be interpreted as “nuances” of a behavioral phenomenon might turn out to be key to understanding how purposely manipulated variables impact behavioral outcomes.

Key words Behavior, Learning, Cognition, Memory, Protocol, Maze, Rat, Mouse, Rodent, Navigation, Place, Spatial, Nonspatial, Reference, Working, History, Brain

1 Introduction

How do learning and remembering happen? The answer to this question is not simple. Like with any scientific query, however, this question can be answered either via a pared-down, elementary way to simplify questions and interpretations, or via an entire book series detailing the scientific data that inform this answer.
There is a lot to talk about—we are now in an exciting time of science when technological advances in the neuroscience field are meeting the demands and eagerness of scientists who wish to study the relationships between the brain and cognition. The biological underpinnings of how learning and remembering happens have a rich history and we have learned much. How does the engram, or memory trace, occur? Where does it occur in the brain?

Of note, while considerable discoveries have been made thus far, there is still a tremendous amount left to discover. Scientific queries in search of the engram, as framed initially by Karl Lashley in 1950 in his summary of research [39], have persisted through the decades. In fact, as occurs in any scientific field that includes sound scientists on a quest to search for the truth in nature, discoveries lead to more questions... and then answers... and then more questions again. This is the glorious cycle of science! Learning and memory experimenters have worked with great resolve to answer the mystery of these processes; indeed, research spans the invertebrate level, such as in the marine mollusk *Aplysia*, to rodents, to nonhuman primates, to humans. Research in rats and mice has been especially prolific. Data from rodents have pioneered dramatic discoveries unlocking many of the mysteries of learning and memory. Chapter 1 takes us down memory lane as we explore the opulent, complex, and rousing history of the science of rodents and mazes to understand learning and remembering. We discuss the first known maze study testing the white rat by Willard Small in 1901, making the landmark contribution of introducing both the maze and the white rat to experimental psychological research. This work was the first to systematically test “mental processes” in the rat, and in doing so acknowledged that rats have a sophisticated form of cognitive processing that can be measured and used to solve problems. We have come far as a field, and now we have a sound basis and understanding of how the experimental analysis of rats and mice yields valuable insights into cognitive processing. It is important to recognize that in addition to utilizing mazes, there are many other ways to test learning and memory in rodents. These methods will not be addressed in detail here, but we would like to note that studies using these procedures have yielded much insight into treatments and factors that impact learning. This includes, for example, research using operant conditioning chambers (also called Skinner boxes, named after its creator, B.F. Skinner) requiring rats to press levers for food, active avoidance boxes utilizing shock, and procedures tapping Pavlovian fear conditioning models.

## 2 Asking Experimental Questions Using Rodents and Mazes

When a scientist performs a study using rodents and mazes, typically the experimental question includes asking whether a particular factor or systematic manipulation, such as a genetic variant, a
drug treatment, or a brain lesion, impacts learning and/or memory. Does one group perform better or worse than another group for learning the new task? Or for memory on the task after learning has occurred? These may seem like simple questions, and a simple experimental task at hand, but once one digs into the reality of how to test learning and memory in the rodent and the many decisions that must be made for accurate measurement and interpretation, the task for the scientist might be initially daunting. The goal for this chapter is to provide the background information necessary to perform a sound maze learning and memory rodent experiment. When studying cognition in rodents, acknowledging the multitude of factors involved in the process of quantifying maze scores in order to properly interpret data as performance measures is critical.

As scientists we must be clear about what we are evaluating; for maze studies this means first defining learning and memory in a broad sense, and then operationally defining the variables we use to quantify types of measurements. A variable can be defined in general terms as something that could impact the outcome of your experiment. Optimally, we will control for as many “extraneous” non-purposefully manipulated variables as we can. Then, there are variables that we purposefully manipulate so we can determine the impact on an outcome measurement. Operational definitions are critical to the interpretation and repeatability of your study; operational definitions detail the specifics of what you are manipulating, how you are manipulating it, and what it means to you as you interpret your results. Being able to differentiate among distinct types of memory is vital to successful translational research testing rodents in mazes. Figures 1 and 2 from Chap. 1 schematically represent some basic operational definitions of different types of memory and task rules.

How do we measure and operationally define learning and memory in a rat or mouse? Memory is traditionally divided into stages: stage 1 (acquisition): information is acquired; stage 2 (consolidation): information is consolidated or stored; and stage 3 (retrieval): information is retrieved or recalled (see also Chap. 1, which discusses these stages as well as reconsolidation). Learning can be defined as the acquisition of knowledge and formation of a memory, and memory can be defined as a recollection and the permanence of learning. If I were to aid you in setting up a maze for your laboratory, I would ask whether you want to test spatial or nonspatial memory, and whether you want to tap working or reference memory processing. Thus, for rodent maze memory, whether setting up your own task or interpreting the literature, you should ask: (1) Is the task spatial or nonspatial? and (2) Is the task working or reference memory? Spatial tasks require the use of cues that are outside of the maze to solve the task, and nonspatial tasks require the use of cues that are within the confines of the maze, that is, within the maze apparatus, to solve the task. For spatial navigation,
rodents learn to navigate through an environment so that a route to the target (and reward) eventually becomes familiar, and cues in the environment that are outside of the maze apparatus form associations to help with overall navigation. This is also referred to as place navigation. The ability to successfully solve a spatial learning and memory maze involves the ability to navigate effectively through space, thereby acquiring, integrating, and retaining features of the world that are outside of the maze, such as landmarks and other prominent cues. For spatial tasks, typically there are no obvious cues inside the maze to indicate the correct answer for the task. Rather, there are spatial, or extra-maze, cues around the room to help the animal navigate through space. These spatial cues can include tables, chairs, and bookshelves, as well as posters, bold patterns, and geometric shapes posted or painted on the walls. Figure 1 shows a typical maze room setup. Mazes that test nonspatial learning and memory can take many forms. In most cases this involves a prominent and notable cue inside the maze, such as a platform visible above the water surface, a flagged platform, or boldly patterned maze walls to identify a correct choice. It is also noteworthy that when solving a task using a nonspatial strategy type, this can additionally involve a motoric strategy whereby animals must learn to alternate turns (e.g., left on one trial, right on the next trial) to obtain the reward and earn the mark of successful performance.

Fig. 1 Schematic showing an example of a spatial maze room setup. Note the many prominent spatial cues in the room. Spatial cues should remain constant throughout testing, unless the goal is to manipulate cues to test cue utilization.
Working memory is a form of short-term memory that requires the rat or mouse to retain information that must be updated and is useful for only a short period of time. This is considered trial-specific information and requires manipulation of information kept “on-line.” The late Dr. Patricia Goldman-Rakic cleverly referred to working memory as “working with memory.” In general, working memory is distinguished from reference memory, which is a form of long-term memory necessary to remember information that remains constant over time. This is considered task-specific information. Any maze apparatus described below can be adapted to test spatial or nonspatial memory, or working or reference memory. The type of memory tested is dictated by the task protocol and rules given to the animal, which they learn as testing trials progress. Figure 2 summarizes many of the mazes used to test rodent learning and memory.

As experimenters interested in asking questions about cognition, we can ask rats and mice what they have learned and remembered by using mazes requiring different types of rules. Rats and mice can be trained to win-stay, or to win-shift. We will use the T-maze task as an example. If an animal is placed in a T-maze at the

![Maze Schematics](https://example.com/maze-diagram.png)
start location and then goes to the east arm, this is a right turn. If we have trained animals to **win-stay**, when the animal is placed in the same start arm again for the next trial, the animal will return to this east arm—it returns back to the maze location where it has just “won.” It “stays” where it has “won.” In contrast, if animals have been trained to **win-shift**, after it goes to the east arm for its first choice, for the next trial after being dropped off in the same start location, the animal will go to the other arm in the west. The animal goes to the maze location where it has not “won”—it “shifts” away from where it has “won.” Studies have shown that both win-shift (do not return to where you were rewarded) and win-stay (do return to where you were rewarded) requirements in maze tasks result in effective learning in rats and mice.

Any maze apparatus can be adapted to be: (1) spatial or non-spatial by requiring use of cues outside or inside the maze to solve the task, respectively, or (2) working or reference memory by requiring memory of updating or constant information, respectively. There are some more complex tasks that require use of multiple types of information to successfully solve the task. For example, the radial-arm maze can require utilization of both spatial and nonspatial components by providing tactile cues (e.g., sandpaper) or bold visual patterns (e.g., stripes) inside a subset of the arms, while the rest of the arms remain neutral on the inside and many extra-maze spatial cues are provided around the room. As another example using the radial-arm maze, it could require utilization of both working and reference memory simultaneously by providing a reward in only a subset of the arms. In this version of the radial-arm maze, the arms with the rewards are kept in the same location across days. When the reward in an arm is located, it is then no longer available on subsequent trials within that specific day so the animal must remember that arm and not go back within that day; this requires working memory and is trial specific. The subset of arms that does not have rewards is the component that requires reference memory; since this information remains constant and requires no updating, it is task specific.

3 Variability: Is it an Evil Red-eyed Beast Throwing Daggers at Your Experiment?

Variability. It is in every experiment, and it can represent a myriad of things happening in a study. When comparing different treatment groups in maze performance using analysis of variance (ANOVA), a large $F$ value for Treatment resulting in a significant Treatment effect means that the variation between the groups is larger than the variation within the groups. A scientist can have a clean, hypothesis-driven question that is addressed in a systematic and sound way, but still have so much variability within groups that the question cannot be clearly answered because it masks the effect.
between the groups. I (HBN) admit, I have made up some quite creative curse words at variability when looking at the error bars in some of my graphs. However, in reality, variability is not necessarily the evil red-eyed beast throwing daggers at your experiment as one might initially be inclined to think. In fact, we can answer questions by capitalizing upon it with specific statistics that use individual variation to understand relationships (such as correlations). Chapter 12 addresses statistics, and dealing with variability, for maze data. We discuss here, in this chapter, that an experimenter should always note and control for as many “extraneous” variables as possible. Some of these factors... well,... we just accept them as inherent variability to the study (see [44], for examples of this).

Indeed, one must have a balanced view of the optimal design of the study and realistic experimental practice and protocol. We must ask, how much can one realistically control?

For instance, it is possible for experimental procedures, including those necessary to implement the experiment, to impact the cognitive scores of animals given specific treatment. An excellent illustration of such an effect is the finding that the handling of rodents necessary for experimental procedures can impact the cognitive effects of hormone treatments. Specifically, we (JD) have shown that increased handling enhances performance on a working memory task and obviates the benefits of estrogen treatment following a delay between trials [12]. The potential of handling effects are especially relevant when comparing different routes of administration as well as when choosing a behavioral task to measure cognition. Moreover, the dependent measures identifying learning and memory performance may interact with the impact of hormone treatment. In fact, it has been shown that a single day of Morris maze testing can abolish estrogen’s ability to increase dendritic spine density in the rat hippocampus [23], an effect that has been replicated many times in animals that were not cognitively tested [67].

What factors should be taken into account when designing your behavior study? Which variations in procedures and protocols impact your behavior data is something that reveals itself as you build a history of behavioral research. The published literature is very informative along these lines, but also, which specific factors are important and salient to your behavior questions will come to light with your own experiences and sensitivity to your data. If you have large variability within your treatment groups (see Chap. 12), make a list of what factors could be increasing this variability. Are animals being tested at different points across the day (Could there be a daily/diurnal rhythm to my learning and memory effects?); Are there many testers running the animals in the mazes (Could testers be handling or scoring animals slightly differently from each other?); Is there variation in the cages in which the rodents are being housed (Could different housing environments impact my behavior data? See [45])? Many subtle details we may not mean to
incorporate into our studies, as well as the treatments that we purposely test, interact with numerous brain systems related to learning, memory, and other functions. As a result, there are ample opportunities for unanticipated interactions to arise. Some of these we may come to figure out, and some we never know about. It is also important to recognize that it is likely that at least some of these factors causing variability, which could be considered “nuances” of your Treatment effects, will one day be key to understanding how your purposely manipulated treatment impacts behavioral outcomes.


As prudent experimentalists, we must acknowledge complexity in our dependent variables, and entertain alternate interpretations of our results. As discussed earlier, in order to test learning and memory, researchers must operationally define performance, and use these definitions to interpret results. We need to, of course, acknowledge that there is the potential for modifications to be made to optimize our working definitions or task designs. An excellent example is the creative research of van Haaren and van de Poll in 1984 [60]. In this study, they demonstrated that the addition of an alternative choice (a third chamber) in a passive avoidance shock task, traditionally offering only two chambers, abolished the well-established sex difference in task performance. This work indicated that the previously observed sex difference of female “impairment” on this task was not due to a memory deficit. Given the established finding that females are more exploratory than males, in the two-chambered task it was plausible that females moved to the shock-paired chamber due to this elevated motor activity (the “need” to move), and not a memory deficit. The results of van Haaren and van de Poll suggest that this was the case, since females no longer returned to the shock-paired chamber when given an alternate option. Instead, the female rats preferred the third chamber that was not previously associated with a foot shock. As a result, the two-chamber version of this test produced a sex difference that had been previously attributed to a lack of memory of the shock location in the females. However, once given another option, it became apparent that female rats preferred to avoid the chamber where the shock had previously been given. Indeed, they moved to the now present optional third chamber instead of the shock chamber, thereby signifying memory of the shock location. Simply put, the operational definition of the memory impairment in female rats in the traditional task was actually an increase in ambulatory and exploratory behavior.
Motivating Animals to Perform

We refer you to Chap. 1 to read about the importance of interpreting maze performance in the context of the motivator. Indeed, while we must motivate rodents to solve the maze task and show us what they learn and remember, the motivator itself can impact performance via non-cognitive factors. This whole idea is very complex, but also critical to accurate interpretation; this is exemplified by the seminal work of Blodgett describing latent learning discussed in Chap. 1.

Both water escape and food deprivation are used to motivate animals to perform in a maze task. While there are some issues with both types of motivators, the reality is that in order to allow animals to inform researchers what they have learned and remembered, they must be motivated to perform the task. For example, as explained in more detail below, traversing or swimming down a radial-arm maze arm, to the researcher, is interpreted as an error. If an animal is not motivated to walk or swim down an arm, and instead floats in the middle arena of the maze and/or makes no arm entries, this could be interpreted as excellent maze performance since no “errors” were made. In reality, performance is not reflective of cognitive prowess in these cases. Instead, the animal may not be motivated to exit or complete the maze task because it is not hungry enough to look for food, the food is not palatable, or the swim water is warm and not uncomfortable enough to warrant interest in escaping. These types of concerns are the reason why control tasks are used in maze studies, and why researchers have gone to great efforts to include appropriate motivators in their maze tasks.

There is an extensive history of rodent experimenters using escape from water as the motivator in maze learning and memory tasks, thereby avoiding the food deprivation necessary when utilizing appetitive motivation or footshock [18, 28, 61]. Water escape motivation capitalizes on the tenet that rodents find immersion in water aversive, and they are therefore motivated to find an escape. Thus, finding the platform serves as a reinforcer (a reinforcer increases the likelihood that a response will occur); the animal locates the hidden platform, climbs on it, is removed from the water-filled maze, and then placed in its heated cage until the next trial.

For water escape tasks, the maze is constructed of a durable material that can withstand being filled with water, such as a thick plastic or plexiglass, or stainless steel. Typically, if the goal is to test an animal’s ability to utilize spatial (extra-maze) cues for navigation, the maze is black in color, and the platform/s are also black. The platform height is designed so it is just under the water surface, about 3 cm under the water level works well for rats, and
1–2 cm under the water level works well for mice. The goal is that the platform will not be seen from the water level. In the Bimonte-Nelson laboratory, our platforms are scored with slight grooves in a checkerboard pattern on the top, as we have noticed that animals stay best on the platform when it is of rough (not smooth and slippery) texture. We currently have our platforms made of plexiglass from a local company that manufactures various plexiglass products; we simply explained what we needed with an adjoining schematic, and they build the platforms to our specifications. In the past, when forced to be more resourceful for various reasons, I (HBN) built platforms by duct taping two cans from the grocery store together. In this case, after many hours sitting on the grocery store floor measuring cans in the dog food aisle (in this particular case, dog food cans were the appropriate width for our maze arms), we found two stacked on top of each other that equaled our needed height. We brought the cans back to the lab and emptied them (as you can imagine, our lab neighbors were thrilled with the odor, which took hours to dissipate), scrubbed them clean, filled the bottom can of each platform with rocks, duct taped the two cans together, covered the top can of each platform with wire mesh, and spray painted them with rust-proof paint to match the maze color. Voila — platforms at the cost of about $3.00 each (and some annoyed lab neighbors)!

You have your scientific question and subjects, you have your protocol, you have your maze, and now you have your platforms. Now… on to the fun part… maze testing to collect your data! For testing, the animal is released from a start point within the maze, and swims to locate a hidden platform. Once the animal locates a platform, it remains on it for a specified amount of time as denoted by the particular protocol being utilized (see the protocol section at the end of this book for specific times). The animal is then removed from the maze and placed into a cage with avoidable heat. This cage is usually heated overhead via a heat lamp that emits heat but no light within the wavelengths thought to be visually perceivable to the animal. This is key, since bright lights are known to be a stressor to rodents. We use red colored, heat emitting bulbs from a pet store.

Food restriction procedures are applied during performance of appetitively motivated tasks that use food as reward. The goal of food restriction is to ensure that animals are motivated to perform, and that motivation levels are controlled for across subjects. Animals are typically food restricted to a target of 85–90% of their free-feeding weights. A target weight for each animal is determined based on an average of 3–5 days of free-feeding weight. To begin food restriction, remove all food. Rats should be checked for health, weighed, and fed daily. Provide food that will result in weight maintenance, reduction, or gain as necessary. For example,
the weight of young adult female rats is typically maintained with three full-size pieces of chow per day. Increase or decrease number of chow pieces from this baseline amount as needed. Rats should be fed after, not before, behavior testing is completed each day. When using very young rats, procedures can be modified to allow for growth. For example, target weights can be adjusted each week and set at approximately 90% of the average free-feeding weight for aged-matched animals according to standard growth charts available from vendors.

Rodents are neophobic with regard to food. To overcome the tendency to avoid new food, in my laboratory (JD) we place pieces of food reward in home cages each day for several days before behavioral training begins. Additionally, one should conduct a habituation trial, during which animals freely explore the maze with food rewards sprinkled throughout, the day before training begins. During early days of training, it is common for an animal to enter an arm and fail to eat a food reward. It may take up to 10 days of training in a radial-arm maze before all rats are consistently eating all food rewards encountered during arm choices. Measures can be implemented to confirm that experimental manipulations are not impacting appetite, motivation, or other non-memory processes associated with the use of food reward. A record of the number of food rewards encountered, but not eaten, during arm entries can be kept and compared across groups to test for group differences in this factor. Furthermore, the speed at which rats transverse the maze can be analyzed by calculating the number of arm entries per minute for the first eight arm choices (see the protocol section at the end of this book for more detail).

6 The Morris Maze

In the early 1980s, Richard Morris published a series of papers that soon came to change the way that researchers studied learning and memory in animal models ([46–48]; see also Chap. 1 for a general history in the context of other maze discoveries, and Chap. 3 by Richard Morris where he chronicles these findings). In 1984, the landmark paper, “Developments of a water-maze procedure for studying spatial learning in the rat,” published in the Journal of Neuroscience Methods and authored by Morris, led the field to a new place for studying rodent learning and memory [47]. The task explained in that paper, and in the others authored by Richard Morris that proceeded it earlier in that decade, describe a task composed of a large round tub filled with water, containing a hidden platform just beneath the water surface (Fig. 2). This task is now referred to as the Morris maze, or “the watermaze.” Many researchers cloud the water with nontoxic (for example, dry tempera) paint, or powdered milk, to be certain the platform cannot be seen by the animal.
In the traditional version, there are no obvious cues inside of the maze (such as stripes on the wall of the maze portion containing the platform) to indicate the location of the platform. Rather, there are spatial, or extra-maze, cues located around the room to help the rodent navigate through space effectively.

To motivate animals to use a spatial strategy rather than, for example, a turn strategy to solve the maze, the animals are dropped off at different locations across trials. Cardinal directions of north, south, east, and west are normally used as the drop-off locations, which make it easier to divide the round tub into quadrants for analysis of behavior (northeast, southeast, northwest, southwest). These drop-off locations are discretely marked on the outside of the maze tub, on areas visible only to the experimenter testing the animals, and not to the subject. Drop-off locations for north, south, east, and west are selected semi-randomly for each trial of each test day so that no two identical drop-off locations are given consecutively. Animals undergoing maze testing are divided into squads of about 8–10 per squad for ease of testing. For testing, the animal is placed in the maze from any of four locations (north, south, east, or west) and has 60 s to locate the hidden platform that remains in a fixed location (for example, northeast quadrant) throughout testing. Once the animal finds the platform, the trial is terminated. After the animal’s platform time (we use a 15 s platform time), the animal is removed from the maze and placed into its heated cage until the next trial. Animals are tested in squads so that trial 1 is completed for each animal in the squad, then trial 2, then trial 3, etc. After the last animal in the squad is tested, the next trial begins and this continues for all trials of the day. Thus, the approximate inter-trial interval for each animal in a squad of 8–10 animals is 10–15 minutes depending on performance levels and the time it takes to clean the maze between animals, etc. When animals in the squad have completed all of the trials for that testing day, the animals are brought back to the colony room, and the next squad of animals is brought into the testing room and testing procedures are repeated. It is important that animals from each treatment group are represented in each squad, thereby counterbalancing for many potential confounding factors, including when testing occurs within a day.

During the first day of testing or so, animals sometimes exhibit thigmotaxic behavior, wherein they circle close to the outside maze perimeter/wall. As trials and days progress, however, animals demonstrate learning by the directionality of their escape behavior, such that a more direct route is taken to the platform rather than a circuitous unsystematic route. This provides evidence that animals are learning the platform location. To determine and analyze an animal’s swim path, a video camera is placed above the maze and a tracking system is used. The video camera records the animal’s performance on the tracking system and simultaneously onto a DVD or separate hard drive. This is a highly recommended back up in case the tracking
system fails (e.g., loses track of the animal, computer freezes, etc.). Indeed, in my (HBN) experience, this happens for at least several subject path tracks in every study, and therefore we access and utilize our back up in some way for every experiment.

The specific protocol for testing rodents on the Morris maze can vary in many ways. The number of trials per day, and the number of days, have each been varied in the literature. For rats, our (HBN) laboratory has used 4 trials a day for 5 days, or 6 trials a day for 3 days. We obtain excellent learning using both protocols, with significant decreases in latency and distance across days, as well as localization to the target previously-platformed quadrant on the probe trial (see Figs. 3 and 4, and below, for further explanation). For mice, we sometimes use more days (4 trials per day for 7 days, for example), deliberately increasing the number of days to allow more time for learning to occur. See also the protocol section at the end of this book for more detail on testing practice.

For both rats and mice, one possible advantage of spreading testing across more days is that there are more overnight intervals for analysis. Overnight forgetting is a variable frequently analyzed, yielding information about an animal’s ability to retain information during the overnight interval. Overnight forgetting analyses are sensitive to many factors, including aging and hormone manipulations \([1, 11, 41, 53]\). Overnight forgetting can be analyzed by comparing performance on the last trial of the previous day, to performance on the first trial of the next day. An increase in distance scores from the last trial of one day to the first trial on the following day indicates overnight forgetting.

The Morris maze has been shown to be hippocampal-dependent. Morris and colleagues have demonstrated that animals with hippocampal lesions were impaired when performing the spatial reference Morris maze, relative to control and cortical-lesioned animals \([48]\). However, all animals, regardless of lesion, generally performed in a similar fashion when the platform was made visible and the task did not need to be solved using spatial navigation. Thus, the data suggest that the hippocampus is necessary for successful performance on the spatial navigation protocol for this task, but not the visible platform nonspatial protocol.

The Morris maze apparatus can also be used with a matching-to-place protocol to test working memory. As described by Steele and Morris \([52]\), the matching-to-place version of the Morris maze requires the location of the platform to be updated within the same environment; this can be contrasted with the original reference memory version where the platform remains in a fixed place in space. For the working memory matching-to-place version, the location of the hidden platform varies across days, but remains in a fixed location within the same day. The first trial is therefore the
The Morris maze

SAMPLE SWIM PATHS

Day 1: 

Day 5:

LATENCY & DISTANCE ARE USUALLY CORRELATED.

IF THEY ARE NOT, IT TYPICALLY INDICATES THAT MOTOR OR VISUAL CHANGES ARE IMPACTING THE SCORES. THIS RESULTS IN SLOWER SWIMMING & A LONGER LATENCY TO FIND THE PLATFORM, BUT A DECREASE IN SWIM DISTANCE TO THE PLATFORM.

DEPENDENT VARIABLE SCORES ACROSS DAYS

How many inches did the animal swim to the platform?

How many seconds did it take until the platform was located?

Latency & Distance decrease similarly

Latency to platform (seconds)

Latency & Distance DO NOT decrease similarly

Days

Days

Fig. 3 Samples swim paths and interpretations in the Morris maze. Care should be taken to decipher actual cognitive ability versus the skill to perform the procedural components of the task.

information trial, “informing” the animal about the new location of the platform, and trial 2 is the working memory test trial. Thus, an animal has to learn the new spatial location of the platform to perform the task successfully on a daily basis. Once an animal learns this rule, latency/distance to reach the platform decreases significantly from trial 1 to trial 2. The animal searches for the platform on trial 1, when the platform location is unknown to the animal. The animal locates the platform, and updates this platform location information for trial 2. This is “match-to-place” since the animal
must match its next response to the place in space where it was just rewarded; it is noted that performance is usually maintained on later trials within that day [52]. This task offers the flexibility of maintaining a fixed inter-trial interval between trials, or instilling delays between trial 1 and trial 2 to test longer-term memory retention. This task is win-stay within a day.

6.1.3 A Dual-Solution Version of the Morris Maze

The Morris maze can be adapted to address questions regarding the strategies animals use to solve tasks. We (JD) have used a dual-solution Morris maze task to test hypotheses regarding how experimental manipulations differentially affect spatial learning and cued learning [17]. This task is based on one previously used to determine the role of the hippocampus in strategy selection [51]. In this dual-solution task rats can use spatial extra-maze cues surrounding the maze (spatial strategy) or an intra-maze cue or landmark (cued strategy) to find the hidden escape platform.

In our version of the dual-solution Morris maze task, we conduct ten acquisition trials during which the submerged escape platform is moved to a new location for each set of four daily trials. During acquisition trials, extra-maze cues surround the maze, and a visible landmark is always located 20 cm to the north of the
escape platform (see Fig. 5). Our landmark is a floating black ping-pong ball attached to a weight by a string. Because we make the water opaque with white tempera paint, we paint the top of the ball white so as not to interfere with the video tracking system. Performance is assessed by averaged swim path distances on each trial across 10 days of acquisition. If a manipulation is biasing rats to use a cued strategy to find the escape platform (i.e., relying on the landmark or black ball), the experimental group should have shorter mean swim path distances as compared to controls on the first trial of each daily session in which the platform is located in a new position and the most effective means to locate the hidden escape platform is to use the landmark. Conversely, if a manipulation is biasing rats to use a spatial strategy, the experimental group should outperform the control group by the fourth trial of each daily session indicating more efficient use of extra-maze cues.

Following the 10-day acquisition period, 1 day of probe trials can be conducted in order to determine the extent to which rats used the landmark to locate the escape platform. The four daily probe trials are identical to the acquisition trials with one exception. During the probe trials, the landmark (the floating black ball) is removed. Swim path lengths across trials 1–4 of the probe trial can be compared to trials 1–4 on Day 10 of acquisition. If rats are predominantly relying on a cued strategy (i.e., the landmark) to find the maze, probe trial performance will be significantly worse as compared to Day 10 of acquisition. However, if rats are predominantly relying on a spatial strategy (i.e., extra-maze cues), probe trial performance should not differ from performance on Day 10 of acquisition.

6.2 Scoring the Morris Maze

How does an experimenter measure performance in the Morris maze? The simplest measure of platform localization is time, or latency, to reach the platform. As days progress there should be a decrease in latency to reach the platform. Speed (distance/time), referring to how fast the animal is moving across the water to the
platform location, can also be used as the dependent variable to measure performance. However, latency and speed can be influenced by other variables such as age and treatment [22]. Thus, studies examining a variety of factors and manipulations can be confounded by measuring latency and speed, since many factors and manipulations can impact these dependent measures.

A more accurate way to measure performance on the Morris maze is to measure swim distance to the platform. This obviates many potential effects by other variables being evaluated or manipulated. Optimally, distance would be interpreted in the context of latency and speed to get a complete picture of performance (see Figs. 3 and 4). This is critically important to understand a more comprehensive profile of your animals, and it can yield much useful information. You should especially note dissociations between latency and distance. I (HBN) have reviewed many papers that have missed or misinterpreted critical findings because of the focus on latency. We will use a drug manipulation as an example here, but take note that this situation can apply to any time you are comparing groups of animals (e.g., different genotypes, ages, hormone states, etc.). Indeed, in my (HBN) own laboratory, in addition to noting dissociations between latency and distance with certain drug manipulations, I have also seen these dissociations with some gonadal hormone manipulations as well as when assessing effects of aging. Some drug manipulations result in a slower swim speed. Thus, this can yield an animal that swims a direct path to a platform, in fact just as direct as the control group; however, because this drug initiates slower swimming, the latency to the platform will be higher than the control group, despite the fact that the distance will be comparable to the control group (note that the scientific reason for the slower swimming is an important, but different, physiological question altogether). Thus, in this case the animals travel the same direct path to the platform, but take longer to get there. If we were to measure latency only, this would result in the interpretation that the drug impaired cognitive performance. However, if we were to measure distance only, it would result in the interpretation that the drug had no impact on cognition. If we consider latency and distance together, this would result in interpretation that the drug had no cognitive impact for spatial reference memory, but it did result in slower swimming. If the researcher is interested in potential drug effects on the motor system, this dissociation between distance and latency implies further study and additional research could yield valuable insights.

At the end of the regular testing trials, a probe trial is given to determine spatial localization of the platform. Figures 3 and 4 detail examples of performance and interpretation of the probe trial for the Morris maze. During this last trial, the platform is removed and the animal is allowed to swim in the maze for the
full 60 s trial. Animals that learn the location of the platform will traverse that location many times. The most common way to measure localization of the platform during the probe trial is to examine total percent swim distance in the quadrant where the platform was compared to the total percent distance in the diagonally opposite quadrant. Animals that learn the platform location should bias swimming to the previously platformed quadrant, relative to the other three quadrants. In particular, they should swim very little in the opposite quadrant, as animals would never have to enter the opposite quadrant to escape. Platform crossings can also be measured during the probe trial. This reflects a more challenging measure as it examines the number of times animals crossed the exact platform location in the absence of the platform. Specific localization and patterns of swimming during the probe trial, when the platform has been removed after learning has occurred, can yield exciting and meaningful results between different groups of interest, such as different genotypes, varied drug manipulations, or brain lesion effects. For example, if on the probe trial an animal swims a large percent of its total distance in the target quadrant that used to contain the platform, but a low percent of its total distance in the quadrant opposite of the quadrant that used to contain the platform, this tells us that the animal knew the quarter of the maze where the platform was; in other words, it knew the general vicinity of the platform location. In my (HBN) experience, most animals (at least those with a functioning hippocampus) do localize to the previously platformed quadrant. However, when looking at a smaller zone than the quadrant, one that is just around the platform (we use a total of 20 cm diameter, centered around the platform location), this allows us to differentiate animals that could find the quadrant but not the more particular platform location. Even more specific still, a quantification of platform crossings allows us to visualize which animals knew exactly where the platform location was. This could be interpreted as, for example, being able to navigate to the correct town (the quadrant), or the correct street (the zone area directly around the platform), or the exact house (the platform).

One last thing to note regarding the probe trials... we (HBN) have noted that some animals that learned very well, for example showing significantly decreased distance and latency scores across days and a steep learning curve, localize search during the probe trial in the platform quadrant and zone directly around the platform, and show numerous platform crossings. However, this typically occurs only during the first 30 s or so during the 1 min long trial, as shown, for example, in Acosta et al. [1]. In fact, animals generally tend to localize search to the platform location initially, but then move on and swim in other locations. If one presumes the animal has learned the rule that
there should be a platform somewhere in the maze, and the platform is now not where it was expected to be, then the animal may be looking for the platform in a new location. This could be interpreted, really, as a “smarter,” more adaptable, and flexible strategy. How do we deal with this complexity when interpreting probe trial data? One way to deal with this situation is to analyze the probe trial in time bins. We (HBN) have found that two 30 s time bins works well, and in fact this removes some of the variability across groups for the probe trial analysis when using all 60 s together. Typically the data are much “tighter” and show less variability in that first 30 s time bin, as compared to the total 60 s probe trial time period.

7 The Barnes Maze

The year 1979 brought the groundbreaking publication, “Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat” by Carol Barnes [6]. In this paper, Barnes describes the circular platform task, which is now referred to as the “Barnes maze.” Barnes’ vision for this task was to test temporal lobe functioning in rats. The apparatus and protocol is clever, creative, and resourceful. The apparatus is a simple round 1.22 m large platform with 18 exit holes that are 9.5 cm in diameter, equally spaced around the perimeter of the platform. The holes serve as “choices” to the animal. This task capitalizes on the known tenet that rodents have a preference for dark tight spaces as compared to bright open spaces, and therefore avoids the use of food or water deprivation and shock to motivate animals to perform. Under predesignated “correct” exit holes is an escape box that is “safe,” and serves as the reinforcement after a correct response. Each incorrect hole choice has a false or blind end, so there is no escape from the circular platform. Thus, the Barnes maze is a large open arena with no walls or barriers. Importantly, the platform spins so that it can be rotated after each choice to dissociate the odor cues derived from the animal’s prior path and any other intra-maze cues (such as scratches on the platform, for example) from the spatial cues/escape holes. In the initial publication, the escape tunnel was in the same place in space for the first portion of testing, thereby evaluating spatial reference memory. This protocol lends itself well to test spatial reversal learning, whereby the correct hole is moved to a different place in space and animals must break the old association of where the exit hole is in space, and form a new one (described in [6]). This task can be tapped to test working memory by altering the protocol used with the apparatus, making the correct exit hole location varied across trials so frequent updating is necessary to solve the task successfully.
Many of the variables measured in the Morris maze can also be assessed in the Barnes maze, including latency to the correct choice, distance traveled, and speed. Learning of the task should be evaluated by decreased latency or distance to the correct hole across days. Furthermore, errors can be assessed by quantifying the incorrect hole choices. Errors should decrease across days if learning has occurred. Potential group comparisons of distance, latency, and errors can each be evaluated to quantify performance differences between groups that have had manipulations of interest. As with the Morris maze, care must be taken to dissociate facets of performance that could skew interpretation, such as thigmotaxic behavior (for an example of appropriate interpretation see: [40]).

In the seminal paper it was noted that initially animals tended to make many incorrect hole choices with many returns to the center after each choice [6]. After multiple trials, animals began to search from hole to hole more readily, without frequent revisits to the center, and then animals went directly to the correct hole right from the start point; errors that occurred at this point were usually limited to incorrect hole choices that were in the near vicinity of the correct holes, that is, one or two holes away from the escape hole [6]. Overall, the search sequence became more efficient and accurate as trials progressed and learning occurred.

Of further note, research has shown that successful performance on the Barnes maze depends on the presence of many extra-maze spatial cues, and that hippocampal lesions impaired performance [7, 43]. While the majority of the research using the Barnes maze has used rats, the task has been adapted for use in mice as well.

### 8 The Radial-Arm Maze

#### 8.1 A Land Version of the Radial-Arm Maze

The land radial-arm maze, made iconic by David Olton in the 1970s [50], is based on the sunburst maze used by Tolman (see Chap. 1), and consists of equally spaced arms radiating out from a center hub. The goal for the animals is to find food rewards located at the end of each arm. The appetitively motivated land maze takes advantage of natural foraging strategies of rodents, in which they tend to avoid places where they have recently depleted a food source. The challenge for the animal as it navigates the maze is to remember which arms it has visited. The land radial-arm maze can be used in similar configurations as the water escape version of the radial-arm maze to test various types of memory, including working and reference memory (see the protocol section at the end of this book for more detail).

Although eight-arm radial mazes are most commonly used, more challenging mazes with up to 17 arms [5] have been successfully used to assess learning and memory in rodent models. Commercially available radial-arm mazes are constructed out of...
various materials, but the materials should allow clear views (from the rodent’s visual perspective) of extra-maze cues that are used to navigate this spatial maze task. To begin a trial, animals are placed in the center hub and are then allowed to freely enter arms to find food rewards. A trial continues until the rodent has found all rewards or until a specified time limit elapses. We (JD) have found 5 min to be a reasonable time limit to terminate trials. To avoid the development of bias in the order of arm choices, we systematically change the orientation of the rodent each day as we place it into the hub to start a trial. Although automated tracking systems are available for radial mazes, we (JD) find hand scoring to be more efficient for this land maze (HBN agrees as well, with reference to the water radial-arm maze). An experimenter, located at a fixed location in the room, records arm choices in real time as the animal enters arms. We define an entry as when a rat crosses the halfway point in an arm. A specified definition of an arm entry is important, as it is common for rats to display vicarious-trial-and-error (VTE) “peeking” behaviors (discussed in more detail below) as they navigate radial-arm maze. Such peeking into arms should not constitute an entry. An error is considered a reentry into a previously entered arm. Rats become very proficient at this land task. We have found that rats reach asymptotic performance, which is a mean of less than one error per trial, in 20–24 days.

Following 20–24 days of training on the maze, delay trials can be conducted during which various delays are placed between the fourth and fifth arm choices. Delay trials allow for repeated testing and provide a greater challenge for the animal as they are required to remember which arms have been entered over increasingly longer delays. During a delay trial, an animal is allowed to visit four arms of its choice, after which it is removed from the maze and placed in a holding cage. After the delay period, the animal is returned to the maze to search for the remaining four rewards. We begin delay training with 1 day of habituation to the procedure using a 1-min delay between the fourth and fifth arm choices. We then institute increasingly longer delays between the fourth and fifth arm choices. The length and number of delays vary depending upon performance on the previous delay as well as practical issues relating to experimental manipulations. We have instituted delays from 1 min to up to 6 h in length [66] with success.

In the late 1990s, Victor Denenberg’s laboratory created a non-automated, win-shift water escape version of the radial-arm maze, designed to efficiently assess working memory and working memory load as items of spatial information incrementally increase along with trial progression [8–10, 33, 34]. The maze is constructed of galvanized steel or plexiglass and filled with water, maintained at room temperature (18–20 °C). It has hidden escape platforms at the ends of the correct arms. The testing room has salient extra-
maze cues that remain constant throughout testing, including the experimenter who sits or stands behind the start arm. The animal is released from the start arm, facing the center, and searches for the platform. If the allotted time expires, the subject is guided with a rod, remaining in the water, to the nearest available platform. Once a platform is found, the animal remains on it for the time dictated by the protocol, and is then returned to its heated home cage until its next trial. During the interval, the just-chosen platform is removed from the maze; this means that the working memory load increases because now animals have to remember the arms that no longer contain a platform, and shift to the arms that still contain a platform. The animal is then placed again into the start arm and allowed to locate another platform. A daily session consists of this sequence of events repeated until all platforms are located. Consequently, for each animal a daily session consists of multiple trials per session, with the number of platformed arms reduced by one on each subsequent trial. Thus, for example, seven arms contain platforms on trial 1, six arms contain platforms on trial 2, etc. This pattern continues so that by trial 7, only one arm contains a platform. Since one platform is removed after every trial, one more item of information needs to be remembered after every trial.

For most studies with rats, animals are tested for 12 days. Each subject is given one session a day, for 12 consecutive days. Day 1 can be considered a training session because the animal has no previous experience in the maze. Days 2–12 are testing sessions. Since we (HBN) previously noted in several studies that errors appear to substantially decrease by day 8, we typically divide the data into the acquisition phase (days 2–7) and the asymptotic phase (days 8–12). This has proven to be a fruitful procedure, yielding much insight into group differences during learning and acquisition of the task, versus the asymptotic portion of the task, measuring primarily memory once task requirements have been learned [10, 34]. However, the data are the best guide in indicating which days will be considered acquisition and asymptotic, as different cohorts will learn at different rates and may require more or less days. Mice can sometimes take longer to learn this maze task; in some cases, we have extended water radial-arm maze testing to 15 days for mouse studies.

For both the land and water radial-arm maze, as an animal progresses through a session, the number of previously reinforced choices, and thus locations to be remembered, increases. Hence, the ability to handle an increasing memory load is required to perform successfully. However, since radial-arm maze data are sometimes summed over choices (or trials) within a session, especially in land versions, the investigation of how groups differ in the ability to handle an increasing memory load is not always addressed. We (HBN) have used the win-shift version of the water radial-arm maze to

8.3 Working Memory Load

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systematically and directly assess memory competence as working memory load increases. To determine when during a session errors are made, we determine the number of errors committed during each trial within each session. This allows evaluation of group differences in working memory competence as trials progress and the working memory load increases.

The land and water versions of the radial-arm maze can be used to assess both working and reference memory simultaneously by placing food or platforms in only a subset of the arms. Each subject has different reward (food or platform) locations that are semi-randomly determined, and that remain fixed throughout the experiment. There is never a reward in more than two adjacent arms nor in the arm from which the animal is released. To solve this version of the task successfully, an animal must learn: (1) to avoid arms that never contain a reward (reference memory arms); this is task-specific information since arms that do not contain platforms remain constant across all testing days, and (2) to visit arms that contain a reward only once (working memory arms); this is trial-specific information since it must be updated every time an animal locates a platform.

For this combined working and reference memory version of the maze, there are many valuable ways to quantify errors to inform the scientist how an animal is performing. Errors have been quantified for each daily session using orthogonal measures of working and reference memory errors, as described by Leonard Jarrard [35], and used by our (HBN) laboratory and others [2, 3, 10, 13, 16]. Working Memory Correct errors are the number of first and repeat entries into any arm from which a platform has been removed during that session. Reference Memory errors are the number of first entries into any arm that never contain a platform. Working Memory Incorrect errors are the number of repeat entries into an arm that never contain a platform (thus, repeat entries into a reference memory arm).

Procedures for the working and reference memory version of the maze are similar to the version of the maze testing working memory only. We will use the four out of eight arms platformed water escape version here as an example. A subject is released from the start location, facing the center, and searches for the platform. If the allotted time expires, the subject is guided with a rod, remaining in the water, to the nearest available platform. Once a platform is found, the animal remains on it for its platform time, and is then returned to its heated home cage until its next trial. During the interval, the just-chosen platform is removed from the maze. The animal is then placed again into the start alley and allowed to locate another platform. A daily session consists of this sequence of events repeated until all four platforms are located. Consequently, for each animal a daily session consisted of four trials per session, with
the number of platformed arms reduced by one on each subsequent trial. Thus, four arms contain platforms on trial 1, three arms contain platforms on trial 2, two arms contain platforms on trial 3, and one arm contains a platform on trial 4. Since one platform is removed after every trial, one more item of information needs to be remembered after every trial; the working memory load increases as trials progress within a day. This version tests the memory for four spatial locations (as compared to seven for the working memory only version). We have also given extended delays (30 min for mice, 6–8 h for rats) between trials 2 and 3 to evaluate the ability to remember multiple items of information across a delayed temporal interval. See the protocol section at the end of this book for specific instructions for testing.

In the traditional task, intra-maze and extra-maze cues remain coupled throughout testing such that animals can use either, or both, cue sets to solve the task. Although we (JD and HBN) and others typically provide no obvious intra-maze cues, it is still possible that animals solve this task by using cues such as odors, scratches on the maze, or some other internal cue that is not obvious to humans. Hence, our (HBN) laboratory wanted to determine whether animals did in fact rely on extra-maze cues to solve the water radial-arm maze task, and, since our questions usually involve hormones, we also wished to determine whether hormonal milieu in adulthood affected such cue dependence. Adult female rats receiving sham, ovariectomy, or ovariectomy plus estrogen treatment were tested on the working and reference memory water radial-arm maze for 12 days, with four of eight arms platformed, followed by a platform rotation procedure designed to test cue dependence. The platform rotation procedure was based on the methods of several studies using the land radial-arm maze [38, 50].

By the last day of testing (day 12) both working and reference memory errors were low. Thus, subjects had learned not to enter an arm where a platform had previously been located (a working memory arm), and not to enter an arm that never contained a platform (a reference memory arm). The procedure for trials 1 and 2 on day 13 was the same as the testing procedure on days 1–12. However, after the completion of trial 2, when two platforms had been chosen and two platforms remained, the intra-maze platform configuration was rotated such that one platform was now in a previously chosen working memory arm (an already chosen extra-maze location), and one platform was in a reference memory arm (an extra-maze location that never corresponded to a platformed arm). Thus, the relationship between the two remaining platforms was identical to the relationship before the rotation. In addition, for trials 3 and 4, animals were released from the start arm, which corresponded to the internal platform configuration. Therefore, the internal configuration of the platforms and start location was
kept constant, but extra-maze information no longer coincided with this intra-maze information. Errors were scored as if following intra-maze information were correct. If animals solved the task following intra-maze information without referencing extra-maze cues, errors should not increase when extra-maze and intra-maze information were dissociated by platform rotation. Conversely, if animals found the platform location by using extra-maze information, there should be an increase in errors after platform rotation. This increase would occur since extra-maze cues that once corresponded to a platformed arm no longer do, and extra-maze cues that never had a platform now do.

We (HBN) found that errors increased from day 12 to day 13 for all groups, demonstrating that platform rotation was detrimental to performance, in turn indicating that animals referenced extra-maze cues to locate platforms. Statistical analyses revealed that there were no significant group differences in the error increase from day 12 to day 13, suggesting that all groups were similarly affected by platform rotation. Thus, all groups appeared to reference extra-maze cues to solve the task, animals did not locate platforms by visualizing platforms or smelling the platforms or odor trails, and internal platform patterns were not effectively utilized to solve the task. Other detailed inspection and quantifications of water radial-arm maze data have shown that rats and mice do not make the same pattern of arm entries from trial-to-trial, or from session-to-session, and platforms are not chosen in any discernable pattern (at least to humans…) within a day. Further, in an identical procedure but with visible platforms, mice did not use extra-maze cues to solve the task, and they learned the task more quickly than mice tested using hidden platforms, as expected [32, 33].

9 Rodents Exhibit “Decision-Making” Behaviors During Maze Testing

While in graduate school conducting water radial-arm maze experiments in rats, I (HBN) noted that during testing many of the rats “peeked” into one or more maze arms before entering an arm, and this peeking behavior occurred most frequently during the middle testing sessions when errors started to decrease. After discussing this with my mentor, Victor Denenberg, he recognized this behavior and sent me to the classic literature of the 1920s–1940s. Indeed, this behavior displayed by the rats appeared remarkably similar to the choice point behavior exhibited during discrimination learning tasks, as originally described by Tolman [54, 56] and Muenzinger [49]. Tolman and Muenzinger each noted that at a choice point, rats hesitated and turned their head back and forth between the stimuli before committing to a choice. This behavior has been termed VTE (see above for discussion with relevance to land radial-arm maze testing) [29].
VTE has been suggested to reflect an animal’s conflicting inclination to approach or avoid a choice point, is related to cognitive competence, is affected by the spatial angle and geometric form of the cues to be discriminated, and varies as a function of hippocampal integrity [4, 24, 29, 30, 31, 55, 57, 56, 58]. Olton and Samuelson [50] noted a VTE-type behavior in rats performing on a land radial-arm maze. This choice point behavior, however, was not quantified. Brown and colleagues [14, 15] have also investigated VTE-type behavior (which was termed “microchoices”) in trained rats performing on a land radial-arm maze.

We (HBN) quantified VTE behavior in male and female rats while they were learning the working memory version of the water radial-arm maze. The sexes differed markedly in VTE behavior. First, females made more VTEs overall. Second, females increased VTEs over the beginning testing sessions and decreased VTEs over the latter testing sessions, resulting in an inverted U-shape function, while males did not exhibit any particular pattern of VTEs across sessions. Further analyses revealed that the sex difference was a result of females VTE-ing into platformed arms more than males, and that as trials increased males selectively VTE-ed into unplatformed arms, while females VTE-ed into both arm types. As such, males must have been able to distinguish unchosen (platformed) arms from chosen (unplatformed) arms before VTE-ing. Since females VTE-ed into both platformed and unplatformed arms, they may not differentiate unchosen from chosen arms until they VTE. Thus, VTEs may facilitate arm identification in females but not males. Consistent with this, VTEs were positively correlated with errors in females, but not males, during the latter portion of testing. Additional work for my doctoral thesis (HBN) showed that VTE behavior is comparable in adult female rats that had ovarian hormone levels manipulated [10].

Tolman [57] has proposed that animals use VTE to actively select, sample, and compare the stimuli guiding choice behavior. To solve the land radial-arm maze, female rats have been shown to attend to both room geometry and landmark cues, whereas males primarily attend to room geometry when both geometrical and landmark cues are available [62]. Thus, females may VTE more than males because they utilize more types of environmental information while learning the radial-arm maze. VTEs may aid females in accumulating and incorporating the several types of cues they need to solve the task efficiently. This interpretation of the VTE findings is further supported by the platform rotation data described above indicating that females, regardless of estrogenic status, utilize extra-maze cues to solve the water radial-arm maze task.

One of the advantages offered by mazes incorporating choice points like the water radial-arm maze or a T-maze (although there are fewer choice points) is that VTEs can be examined. Mazes consisting of an open arena like the Morris maze or the Barnes maze...
do not offer this type of measure, as the animal is not forced to make a distinct arm choice and therefore there is no choice point, per se. Rather, for these mazes, animals are able to swim or walk in the arena without necessarily committing to a specific arm or alley. Animals can know the general vicinity of the platform or exit hole and still easily find an escape. On the water radial-arm maze, however, a wrong arm entry means having to go into a completely different arm, which may extensively prolong escape.

10 The T-Maze and the Plus-Maze

The T-maze, as its name implies, is shaped in the form of a T. It consists of a start arm that terminates at a choice point. The maze is normally made of plexiglass or stainless steel, and can be used as an appetively motivated task whereby animals are tested in a dry maze with food deprivation and food given as a reinforcement, or as a water escape task whereby hidden platforms are located at the ends of the two T-choice arms. This maze can be used to test spatial (extra-maze cues) or nonspatial (intra-maze cues or turn strategy), or working memory (updating correct location) or reference memory (correct location remains constant), protocols.

The T-maze is often employed to test delayed matching-to-position (win-stay) measuring working memory and delayed retention. For example, Gibbs [25] trained rats by administering eight trial pairs per day. The first trial of the pairs was a forced “choice” trial with one goal arm blocked off, forcing the animal to enter the arm containing a food pellet reward. The second trial occurred immediately after the first, with both of the arms accessible to the animal. However, an animal was only rewarded if it returned to the same arm it was previously rewarded in during the first trial (thereby making this a win-stay task). Entering the incorrect arm resulted in no food reward and confinement for 10 s. The rewarded forced trial varied randomly and arms were wiped down between trials to minimize odor cues. Animals were returned to their testing cage after each trial pair, and other animals were tested on that trial. Thus, the inter-trial interval was about 5–10 min (enough time to test other animals in the squad). Testing continued until animals reach a criterion of 15/16 correct choices over two consecutive days. After animals had acquired the task, increasing delays were given between trial 1 and trial 2 (10, 30, 60, 90 s).

Although the T-maze is a seemingly simple task, a disadvantage to its simplicity is that animals can relatively easily use a nonspatial response strategy to solve the task, even though the experimenter might want to test spatial memory. Because the maze only contains two choice points, in some cases an animal remembers which turn it took on the forced trial, and then uses a response strategy (e.g., using turn direction) instead of a place strategy (e.g.,
using extra-maze cues). To examine whether animals are using a place or response strategy on the T-maze, the maze can be rotated on the test trial (trial 2). Animals will either use the cues to go to the place in space where reinforcement occurred, or they can take the same turn used previously on the forced trial. Thus, on this task, both strategies are possible. Gibbs and colleagues [26, 36] demonstrated, in females and males, that cholinergic integrity is necessary for learning the delayed matching-to-sample T-maze task, as cholinergic lesions in the medial septum and vertical limb of the diagonal band of Broca impaired acquisition of the maze. More specifically, cholinergic neurons are likely involved in using a place strategy to solve the task as basal forebrain cholinergic lesions increased perseveration of a response strategy over a place strategy on the delayed matching-to-position T-maze [21, 27]. Moreover, cholinergic lesions increased the amount of time rats persisted with the response strategy before adopting the spatial strategy.

While the radial-arm maze typically has eight arms or more with rewards, and the T-maze has two arms with rewards, there are some researchers that have chosen an intermediate task by using a maze with four arms. This is typically called a plus-maze. It is a symmetrical plus-shaped maze constructed similarly to both the radial-arm maze and the T-maze, made of stainless steel or plexiglass. Procedures are notably similar to the T-maze described above. Spatial cues are placed throughout the room to enable spatial navigation, if spatial evaluation is the goal. The animal is placed within a start arm and allowed to walk or swim through the maze to seek the food reward or hidden platform. Training can take place across several days, or all trials can be given in one day; the latter has been done in the laboratory of Donna Korol, and results have been clean and replicable [37]. In this massed trial task, rats were given a 3 min maximum latency to find the arm with the food reward, and training trials continued until the criterion, 7/8 arms was reached or to 100 trials. In this case, Korol and Kolo [37] were interested in examining whether the estrogen 17β-estradiol biases the strategy used to solve a task, so their question focused on hormone milieu within one day. Using the plus-maze, rats were trained on either a place task requiring the animal to find food in a fixed location of the goal arm using extra-maze cues, or a response task for which the animal had to make a specific arm turn (right or left) to find the goal arm. Results demonstrated that for the place learning task, ovariectomized female rats receiving 17β-estradiol 48 and 24 h prior to testing showed enhanced performance relative to rats receiving vehicle. However, on the response task, the opposite pattern was observed, with 17β-estradiol impairing performance on the response task, suggesting that 17β-estradiol biases animals to use a place, rather than response, strategy. The cholinergic system may also be interacting with 17β-estradiol treatment, as the Korol
laboratory later showed that 17β-estradiol also potentiated acetylcholine release during place learning [42]. In this regard, Robert Gibbs also found that 17β-estradiol enhanced acquisition of the delayed matching-to-place T-maze task in ovariectomized female rats, but 17β-estradiol had no effects in rats with cholinergic lesions, suggesting cholinergic integrity is necessary for 17β-estradiol-induced effects [26].

We (HBN) have utilized a delayed match-to-sample task using a water version of the plus-maze ([1, 2]; see also the protocol section of this book for detailed testing procedures). This task measures spatial working and short-term memory retention using a win-stay (within a day) strategy. As detailed in the protocol section at the end of this book, the water radial-arm maze apparatus can be used for this task. The maze is constructed of black plexiglass and filled with water made opaque with black nontoxic paint. There is a hidden platform at the end of one of the four arms. The start location varies across trials and the platform location changes every day. Within a day, the platform remains in the same spatial location. Animals receive six consecutive trials within a daily session. Trial 1 is the information trial informing the animal where the platform is on that day, trial 2 is therefore the working memory test trial, on which information needs to be updated, and trials 3–6 are recent memory test trials since this information is recent but does not need updating per se. Animals are given a certain amount of search time to find the platform (we use 90 s). Once on the platform, the animal remains on it for its allotted platform time (we use 15 s), followed by placement into a heated cage for an inter-trial interval (we use 30 s). Entry into any non-platformed arm is counted as an error and the total number of errors is analyzed for each trial. Prior to the published Acosta et al. [1] study, we conducted several pilot studies to optimize maze acquisition parameters. We found that animals were inclined to swim straight ahead on the first trial. When the start arm for trial 1 was a straight swim for the platform, rats perseverated on the straight swim response strategy on the later trials. Thus, we devised rules so that animals would have to take a turn to locate the platform. We also noted that animals perseverated on the start arm if the platform had been in that arm the previous day. This is not surprising since the animal had been reinforced to escape in that particular arm the previous day for six trials. To optimize the maze protocol and correct for these observations which could lead to nonspatial solving strategies, we initiated the following rules: only use two of the same start locations within the maze, the start arm pattern cannot be the same across days, the platform cannot be where the platform was yesterday, the start arm cannot be a straight swim from the platform, and the start arm for trial 1 cannot be where the platform was on the previous day.
After animals demonstrate learning and stable performance on the delayed match-to-sample plus-maze, delays can be introduced to test memory retention. We (HBN) have noted that plus-maze training typically takes between 5 and 7 days of testing (there can be variations in markers of successful learning depending on certain factors such as age, hormone status, etc.). For one of the first studies using this maze [1], we trained animals for 5 days at a 30 s inter-trial interval, and increased time delays were given on subsequent days. Four- and six-hour delays were initiated between trials 5 and 6, to assess retention of recent memory. Because these delays did not influence performance, we then gave delays between trials 1 and 2 after only one exposure to the correct platform location. Indeed, since the second trial is the first trial to test recall of the updated information (working memory), the next series of delays were given between trial 1 and trial 2 to determine whether the increasing delays impacted memory retention. Using this procedure, delays of 4-, 6-, and 7-h were given to rats. After the 7 h delay, rats were given a probe trial after trial 2, whereby the platform was removed. This was initiated to confirm that animals localized the spatial location of the platform, and that an animal’s choice was not due to platform visualization or another strategy, i.e., response strategy. Of note, for mice, the delays should be much less; typically 30 min is the maximum delay mice can withstand on this task and still show some memory for the reward location.

Studies done with human subjects demonstrate that experimental interference can decrease accuracy of memory recall. Classically, interference trials have been administered to people to examine processes of forgetting in short-term memory, which is driven by limited capacity [59]. Some animal research has imposed task-related interference on passive avoidance, operant behavior, and visual discrimination tasks [19, 20, 63–65]. We (HBN) performed a series of interference manipulations with the delayed match-to-sample plus-maze using an alternate room to conduct the interference trials [1]. We gave the information trial in the original room, rats received interference trials in a new room on an identical maze with different spatial cues, and were then brought back to the original room for the test trial. Performance on the test trial in the original room was a measure of retroactive interference, with new information interfering with previously learned information. Each rat received an information trial in the original room, was immediately transported to the new room for one interference trial, and then was transported back to the original room for the test trial. The last interference test tested susceptibility to proactive interference, or to more retroactive interference trials. Rats were administered three consecutive trials in the original room (this was the proactive interference, previously learned information interfering with new information being learned), followed by three consecutive trials in the new room.
trials in the new room (here the first trial was the “information trial” and the second trial was the proactive interference “test trial”). Next, the rat received the test trial in the original room (this was the retroactive interference “test trial”). Three trials were given in the new room so that there were three trials of retroactive interference before the retroactive interference test trial in the original room. Since the parameters that were optimal for spatial performance on the delayed match-to-sample plus-maze limited us to use only two of the four arms as the start arms (one arm contained the platform and the other arm was a straight swim from the platform, leaving only the other two arms animals could turn into), an asymmetrical maze with four arms was constructed. This new asymmetrical version allowed for use of all three non-platformed arms as the start location (see the protocol section at the end of this book for more information).

11 Object Recognition

Object recognition is tested in an apparatus that is an “open field,” or simply put, an empty dry arena. This task is not a maze per se. Object recognition is sometimes used to test memory as the sole task in some manuscripts, and it has been used as part of a battery of memory tests as well. This task is relatively time efficient, is being used with more frequency as of late, and it can yield very useful information. It is discussed in more detail in Chap. 7 by Fortess and Frick, along with some excellent discussions of caveats, cautions, and interpretations of scores. The reader is referred to this chapter for more information on object recognition procedures.

12 Reliability

In science, reliability is consistency of a measurement. In the context of behavioral maze research, reliability can be broadly defined as measuring rodent performance in a consistent manner for a given maze task. It is imperative that experimenters administering behavioral tasks are reliable with each other within, as well as across, studies. This minimizes error and reduces variability in the data, which allows for dependable and replicable results. In our (HBN) laboratory, we ensure that all researchers are supervised and trained in the same manner, strictly following guidelines laid out by the testing protocol. Each experimenter is required to practice extensively with non-experimental animals before approval from the principal investigator. Furthermore, before we begin behavior testing for each and every study, we have a reliability meeting, wherein all researchers in the laboratory, including undergraduates students, graduate students, technicians, post-doctoral fellows, and the principal investi-
gator (HBN) watch the tester(s) for the upcoming study run several mock trials with non-experimental animals. During this meeting, we verify that all procedures among testers are identical. This includes knowledge of the task rules, proper animal handling for the maze, arm entry scoring (if applicable), and overall consistency in daily setup of the maze room. To do this, the laboratory team runs through a written checklist at each reliability session to establish inter-rater reliability. In the framework of behavioral testing, inter-rater reliability is a consensus among experimenters for behavioral data collection procedures. For example, if there is inconsistency with regard to defining an arm entry, this could severely impact the overall outcome of the experiment. Even seemingly small details such as the tone and number of beeps made by the stopwatch during maze testing, or whether the nose versus the neck of the animal crossing the designated arm entry line constitutes an arm entry, could impact outcome scores if procedures are not identical for every maze tester. Therefore, it is an absolute necessity to establish inter-rater reliability for all details of behavioral maze data collection. In our (HBN) laboratory, we are sure to confirm that all of the researchers involved are knowledgeable about each step of the protocol. Producing reliable measures allows for replication within the laboratory, as well as the ability to compare research to other labs using the same task. Below we give examples from the inter-rater reliability quiz we give each tester before every maze study. The answers to these questions are in the protocol section at the end of this book.

Examples from the Bimonte-Nelson laboratory inter-rater reliability quiz for each tester:

- What constitutes an arm entry, exit, and reentry and how do you record this on the testing sheet?
- What do you do if an animal touches a platform and swims away?
- What do you do if an animal is floating?
- What do you do if an animal is in distress and is unable to keep its nose above water?
- What do you do if an animal does not find a platform during the allotted trial time?
- How long does the animal sit on the platform?
- What should you do during the time that the animal is on the platform?
- What do you do if an animal jumps off of the platform the first time?
- What should you do if an animal repeatedly jumps off of the platform?
• Approximately what temperature should the maze water be at the beginning of each testing day?
• Describe how you should clean the maze between trials and animals.
• Describe how you should clean the maze at the end of a testing day.
• Describe how you should clean the maze room at the end of a testing day.
• Describe how you should clean the colony room at the end of a testing day.
• What do we mean when we say “escapable heat” and why is it important?
• What do you need to remember about your daily habits at home while testing (i.e., showering, getting ready, interacting with things outside of lab)?

13 Summary: Using Rodents and Mazes to Answer Your Questions About Treatments and Factors Impacting Learning and Memory

Learning and memory processing is multidimensional and complex, and rodent mazes can tap the different stages and depths of this processing by varying maze types and protocols. In doing this, experimenters can answer their questions about how certain treatments and factors impact learning and memory. We can test detriments in spatial or nonspatial memory, and working or reference memory, evaluating acquisition, consolidation, or retrieval specifically, as well as performance when task demand is easy or elevated. How does my genetic manipulation impact learning and memory? How does my hormone of interest impact learning and memory? How does the drug I created impact learning and memory? How does the sleep pattern I noted impact learning and memory? The experimental questions that can be answered by testing rodents and mazes is only limited by nature, and by the creativity of the scientists seeking the truth within it. As treatments and factors that alter maze performance are revealed, the experimenter can evaluate whether those treatments and factors are also potent modulators of brain structure and function, including in brain regions well-documented to modulate learning and memory.

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The Maze Book
Theories, Practice, and Protocols for Testing Rodent Cognition
Bimonte-Nelson, H.A. (Ed.)
2015, XVIII, 457 p. 75 illus., 46 illus. in color., Hardcover
ISBN: 978-1-4939-2158-4
A product of Humana Press