Video Article

Use of an Eight-arm Radial Water Maze to Assess Working and Reference Memory Following Neonatal Brain Injury

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Abstract

Working and reference memory are commonly assessed using the land based radial arm maze. However, this paradigm requires pretraining, food deprivation, and may introduce scent cue confounds. The eight-arm radial water maze is designed to evaluate reference and working memory performance simultaneously by requiring subjects to use extra-maze cues to locate escape platforms and remedies the limitations observed in land based radial arm maze designs. Specifically, subjects are required to avoid the arms previously used for escape during each testing day (working memory) as well as avoid the fixed arms, which never contain escape platforms (reference memory). Re-entries into arms that have already been used for escape during a testing session (and thus the escape platform has been removed) and re-entries into reference memory arms are indicative of working memory deficits. Alternatively, first entries into reference memory arms are indicative of reference memory deficits. We used this maze to compare performance of rats with neonatal brain injury and sham controls following induction of hypoxia-ischemia and show significant deficits in both working and reference memory after eleven days of testing. This protocol could be easily modified to examine many other models of learning impairment.

Video Link

The video component of this article can be found at http://www.jove.com/video/50940/

Introduction

Working memory (WM) corresponds to a critical cognitive domain required for the representation of objects or places during goal directed behavior[1]. Alternatively, reference memory (RM) is required for temporally stable representations of those objects or places. Working and reference memory have long been assessed in rodents using land based radial arm maze paradigms[2,3]. However, these tasks frequently require pretraining, as rats are not predisposed to spontaneous maze running. This can significantly increase the time needed to complete an experiment and can interfere with time sensitive longitudinal designs. Other limitations of the land based eight-arm maze include requirements for food deprivation and difficulty controlling changes in scent cues left on the maze after each trial. Many of these limitations have been overcome by using the Morris water maze paradigm[4], however historically these designs have been limited to testing reference memory and spatial learning (exceptions[5-9]). The eight-arm radial water maze is a modified version of the eight-arm radial land maze, which has been used to assess both reference and working memory in rats and mice[5-7]. In contrast to traditional land based mazes, the eight-arm radial water maze does not require food deprivation, minimizes potential confounding scent cues and utilizes the subjects' motivation for escape as an effective means to assess working and reference learning and memory simultaneously without the need for pretraining[5-7].

One application for this paradigm is to test working memory deficits following neonatal brain injury. Research in humans has shown that preterm infants at risk for brain injury exhibit working memory deficits later in life[10,11]. Neonatal brain injury can be modeled in rodents by inducing hypoxia/ischemia (HI) early in postnatal development[12-15]. More importantly, spatial learning and working memory deficits found in at risk infants are paralleled in rodents using the radial arm water maze, making it a robust model for the study of such impairments[8,9]. The eight-arm radial water maze also allows for simultaneous quantification of reference and working memory making it ideal for the comparison of brain injured and noninjured subjects in time sensitive models (*i.e.* during discrete developmental widows) or for counter balanced longitudinal designs.

In this protocol, we describe testing procedures using an eight-arm radial water maze (see **Figure 1**) and example data for rats with and without neonatal hypoxic-ischemic injury. Hypoxia-ischemia (HI) was induced on postnatal day 7 following cauterization of the right common carotid artery and 120 min of 8% oxygen exposure. This surgical procedure has been extensively used to model pathology of prematurity and neonatal brain injury (for details see[12,13,16,17]). We show that the radial arm water maze paradigm reveals deficits in reference memory and working memory capabilities following neonatal HI injury. In the current protocol, reference memory is assessed using four specific arms of the eight-arm radial water maze, which remain constant to extra maze cues throughout testing. These arms never contain an escape platform, thus, subject entries into these arms reflect poor reference memory performance and deficits in long-term learning. The remaining four arms all contain escape platforms at the beginning of the day, however each platform is removed once the animal successfully enters an arm and escapes the water[5-7]. This requires the animal to remember which arms it entered over the four successive trials, increasing the working memory demand as

each additional platform is removed. Working memory is assessed by examining re-entries into arms previously used for escape within the same testing day or re-entries into reference memory arms. Additionally, this design utilizes extra-maze cues to locate the escape platforms reflecting hippocampal dependent spatial learning. This method for assessing working and reference memory errors was originally used by Jarrard *et al.*[18] and has since been used extensively to assess rodent models of aging related neurological disorders, learning disabilities and following hormonal manipulations[5,6,7,19,20].

The primary objective of this paradigm is to assess reference and working memory, thus, the eight-arm radial water maze design should not be limited for testing deficits due to developmental brain injury. Instead, previous studies using this paradigm show that a variety of rodent models can be evaluated to assess pathology of learning and memory across many experimental contexts[5,6,7,18,19,20].

Protocol

All procedures were approved by the Rhode Island College Institutional Animal Care and Use Committee and adhere to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

1. Maze Configuration

1. Place clear, static, salient extra-maze cues (painted shapes, furniture or other stationary room features) around the maze.

   Note: Any extra-maze cues must remain in the same location for the duration of the experiment.
2. Place the hub and arms of the radial arm maze in a plastic tub (See equipment table for example).

   Note: The maze used in this protocol consists of eight removable stainless steel arms attached to eight central supports affixed to a circular stainless steel base which has been painted black (see **Figure 1** for diagram and corresponding dimensions). The plastic tub exemplified in **Figure 1** has an inner diameter of 122 cm and a height of 60 cm.
3. Fill the tub with water warmed to room temperature (22-26 °C)[7].
4. Place platforms 1 cm below the water surface at the ends of four of the eight arms.

   Note 1: For each subject the platform locations are fixed for the duration of testing. Regardless of the configuration, care should be taken to avoid placement of the platforms in more than two adjacent arms (no more than two platforms should be located in adjacent arms). Do not place a platform in the starting arm.

   Note 2: The hidden platforms must be painted the same color as the maze insert to blend in with the maze background.

2. Transferring Subjects for Testing

1. Transfer subjects from the vivarium to the testing room maintaining the designated testing order.

   Note: Animals should be placed into an opaque cage to help reduce anticipatory stress and prevent viewing of testing room cues.
2. Once in the testing room, place all of the opaque cages from group 1 onto a staging bench or table maintaining the testing order.

   Note: In a large study (*e.g.* 30 subjects) it may be best to break the subjects into testing groups (*e.g.* 3 groups of 10 subjects each). This will depend on the size of your testing room and the feasibility of subject transport.

3. Digital Tablet Protocol for Recording Maze Arm Entries

1. Open a PDF writer program on the digital tablet.
2. Import all presaved data recording templates for subjects in group 1.

   Note: Each recording template should be presaved and labeled with specific subject and trial numbers (*e.g.* subject 1_trial 1, subject 1_trial 2, *etc.*) with a total of four templates for each subject (trials 1-4). The template should have a blank representation of the maze and platform locations as well as a place to record the subject number, trial number, testing day, escape platform used and trial latency (see **Figure 2**).
3. Open the template for the first trial of your first subject.

   Note: This system can also be used with paper templates for researchers without access to tablets or tablet based note-taking programs.

4. Radial Arm Water Maze Testing

1. If animals are pair housed, separate the subjects prior to the testing session. Transfer the first testing subject into a separate opaque warming cage.

   Note: The cage should be warmed to 37 °C using heating pad to maintain body temperature between testing trials.
2. Prior to the first trial of each subject, confirm that the subject identification number matches the subject number on the data recording sheet and ensure that platforms are correctly positioned in the eight-arm water maze.
3. Place the subject into the start arm facing the wall of the maze and start the timer.

Note: This protocol was designed for two experimenters, one to record the data and one to handle the subjects. During this collaboration, communication between the experimenters is paramount. Confirmation of start times, subject numbers, and proper platform placement prior to each trial is essential.

4. Remain stationary during each trial in a designated position in the room to maintain cue consistency during each trial.

   Note: Care should be taken to avoid salient sent cues (*e.g.* perfumes) during the experiments and the experimenters should wear the same garments (lab coats) each day to maintain cue consistency.
5. Record each entry into an arm by placing a mark on the corresponding arm in the recording template on the digital tablet.

   Note: An entry occurs when a subject's shoulders break the opening plane of the arm.
6. Allow a maximum of 120 sec for the subject to locate one of the four platforms. Once the animal reaches the platform, stop the timer, the trial is complete.

   Note: To maintain consistency, a subject is said to have reached the target when its front paws are on the platform.
7. If the subject is unable to locate the platform within 120 sec, carefully guide them to the closest platform and allow them to remain on the platform for 10 sec before removal to ensure that the animal is aware of the target location.
8. *On day one only*, allow the subject to sit on the platform for 10 sec following each of all four trials so the subject can reference the spatial cues.
9. Following trial completion, remove the subject from the platform by gently lifting them by the body.
10. Begin the timer for the 90 sec intertrial interval.
11. Place the subject back into the individual cage over the thermoregulation pad.
12. Record the specific platform used for escape by circling the corresponding location on the data-recording template.
13. During this interval remove the escape platform used by the subject in the previous trial.
14. Open the template for the next trial and mark all platform(s) that have been used for escape by the subject, and thus removed during previous trials, with an **X**.

    Note: Marking all missing platforms prior to a subsequent trial is critical for defining error types (*e.g.* reference memory and/or working memory correct).
15. Prior to starting a subsequent trial, confirm the subject number, the trial number and the remaining platform locations.
16. After the 90 sec intertrial interval has elapsed, place the subject into the start arm facing the wall of the maze and start the timer for the next trial.
17. Repeat this process for trials 2-4, removing the escape platform reached after each trial and marking the template appropriately during and after each trial (*i.e.* place and X over removed platforms, indicate arms entered, and circle the platform used for escape).
18. Once all four trials for a subject have been completed, place the escape platforms back in the appropriate arms of the maze and repeat the testing process for the next subject (testing steps 4.1-4.17).
19. Once testing is completed for group 1, export the data recording sheets to a folder for that testing day (*e.g.* Day 1) and save it to a file storage system.

    Note: The data recording sheets should be exported prior to starting the next testing group to minimize the chance of data loss.
20. Repeat for each group (testing steps 2.1-4.19).

5. Dependent Variables

1. For each trial, count the number of first and repeat entries into any arm that previously had a platform. This will be indicated by an X over the platform on the data recording sheet. These are the working memory correct errors.

   Note: Because working memory correct errors are dependent on remembering the location of a platform that has already been used for escape and avoiding that arm, you cannot have a working memory correct error on the first trial.
2. For each trial count the number of first entries into any arm that never contained a platform from the data recording sheet. These are reference memory errors.
3. For each trial count the number of repeat entries into any arm that never contained a platform from the data recording sheet. These are working memory incorrect errors.
4. Record the latency to reach the platform for each trial.

Representative Results

Sample data from our lab show significantly more working memory incorrect errors in HI animals as compared to shams by day eleven of testing ($t = 2.124$, $p<0.05$, (see **Figure 3A**)). HI animals also showed significantly more reference memory errors as compared to sham subjects by the eleventh day of testing ($t = 2.303$, $p<0.05$, (see **Figure 3B**)). Working memory incorrect and reference errors include the total number of cumulative errors across all four trials for each subject on day eleven. These results support the validity of this task to assess working and reference memory in neonatal brain injury models and further support the use of this paradigm to assess other models of neurobehavioral pathology.

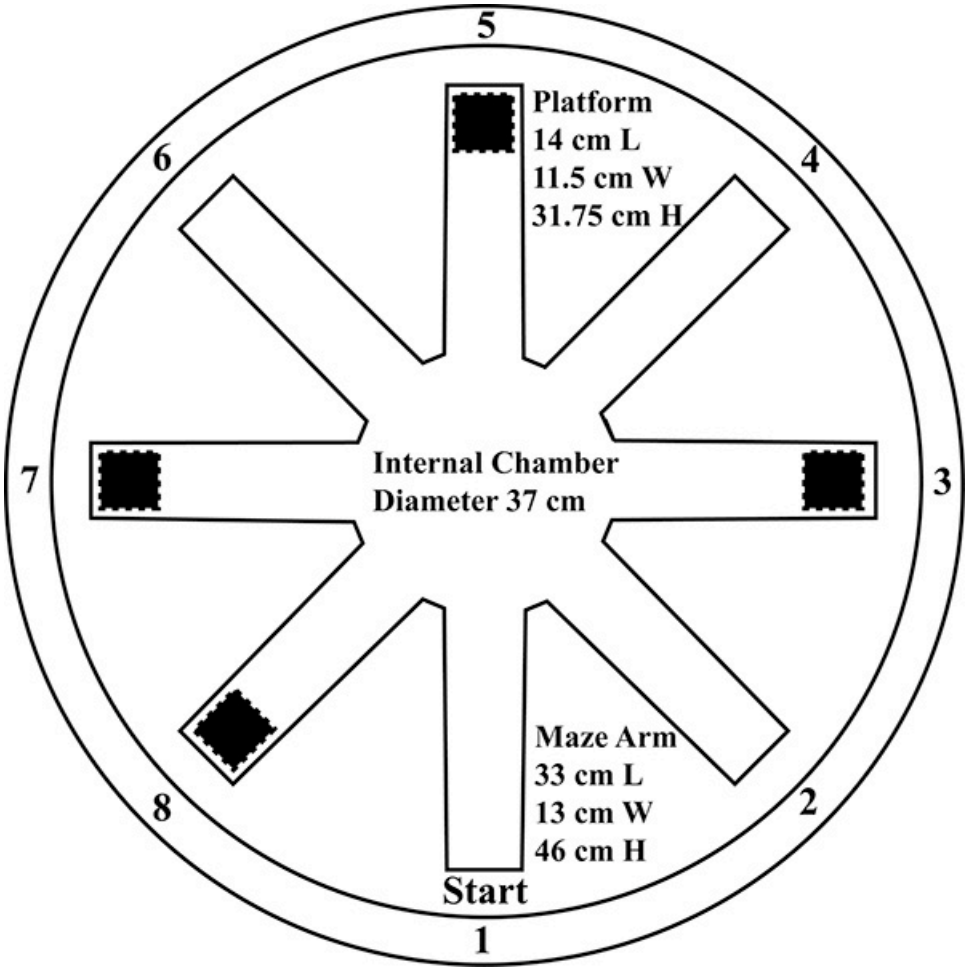

Figure 1. The eight-arm radial water maze. A schematic diagram showing a top view of the eight-arm radial water maze with each arm number and start position labeled. The black squares indicate locations of hidden platforms within the maze. These platform locations will remain constant to extra-maze cues throughout the testing protocol. The dimensions of the maze insert and platforms are included (L = length, W = width, H = height). The diameter of the plastic tub was 122 cm and the depth of the water was 33 cm. Click here to view larger image.

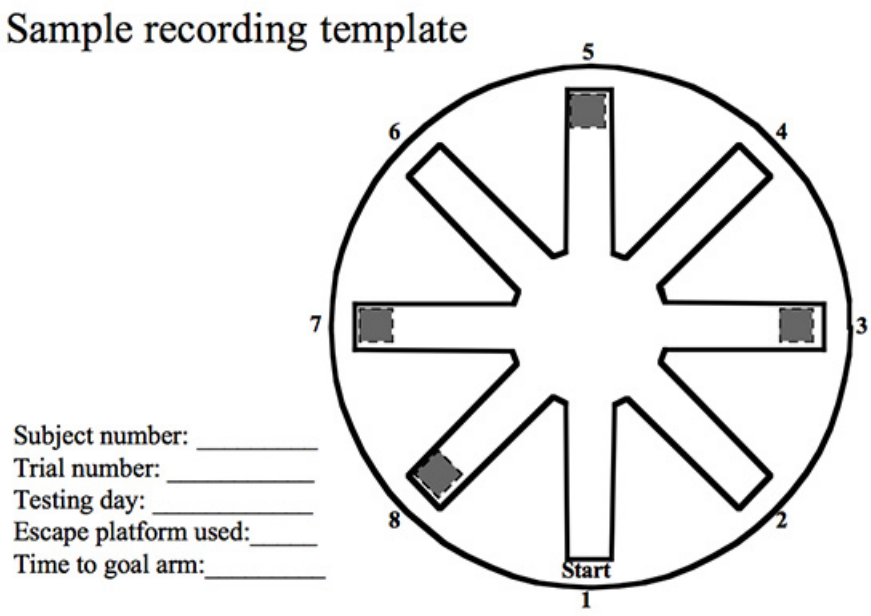

Figure 2. Sample recording template for the data collection. An example of the data sheet used to record subject arm entries and escape patterns. This file can be converted to a PDF for use with a digital tablet and PDF writer program. Data sheets should include a place to record the subject number, trial number, day of testing, which escape platform was used during the trial, and latency to reach platform. Prior to a trial any removed platforms should be marked with an **X**. At the end of a trial the platform used for escape should be circled to indicate the target arm for that run. Thus, a new template used on trial four would have three platforms with an **X** and one open target left on the template. Click here to view larger image.

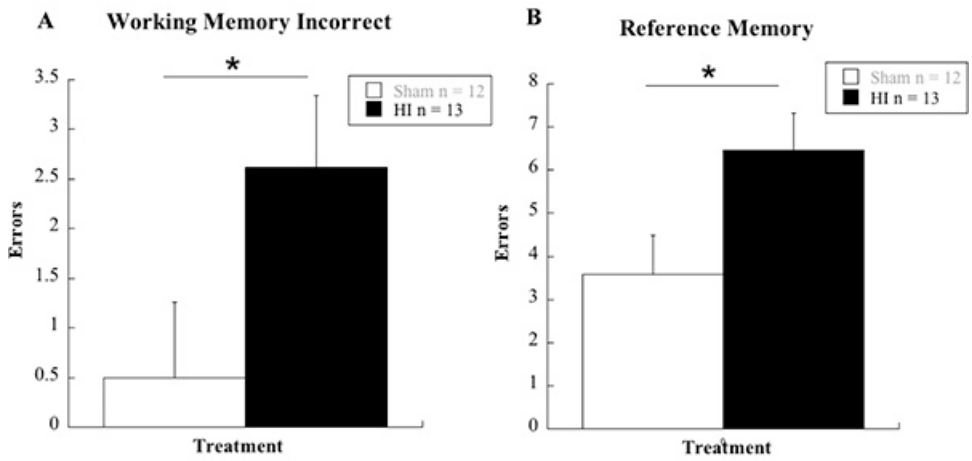

Figure 3. The effect of neonatal brain injury on reference and working memory. Using the eight-arm radial arm water maze we were able to show **A)** a significant increase in the number of working memory incorrect errors in rodents with hypoxic-ischemic (HI) injury by the eleventh day of testing. This is indicative of a working memory deficit. Further, we found **B)** a significant effect of HI injury on reference memory errors as compared to shams by the eleventh day of testing. Errors include the total cumulative errors across all four trials of day 11 (* $p<0.05$). Click here to view larger image.

Discussion

The eight-arm radial water maze paradigm has been utilized successfully in our lab and by others to assess working and reference memory performance in rats with and without neonatal brain injury[5-7,18-20]. In the current paradigm, removal of an escape platform after each trial increases working memory demand (subjects have fewer escape options) on subsequent trials. Therefore, on trial four, only one platform remains and working memory demand is at its highest, increasing the probability that an animal will re-enter a previously visited arm (working memory error). Since escape arms remain constant for all days of testing, reference memory can be easily analyzed by recording first entries

into an arm that never contained a platform. Other significant advantages of this paradigm include no requirement for food deprivation, less risk of potential confounding scent cues and no need for pretraining unlike with traditional land based mazes[5-7,18-20].

With respect to the length of testing (number of days), researchers should be aware of the cognitive limitations and requirements of the species or strain of animal being evaluated before determining the number of days required for optimal results. As with land based mazes, young rats, those used in models of neurological disease, and mice may take longer to reach asymptotic performance on the eight-arm radial water maze as compared to adult or typical rats. The data presented in the present protocol utilized eleven days of testing. Even with the observed significant effects in the present data, we recommend that testing be extended to 15-20 days in order to reduce variability when investigating deficits between experimental and control groups.

The use of a digital tablet and PDF maze templates for recording arm entries make this a practical and economical option for labs beginning to explore domains of working and reference memory. Further, this system can also be used with paper templates, making data collection more feasible for researchers without access to tablets or tablet based note-taking programs.

Finally, while we specifically used the radial arm water maze to assess working and reference memory in a rodent model of neonatal brain injury this protocol has been used to examine working and reference memory following hormone manipulation, in models of aging related cognitive decline and to examine other models of developmental disorders[5-7,18-20]. However, as with any testing design there are some limitations to keep in mind when using this specific water maze protocol. For example, to assess working memory with this water maze task, subjects are required to employ a win-shift strategy in the four arms with escape platforms (once an escape platform is located, it is removed for the following trial, thus testing working memory within a day)[18]. This is a common strategy for rodents when food reward is used in land based mazes[21], but it is counter to typical behavioral tendencies of rodents in water mazes[22]. For this reason, the working memory component of the eight-arm radial water maze may be more challenging for some subjects to learn as compared to other maze tasks[22]. Additionally, it is important that researchers are aware of the physical limitations of the species or model being evaluated such as visual abilities or thermoregulation. Any known motoric deficits could also affect comparisons of trial latency so researchers should use errors as the primary dependent measure. For this reason it may also be advisable to use a visible platform escape trial to assess motor differences before initiation of this water maze protocol. Despite these minor limitations, the eight-arm radial water maze is a robust tool for examining working and reference memory across many animal models[5-7,19,20].

Disclosures

The authors declare that they have no competing financial interest.

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