Video Article

Virus-induced Gene Knockdown in the CA3 and Subsequent Spatial Relocation Test to Assess CA3-dependent Cognitive Function

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Abstract

Investigating the role of a gene of interest in a specific brain region allows further understanding of brain physiology and pathophysiology. Modulation of gene expression by local injection of adeno-associated virus (AAV) has been proven to be efficient and safe. The stability and long-term expression of the AAV construct allows the use of a battery of behavioral tests to screen the animals for a region-specific involvement of the target gene in shaping performance in different behavioral domains.

The spatial object relocation (SOR) test is a hippocampal-dependent one-trial memory test based on the natural spontaneous exploratory behavior of rodents. This test gives robust information on memory function and can be easily integrated in a battery of behavioral testing for phenotype screening.

In this video-article, we provide a detailed protocol to assess the role of a particular target gene in shaping hippocampus-dependent spatial memory function. The protocol includes stereotactic AAV-induced gene transfer specifically into the mouse hippocampal CA3 region and combines this with the SOR test. Due to the variability in SOR protocols in the literature, we carefully described relevant aspects of the protocol to ensure the optimal behavioral protocol and setup selection. Also, detailed analyses of the results are described to guarantee the proper interpretation of the results.

Video Link

The video component of this article can be found at http://www.jove.com/video/53054/

Introduction

Dissecting the specific function of an individual gene, expressed in a defined brain region, is a key milestone to a better understanding of brain physiology and pathophysiology. One valuable approach is to investigate the behavioral consequences of local gene expression changes in the brain. Adeno-associated virus (AAV) based gene transfer has been proven to be efficient, safe and to induce long-term gene expression in the central nervous system[1–5]. In rodents, the stability of AAV-induced gene expression is suitable for extensive behavioral characterization, which usually requires several sessions in different days.

The rationale to select the most suitable behavioral tests to decipher the region-specific function of a target gene might depend on several factors. First, the region of interest might be associated with a prominent behavioral function and measured using a specific set of behavioral tests: *e.g.* the hippocampal CA3 area is linked to spatial memory[6], prefrontal cortex is related to executive function[7], amygdala is related to fear[8], *etc.* Second, the gene (and corresponding protein) of interest might be associated with specific functions, such as the glucocorticoid receptor is related to stress[9], the serotonin transporter is related to depression[10], *etc.* Also, a battery of behavioral tests could be performed to study different aspects of behavior. However, repeated testing might influence behavior[11], therefore considering the order and minimizing the number of tests is important for the validity of the results.

The spatial object relocation test (SOR) is an interesting test to specifically monitor changes in hippocampus-dependent spatial memory function. Similar to the novel object recognition test, the SOR is a one-trial memory test based on rodents spontaneous exploratory behavior[12–17] where the hippocampus plays a prominent role[18,19]. Compared to other spatial memory tests (*i.e.* Morris water maze[20,21], radial arm maze, Barnes maze[22]), the SOR is short, less stressful (*e.g.* compared to the swimming effort demanded in the Morris Ware maze), does not require food deprivation (such as the radial arm maze) or repeated training (*e.g.* Morris water maze, radial arm maze, Barnes maze), and provides a clear readout on the memory function of the animal. For these reasons, it can be easily added to a battery of tests to assess the role of a particular target gene in shaping brain-region dependent changes in cognitive behavior.

The SOR consists of the presentation of two similar objects during an acquisition trial, and after a defined inter-trial-interval (ITI), the animal is exposed to the same arena with one of the objects placed in a new location (retrieval). Based on the natural exploratory behavior of rodents, animals that remember the old location of the objects are expected to explore more the object placed in the new location. However, several protocols have been used in the literature [16,23–27], which show considerable variability in the use of arenas (open field of different sizes, T-maze, Y-maze, other); objects (number of objects presented to the animal, shapes, material, colors); number of habituation and acquisition trials; duration of the different trials; and also the length of the inter-trial-interval time, which is useful to identify changes in short (1 min up to 3 hr) or long-term memory (24 and 48 hr). This variety of different conditions and potential influencing parameters makes it difficult to select the best conditions for a particular experiment and, in particular, to compare results from different laboratories.

In a recent study[28], we were interested in further understanding the functional role of the down-regulated in renal-cell carcinoma 1 gene (DRR1) in the hippocampal CA3 region. DRR1 is a glucocorticoid-regulated gene recently suggested to promote stress resilience[28,29]. This gene shows particularly high constitutive expression in the hippocampal CA3 region[28,30,31]. In order to study the role of DRR1 in the CA3, we used an AAV containing a short hairpin RNA against DRR1 (shDRR1) to knock-down DRR1 expression and a scrambled version (shSCR) as control[28]. The phenotype of these mice was characterized using a battery of tests including cognitive (Y-maze, novel object recognition test, SOR and cross water-maze) and anxiety-like domains (open field, elevated plus-maze and forced swimming test). In this particular context, the SOR test was robust and efficient to detect the behavioral changes induced by DRR1 knock-down.

For this reason, the SOR test was chosen to be presented in this video article, in combination with providing detailed information on the stereotactic delivery of AAV-shDRR1 and AVV-shSCR to the mouse hippocampal CA3 region. Moreover, we carefully describe the protocol to perform the SOR and its subsequent analysis. We also provide the rationale used to select our optimal conditions for the SOR test, including data from the pre-test optimization phase. Finally, we show how the knock-down of DRR1 impacts on hippocampus-dependent cognitive function by reducing the performance of the mice in the SOR test.

Protocol

C57Bl6/N male mouse (<8 weeks old) were used for all the procedures. Animals were individually housed and kept on a 12-hr light/dark cycle (lights on at 7:00 AM), at room temperature of 23 ± 2 °C with food and water *ad libitum*. All experiments were conducted in accordance with European Communities Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

1. Stereotactic Adeno-associated Virus Injection in the Mouse CA3

1. Pull capillary glass tubes to create the injection glass pipettes. Cut the tips of the pipette formed to ensure an open end of approximately 45 µm outer and 15 µm inner diameters. The length of the pipette should be enough to allow reaching deep structures with minimum brain tissue damage.
2. Fill the isoflurane vaporizer system with isoflurane (approx. 2/3 of one bottle).
3. Quickly anaesthetize the mouse in a isoflurane induction chamber or using a closed container. In the second case, add 1-2 ml isoflurane to the closed container, with paper at the bottom, and let the isoflurane vapor fill the container. Place the mouse in the induction chamber or the container until the mouse is completely anaesthetized (approximately 10-20 sec).
4. Quickly transfer the animal to the stereotactic frame equipped with isoflurane vaporizer system. Use high isoflurane percentage while fixing the mouse head with the ear bars of the stereotactic apparatus (*e.g.* 4% vol) and, once the mouse head is correctly fixed, reduce and keep it constant until the end of the surgery (*e.g.* 1.5% vol). Maintain the mouse body temperature at 37 °C with a heating pad and use a cold light source for illumination.
5. Administer a systemic pain killer (non-steroidal anti-inflammatory drug) to prevent postsurgical pain and inflammation.
6. Apply a humectant, emollient and moisturizer to the eyes to prevent them from drying.
7. Remove the fur on the head with scissors or shaver.
8. Administer local anesthesia and disinfect the scalp with povidone-iodine.
9. Cut the skin along the midline with a scalpel. Be careful not to harm the muscles from the external occipital crest on the back of the skull.
10. Push aside the skin and the membranes above the skull from the midline with cotton swabs, cleaning specially bregma, lambda and around the future injection site.
11. Measure bregma and lambda coordinates. If the head of the mouse is properly fixed in the stereotactic frame, the lateral and ventral coordinates should be the same for both bregma and lambda. Anterior-posterior coordinates might vary depending on the age/body weight of the mouse. For mice around 8-10 weeks, expect around 3.9 mm between both anatomical points.
12. Use the measured bregma coordinates to calculate the injection sites coordinates. For bilateral hippocampal CA3 injection the coordinates are 1.9 mm anterior to bregma, 2.2 mm lateral from midline and 1.8 mm below dura mater [32].
13. Mark the injection sites, and make a corresponding hole through the skull using a micro drill. Avoid high pressure onto the skull to minimize inflammation. Also, discontinuous drilling facilitates heat dissipation and avoids excessive heating of the area.
14. Connect the injection system with the pulled pipette and take 0.5-1 µl of viral solution.
15. Lower the tip of the pipette containing the virus solution into the drilled hole until the surface of the *dura mater*. Measure the ventral coordinate and calculate the depth of the injection.
16. Carefully descend the pipette using the micromanipulator of the stereotactic system to the desired coordinates and when in position, slowly inject the viral solution (0.5 µl ~ 5 min).
    NOTE: The injection speed is important to minimize tissue damage and allow diffusion of the solution through the tissue. Leave the pipette additional 5 min after each injection to avoid solution reflux through the injection site and remove the pipette slowly afterwards.
17. Discard the pipette in the waste.
18. Repeat the steps 1.14 to 1.17 for the second injection site.
19. Stitch the skin with surgical suture and anaesthetize again locally.
20. Remove the animal from stereotactic system and place it in the home cage over a heating pad until recovery from the anesthesia.

21. Dilute an anti-inflammatory such as Metacam 1:1,000 in the drinking water (0.5 ml in 0.5 L tap water) for post-surgical pain killer treatment, change water after 1 week.
22. AAV infection is stable 4 weeks after surgery.

2. Behavioral Test

1. Preparation of the behavioral setup.
    1. Start the behavioral testing at least 4 weeks after the surgery. Allow the mice to habituate to the testing room: when possible, house the animals in the room 1 week in advance or move the animals to the room at least 1 hr prior the test.
    2. Handle the mice daily one week before testing to habituate the animals to the experimenter. Handling involves repeating the procedure of taking out the mouse from the home cage, let the mice walk on the subjects hand and arms and return them to the home cage again.
    3. Place the Y-maze, consisting of three arms (30 x 10 x 15 cm) at 120° from each other made of gray PVC, in the center of the testing chamber. We used four different chambers (110 x 110 x 130 cm) in order to test four mice at the same time.
    4. To avoid arm preferences of the animals, make sure none of the arms is perpendicular to any chamber wall and the light in the arena is evenly distributed with a peak around 15-20 Lux in the center of the arena. Optionally, pre-test the arm preference by measuring the distance and times spend in each arm during habituation or priory using another group of mice.
    5. Locate extra-maze spatial cues at the top of the testing chamber walls. These spatial cues could be white DIN A4 paper cut in different shapes (*e.g.* two triangles, one rectangle and one rhomboid respectively in three of the chamber walls) to contrast with the black wall of the chamber and visible for the animals performing the test.
    6. Include also intra-maze cues at the side wall and at the end of each arm in the Y-maze to easily distinguish between them. These cues could be made of white tape in different shapes: *e.g.* one long vertical stripe, one cross and one triangle, respectively on each arm.
    7. When more than one apparatus is used at the same time, counterbalance the position of the arms within the chambers.
    8. Setup the videotracking software to record the tracking and videos of the test.

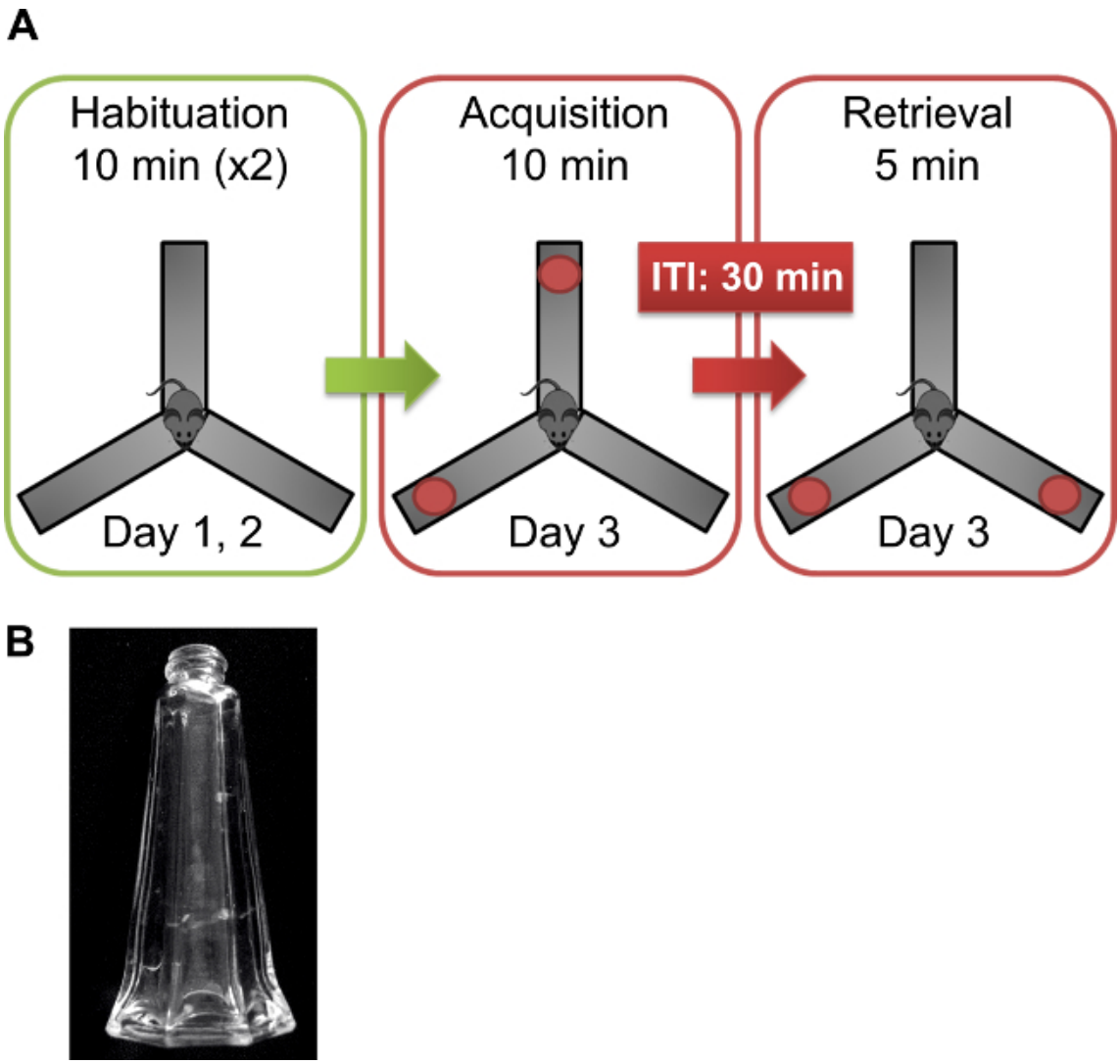

Figure 1. Spatial object relocation protocol. (**A**) Mice are placed in the center of the Y-maze on two consecutive days (days 1 and 2) for habituation to the arena (10 min/day). On the day of the test (day 3), mice are placed again in the center of the Y-maze containing two similar objects at the end of two of the arms and allowed to explore the arena for 10 min (Acquisition). After an inter-trial interval (ITI), the mice are returned to the Y-maze containing two clean similar objects, one of them in a new location (Retrieval). The arena and objects are thoroughly cleaned after each trial with water and dried before the next trial. (**B**) The objects used in the SOR test were glass salt shakers.

2. Test
    1. Habituate the mice to the empty arena on two consecutive days. Each day, place the mice in the center of the Y-maze and let them explore for 10 min. Then, return the mice to the respective home cages and clean the surface of the Y-maze thoroughly with water and dry it with paper afterwards.
    2. On the day of the test, put two identical objects at the end of two of the arms in the Y-maze. Place the objects at the end of the arm approximately 5 cm to the wall, allowing the mice to travel around the object. Counterbalance the position of the objects between chambers and mazes.
    NOTE: Ensure that these objects are attractive for the mice, evoking exploratory activity. This might be pre-tested in a group of mice to ensure the mice explore the objects more than 10 sec during 5 min. Glass salt shakers were used in this study as objects.
    3. Let the mice explore the arena with the objects for 10 min → **Acquisition trial.**
    4. Return the mice to the respective home cage for the desired **inter-trial-interval** (in our case 30 min).
    5. Clean the arena and the objects thoroughly with water and dry them. Place two new clean objects, one in the previous position (old object location) and the second one in the previously empty arm (new object location).
    6. Let the mice explore the arena with the objects for 5 min → **Retrieval trial**.
    7. Return the mice to the respective home cage and clean the arena and the objects thoroughly with water and dry them.
    8. Disinfect the objects and arenas with *e.g.* 70% ethanol at the end of the experiment.

3. Analysis

1. For each experimental group, measure the total distance travelled. Different distance travelled might bias exploration time of the objects and mask the results.
2. Analyze the videos measuring the exploration time of each of the objects. Consider exploration of the objects when the mice touch the objects with the nose, forepaws or vibrissae. Exclude mice when they do not explore both objects. Also, data from mice that explore in total less than 5 sec might be discarded. Total exploration time of the objects should not differ between groups.
3. Calculate the index of recognition as a measure of memory function for each mouse, *i.e.* percentage of exploration of the object in the new location compared to total exploration time during the retrieval phase:

$$Index\ of\ recognition\ (\%) = \frac{new\ location\ exploration\ time}{total\ exploration\ time} * 100$$

4. Validation of AAV-injection

1. Evaluate the correctness of the AAV-injection site and the efficacy of the AVV-induced gene modulation. Most of the AAV co-express fluorescent markers allowing *ex vivo* fluorescence visualization in brain slices. Also, the efficacy of the AAV-induced gene expression modulation can be quantified by *in situ* hybridization.

Representative Results

The functional role of DRR1 expression was studied using a combination of AAV-induced mRNA changes in the CA3 region and spatial memory assessment using the SOR test in C57Bl/6N mice [28]. We used an AAV containing a short hairpin RNA against DRR1 (shDRR1) to knock-down DRR1 expression and a scrambled version (shSCR) as control. Here we present (1) data showing how the SOR test conditions were previously selected, using naive C56Bl/6N mice; (2) the results of the SOR in mice injected in the CA3 with AAV-shDRR1 or AAV-shSCR, and (3) the *ex vivo* validation that the AVV-injection was correctly placed –by means of EGFP expression- and that shDRR1 was effective in reducing DRR1 mRNA expression in the CA3 region — by means of *in situ* hybridization.

Selection of SOR test conditions:

Arena: Three different arenas were tested for suitability to perform the SOR: (1) an open field (50 x 50 x 40 cm of light grey PVC)[29]; (2) a Type III rodent cage (38.2 x 22 x 15 cm), with a thin layer of clean sawdust and surrounded by black cartoon paper 25 cm height to avoid the mouse jump out of the cage (similar to [33]) and (3) a Y-maze consisting of three arms (30 x 10 cm) at 120° from each other made of grey PVC (similar to [23]). Mice were placed in the center of the arena and allowed to freely explore two objects for 10 minutes.

On average, mice explored each of the objects of the arena 4.5 ± 0.7 sec in the OF (50 x 50 cm), 25.9 ± 2.4 in the Type III Rodent cage, and 11.3 ± 2.8 sec in the Y-maze (**Figure 2**). Although the exploration time of the objects in the Type III Rodent Cage was higher than the other arenas, the positions of the objects for the retrieval session could not be clearly changed. Thus, the Type III Rodent cage might be useful arena for novel object recognition test but not appropriate for SOR test. For this reason, the Y-maze arena was selected for the SOR test.

Objects: Glass salt shakers were used as objects as they allowed the mice to travel around the object to optimize the exploration. Additionally they were easy to clean.

Number and duration of habituation, acquisition and retrieval trials: Based on literature research, 10 min habituation at two consecutive days, a single acquisition trial (10 min) and retrieval (5 min) was chosen[15,34–36].

Length of the ITI: 30 min ITI was selected according to previous results[30]. In the previous published data[30], over-expression of DRR1 improved memory performance after 15 min ITI. In the current experiment using DRR1 knock-down mice (shDRR1) memory impairment was expected. For this reason, the difficulty of the task was increased by using a higher ITI.

SOR test results:

Spatial memory function was impaired in AAV-shDRR1 injected mice compared to AAV-shSCR mice in the SOR test (shSCR n=18; shDRR1 n=19). Only AAV-shSCR mice were able to discriminate between the old and new object position, as shown by the different exploration time of the old/new location of the objects (**Figure 3A**) and the significantly reduced index of recognition in AAV-shDRR1 compared to AAV-shSCR mice ($p<0.01$) (**Figure 3B**). Two-way ANOVA showed object-location exploration time effect ($F_{(1,70)}=10.44$; $p<0.01$) and exploration time-virus interaction effect ($F_{(1,70)}=6.267$; $p<0.05$). Bonferroni post-hoc analysis showed significantly different time exploring the old object location between AAV-shDRR1 and AAV-shSCR mice ($p<0.01$).

Total exploration time and total distance travelled in the arena were not significantly different between groups (**Figure 3C and 3D**). The results showed that control AAV-shSCR mice explored more the object in the new location compared to the old one. However, AAV-shDRR1 injected mice were not able to discriminate between positions and explored similarly both objects.

Validation of AAV-injection:

EGFP expression in the dorsal hippocampus was used to evaluate the accuracy of the AAV injections to the CA3 region in all the mice used. The spreading of the virus was drawn (shSCR n=10; shDRR1 n=12) and the overlay was represented (**Figure 4A**). Moreover, *in situ* hybridization

was used to quantify the changes in DRR1 mRNA expression in the CA3 in order to validate the DRR1-induced knock-down. DRR1 mRNA was significantly decreased (about 20%) in the CA3 of mice with shDRR1 compared to shSCR mice ($p < 0.05$) (**Figure 4B**).

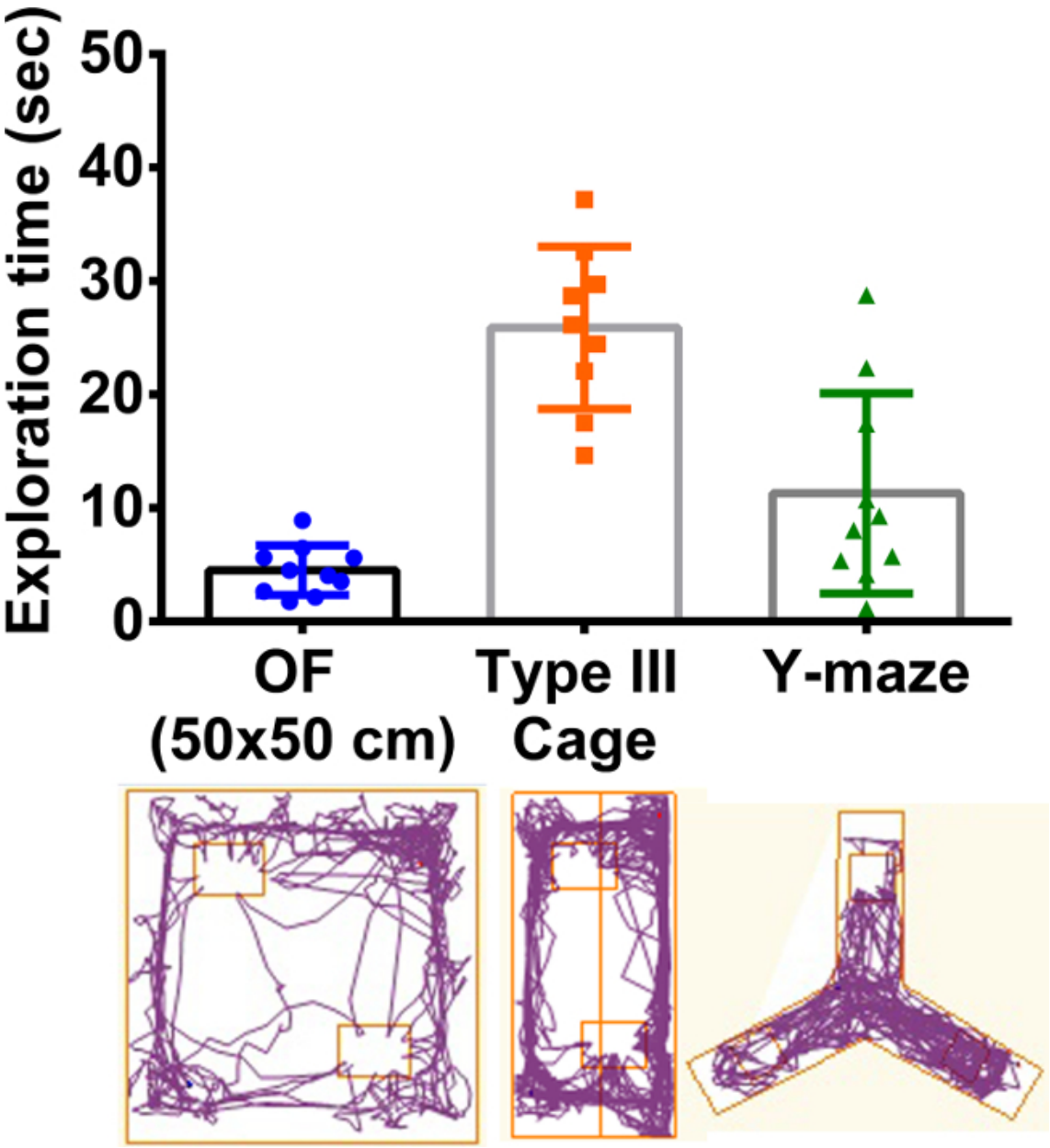

Figure 2. Selection of the arena for the SOR. Two objects were placed in each arena and object exploration time was measured. The graph showed the mean exploration time per object towards the objects in the different arenas. Tracking plot of an example mouse is depicted below each bar. Individual values for each mouse are represented in blue circles, orange squares and green triangles for the open field (50 x 50 x 40 cm), Type III Rodent Cage (38.2 x 22 x 15 cm) and Y-maze arena (three arms, 30 x 10 cm, at 120°), respectively. Bars show mean ± SEM. n=9-10 mice/group.

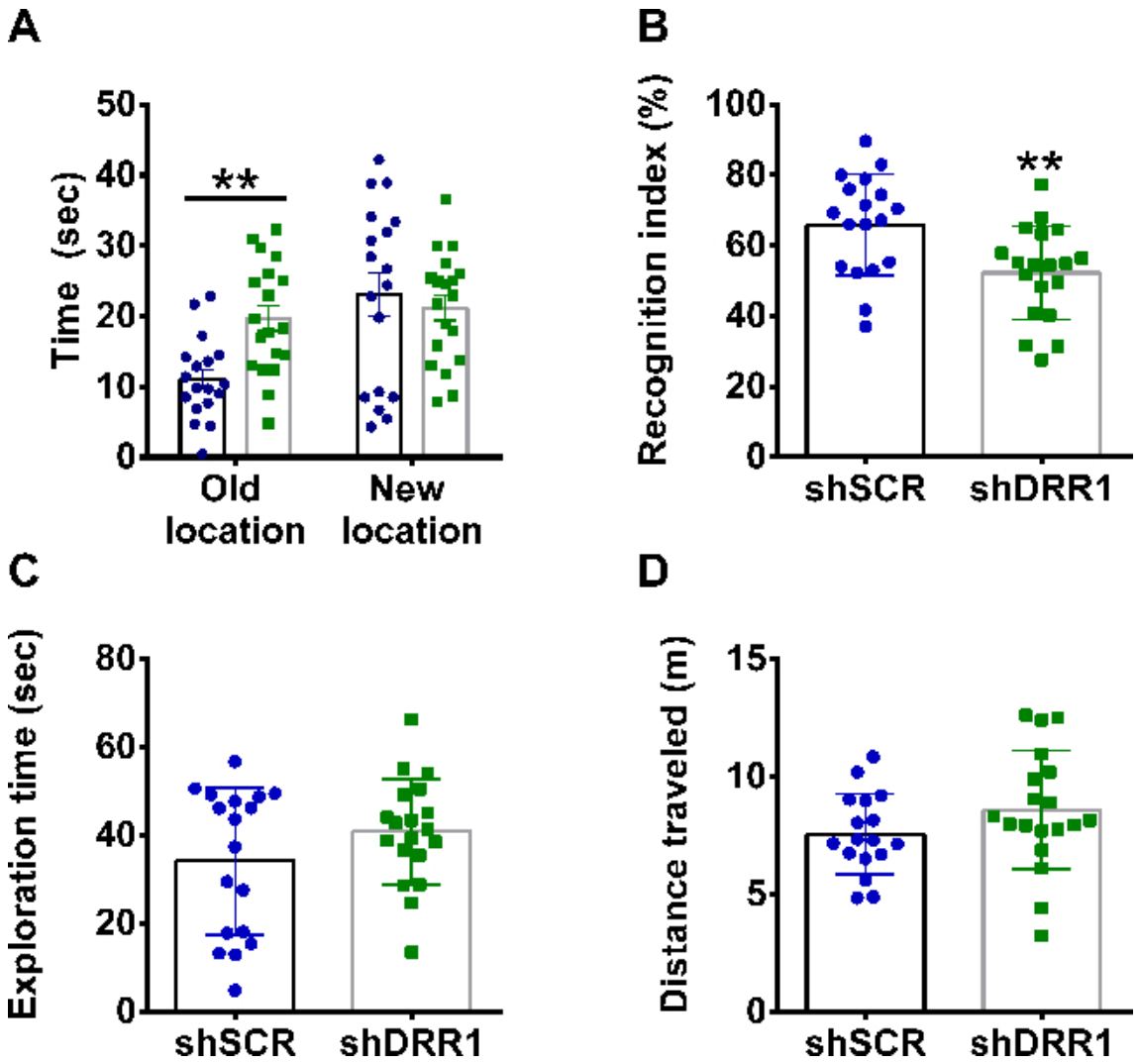

Figure 3. Representative results of the spatial object relocation. (**A**) Exploration time of the objects during retrieval trial. (**B**) Index of recognition. (**C**) Total exploration time during retrieval. (**D**) Total distance travelled in the arena during retrieval. Individual values for shSCR and shDRR1 mouse are represented in blue circles and green squares, respectively. Bars show mean ± SEM. **p<0.01. n=18-19 mice/group. Figure modified from[28].

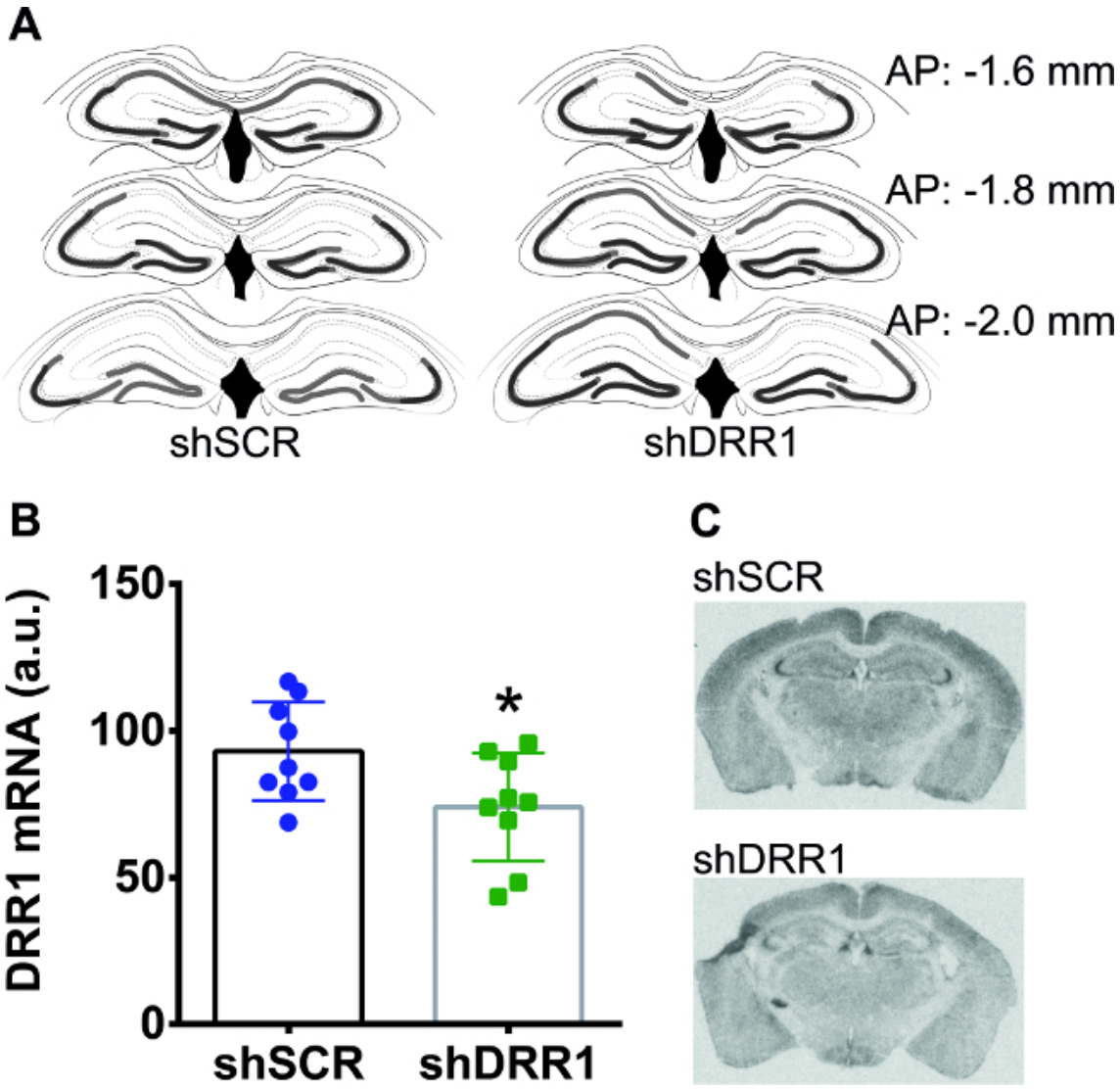

Figure 4. Validation of AAV injection and DRR1 mRNA knock-down. (**A**) Virus spreading in shSCR and shDRR1 injected mice (overlay of n=10-12 mice group). (**B**) Densitometric quantification of DRR1 mRNA expression in the CA3 (n=9 mice/group). (**C**) Representative *in situ* hybridization autoradiographs of [^{35}S]-labeled DRR1 mRNA. Figure modified from[28].

Discussion

The SOR is an robust and valid test to investigate changes in hippocampus-dependent cognitive function. The test can be easily included in a screening battery of tests because it is short, non-aversive, and does not require food deprivation or repeated training. In the present video article, we combined the SOR test with an AAV-mediated local gene knock-down of DRR1. The results of the study showed that the SOR was efficient in detecting memory impairment induced by DRR1 knock-down locally in the CA3.

Previous studies show that DRR1 expression is high in the CA3 region under basal conditions[30], is regulated by stress and glucocorticoids[29,30] and its over-expression in the mouse CA3 improves cognitive flexibility[30]. As all the previous data indicate that DRR1 in the CA3 is related to cognition[30], cognitive tests were the main behavioral readout chosen for the present experiments. An AAV containing a short hairpin RNA against DRR1 (shDRR1) to knock-down DRR1 expression, or a scrambled version (shSCR) as control, were used. Adeno-associated virus injection in the brain was performed locally in the hippocampal CA3 following a similar protocol previously described in JoVE[37]. Using this virus-induced knock-down strategy, we expect that DRR1 expression would be only reduced in infected cells expressing the gene. Therefore, this manipulation is closer to a physiological modulation than a virus-induced DRR1 overexpression strategy[29,30], where DRR1 could be expressed in all infected cells, including those where DRR1 is not normally expressed. The expression of a reporter gene (*e.g.* EGFP) together with the gene of interest might be used to confirm the correctness of the injection sites and to study morphological changes related to the expression of the gene of interest in the infected cells[28,29,37]. In addition, using pharmacological[27,38], optogenetics[39], and other manipulations prior to acquisition, after acquisition and before retrieval in the SOR, can be also used to specifically dissect the different domains of memory function such as acquisition, consolidation and retrieval.

Mice injected with the shDRR1 and shSCR were screened for spatial memory function using the SOR. Several aspects of the SOR test were carefully considered prior the selection of the conditions for the test, due to the diversity of protocols in the literature[16,23–27]. (1) Arena used: Y-

maze was selected among other arenas because the exploration time of the objects was higher compared to the open field, probably because the mice travel in a closer environment where it may feel safer, and consequently increases the accuracy of the results. This facilitates the combination of this approach with mice that underwent stress procedure, which usually reduce exploration time towards the objects. Although the exploration time of the objects was higher in the Type III Rodent cage arena, this arena was not suitable for detection of novel object locations. Moreover, the habituation to the Y-maze, when the arena is presented for the first time to the mice, could serve as a test for working memory (spontaneous alternation task). (2) The objects used: for the SOR is not as critical as it is for the novel object recognition[40], because objects should be similar and therefore no object-preference should be observed. Still, the objects need to be attractive for the mice to encourage their exploration. (3) Number of habituations: habituation to the empty arena is necessary to reduce the novelty to the arena, decrease freezing and increase the exploratory behavior towards the objects. Too many habituation trials might increase the length of the test and decrease exploration, but only one might not be enough in some cases (*e.g.* in stressed mice). For this reason we selected two habituation trials on consecutive days. (4) Number of acquisition trials: only one acquisition trial was used to avoid confounding effects of memory reconsolidation before the retrieval phase. (5) Duration of trials: 10 min habituation, 10 min acquisition and 5 min retrieval trial times are the most commonly used. And finally, (6) length of the ITI: a pre-test with naïve mice is recommended, as it is necessary to ensure that naïve mice are able to learn and perform the task for a specific short ITI and they lose this ability in a longer ITI. Then, the short ITI will be used for testing deficits in cognition and the longer ITI to test memory improvement.

Finally, several aspects need to be considered for the interpretation of the behavioral analysis. (1) Exploration time: Mice that remember the locations of the objects during acquisition are expected to explore more time the object in the new location during the retrieval trial. (2) Index of recognition: Is a relation of the time spend exploring the object in the new location compared to the old one for each single mouse. When the index is above 50 % it indicates the overall group of mice remember the previous objects locations. However, an index of recognition below 50 % might reflect preference for the familiar object location rather than cognitive impairment, and only an index around 50 % indicates no preference for neither of the objects location. (3) Total exploration time: Needs to be similar between the tested groups as more/less exploration could mask the results. (4) Total distance travelled: similar to total exploration time, hyper-/hypo-active mice might show more/less exploration, respectively. When differences are found, the results need to be carefully interpreted. (5) Preference for object/arm: The exploration time of the objects during acquisition must be on average similar for each mice group and all mice should explore the location of both objects, so they are able to distinguish a new location. Also, measurement of the time spent in each arm during habituation might indicate if there is an arm preference. Finally, the position of the objects in the old and new location might be counterbalanced for the different mice within the group to avoid this issue.

In summary, our data show that the combination of virus-mediated, brain-region specific modulation of a target gene with specific behavioral tests is a valid approach to advance our understanding of brain function. In particular, the SOR is a powerful test to study spatial memory changes induced by AAV-gene expression modulation, it gives robust results and can be easily added to a battery of behavioral testing. Similar to other behavioral tests, several aspects need to be considered in order to select the appropriate conditions and also to properly interpret the results. Here, we provide detailed information on the protocols for both stereotactic injection of the virus and behavioral testing. In addition to the basic protocols, we provide further information so as to enable the selection of the best experimental conditions and adaptation of the methods described for a particular experiment.

Disclosures

The authors have nothing to disclose.

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