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Virus-induced gene knockdown in the CA3 and subsequent spatial relocation test to assess CA3-dependent cognitive function

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Corresponding Author:	Mercè Masana, Ph.D Johannes Gutenberg University Medical Center Mainz, GERMANY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	merce.masana@unimedizin-mainz.de;merce.masana@gmail.com
Corresponding Author's Institution:	Johannes Gutenberg University Medical Center
Corresponding Author's Secondary Institution:	
First Author:	Mercè Masana, Ph.D
First Author Secondary Information:	
Other Authors:	Ulrich Schmitt, Prof.
	Konstantin Radyushkin, MD
	Andrés Uribe-Mariño
	Marianne B Müller, MD
Order of Authors Secondary Information:	
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.
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Department of Psychiatry and Psychotherapy
& Focus Program Translational Neurosciences

Journal of Visualized Experiments (JoVE)
Jaydev Upponi, Ph.D.
Science Editor
JoVE

Mercè Masana, PhD

Hanns-Dieter-Hüsch Weg 19
55128 Mainz
Telefon: ++49(0)6131 39-21345
Telefax: +49 (0) 6131 17-6690
E-Mail: Merce.Masana@unimedizin-mainz.de
www.klinik.uni-mainz.de/Psychiatrie

Mainz, 25th March 2015

Dear Dr. Jaydev Upponi,

We are pleased to submit the revisions of the JoVE53054 manuscript and we thank you and the reviewers for the helpful and constructive criticism.

According to the reviewers' suggestions, we corrected and improved the protocol, and we added further background information on DRR1 to better describe the rationale of the study. In addition, we added a new section under the representative results with the corresponding new figure, were validation of the injection site and efficacy is shown.

Having answered point-by-point all of the reviewer's questions in the rebuttal letter, we feel that our manuscript is now considerably improved, and we hope that it will be considered acceptable for publication in *JoVE*.

We are looking forward to hearing from you.

Sincerely,

Mercè Masana

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TITLE:

Virus-induced gene knockdown in the CA3 and subsequent spatial relocation test to assess CA3-dependent cognitive function

AUTHORS:
Mercè Masana, Ulrich Schmitt, Konstantin Radyushkin, Andrés Uribe-Mariño, Marianne B. Müller

Masana, Mercè
[1]Department of Psychiatry and Psychotherapy & Focus Program Translational Neuroscience (FTN)
Johannes Gutenberg University Medical Center
Mainz, Germany
Merce.masana@unimedizin-mainz.de

[2]Max-Planck Institute of Psychiatry
Munich, Germany

Schmitt, Ulrich
[1]Department of Psychiatry and Psychotherapy & Focus Program Translational Neuroscience (FTN)
Johannes Gutenberg University Medical Center
Mainz, Germany
Ulrich.Schmitt@unimedizin-mainz.de

Radyushkin, Konstantin
[3]Focus Program Translational Neurosciences (FTN)
Johannes Gutenberg University of Mainz
Mainz, Germany
radyushkin@uni-mainz.de

Uribe-Mariño, Andrés
[2]Max-Planck Institute of Psychiatry
Munich, Germany
andres_uribe@psych.mpg.de

Müller, Marianne B.
[1]Department of Psychiatry and Psychotherapy & Focus Program Translational Neuroscience (FTN)
Johannes Gutenberg University Medical Center
Mainz, Germany
Marianne.Mueller@unimedizin-mainz.de

[2]Max-Planck Institute of Psychiatry

Munich, Germany

CORRESPONDING AUTHOR: Masana, Mercè
Department of Psychiatry and Psychotherapy & Focus Program Translational Neuroscience (FTN)
Johannes Gutenberg University Medical Center
Mainz, Germany
Merce.masana@unimedizin-mainz.de
Tel: ++49(0)6131 39-21345
Fax: ++49(0)6131 39-21386

KEYWORDS:
Mouse; behavior; spatial relocation task; spatial object recognition test; memory; cognition; neuroscience; AAV; gene transfer; hippocampus; CA3; stereotactic surgery.

SHORT ABSTRACT:
Hippocampal cognitive function can be modulated by locally manipulating gene expression. The spatial object relocation test is a short and robust spatial memory test. We combined this test with virus-induced manipulation of gene expression in the CA3 region to assess the impact of the target gene in shaping cognitive performance.

LONG 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.

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, e.g.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, e.g. 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 3hrs) or long-term memory (24 and 48h). 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-h 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

1.1) 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.

1.2) Fill the isoflurane vaporizer system with isoflurane (approx. 2/3 of one bottle).

1.3) 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).

1.4) Quickly transfer the animal to the stereotactic frame equipped with soflurane 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.

1.5) Administer a systemic pain killer (non-steroidal anti-inflammatory drug) to prevent postsurgical pain and inflammation.

1.6) Apply a humectant, emollient and moisturizer to the eyes to prevent them from drying.

1.7) Remove the fur on the head with scissors or shaver.

1.8) Administer local anesthesia and disinfect the scalp with povidone-iodine.

1.9) 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.

1.10) 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.

1.11) 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.

1.12) 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].

1.13) 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.

1.14) Connect the injection system with the pulled pipette and take 0.5-1 µl of viral solution.

1.15) 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.

1.16) 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.

1.17) Discard the pipette in the waste.

1.18) Repeat the steps 1.14 to 1.17 for the second injection site.

1.19) Stitch the skin with surgical suture and anaesthetize again locally.

1.20) Remove the animal from stereotactic system and place it in the home cage over a heating pad until recovery from the anesthesia.

1.21) Dilute an anti-inflammatory such as Metacam 1:1000 in the drinking water (0.5 ml in 0.5 liter tap water) for post-surgical pain killer treatment, change water after 1 week.

1.22) AAV infection is stable 4 weeks after surgery.

2. Behavioral test

2.1) **Preparation of the behavioral setup.**

2.1.1) 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 hour prior the test.

2.1.2) 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.

2.1.3) 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.

2.1.4) 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.

2.1.5) 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.

2.1.6) 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.

2.1.7) When more than one apparatus is used at the same time, counterbalance the position of the arms within the chambers.

2.1.8) Setup the videotracking software to record the tracking and videos of the test.

2.2) **Test** *[Place Figure 1 here]*

2.2.1) 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.

2.2.2) 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.

2.2.3) Let the mice explore the arena with the objects for 10 min → **Acquisition trial**.

2.2.4) Return the mice to the respective home cage for the desired **inter-trial-interval** (in our case 30 min).

2.2.5) 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).

2.2.6) Let the mice explore the arena with the objects for 5 min → **Retrieval**.

2.2.7) Return the mice to the respective home cage and clean the arena and the objects thoroughly with water and dry them.

2.2.8) Disinfect the objects and arenas with e.g. 70% ethanol at the end of the experiment.

3. Analysis

3.1) For each experimental group, measure the total distance travelled. Different distance travelled might bias exploration time of the objects and mask the results.

3.2) 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.

3.3) 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

4.1) 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 cab 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 LEGENDS:

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.

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 [35S]-labelled 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.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. McCown, T. J., Xiao, X., Li, J., Breese, G. R. & Samulski, R. J. Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain research* **713** (1-2), 99–107, doi:10.1016/0006-8993(95)01488-8 (1996).
2. Tenenbaum, L., Chtarto, A., Lehtonen, E., Velu, T., Brotchi, J. & Levivier, M. Recombinant AAV-mediated gene delivery to the central nervous system. *The journal of gene medicine* **6 Suppl 1**, S212–22, doi:10.1002/jgm.506 (2004).
3. Burger, C., Gorbatyuk, O. S., *et al.* Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Molecular therapy : the journal of the American Society of Gene Therapy* **10** (2), 302–17, doi:10.1016/j.ymthe.2004.05.024 (2004).
4. Davidson, B. L., Stein, C. S., *et al.* Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proceedings of the National Academy of Sciences of the United States of America* **97** (7), 3428–32, doi:10.1073/pnas.050581197 (2000).
5. Burger, C., Nash, K. & Mandel, R. J. Recombinant adeno-associated viral vectors in the nervous system. *Human gene therapy* **16** (7), 781–91, doi:10.1089/hum.2005.16.781 (2005).
6. Kesner, R. P. Behavioral functions of the CA3 subregion of the hippocampus. *Learning & memory (Cold Spring Harbor, N.Y.)* **14** (11), 771–81, doi:10.1101/lm.688207 (2007).
7. Chudasama, Y. & Robbins, T. W. Functions of frontostriatal systems in cognition:

comparative neuropsychopharmacological studies in rats, monkeys and humans. *Biological psychology* **73** (1), 19–38, doi:10.1016/j.biopsycho.2006.01.005 (2006).
8. Johansen, J. P., Cain, C. K., Ostroff, L. E. & LeDoux, J. E. Molecular mechanisms of fear learning and memory. *Cell* **147** (3), 509–24, doi:10.1016/j.cell.2011.10.009 (2011).
9. Reul, J. M. & de Kloet, E. R. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* **117** (6), 2505–11at doi:10.1210/endo-117-6-2505 (1985).
10. Levinson, D. F. The genetics of depression: a review. *Biological psychiatry* **60** (2), 84–92, doi:10.1016/j.biopsych.2005.08.024 (2006).
11. Voikar, V., Vasar, E. & Rauvala, H. Behavioral alterations induced by repeated testing in C57BL/6J and 129S2/Sv mice: implications for phenotyping screens. *Genes, Brain and Behavior* **3** (1), 27–38, doi:10.1046/j.1601-183X.2003.0044.x (2004).
12. Ennaceur, a & Delacour, J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural brain research* **31** (1), 47–59, doi:10.1016/0166-4328(88)90157-X (1988).
13. Ennaceur, a One-trial object recognition in rats and mice: methodological and theoretical issues. *Behavioural brain research* **215** (2), 244–54, doi:10.1016/j.bbr.2009.12.036 (2010).
14. Dere, E., Huston, J. P. & De Souza Silva, M. a The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. *Neuroscience and biobehavioral reviews* **31** (5), 673–704, doi:10.1016/j.neubiorev.2007.01.005 (2007).
15. Antunes, M. & Biala, G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive processing* **13** (2), 93–110, doi:10.1007/s10339-011-0430-z (2012).
16. Dix, S. L. & Aggleton, J. P. Extending the spontaneous preference test of recognition: evidence of object-location and object-context recognition. *Behavioural brain research* **99** (2), 191–200, doi:10.1016/S0166-4328(98)00079-5 (1999).
17. Murai, T., Okuda, S., Tanaka, T. & Ohta, H. Characteristics of object location memory in mice: Behavioral and pharmacological studies. *Physiology & behavior* **90** (1), 116–24, doi:10.1016/j.physbeh.2006.09.013 (2007).
18. Barker, G. R. I. & Warburton, E. C. When is the hippocampus involved in recognition memory? *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31** (29), 10721–31, doi:10.1523/JNEUROSCI.6413-10.2011 (2011).
19. Warburton, E. C. & Brown, M. W. Findings from animals concerning when interactions between perirhinal cortex, hippocampus and medial prefrontal cortex are necessary for recognition memory. *Neuropsychologia* **48** (8), 2262–72, doi:10.1016/j.neuropsychologia.2009.12.022 (2010).
20. Nunez, J. Morris Water Maze Experiment. *Journal of visualized experiments : JoVE* (19), e897, doi:10.3791/897 (2008).
21. Bromley-Brits, K., Deng, Y. & Song, W. Morris water maze test for learning and memory deficits in Alzheimer's disease model mice. *Journal of visualized experiments : JoVE* (53), e2920, doi:10.3791/2920 (2011).
22. Rosenfeld, C. S. & Ferguson, S. A. Barnes maze testing strategies with small and large rodent models. *Journal of visualized experiments : JoVE* (84), e51194, doi:10.3791/51194

(2014).
23. Zlomuzica, a, Viggiano, D., *et al.* Behavioral alterations and changes in Ca/calmodulin kinase II levels in the striatum of connexin36 deficient mice. *Behavioural brain research* **226** (1), 293–300, doi:10.1016/j.bbr.2011.08.028 (2012).
24. Assini, F. L., Duzzioni, M. & Takahashi, R. N. Object location memory in mice: pharmacological validation and further evidence of hippocampal CA1 participation. *Behavioural brain research* **204** (1), 206–11, doi:10.1016/j.bbr.2009.06.005 (2009).
25. Heyward, F. D., Walton, R. G., Carle, M. S., Coleman, M. A., Garvey, W. T. & Sweatt, J. D. Adult mice maintained on a high-fat diet exhibit object location memory deficits and reduced hippocampal SIRT1 gene expression. *Neurobiology of Learning and Memory*, **98** (1), 25-32, doi: 10.1016/j.nlm.2012.04.005 (2012).
26. Kenney, J. W., Adoff, M. D., Wilkinson, D. S. & Gould, T. J. The effects of acute, chronic, and withdrawal from chronic nicotine on novel and spatial object recognition in male C57BL/6J mice. *Psychopharmacology* **217** (3), 353–65, doi:10.1007/s00213-011-2283-7 (2011).
27. Oliveira, A. M. M., Hawk, J. D., Abel, T. & Havekes, R. Post-training reversible inactivation of the hippocampus enhances novel object recognition memory. *Learning & memory (Cold Spring Harbor, N.Y.)* **17** (3), 155–60, doi:10.1101/lm.1625310 (2010).
28. Masana, M.,Dine, J., *et al.* Cognitive performance and synaptic function are dependent on the resilience-promoting protein DRR1. In preparation
29. Masana, M., Su, Y.-A., *et al.* The stress-inducible actin-interacting protein DRR1 shapes social behavior. *Psychoneuroendocrinology* **48**, 98–110, doi:10.1016/j.psyneuen.2014.06.009 (2014).
30. Schmidt, M. V, Schülke, J.-P., *et al.* Tumor suppressor down-regulated in renal cell carcinoma 1 (DRR1) is a stress-induced actin bundling factor that modulates synaptic efficacy and cognition. *Proceedings of the National Academy of Sciences of the United States of America* **108** (41), 17213–8, doi:10.1073/pnas.1103318108 (2011).
31. Masana, M., Jukic, M. M., *et al.* Deciphering the spatio-temporal expression and stress regulation of Fam107B, the paralog of the resilience-promoting protein DRR1 in the mouse brain. *Neuroscience* , doi:10.1016/j.neuroscience.2015.01.026 (2015).
32. Paxinos, G. & Franklin, K. B. J. *Mouse Brain in Stereotaxic Coordinates*. (Academic Press: New York, 2008).
33. Knapman, a, Heinzmann, J.-M., Hellweg, R., Holsboer, F., Landgraf, R. & Touma, C. Increased stress reactivity is associated with cognitive deficits and decreased hippocampal brain-derived neurotrophic factor in a mouse model of affective disorders. *Journal of psychiatric research* **44** (9), 566–75, doi:10.1016/j.jpsychires.2009.11.014 (2010).
34. Kamei, H., Nagai, T., *et al.* Repeated methamphetamine treatment impairs recognition memory through a failure of novelty-induced ERK1/2 activation in the prefrontal cortex of mice. *Biological psychiatry* **59** (1), 75–84, doi:10.1016/j.biopsych.2005.06.006 (2006).
35. Mizoguchi, H., Takuma, K., *et al.* Improvement by minocycline of methamphetamine-induced impairment of recognition memory in mice. *Psychopharmacology* **196** (2), 233–41, doi:10.1007/s00213-007-0955-0 (2008).
36. Koike, H., Ibi, D., *et al.* Behavioral abnormality and pharmacologic response in social isolation-reared mice. *Behavioural brain research* **202** (1), 114–21, doi:10.1016/j.bbr.2009.03.028 (2009).

37. Lowery, R. L. & Majewska, A. K. Intracranial injection of adeno-associated viral vectors. *Journal of visualized experiments : JoVE* (45), e2140, doi:10.3791/2140 (2010).
38. Lamirault, L. & Simon, H. Enhancement of place and object recognition memory in young adult and old rats by RS 67333, a partial agonist of 5-HT4 receptors. *Neuropharmacology* **41** (7), 844–53, doi:10.1016/S0028-3908(01)00123-X (2001).
39. Rolls, A., Colas, D., *et al.* Optogenetic disruption of sleep continuity impairs memory consolidation. *Proceedings of the National Academy of Sciences of the United States of America* **108** (32), 13305–10, doi:10.1073/pnas.1015633108 (2011).
40. Bettis, T. & Jacobs, L. F. Sex differences in object recognition are modulated by object similarity. *Behavioural brain research* **233** (2), 288–292, doi:10.1016/j.bbr.2012.04.028 (2012).

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Figure 1

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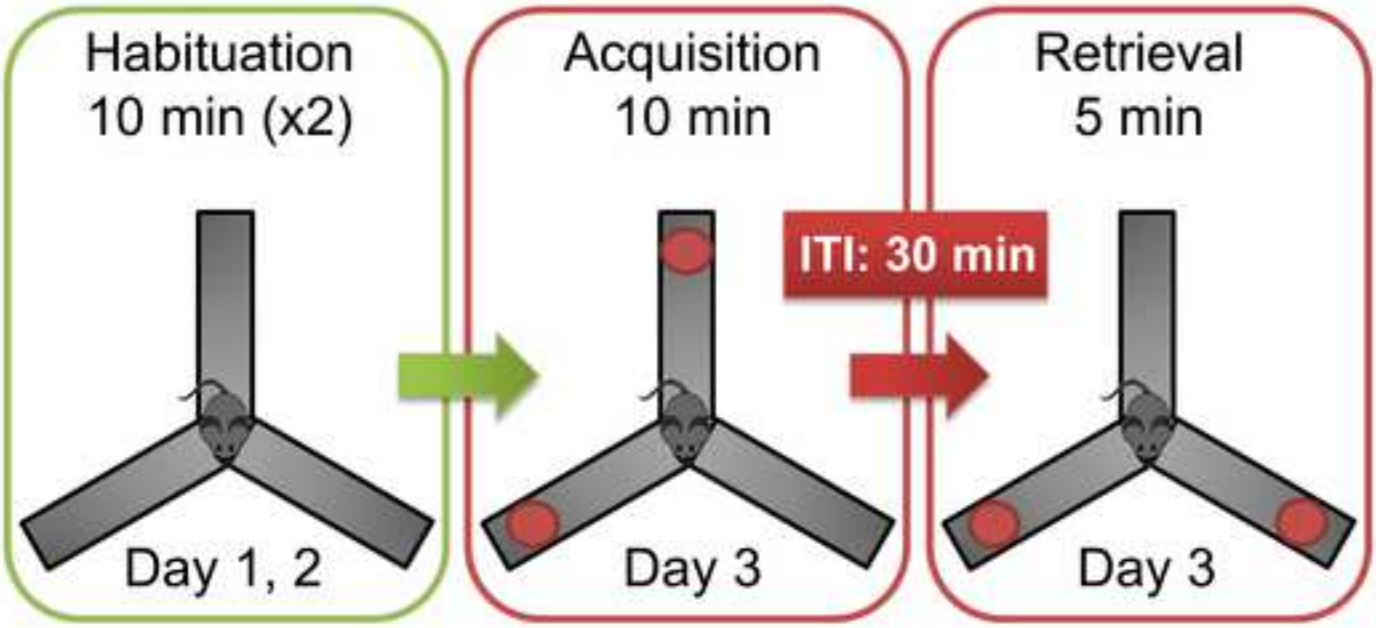

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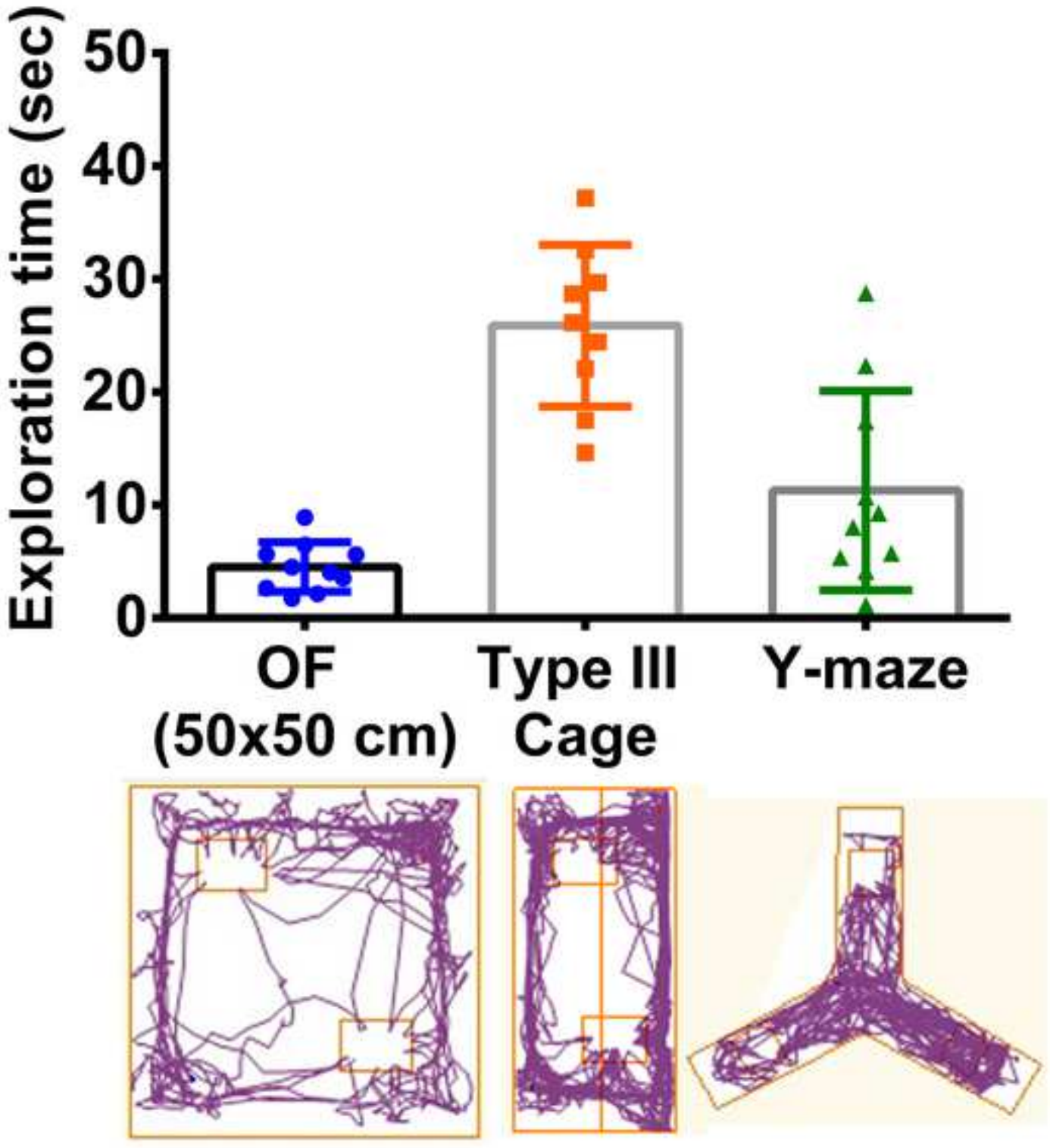

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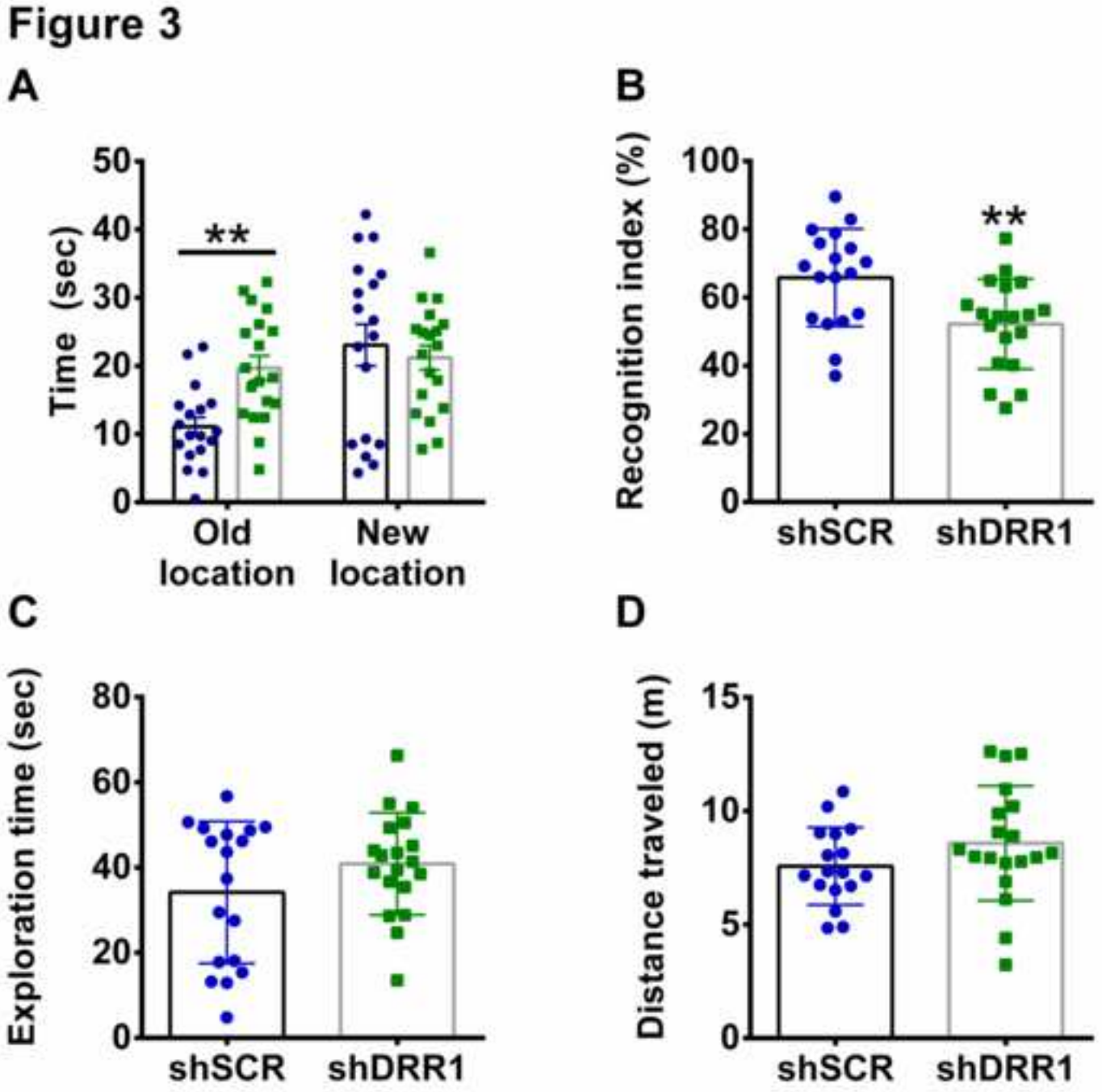

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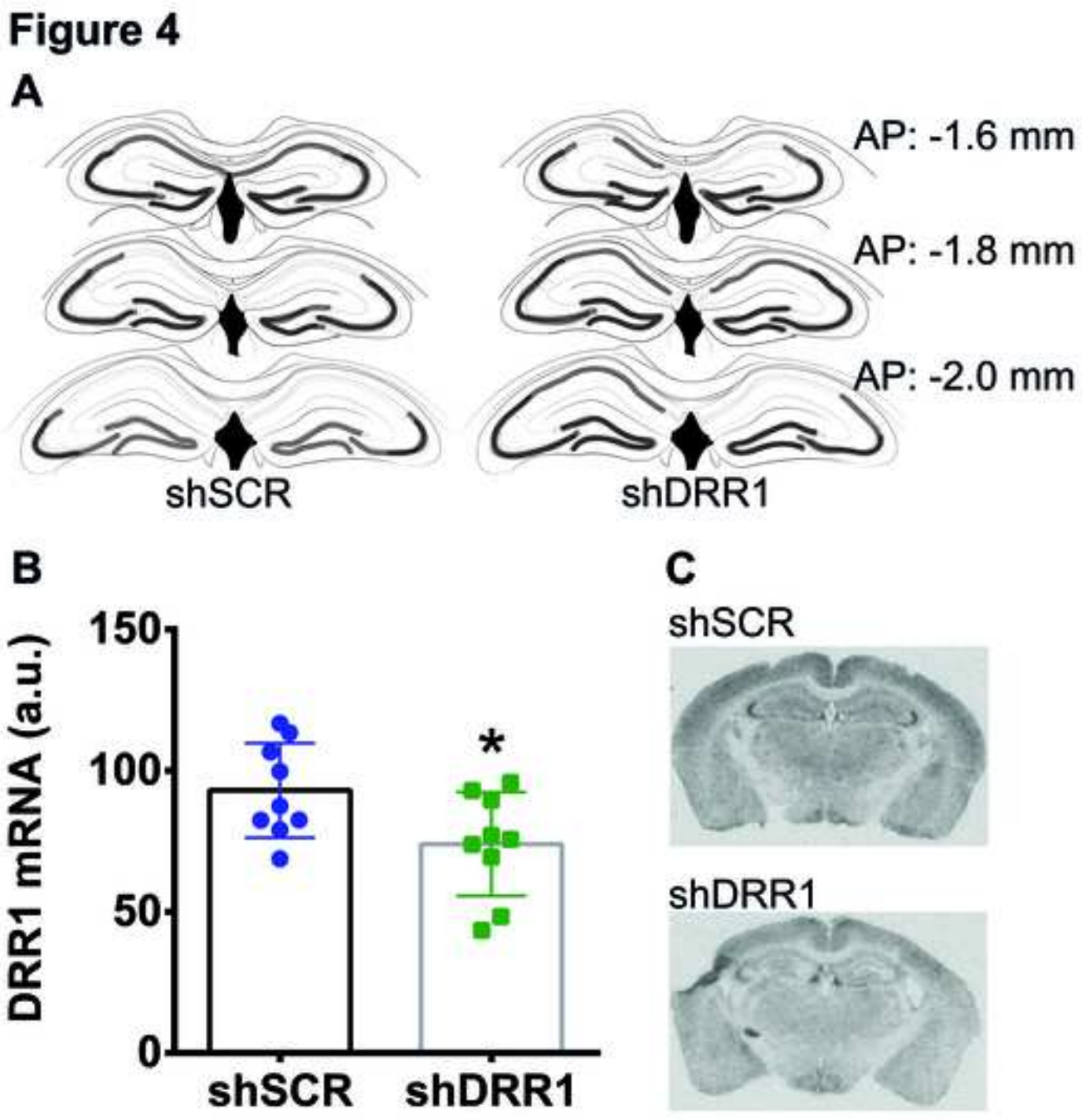

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
LightCycler Capillaries	Roche	4929292001	1-5 µl glass capillar
Micropipette puller	Narishige	PC-10	
Warming pad			
Oxygen/Isofluran system			General anesthesia
Stereotactic system for mouse	Kofp		
Cold light source	Leica/SCHOTT	KL1600LED	
Micro drill: Ultimate-XL, UMXL-T + UHRXL-T	NSK	Y141440 + H269	
Stereo Microscope	Leica	M80	
Panthenol	Bepanthen, Bayer		Humectant, emollient and moisturizer
Lidocain	Xylocain		Local anesthesia
Povidone-Iodine (or propanol)	Betadine (or Kodan)		Antiseptic for desinfection
Meloxicam	Metacam, Heiland		Pain killer
Cotton swabs			
Ethilon sutures	Ethicon	EH7500H	
Y-maze	Custom made		Three arms (30 x 10 x 15 cm) at 120° from each other made of grey PVC
Objects			Glass salt shakers
AAV-shDRR1/AVV-shSCR	Gene Detect	AAV1/2-U6-DRR1 shRNA-terminator-CAG-EGFP-WPRE-BGH-polyA	DRR1 knock-down virus expression cassette
AAV-shshSCR	Gene Detect	AAV1/2-U6-scrambled shRNA-terminator-CAG-EGFP-WPRE-BGH-polyA	Control virus expression cassette

Videotracking software	ANY-maze	Any videotracking software can be used

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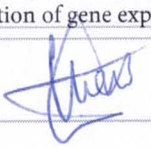
CORRESPONDING AUTHOR:

Name:	Mercè Masana
Department:	Department of Psychiatry and Psychotherapy & Focus Program Translational Neuroscience (FTN)
Institution:	Johannes Gutenberg University Medical Center
Article Title:	~~Spatial object recognition test to evaluate CA3-dependent cognitive function following region-specific~~ regulation of gene expression in the mouse brain
Signature:	
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Rebuttal Comments
Click here to download Rebuttal Comments: Masana et al_reviewers answers_R1.docx

Reply to the reviewer's comments:

Manuscript: JoVE53054
Title: Spatial object recognition test to evaluate CA3-dependent cognitive function following region-specific regulation of gene expression in the mouse
Corresponding Author: Dr. Mercè Masana
Section: JoVE Behavior

Editorial comments:

Please note that the reviewers have raised some serious concerns about aspects of your manuscript. Please thoroughly address or rebut each individual comment below to further strengthen and clarify your submission.

1. The manuscript would benefit from copyediting to fix the occasional grammatical errors.

Reply: The manuscript was now carefully corrected for language mistakes.

2. Formatting: Line 177: Remove personal pronoun "we".

Reply: Done as requested

3. References: Ref 25 is incomplete; journal titles are not abbreviated.

Reply: Reference 25 is now complete:
Heyward, F. D., Walton, R. G., Carle, M. S., Coleman, M. A., Garvey, W. T. & Sweatt, J. D. Adult mice maintained on a high-fat diet exhibit object location memory deficits and reduced hippocampal SIRT1 gene expression. *Neurobiology of Learning and Memory*, **98** (1), 25-32, doi: 10.1016/j.nlm.2012.04.005 (2012).

4. Additional detail is required:
-1.8: How is the skin cut? Scalpel is required for recovery surgeries.
Reply: The skin is cut using a scalpel, as pointed by the reviewer (corrected in pag 5, 1.9)

-Results: Definitions of the constructs used should appear here, not the Discussion.
Reply: The definition of the constructs was now added to the results section (pag. 8 line 337). Also, further details are added in the introduction (pag 4, 1st paragraph), in order to clearly describe the context in which the original study was made.

-Fig. 3A Legend: What is the difference between black and gray bars? Should be explicitely written.
Reply: The new Figure 3 legend now includes the color codes for the bar graphs.

5. Unnecessary branding should be removed:
-1.5: Metacam
-1.6: Panthenol
Reply: In 1.5 (pag 5), the text was modified to emphasize the administration of a pain killer

regardless of the brand name, but still I add as an example the possible administration of Meloxicam with the corresponding dosage (which is related uniquely to this compound) in 1.21, pag 6.
In 1.6 the brand name was removed as suggested by the reviewer, as in this case dosage associated to the brand name is not relevant.
As suggested, unnecessary branding was removed from the protocol (1.5, 1.6, 1.7 and 1.8).

6. Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.
7. Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Reply: The manuscript was now carefully corrected for language mistakes.

8. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

Reply: As previously discussed with the behavior editor Emma Pennock, part of the data presented in this video-article is not yet published. For this reason, Emma Pennock agreed on to hold the publication until the data is published and later add the corresponding final reference from the original paper. Once the paper is published, we will ask and send you the corresponding permission for re-print. At the moment, we marked with grey color the aforementioned reference, which needs to be edited prior to the publication.

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Reply: There were some references lacking the DOI, and they were added as requested (reference 1, 9, 12, 16, 25 and 38).

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this manuscript by Masana et al., authors describe a detailed protocol for intracerebral microinjection of a viral vector and the spatial object recognition to evaluate CA3-dependent cognitive function in the mouse.

Major Concerns:

Although the manuscript is written in a clear and easy to follow manner, there are several concerns that called my attention.

Reply: We were glad to read that reviewer #1 found the manuscript "written in a clear and easy to follow manner".

The methodology described involves two different procedures. One would be the in vivo injection of viral vectors (adeno-associated virus, AAV), and the other would be the cognitive assay. There would be no need to explain both methods together, and both methods can be used independently for numerous purposes. In fact, the title is misleading since authors mention that their test evaluates CA3-dependent cognitive function, but they do not show whether the manipulation of down-regulated in renal cell carcinoma 1 (DRR1) expression is effective (they do not show the expression level of DRR1 after AAV-shDRR1 injection) or if the injection of the AAVs is restricted to the area of interest (hippocampal CA3). This lack of data makes the title not very accurate.

Reply: We agree with reviewer #1 that both methods: in vivo AAV injection and cognitive assay can be used together or independently. We also do not pretend to say that this test is better than others to assess memory, but in particular, we consider the object relocation test as an excellent test of choice to be included in a battery of behavioral tests to assess cognition, because this test is short, less stressful and does not require food deprivation or repeated training such as other cognitive tests.
The data presented here are part of an in depth investigation using AAV-mediated knock-down of DRR1 to characterize in detail the impact of a loss-of-function of DRR1 on the behavioral phenotype. Here, the spatial object recognition test was included into a battery of different behavioral assessments to investigate cognitive performance (Masana et al, in preparation). The lack of information, as pointed by the reviewer, could be misleading, and for this reason we added more information about the context of the study in the introduction (pag 4, 1st paragraph), explicitly citing the paper which will provide full information on the in-depth analysis. In addition, we further provide evidence of the validation of the injection site and quantification of the reduction of DRR1 mRNA expression in CA3 as suggested (Figure 4 and results section, pag 9-10). Also, a new section in the protocol was added, mentioning the need to validate the AAV-mediated gene knockdown and the correctness of the injection site (protocol, new section 4, pag 8). Finally, we also modify the title as suggested.

The manuscript does not display crude data or scattered plots for all the data (Figure 3, for example) that could be interesting to have an idea of the expected variability of the measures.

Reply: As suggested by the reviewer, we now included the scattered plots for all the data. The new figure clearly show how the shSCR mice generally learn the task, meaning the animals explore more the new object location compared to the old (known) one, with exception of few mice. Instead, shDRR1 explore similarly both new and old objects, indicating that they do not learn the new position of the object.

In this regard, a relevant issue is the number of mice used. This study used 18-19 mice per group for the behavioral test. This number is very high for a method that wants to be easy to perform.

Reply: We agree with the reviewer that n=18-19 mice per group seems to be big. However, dealing with a larger variability in behavioral outcomes than, e.g. in other laboratory measures, it is quite reasonable not to use smaller groups for behavioral assessments (see e.g. Dell R.B., Holleran S., Ramakrishnan R. (2002) Sample size determination. ILAR J. 43: 207-213. Institute for Laboratory Animal Research (U.S.), Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, and National Academies Press (U.S.) (2003) Guidelines for the care and use of mammals in neuroscience and behavioral research. Washington, D.C., The National Academies Press, ISBN 0309089034). The original study included all these mice (as the group was divided in two groups later), and for this reason we think it is appropriate to include all the animals in the results. In addition, with the new Figure 3, we can see that there is not much variability between the groups.

Authors seem to test three mazes for the cognitive test, but they do not show data supporting that the one they describe in detail (Y-maze) is the best one at the time of testing memory for object location.

Reply: Only total exploration time of objects during 10 min was obtained using the three different mazes (single object exploration time: 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 arena). The rationale to select the Y-maze was based on this measure and the possibility to distinguish between changes in location of objects (we excluded the Type III Rodent cage for this reason). From the use of OF or Y-maze, we selected Y-maze because the exploration time of the objects was higher in that arena.
The reason of that is that the greater the time explored, the greater the accuracy of the results. We can assume that exploration time during retrieval (5 min compared to the 10 min measured here) would be lower than 4.5 sec. Considering the error of the time measures of 0.1 sec, the exploration time value of 4.5 sec has a relative error of 0.1/4.5=0.02 → 2%; while exploration time value of 11.3 sec has a relative error of 0.1/11.3=0.009 → 0.9%; these relative errors will respectively increase when time measurements are smaller. Moreover, changes in exploration time of e.g. 2 and 4 sec for old and new object's location (2 sec difference, 66% index recognition) will not have the same impact as changes from 15 to 17 sec (2 sec different, 53% index recognition). Therefore the results are more accurate when total exploration time is increased and possibly animal numbers can be reduced as a consequence of it. In fact, our group performed the SOR in the open field and this did work (Schmidt et al 2011 PNAS, Masana et al 2014 PNEC), but with the use of the Y-maze arena we were able to increase the exploration time of the animals, making the results more robust and accurate (Masana et al, in preparation). Therefore using the Y-maze for the SOR test increases the accuracy of the time measurements, despite the ability of both arenas to detect changes in spatial memory function.
In any case, it is well known that different cognitive tests might yield slightly different results, because they involve other additional aspects (anxiety, motivation, etc) and it is recommended to perform more than one test to check for a behavioral domain, such as e.g. cognition. Thus, we feel not convenient to claim that one arena is generally better than another in a particular inter-trial interval, as this might depend on the particular details of each experiment. Still, we showed that our selected conditions were able to detect changes in cognitive function in our model.
In order to clarify the rationale of the selection of the arena, we added more information (pag 11, 2nd paragraph, discussion).

Another concern that is not clear, and perhaps authors know about, is the necessity of running two habituation sessions in consecutive days. This makes the test more laborious and should be

documented whether these two days of habituation in a row are really necessary for the test to work. If the second day is not really necessary, the procedure would be significantly shorter than the one proposed. It should be taken into account that other protocols in the literature manage to obtain solid data using a single habituation session.

Reply: In the literature there are several protocols from different laboratories with different numbers of habituation sessions. In some conditions one habituation might be sufficient, but as our lab uses stress models, we prefer to have a second habituation to ensure that the animal will explore (and not freeze) on day of the test (this was already mentioned in the Discussion, line 475). Moreover, increasing the number of habituation sessions ensures that the animal will be familiar with the arena, and therefore the novelty will be restricted to the objects. With optimal habituation of the animal, we finally are able to minimize other aspects such as novelty, arousal and anxiety and to focus on the cognitive performance.

Finally, authors only show that the cognitive test works with a 30 min inter-trial interval (ITI), making the test suitable only for assessing short-term memory. The behavioral test would increase their applicability in the field of cognition if the authors could demonstrate that the test works when the ITI is 24h or 48 h, or as long as the object position memory trace lasts.

Reply: We agree with the reviewers that the applicability of the test would be greater if the test works also at different ITI, including 24 or 48h, but we did not test these other time points in order to extend the test. Still, we hope that with all the information provided in the paper, anyone could be able to test this.

The general impact of the article is limited since there is no clear description of the advantages in performing the cognitive test in the way proposed by the authors, compared to other possible settings. There are no clear explanations for some of the steps performed. For example authors mention to habituate mice to the room if possible for one week, but they also say that 1hour might be enough. Another issue that is not clear is the necessity of two habituation sessions in two consecutive days.

Reply: We agree with the reviewers that some descriptions were not precise. As mentioned before, the objective of the article was to show one possibility to perform the test, but also to discuss all the steps necessary to take into account when setting up the test in a new lab. Some of the descriptions are vague, like the habituation time of the mice to the testing room, because not all the testing rooms allow housing the animals, and therefore, a minimum time for the animal to habituate to the room is needed.
About the need of 2 habituations we have evidence to suggest that 2 habituations might indeed be recommended: In another experiment using a different way of treatment, we used only one habituation by mistake. Those animals (vehicle treated) explored 20±2 sec (n=20) but drug-treated mice only explored 11±2 (n+18) sec in total, making it impossible to compare the groups, as drug-treated mice did not explore similar to control mice. Thus, we think that a second habituation would have helped to further habituate, specially the drug-treated group, in order to increase its exploration time on the day of the test. However, these arguments are not conclusive enough to state that 2 habituation are always needed.

Authors do not mention key details such as the mouse strain, or if the behavioral approach would work in several mouse strains.

Reply: We added the information regarding the mouse strain, gender and age of the mice used in the present study (pag 4, protocol section, and pag 8, results section; male C57Bl/6N (>8 weeks old) were used). Unfortunately, we did not test for gender or strain difference. It is know that in the novel object recognition gender is relevant for the perception of the objects (Betis and Jacobs Behavioural Brain Research 233 (2012) 288– 292). In this case, the objects in SOR are the same, so we assume the test would be suitable for both genders, but more data would be needed to ultimately prove it. However, different strains can show different stress levels, locomotor activity, exploratory behavior, motivation, etc. and have been shown to have different phenotypes. A comparison of strain-specific differences for this protocol would be beyond the scope of this paper.

Reviewer #2:

Manuscript Summary:

In their manuscript "Spatial object recognition test to evaluate CA3-dependent cognitive function following region-specific regulation of gene expression in the mouse" Masana and co-authors describe the procedures of the stereotactic surgery to inject a viral solution into the CA3 region of the mouse and of the performance of a spatial object relocation test in a Y-Maze.

While the idea of developing and precisely describing a reliable short test that gives robust information on memory function is intriguing, I find several aspects of this paper problematic, in particular in the methods section and in view of JoVE's mission to improve the reproducibility of scientific experiments.

Reply: We are glad that the reviewer likes the idea of precisely describing a reliable short test that gives robust information. Our aim was not to develop a "new" behavioral test, but to facilitate the concepts and tools to perform and analyze the test correctly. Also, to give insights into small aspects that could significantly influence the test and the interpretation of the results.

Major Concerns:

1. The terminology is not precise. Since identical objects are used, and the animal is asked to notice a new location of one of the objects, this is not an object recognition, but an object relocation test. This must be consistent throughout the manuscript.

Reply: In the literature we can find both object recognition and object relocation test referring to the same test, but we agree with the reviewer that the use of object relocation test is more precise. Thus, we changed the terminology accordingly.

2. The rationale of the choice of the gene that is knocked down to induce the memory impairment to demonstrate the validity of the methods described is not adequately explained. I can not even find an explanation for the abbreviation DRR1 anywhere in the manuscript. More background information is needed for the reader to follow the line of argumentation.

Reply: The DRR1 gene study was used to illustrate the methods used, but was not specifically chosen in order to validate the SOR test, for this reason we avoided the information from the relevance of the gene and focused mainly on the methods. Nonetheless, we added further information in the

introduction (pag 4, 1st paragraph) on the DRR1 constitutive expression in the CA3, and provided further data with the need of post-mortem validation of the knock-down in the CA3 (protocol pag 8, new section 4; Results pag 9-10, new section: validation of AAV-injection; new Figure 4 and corresponding Figure 4 legend, pag 10). Moreover, we provide further details on the context of the study, which was an in-depth investigation on the loss of function of DRR1 in the hippocampal CA3, and we explicitly refer to the article where the data presented here will be published as part of the dataset (Masana et al in preparation). We hope that with all these further details, we clarify that the rationale of the video-article was to describe an easy test to be included in a battery of behavioral tests and because of the non-aversive condition of the test. Our results based on the SOR further support the relevance of this test for CA3-dependent cognitive function.

3. The authors do not show any histological evidence that their injection sites were correct or that their gene knock down worked. The claim that DRR1 expression was only reduced in neurons where it is actually expressed (p. 9, lines 376-377) is completely unproven.

Reply: Our new results section (pag 9-10) and the corresponding new Figure 4 + Legend (pag 10) showed the validation of the virus injection and the quantification of the DRR1 knock-down.
The reviewer is right that we did not prove specifically in which cells DRR1 was reduced. For this reason, we modify the sentence to: "Using this virus-induced knock-down strategy, we expect that DRR1 expression would be only reduced in infected cells expressing the gene" (pag 11, line 446-7).

4. p. 4, line 168: describe the instrument to be used to cut the skin.

Reply: The information is added to pag 5, 1.9, line 197 (the skin is cut using a scalpel).

5. p. 5: The time line of single housing, surgery and behavioural testing is unclear. It reads as if the animals were still group housed after surgery and single housed one week before the start of behavioural testing. That does not make sense to me. Also, it is stated that virus infection is stable for 4 weeks after surgery, and then recommended to start the behavioural testing at least 4 weeks after the surgery. Does that mean one should start behavioural testing when the virus infection is no longer stable? That also does not seem to make sense. Does starting "at least" 4 weeks after the surgery mean one should wait at least 4 weeks before starting? This part needs to be explained more clearly, also with more background information.

Reply: We apologize for the mistake in the protocol. We corrected this and hope to have clarified section 1.22 (pag 6, line 244) and 2.1.1 (pag 6, line 149-252). In section 1.22 it should read as AAV infection is stable 4 weeks after surgery, meaning we need to wait 4 weeks to allow the virus to infect and finally show a stable infection over time. Then, we need to wait 4 weeks before starting the behavioral testing.
Animals were single housed during all the procedure (surgery, surgery recovery and behavioral testing). We delete the sentence on the housing here, but added a sentence at the beginning of the protocol stating that the animals where individually housed during all the procedure to avoid any misunderstanding (pag 4, line 155-7).

6. The lack of use of a disinfectant for cleaning the equipment is not compatible with the hygiene conditions required in animal facilities, let alone the reliable erasure of odour traces left by the

animals. Likewise, the use of white tape for intra-maze cues is not ideal and raises the question how that could be kept clean and free from odour traces in long-term usage.

Reply: We agree with the reviewer that cleaning of behavioral test equipment between individual animals is crucial to avoid the spread of odor traces which otherwise might influence test results. Apart from obvious olfactory cues like feces and urine, rodents secrete fluids from their foot pads which might influence subsequent testing. However, as all our animals are housed under identical conditions and share the same (identical) hygiene status, we consider careful cleaning with water to be sufficient as between-tests procedure, as there is no reason to be afraid of spreading contagious diseases between animals that anyway share the same housing conditions. With this, we can make sure that we do not introduce an additional (novel) odor to the animal and the test arena which could be the case when cleaning with alcohol.
Having finished the behavioral testing with the two experimental groups, the test arenas are carefully cleaned and disinfected with e.g. 70% alcohol. This aspect was added in the protocol as suggested (pag 8, 2.2.8, line 312).

7. The features of the objects are key in an object-based task. Therefore easily obtainable objects should be used if a reproducible method is to be described. The authors don't even mention the manufacturer of the glass salt shakers they used as objects. In general, the list of materials could be more precise.

Reply: We agree that the objects are key in object-based test. For this reason, we provide a picture of the objects in Figure 1B.

Minor Concerns:
8. p. 9, line 359: in Fig. 3 bars do not show mean ± SEM, but mean + SEM only.

Reply: With the new figure this annotation is now correct.

9. The language should be checked carefully by a native speaker.

Reply: The manuscript was now carefully corrected for language mistakes.

Additional Comments to Authors:
N/A