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Molecular Mechanisms in Perirhinal Cortex Selectively Necessary for Discrimination of Overlapping Memories, but Independent of Memory Persistence.

BDNF, Arc, pattern separation in perirhinal cortex

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Molecular mechanisms in perirhinal cortex selectively necessary for discrimination of overlapping memories, but independent of memory persistence.

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Abstract

Successful memory involves not only remembering over time but also keeping memories distinct. The ability to separate similar experiences into distinct memories is a main feature of episodic memory. Discrimination of overlapping representations has been investigated in the dentate gyrus of the hippocampus (DG), but little is known about this process in other regions like the perirhinal cortex (Prh). We found in male rats that perirhinal Brain-derived neurotrophic factor (BDNF) is required for separable storage of overlapping, but not distinct, object representations, which is identical to its role in the DG for spatial representations. Also, Activity-regulated cytoskeletal-associated protein (Arc) is required for disambiguation of object memories, as measured by infusion of antisense oligonucleotides. This is the first time Arc has been implicated in the discrimination of objects with overlapping features. Although molecular mechanisms for object memory have been shown previously in PRh, these have been dependent on delay, suggesting a role specifically in memory duration. BDNF and Arc involvement were independent of delay — the same demand for memory persistence was present in all conditions — but only when discrimination of similar objects was required were these mechanisms recruited and necessary. Finally, we show that BDNF and Arc participate in the same pathway during consolidation of overlapping object memories. We provide novel evidence regarding the proteins involved in disambiguation of object memories outside the DG and suggest that, despite the anatomical differences, similar mechanisms underlie this process in the DG and Prh that are engaged depending on the similarity of the stimuli.

Significance statement

In this manuscript we show, outside of the hippocampus, the molecular mechanisms underlying the ability to separate similar experiences into distinct memory representations (thought to result from the computational mechanism of pattern separation). The dentate gyrus (DG) is thought to disambiguate representations belonging to any domain, but other regions could also perform this operation. Although molecular mechanisms have been shown previously in the perirhinal cortex (Prh), these have always been dependent on delay, suggesting a role specifically in memory persistence. We report that, despite the profound anatomical differences between the perirhinal cortex (Prh) and the DG, the discrimination of overlapping memories in these regions relies on the same molecular mechanisms.

Introduction

Two similar stimuli could be associated with two very different experiences: a cat inside your house may be friendly while a puma could be threatening to your life. It is thought that the brain creates unique representations of similar events, which are less confusable and can be associated with different outcomes, through a process called pattern separation (Treves and Rolls, 1994; Gilbert et al., 1998; Leutgeb et al., 2007). The original computational models define the process in terms of a transformation of input representations into output representations that are less correlated with each other (Marr, 1971; Treves and Rolls, 1994; McClelland et al., 1995). Thus, pattern separation increases the likelihood of accurate encoding and subsequent retrieval. It has been

studied effectively using electrophysiology (Leutgeb et al., 2007; Neunuebel and Knierim, 2014), and we and others have developed tasks to demonstrate the relevance of pattern separation processes to cognition (Gilbert et al., 1998; Kirwan and Stark, 2007; Clelland et al., 2009; Toner et al., 2009; Creer et al., 2010; Bekinschtein et al., 2013).

Since episodic memory involves the recollection of unique events, separation of similar experiences is proposed to be an essential component for the storage of non-confusable representations of these episodes and has been studied mainly in the hippocampus (Ranganath, 2010). Indeed, the computational models focus specifically on DG granule cells, which are thought to be a domain-general pattern separator (Yassa and Stark, 2011), well-suited for performing pattern separation on overlapping inputs from the entorhinal cortex. Adult neurogenesis in the DG, has been shown to be required for discrimination of overlapping representations in the spatial domain (Gilbert et al., 1998; Clelland et al., 2009; Bekinschtein et al., 2014a), and some studies have begun to elucidate the molecular basis involved in this process (Bekinschtein et al., 2013, 2014b).

Because the hippocampus is known to mediate spatial memory in rodents, with the exception of a few studies (e.g. (Johnson et al., 2017), most tasks used to evaluate the behavioural outputs thought to result from discrimination of overlapping representations in rodents have involved some kind of contextual or spatial manipulation (Gilbert et al., 1998; Clelland et al., 2009; Kheirbek et al., 2012; Nakashiba et al., 2012; Bekinschtein et al., 2013). However, this type of disambiguation could, in principle, occur during encoding of representations other than spatial, for example for objects in Prh (Kent et al., 2016).

Indeed, disambiguation of object representations has been shown to require Prh (Bussey et al., 2002; Bartko et al., 2007), and it has been proposed that Prh discriminates similar objects by storing unique conjunctive representations of these items (Bussey et al., 2002; Bartko et al., 2007). However, it has been suggested that the DG is a domain-general discriminator of both spatial and object representations, among other types. Although molecular mechanisms have been shown previously in PRh, these have always been dependent on delay, suggesting a role specifically in persistence (Winters and Bussey, 2005b; Seoane et al., 2012). Manipulation of the Prh during acquisition or after learning, produced delay-dependent effects on memory, but this does not indicate a specific effect on the ability to disambiguate similar input stimuli. It is not known whether a putative function of Prh in object disambiguation operates via the same molecular mechanisms as those shown within the DG (Bekinschtein et al., 2013). In this work, we tested whether Prh is involved in the consolidation of overlapping object memories through plasticity-related mechanisms such as BDNF that have been implicated during discrimination of overlapping spatial memories. We found that BDNF, a protein essential for memory storage (Bekinschtein et al., 2014a), is required for disambiguation of memories for similar objects in Prh, just as it is for spatial memories in the hippocampus. In addition, we found that Arc, a molecule important for plasticity and memory (Bramham et al., 2010), is also required. This immediate early gene product, has emerged as a key protein in memory formation and different types of synaptic plasticity including long-term potentiation (LTP), long-term depression (LTD) and homoeostatic synaptic scaling (Bramham et al., 2010). Arc is strongly associated with neuronal activity related to behaviourally relevant experiences

(Guzowski et al., 2005). In addition, this molecule has been shown to be required in various structures for different types of learning such as fear conditioning (Ploski et al., 2008) and inhibitory avoidance (Martinez et al., 2012). Arc-deficient mice present deficits in several learning tasks such as the water maze fear conditioning, conditioned taste aversion and novel object recognition (Plath et al., 2006). These evidences pointed at Arc as a possible target of BDNF action. Finally we demonstrated that BDNF is likely to act upstream of Arc during the consolidation of "pattern-separated" object memories. We suggest that discrimination of similar, but not distinct, stimuli in the medial temporal lobe occurs not only in the DG, but also in the Prh, depending on the nature of the representations. Importantly, similar mechanisms underlie the discrimination of overlapping memories wherever it occurs, and these mechanisms are different from those that vary with demand on memory persistence.

Materials and methods

Subjects

The subjects were 201 male Long Evans rats from our breeding colony, weighing approximately 250-300 g at the start of testing. The rats were housed on a reversed 12 h light/12 h dark cycle (lights on 19:00-07:00), in groups of two or four. All behavioral testing was conducted during the dark phase of the cycle. Rats were food deprived to 85-90% of their free feeding weight to increase spontaneous exploration, except during recovery from surgery, where food was available *ad libitum*. Water remained available *ad libitum* throughout the study. All experimentation was conducted in accordance with the

National Animal Care and Use Committee of the University of Buenos Aires (CICUAL) and strict compliance with the guidelines of the University of Cambridge and United Kingdom Animals (Scientific Procedures) Act 1986 and the Amendment Regulations 2012.

Surgery and cannulation

All rats were implanted bilaterally in Prh with 22-gauge indwelling guide cannulas. Subjects were anaesthetised with ketamine (Holliday, 74 mg kg^{-1}, i.p.) and xylazine (Konig, 7.4 mg kg^{-1}, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the incisor bar set at −3.2 mm. Guide cannulas were implanted according to the following coordinates, measured relative to the skull at bregma (Paxinos and Watson, 1998): anteroposterior −5,5 mm, lateral ±6.6 mm, dorsoventral −7.1 mm. The cannulas were secured to the skull using dental acrylic and three jeweller screws. Obturators, cut to sit flush with the tip of the guide cannulas and with an outer diameter of 0.36 mm, were inserted into the guides and remained there except during infusions. At the completion of each surgery, an antibiotic was applied for three days (Enrofloxacin; 0.27 mg kg^{-1}, Vetanco, Arg). Animals were given at least 7 days to recover prior to drug infusions and behavioural testing.

Infusion procedure

Depending on the experiment, rats received bilateral infusions of oligonucleotides (ODNs, 4 nmol μl^{-1}/0.5 µl side; Genbiotech), human recombinant BDNF (0.5 µg μl^{-1}/0.5 µl side; Byoscience), emetine (50 µg μl^{-1}/ 0.5 µl side; Sigma-Aldrich) or saline at different times during the behavioural task. The injection volume was always 0.5 µl/side. ODNs were HPLC-purified phosphorothioate end-capped 18-mer sequences, dissolved in sterile saline to a concentration of 4 nmol μl^{-1}. All ODNs were phosphorothioated on the three terminal bases of both 5' and 3' ends. This modification results in increased stability and less toxicity of the ODN. BDNF ASO, 5'-TCTTCCCCTTTTAATGGT-3'; BDNF MSO, 5'-ATACTTTCTGTTCTTGCC-3'. Arc ASO, 5'-GTCCAGCTCCATCTGCTCGC-3'; Arc MSO, 5'-CGTGCACCTCTCGCAGCTTC-3'. All ODN sequences were subjected to a BLAST search on the National Center for Biotechnology Information BLAST server using the Genbank database. Control MSO sequence, which included the same 18 nucleotides as the ASO but in a scrambled order, did not generate any full matches to identified gene sequences in the database. Bilateral infusions were conducted simultaneously using two 5-µl Hamilton syringes that were connected to the infusion cannulas by propylene tubing. Syringes were driven by a Harvard Apparatus precision syringe pump, which delivered 0.5 µl to each hemisphere over 2 min. The infusion cannulas were left in place for an additional minute to allow for diffusion. At least 3 days were allowed for washout between repeated infusions.

Immunoblot assays

206 After rats were sacrificed, brains were immediately frozen and the Prh was
207 microdissected. Tissue was homogenized in ice-chilled buffer (20 mM Tris-HCL [pH 7.4],
208 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mg/ml aprotinin, 15 mg/ml
209 leupeptin, 10 mg/ml bacitracin, 10 mg/ml pepstatin, 15 mg/ml trypsin inhibitor, 50 mM
210 NaF, and 1 mM sodium orthovanadate). Samples of homogenates (15 μg of protein) were
211 subjected to 10% or 12% SDS-PAGE under reducing conditions. Proteins were transferred
212 onto nitrocellulose membranes (Biorad) in transfer buffer (25 mM Tris, 192 mM glycine,
213 10% v/v methanol) for 2 h at 100V. Western blots were performed by incubating
214 membranes first with anti-BDNF antibody (N20, 1:1000, Santa Cruz Biotechnology Inc),
215 with anti-Arc antibody (1:2000, Santa Cruz Biotechnology Inc, Santa Cruz, CA) and anti-
216 actin antibody (1:5000, Santa Cruz Biotechnology Inc). One nanogram of recombinant
217 human BDNF was used as a standard for Western blot (rhBDNF, Alomone). Blots were
218 developed using enhanced chemiluminescence (GE Healthcare), visualized by Storm 845
219 phosphorimager (GE Healthcare Life Sciences) and quantified using ImageJ software (NIH,
220 USA). For analysis, optical density (OD) values and the band areas were obtained for each
221 microdissected hippocampal sample for both the target protein (BDNF, Arc) and the actin
222 loading control. Each target OD value was normalized to its corresponding actin OD value
223 and normalized levels were averaged for each condition. Data were analysed using a one-
224 way ANOVA followed by Newman-Keuls post-hoc comparisons. Data depicted in Fig 2D
225 was transformed before the analysis.
226
227 **Apparatus**

The triangular open field used for the spontaneous object recognition task (SOR) was made of white foam board. Each wall had 60 cm long x 60 c m high. The circular open field (90 cm diameter x 45 cm high) used for the spontaneous location recognition task (SLR) was made of black plastic. Both open fields were situated in the middle of a dimly lit room. The walls of the triangular open field were higher in order to minimize the visual access to the distal cues in the room. The circular open field was surrounded by four spatial cues and standard furniture. The open field floor was always covered with wood shavings. A video camera was positioned over the arena and sample and choice phases were recorded for later analysis. The objects for the SOR task were made of two different smaller objects, except for the extra-similar condition in which they were made by three smaller objects. Composite objects were made by simply attaching together two or three of the smaller items in the conditions described in the 'results' section (Fig. 1). We always used different objects for our within subject design, examples can be seen in Fig 1). For the SLR, the objects used were either soda cans or beer bottles from which the label had been removed. All objects were fixed to the floor of the open field with Blu-tack ™ and cleaned with a 50% ethanol solution between sample and choice trials. For the SOR task, all three composite objects were aligned close to one of the walls of the arena and positions within this line were pseudorandomnly assigned. Other tasks that evaluate object discrimination have used objects built with LEGO™. While LEGO-constructed objects offer some versatility when trying to manipulate the similarity between them, they could also cause more interference, as the texture would be the same between the different objects made of

the same material. In fact, it has been shown that merely the fact that an object is built with LEGO can cause interference with another LEGO object that is not particularly similar (Bartko et al., 2010). Junk object features offer different textures and curvy shapes that are not present in LEGO-based objects.

For the SLR task (Fig 5D-E), positions varied according to the condition tested, with objects always placed along a circumference 15 cm away from the wall and 30 cm away from the center of the arena. For the similar condition, objects were separated by a 50° angle; and for the dissimilar condition, they separated by an angle of 120°.

Behavioural procedures

For the SOR task (Fig. 1) each rat was handled for 3 days and then habituated to the arena for 5 min a day for 3 days before exposure to the objects (Fig 2, 3, 5, 6 and 8). For SLR task (Fig 5D-E), each rat was handled for 3 days and then habituated to the arena for 10 min a day for 5 days before exposure to the objects. For the SOR task, after habituation the rats were exposed, during a 5-min duration sample phase, to three objects made of either two or three features depending on the condition. For the similar condition, two of the objects shared one feature (AB and BC) and the third object was made of two other different features (EF). For the dissimilar condition all three objects were made of different features (AB, CD and EF). For the extra-similar condition (Fig 8A-D), two of the objects shared two of three features (ABB and BBC), and the third one was different (EFG). The choice phase lasted 3 min and was carried out 24 h after the finalization of the sample phase. In this

case, the animals were exposed to two objects, one novel and one familiar, that varied in composition according to the condition evaluated. For the similar condition, the novel object was made of the two non-shared features of the objects presented in the sample phase (AC) and the familiar object was a copy of the third object (EF). For the dissimilar condition, the novel object was made of two novel features (GH) and the familiar object was a copy of one of the objects presented during the sample phase (AB, CD or EF). Since most of the experiments involved a within-subject design, the letters do not indicate that we used the same object or feature. We always used different objects and features for the different trials. The rationale behind the task was that if the rats were able to separate the two similar objects, their representations should be distinct and resistant to confusion; therefore, the rats should show preference for the novel object during the retrieval phase. However, if the representations of the two similar objects were not sufficiently separated, presentation of the new object would activate a familiar representation in memory and would thus not be distinguishable. The result would be that rats should behave as if the new object was familiar. As this process is thought to happen during encoding/consolidation stages of memory formation, the similarity of the to-be-remembered objects was varied during encoding/consolidation, rather than the retrieval phase of the task. Unlike other tests of discrimination (Gilbert et al., 1998; Clelland et al., 2009; Nakashiba et al., 2012), the use of a continuous variable as a measure of performance yields sufficient data within a single trial to allow manipulations at different stages of memory. In contrast, previous tasks using discrete trial procedures require many trials to collect sufficient data, and thus such manipulations would have to be repeated an

impracticable number of times.

For the extra-similar condition (Fig 8A, 8, 8C and 8D), the novel object was made of a novel combination of familiar features (ABC) and the familiar object was a copy of the third object presented in the sample phase (EFG). Exploration was recorded and later scored manually for both the sample and choice phases. For all experiments, exploration of a particular object was defined as the rat having its nose directed at the object at a distance of 2 cm or less, or touching the object with its nose. Rearing with the head oriented upward did not count as exploration. Climbing over or sitting on the objects was not included. Two people scored the videos; one was blind to the novel and familiar objects. There was no significant inter-rater variability.

For the SLR task (Fig 5D-E), after habituation, rats were exposed to three identical objects A1, A2 and A3, during a sample phase that lasted for 10 min. For the similar SLR (s-SLR), objects A2 and A3 were placed 50° apart (20.5 cm between them) and object A3 at an equal distance from the other two. For the dissimilar SLR (d-SLR), objects A1, A2 and A3 were equidistant, 120° (49 cm between them) apart from each other. Twenty-four hours after the sample phase, rats were exposed to two new identical copies of the objects, named A4 and A5, for 5 min. New identical copies were used to prevent the use of olfactory cues. During this choice phase, object A4 was placed in a familiar location (same position as in the sample phase) and object A5 was placed in a novel location. For the s-SLR task, the novel location was defined as a position exactly in between the ones in which objects A2 and A3 were located during the sample phase (see schemes in Fig 5D). For the

d-SLR task, object A4 was placed in a familiar location and object A5 in a position equidistant to the previous locations of A2 and A3 (see schemes in Fig 5D).

Experimental Design and Statistical Analysis

For all the experiments, the results were expressed as a discrimination ratio that was calculated as the time exploring the novel object (SOR) or the object in the novel location (SLR) minus the time exploring the familiar object (SOR) or the object in the familiar location (SLR) divided by total exploration time ($(t_{novel}-t_{familiar})/t_{total}$). In Fig. 2C, one sample t test were used to compare discrimination ratio from the similar and dissimilar conditions to verify that the ratio was different from zero. For the experiment shown in Fig 2C, half of the rats were tested first in the "novel condition" and then in the "familiar condition", and the other half were tested first for the familiar and then for the novel conditions. Discrimination ratios were compared within subject using a paired *t* test. For experiments shown in Figures 3C, 3D, 4C, 5A and 5E, and 6B rats were tested twice. In the first trial half of the animals received ASO injection and the other half received MSO injection. In the second trial they were injected with either ASO or MSO depending on what they had received in the first trial. For the sample phase, the percentage of time exploring each object was compared using a repeated measures two-way ANOVA, with time and object as the repeated measures. For the choice phase, discrimination ratios were compared within subject using a paired *t* test. Different features (A, B, C, etc) were used to reproduce the same task conditions in the consecutive trials of the within subject design.

For the experiment in Figure 8F, animals were tested twice, once injected with Arc-ASO and BDNF-ASO in the hemisphere and once with Arc-ASO and BDNF-ASO in different hemispheres. Control MSO was injected in the other hemisphere. Discrimination ratios were compared within subject using a paired *t* test. For the experiments shown in Figures 8B and 8D, animals were tested only once, discrimination ratios were analyzed using a *t* test or a Two-way ANOVA followed by Bonferroni post-hoc comparisons. In all experiments drug and vehicle or ASO and MSO injections were counterbalanced. We performed one-sample t tests for every discrimination ratio in order to analyze whether control animals learned the task.

Histology

At the completion of behavioral testing, all rats except the ones used for further experiments were anaesthetized by IP injection with 2 ml of Euthatal (Rhône Merieux) and perfused transcardially with phosphate buffered saline (PBS), followed by 10% neutral buffered formalin. The brains were removed and postfixed in formalin for at least 24 hours before being immersed in 20% sucrose solution until they sank. Sixty-µm sections were cut on a freezing microtome encompassing the extent of the injector track. Every fifth section was mounted on a gelatin-coated glass slide and stained with cresyl violet. Slides were examined under a light microscope to verify the location of the injections. For analysis of oligonucleotide (ODN) spread after injection, rats were injected with 2 nmol/µl (0,5 µl/side) of biotinylated Arc ASO ODN 2 h later, they were anesthetized and perfused

transcardially with 0.9% saline followed by 4% paraformaldehyde. The brains were isolated and sliced, and the ASO was detected by avidin–biotin staining (Bekinschtein et al., 2007)

Results

In the original spontaneous object recognition (SOR) task (Ennaceur and Delacour, 1988; Warburton et al., 2000), rats are exposed during a sample phase to two identical objects placed within an arena. After a variable delay, rats are given a choice phase in which one of the objects was replaced by a completely novel object. Since rats naturally prefer novelty, rats with intact memory spend significantly more time exploring the novel object than the familiar one (Warburton et al., 2000). A detailed description of the modified task we used in this study can be found in the 'Methods' section. Briefly, it consisted of a sample (study) phase in which rats were exposed to three objects; two of them were similar to each other (AB and BC) and the third object was dissimilar (EF) (Fig 1). This task is analogous to our SLR, which was developed as a test for spatial discrimination of overlapping memories (Bekinschtein et al., 2013). In SLR, the similarity between the spatial representations was manipulated by varying the distance between identical objects. In the analogous task used in the present study to evaluate discrimination of overlapping object memories during consolidation, the similarity between objects was manipulated by varying the number of features shared by them at the encoding phase (Fig 1).

Object exploration and preference is driven by novelty in the modified SOR task

There were no differences in the percentage of time the animals spent exploring the three objects during the sample phase for the similar or the dissimilar conditions (Fig 2A). In addition, the total amount of time rats spent exploring did not differ between conditions (Similar vs. Dissimilar: paired *t* test, p=0.943). The "choice" phase or test was carried out 24 h after the "sample" phase and memory was evaluated by comparing the amount of time spent exploring a novel object and a familiar object. In the "similar" condition, the novel object was made of the non-overlapping (AC) features of the two similar objects from the "sample" phase (AB and BC) and the familiar object was a copy of the third one presented in the sample phase (EF) (Fig 1). Rats spent significantly more time exploring the novel than the familiar object (Fig 2B, Table II), indicating that they were able to store separate representations of the similar objects presented during the sample phase and to recognize the new object as novel despite it being made of familiar features. A similar result was obtained for the dissimilar condition in which a novel object made of two completely new features (KI) was paired against a familiar object seen during the sample phase (AB, CD or EF) (Fig 2B).

These results indicate that intact animals were able to spontaneously disambiguate the representations of two similar objects seen 24 h before the test. However, there was a possibility that the rats explored the novel object more during the choice phase due to a change in the number of items from three to two between the sample and the choice phases. To rule out that the difference in the novelty coming from the change in the

number of objects was driving exploration preferentially to one of them, we presented two familiar objects during the choice phase and compared either AB or BC against EF (Fig 2C). There was no preference for any of the two objects after this manipulation, indicating that item novelty was the main driver for exploration in this task (Fig 2C, Table II). While in the novel condition the discrimination ratio was different from zero, this was not the case for the familiar condition ($p_{(fam)}$=0.68, t=0.43; $p_{(novel)}$=0.016, t=3.97; One sample t test). Object location was always pseudorandomly assigned in case there was a bias for location within the arena.

BDNF and protein synthesis are required for the discrimination of overlapping object representations in Prh

Long-term storage of information in the brain is thought to require structural changes at the synapses (Kandel, 2001). Stable forms of synaptic plasticity and memory have long been known to depend on neuronal activity-induced protein synthesis (Davis and Squire, 1984; McGaugh, 2000). BDNF is a neurotrophin shown to be essential for memory consolidation in different learning tasks, including object recognition (Bekinschtein et al., 2014a). In addition, BDNF can induce long-term potentiation in the DG (Messaoudi et al., 2007). We have previously demonstrated that BDNF is required for consolidation of overlapping spatial memories in the DG (Bekinschtein et al., 2013), thus we hypothesized that it may participate in this process in Prh as well.

To evaluate the requirement of BDNF in the SOR task, we injected an antisense oligonucleotide for BDNF (BDNF-ASO) or a missense control oligonucleotide with the same

base composition but in a random order (BDNF-MSO) in Prh 2 h before the sample phase for the similar and dissimilar versions of the SOR task (Fig 3A). To first ensure that BDNF-ASO was efficiently blocking BDNF expression in Prh, we infused either ASO or MSO 2 h before injection of kainic acid or vehicle into the Prh of naive animals. This method was previously used to induce immediate-early genes (Nakayama et al., 2015). Thirty minutes after kainic acid injection, the Prh was dissected out and processed for western blot analysis of BDNF protein content. BDNF-ASO, but not BDNF-MSO was able to block the increase in BDNF expression caused by kainic acid (Fig 3B), indicating that the ASO was effectively preventing BDNF expression. It is unlikely that BDNF-ASO reduced steady-state levels at the time of the sample phase. Previous experiments using fear learning have shown an amnesic effect on long-term memory of pre-sample BDNF blocking antibodies, but not of BDNF-ASO, suggesting that BDNF-ASO only acts on de novo BDNF synthesis (Slipczuk et al., 2009). Although in this work we did not perform a dose-response curve of BDNF-ASO on BDNF protein levels, previous work showed that 2h post injection, there were no differences in BDNF steady-state levels between BDNF-ASO and BDNF-MSO in the dorsal hippocampus (Bekinschtein et al., 2007). This also suggests that in these experiments, BDNF-ASO blocks BDNF expression induced by learning. Animals in both groups explored the three objects equally (Fig 3C, inset, Table I). When the animals were evaluated 24 h later, we found a significant difference in the discrimination ratio between BDNF-ASO and BDNF-MSO- injected animals only for the similar SOR (Fig 3C), but no differences in total exploration times (see Table IV; paired *t* test, $p_{similar}$=0.945, $p_{dissimilar}$=0.523,). One sample t test indicate that BDNF-MSO injected animals did learn the

s-SOR and d-SOR tasks ($p_{similar}$=0.01, t=3.38; $p_{dissimilar}$ <0.0001, t=8.55), while BDNF-ASO-injected animals only learned the d-SOR task ($p_{similar}$=0.16, t=3.14; $p_{dissimilar}$=0.006, t=3.35). We have seen negative discrimination ratios before, but see discussion for an interpretation of this particular result. This indicates that BDNF is required for acquisition and/or consolidation of overlapping object memories in Prh. If BDNF was specifically involved in consolidation, then infusion of the BDNF ASO should not affect short-term memory. To evaluate this, we injected BDNF ASO or MSO into Prh and tested short-term memory in the similar version of the task. We did not find a significant difference between ASO and MSO. Both ODNs were infused 2 hr before the sample phase and memory evaluated 3 hr post-acquisition. We found that both groups remembered equally (BDNF MSO DR 0.23±0.03 vs. BDNF ASO DR 0.24±0.03, n=7, p=0.63, t_6=0.50, paired t test). We next asked whether specific expression of BDNF was involved in the process of consolidating overlapping memories and whether other molecules could participate in a process of storing non-overlapping memories in Prh. If this were the case, contrary to the effects of BDNF blockade, general inhibition of protein synthesis in Prh should impair SOR both in the similar and the dissimilar condition. To block protein synthesis, we injected the translation inhibitor emetine (Sigma-Aldrich) into Prh, 15 min before the sample phase in both the similar and dissimilar conditions. When memory was evaluated 24 h later, we found a deficit for the emetine-injected group only in the similar condition (Fig 3D, left panel). No memory impairment was observed in emetine-injected animals that were evaluated in the dissimilar condition (Fig 3D, right panel). One sample t tests indicated that vehicle-injected animals were able to learn both the s-SOR and the d-SOR

($p_{similar}$=0.001, t=4.75; $p_{dissimilar}$<0.0001, t=6.67), while emetine-injected animals only the d-SOR version ($p_{similar}$=0.16, t=1.5; $p_{dissimilar}$=0.01, t=3.22). These results suggest that protein synthesis in Prh is required for consolidation of overlapping, but not of non-overlapping memories and that BDNF participates in a general protein synthesis-dependent mechanism of disambiguation of object memories in Prh.

Arc/Arg3.1 expression is required for the discrimination of overlapping object memories in Prh

We then decided to look for a potential effector of BDNF in Prh. Most studies have focused on the study of Arc in brain regions such as the hippocampus and amygdala, and there is no information regarding the role of Arc in object recognition in Prh or specifically in pattern separation. In addition, BDNF-induced long-term potentiation in the DG is dependent on Arc synthesis (Messaoudi et al., 2007). Thus we hypothesized that Arc expression could be induced by BDNF in Prh during consolidation of similar object memories.

We focused this set of experiments on the function of the Arc protein in Prh during storage and disambiguation of object representations. As with BDNF, the expression of Arc can be efficiently blocked by the application of antisense oligonucleotides (ASO) that bind specifically to the Arc mRNA (Messaoudi et al., 2007; Ploski et al., 2008; Martinez et al., 2012; Nakayama et al., 2015). We infused Arc-ASO or a control missense oligonucleotide (Arc-MSO) in Prh 2 h before the sample phase and tested the animals 24 h later. Infusion of the ODNs did not affect total exploration times during the sample phase

(see Table IV; ASO vs MSO, Paired *t* test, $p_{similar}$=0.585; $p_{dissimilar}$=0.919), and rats spent an equal amount of time exploring each one of the three objects (Fig 4B, Table I). However, infusions of the ODNs impaired object recognition memory for the similar, but not for the dissimilar condition (Fig 4C). One sample t tests indicate that Arc-MSO-injected animals were able to learn both the s-SOR and the d-SOR ($p_{similar}$<0.0001, t=7.14; $p_{dissimilar}$<0.0001, t=11.8), while Arc-ASO-injected animals only the d-SOR version ($p_{similar}$=0.13, t=1.64; $p_{dissimilar}$<0.0001, t=10.8). No memory impairment was observed when the Arc-ASO was infused 2h before the sample phase and the animals were evaluated after 3 h (Fig 5A). One sample t tests indicate that both Arc-MSO- and Arc-ASO-injected animals were able to remember the s-SOR task at 3h ($p_{similar\ MSO}$=0.04, t=2.8; $p_{similar\ ASO}$=0.02, t=3.3). There were no differences in total exploration times between ASO- and MSO-injected animals during the choice phase (see Table III; Paired *t* test, $p_{similar}$= 0.206; $p_{dissimilar}$=0.875). This indicates that initial acquisition of the task was not affected by Arc blockade and that the effect of this manipulation was dependent on the delay between "sample" and "choice", suggesting that the effect was happening during the consolidation phase. To ensure that Arc-ASO was efficiently blocking Arc expression in Prh, we infused either ASO or MSO 2 h before injection of kainic acid or vehicle into the Prh of naive animals. Thirty minutes after kainic acid injection, the Prh was dissected out and processed for western blot analysis of Arc protein content. Arc-ASO, but not Arc-MSO was able to block the increase in Arc expression caused by kainic acid (Fig 5B), indicating that the ODN was effectively preventing Arc expression.

These results cannot be explained by unspecific damage to Prh by the oligonucleotide Arc-ASO, because no change in performance was seen after administering Arc-MSO, and staining did not reveal any lesion to the site of infusion (Fig 5C). In addition, the experimental design was within-subject, so every rat was both injected with ASO and MSO. Thus, it is very unlikely that ASO and MSO had differential toxic effects that were somehow reversible. We evaluated ODN spread 2 h after injection of biotinilated Arc-ASO into Prh. We found little spread outside Prh, indicating that the observed deficit was not caused by blocking Arc expression in other structures (Fig 5C).

Arc expression in Prh is not necessary for DG-dependent discrimination of overlapping spatial representations

Another interpretation of these results could be that Arc is required in Prh for discrimination of similar information of any kind or that the impairment is evident or not depending on the difficulty of the task. If this were the case, then disambiguation of similar information, regardless of the type of stimuli involved, should also be affected by injection of Arc-ASO into Prh. To evaluate this possibility we tested the rats in a spontaneous spatial discrimination task that is particularly sensitive to manipulations of the DG (Bekinschtein et al., 2013, 2014b)(Fig 5D). As with our version of the SOR, the spontaneous location recognition task (SLR) can be run in two different conditions, the similar (s-SLR) and the dissimilar (d-SLR) configurations (Fig 5D). Similarity of the locations can be manipulated by varying the distance between the objects within a circular arena surrounded by distal spatial cues. The s-SLR, but not the d-SLR is sensitive to DG

manipulations like blockade of BDNF (Bekinschtein et al., 2013) or adult neurogenesis (Bekinschtein et al., 2014b; Reichelt et al., 2016). Infusion of Arc-ASO in Prh 2 h before the sample phase did not produce any observable deficit in the SLR task for any of the conditions (Fig 5E, Table II). One-sample t tests indicate that both Arc-MSO- and Arc-ASO-injected animals were able to learn the s-SLR and d-SLR task ($p_{\text{similar MSO}}$=0.006, t=3.86; $p_{\text{similar ASO}}$=0.007, t=3.76; $p_{\text{dissimilar MSO}}$=0.002, t=4.73; $p_{\text{dissimilarASO}}$=0.04, t=2.56). These results indicated that disambiguation of spatial overlapping information does not require Arc in Prh.

Arc expression is necessary for discrimination of overlapping object memories in Prh during a time-restricted window

Memory consolidation is a time-restricted process, with amnestic agents being effective only during a limited time window (McGaugh, 2000; Winters and Bussey, 2005a). To test whether Arc requirement for LTM of the similar SOR was limited to the first few hours after the sample phase, Arc-ASO was injected into Prh either immediately or 3 h after the sample phase and the rats tested 24 h after acquisition. We found a significant effect of Arc-ASO, compared to Arc-MSO when the injection was made immediately after the sample phase, but only for the similar condition (Fig 6B). One-sample t tests indicated that MSO-injected animals were able to learn both the s-SOR and the d-SOR (p_{similar}=0.0001, t=6.2; $p_{\text{dissimilar}}$=0.0049, t=4.04), while ASO-injected animals only the d-SOR version (p_{similar}=0.43, t=0.81; $p_{\text{dissimilar}}$<0.0001, t=9.1). We did not observe any memory impairment in the similar SOR when the Arc-ASO was injected in Prh 3 h after the sample phase (Fig

6B, bottom panel), indicating that the effect of Arc-ASO was time-restricted. Injection of the Arc-ASO did not change total exploration times compared to Arc-MSO (see Table IV; paired *t* test, $p_{similar}$= 0.837; $p_{dissimilar}$=0.654). In addition, one-sample t tests indicated that both Arc-MSO- and Arc-ASO-injected animals were able to learn the s-SOR ($p_{similar\ MSO}$=0.009, t=3.75; $p_{similar\ ASO}$=0.005, t=4.26). The timing of infusion was conducted as previously described for this and other ODNs. The pre-sample time was chosen because ODNs are slowly taken by cells, so for them to have an effect on de novo synthesis they need to be injected at least 1,5 h before the experience. Thus, the ODNs inhected 3 h post-sample might affect protein synthesis at around 4,5 h post-sample, when consolidation seems to have ended. These results are similar to the ones obtained when infusiing Arc-ASO into the amygdala to block fear extinction (Onoue et al., 2014), pre-extinction infusion caused an inpariment, but infusion 3 h post- extinction training did not produce any effect.

Arc expression in Prh increased "as-needed"

The findings of these experiments provide compelling evidence that Arc in Prh is involved in the molecular mechanisms underlying the disambiguation of overlapping object memories. Moreover, these findings isolate the action of Arc to the consolidation phase of memory, specifically. Particularly interesting is the finding that post-sample injections, made after initial encoding of the to-be-remembered objects, disrupt memory only in the similar SOR but not in the dissimilar SOR. This finding raises the question of whether Arc is expressed equally in both conditions but only needed in the first, or whether Arc is

expressed on an "as-needed" basis, that is, spontaneously in response to encountering similar objects – the representations of which need to be separated before storage in memory. We have previously found that BDNF was expressed in this manner in the DG after exposure to similar locations (Bekinschtein et al., 2013).

To test this possibility, we exposed rats to two similar objects or two dissimilar objects within the training arena and a control group to the empty arena (Fig 7A). One hour after the exposure, rats were sacrificed and the Prh was dissected and homogenized for Western blot analysis of Arc protein content. There were no differences in total exploration times, and rats spent an equal amount of time exploring each object in the similar and the dissimilar conditions (two way ANOVA (%time) $p_{position}$=0.943, $p_{condition}$= 0.673, $p_{interaction}$=0.591; *t* test (total time) *p*=0.943) (Fig 7B). Immunostaining revealed a one-fold increase in Arc expression in the animals exposed to the two similar objects compared with the ones exposed either to the two dissimilar objects or to the empty arena (Fig 7C). These findings provide evidence that Arc is expressed on an "as-needed" basis, such that Arc is increased spontaneously when separating the representations of similar objects. Although we tried measuring BDNF, its levels were proven difficult to measure, because of its low expression in Prh. Nonetheless, BDNF-ASO only caused amnesia for the similar condition, indicating that synthesis of BDNF was only required to consolidate overlapping memories.

BDNF enhances discrimination of overlapping object memories in Prh through Arc expression

We then asked whether BDNF and Arc expression in Prh during consolidation of overlapping memories were part of the same or different pathway. Since BDNF has been shown to enhance memory consolidation when injected exogenously (Alonso et al., 2002; Peters et al., 2010; Bekinschtein et al., 2013), we reasoned that this putative enhancing effect should be prevented if Arc expression was blocked. In addition, it has already been shown that hrBDNF induces Arc expression in the hippocampus (Ying et al., 2002; Lee et al., 2004). To be able to see memory enhancement, we brought control animals' performance down to chance levels by making the discrimination more difficult. Thus, we modified the task by making the objects more similar during the sample phase. For this extra-similar SOR (xs-SOR), we used objects made of three features, two of these objects shared two of the features (ABB and BBC) and the third object was completely different from the other two (EFG) (Fig 8A, see also Fig 1). We evaluated memory 24 h after the sample phase using one novel object made of the repeated feature and the other two non-shared features (ABC) and a familiar object (EFG) (Fig 8B). There were no differences in exploration of the three objects during the sample phase, indicating that making two objects even more similar did not affect visual or tactile perception of them (Fig 8A, bottom panel, Table I). The discrimination ratio for control saline-injected rats was not significantly different from zero, indicating that they could not store the representations of the two similar objects as different (Fig 8B, p_{xsVeh}=0.08, t=2.02, one-sample t test). However, injection of human recombinant BDNF (hrBDNF) into Prh 5 min after the sample phase, enhanced performance compared to the control group (Fig. 8B, Table II). In addition, a one-sample t test revealed that the discrimination ratio of BDNF-injected

animals was significantly different from zero (p_{xsBDNF}=0.0015, t=5.06). This indicates that infusion of BDNF into Prh improved the consolidation of overlapping object memories.

To analyze whether Arc expression was required for this enhancing effect of BDNF, we combined injection of hrBDNF with Arc-ASO into Prh. Arc-ASO or Arc-MSO were injected 2 h before the sample phase and hrBDNF or saline were injected 5 min after the sample phase (Fig 8C). There were no differences in exploration time during the sample phase between Arc-ASO- and Arc-MSO-injected animals (Fig 8C, bottom panel). Arc-ASO, but not Arc-MSO infusion prevented the BDNF-dependent enhancement in performance during the choice phase carried out the following day (Fig 8D). In addition, one-sample t tests indicated that the only group with a discrimination ratio significantly above zero was the BDNF/MSO group ($p_{Veh/MSO}$=0.0002, t=9.47; $p_{BDNF/MSO}$=0.03, t=0051; $p_{Veh/ASO}$=0.96, t=3.01; $p_{BDNF/ASO}$=0.9, t=0.9). These results indicate that Arc expression is required for BDNF-induced increase in consolidation of highly overlapping memories.

"Molecular disconnection" suggests Arc is a critical effector of BDNF during discrimination of overlapping object memories in Prh

We next sought to determine whether BDNF and Arc interacted during consolidation of the similar SOR task. Thus, we carried out a "molecular disconnection" experiment. The rationale for this can be found in a typical brain disconnection experiment in which one wants to determine if two brain structures are connected during a particular behavioral manipulation (Gaffan and Harrison, 1987; Ito et al., 2008). Assuming that the main connections between the two structures are ipsilateral, inactivation of the two regions in

the same hemisphere would leave behaviour intact, but contralateral inactivation would hamper performance. If instead of two regions, we think of two molecular or gene expression pathways within a given structure, we can apply a similar line of reasoning. If the two molecular pathways interact to produce behaviour, then blocking both of them in that region of one hemisphere would not have any effect, but blockade of one molecule in one hemisphere and the second molecule in the other hemisphere would produce a deficit.

Thus, to evaluate whether BDNF and Arc signaling pathways are connected in Prh, we blocked BDNF and Arc expression in the Prh of the same hemisphere or blocked BDNF expression in the Prh of one hemisphere and Arc expression in the Prh of the other hemisphere (Fig 8E). We found no effect in the similar SOR task evaluated at 24 h if BDNF-ASO and Arc-ASO were injected into the same Prh, while injecting BDNF-MSO and Arc-MSO into the other Prh 2 h before the sample phase (Fig 8F). However, when BDNF-ASO/Arc-MSO and BDNF-MSO/Arc-ASO were injected into Prh in different hemispheres, there was a significant impairment in the similar SOR task (Fig 8F). There were no differences in total exploration times between the two groups (see Table II). In addition, one-sample t tests revealed that the discrimination ratio from the “same” group was different from zero, while the discrimination ratio from the “different” group was not (p_{same}=0.0023, t=5.73; $p_{different}$=0.29, t=1.17). This result suggests that BDNF and Arc interact during consolidation of overlapping memories in Prh.

Discussion

In this work, we have shown that BDNF and Arc are required for consolidation of overlapping object memories in Prh. Several of our results point at the BDNF- Arc pathway as an important player underlying disambiguation of overlapping object representations: 1) Both BDNF and Arc ASO impaired memory only for the similar condition of the SOR task; 2) the effect of Arc-ASO is time restricted, suggesting that Arc is mainly involved in consolidation: 3) the amnesia caused by Arc-ASO is dependent on the delay between “sample” and “choice”, not affecting memory at short delays such as 3h, but causing amnesia at 24 h; 4) Arc is expressed in an "as needed" manner after encountering similar objects; 5) Arc in Prh is not required for acquisition/consolidation of overlapping spatial memories, indicating that these molecular processes in this structure are dependent on the type of representations that are necessary to solve the task; 6) the memory enhancement induced by hrBDNF is abolished completely by Arc-ASO, suggesting that Arc is one of the molecules required for the effect of BDNF; and finally 7) BDNF and Arc molecular pathways interact during acquisition/consolidation of overlapping object memories as shown by the “molecular disconnection” experiment.

We used a modified version of the spontaneous object recognition task and thus, there could be a concern regarding a change in motivation to explore the objects after a particular pharmacological manipulation (i.e., manipulations could change the animals’ preference for novel items to familiar items). In our experiments, this factor could not account for the differences in the discrimination ratios, because that would mean that our manipulations of the Prh somehow affected motivation only in the similar condition but

677 not in the dissimilar condition. Moreover, the fact that infusion of the Arc-ASO 3 h after
678 the sample phase did not affect novelty preference in the similar SOR condition effectively
679 rules out the possibility that a change in motivation explains these results. Also, infusion
680 of ODNs in Prh did not change exploration or caused memory impairment in a spatial
681 object exploration task. In the experiment depicted in Fig 3C, BDNF-ASO treated animals
682 show a negative discrimination ratio. We have seen these type of results before using our
683 spatial discrimination task (Bekinschtein et al., 2013) and it could be explained if the
684 animals could not store separate representations of the two similar objects, then during
685 the choice phase, it might seem that the novel object (made of two familiar features)
686 would have been explored twice as long during sample, increasing familiarity during test.

687 These results indicate that BDNF and Arc take part in a protein synthesis-dependent
688 mechanism important for consolidation of certain types of memories. This is remarkably
689 similar to our findings in the DG of the hippocampus (Bekinschtein et al., 2013). Our
690 results also suggest that there is interaction between BDNF and Arc during consolidation
691 of overlapping object memories, indicating that Arc is likely an effector of the plasticity
692 induced by BDNF. Importantly, we compared the similar and dissimilar conditions for all
693 experiments and the memory test was always carried out after the same delay for both of
694 them (i.e. 24 hr after acquisition). Since the effects were observed only for the similar
695 condition, they were dependent on the similarity, but not on the delay of testing. Thus,
696 these mechanisms are specifically involved in discrimination of overlapping memories, but
697 not on their persistence. However, we cannot conclude from these results that BDNF and

Arc are not involved in the mechanisms of longer lasting maintenance of non-overlapping memories in Prh or that other known plasticity molecules such as Zif268 are required for consolidation of non-overlapping memories in this structure.

There is convincing evidence to indicate that Prh, rather than storing simple features of objects, stores conjunctive representations that can later be used to disambiguate particular objects during memory retrieval. This hypothesis has been previously tested by examining the role of Prh during discrimination of objects that shared overlapping features at the moment of retrieval (Norman and Eacott, 2004; Bartko et al., 2007). In this sense, Prh could be thought of as a structure that acts as a 'pattern separator' for representations of objects, disambiguating overlapping information into separate and less confusable representations. In fact, recordings of single units from the Prh showed populations of neurons whose firing rate changed gradually as the originally learned objects were ambiguously morphed to varying degrees, and other neurons whose firing rate changed abruptly according to the rewarded response categories associated with the objects. They suggest that this abrupt change in the firing rate could be a result of the orthogonalization of the original morphing continuum (Ahn and Lee, 2017). This neural perirhinal population with orthogonalized responses that correlate with their memory guided choices could be the neural substrate that supports the consolidation of similar objects into non-overlapping representations that guide behaviour in the SOR task.

Our experiments suggest that, at least for storage of object representations, but not of spatial representations, BDNF and Arc are essential for consolidation of separate

719 memories and a part of a time-restricted protein synthesis-dependent mechanism of
720 memory stabilization in Prh. These results are in line with the evidence indicating that
721 structures in the medial temporal lobe are specialized in processing different types of
722 representations. Since the Prh receives prominent afferents from the ventral visual stream
723 (the “what” pathway), it has been suggested to be at the top of a hierarchical network of
724 object processing (Kent et al., 2016). This idea is compatible with the thought of Prh as
725 being a pattern separation structure. On the other hand, the postrhinal cortex (Pc) lies
726 posterior to the Prh and receives afferent projections primarily from the dorsal (“where”)
727 processing system (Suzuki and Amaral, 1994) that has been implicated in visuospatial
728 processing (Kravitz et al., 2011). Since the “what” and “where” features are essential to
729 episodic memory, information from Prh and Pc has to be integrated into an experience. In
730 fact, efferents from these structures project preferentially to different regions of the
731 entorhinal cortex (EC), which, in turn, project to the hippocampus (Witter, 2007). While
732 Prh primarily projects to the lateral entorhinal cortex (LEC), the Pc projects to the medial
733 entorhinal cortex (MEC) (Suzuki and Amaral, 1994). This pattern of connectivity suggests a
734 segregation of object and spatial information processing in EC that could be integrated
735 within the EC or in the hippocampus via de perforant path (Witter, 2007). Thus plasticity in
736 the Prh could occur at the synapses connecting to the LEC, facilitating object information
737 processing necessary for episodic memory. It is highly unlikely that our manipulation of
738 Prh, such as infusion of ASO, reached Pc, since the infusion site was far away from this
739 structure and we observed no spreading of the oligonucelotides outside Prh.

It is widely believed that changes in synaptic strength support long-term memory storage in the brain (Kandel, 2001). *In vitro* studies have found that Prh neurons can develop both long-term potentiation (LTP) and long-term depression (LTD) (Bilkey, 1996; Ziakopoulos et al., 1999; Cho et al., 2000; Massey et al., 2001). *In vivo* experiments have strongly associated object recognition memory with LTD induction and maintenance in Prh (Griffiths et al., 2008). This type of plasticity has been found to be dependent on internalization of AMPA receptors in Prh. In this sense, Arc KO mice have deficits in many learning tasks, including object recognition and they have diminished LTD in the hippocampus (Plath et al., 2006). In another study, Jakkamsetti et al. (Jakkamsetti et al., 2013) observed that Arc-expressing neurons preferentially develop LTD in response to activation of group I metabotropic receptors in CA1, and that this molecule is required for mGlurR-dependent LTD. It is possible that similar mechanisms are involved in Arc-dependent consolidation of overlapping object memories in our behavioural paradigm. Arc has been implicated in AMPA receptor trafficking at the synapses (Rial Verde et al., 2006; Shepherd et al., 2006; Waung et al., 2008), thus it seems logical that this could be a possible mechanism for object memory storage in Prh.

One previous study used BDNF ASO to block BDNF expression in Prh either before or after the sample phase in a spontaneous object recognition paradigm (Seoane et al., 2012). BDNF-ASO injected 1h before or immediately after acquisition impaired familiarity discrimination at 24 h, but not 20 min after acquisition. Infusion of the ASO 6 h post-acquisition did not impair memory 24 h later. However, we believe the results of our study

761 do not generalized to the molecular mechanisms of recognition memory but rather the
762 mechanisms underlying storage of unique representations of objects in Prh. In our
763 experiments, we only found a memory impairment caused by BDNF-ASO in the similar,
764 but not in the dissimilar condition. Our results are consistent with a role of Prh in storage
765 of non-confusable object representations.

766 Given that adult neurogenesis in the DG has been implicated in the discrimination of
767 overlapping spatial representations (Clelland et al., 2009; Kheirbek et al., 2012; Nakashiba
768 et al., 2012; Bekinschtein et al., 2014b) and that adult neurogenesis is absent in Prh, it is
769 clear that the underlying cellular mechanisms of pattern separation are different between
770 structures such as the DG and Prh. However, despite these anatomical differences, several
771 molecular mechanisms that influence plasticity changes at synapses seem to be similar
772 and common to memory storage processes. Synaptic mechanisms for memory
773 consolidation are widely conserved across species despite the differences in their brain
774 anatomy. Molecules such as cAMP response element-binding protein (CREB) are essential
775 in consolidation of many types of learning in invertebrates and vertebrates (Carew and
776 Sahley, 1986; Abel and Lattal, 2001; Schafe et al., 2001; Barco et al., 2006) and compounds
777 such as BDNF are important parts of the machinery involved in plasticity of many sorts,
778 from synaptic plasticity and memory, to development and pain (Lu and Chow, 1999;
779 McAllister et al., 1999; Bramham and Messaoudi, 2005; Pezet and McMahon, 2006;
780 Bekinschtein et al., 2008). Thus, from an evolutionary perspective, it seems logical that
781 different regions of the brain became specialized to process particular types of

representations, but the underlying plasticity mechanisms were conserved. In light of this argument, it makes sense that some of the main players in the intracellular molecular plasticity mechanisms driving consolidation of overlapping memories appear to be identical across different brain regions. Adult neurogenesis, therefore, might have evolved at least in part as a cellular mechanism that prevents interference specifically between spatial and episodic representations—and not representations involving only objects—because the increased excitability and plasticity of adult-born neurons in the DG is necessary for the processing of highly complex information present in places and episodes.

To our knowledge, the present study is the first to provide evidence regarding the molecular pathways involved in the consolidation of overlapping memories outside the DG and, together with our previous studies, to demonstrate that BDNF is an important plasticity molecule involved in this process in multiple brain regions. In addition we show, for the first time, that under certain conditions Arc protein is required for spontaneous object recognition in Prh and in particular for storage of separated representations of overlapping objects. Our results point toward an evolutionary convergence of the molecular mechanisms involved in plasticity required for storage of unique representations across different regions of the brain. Importantly, these molecular mechanisms are not general to all conditions of object (or location) recognition; they were required only when similar memories had to be kept distinct.

References

Figure legends

Figure 1. **(A)** (Left) Cartoon depicting the apparatus and the spontaneous object recognition task (SOR). **(B)** Representative objects for the trials 1 and 2 for the similar and dissimilar versions of the SOR task and the extra-similar version of the SOR task.

Figure 2. The spontaneous object recognition task. (A) Percentage of time spent exploring each of the objects in the sample phase in the dissimilar (left) and similar condition (right). Rats spent an equal amount of time exploring each of the three objects during the sample phase. Similar: RM one way ANOVA (%time), F_{obj}=2.829 p=0.125, F_{ind}=1.624e-13 p>0.999; Dissimilar: RM one way ANOVA (%time), F_{obj}=1.456 p=0.274, F_{ind}=1.014e-13 p>0.999. **(B)** Discrimination ratios during the choice phase, 24h after the sample phase, in the similar and dissimilar condition. One sample t test (similar, t= 8.11) p<0.0001; one sample t test (dissimilar, t=4.361) p=0.003; Similar vs Dissimilar paired t test (t=1.521) p=0.172, n=8. **(C)** (Left) Control task. (Right) Discrimination ratios during the choice phase for the novel and familiar conditions. Paired t test (t=2.861) p=0.0187, n=10, d=0.054. Data are expressed as the mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.

Figure 3. BDNF expression and protein synthesis in the Prh are required for consolidation of similar, but not dissimilar, object memory representations. (A) Schematic illustration of the two configurations of the SOR task depicting the time point at which BDNF ASO was infused. **(B)** BDNF protein levels in the Prh of non trained animals infused with either oligonucleotide antisense of BDNF (BDNF ASO) or missense (BDNF

MSO) 2 h before injection of kainic acid into the Prh Unpaired t test (t=2.334), p=0.0322, n=9-10, d=0.377. **(C)** Effect of BDNF ASO or BDNF MSO injections on the discrimination ratios for the similar (s-SOR) and the dissimilar (d-SOR) version of the task. Paired t test (t=4.284) p=0.0036, n= 8-13, d=2.284. ***Inset:*** Percentage of time spent exploring each of the objects in the sample phase in the s-SOR (left) and d-SOR (right), 2 h after BDNF MSO (light color) BDNF MSO (dark color). Similar: RM two way ANOVA; F=0.652 p(drug)=0.440, F=0.957 p(object)=0.403, F=0.135 p(interaction)=0.875. Dissimilar: RM two way ANOVA F=0.055 p(drug)=0.818, F=1.388 p(object)=0.269, F=0.001 p(interaction)=0.999. **(D)** The injection of Emetine in the PRH 15 min before the sample phase impaired performance on the s-SOR task during the choice phase 24 h later relative to Vehicle-injected rats (left), while there was no effect of Emetine on the d-SOR version of the task (right). Paired t test (s-SOR, t= 3.540) p=0.0076, n=9, d=1.698; paired t test (d-SOR, t=1.284) p=0.231, n=10. Data are expressed as the mean ± SEM; *p<0.05, **p< 0.01.

Figure 4. Arc expression in the Prh is required for consolidation of similar, but not dissimilar, object memory representations. (B) Percentage of time spent exploring each of the objects in the sample phase, 2 h after MSO (light color) or ASO (dark color) of Arc injection. Similar: RM two way ANOVA; F= 0.026 p(drug)=0.875, F= 1.561 p(object)=0.240, F= 0.256 p(interaction)=0.777. Dissimilar: RM two way ANOVA; F= 4615 p(drug)=0.522, F=0.1971 p(object)=0.824, F=0.2516 p(interaction)=0.782. **(C)** Effect of pre-sample injection of Arc-ASO or Arc-MSO into the Prh in the choice phase at 24 h in the s-SOR (left) or the d-SOR (right) version of the task. Paired t test (s-SOR, t=5.762) p=0.0002, n =11,

d=7,599; paired t test (d-SOR, t=0.421) p=0.683, n =11. Data are expressed as the mean ± SEM; ***p < 0.001.

Figure 5. Arc expression in the Prh is not necessary for discrimination of overlapping spatial representations or for short-term memory. (A) Short term memory test after the injection of Arc ASO or MSO 2 h previous to the s-SOR. Paired t test p=0.974, t= 0.0343, n=6. Data are expressed as the mean ± SEM. **(B)** Arc protein levels in the Prh of non trained animals infused with either Arc ASO or MSO 2 h before injection of kainic acid into the Prh. Unpaired t test p= 0.046, t= 2.317, n=5-6, d=1.644. **(C)** (Upper panel) Coronal section showing the track of the cannula and indicating representative infusion sites in the Prh. (Lower panel) Representative spread of a biotinilated Arc ASO in the Prh 2 h after injection of 2 nmol. **(D)** Schematic representation of the similar configuration (s-SLR, left) and dissimilar configuration of the spontaneous location recognition task (d-SLR, right) showing the time of infusion of Arc ASO or MSO. **(E)** Effect of Arc ASO or Arc MSO infusion into Prh in the SLR task. Paired t test (s-SLR, t= 0.521) p= 0.618; paired t test (d-SLR, t= 0.713) p=0.499, n= 8. Data expressed as the mean ± SEM; *p<0.05.

Figure 6. Arc expression in the Prh is required in a time-restricted window for consolidation of similar object memory representations. (A) Schematic illustration of the similar (s-SOR, left) or dissimilar (d-SOR right) task configurations depicting the time points at which Arc MSO or ASO was infused. **(B)** Effect of the injection of Arc ASO or Arc MSO into the Prh 5 min or 3 h after the sample phase in the s-SOR (left) or the d-SOR (right) version of the task evaluated in a choice phase 24 h later. Paired t test (similar 0h,

t=2.274) p=0.046, n=11, d=1.611; paired t test (dissimilar 0h, t=0.999) p=0.351, n=8; paired t test (similar 3h, t=0.459) p=0.663, n =7.Data are expressed as the mean ± SEM; **p < 0.01.

Figure 7. Exploration of similar objects, but not dissimilar objects, is associated with an increase in the protein levels of Arc in the Prh. (A) Schematic representations of the task configurations. **(B)** Percentage of exploration of the objects used during the similar and dissimilar task, considering the location (left or right) of the object during the task. **(C)** Arc protein levels in the Prh after exposure to the objects. One-way ANOVA, F=3.818 p=0.038, n=8. Control vs Similar: d=2.407; Dissimilar vs Similar: d=2.073. Data are expressed as the mean ± SEM; *p < 0.05.

Figure 8. Arc and BDNF molecular pathways interact during consolidation of similar object representations in Prh. (A) (Bottom) Percentage of time spent exploring each of the objects in the sample phase in the xs-SOR. One-way ANOVA (%time), F=0.845, p=0.436 **(B)** Rats injected with recombinant BDNF in the Prh 5 min after the sample phase. Unpaired t test (t=5.224) p=0.0001, n =8, d=2.612. **(C)** Percentage of time spent exploring each of the objects in the sample phase in the xs-SOR after the injection of Arc MSO (light color) or ASO (dark color). Two way ANOVA (%time) F=1.496 p(drug)=0.235; F=0.098 p(object)=0.907; F=1.358 p(interaction)=0.269. **(D)** Effects of the combined injection of BDNF and Arc ASO on the discrimination ratio in the xs-SOR . Two way ANOVA F=14.95 p(BDNF)=0.001; F=1.627 p(Arc ASO)=0.217 ; F=14.29 p(interaction)=0.0012; n =6. BDNF/MSO vs BDNF/ASO: d=1.796; Veh/MSO vs BDNF/MSO: d=1.411; Veh/ASO vs

889 BDNF/MSO: d=0.294. **(E)** Schematic illustration of the s-SOR task and infusion time points.
890 **(F)** Effects of the injection of an Arc ASO and BDNF ASO in the Prh of the same or opposite
891 hemispheres on performance of the s-SOR task. Paired t test (t=4.338) p=0.0074, n =6,
892 d=7.383. Data are expressed as the mean ± SEM; *p<0.05, **p < 0.01, ***p<0.001.

893 **Table I**. Total exploration times during the sample sessions are shown for the
894 experiments. Results are expressed as mean ± SEM in seconds. The first column indicates
895 the figure number, the specific sub-index of the corresponding experiment and the
896 version of the task (s-SOR and d-SOR). On the top row, AB, BC (or CD) and EF represent
897 the different object compositions used in the respective tasks.

898 **Table II**. Total exploration times during the choice session of the SOR and SLR tasks are
899 shown for all the experiments. Results are expressed as mean ± SEM in seconds. The first
900 column indicates the figure number, the specific sub-index of the corresponding
901 experiment . For each experiment, light grey rows indicate the corresponding
902 experimental groups. Left panel corresponds to s-SOR or s-SLR version of the task, while
903 right panels to d-SOR or l-SLR, except in the case of Fig. 8, where both left and right panel
904 correspond to the xs-SOR version of the task for each experimental group depicted. On
905 the top row, “novel” and “familiar” indicate to which of the two objects present during
906 the choice phase the exploration time corresponds to (“novel location/identity” object or
907 “familiar location/identity” object).

908 **Table III**. Total exploration times during the choice session of the SOR task are shown for
909 all the experiments. Results are expressed as mean ± SEM in seconds. The first column

910 indicates the figure number, the specific sub-index of the corresponding experiment and
911 the version of the task (s-SOR and d-SOR). The second column indicates the
912 corresponding p-values for the comparison between total exploration times during the
913 choice session for each experimental group depicted in the same row. Paired t test were
914 used for these comparisons, except in the case of Fig. 8b and d, for which Unpaired t test
915 and One Way ANOVA were used. For each experiment, light grey rows indicate the
916 corresponding experimental groups or condition.

917 **Table IV**. Total exploration times during the sample session of the SOR task are shown for
918 all the experiments. Results are expressed as mean ± SEM in seconds The first column
919 indicates the figure number, the specific sub-index of the corresponding experiment and
920 the version of the task (s-SOR and d-SOR). For each experiment, light grey rows indicate
921 the corresponding experimental groups or condition.

922

923 **Tables**

Table I

Fig n°	AB	BC/CD	EF	AB	BC/CD	EF
2a *s-SOR*	32,40±1,72	37,75±2,98	36,22±2,25			
d-SOR	34,23±2,45	33,79±2,12	38,42±2,91			
	BDNF MSO			BDNF ASO		
3c *s-SOR*	27,76±4,06	27,59±4,00	34,14±4,09	31,06±4,83	27,54±5,30	32,04±5,41
d-SOR	26,07±3,06	24,64±3,70	23,26±3,77	24,93±2,94	27,75±3,72	25,65±3,12
	Veh			Eme		
d *s-SOR*	31,82±2,99	25,98±2,92	28,81±3,27	30,86±5,35	25,21±3,91	29,84±4,10
d-SOR	32,58±3,70	30,92±3,94	31,38±3,28	34,37±4,05	31,98±5,09	30,48±3,92
	Arc MSO			Arc ASO		
4b *s-SOR*	36,06±3,07	43,03±2,87	38,44±3,54	39,24±3,36	39,27±3,82	36,21±4,51
d-SOR	38,74±2,63	38,19±1,66	42,46±4,46	37,39±2,48	33,49±2,81	36,15±3,51
	ABB	BBC	EFG	ABB	BBC	EFG
8a	37,52±8,03	37,56±10,46	38,62±9,59			
	Arc MSO			Arc ASO		
c	36,75±2,67	39,66±3,14	41,47±2,64	41,80±2,39	39,02±3,20	40,01±3,37

Table II

Fig.	Novel	Familiar	Novel	Familiar	Novel	Familiar	Novel	Familiar
2b	Similar		Dissimilar					
	24,9±1,6	15,6±1,3	32,3±2,6	15,4±1,8				
2c	Familiar condition		Novel condition					

	28,4±2,2	27,0±3,2	32,5±3,2	23,6±1,6				
3d-e	s-SOR				d-SOR			
	BDNF MSO		BDNF ASO		BDNF MSO		BDNF ASO	
	30,5±2,8	20,2±2,4	23,5±4,7	28,3±4,3	37,3±3,1	20,2±2,1	38,3±3,7	24,2±2,7
3f-g	Vehicle		Emetine		Vehicle		Emetine	
	31,6±3,6	19,7±2,2	23,3±3,9	29,1±3,3	38,8±4,5	22,0±2,5	28,9±4.0	19,1±2,2
4c	s-SOR				d-SOR			
Left	Arc MSO		Arc ASO		Arc MSO		Arc ASO	
	20±0,9	12,8±0,7	16,5±1,7	20,0±1,8	25,0±1,9	14,1±1,8	25,3±1,9	13,4±1,1
Right	Arc MSO		Arc ASO					
	28,9±3,0	17,3±2,9	32,4±4,7	18,1±2,8				
5e	s-SLR				l-SLR			
	Arc MSO		Arc ASO		Arc MSO		Arc ASO	
	36,5±4,7	26,0±3,6	39,6±5,8	24,5±2,0	30,5±3,8	19,6±2,0	34,3±4,3	24,6±5,3
6b	s-SOR				d-SOR			
	Arc MSO		Arc ASO		Arc MSO		Arc ASO	
5min	28,0±3,2	18,0±2,2	23,0±2,4	21,9±3,0	41,8±3,4	25,0±2,4	38,1±6,2	22,8±3,2
3h	25,66±4,50	13,65±1,26	28,96±3,96	17,29±1,92				
8b	xs-SOR							
	Vehicle		BDNF					
	32,3±2,5	33,1±4,4	33,7±4,8	22,0±2,2				
8d	Veh, Arc MSO		Veh, Arc ASO		BDNF, Arc MSO		BDNF, Arc ASO	
	25,8±3,1	32,8±3,4	28,2±3,5	27,7±3,0	32,3±2,1	19,9±3,0	29,1±1,9	28,7±2,0
8f	s-SOR							
	Same		Different					

37,4±6,5	19,0±4,0	19,5±1,6	24,0±3,1	

Table III

Fig n°	*P value*				
		T total similar	*T total dissimilar*		
2b	p=0,1100	40,66±2,82	47,77±2,02		
		T total familiar	*T total novel*		
c	p=0,3593	60,76±4,30	53,1±7,21		
		T total MSO	T total ASO		
3c *s-SOR*	p=0,902	50,66±4,17	51,80±8,92		
d-SOR	p=0,354	57,53±5,41	62,47±5,16		
d *s-SOR*	p=0,823	51,23±5,52	52,40±6,26		
d-SOR	p=0,077	60,80±6,51	48,01±5,56		
4c *s-SOR*	p=0,2059	32,84±1,16	36,47±2,76		
d-SOR	p=0,8750	39,18±3,21	38,65±2,81		
5a	p=0,174	46,22±5,05	50,50±5,66		
e *s-SLR*	p=0,419	63,61±6,89	55,00±6,38		
d-SLR	p=0,310	50,10±5,28	58,95±8,30		
6b up *s-SLR*	p=0,837	46,02±5,13	44,92±5,05		
d-SLR	p=0,654	65,28±5,47	62,50±7,70		
bottom	p=0,663	39,31±12,78	46,25±5,39		
8b	p=0,173	68,37±5,98	55,73±6,41		
f	p=0,273	56,48±9,63	43,59±3,50		
		Veh MSO	Veh ASO	BDNF MSO	BDNF ASO
d	p=0,825	58,59±6,47	55,88±6,60	52,19±3,93	57,85±2,96

952 **Table IV**

953

Fig n°				
	Similar		Dissimilar	
2 b	106,4±6,16		106,4±6,73	
	Familiar		Novel	
c	113±14,20		97,81±12,74	
	BDNF MSO		BDNF ASO	
3c *s-SOR*	89,49±8,86		90,65±14,98	
d-SOR	73,97±9,18		78,33±9,14	
	Veh		Eme	
d *s-SOR*	86,62±8,58		85,91±12,36	
d-SOR	94,88±9,26		96,83±11,71	
	Arc MSO		Arc ASO	
4b *s-SOR*	107±9,56		114,7±10,68	
d-SOR	108,7±7,25		120,7±5,62	
	BDNF	Veh		
8a	120,7±4,57	114±10,12		
	Veh MSO	Veh ASO	BDNF MSO	BDNF ASO
c	108,3±9,80	118,5±10,97	129,5±4,30	123,7±8,86

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Fig.1

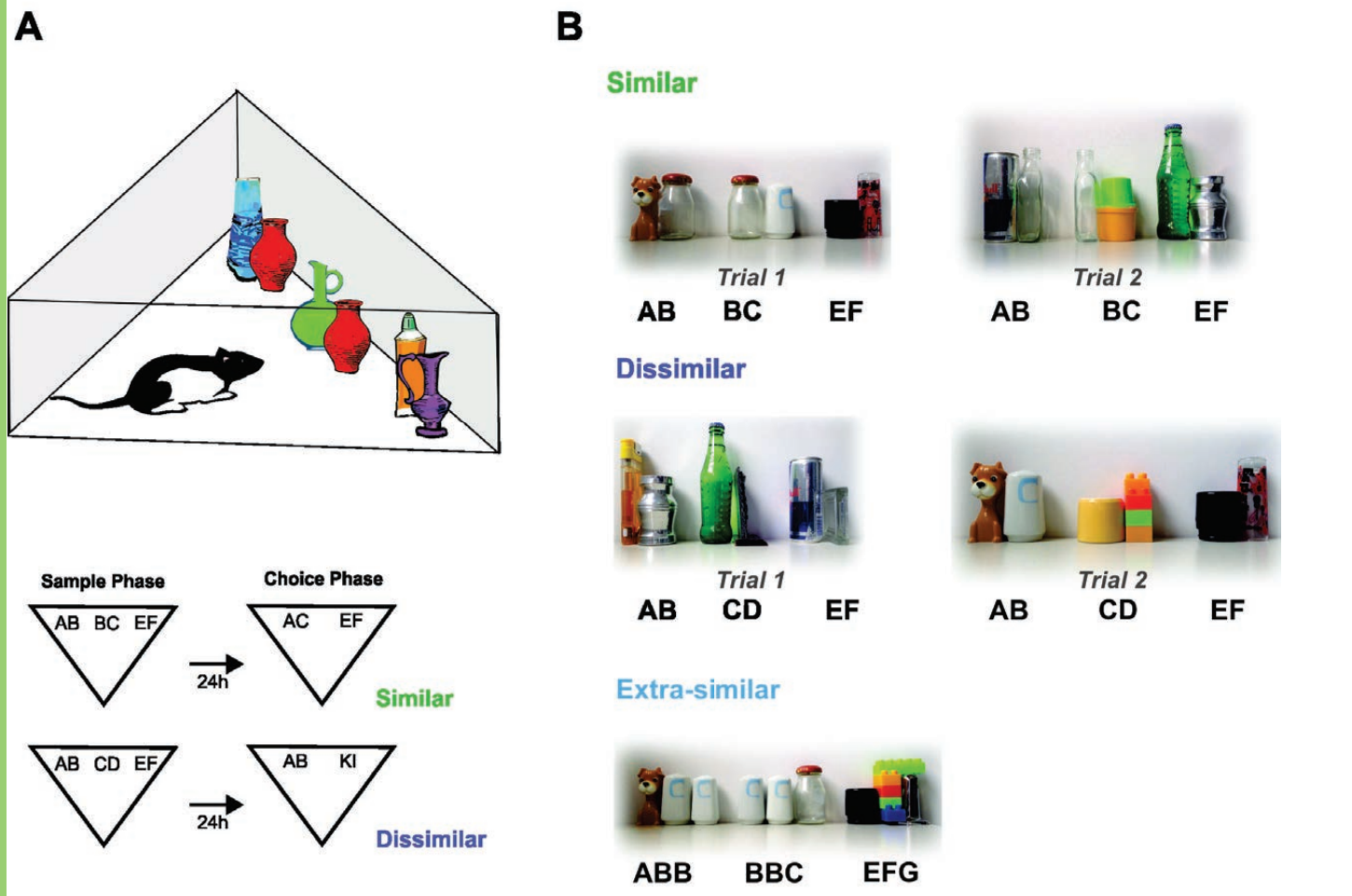
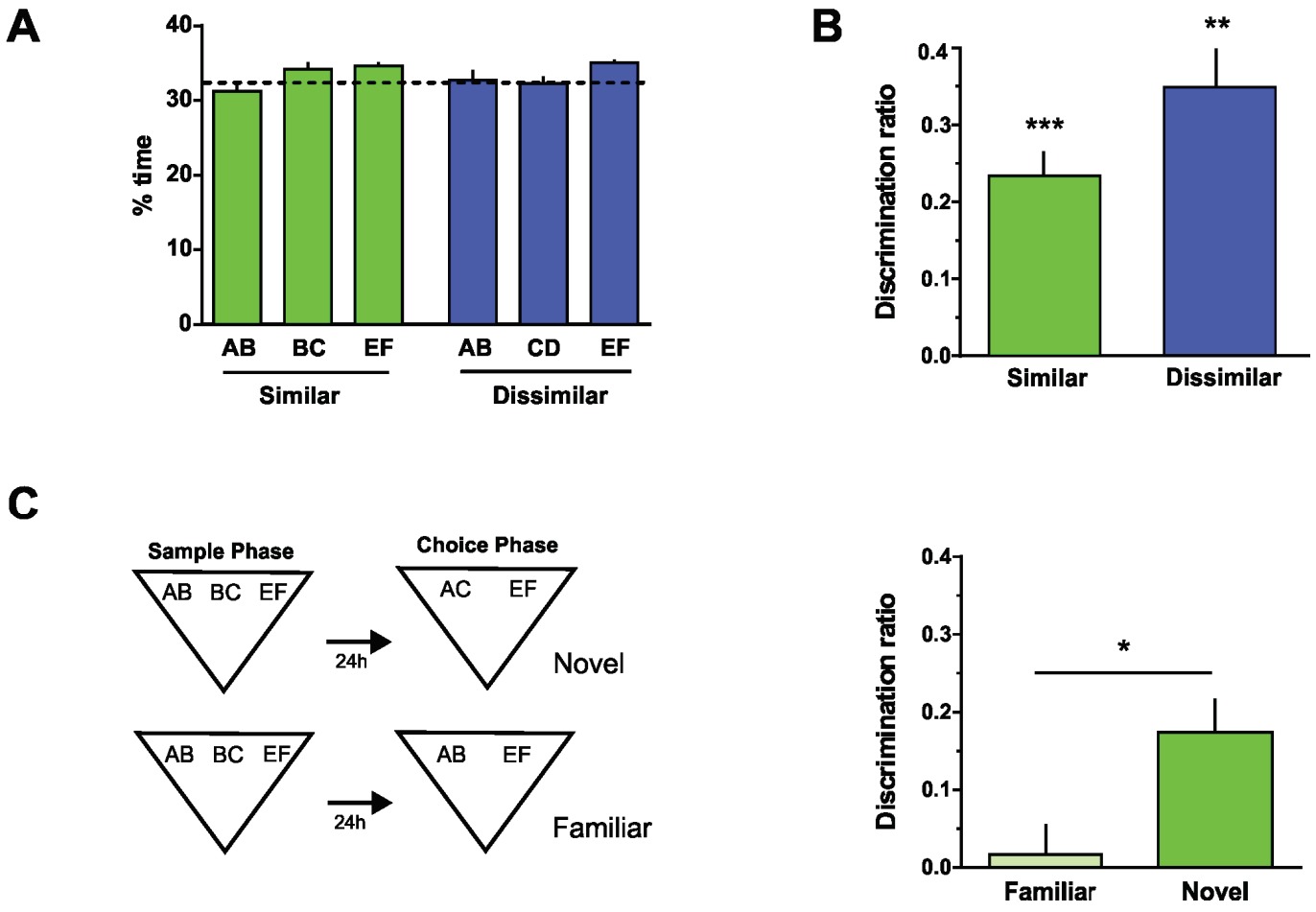


Fig. 2



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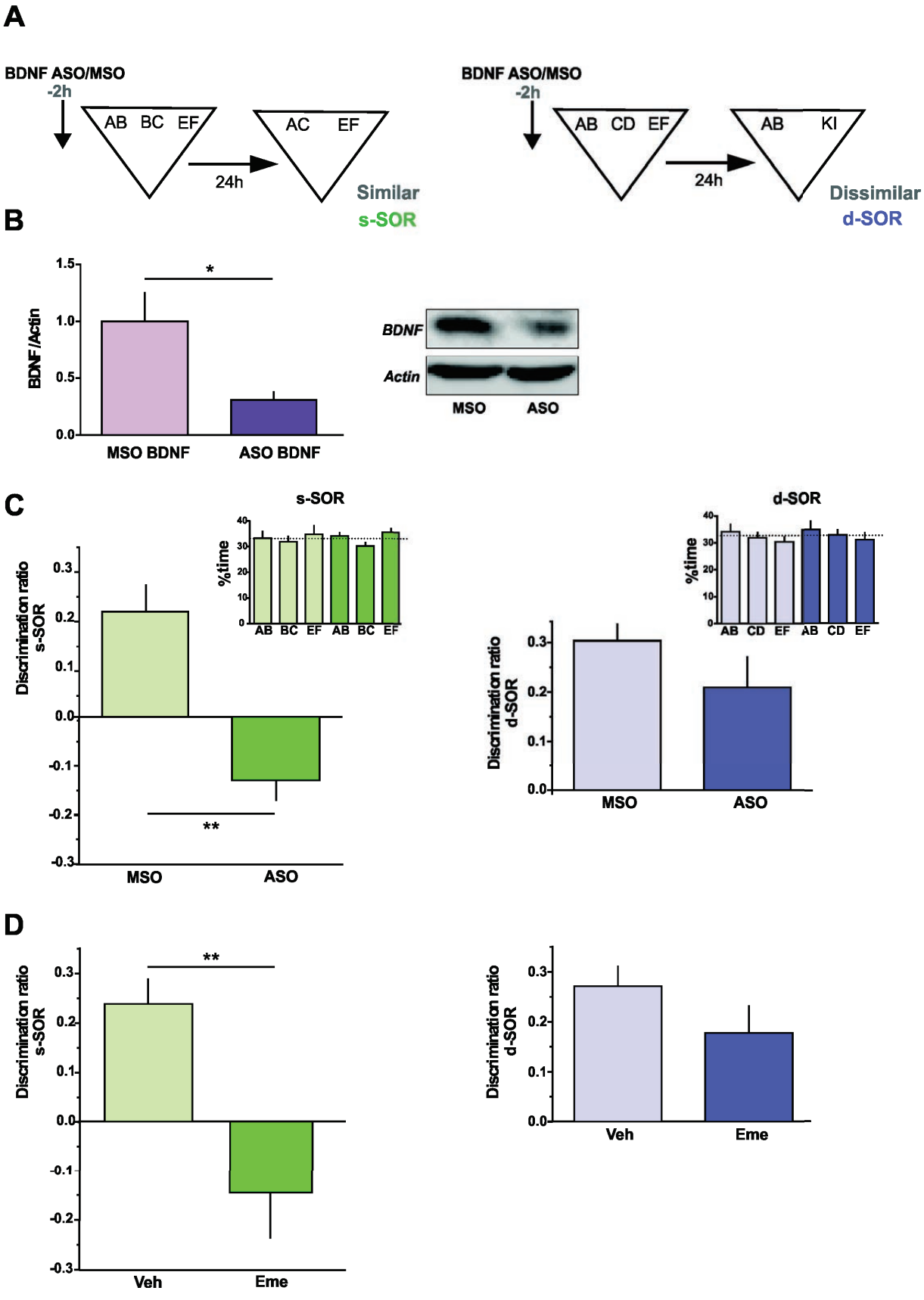

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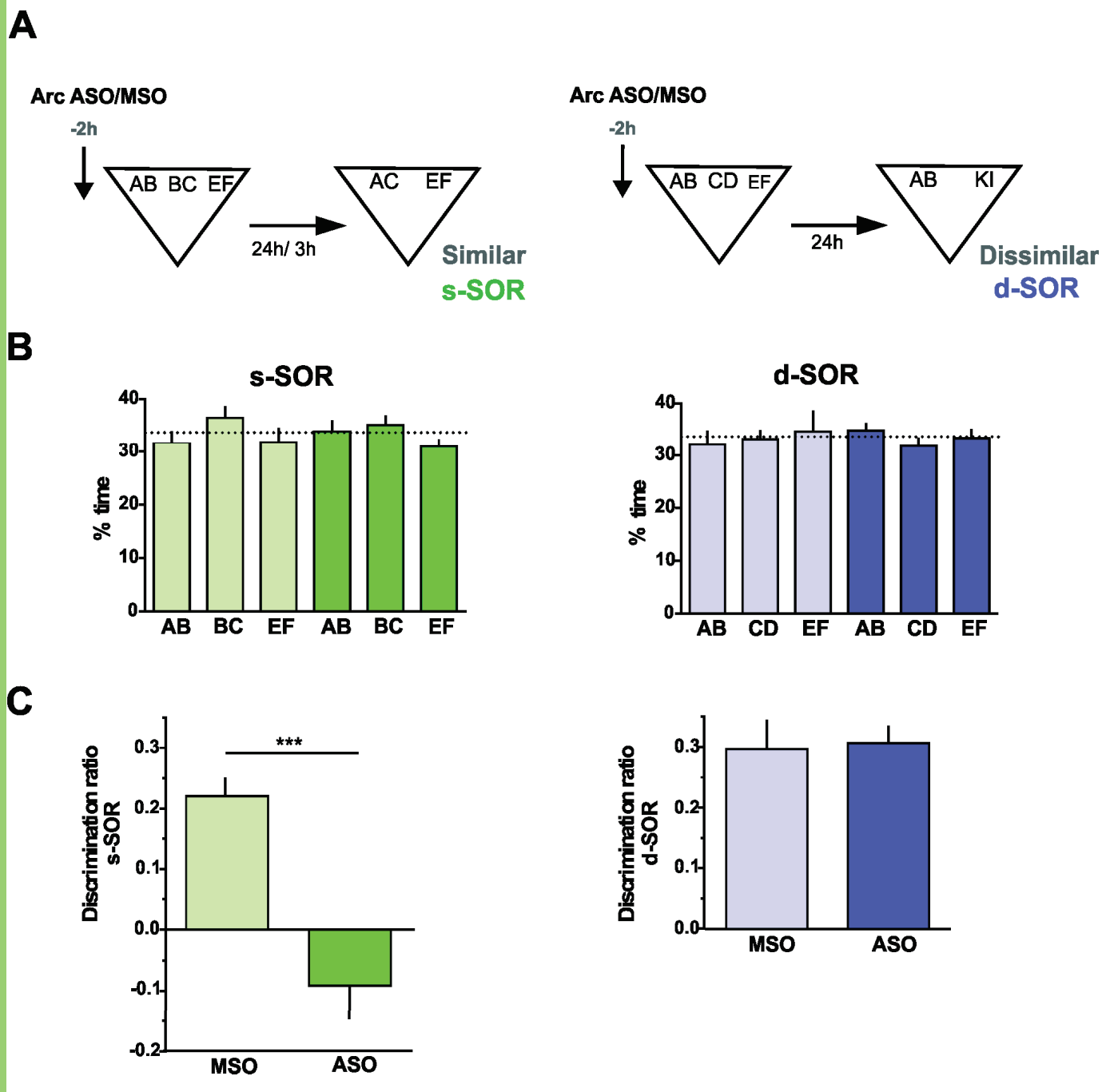

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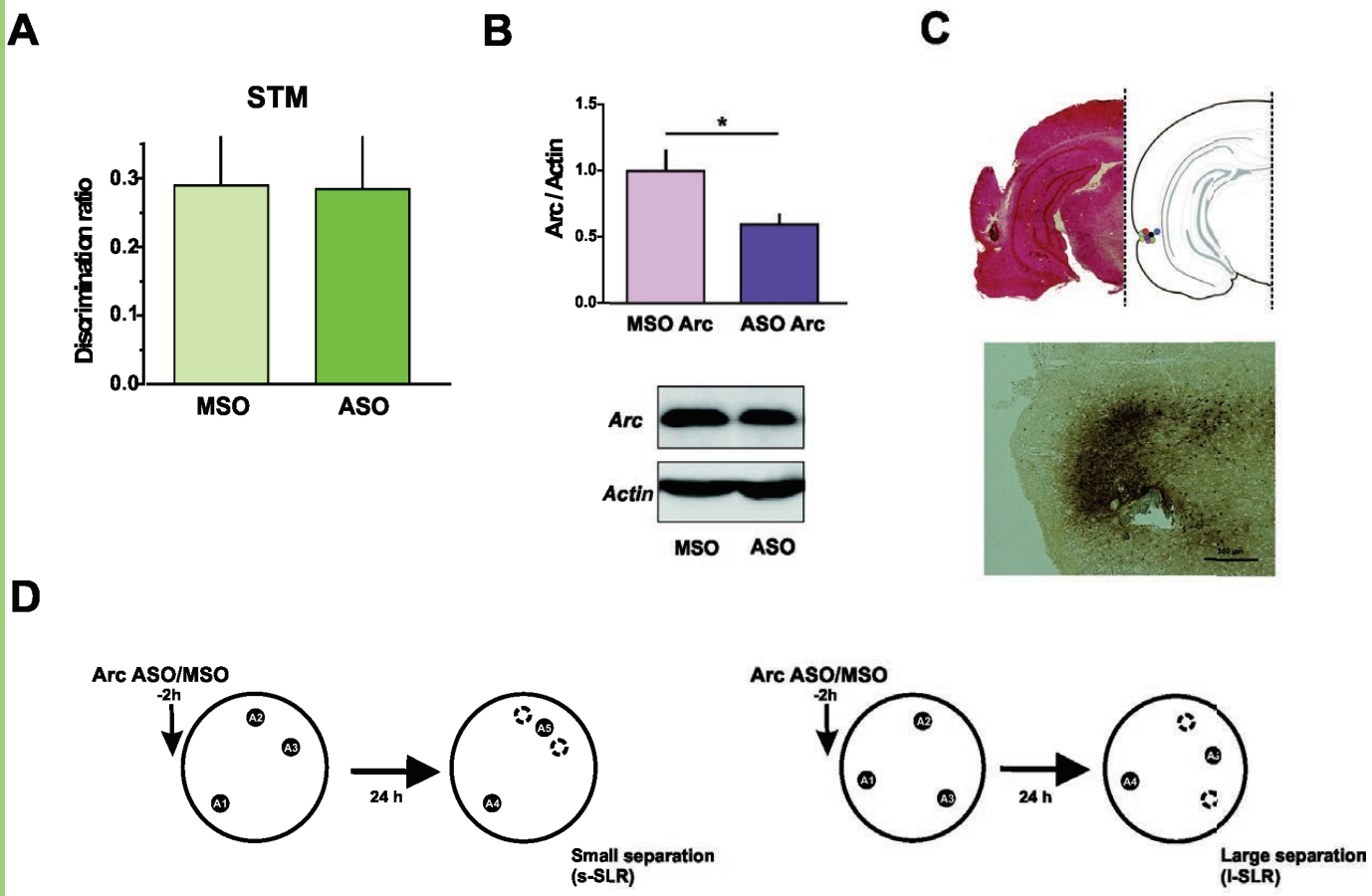

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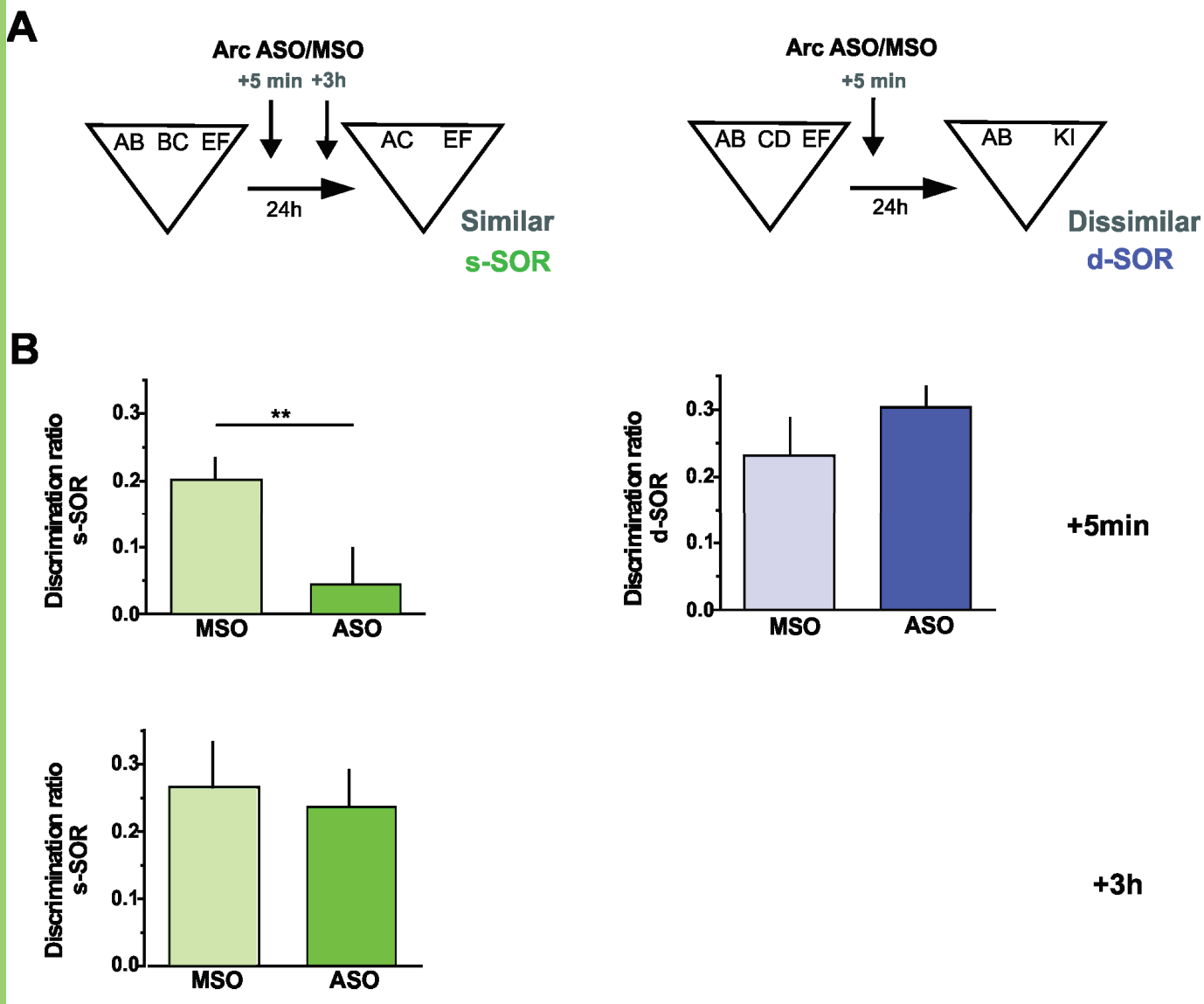

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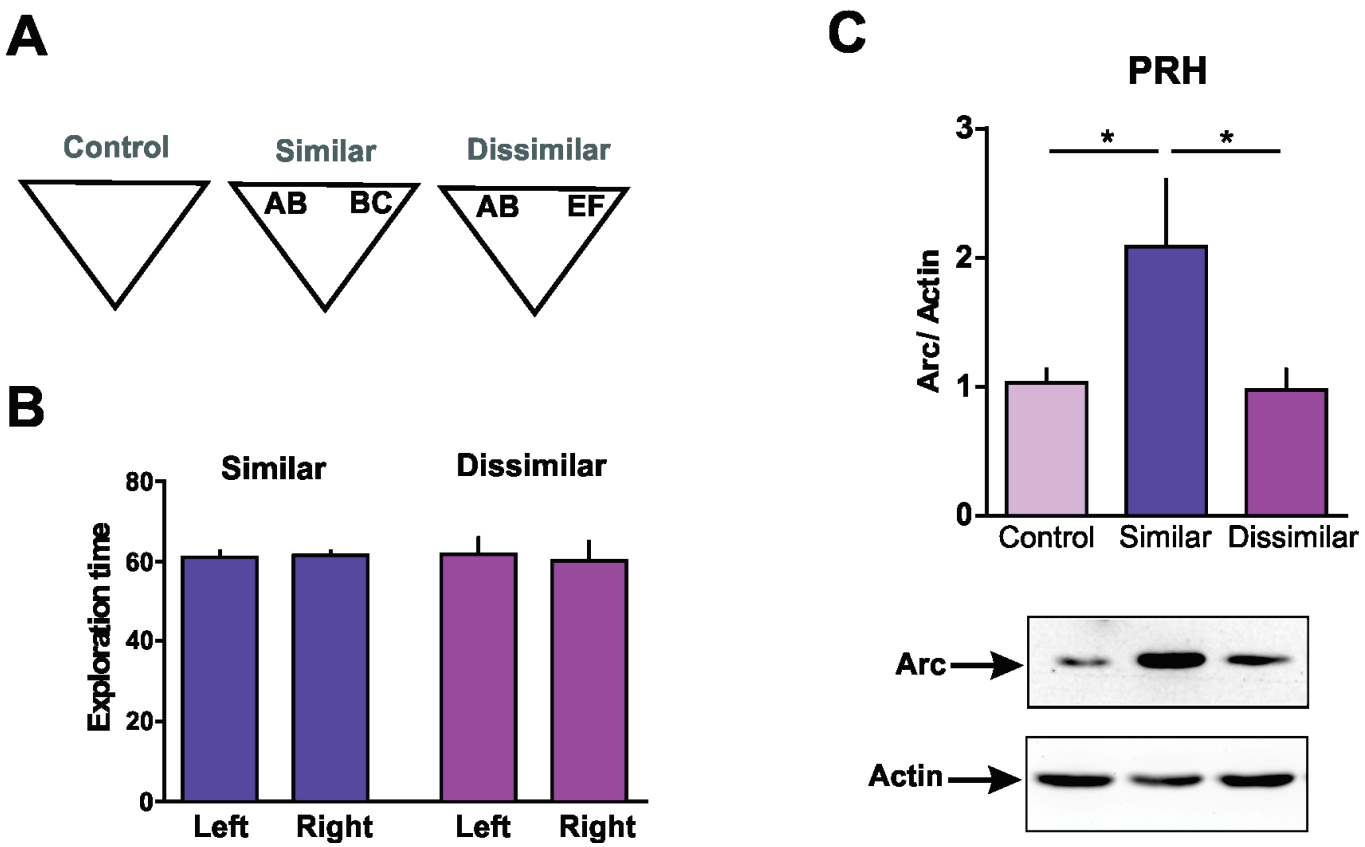

Fig. 8