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On the Role of Retrosplenial Cortex in Long-Lasting Memory Storage

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ABSTRACT: The retrosplenial cortex (RSC) is involved in a range of cognitive functions. However, its precise involvement in memory processing is unknown. Pharmacological and behavioral experiments demonstrate that protein synthesis and c-Fos expression in the anterior part of RSC (aRSC) are necessary late after training to maintain for many days a fear-motivated memory. Long-lasting memory storage is regulated by D1/D5 dopamine receptors in aRSC and depends on the functional interplay between dorsal hippocampus and aRSC. These results suggest that the RSC recapitulates some of the molecular events that occur in the hippocampus to maintain memory trace over time. © **2013 Wiley Periodicals, Inc.**

KEY WORDS: protein synthesis; c-Fos; hippocampus; memory persistence; dopamine

INTRODUCTION

To last, fear memories require a protein synthesis- and brain-derived neurotrophic factor (BDNF)-dependent late post training phase, involving activation of the ventral tegmental area (VTA)/hippocampus dopaminergic loop (Bekinschtein et al., 2007, 2008a; Rossato et al., 2009). However, although cortical plasticity seems to be crucial for the lasting storage of different memory types (Frankland et al., 2001; Lesburgueres et al., 2011; Martinez-Moreno et al., 2011), little is known about the molecular and cellular events underlying fear memory persistence in the cortex. The retrosplenial cortex (RSC) is one of the largest cortical areas in the rat, comprising the entire posterior cingulate cortex (Vogt and Peters, 1981). Situated at the crossroads between the hippocampal formation and many neocortical areas, it has attracted much attention especially for its role in cognition (Vann et al., 2009). For example, temporary inactivation of the RSC causes transient reorganization of spatial memory encoding in the hippocampus (Cooper and Mizumori, 2001), and it is known that functional integrity of this cortical area is necessary for the normal processing of hippocampus-dependent memories such as cue-specific and contextual fear conditioning (Keene and Bucci, 2008). RSC lesions produce memory impairments similar to those caused by hippocampal lesions, suggesting that these interconnected brain areas might cooperate in processing remembered information (Iaria et al., 2007). Indeed, several studies indicate that RSC function is severely compromised in most of the neurological disorders associated with memory impairment and that metabolic deregulation of the RSC is, in fact, a common feature of Alzheimer's disease and Korsakoff syndrome patients (Yasuno et al., 1998; Aupée et al., 2001; Villain et al., 2008; Pengas et al., 2012; Savage et al., 2012). To study the precise role of RSC in long-lasting fear-motivated memory storage, we used the hippocampus-dependent one-trial inhibitory avoidance (IA) learning task in rats. This paradigm permits an uncontaminated analysis of the different stages of memory processing initiated by a single training experience (Izquierdo and Medina, 1997; Taubenfeld et al., 1999; Bekinschtein et al., 2007) and has been successfully used previously to study the environmental attributes and physiological variables that make some memories last more than others (Bekinschtein et al., 2008b). In this study, we set to determine whether there is a late protein synthesis- and D1/D5-dependent phase involved in long-lasting memory storage and whether c-Fos expression in the RSC is functionally associated with that phase.

MATERIALS AND METHODS

Subjects

Male Wistar rats (2.5 months/220–250 g) from our own breeding colony were used. Animals were housed five to a cage at 23°C, with water and food ad libitum, under a 12 h light/dark cycle (lights on at 7:00 a.m.). All procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of the School of Medicine, University of Buenos Aires and the Pontifical Catholic University of Rio Grande do Sul.

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Behavioral Procedures

Animals were trained in a one-trial step-down IA task, between 7:00 a.m. and 9:00 a.m, as described (Benkinschtein et al., 2007). Briefly, the apparatus was a 50 × 25 × 25 cm acrylic box with a 5-cm high, 7-cm wide, and 25-cm long platform on the left end of a series of stainless steel bars that made up the floor of the box. For training, animals were gently placed on the platform; as they stepped down to the grid, they received either a 3-s, 0.4 mA (weak training), or 0.7 mA (strong training) scrambled footshock. Rats were tested for retention 2, 7, or 21 days after training. All animals were tested only once. In the test session, the footshock was omitted. Significant differences on latency to step down between training and test sessions were taken as a measure of retention.

Surgery and Infusion Procedures

Rats were implanted under deep thionembutal anesthesia with 22-g guide cannulas stereotaxically aimed to anterior part of RSC (aRSC) and/or the CA1 region of the dorsal hippocampus (coordinates A −4.3, L ±0.5, V −0.4 and A −4.3, L ±3.0, V −1.4, respectively, taken from the atlas of Paxinos and Watson, 1997). Cannulas were fixed to the skull with dental acrylic. After recovery from surgery, the animals were handled once a day for 2 days and then trained in IA. In all cases, infusions (1 μl) were bilateral. The entire infusion procedure took ∼2 min, including 45 s for the infusions themselves, first on one side and then on the other, and the handling. Histological examination of cannulas placement was performed as previously described (Bekinschtein et al., 2007; Rossato et al., 2009) and only the behavioral data from animals with the cannulas located in the intended site were included in the final analysis. Rats received a local infusion of 2 nmol of biotinylated *c-fos* antisense oligonucleotides (ASO) and sacrificed 2, or 24 h later. Two hours later, *c-fos* ASO is observable in a 3D volume of 1.6 mm^3 and became undetectable at 24 h after infusion of the biotinylated ASO (Fig. 1).

Drugs

Oligonucleotides (ODN) (Genbiotech, S.R.L.) were high-performance liquid chromatography (HPLC) purified phosphorothioated end-capped 15-mer sequences, resuspended in sterile saline to a concentration of 2 nmol/μl. *c-fos* ASO 5′-GAA CAT CAT GGT CGT-3′, *c-fos* missense oligonucleotides (MSO) 5′-GTA CCA ATC GGG ATT-3′. Both ODN sequences were subjected to a BLAST search on the National Center for Biotechnology Information BLAST server using the Genbank database. ASO is specific for rat *c-fos* mRNA. Control MSO sequence, which included the same 15 nucleotides as the ASO but in a scrambled order, did not generate any full matches to identified gene sequences in the database. Anisomycin was prepared in sterile saline (80 μg/side; Sigma, St. Louis, MO). Emetine was prepared in sterile saline (50 μg/side; Sigma, St. Louis, MO). SKF38393 was dissolved in DMSO 0.1% in sterile saline (12 μg/side; Sigma, St. Louis, MO). SCH23390 was prepared in sterile saline (1.5 μg/side; Sigma, St. Louis, MO).

Immunoblot Assays

Tissue was homogenized and samples were subjected to SDS-PAGE and immunoblot. Polyvinylidene fluoride (PVDF) membranes were incubated first with anti-c-Fos antibody (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-Egr-1 antibody (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA), then stripped and incubated with anti-Actin antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). Film densitometry analysis was performed by using Gel-Pro Analyzer (version 4.0, Media Cybernetics, MD).

Immunohistochemistry

Rats were anesthetized 12 h and 24 h after IA training and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde. Brains were removed and placed in 10% buffered formalin with 30% sucrose and slices. The brain sections (50 mm thickness) were subjected to an immunohistochemical assay with an anti-c-Fos antibody (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) as previously described. The reaction product was detected by avidin-biotin staining. For analysis of ODN spread after injection, rats were injected with 2 nmol of biotinylated *c-fos* antisense ODN (ASO), and 2 or 24 h after injection, the animals were anesthetized and perfused with 4% paraformaldehyde. The brains were isolated and sliced, and the ASO was detected by avidin-biotin staining.

Data Analysis

Data are presented as mean ± SEM. Statistical analysis was performed by unpaired Student's *t* test or one-way ANOVA followed by Newman-Keuls multiple comparison test, as appropriate.

RESULTS

Using a strong training protocol, which produces a persistent IA memory lasting more than 2 weeks (Bekinschtein et al., 2008a; Rossato et al., 2009), we found that two different protein synthesis inhibitors, emetine (50 μg/μl) and anisomycin (80 μg/μl), infused in aRSC 12 h, but not 24 h after training, impaired memory retention 7 but not 2 days later (Fig. 1A). No spontaneous recovery of the IA response was found up to 21 days post training (Fig. 1B). Confirming previous findings of our group (Bekinschtein et al., 2007), anisomycin infusion into the CA1 region of dorsal hippocampus impaired memory retention 7 but not 2 days after training (Fig. 1C).

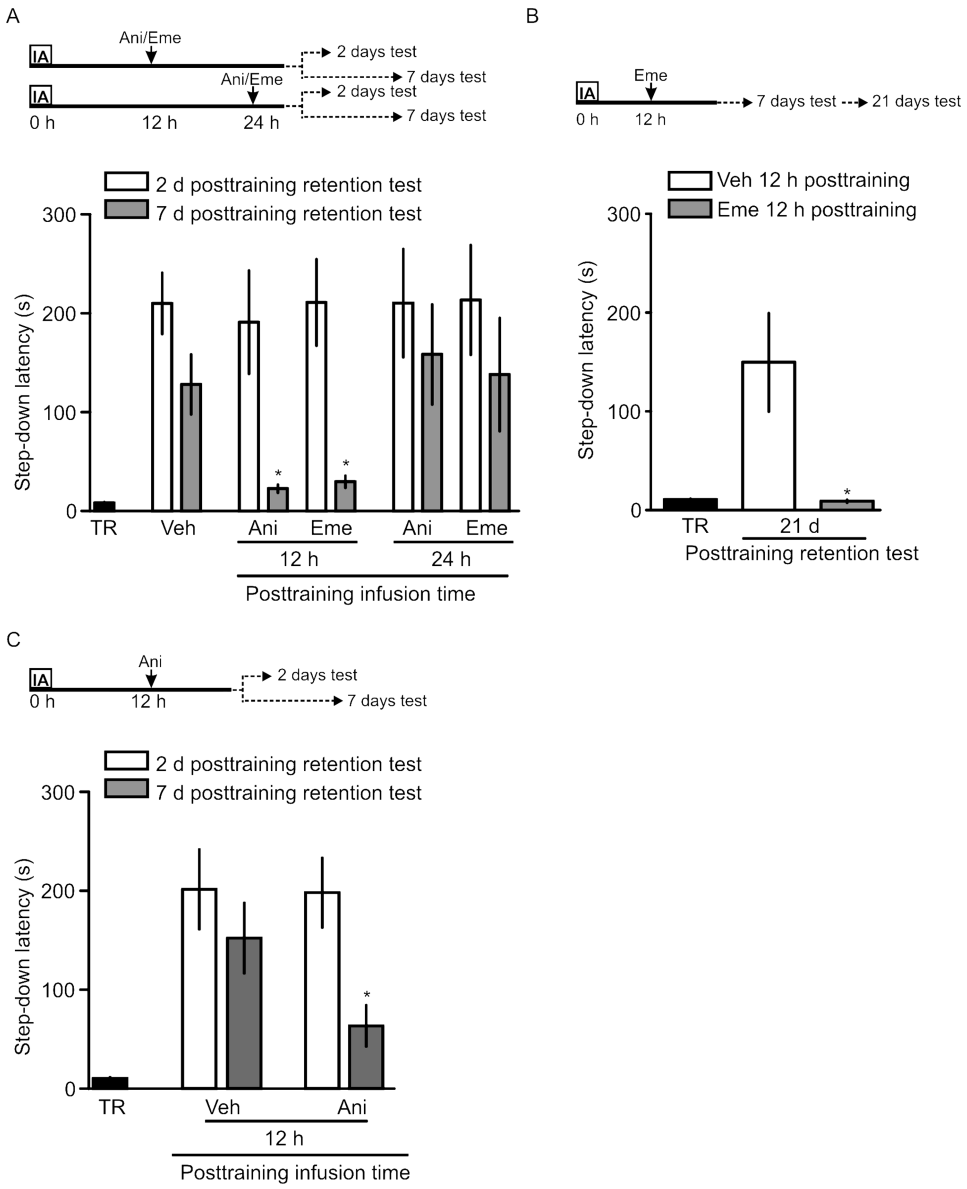

FIGURE 1. Delayed protein synthesis is required for long-lasting memory storage. A: Protein synthesis inhibition 12 h after training impaired memory persistence. Animals were infused into aRSC with vehicle (Veh) or anisomycin (80 µg/1 µl/side; Ani) or emetine (50 µg/1 µl/side; Eme) 12 or 24 h after training. Data are expressed as mean ± SEM of training (TR, black bar) or test session step-down latency, 2 days (white bars) or 7 days (gray bars) after IA training. *$P < 0.05$, vs. Veh; Two-tailed Student's t test, $n = 10–12$ per group. B: No spontaneous memory recovery after protein synthesis inhibition 12 h after IA training. Animals were infused in the aRSC with vehicle (Veh, white bar) or emetine (50 µg/1 µl/side; Eme, gray bar) 12 h after training were tested 7 days and retested 21 days after IA training. Data are expressed as mean ± SEM of training (TR, black bar) or test session step-down latency 21 days after IA training. *$P < 0.05$, vs. Veh; Two-tailed Student's t test, $n = 10–12$ per group. C: Protein synthesis inhibition 12 h after training impaired memory persistence. Animals were infused into CA1 region of dorsal hippocampus with vehicle (Veh) or anisomycin (80 µg/1 µl/side; Ani) 12 h after training. Data are expressed as mean ± SEM of training (TR, black bar) or test session step-down latency, 2 days (white bars) or 7 days (gray bars) after IA training. *$P < 0.05$, vs. Veh; Two-tailed Student's t test, $n = 8–10$ per group.

Immediate early genes, including those coding the inducible transcription factors c-Fos and Egr-1, are the first group of genes expressed after synaptic activation (Guzowski, 2002). Therefore, we reasoned that c-Fos and/or Egr-1 could be part of the general program of protein synthesis required for long-lasting memory storage. Immunoblot analyses (Fig. 2A) showed that aRSC c-Fos levels increased 12 h and 24 h, but not 6 h or 30 h post training. This late post training wave of c-Fos expression was confirmed by immunohistochemistry (Fig. 2B). We did not find any modification in c-Fos levels in the aRSC of nontrained animals that received a footshock identical to that given to IA trained rats (Figs. 2A,B). No change in aRSC Egr-1 levels was detected at any post training time studied, which is in agreement with recent findings in spatial learning tasks

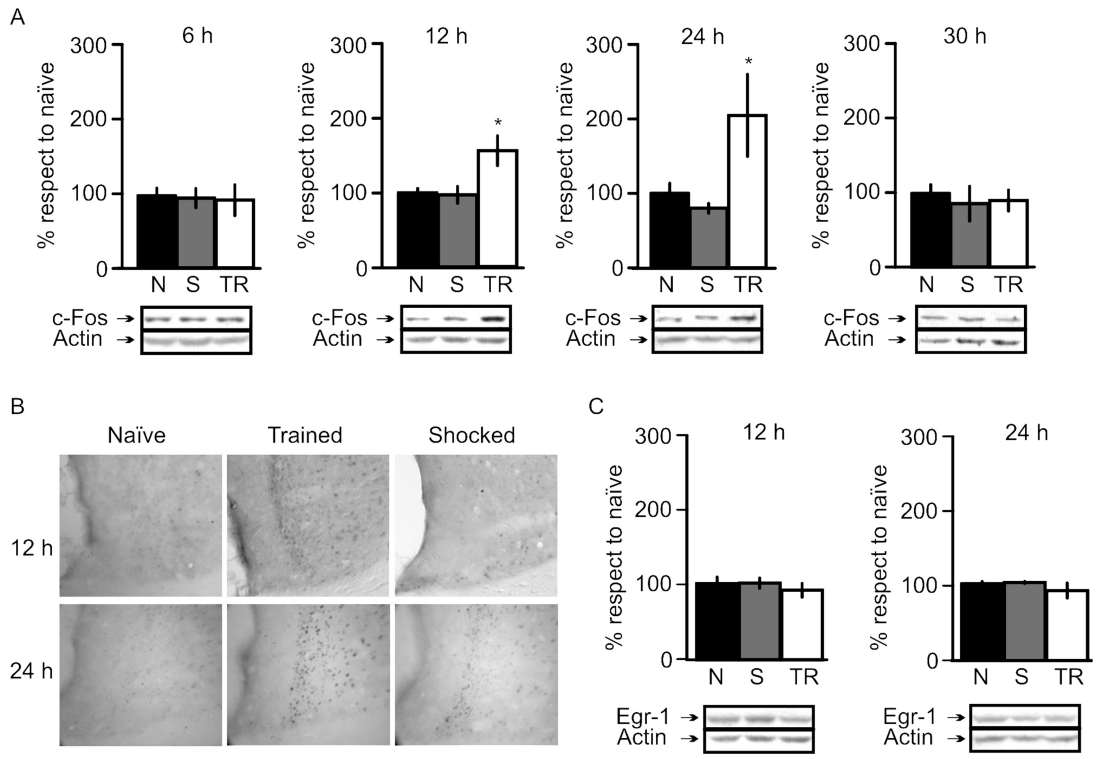

FIGURE 2. Memory processing is associated with c-Fos expression late after training in aRSC. A: Time course of aRSC c-Fos levels after strong IA training in the aRSC. The aRSC was dissected out and total homogenates were subjected to SDS-PAGE followed by western blot analysis with antibodies against c-Fos or Actin. Bars indicate the percentage of change with respect to the naïve group (N, black bars) for rats shocked (S, gray bars) or trained (TR, white bars) and sacrificed 6, 12, 24, or 30 h after the behavioral procedure. Data are expressed as mean ± SEM. *$P < 0.05$ in Newman-Keuls test after one-way ANOVA, $n = 5$–8 per group. B: IA training is associated with an increased in c-Fos immunoreactivity in aRSC. Rats were sacrificed 12 or 24 h after IA training, coronal sections of the brain were subjected to immunohistochemical assays using antibodies against c-Fos. C: aRSC Egr-1 levels after strong IA training in the aRSC. The aRSC was dissected out and total homogenates were subjected to SDS-PAGE followed by western blot analysis with antibodies against Egr-1 or Actin. Bars indicate the percentage of change with respect to the naïve group (N, black bars) for rats shocked (S, gray bars) or trained (TR, white bars) and sacrificed 12 or 24 h after the behavioral procedure. Data are expressed as mean ± SEM. $P > 0.05$ in Newman-Keuls test after one-way ANOVA, $n = 5$–8 per group.

(Tse et al., 2011). Intra-aRSC injection of *c-fos* ASO 8 h or 12 h post training did not affect memory as evaluated 2 days later, but impaired retention when animals were tested 7 days thereafter (Figs. 3A,B). No effect on memory was seen when *c-fos* ASO was infused immediately or 24 h post training. Equally, c-fos ASO given in dorsal CA1 8 h after training did not affect memory as assessed 2 days or 7 days later (Figs. 3A,C). Intra-aRSC infusion of *c-fos* MSO had no effect whatsoever on memory (Fig. 3B).

IA memory persistence depends on the late post training activation of the VTA/hippocampus dopaminergic loop (Rossato et al., 2009). Because aRSC is innervated by the VTA (Descarries et al., 1987), we investigated whether long-lasting storage of IA memory is also controlled by aRSC D1/D5 dopamine receptors. Intra-aRSC infusion of the specific D1/D5 receptor antagonist SCH23390 (1.5 μg/side) 12 h, but not immediately after strong IA training, hindered retention when memory was tested 7 days, but not 2 days post training (Fig. 4A). Conversely, intra-aRSC administration of the selective D1/D5 receptor agonist SKF38393 (12 μg/side) 12 h after a weak IA training session, which generates a short-lived memory lasting no more than 2 days, promoted the persistence of IA memory for at least 7 days (Fig. 4B).

We next investigated the possible interplay between aRSC and the hippocampus to maintain IA memory storage. We found that the impairment in IA memory persistence caused by emetine given in aRSC 12 h after training was rescued by the concomitant activation of hippocampal D1/D5 dopamine receptors with SKF38393 (Fig. 3C). In addition, the amnesic effect of emetine given in dorsal CA1 12 h after strong IA training was reversed by co-infusion of SKF38393 in aRSC (Fig. 4D). This set of results suggests that aRSC and the hippocampus work in concert during the memory persistence phase. If that were the case, then blocking c-Fos expression in one of these structures should affect c-Fos expression in the other one. We found that infusion of *c-fos* ASO in aRSC 12 h after training prevented the learning-induced increase in c-Fos expression both in aRSC and in dorsal hippocampus (Figs. 5A,B). Likewise, intra-CA1 infusion of *c-fos* ASO blocked the late post training increase in c-Fos levels in both structures (Figs. 5C,D).

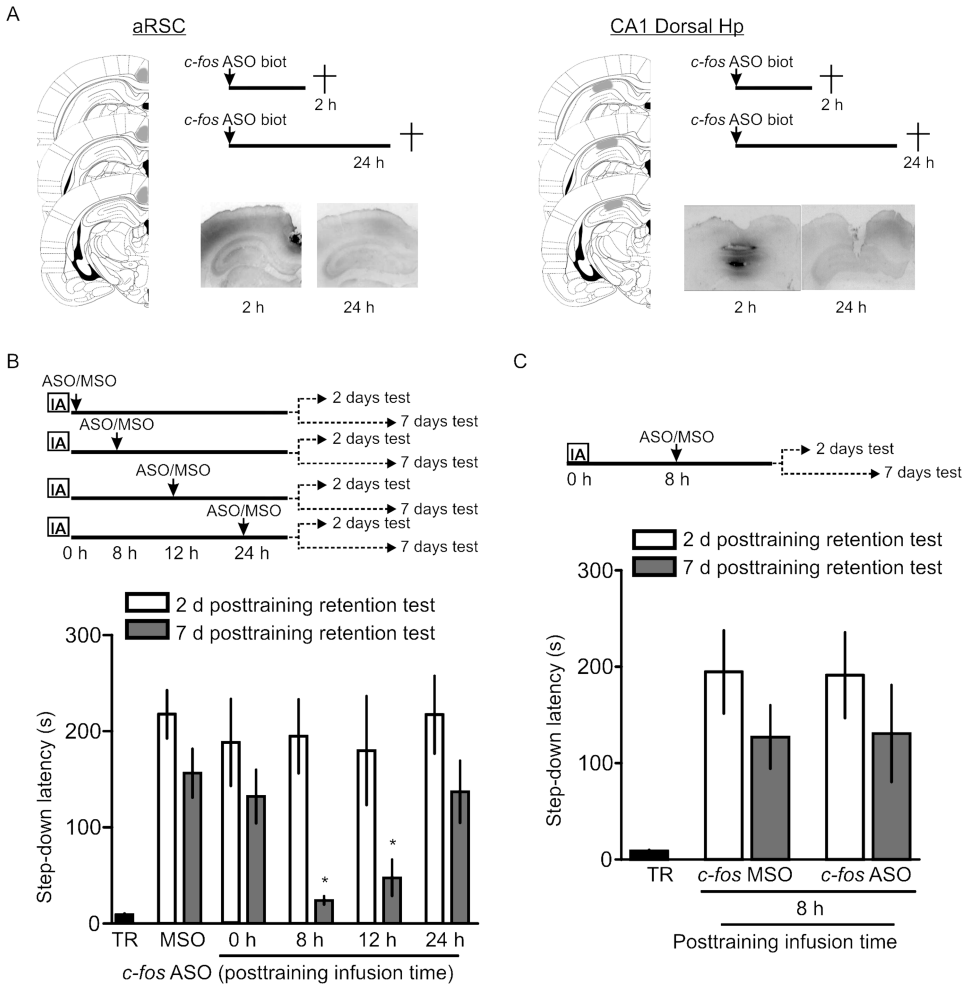

FIGURE 3. c-Fos expression is required for maintenance of memory storage in aRSC. A: Biotinylated *c-fos* antisense oligonucleotides (ASO) anatomical distribution and relative concentrations at different times after infusion. Schematic representations of rat brain sections at three rostrocaudal planes (−3.80, −4.30, and −4.80 from bregma) taken from the atlas of Paxinos and Watson (1997). In stippling, the extension of the area reached by the infusions in the aRSC (left) and in the CA1 of dorsal hippocampus (Hp) (right), and photomicrograph of a representative coronal brain section showing the infusion cannula track terminating in the aRSC (left) and into the CA1 region of the dorsal hippocampus (right). B: Disruption of c-Fos expression in the aRSC impairs memory persistence. Animals were infused into aRSC with *c-fos* missense oligonucleotides (MSO) or *c-fos* ASO (2 nmol/1 µl/side), 0, 8, 12, or 24 h after IA training. Data are expressed as mean ± SEM of training (TR, black bar) or test session step-down latency, 2 days (white bars) or 7 days (gray bars) after IA training. *$P < 0.05$ vs. MSO; Two-tailed Student's *t* test, $n = 10–12$ per group. C: Infusion of *c-fos* ASO in the CA1 region of dorsal hippocampus 8 h after training does not impair memory persistence. Animals were infused in the CA1 of the dorsal hippocampus with *c-fos* MSO or *c-fos* ASO (2 nmol/1 µl/side), 8 h after training. Data are expressed as mean ± SEM of TR (black bars) or test session step-down latency 2 (white bars) or 7 days (gray bars) after IA training. $P > 0.05$ vs. MSO group; Student's *t* test; $n = 10–12$ per group.

DISCUSSION

The main findings of this study are: (1) a late protein synthesis-dependent phase in aRSC is mandatory for maintenance, but not formation, of fear memory; (2) maintenance of the fear memory trace depends on late post training activation of c-Fos in aRSC; (3) long-lasting storage of fear memory is under the control of aRSC D1/D5 receptors and involves the functional interplay between aRSC and dorsal hippocampus. These assertions are based on findings showing that infusion of two different protein synthesis inhibitors in aRSC 12 h after IA training impaired memory persistence, but did not affect memory formation, as memory expression was intact 2 days after training; by blocking the learning-induced increase of c-Fos expression 12–24 h after training, we found an impairment on memory persistence at 7 days, but not in memory formation; and finally, we showed that in the aRSC, SCH23390 infusion, a D1/D5 receptor antagonist, impaired long-term memory persistence; on the other hand, SKF38393 infusion, a D1/D5 receptor agonist, transformed a short-live memory into a lasting one. In addition, impairment in long-lasting memory persistence produced by the infusion of emetine into CA1 of the dorsal hippocampus was rescued by the infusion of SKF38393 into aRSC, and vice versa. These results reveal that a late post

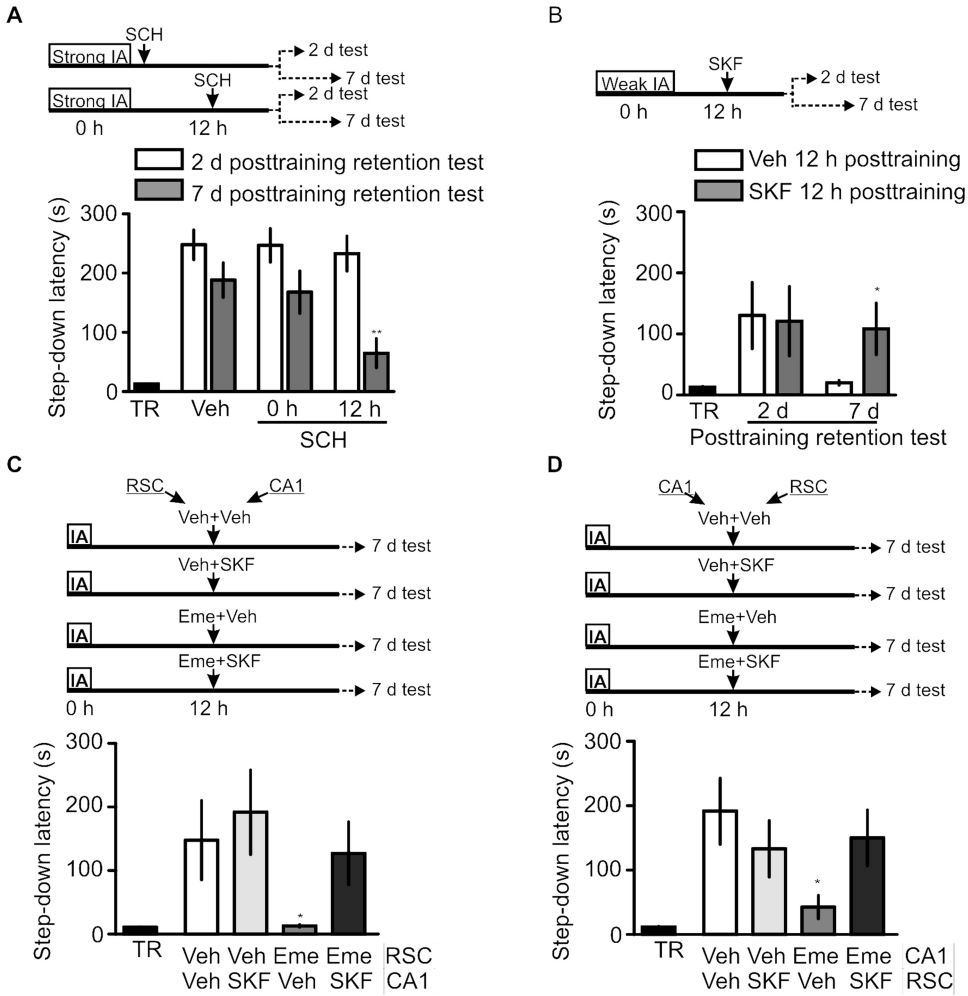

FIGURE 4. D1/D5 receptors are required for long-lasting memory storage in the aRSC and are involved in its interplay with the hippocampus. A: D1/D5 antagonist impairs memory persistence. Animals were infused into aRSC with vehicle (Veh) or SCH23390 (1.5 μg/1 μl/side; SCH) immediately or 12 h after training. Data are expressed as mean ± SEM of training (TR, black bar) or test session step-down latency, 2 days (white bars) or 7 days (gray bars) after IA training. **$P < 0.01$, vs. Veh; Two-tailed Student's t test, $n = 10–13$ per group. B: D1/D5 agonist promotes memory persistence. Animals were infused into aRSC with vehicle (Veh) or SKF38393 (12 μg/1 μl/side; SKF) immediately or 12 h after weak IA training. Data are expressed as mean ± SEM of training (TR, black bar) or test session step-down latency 7 days (gray bars) after IA training. *$P < 0.05$, vs. Veh; Two-tailed Student's t test, $n = 7–9$ per group. C: Activation of D1/D5 receptors in the hippocampus rescues the impairment in memory persistence caused by inhibition of protein synthesis in the aRSC. Animals were infused into aRSC with vehicle (Veh) or emetine (50 μg/1 μl/side; Eme) and into CA1 of the dorsal Hp with vehicle (Veh) or SKF38393 (12 μg/1 μl/side; SKF) 12 h after IA training. Data are expressed as mean ± SEM of training (TR, black bar) or test session step-down latency, 2 days (white bars) or 7 days (gray bars) after IA training. *$P < 0.05$, vs. Veh/Veh, Veh/SKF, and Eme/SKF; in Newman-Keuls test after one-way ANOVA, $n = 7–9$ per group. D: Activation of D1/D5 receptors in the aRSC rescues the impairment in memory persistence caused by inhibition of protein synthesis in the hippocampus. Animals were infused into CA1 of the dorsal Hp with vehicle (Veh) or emetine (50 μg/1 μl/side; Eme) and into aRSC with vehicle (Veh) or SKF38393 (12 μg/1 μl/side; SKF) 12 h after IA training. Data are expressed as mean ± SEM of training (TR, black bar) or test session step-down latency 7 days after IA training. *$P < 0.05$, vs. Veh/SKF and vs. Eme/SKF; **$P < 0.01$, vs. Veh/Veh; Two-tailed Student's t test, $n = 7–9$ per group.

training phase involving activation of D1/D5 dopamine receptors is important for the maintenance of memory storage in both the hippocampus (Rossato et al., 2009) and aRSC.

Thus, the role of aRSC in the persistence of the fear memory trace closely overlaps that of the hippocampus. For instance, de novo protein synthesis is needed in both structures several hours after training (Bekinschtein et al., 2007; Alberini, 2009), which, incidentally, is in line with correlative studies in humans showing that successful memory storage is associated with increased connectivity between the hippocampus and some cortical regions, including RSC (Ranganath et al., 2005). Furthermore, it has been recently published that the hippocampus and RSC play distinct, but complimentary roles in mediating memory context in rats (Smith et al., 2012).

The maintenance phase for long-lasting memory storage is not restricted to aRSC and hippocampus. Recently, it has been shown that in conditioned taste aversion there is also a late protein synthesis phase in the insular cortex important for

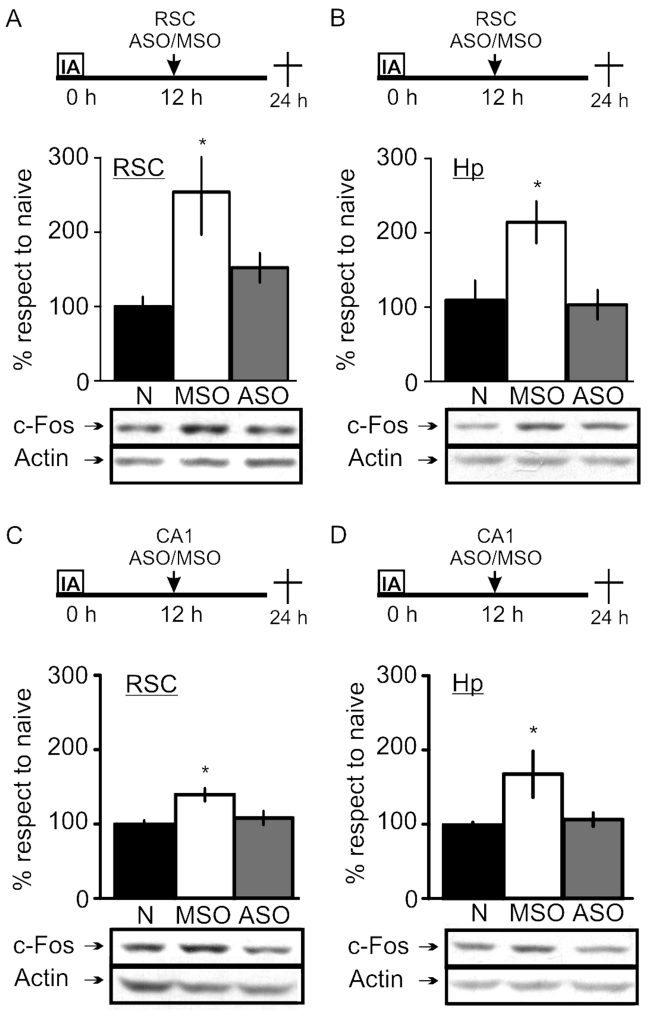

FIGURE 5. Interplay between aRSC and dorsal hippocampus mediated by c-Fos expression. A and B: Infusion of *c-fos* ASO in aRSC 12 h after training prevented the learning-induced increase in c-Fos expression in both the aRSC and the dorsal hippocampus. Bars indicate the percentage of change in the aRSC (A) and in the dorsal hippocampus (B) respect to the naïve group (N, black bars) for rats infused in the aRSC with *c-fos* MSO (white bars) or *c-fos* ASO (gray bars) (2 nmol/1 µl/side), 12 h after training and sacrificed 24 h after the behavioral procedure. Data are expressed as mean ± SEM. *$P < 0.05$, in Newman-Keuls test after one-way ANOVA, $n = 5–8$ per group. C and D: Infusion of *c-fos* ASO into the CA1 region 12 h after training prevented the learning-induced increase in c-Fos expression in both the aRSC and the dorsal hippocampus. Bars indicate the percentage of change in the aRSC (C) and in the dorsal hippocampus (D) respect to the naïve group (N, black bars) for rats infused into the CA1 region of the dorsal hippocampus with *c-fos* MSO (white bars) or *c-fos* ASO (gray bars) (2 nmol/1 µl/side), 12 h after training and sacrificed 24 h after the behavioral procedure. Data are expressed as mean ± SEM. *$P < 0.05$, in Newman-Keuls test after one-way ANOVA, $n = 5–8$ per group.

long-lasting memory storage (Martínez-Moreno et al., 2011). The time window is about 5–7 h after training. In addition, Ou et al. (2010) demonstrated that fear conditioning is associated with increased expression of BDNF in the amygdala 12 h after training. Blocking BDNF activity impaired memory retention at 7, but not at 1 day post training, suggesting that a late BDNF-dependent phase in the amygdala is important for long-lasting fear memory storage.

It is known that the rapid induction of immediate-early genes (IEGs) in the brain plays an important role in regulating synaptic plasticity, and some IEGs, including c-Fos, have been associated with the first transient transcriptional steps involved in memory formation (Lamprecht and Dudai, 1996; McGaugh, 2000; Kandel, 2001). Several studies have used c-Fos expression in the RSC as neural activity marker of remote memories (Frankland et al., 2001, 2004; Maviel et al., 2004) and fear-associated memories (Radwanska et al., 2010). Nevertheless, none of these works have revealed that c-Fos has a key role in memory persistence in this structure. Here, we showed that delayed post training c-Fos expression in aRSC is necessary for the persistent storage of long-lasting fear memory. Similar results were obtained blocking delayed c-Fos expression in the hippocampus (Katche et al., 2010). However, the dynamics of the mechanism involved in memory persistence in both structures appears to be different. While infusion of *c-fos* ASO in dorsal CA1 8 h after training has no effect on memory persistence, it caused a clear-cut impairment in memory persistence when given in aRSC at the same post training time. This difference has an important implication. It rules out the possibility that the amnesic effect of intra-aRSC ASO was due to its diffusion into the hippocampus.

In conclusion, our findings are consistent with those showing that RSC is profusely interconnected with the hippocampal formation (Vann et al., 2009; Sugar et al., 2011) and indicate that aRSC and the hippocampus require each other to preserve fear memory, suggesting that they influence a common downstream component of the neural circuit responsible for long-lasting memory storage.

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