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Neurogenesis Inhibition Prevents Enriched Environment to Prolong and Strengthen Social Recognition Memory, But Not to Increase BDNF Expression

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Abstract Hippocampus-dependent memories, such as social recognition (SRM), are modulated by neurogenesis. However, the precise role of newborn neurons in social memory processing is still unknown. We showed previously that 1 week of enriched environment (EE) is sufficient to increase neurogenesis in the hippocampus (HIP) and the olfactory bulb (OB) of mice. Here, we tested the hypothesis that 1 week of EE would enhance SRM persistence and strength. In addition, as brain-derived neurotrophic factor (BDNF) may mediate some of the neurogenesis effects on memory, we also tested if 1 week of EE would increase BDNF expression in the HIP and OB. We also predicted that neurogenesis inhibition would block the gain of function caused by EE on both SRM and BDNF expression. We found that EE increased BDNF expression in the HIP and OB of mice; at the same time, it allowed SRM to last longer. In addition, mice on EE had their SRM unaffected by memory consolidation interferences. As we predicted, treatment with the anti-mitotic drug AraC blocked EE effects on SRM. Surprisingly, neurogenesis inhibition did not affect the BDNF expression, increased by EE. Together, our results suggest that newborn neurons improve SRM persistence through a BDNF-independent mechanism. Interestingly, this study on social memory uncovered an unexpected dissociation between the effect of adult neurogenesis and BDNF expression on memory persistence, reassuring the idea that not all neurogenesis effects on memory are BDNF-dependent.

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Introduction

In rodents, adult neurogenesis occurs in the subventricular zone (SVZ) of the lateral ventricle and in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. Newborn neurons from the SVZ migrate to the olfactory bulb, playing a major role in olfaction [1], while newborn neurons from the SGZ are associated to learning and memory in the hippocampus [2]. However, the precise role of these newborn neurons in memory processing is still under investigation.

Evidence indicates that immature neurons are more easily excitable than mature neurons and process input information independently from changes in synaptic plasticity [3]. In fact, DG immature neurons may be crucial to the process of pattern separation, which is an important memory function [4].

One of the possible mediators of neurogenesis effects is the brain-derived neurotrophic factor (BDNF). BDNF increases the number and survival of newborn neurons, while affects adult-born GCs morphogenesis [5]. Furthermore, BDNF acts on immature DG neurons to promote consolidation of "pattern-separated" memory [6, 7] suggesting that, through modulation of BDNF signaling, newborn cells may be involved in memory formation even before their complete maturation.

In mice, social recognition memory (SRM) requires functional participation of the hippocampus [8, 9]. Previously, we demonstrated that only 1 week of social isolation impairs SRM, while concomitant exposure to enriched environment (EE) reverses this amnesia through a mechanism involving induction of hippocampal neurogenesis [10]. Interestingly, the same study showed that 1 week of EE is sufficient to increase neurogenesis in the hippocampus and olfactory bulb

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of group-housed mice [10]. However, it still remains to be investigated if these newborn neurons are playing a role on SRM. Here, we hypothesized that 1 week of EE would enhance SRM persistence and strength, in addition to increase BDNF expression. Furthermore, we tested if the treatment with a neurogenesis inhibitor would block the gain of function caused by EE.

Material and Methods

Subjects and Environment Conditions Adult (8–12-week old) and juvenile (21–30-day old) male Swiss mice were used. All animals were maintained in a climate-controlled environment (22 ± 2 °C, humidity at 55 ± 10 %) under a 12-h dark-12-h light cycle. All behavioral experiments were performed during the light phase. Both food and water were available ad libitum. Animals were housed in groups of five, under either standard (SE) or enriched environment (EE) conditions. SE mice were kept in a standard plastic cage ($28 \times 17 \times 12$ cm^3) with lining, while EE mice were kept in a larger plastic cage ($40 \times 33 \times 16$ cm^3) containing ribbons, pieces of plastic, cardboard rolls, and toys. Animals stayed in EE for only 7 days. The Animal Use Ethic Committee of the Universidade Federal de Minas Gerais (CEUA 44/2013) approved all the experimental procedures.

Social Recognition Test Habituation phase consisted on introducing the subject mouse inside a clean standard cage containing an empty cylinder, with 60 evenly spaced holes on its walls, for a period of 30 min. During the last 5 min, a juvenile mouse was introduced into an identical cylinder, within its own cage. Training session (TR) lasts 5 min and consisted in replacing the empty cylinder by the one containing the juvenile mouse. Social exploration time was counted when the subject's nose or whiskers were introduced in the cylinder's holes. Test session (TT) lasts 5 min and was performed either 24 h or 10 days after TR and was identical to TR [10].

Interference Stimulus We used two common behavioral tests to interfere in social memory encoding at a time point known to be important for memory consolidation [9, 11]. Open-field test was performed in a box ($50 \times 50 \times 50$ cm) with its floor subdivided into 16 identical squares. During 5 min, the animal was allowed to freely explore the arena. Number of segments crossed and number of rearing were counted. Tail suspension test (TST) was performed by attaching the mouse's tail to a support by sticky tape. The animal stayed upside down throughout 6 min, and we measured immobility time, which was considered as absence of movements (except for respiratory ones) [12].

BrdU Administration Bromodeoxyuridine (BrdU—Sigma) was dissolved in 0.9 % NaCl and administered intraperitoneally at 75 mg/kg, once a day for a total of 7 days. This protocol allowed verification of proliferation and adult newborn cell survival [10, 13].

Western Blotting Samples were homogenized on ice in RIPA buffer (150 mM NaCl, 50 mM Tris, pH = 7.4, 1 mM EDTA, 1 % Nonidet P40, 1 mM phenylmethylsulfonyl fluoride, 0.5 % sodium deoxycholate) and centrifuged at 2040.35*g* (Eppendorf) for 15 min at 4 °C. Protein content was determined as previously described [14]. Proteins (50 μg) were denatured in sample buffer (100 mM Tris–HCl pH 5 6.8, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 20 % H_2O, 0.5 % *b*-mercaptoethanol) at a temperature of 100 °C for 4 min. Proteins were loaded and separated on 15 % SDS-polyacrylamide gels, followed by transfer to polyvinylidene dixuoride membrane (PVDF, Immobilon-P, Millipore) in a Bio-Rad electrophoresis system. Membranes were blocked with TBST (Tris-buffered saline with 0.1 % Tween) containing 5 % non-fat milk and 0.3 % BSA. Membranes were incubated overnight at 4 °C with primary antibodies against BDNF (1:500, Santa Cruz Biotechnology, sc-546). After, membranes were incubated with anti-mouse antibody conjugated with HRP for 1 h. β-Actin (1:2500, Santa Cruz Biotechnology, sc-69879) was used as the normalization protein. Target protein bands were detected using ECL Western blotting detection system (Pierce). β-Actin and BDNF blotting band densities were accessed by ImageJ software (Version 1.44p).

Immunofluorescence Animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), followed by transcardial perfusion with 0.01 M phosphate-buffered saline (PBS) and subsequently 4 % paraformaldehyde (PFA in PBS). Brains were removed, fixed overnight in 4 % PFA, and maintained in a 30 % sucrose solution at 4 °C during 3 days. Coronal 40-μm brain sections were obtained and stored at −22 °C in PBSAF (PBS, 20 % sucrose, 15 % ethylene glycol, 0.05 % NaN_3). For each animal, we selected as many as possible olfactory bulb slices, six slices of dorsal hippocampus (1.70 mm to −2.30 mm from Bregma) and five slices of ventral hippocampus (−3.16 to −3.52 mm from Bregma) [15]. Slices were washed sequentially in PBS, PBST, and 0.9 % NaCl. Subsequently, slices were incubated at 37 °C in HCl and washed in 0.1 M borate buffer and PBST. Slices were placed for 1 h in 5 % normal goat serum (NGS) and incubated for 72 h at 4 °C with anti-BrdU (1:800, Abcam) and anti-NeuN (1:500, Millipore). Anti-BrdU marked newborn cells, while anti-NeuN labeled neurons. After, slices were washed in PBS and incubated for 90 min at room temperature with Alexa Fluor 488 (1:400, Invitrogen) and Alexa Fluor 568 (1:400, Invitrogen). Lastly, slices were washed in PBS, mounted, fixed in Vectashield (Vector Laboratories), and covered [10].

Imaging Acquisition and Analysis Slides were photographed using an epifluorescence microscope (Zeiss) and Axiovision 4.8 software. A cell was considered double-labeled if (1) co-labeled with relevant morphology and (2) viewed in x–y, x–z, and y–z cross-sections produced from orthogonal z-series reconstructions. BrdU or BrdU/NeuN cells were counted manually using ImageJ Software (NIH, USA). Proliferation ($BrdU^+$) and neurogenesis ($BrdU^+$ and $NeuN^+$) were expressed in number of cells per square millimeter. Neurogenesis rate was expressed as number of $BrdU^+$ and $NeuN^+$ cells/$BrdU^+$ cells.

AraC Infusion In order to implant a single cannula into the right lateral ventricle, mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and positioned in a stereotaxic apparatus. The following coordinates were used: 20.5 mm AP, 21.0 mm LL, and 22.0 mm DV [15]. A polypropylene catheter was attached to a cannula, allowing it to be coupled to a mini-osmotic pump (Alzet; model 1007D; flow rate 0.5 μl/h, 7 days), which was implanted subcutaneously in the dorsal region of the mouse. Mini-osmotic pumps were filled with saline or cystosine-β-D-arabinofuranoside (AraC, 2 %; Sigma) [10].

Statistical Analysis Data were expressed as mean ± SEM. Differences between groups were assessed by either paired and unpaired *t* tests or two-way ANOVA with Bonferroni's post-test, as stated in results. Significance level was set at $p < 0.05$.

Results

As expected [10], both SE and EE groups remembered the juvenile 24 h after TR at similar levels ($F_{(1,10)} = 59.99$, $p < 0.0001$ for trial and $F_{(1,10)} = 0.26$, $p = 0.62$ for housing condition) (Fig. 1a). Next, we tested whether EE would prolong SRM duration. In mice, SRM measured by social discrimination test can last up to 7 days [8]. To test our hypothesis by more thoroughly challenging SRM, we adopted a protocol of 10 rather than 7 days as delay between memory acquisition and retrieval. Only the EE group was capable of maintaining social memory 10 days after TR ($F_{(1,13)} = 95.34$, $p < 0.0001$ for housing condition X trial and $F_{(1,13)} = 5.92$, $p = 0.001$ for trial) (Fig. 1b). Others and we had showed 6 h post-training to be an important time-point for SRM consolidation [9, 11]. Therefore, we predicted that EE would protect social memory consolidation from interferences, such as TST or OF exposure at 6 h post-training. In fact, only EE mice showed 24-h social memory after being exposed to TST and OF. For the OF experiment, we observed no interaction between factors ($F_{(1,10)} = 0.10$, $p = 0.75$) or main effect of housing condition ($F_{(1,10)} = 3.34$, $p = 0.09$), though a main effect of trial was observed ($F_{(1,10)} = 13.01$, $p = 0.004$) (Fig.1c). TST presented a more consistent effect on impairing SRM of SE mice, though it had no effect on EE mice. Two-way ANOVA showed interaction between factors ($F_{(1,9)} = 21.58$, $p = 0.001$) and main effect of trial ($F_{(1,9)} = 21.58$, $p = 0.001$), without main effect of housing ($F_{(1,9)} = 9.03$, $p = 0.99$) (Fig. 1c). No difference between groups was found in OF parameters (crossing: $t(10) = 1.2$, $p = 0.24$; rearing: $t(10) = 0.16$, $p = 0.86$) (Fig. 1d). However, in TST, we observed a reduction in the immobility time for the EE group ($t = 2.74$, $p = 0.02$), indicating an antidepressive effect of EE [16, 17] (Fig. 1e).

To verify whether EE effect on extending SRM is neurogenesis-dependent, we treated animals with AraC during the whole period of EE. AraC animals did not remember juvenile 10 days after TR ($F_{(1,24)} = 35.24$, $p < 0.0001$ for treatment X trial and $F_{(1,24)} = 23.36$, $p < 0.0001$ for treatment), while the saline-treated animals did (Fig. 2a). Immunofluorescence confirmed AraC treatment reduced proliferation in the dentate gyrus of the dorsal ($t = 4.74$, $p = 0.0008$) and ventral ($t = 6.88$, $p < 0.0001$) hippocampi, as well as in the olfactory bulb ($t = 6.44$, $p < 0.0001$) (Fig. 2g). Neurogenesis was also impaired by AraC in the dorsal ($t = 4.31$, $p = 0.0015$) and ventral hippocampi ($t = 8.47$, $p < 0.0001$), and in olfactory bulb ($t = 7.19$, $p < 0.0001$) (Fig. 2f). Surprisingly, neurogenesis rate was reduced in the ventral hippocampus of AraC group ($t = 4.55$, $p = 0.0002$) and olfactory bulb ($t = 5.27$, $p = 0.0003$) but remained the same in the dorsal hippocampus ($t = 1.10$, $p = 0.29$) (Fig. 2d).

We also treated mice with AraC to verify if EE compensates the deleterious effect of TST on SRM through neurogenesis-dependent mechanism. The AraC group had impaired SRM ($F_{(1,20)} = 22.18$, $p = 0.0001$ for treatment X trial and $F_{(1,20)} = 34.68$, $p < 0.0001$ for treatment) (Fig 3a). There was no difference between both groups in time of immobility (Sal = 20.1 ± 3.9 s, $n = 6$; AraC = 19.5 ± 3.7, $n = 6$; $t = 0.12$, $p = 0.90$), showing AraC treatment did not promote a depressive-like phenotype in EE animals. Immunofluorescence confirmed AraC treatment decreased proliferation in the dentate gyrus of the dorsal ($t = 9.05$, $p < 0.0001$) as well as in ventral ($t = 6.11$, $p = 0.0002$) hippocampus and olfactory bulb ($t = 12.80$, $p = 0.0001$) (Fig. 3b). Neurogenesis was also smaller in the AraC group, for the dorsal hippocampus ($t = 6.46$, $p < 0.0001$), ventral hippocampus ($t = 6.12$, $p = 0.0002$), and olfactory bulb ($t = 20.79$, $p < 0.0001$) (Fig. 3c). Interestingly, we observed that neurogenesis rate was lower in AraC-treated animals, though only in the olfactory bulb ($t = 11.05$, $p < 0.0001$) (Fig. 3d).

As EE increases BDNF expression [18, 19], we tested whether the EE condition used here was sufficient to alter BDNF expression. EE increased BDNF expression in CA1 ($t = 5.13$, $p = 0.0009$), dentate gyrus ($t = 6.30$, $p = 0.0002$), and olfactory bulb ($t = 3.72$, $p = 0.005$). In hippocampal CA3, no difference between groups was noted ($t = 0.45$, $p = 0.66$) (Fig. 4a). As AraC inhibited neurogenesis induced by EE and abolished SRM's persistence, we tested whether

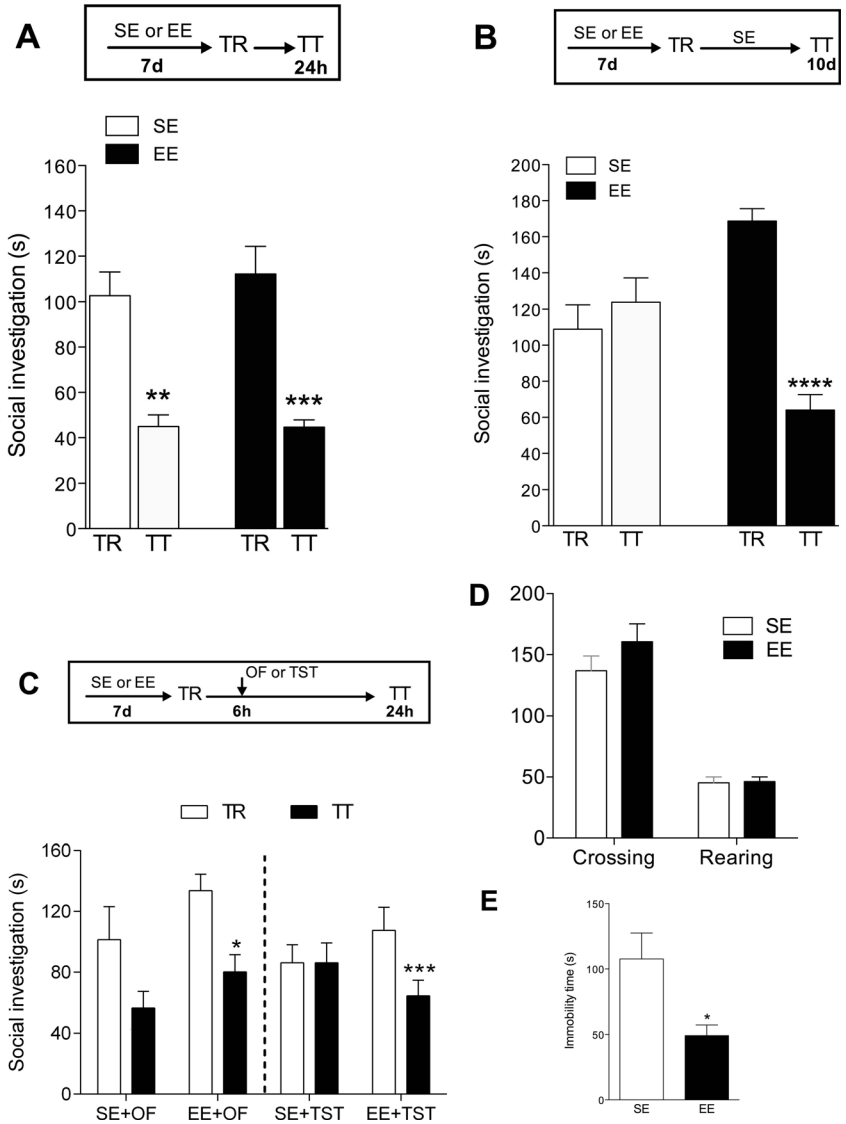

Fig. 1 Enriched environment (EE) increases social recognition (SRM) memory strength and persistence. **a** Standard environment (SE, $n = 5$) and EE animals ($n = 5$) remained seven days (7d) in their cages. On the eighth day, training (TR) was conducted and 24 h later, testing (TT) in social recognition task was performed. **b** Time of juvenile social investigation in training and testing. ** and *** indicate a significant difference between training and testing within groups ($p < 0.05$). **c** SE ($n = 8$) and EE ($n = 8$) animals remained 7 days in their cages. On the eighth day, training in social recognition task was performed and mice returned to a standard cage for 10 days. Then, mice underwent testing in social recognition task. **d** Time of juvenile social investigation in training and testing. **** indicate significant difference between training and testing within groups ($p < 0.05$). **e** SE and EE animals remained 7 days in their cages. On the eighth day, training in social recognition task was performed, and 6 h after, mice were submitted to 8 min in the tail suspension test (TST) or to 5 min in the open field (OF). After, animals returned to the same environment condition they were before. Twenty-four hours after training, mice were tested for long-term social memory ($n = 5$-6/group). **f** SE animals exposed to both stressors showed impaired 24 h SRM, while EE animals were able to recognize the juvenile even after exposure to the stressing conditions. * and *** indicate difference between training and testing within groups ($p < 0.05$). **g** No difference between groups could be seen in the open-field test for rearing and crossing parameters. **h** EE mice spent less time in immobility during TST. * indicates difference between groups ($p < 0.05$). All data are expressed as mean ± SEM

AraC effect would be extensive to BDNF expression. No change in BDNF expression was observed (CA1: $t = 0.48$, $p = 0.63$; dentate gyrus: $t = 1.85$, $p = 0.09$; CA3: $t = 0.11$, $p = 0.91$; and olfactory bulb: $t = 0.12$, $p = 0.90$) (Fig. 4b). Taken together, these results suggest the beneficial effect of EE on SRM persistence, and strength relies on neurogenesis, but probably not on BDNF expression.

Discussion

Our study provides the first evidence that neurogenesis promotes a gain-of-function on social recognition memory (SRM), since we showed that after increasing neurogenesis, SRM lasted longer and was resistant to interferences.

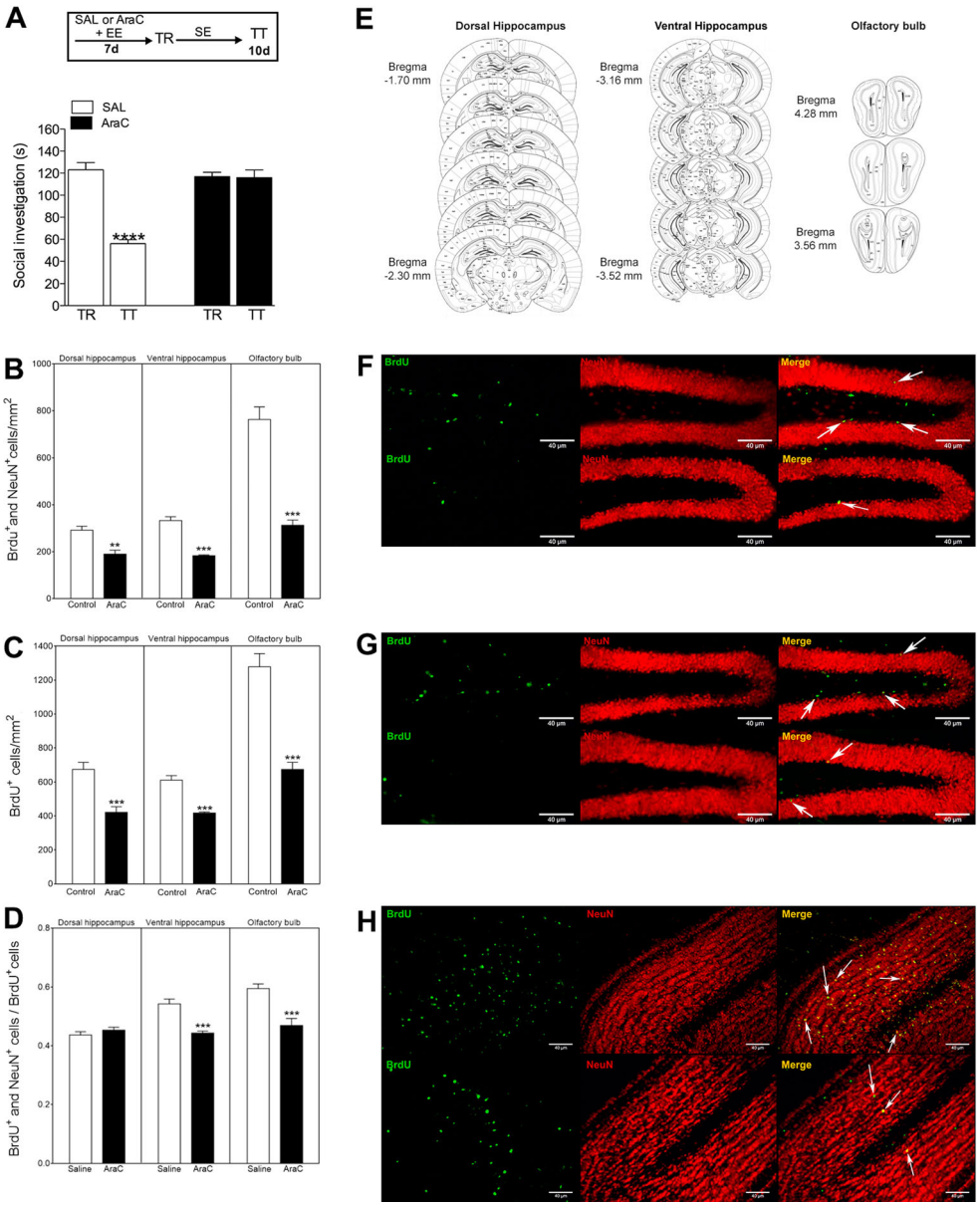

Fig. 2 Enriched environment effect on social memory persistence depends on neurogenesis. **a** Animals went to surgery to received the mini-pump implant containing saline (SAL, $n = 8$) or the anti-mitotic drug AraC ($n = 8$) and after, remained 7 days (7d) in enriched environment (EE). On the eighth day, training (TR) in social recognition task was performed and mice returned to a standard cage for 10 days. Then, mice underwent testing (TT) in social recognition task. Time of juvenile social investigation in training and testing are depicted. **** indicate significant difference between training and testing within groups ($p < 0.05$). AraC treatment decreased **b** neurogenesis, **c** cell proliferation, and **d** neurogenesis rate. ** and ***$p < 0.0001$ indicates difference between SAL and AraC. **e** Images from Paxinos (2011) showing the analyzed brain areas. Immunofluorescent representative images from the **f** dorsal hippocampus, **g** ventral hippocampus, and **h** olfactory bulb. *Arrows* indicate doubled-labeling newborn neurons. All data are expressed as mean ± SEM

Social memory persistence depends on protein synthesis at 6 h post-training [9, 11] and here, we demonstrated SRM is particularly sensitive to interferences, such as tail suspension test and open field, performed 6 h after training. These findings corroborate one recent study that showed loud tone caused retroactive interference at 6 h, but not 3 and 22 h, after sampling [20]. Remarkably, our study showed that EE, through increasing neurogenesis, was sufficient to protect SRM against interference. This result suggests newborn neurons may be playing a role in shielding social memory from stressors within a narrow critical time-point during its consolidation.

In the present study, the behavioral effects of EE or the combination of EE + AraC were observed between 8 and 17 days after the first BrDU injection, which seems too short time to explain the behavioral consequences as a product of adult neurogenesis. However, several studies have shown that immature newborn neurons can be behaviorally useful. In a very interesting study, authors found that the exposure to male pheromones during 7 days increased neurogenesis in the female's brain. Furthermore, they inhibited neurogenesis with AraC during the 7 days of odor exposure, as we did in our study, and found that neurogenesis inhibition decreased female preference for male dominant odor. The effect of AraC was not related to side effects on olfaction or locomotor activity [21]. Recently, it was demonstrated that reducing the immature newborn neurons population lead to a delayed habituation to a novel environment, impaired contextual learning, and enhanced hyponeophagia in a novelty-suppressed feeding test [22]. All together, these results suggest that newborn neurons do not need to be mature to be behaviorally relevant.

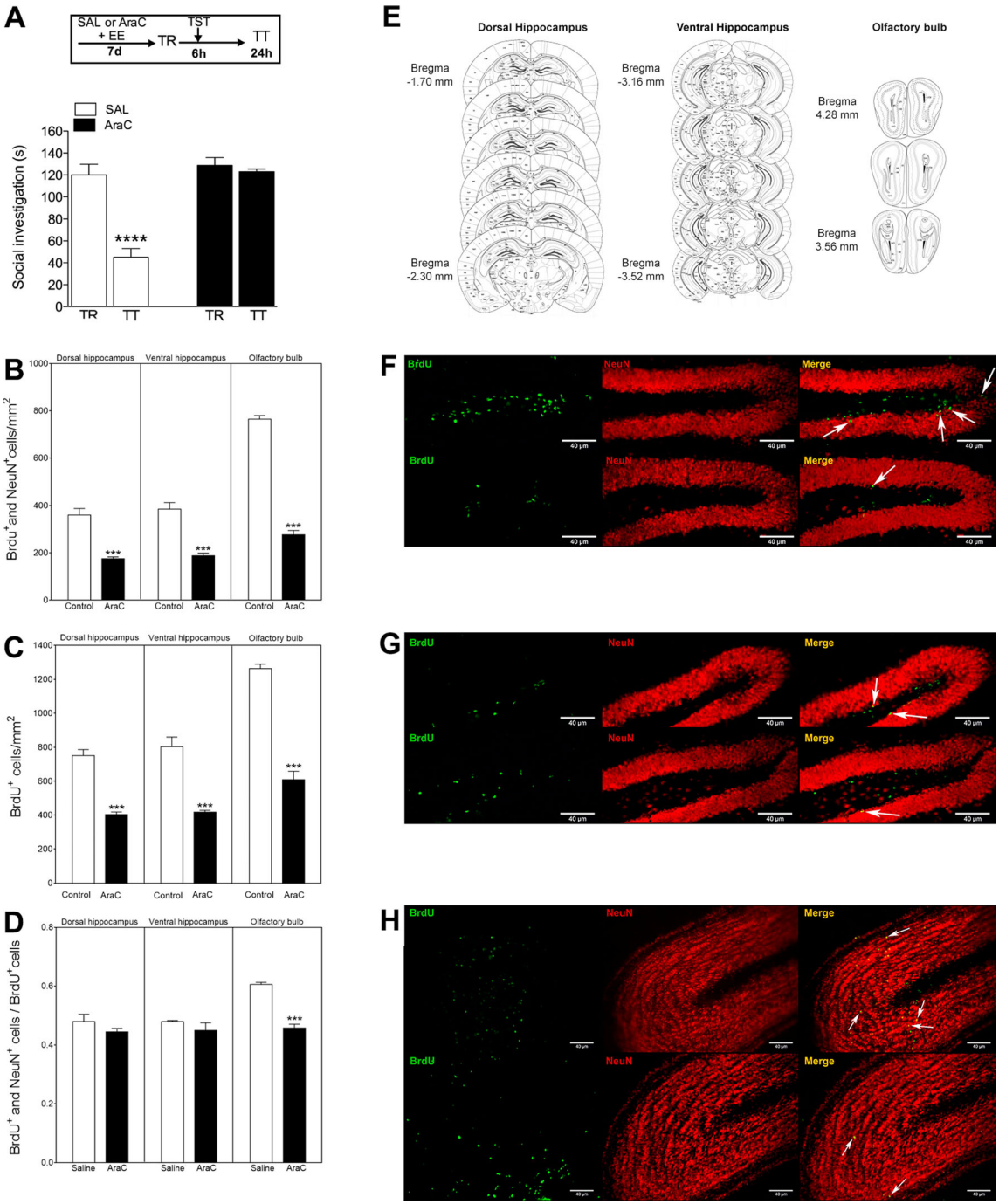

Fig. 3 Enriched environment-effect on social memory strength depends on neurogenesis. **a** Animals went to surgery to received the mini-pump implant containing saline (SAL, $n = 6$) or the anti-mitotic drug AraC ($n = 6$) and after remained 7 days (7d) in enriched environment (EE). On the eighth day, training (TR) in social recognition task was performed, and 6 h after, all animals were submitted to 8 min in the tail suspension test (TST) Twenty-four hours later, mice underwent testing (TT) in social recognition task. Time of juvenile social investigation in training and testing are depicted. **** indicate significant difference between training and testing within groups ($p < 0.05$). AraC treatment decreased **b** neurogenesis, **c** cell proliferation, and **d** neurogenesis rate. ***$p < 0.0001$ indicates difference between SAL and AraC. **e** Images from Paxinos (2011) showing the analyzed brain areas. Immunofluorescent representative images from the **f** dorsal hippocampus, **g** ventral hippocampus, and **h** olfactory bulb. *Arrows* indicate doubled-labeling newborn neurons. All data are expressed as mean ± SEM

Although DGCs were not mature enough to engage a neural circuit [23] at the moment of social memory encoding, both learning [24] and EE [25] promote DGC survival. During memory retrieval, then, those survivor cells could be playing a role on accessing social memory. In fact, as early as 2 weeks after birth, surviving DGCs extend dendrites and receive

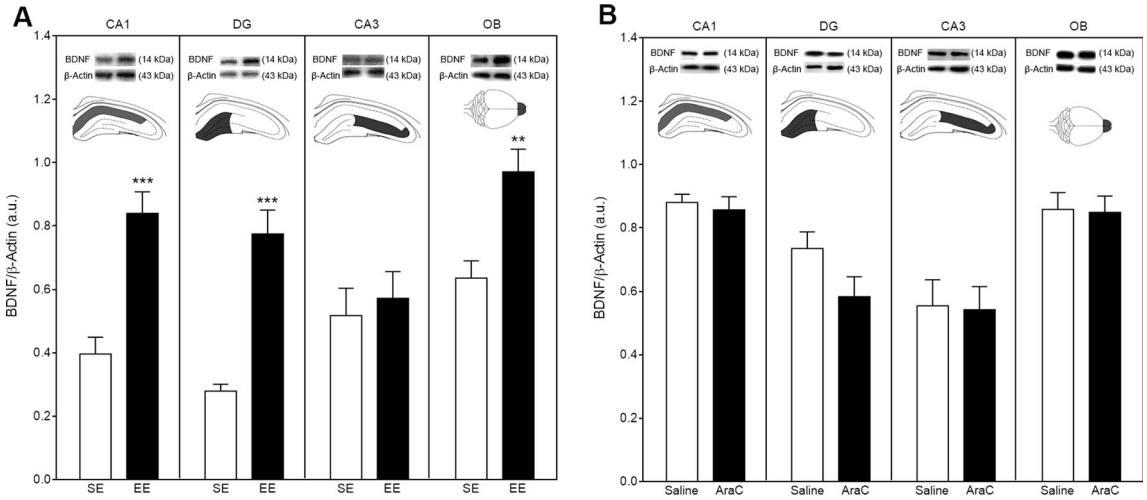

Fig. 4 Enriched environment effect on increasing BDNF expression is not affected by AraC. **a** Animals were maintained in standard environment (SE) or enriched environment (EE) for 7 days. After, animals have their olfactory bulb (OB) and hippocampi dissected in CA1, DG, and CA3 regions. $^{**}p = 0.002$ and $^{***}p < 0.0001$ indicate difference between SE and EE in the same brain area. **b** Animals went to surgery to receive the mini-pump implant containing saline (SAL) or the anti-mitotic drug AraC and were maintained 7 days in enriched environment. After, animals have their olfactory bulb (OB) and hippocampi dissected in CA1, DG, and CA3 regions. All data are expressed as mean ± SEM ($n = 5$–6 per group)

functional input from existing neural circuits [26, 27]. Moreover, 4-week-old newborn neurons seem to be fundamental to retrieval of contextual fear and spatial memories [28]. Therefore, the beneficial effect of neurogenesis on social memory persistence could be related to its effect on memory retrieval.

Regardless of evidences EE needs at least 3 weeks to increase bdnf mRNA expression [29], our results are in accordance with others that have shown how sensitive BDNF protein expression can be to EE [19]. Thus, we asked whether newborn cells would be the source of BDNF. If so, AraC treatment should decrease BDNF expression. However, we observed no such result. BDNF is secreted in an activity-dependent manner [30], then, AraC treatment effect might be seen only after learning. Furthermore, AraC did not ablated proliferation and neurogenesis completely. Thus, it could be that the level of inhibition caused by AraC was not sufficient to affect EE-induced BDNF expression. Finally, we may also speculate the BDNF source is the resident mature neuronal population. If AraC is not affecting those cells, it should not affect their BDNF expression either. Supporting this idea is the result showing AraC treatment for longer periods (25–28 days) did not change the resident granule cell density in the olfactory bulb [31]. However, further studies are necessary in order to better explore this hypothesis.

Our results contradict the assumption that BDNF expression is related to memory persistence. Indeed, BDNF controls a late memory consolidation phase in the hippocampus, which is essential to memory persistence [32]. However, this study used a different memory task. Thus, BDNF expression may not be related to social memory persistence, at least not in a condition where its expression is increased by EE. Perhaps, in a different scenario where BDNF expression is not boosted by EE, inhibiting its learning-dependent signaling may compromise social recognition memory persistence. Nevertheless, our results reassure the idea that not all neurogenesis effects on memory are BDNF-dependent.

Finally, our study showed for the first time that EE increases social memory persistence in a neurogenesis-dependent manner. Furthermore, we uncovered a previously unexpected dissociation between the effect of enriched environment on increased adult hippocampal neurogenesis and BDNF expression.

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Compliance with Ethical Standards The Animal Use Ethic Committee of the Universidade Federal de Minas Gerais (CEUA 44/2013) approved all the experimental procedures.

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