Maturation of newborn neurons predicts social memory persistence in mice

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HIGHLIGHTS

- Social recognition memory is prolonged by enriched environment and memantine.
- Inhibition of actin polymerization blunted the promnesic effect of EE.
- The mechanisms used by EE to improve SRM are beyond to increase the number of newborn neurons.
- Maturation of newborn neurons predicts social memory persistence in mice.

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ABSTRACT

Memory transience is essential to gain cognitive flexibility. Recently, hippocampal neurogenesis is emerging as one of the mechanisms involved in the balance between persistence and forgetting. Social recognition memory (SRM) has its duration prolonged by neurogenesis. However, it is still to be determined whether boosting neurogenesis in distinct phases of SRM may favor forgetting over persistence. In the present study, we used enriched environment (EE) and memantine (MEM) to increase neurogenesis. SRM was ubiquitously prolonged by both, while EE after the memory acquisition did not favor forgetting. Interestingly, the proportion of newborn neurons with mature morphology in the dorsal hippocampus was higher in animals where persistence prevailed. Finally, one of the main factors for dendritic growth is the formation of cytoskeleton. We found that Latrunculin A, an inhibitor of actin polymerization, blunted the promnesic effect of EE. Altogether, our results indicate that the mechanisms triggered by EE to improve SRM are not limited to increasing the number of newborn neurons.

1. Introduction

From birth to full maturity, adult-born neurons interfere in hippocampal excitability and plasticity (Alvarez et al., 2016; Ikrar et al., 2013; Toni and Schinder, 2016). For instance, as early as two weeks after birth, newborn neurons extend dendrites and receive functional input from pre-existing neural circuits (Ge et al., 2006; Schmidt-Hieber et al., 2004; van Praag et al., 2002).

The maturation and integration of new neurons are linked to the greater capacity of these cells to stablish synaptic connections (Ambrogini et al., 2004; Cope and Gould, 2019; Ehninger and Kempermann, 2008; van Praag et al., 2002). Morphological aspects of dendritic branches are key structural elements connecting neurons (Major et al., 2013; Papoutsi et al., 2014; Sjöström et al., 2008) and integrating multiple synaptic elements while forwarding signals to the soma (Kastellakis et al., 2015; Papoutsi et al., 2014; Polsky et al., 2004).

Dendritic branching is dynamically changed during neural development (for review see Lefebvre et al., 2015). However, as the brain develops, dendritic arborizations become more stable which is crucial for long-term stability of circuits, allowing mature neurons the ability to fine-tune synaptic connections (Fujino et al., 2011; McAllister, 2007; Meyer, 2006; Ruthazer, 2006). Interestingly, enriched environment (EE), an experimental strategy used to increase neurogenesis, enhances dendritic arborization (Bechard et al., 2016; Bindu et al., 2007; Leger et al., 2014; Leggio et al., 2005). Similarly, memantine (MEM), an antagonist of N-methyl-ᴅ-aspartate receptors (NMDA) also known to increase neurogenesis (Jin et al., 2006; Namba et al., 2009; Akers et al., 2014; Ishikawa et al., 2016; Cahill et al., 2018), acts on stabilizing (Vlachos et al., 2013) and maturating (Wei et al., 2012) dendritic spines.

In adult rodents, the production of new neurons in the dentate gyrus of the hippocampus (DG) is a continuous physiological process (Toda et al., 2018) that has been suggested to play a role in learning and memory (Aimone et al., 2011; Arruda-carvalho et al., 2014; Bruel-

Jungerman et al., 2007; Deng et al., 2010; Drew et al., 2013; Frankland et al., 2013; Goodman et al., 2010; Josselyn and Frankland, 2012; Lazarov and Hollands, 2016; Leger et al., 2014; Marín-Burgin and Schinder, 2012; Yau et al., 2015). Typically, increasing neurogenesis improves memory (Aimone et al., 2011; Deng et al., 2010; Drew et al., 2013; Frankland et al., 2013; Goodman et al., 2010; Josselyn and Frankland, 2012; Wang et al., 2018) while decreasing it has the opposite effect (Akers et al., 2010; Arruda-carvalho et al., 2014; Ko et al., 2009; Pereira-caixeta et al., 2018; Saxe et al., 2006). It was not until recently that a new perspective about the role of neurogenesis on learning and memory emerged (Akers et al., 2014; Frankland et al., 2013; Ishikawa et al., 2016). In this new model, boosting neurogenesis after a memory trace has been formed leads to forgetting (Akers et al., 2014b; Ishikawa et al., 2016), suggesting a phase-dependent effect of neurogenesis on memory.

In addition to fear and spatial memories, social recognition memory (SRM) is also sensitive to the neurogenesis levels (Ishikawa et al., 2014; Opendak et al., 2016; Pereira-caixeta et al., 2018). SRM is a hippocampus-dependent memory (Chiang et al., 2018; Dias et al., 2016; Kogan et al., 2000; Meira et al., 2018; Okuyama et al., 2016; Pena et al., 2014; Tanimizu et al., 2017). SRM is crucial to territorial defense and establishment of hierarchies in natural contexts (Arakawa et al., 2008; Bickle, 2008; Chiang et al., 2018; Ferguson et al., 2002; Okuyama, 2018; Richter et al., 2005). Experimentally, SRM can be accessed by the resident-intruder paradigm (Thor and Holloway, 1982). The decrease in the number of social investigation behaviors between the first and second exposures to a juvenile mouse provides the metrics to measure SRM. The long-term persistence of SRM is highly dependent on the trial's duration (Tanimizu et al., 2017).

We showed previously that neurogenesis increases the persistence of SRM (Pereira-Caixeta et al., 2017). In the present study, we tested whether increasing neurogenesis in distinct phases of SRM formation may favor forgetting over persistence. Additionally, we tested whether dendritic branching and maturation of newborn neurons would play a role on SRM persistence.

2. Materials and methods

2.1. Mice

Two strains of adult (8–12-week old) and juvenile (21–33-day old) mice were used. Swiss mice were used in the experiments with enriched environment (EE), to reproduce previous results from our laboratory showing the effect of EE on prolonging social memory trough a neurogenesis-dependent mechanism (Pereira-Caixeta et al., 2017). To increase neurogenesis with memantine (MEM; Sigma-Aldrich, USA), C57/BL6 mice were used, because it is a strain sensitive to MEM (Akers et al., 2014). Animals had free access to food and water. Behavioral testing was performed during the light phase of the 12h light/dark cycle.

All experiments were performed in compliance with the guidelines from the National Council for Animal Experimentation Control (CONCEA-BRAZIL). All protocols were approved by the Institutional Ethics Committee on the Use of Animals at the Universidade Federal de Minas Gerais (CEUA/UFMG) (nº 365/2015 and 261/2017). The investigators were blind to the group allocation during data collection and analysis.

2.2. Neurogenesis level manipulation

We used two strategies to increase neurogenesis: EE and MEM. EE was provided by maintaining animals in large cages $(40 \times 33 \times 16 \text{ cm}^3)$, in groups of five, with multisensorial stimulation with plastic objects, tunnels and burrows (Pereira-Caixeta et al., 2017). Control animals were maintained in standard environment (SE) $(28 \times 17 \times 12 \text{ cm}^3)$. MEM was dissolved in 0.9% saline and administered once, intraperitoneally (i.p), at a dose of 25 mg/kg body

weight (Akers et al., 2014). Control group received the same volume of 0.9% saline (SAL).

2.3. Social recognition test

To assess social memory we used a modified version of social recognition test (Thor and Holloway, 1982) described previously by our group (Gusmão et al., 2012; Monteiro et al., 2014). In the habituation phase, that lasted 20min, the adult mouse was introduced inside a clean standard cage containing an empty cylinder, with 60 evenly spaced holes on its walls. The training session (TR) was initiated by introducing the juvenile into the cylinder. Social investigation was measured with a stopwatch during 5 min. Test phase (TT) occurred 24 h or 10 days later, and was similar to the TR. We considered as social investigation every time the adult mouse introduced its whiskers and/or nose inside the cylinder's holes. The cylinder was always positioned at the same location inside the cage and was cleaned with 70% alcohol between the trials and animals (Pereira-Caixeta et al., 2017). Results were expressed as social recognition index [time exploring the juvenile during TT/time exploring the juvenile during TR + TT].

2.4. Immunohistochemistry

Some of the mice from the behavioral analysis were used to quantified neurogenesis, therefore the sample size of immunohistochemistry was smaller. Animals were anaesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) injected i.p. and were perfused transcardially with 20 mL of 0.01M phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Next, brains were removed and fixed in 4% PFA for 24h until dehydration and stored in 30% sucrose for cryoprotection. Tissue sections of 40um were then processed for free-floating immunohistochemistry. Slices from coordinates (1) Bregma −1.34 to Bregma −2.30 and (2) Bregma −2.92 to Bregma −3.64 were considered as dorsal and ventral hippocampus, respectively.

Sections were incubated for 48h in rabbit anti-doublecortin (DCX) primary antibody (Santa Cruz, sc8067, RRDIDAB2088491) diluted 1:200 in 0.3% Triton-X PBS (PBST) and 3% normal goat serum, at 4 °C. A different group of sections were incubated for 20h in rabbit anti-Ki-67 primary antibody (Millipore Cat# AB9260, RRID:AB_2142366) diluted 1:500 in 0.1% PBST and 5% normal goat serum, at 4 °C. DCX and Ki-67 labeled sections were incubated for 1h in anti-rabbit secondary antibody (Vector Laboratories, Cat# PK-6105, RRID:AB_2336824) diluted 1:500 and 1:200 in PBST, respectively. After incubation with ABC (Vector Laboratories), sections were incubated in a solution containing 0.02 mg/mL 3,3' –diaminobenzidine (DAB), 95 mM NiSO4.6H2O and 0.03%H_2O_2 in acetate buffer, for 10min. Sections were mounted on gelatin-coated microscope slides, and coverslipped with Vectashield (Vector Laboratories).

2.5. Image acquisition and analysis

Images were captured by a digital camera (Axio Cam MRm, Zeiss) coupled to an Axio Imager.Z2 microscope, at bright-field function. DCX and Ki-67 positive cells were quantified using 20X objective. The granular layer of the dentate gyrus (DG) was delimited manually and cells were automatically counted, bilaterally, using a non-parametric thresholding, establish after removing the noise (excluding label of less 10um).

We used 3–4 slices for each structure. Dendritic arborization was analyzed using NeuroJ, a semiautomatic plugin that facilitate the tracing and the quantification of the morphology of neurons in two-dimensional (2D) images (Meijering, 2010; Meijering et al., 2004). Following the developers' instructions, the dendrites were traced and then classified as primary, secondary or tertiary in relation to their position from the soma cell body (Popko et al., 2009). We used a 150% zoom to improve the visualization and to ensure that the traced dendrites were

from the same cell body.

Dendrites were defined and identified as following: (1) primary dendrite between soma and first branching point, (2) secondary, branching from primary dendrite and (3) tertiary emanating from secondary dendrites (Popko et al., 2009).

To determine the degree of maturation, DCX^+ cells were allocated in one of the three types, according to their morphology: proliferative, intermediate and post-mitotic (Plümpe et al., 2006).

The number of DCX^+ cells was normalized by the selected area (mm^2). The number and the length of primary, secondary and tertiary dendrites were normalized by the total number of DCX^+ cells.

2.6. Surgery and drug administration

Mice were anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and, subsequently, underwent stereotaxic surgery for cannulation. Bilateral cannulas were placed into the dHIP (antero-posterior, +1.9 mm; medio-lateral, ± 1.6 mm; dorso-ventral, -1 mm) (Paxinos and Franklin, 2001). Mice recovered from surgery for 3 days and then moved to EE. During five days, mice were microinjected with Latrunculin A (100 ng in 0.25 μL/side) or vehicle (4% DMSO in saline) at 9am (Fisher, 2004).

2.7. Statistical analyses

Data were analyzed using Prism 5.0b (GraphPad Software). Social recognition indexes were compared to 0.5 by one-sample *t*-test and unpaired *t*-test (MEM) or One-way ANOVA followed by Tukey's multiple comparison test (EE). For dendrites analysis we used a two-way repeated measures ANOVA followed by Tukey's multiple comparison test. Pearson's correlation was used to analyze the relationship between the percentage of proliferative, post-mitotic cells or neurogenesis and social memory performance. To evaluate the effect of latrunculin A, two-way repeated measures ANOVA followed by Tukey's multiple comparison test was used.

3. Results

3.1. MEM prolongs SRM duration

To reinforce that SRM persistence is sensitive to neurogenesis (Pereira-Caixeta et al., 2017), we used C57/BL6 mice, which is a strain sensitive to the effect of MEM on increasing neurogenesis (Akers et al., 2014) (Fig. 1A).

Our results showed that animals treated with MEM ($t_{(7)}$ = 6.2,

p = 0.0004), but not the control (SAL: $t_{(7)}$ = 1.7, p = 0.12) present a social recognition index different from the chance, indicating SRM. Furthermore, there was difference between SAL and MEM ($t_{(14)}$ = 3.3, p = 0.004) (Fig. 1B). Additionally, we confirmed that MEM increases neurogenesis in dorsal ($t_{(9)}$ = 2.8, p = 0.01) (Fig. 1C) and ventral hippocampus ($t_{(9)}$ = 2.4, p = 0.03) (Fig. 1D).

3.2. SRM persistence is favored by the presence of EE during memory consolidation

Next, we asked whether boosting neurogenesis after SRM acquisition might favor forgetting over persistence.

Animals were exposed to EE before and/or after training session (Fig. 2A). We reproduced our previous results (Pereira-Caixeta et al., 2017) by showing that animals maintained in standard environment (SE) did not remember the juvenile ($t_{(8)}$ = 0.8, p = 0.0009), while SRM persisted after EE pre-training ($t_{(8)}$ = 5.1, p = 0.39). Interestingly, the EE post-training did not affect its pre-training promnesic effect ($t_{(8)}$ = 7.2, p < 0.0001). EE also prolonged the SRM when applied only after training ($t_{(7)}$ = 3.1, p = 0.01). Further analysis by One-way ANOVA showed difference between SE + SE and EE + SE ($F_{(3,31)}$ = 3.5, p = 0.02) (Fig. 2B).

Next, we designed a protocol where the post-training EE exposure was performed only 3 days after learning. We chose this time to ensure that memory was already consolidated by the time neurogenesis had been increased (Fig. 2C). We reproduced once again the results with SE + SE ($t_{(9)}$ = 1.4, p = 0.1) and EE + SE ($t_{(10)}$ = 3.9, p = 0.002). The combination of EE pre and post-training favored persistence ($t_{(10)}$ = 2.9, p = 0.01). And finally, we found that EE 3 days post-training was unable to allow persistence. One-way ANOVA detected difference between SE + SE and the following groups: EE + SE and EE + EE ($F_{(3,40)}$ = 5.1, p = 0.004) (Fig. 2D).

3.3. The combination of pre- and post-training EE does not increase neurogenesis or cell proliferation

At the behavioral level, our results showed two conditions where SRM did not persist. For instance, in SE + SE there was a natural forgetting of SRM, while in SE + EE (3 days) the EE had no effect on memory persistence. Unexpectedly, SRM persisted in EE + EE group. Thus, we predicted that hippocampal neurogenesis would be increased specifically in the groups were memory persisted.

In the dentate gyrus of the dorsal hippocampus (dHIP) (Fig. 3A), the number of DCX^+ cells was higher only in the EE + SE group ($F_{(3,18)}$ = 7.6, p = 0.001) (Fig. 3B), while no difference between groups
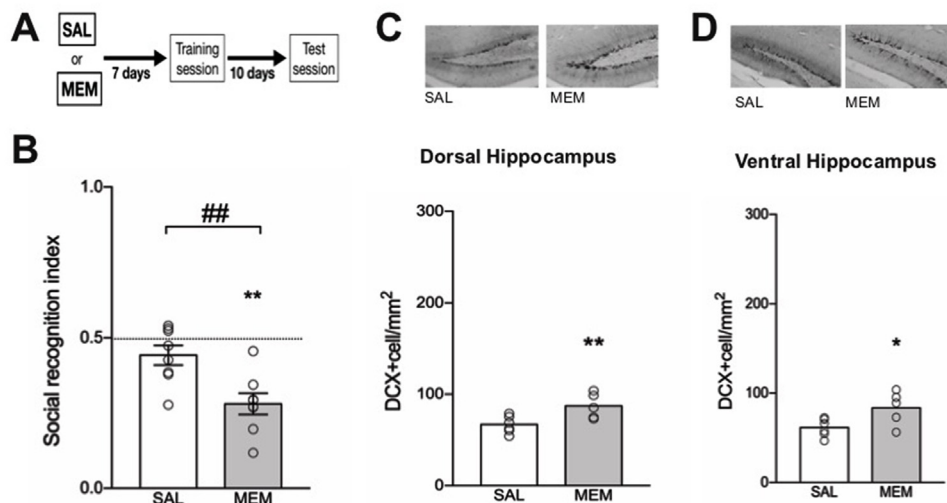

Fig. 1. Memantine (MEM) enhances social recognition memory (SRM) and increased neurogenesis in the hippocampus. **(A)** Animals received saline (SAL, n = 8) or MEM (n = 8) once and after 7 days were submitted to training session and 10 days later to the test session. **(B)** SRM persisted only in the MEM group. **p < 0.01, compared to the hypothetical value 0.5 and ##p < 0.01 indicates difference between groups. MEM increased DCX^+ cells in the **(C)** dorsal and **(D)** ventral hippocampus (SAL, n = 6; MEM, n = 5).
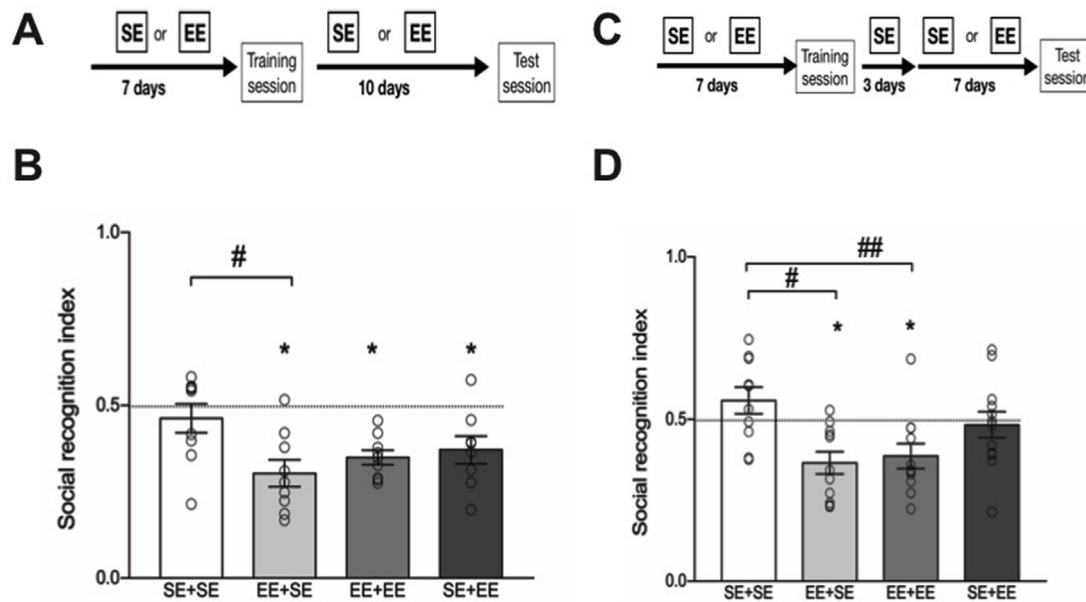
Fig. 2. Social recognition memory (SRM) is enhanced by the exposition to enriched environment (EE) before training. (A) Animals were exposed to EE or maintained in standard environment (SE) for 7 days and then were submitted to training session. Immediately after, they were kept in the same condition (SE + SE, n = 9; EE + EE, n = 9) or were moved to a different environment (EE + SE, n = 9; SE + EE, n = 8) and 10 days later, the test session was performed. **(B)** EE before and/or after training enhanced SRM. *p < 0.05, compared to the hypothetical value 0.5. #p < 0.05 indicates difference between groups. **(C)** Animals were exposed to EE or maintained in standard environment (SE) for 7 days and then were submitted to training session. Three days after, they were kept at the same condition (SE + SE, n = 10; EE + EE, n = 11) or were moved to a different environment (EE + SE, n = 11; SE + EE, n = 12) and 10 days later, the test session was performed. **(D)** EE after training did not enhance SRM. *p < 0.05, compared to the hypothetical value 0.5. #p < 0.05 and ##p < 0.01 indicates difference between groups.

was observed in ventral hippocampus (vHIP) ($F_{(3,18)}$ = 1.0, p = 0.3) (Fig. 3C and D).

Next, we evaluated cell proliferation. Then again, the number of Ki-67^{+} cells did not follow the behavior results. Cell proliferation was statistically increased only in the dHIP of EE + SE mice ($F_{(3,6)}$ = 5.1, p = 0.01) (Fig. 3E and F), similar to what occurred in the vHIP ($F_{(3,16)}$ = 4.6, p = 0.02) (Fig. 3G and H). Altogether, our results do not show a direct association between SRM and the number of newborn neurons or cell proliferation.

3.4. Newborn neurons have broader dendritic arborization in EE + SE group

In addition to increasing the number of newborn neurons, EE stimulates dendritic arborization (Bindu et al., 2007; Leggio et al., 2005). DCX is a microtubule protein and it is considered a suitable marker to examine the dendritic growth of newborn neurons (Rao and Shetty, 2004). Thus, we decided to quantify the number and the length of dendrites in DCX^{+} cells.
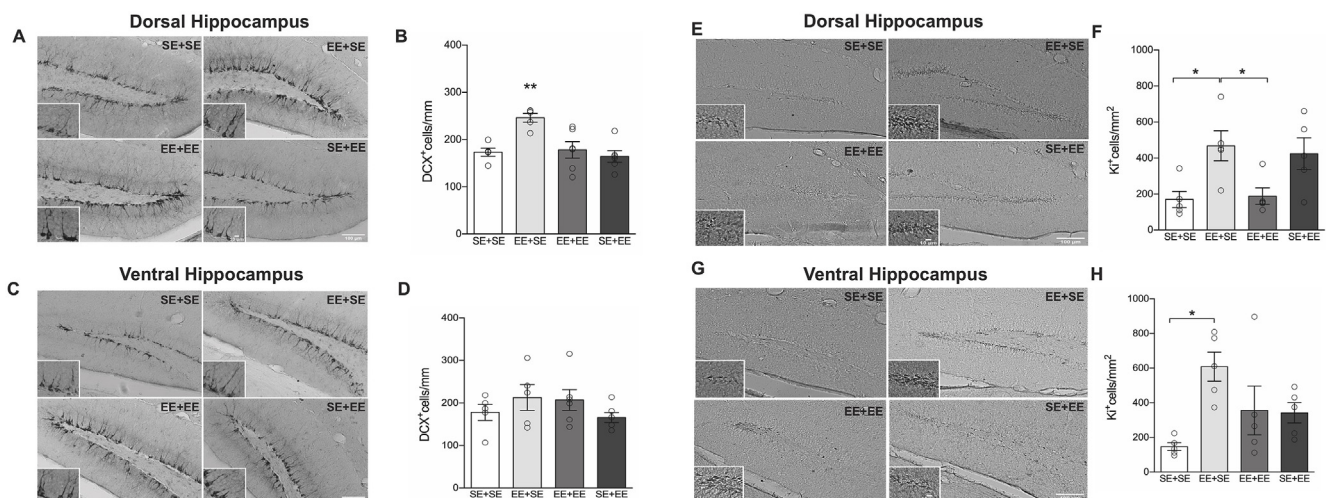

Fig. 3. Pre-training enriched environment (EE) increases neurogenesis and cell proliferation. Some of the animals from Fig. 1 were used to analyze neurogenesis and cell proliferation. Doublecortin positive cells (DCX^{+} cells) were quantified in **(A)** the dorsal (dHIP) and **(C)** ventral hippocampus (vHIP) of SE + SE (n = 5), EE + SE (n = 5), EE + EE (n = 6) and SE + EE (n = 6). **(B)** Neurogenesis increased in the dHIP of EE + SE group. **p < 0.01, compared to all other groups. **(D)** There was no difference between groups in the vHIP. Cell proliferation was measured by quantification of Ki-67^{+} cells in the **(E)** dHIP and **(G)** vHIP of SE + SE (n = 5), EE + SE (n = 5), EE + EE (n = 5) and SE + EE (n = 5). Cell proliferation was higher in the **(F)** dHIP and **(H)** vHIP of EE + SE group. *p < 0.05 indicates difference between groups. Data are expressed as mean ± SEM.

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Dorsal Hippocampus
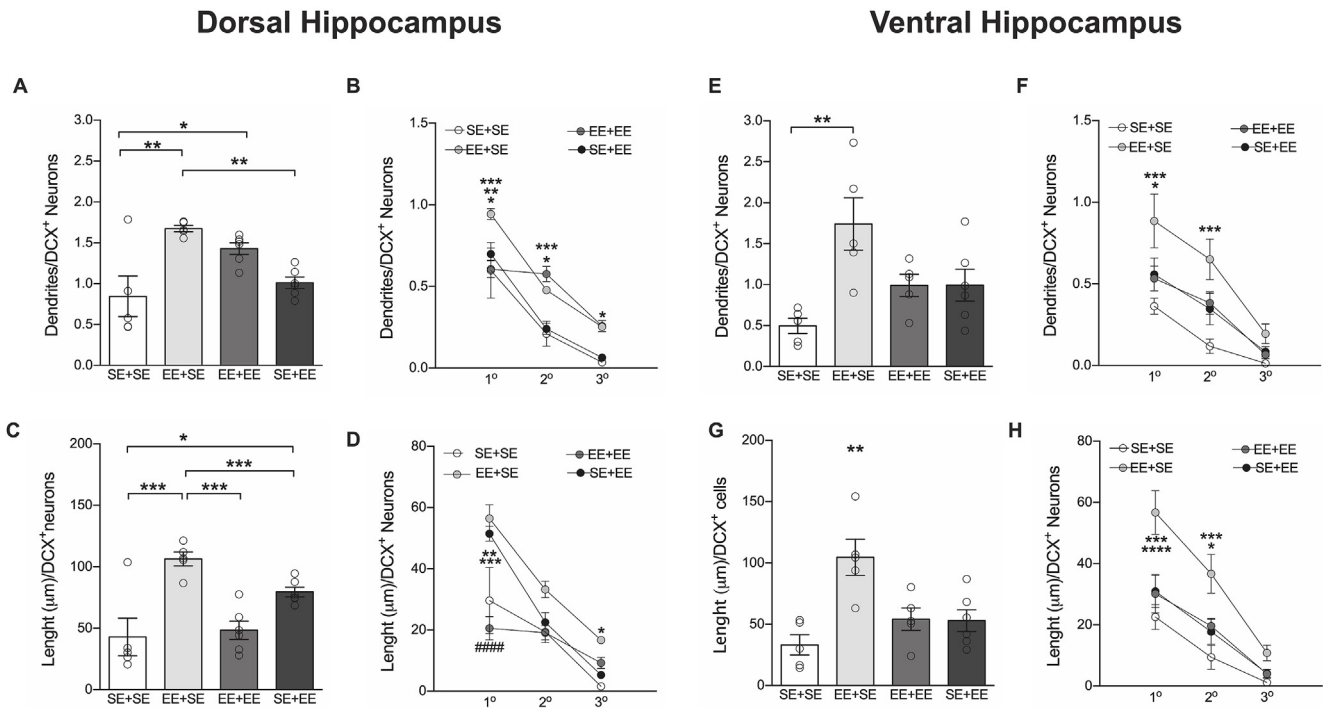
Ventral Hippocampus

Fig. 4. Memory phase-dependent effect of enriched environment (EE) on dendritic arborization of newborn neurons. DCX^+ cells from Fig. 3 were used to analyze dendritic arborization. **(A)** Number of total dendrites in dorsal hippocampus (*$p < 0.05$ and **$p < 0.01$ indicate difference between groups). **(B)** Number of primary (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate difference from EE + SE), secondary (*$p < 0.05$ and ***$p < 0.001$ indicate difference from EE + SE and EE + EE) and tertiary (*$p < 005$ indicates difference between SE + SE and EE + EE) dendrites. **(C)** Length of dendrites in dorsal hippocampus (*$p < 0.05$ and ***$p < 0.001$ indicate difference between groups). **(D)** Length of primary (**$p < 0.01$ and ***$p < 0.001$ indicate difference from EE + SE and SE + EE; ####$p < 0.0001$ indicates difference from EE + SE and SE + EE), secondary and tertiary (*$p < 0.05$ indicates difference between SE + SE and EE + SE) dendrites in dorsal hippocampus. **(E)** Number of total dendrites in ventral hippocampus (*$p < 0.05$ indicate difference between groups). **(F)** Number of primary (*$p < 0.05$ indicates difference from EE + EE and SE + EE, ***$p < 0.001$ indicates difference from SE + SE), secondary (***$p < 0.001$ indicates difference between SE + SE and EE + SE) and tertiary dendrites in ventral hippocampus. **(G)** Length of dendrites in ventral hippocampus (**$p < 0.01$ indicate difference from all the other groups). **(H)** Length of primary (***$p < 0.001$ and ***$p < 0.0001$ indicate difference from EE + EE, SE + EE and SE + SE), secondary (*$p < 0.05$ and ***$p < 0.001$ indicate difference from EE + EE, SE + EE and SE + SE) and tertiary dendrites in ventral hippocampus. Data are expressed as mean ± SEM.

In the dHIP, dendrites from DCX^+ cells were more numerous in the groups that received EE previous to training session ($F_{(3,18)} = 8.6$, $p = 0.002$) (Fig. 4A). The separate analysis of dendrites by Two-Way repeated measures ANOVA detected an interaction between factors ($F_{(6,36)} = 5.2$, $p = 0.0005$) and a main effect for both ramification ($F_{(2,36)} = 135.5$, $p < 0.0001$) and housing ($F_{(3,18)} = 8.6$, $p = 0.0009$). In general, multiple comparison analysis showed that EE + SE and EE + EE groups presented more secondary and tertiary dendrites (Fig. 4B).

Next, we measured the dendrite's length and the higher value was observed in EE + SE group ($F_{(3,18)} = 10.9$, $p = 0.0003$) (Fig. 4C). The separate analysis of dendrites showed again an interaction between factors ($F_{(6,36)} = 5.78$, $p = 0.0003$), with main effect for ramification ($F_{(2,36)} = 79$, $p < 0.0001$) and housing ($F_{(3,18)} = 12$, $p = 0.0001$). Post-hoc analysis showed longer primary dendrites in EE + SE and SE + EE. However, tertiary dendrites were longer only in EE + SE group (Fig. 4D).

The analysis of the vHIP showed more dendrites in EE + SE group ($F_{(3,17)} = 6$, $p = 0.005$) (Fig. 4E). Two-way ANOVA did not detect interaction between factors ($F_{(6,34)} = 2$, $p = 0.08$). However, a main effect for ramification ($F_{(2,34)} = 72.1$, $p < 0.0001$) and housing was detected ($F_{(3,17)} = 59$, $p = 0.005$). The multiple comparison test indicated that EE + SE present more primary and secondary dendrites (Fig. 4F).

Dendrite's length presented a similar profile. For instance, EE + SE group showed longer dendrites ($F_{(3,17)} = 8.1$, $p = 0.001$) (Fig. 4G). The analysis of the dendrites length in vHIP by two way ANOVA showed an

interaction between factors ($F_{(6,34)} = 30.6$, $p = 0.01$), and main effect for ramification ($F_{(2,34)} = 91.7$, $p < 0.0001$) and housing ($F_{(3,17)} = 8.1$, $p = 0.001$). The multiple comparison test indicated that EE + SE present longer primary and secondary dendrites (Fig. 4G).

3.5. MEM did not change the number and length of dendrites in hippocampal newborn neurons

EE and MEM increased neurogenesis and improved SRM persistence. We also found that the mechanism used by the EE to improve persistence seems to relate to dendritic arborization. Thus, we also quantified the number and the length of dendrites in DCX^+ cells after MEM treatment.

In dHIP, we found no difference between groups in the dendrites number ($t_{(9)} = 1.4$, $p = 0.1$) (Fig. 5A). Two-way repeated measures ANOVA showed no interaction ($F_{(2,18)} = 0.1$, $p = 0.08$) or main effect for treatment ($F_{(1,9)} = 3$, $p = 0.1$). As expected, a main effect for ramification was found ($F_{(2,18)} = 176.1$, $p < 0.0001$) (Fig. 5B). Regarding total dendrite's length, no effect of MEM was observed ($t_{(9)} = 0.3$, $p = 0.7$) (Fig. 5C). Two-way ANOVA showed no interaction ($F_{(2,18)} = 0.08$, $p = 0.92$) or main effect for treatment ($F_{(1,9)} = 0.5$, $p = 0.47$). As expected, a main effect for ramification was found ($F_{(2,18)} = 129.3$, $p < 0.0001$) (Fig. 5D).

In the vHIP, no difference between groups was found in the number of total dendrites ($t_{(9)} = 0.5$, $p = 0.5$) (Fig. 5E). Two-way repeated measures ANOVA showed no interaction ($F_{(2,18)} = 0.38$, $p = 0.6$) or main effect for treatment ($F_{(1,9)} = 0.2$, $p = 0.6$). As expected, a main
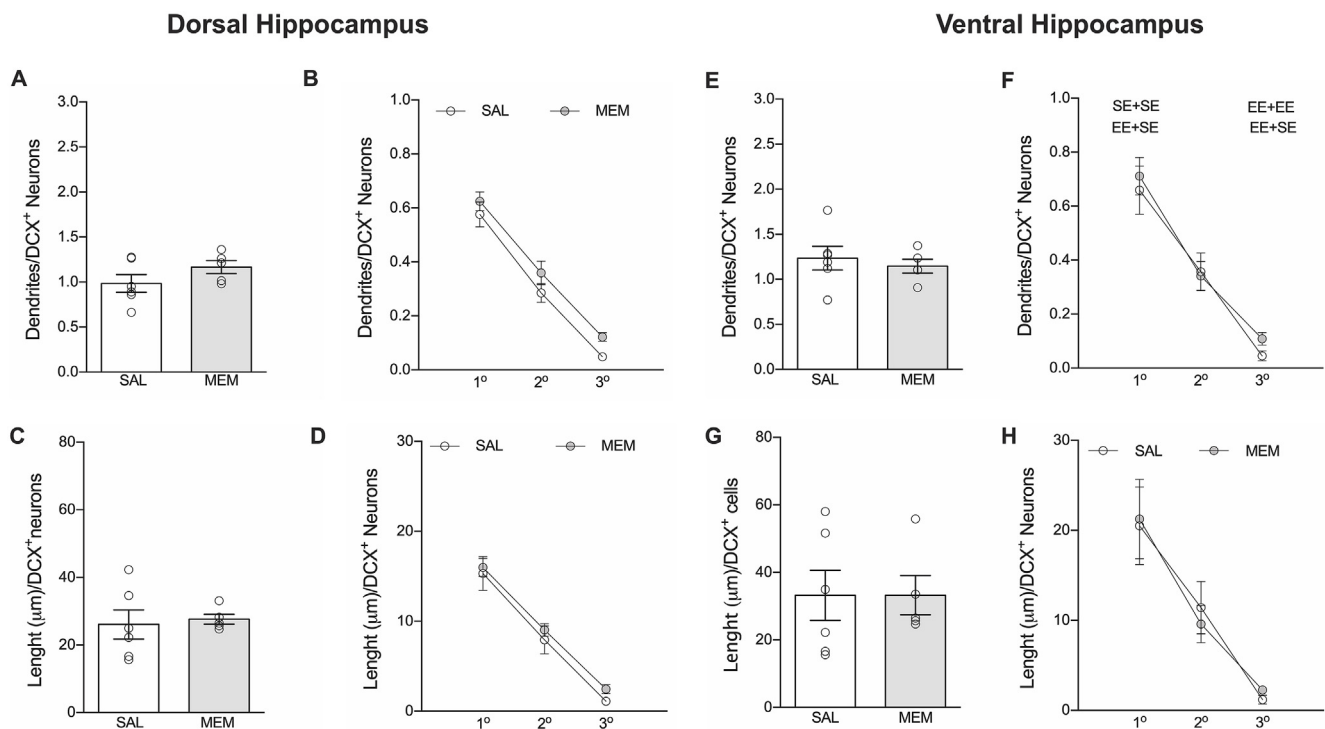
Dorsal Hippocampus   Ventral Hippocampus

Fig. 5. Effect of memantine (MEM) in the dendritic arborization of newborn neurons. There was no difference between groups in the **(A, B)** number and **(C and D)** length of dendrites from dorsal hippocampus. There was no difference between groups in the **(E, E)** number and **(G and H)** length of dendrites from ventral hippocampus. Data are expressed as mean ± SEM.

effect for ramification was found ($F_{(2,18)}$ = 77, p < 0.0001) (Fig. 5F). Similar results were observed for dendrite's length ($t_{(9)}$ = 0.0, p = 0.9) (Fig. 5G). Two-way ANOVA showed no interaction ($F_{(2,18)}$ = 0.24, p = 0.7) or main effect for treatment ($F_{(1,9)}$ = 5.6, p = 0.99). As expected, a main effect for ramification was found ($F_{(2,18)}$ = 35.9, p < 0.0001) (Fig. 5H).

3.6. Higher percentage of mature newborn neurons is concomitant with SRM persistence

It has been suggested that the number and the length of secondary and tertiary dendrites shapes the newborn neurons morphology, which in turn predicts their maturation state (Plümpe et al., 2006). Thus, we decided to analyze the morphological profile of DCX⁺ neurons. We established three distinct morphologies: proliferative, intermediate and post-mitotic (Fig. 6A).

In dHIP, there was no difference between groups on the percentage of proliferative cells ($F_{(3,18)}$ = 1.2, p = 0.32). The percentage of intermediate cells was higher in the SE + EE group ($F_{(3,18)}$ = 8.2, p = 0.001), while post-mitotic cells corresponded to approximately one third of DCX⁺ cells in EE + SE group ($F_{(3,18)}$ = 9.8, p = 0.005). Similarly, the percentage of post-mitotic cells was higher in EE + EE group (Fig. 6B). In MEM treated mice we also observed a greater proportion of post-mitotic cells compared to control ($t_{(9)}$ = 2.8, p = 0.01), with no difference between groups in the proportion of proliferative ($t_{(9)}$ = 0.7, p = 0.4) and intermediate cells ($t_{(9)}$ = 0.006, p = 0.9) (Fig. 6C).

In vHIP, there was a lower proportion of proliferative-like morphology in EE + SE and SE + EE groups ($F_{(3,16)}$ = 10.9, p = 0.0004). No difference between groups was observed in intermediate cells ($F_{(3,16)}$ = 1.4, p = 0.26), while EE + SE and SE + EE showed more DCX⁺ cells compatible with a post-mitotic morphology ($F_{(3,16)}$ = 5.3, p = 0.009) (Fig. 6D). MEM increased the proportion of intermediate cells ($t_{(9)}$ = 3, p = 0.01), with no effect on proliferative ($t_{(9)}$ = 1.7, p = 0.1) and post-mitotic cells ($t_{(9)}$ = 0.5, p = 0.6) (Fig. 6E).

3.7. Newborn neurons maturation predicts SRM persistence

To examine whether SRM persists the more mature the newborn neurons are, we performed a linear regression using data from social recognition index versus the percentage of post-mitotic cells from the dorsal hippocampus of EE and MEM groups. We observed a positive correlation between variables (Pearson r = 0.52; p < 0.001) (Fig. 7A). However, as expected, the percentage of proliferative cell showed no correlation with SRM (Person r = 0.06; p = 0.7) (Fig. 7B).

Next, we hypothesized that the maturation of newborn neurons rather than their total number is dictating the persistence of SRM for 10 days. To test that, we calculated the correlation between the number of DCX⁺ neurons and the social recognition index. As predicted, we found no correlation between those two variables (Person r = 0.01; p = 0.9) (Fig. 7C).

3.8. Inhibition of actin polymerization dampened EE effect on prolonging SRM duration

Finally, we designed a cause-effect relationship experiment to verify whether the level of newborn neurons maturation is determinant to SRM persistence. One of the main factors for the dendrite's growth is the formation of the cytoskeleton. We took advantage of Latrunculin A, which is a drug that inhibits actin polymerization (Coué et al., 1987; Jefford et al., 1996; Kashman et al., 1980) and injected it intra-hippocampus during the last 5 days of EE. Afterwards, animals were trained in the social recognition task. SRM was tested 24h and 10 days after. Two-way ANOVA showed interaction between factors ($F_{(1,14)}$ = 15.3, p = 0.001) and a main effect of time ($F_{(1,14)}$ = 37.7, p < 0.0001). No effect of treatment was found ($F_{(1,14)}$ = 3.5, p = 0.07). Post-hoc analysis showed difference between vehicle and Latrunculin A at TT2 (p < 0.01), meaning that the inhibition of actin polymerization blunted the promnesic effect of EE, without interfering in the 24h memory (Fig. 8).
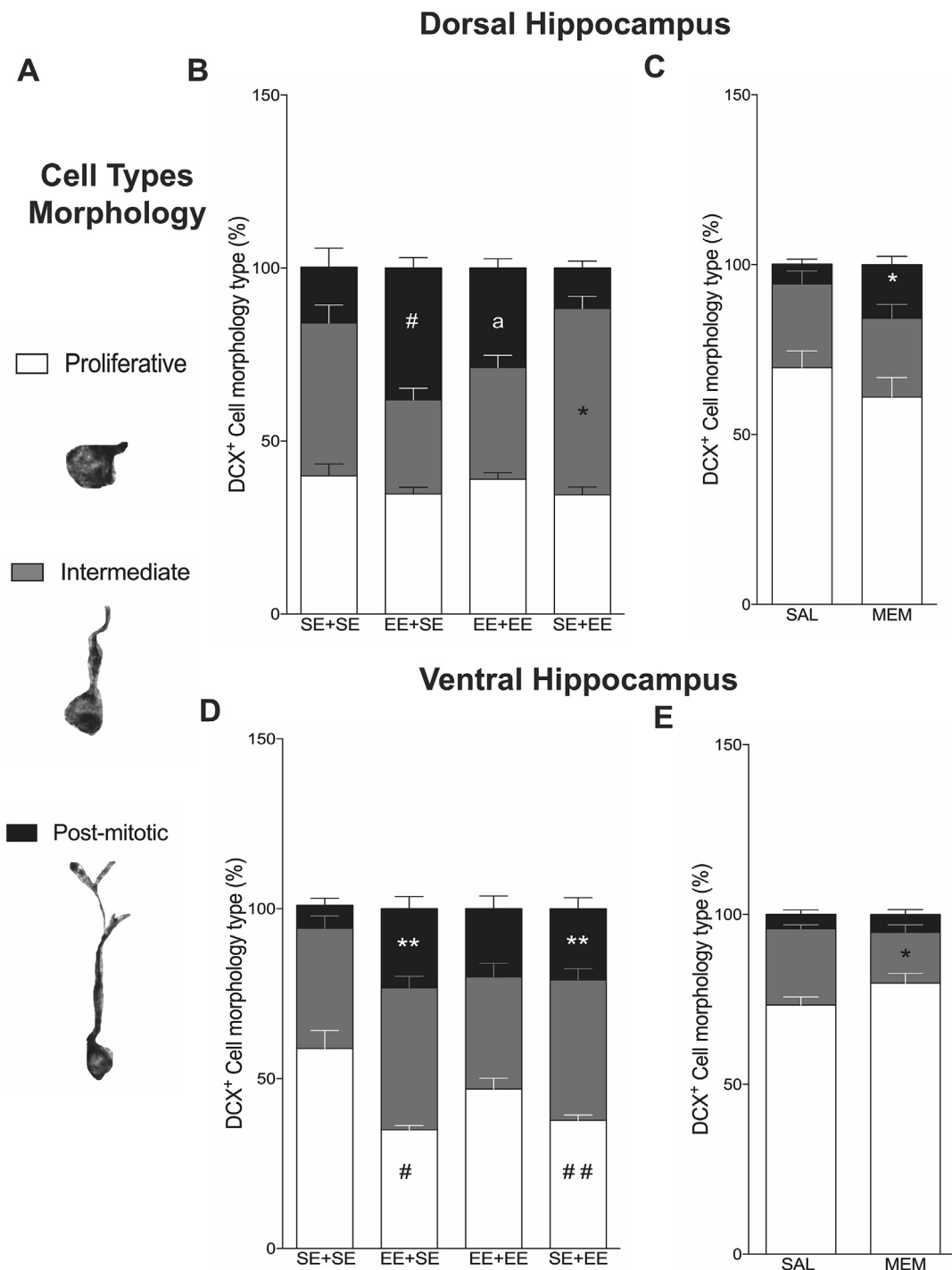
Dorsal Hippocampus

Fig. 6. Effect of enriched environment (EE) and memantine (MEM) on the morphological profile of newborn neurons. (A) Representative figures of proliferative, intermediate and post-mitotic morphology of DCX$^+$ cells. In the dHIP **(B)** The percentage of cell with intermediate morphology was higher in the SE + EE (n = 5) compared to EE + EE (n = 6) and EE + SE (n = 5). The percentage of cells with mature morphology was higher in EE + SE compared to SE + SE (n = 6) and SE + EE ($^#$p < 0.05), while in EE + EE was higher compared to SE + EE only (^{a}p < 0.05). **(C)** MEM increased the proportion of post-mitotic cells (*p < 0.05). In the vHIP **(D)** The percentage of cell with proliferative morphology was lower in EE + SE (n = 5) and SE + EE (n = 5) compared to SE + SE (n = 5) ($^#$p < 0.05 and $^{##}$p < 0.01, respectively). The percentage of cells with mature morphology was higher in EE + SE and SE + EE compared to SE + SE (**p < 0.01). No difference from EE + EE (n = 5) group was detected. **(E)** MEM decreased the proportion of intermediate cells (*p < 0.05). Data are expressed as mean ± SEM.

4. Discussion

The present study reproduced our previous results (Pereira-Caixeta et al., 2017) and also found that MEM improved SRM and increased neurogenesis. To our knowledge there is only one study with MEM and SRM (Ishikawa et al., 2014). In this study, animals received one single dose of MEM (50 mg/kg) and after 3 days, 3 weeks or 4 months were trained and 24h later tested for SRM. MEM was effective when behavior was performed 3 weeks after its administration. Furthermore, the effectiveness of MEM appeared only after a short training session, that normally do not result in memory (Ishikawa et al., 2014). Our study complements that MEM can be effective 1 week after its administration,
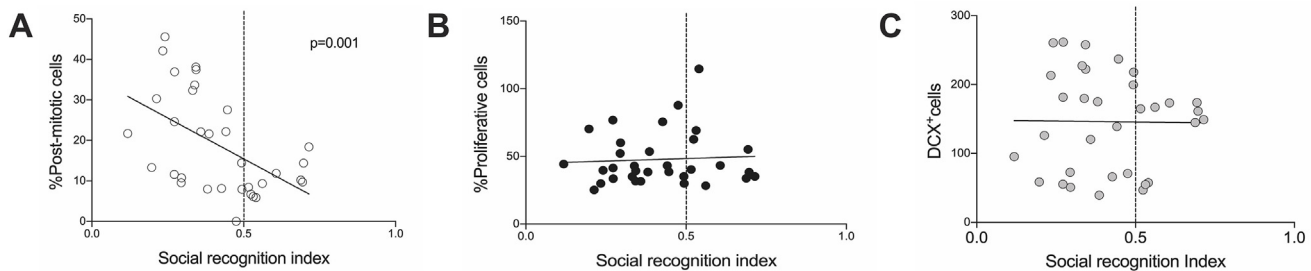
Fig. 7. The percentage of DCX$^+$ cells with post-mitotic morphology and not their absolute number predicts the persistence of social recognition memory. Correlation between social recognition index and **(A)** percentage of post-mitotic cells (n = 33), **(B)** percentage of proliferative cells (n = 33) and **(C)** number of DCX$^+$ cells (n = 33), all from dorsal hippocampus.
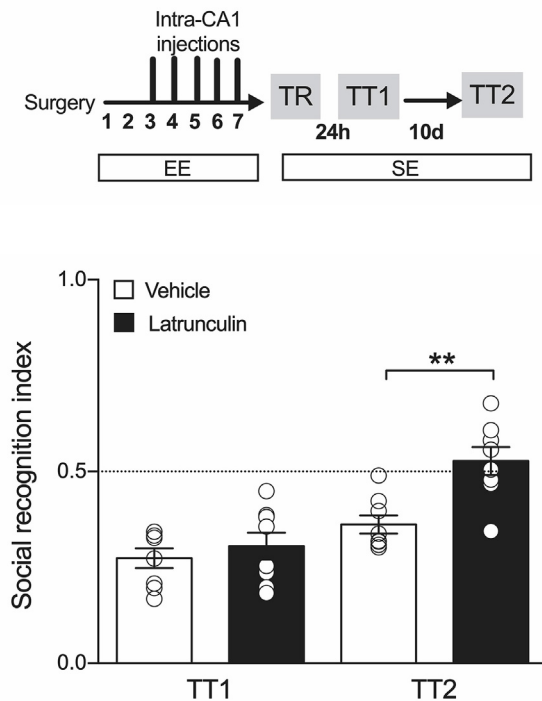

Fig. 8. Inhibition of actin polymerization blocked the pre-training effect of enriched environment (EE) on enhancing social recognition memory (SRM) persistence. Bilateral cannulas were surgically inserted in the dorsal hippocampus (CA1 region), and animals were kept in EE. In the third day, animals received latrunculin (n = 8) or vehicle (n = 8) daily for 5 days. All animals were submitted to training session (TR) and after kept in standard environment (SE) Animals were tested twice. First 24h after TR (TT1) and second 10 days after TR (TT2). **p < 0.01 indicates difference between groups.

specifically to allow SRM to last as long as 10 days.

The genetic background of mice may influence the constitutive (Kempermann and Gage, 2002) as well as the exercise-induced neurogenesis (Clark et al., 2011; Kim et al., 2017). In the present study we used the isogenic mice C57/BL6, and the outbred lineage Swiss. In general, the number of DCX$^+$ cells were higher in Swiss compared to C57/BL6 mice. However, in both strains, the groups showing 10 days SRM were the ones with the higher proportion of newborn neurons in the dorsal hippocampus with post-mitotic morphology. In fact, to SRM persist in a condition that normally do not – 10 days – the maturation of newborn neurons is more relevant than their number.

Manipulation of neurogenesis levels in different phases of memory processing is essential to understanding the contribution of newborn neurons in shaping memories (Tran et al., 2019). Specifically, recent studies had suggested that increasing neurogenesis after learning

promotes forgetting (Epp et al., 2016; Gao et al., 2018). In other words, after increasing neurogenesis, a memory that is usually recalled is no longer retrieved (Frankland et al., 2013). Here, we did not address forgetting directly because the SRM evaluated correspond to a memory that cannot be recalled unless neurogenesis had been increased before training (Pereira-Caixeta et al., 2017). Instead, we tested whether EE after training would blunt the pre-training effect of EE. Surprisingly, animals continued to recall the juvenile after combining pre and post-training EE. At least two possibilities might account for the persistence of SRM. First, neurogenesis was not increased enough to cause forgetting of SRM. In fact, the number of DCX$^+$ neurons in the dorsal hippocampus of EE + EE group was similar to control. Likewise, the proliferation level, represented here by the number of Ki-67$^+$ cells, was also similar to control. However, we did not always observe a direct relation between the number of DCX$^+$ neurons and SRM persistence. For instance, neurogenesis in EE + SE group was similar to control and even so the animals showed SRM 10 days after training. Furthermore, we did not find significant correlation between DCX$^+$ neurons and social recognition index.

Second, increasing neurogenesis after training do not promote forgetting of SRM, as it does for other memories (Akers et al., 2014; Gao et al., 2018; Epp et al., 2016; Ishikawa et al., 2019). We may speculate that the nature of the memory trace can be a factor influencing the neurogenesis effect on forgetting. SRM is a non-aversive and non-associative type of memory, and it can be considered a declarative-like memory (Camats Perna and Engelmann, 2015; Engelmann et al., 2011). Contextual fear conditioning, for instance, establishes an association between the context and the aversive stimulus, and is a non-declarative memory (Squire, 1992, 2004).

One interesting result was the lack of EE's promnesic effect, caused by initiating EE 3 days after training. This result strongly suggest that EE must be presented prior to acquisition or during the early stages of consolidation to prolong SRM. Delaying the exposition to voluntary exercise also compromised its effect on inducing forgetting of contextual fear memory (Gao et al., 2018). Alternatively, we may suggest that 7 days is not time enough for a post-training effect of EE on SRM. In fact, EE applied immediately after training, which rendered 10 days of EE instead of 7, was promnesic. Interestingly, the ability of voluntary exercise to induce forgetting of contextual fear memory potentiates as longer it is its duration (Gao et al., 2018).

Surprisingly, neither social memory duration nor neurogenesis increased after 2 weeks of EE. Specifically, the number of DCX$^+$ cells in the dorsal hippocampus of EE + EE group was similar to control (SE + SE). We may suggest that the second phase of EE could lead to cell death and this could be an explanation why there is no net increase in newborn neurons number. However, there was no running in the EE, which may be suggested as a possible apoptotic cell death inducer (Kitamura and Sugiyama, 2006; Kerr and Swain, 2011). Alternatively, some evidences suggest that maintaining the novelty by changing the stimulus during prolonged periods of EE is essential to enhance neurogenesis (Lemaire et al., 1999; Veyrac et al., 2009) and cognition

(Clemenson et al., 2015; Kempermann, 2019; Kuczaj et al., 2002; Nithianantharajah and Hannan, 2006; Sztainberg and Chen, 2010; van Praag et al., 2000). In our study, no new stimulus was introduced during the whole period of EE, which may be the reason for the results observed in EE + EE group.

The dendrite-centered theory of memory (reviewed by Kastellakis et al., 2015) supports the idea that dendritic branches are the primary functional units for long-term memory storage (Govindarajan et al., 2011). As well as analytical models of recognition memory indicate that in comparison to entire neurons, dendrites increase storage capacity by providing a large number of better sized learning units (Wu et al., 2019). We found secondary and tertiary dendrites to be more numerous in the dHIP of animals from EE + SE group. Similarly, the percentage of cells with morphology compatible with post-mitotic cells was higher in the groups with SRM. And finally, we found a high correlation between the percentage of post-mitotic cells and social recognition index. Altogether these results encourage us to propose that the maturation of newborn neurons is the determinant factor that allows SRM to last longer. In fact, EE triggers the maturation of newborn neurons (Llorens-Martin et al., 2016; Zhao et al., 2014). Similarly, MEM may also act on maturating dendritic spines (Wei et al., 2012).

The exact function of post-mitotic cells remains to be elucidated. However, some studies suggest that even before reaching complete maturity, the new immature neurons make connections with mature neurons (Ambrogini et al., 2004; Overstreet Wadiche et al., 2005; Toni et al., 2007), probably because immature granular cells show a differentiated electrophysiological profile characterized by reduced GABA transmission, high excitability and a low threshold for LTP induction (Schmidt-Hieber et al., 2004; Snyder et al., 2009; Wang et al., 2000). Our study did not address the functionality of the post-mitotic cells. However, our behavioral and morphological results open for the possibility of these cells playing an active role in the persistence of SRM.

Hippocampal neurogenesis comprehends the generation and maturation of newly generated neurons. While neurogenesis is presumably ongoing continuously, it takes a considerable amount of time until the newly generated neurons reach a complete mature morphology and function (Gozel, 2019; Mouret et al., 2008; Opendak and Gould, 2015) Snyder (2019). In our study, MEM was administered once, in a dose sufficient to increase neurogenesis. On the other hand, EE was continuously applied for 7 or 14 days. Considering the timing and duration of MEM and EE, the latter is probably affecting both generation and maturation of newborn neurons, which may explain why the effect of EE on dendritic arborization of newborn neurons was more robust than MEM.

Memories that endure overtime may have particular signatures in cellular and molecular level (Bekinschtein et al., 2010; Fioriti et al., 2015). Here, the inhibition of actin polymerization with Latrunculin A during EE impaired SRM tested 10 days, but not 24h, which suggest the maintenance and renovation of actin polymerization as one of the mechanisms involved in the persistence of SRM. Previous studies have shown that the homeostatic change of the actin dynamics may affect distinct memories. The insular cortex (IC) had their post-synaptic density enlarged after training in the conditioned taste aversion task (CTA); while latrunculin A administered intra-IC impaired the acquisition and consolidation of CTA (Bi et al., 2010). Drug-associated memories are impaired by intra-amygdala administration of latrunculin A (Young et al., 2014). Furthermore, post-training intra-hippocampal latrunculin A impaired contextual fear memory acquisition and extinction (Fisher et al., 2014). Our study adds that the persistence of SRM, a non-associative memory, also relies on actin polymerization.

Our results suggest dHIP rather than vHIP as a target region for the effects of EE and MEM on neurogenesis and SRM. Accordingly, others have identified a lack of EE effect on vHIP (Tanti et al., 2012; Tanti and Belzung, 2013), while no studies had quantified neurogenesis in the vHIP after MEM injection. It is not surprising that we found a difference between vHIP and dHIP (Fanselow and Dong, 2010). For instance, dHIP

received mainly sensory inputs from the associative and entorhinal cortices (Amaral and Witter, 1989; Witter and Groenewegen, 1984), while vHIP receives signals from the amygdala, hypothalamus and nucleus accumbens (Fanselow and Dong, 2010; Petrovich et al., 2001). In fact, the present results complement our previous study that showed that the degree of neurogenesis inhibition must be higher in vHIP compared to dHIP in order to correlate with SRM tested 24h after training (Pereira-caixeta et al., 2018).

Memory persistence is not the main focus of most studies investigating neurogenesis (Bouet et al., 2011; Bruel-Jungerman et al., 2007; Clemenson et al., 2015; Leger et al., 2014; Tarou and Bashaw, 2007; Wang et al., 2012). Our study provides a novel framework on the necessary functional requirements for neurogenesis to improve SRM. In other words, the maturation of newborn neurons, and not simply their number, is determinant to elicit SRM persistence for 10 days.

CRediT authorship contribution statement

Laura F. Jaimes: Data curation, Formal analysis, Writing - original draft. **Lara M.Z. Mansk:** Formal analysis. **Ana F. Almeida-Santos:** Formal analysis. **Grace S. Pereira:** Writing - original draft.

Declaration of competing interest

The authors declared no competing interests.

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