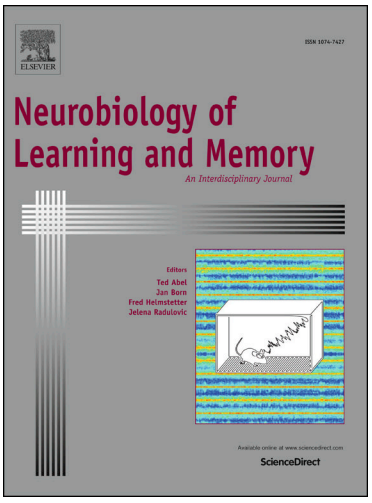

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Inhibiting constitutive neurogenesis compromises long-term social recognition memory

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Abstract

Although the functional role for newborn neurons in neural circuits is still matter of investigation, there is no doubt that neurogenesis modulates learning and memory in rodents. In general, boosting neurogenesis before learning, using genetic-target tools or drugs, improves hippocampus-dependent memories. However, inhibiting neurogenesis may yield contradictory results depending on the type of memory evaluated. Here we tested the hypothesis that inhibiting constitutive neurogenesis would compromise social recognition memory (SRM). Male Swiss mice were submitted to three distinct procedures to inhibit neurogenesis: (1) intra-cerebral infusion of Cystosine-β-D-Arabinofuranoside (AraC); (2) intra-peritoneal injection of temozolomide (TMZ) and (3) cranial gamma irradiation. All three methods decreased cell proliferation and neurogenesis in the dentate gyrus of the dorsal (dDG) and ventral hippocampus (vDG), and the olfactory bulb (OB). However, the percentage inhibition diverged between methods and brain regions. Ara-C, TMZ and gamma irradiation impaired SRM, though only gamma irradiation did not cause side effects on weight gain, locomotor activity and anxiety. Finally, we examined the contribution of cell proliferation in vDG, dDG and OB to SRM. The percent of inhibition in the dDG correlates with SRM, independently of the method utilized. This correlation was observed for granular cell layer of OB and vDG, only when the inhibition was induced by gamma irradiation. Animal's performance was restrained by the inhibition of dDG cell proliferation, suggesting that cell proliferation in the dDG has a greater contribution to SRM. Altogether, our results demonstrate that SRM, similarly to other hippocampus-dependent memories, has its formation impaired by reducing constitutive neurogenesis.

Key-words: social recognition memory; neurogenesis; hippocampus; olfactory bulb.

1. Introduction

Identifying the elements that dictate whether a memory's fate is to persist or to be forgotten has been a challenge to neuroscientists (reviewed by Richards and Frankland 2017). Compared to memory consolidation, the neurobiological bases of forgetting still need to be thoroughly investigated (Davis and Zhong 2017). However, some studies have been suggesting that rather than a passive event, forgetting may be an active process, with specific molecular markers (Shuai et al. 2010; Shuai et al. 2015; Sachser et al. 2016).

Neurogenesis has been rising as a possible key element in the balance between persistence and forgetting. In general, boosting neurogenesis before the acquisition improves memory persistence of several hippocampus-dependent memories (Pan et al. 2012; Monteiro et al. 2014; Gradari et al. 2016; Pereira-Caixeta et al. 2017). In contrast, enhancing neurogenesis after memory acquisition may lead to forgetting (Akers et al. 2014; Epp et al. 2016). Nevertheless, whether varying constitutive neurogenesis would impact the formation and persistence of hippocampus-dependent memories seems controversial. For instance, fear memory can be impaired (Son et al. 2016), improved (Kugelman et al. 2016), or be unaffected (Ko et al. 2009) by the inhibition of hippocampal neurogenesis with gamma-ray. However, gamma-ray compromised animal's performance in the radial arm water maze (Ungvari et al. 2017).

Considered a hippocampus-dependent memory (Kogan et al. 2000; Pena et al. 2014), social recognition memory can be altered by distinct environment conditions. Enriched environment-induced neurogenesis can both extent to 10 days and avoid

interference-based forgetting of social recognition memory (Pereira-Caixeta el al. 2017). Furthermore, in a scenario where the social recognition memory did not last more than a few hours, named social isolation, increasing neurogenesis allowed this memory to last at least 24h (Monteiro et al. 2014). Based on our previous studies indicating that social recognition memory is particularly sensitive to neurogenesis, here, we tested the hypothesis that decreasing constitutive neurogenesis would compromise social memory formation.

2. Material and Methods

2.1. Subjects

Adult (8-12 weeks of age) Swiss male mice were used. As stimuli for social recognition test, Swiss male juvenile mice were used (21 to 35 days of age). All animals were kept in an environment with controlled temperature (22 ± 2°C) and humidity (55 ± 10%) under a light-dark cycle of 12-12 h. All behavioral experiments were performed during the light phase. Mice were kept in groups (4-5 animals) in a polypropylene cage (28 × 17 × 12 cm^3). Food and water were available *ad libitum*. All experiments were approved by the Animal Use Ethic Committee of the Universidade Federal de Minas Gerais (CEUA 53/2014).

2.2. BrdU injection

Progenitor cells in the S-phase of mitosis were labeled with 5-bromo-2'-deoxyuridine (BrdU) (van Praag et al. 1999). Animals received a daily 75 mg/kg BrdU

injection (Sigma, diluted in 0.9% NaCl solution), intraperitoneally (i.p.), during 7 days (Monteiro et al. 2014; Pereira-Caixeta et al. 2017).

2.3. AraC administration

Animals were anesthetized with ketamine (80 mg/Kg, i.p.) and xylazine (10 mg/Kg. i.p.) and positioned in the stereotaxic apparatus (Digital Stoelting, 51730D, USA). A single cannula was implanted in the right lateral ventricle, using the following coordinates: -0.5 mm AP, -1.0 mm LL and -2.0 mm DV (Paxinos and Franklin, 2004). A miniosmotic pump (Alzet, model 1007D, flow rate 0.5 µl/h, 7 days) was attached to the cannula through a polypropylene catheter that was positioned subcutaneously, on the animal's back. The mini-osmotic pump was filled beforehand with Cystosine-β-D-Arabinofuranoside (AraC, 2%; Sigma) or 0.9% saline solution (Alonso et al. 2008; Monteiro et al. 2014, Pereira-Caixeta et al. 2017) (Figure 1A). Animals were weighted before the surgery and for seven days post-surgery.

2.4. Temozolomide (TMZ) administration

TMZ (Sigma) was dissolved in dimethylsulfoxide (DMSO) and PBS at a concentration of 2.5 mg/mL (10% DMSO). TMZ at the dose of 25 mg/kg or the relative volume of 10% DMSO/PBS solution were administered intraperitoneally. Animals received one injection per day during three consecutive days (Garthe et al. 2009) (Figure 1B). Animals were weighted before the TMZ injections and during seven days after completed TMZ treatment.

2.5. Gamma irradiation

Mice were anesthetized with ketamine (100 mg/Kg) and xylazine (10 mg/Kg) and then were immobilized inside a plastic tube (50 mL), properly perforated to allow ventilation. Subsequently, this tube was positioned vertically inside a lead brick to protect the animals' body from irradiation. The lead brick has a 0.8 cm hole on one side, that allowed the passage of radiation towards the skull of the animal. Animals received one dose of 7Gy (4.7Gy/min) in the whole brain, using a Cobalt-60 source (Category II Multiple Panoramic Irradiator series IR-214 - type GB-127, equipped with a source of Cobalto-60 dry stock with activity maximum of 2,200 TBq or 60,000 Ci manufactured by MDS Nordion / Canada - CDTN, Brazil) (Tada et al. 1999). Sham mice underwent the same procedure of anesthesia and positioning inside the plastic tube, though were not irradiated. Behavioral tests were conducted 30 days after the irradiation procedure (Figure 1C). Animals were weighted before the irradiation and during the seven days following irradiation.

2.6. Magnetic Resonance Imaging (MRI)

A 4.7T NMR magnet (Oxford Systems) controlled by UNITY Inova-200 image console (Varian) was used (CTPMAG/UFMG, Brazil). MRI images of the whole brain were obtained before the irradiation session (basal), 48 h and 21 days after the irradiation session. Animals were anesthetized with isoflurane (3% induction, 1.5%

maintenance) mixed with oxygen (1.5 l/min) delivered by a nose mask. Mouse's head was fixed to avoid movement artifacts during the imaging acquisition. We obtained images from coronal T2-weighted (TR = 3000 ms, TE = 50 ms) spin echo multi-slice scans, forming 20 continuous slices of 1 mm thickness. After choosing three images of the same animal (slice anteroposterior -1.8, referenced from bregma), one for each specific moment, we compared the optical density (OD) and whole volume by using NIH ImageJ program. The OD gray scale of each slice was normalized by the respective background (1mm square on the upper left of the figure).

2.7. Behavioral analysis

Behavioral testing commenced at least 24h after the last BrdU injection. Distinct batches of animals were used. The first batch was used to evaluate the weight gain, social recognition memory and immunohistochemistry. The second batch was used to MRI and all the other behaviors.

2.7.1. Locomotor activity

Animals had their locomotor activity monitored in an open field apparatus (LE 8811 IR Motor Activity Monitors PANLAB, Harvard Apparatus; Spain), with acrylic box dimension of 450 x 450 x 200 mm (width x depth x height). The total distance traveled during 5 min was monitored automatically using the ACTITRACK software (Actitrack v2.7.13.).

2.7.2. Elevated plus-maze

Anxiety-like behavior was measured in the elevated plus-maze, which consisted in two open (30cmx6cm) and two closed (30cmx6cmx16cm) arms elevated 45cm above the floor (Handley and Mithani, 1984; Pellow et al. 1985; Pellow and File, 1986; Duncan et al. 1990). During 5 minutes, animals freely explored the labyrinth in the dark (3 lux of luminous intensity in open arms). Time and entrances in the open arms were used as an indicative for anxiolytic behavior, while entrances in the closed arms were used as an indicative of exploratory behavior (Pádua-Reis et al. 2017).

2.7.3. Object location test

Animals were habituated to a rectangular box (50cm X 40cm X 20cm) during 20 min. Twenty-four hours later, animals were re-habituated for 1 min in the same box, and then two identical objects, A and B, were inserted in it. During the training phase, which lasted 5 min, animals were allowed to investigate objects freely. An hour and a half later, in the test phase, the same procedure was repeated, though one of the objects had its location changed. Time exploring each object was recorded. Object location memory was expressed as recognition index, calculated by: time exploring the object in the new location/total time of exploration (Gresack and Frick, 2006).

2.7.4. Contextual fear conditioning

The apparatus was a Plexiglas box (23cmx23x30cm) with black walls and a floor containing stainless-steel grid rods (0.4cm in diameter, spaced 0.6cm part) (Insight Equipamentos, Ribeirão Preto, Brazil). The box was surrounded by a soundproof compartment. Conditioning started by putting the animal inside the box and allowing the

mouse to freely explored the box for 150s. After, 3 foot-shocks (unconditioned stimulus, US: 0.7mA, 2s) were delivered, with 90s intervals between them. After the last US, mice remained for 30s inside the box. Twenty-four hours later, animals returned to the same box for a period of 5 min, but no US was delivered (Lazaroni et al. 2016). Both sessions were recorded to perform the offline quantification of freezing. Freezing consisted in the absence of movements, except respiratory.

2.7.5. Morris water maze

The apparatus consisted of a circular tank (50 cm in diameter and 90 cm high), with a transparent acrylic platform (28 cm high), always positioned at the same quadrant and submerged by 2 cm of water. The water temperature was adjusted to 22 ºC and the turbidity increased with non-toxic white paint, to ensure the mouse did not maintain visual contact with the platform. Surrounding the maze there were visual cues on the walls to aid spatial orientation. Animal was placed in the water, always with its head facing the edge of the tank and allowed to reach the platform for up to 60 s, remaining at the platform for 10 s before being removed. If the time to reach the platform exceeded 60 s, the animal was gently conducted to the platform, then being removed after 10s. The learning protocol consisted of 4 daily training sessions, each one containing 4 trials, alternating start quadrants. Latency to reach the platform was recorded for each trial and the average represented the training session latency.

On the 5th day, spatial memory was tested. The platform was removed during the probe test, and the animal was released from the quadrant opposite the target. Time spending in each quadrant was recorded during 120s (Santin et al. 2009).

2.7.6. Social recognition test

We measured social recognition memory in the social recognition test (Gusmão et al. 2012; Monteiro et al. 2014; Pereira-Caixeta et al. 2017). Mice were individually habituated for 20 min to a clean regular cage, containing one transparent acrylic cylinder (10 cm in diameter, with 60 equally distributed holes). After, a juvenile Swiss mouse (21 to 35 days of age) was introduced into the cylinder and social investigation was measured during 5 minutes, in a trial named training (TR). Long-term memory (LTM) was tested 24 hours after training. Test session (TT) lasted 5 minutes and was preceded by a period of habituation, exactly as described before. Sessions were filmed and videos watched and analyzed offline. Every time the adult mouse put its nose and/or whiskers inside one or more holes in the transparent acrylic cylinder where the juvenile was, we recorded as social investigation. To avoid constrain-related stress in the juvenile mice, they were used no more than three times each.

Data were expressed as total time (social investigation) exploring the juvenile during the TR and TT sessions or as social recognition index, calculated by the following formula: time exploring the juvenile during TT/ time exploring the juvenile during TR + time exploring the juvenile during TT.

2.7.7. Spatial odor task (SOT)

The open field's (50 x 50 x 40 cm) floor was covered with 3 cm of bedding and virtually divided into four quadrants: Target (TG), Right Adjacent (RA), Left Adjacent (LA) and Opposite (OP). The task consisted of two phases: habituation and testing.

During habituation, animals freely explored the apparatus for 10 min. Subsequently, the mouse was removed from the open field, while 100 cm^3 of the mouse's home-cage bedding were hidden underneath the clean bedding of the target quadrant (TG). Immediately after, the mouse returned to the open field and was allowed to freely explore it for 10 min. The total time spend in each quadrant was measured during the habituation and test phases. The apparatus was cleaned and bedding was changed between each animal and session. The quadrants and the relative position of the box in the room were randomized. Session was filmed and videos analyzed offline, using the freeware computer rat-racking software (X-Plot Rat, Laboratório de Comportamento Exploratório USP-RP Brazil. (Pena et al. 2017). Results are expressed as the percentage time in the target quadrant during the test phase.

2.8. Immunofluorescence

Twenty-four hours after the last injection of BrdU, animals were anesthetized with ketamine (80 mg/Kg) and xylazine (10 mg/Kg) and underwent transcardial perfusion with 0.01M PBS, followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed overnight with 4% PFA for 24 h and afterwards in 30% sucrose in PBS at 4°C for 3 days. Coronal brain slices (40 mm) were obtained using cryostat and then stored at a temperature of 4°C in PBSAF (PBS, 20% sucrose, 15% ethylene glycol, 0.05% NaN3). From each animal, we selected 8 slices of the olfactory bulb (4.28 mm to 3.56 mm from bregma), 6 slices containing dorsal hippocampus (-1.70 mm to -2.30 mm from bregma) and 6 slices containing ventral hippocampus (-3.16 mm to -3.52 mm from bregma) (Paxinos and Franklin, 2004).

To analyze cell proliferation, neurogenesis and astrogenesis, cells were labeled for BrdU, NeuN and GFAP, respectively. Slices were washed three times in 0.01M PBS, followed by three washes in PBS-T (0.01M PBS containing 0.3% Triton X-100) at room temperature. Afterwards, slices were washed three times in 0.9% NaCl. Next, slices were incubated for 10 min in 2M HCl followed by 30 min in 3M HCl, at 37 °C. Subsequently, slices were washed three times in 0.1M borate buffer and three times in PBS-T. Blocking was made with 5% normal goat serum (NGS) in PBS-T for 1 h. Slices were incubated either with a combination of Anti-BrdU (1: 800, rat monoclonal, Abcam) and anti-NeuN (1: 500, mouse monoclonal, Millipore) or anti-BrdU (1: 800, Abcam) and anti-GFAP (1: 500, mouse polyclonal, Millipore) for 72 h at 4 °C under gentle shaking. Subsequently, slices were washed three times in 0.01M PBS and incubated with Alexa Fluor 488 (1: 400, anti-rat, Invitrogen) in combination with Alexa Fluor 647 (1: 400, anti-mouse, Invitrogen) for 90 min at room temperature. Finally, slices were washed four times in 0.01M PBS (Lu et al. 2003; Monteiro et al. 2014; Pereira-Caixeta et al. 2017). Slides were mounted and fixed in Vectashield (Vector Laboratories), covered with cover slips, kept in a container protected from light and maintained at 4°C until the following day, when were photographed.

2.9. Imaging acquisition and analysis

Images were obtained using an epifluorescence microscope (Axio Imager M2 - Zeiss system) and Carl Zeiss Axiovision 4.8 software, with 20x objective lens. The regions were photographed using the Z series, which consists in making a series of photographs from the top focus of the slice to the lower focus, at regular intervals. Two

different filters were used, one to visualize positive cells to BrdU (excitation 450-530 nm / emission 500-610 nm for Alexa Fluor 488), and a second one to visualize positive cells to GFAP and NeuN (excitation 590-680 nm / emission 630-800 nm for Alexa Fluor 647). The exposure time for each filter was determined based on the pixel saturation histogram, using the maximum possible pixels below the saturation limit. Cells were considered double-labeled if co-labeling and relevant morphology were seen throughout the extent of the cells, viewed in x-y section, as well as in x-z and y-z cross-sections produced by orthogonal reconstructions from z-series. $BrdU^+$, $BrdU^+/NeuN^+$ or $BrdU^+/GFAP^+$ cells were counted using ImageJ Software (NIH, USA).

2.10. Statistical analysis

All data were expressed as mean ± standard error of the mean. The weight gain and social recognition memory were analyzed by two-way ANOVA with repeated measures, followed by Bonferroni post-hoc test. Data from locomotor activity, EPM, contextual fear conditioning, probe test in Morris water maze, proliferation, neurogenesis and astrogenesis were analyzed by unpaired t-test. MRI imaging and latency to find the platform in the Morris water maze were analyzed by one-way ANOVA with repeated measures and Tukey's multiple comparison test. Data from percent of inhibition were analyzed by one-way ANOVA and Tukey's multiple comparison test. Object location were analyzed by one sample t-test with a hypothetical value equal to 0.5. Linear relationship between $BrdU^+$ cells and social recognition indexes was determined by Pearson correlation coefficient (r).

3. Results

3.1. Neurogenesis inhibition and weight gain

We took advantage of three different and well-known methods to inhibit neurogenesis. The antimitotic drug AraC was administered intracerebroventricular (Figure 1A), while TMZ was injected intraperitoneally (Figure 1B). We also used cranial gamma irradiation (Figure 1C). In general, weight gain was reduced in animals treated with AraC during the whole treatment (Interaction: $F(6,84)=2.8$, $p=0.01$; Time: $F(6,84)=11.9$, $p<0.0001$; Treatment: $F(1,14)=29.4$, $p<0.0001$), though statistical difference between groups was reached on day 1 and 6 after the beginning of the treatment (Figure 1D). TMZ did not alter the animal weight gain compared to control group, though both groups gained less weight in the first day of the treatment (Interaction: $F(6,36)=0.1$, $p=0.99$; Time: $F(6,36)=3.0$, $p=0.01$; Treatment: $F(6,36)=0.3$, $p=0.92$) (Figure 1E). Gamma irradiation also affected the weight gain, but only in the first day after the procedure (Interaction: $F(6,48)=2.4$, $p=0.03$; Time: $F(6,48)=0.8$, $p=0.56$; Treatment: $F(1,8)=8.9$, $p=0.01$) (Figure 1F).

To verify whether the reduced weight gain observed in the first day post-irradiation was an outcome of inflammatory processes (Pillai et al. 2011; Begolly et al. 2016; Tang et al. 2017), we imaged the brain of irradiated animals before, 48h and 21 days after the procedure. We observed an increase in the normalized grey scale 48h after irradiation, ($F(2,6)=9.9$, $p=0.03$) (Figure 1G), that returned to basal level 21 days after. No significant difference was noted for slice volume (data not shown). Altogether, we may conclude that cranial irradiation induced brain inflammation, that was recovered by the time the behavioral analysis was made, suggesting that neurogenesis inhibition

more than inflammatory process is playing a role on the effect of cranial irradiation on memory.

3.2. Anti-mitotic drugs compromised locomotion and increased anxiety-like behavior

We tested whether the neurogenesis inhibitors would affect the locomotor activity, which is essential to most of the paradigms used to evaluate learning and memory. AraC treatment decreased the total distance travelled in the open field ($t(10)$, $p=4.3$, $p=0.001$) (Figure 1H), while TMZ ($t(13)=0.6$, $p=0.54$) (Figure 1I) and irradiation ($t(22)=0.4$, $p=0.68$) (Figure 1J) did not affect locomotor activity.

Learning and memory processes can be potentially affected by emotional states (Kheirbek et al. 2013; Rubin et al. 2014; Anacker and Hen, 2017). Furthermore, it has been suggested that neurogenesis may impact the neural circuits supporting emotional states (Eisch and Petrik, 2012; Gulbins et al. 2015; Culig et al. 2017; Ryu et al. 2018; Sun et al. 2018). We found that TMZ reduced the time in the open arms of the elevated plus maze (EPM) ($t(11)=4.7$, $p=0.0006$) (Figure 1K). However, no effect of irradiation was detected in this paradigm ($t(7)=0.1$, $p=0.9$) (Figure 1L). We did not test animals under AraC treatment since they already presented reduced locomotor activity, which could compromise their performance in the EPM.

3.3. Hippocampus-dependent memories and neurogenesis inhibition

It has been demonstrated that hippocampus-dependent memories are particularly sensitive to neurogenesis (Motta-Teixeira et al. 2016; Klein et al. 2016;

Pereira-Caixeta et al. 2017; Urbach et al. 2017; Drew and Huckleberry, 2017). Therefore, we tested whether the methods used here to inhibit neurogenesis would impact on 3 distinct hippocampus-dependent paradigms: object location, contextual fear conditioning and Morris water maze.

All treatments, AraC (Interaction: $F(1,11)=14.3$, $p=0.003$; Trial: $F(1,11)=12.6$, $p=0.004$; Treatment: $F(1,11)=18.8$, $p=0.001$); TMZ (Interaction: $F(1,18)=5.3$, $p=0.03$; Trial: $F(1,18)=3.4$, $p=0.08$; Treatment: $F(1,18)=2.7$, $p=0.11$) and irradiation (Interaction: $F(1,18)=8.3$, $p=0.009$; Trial: $F(1,18)=8.9$, $p=0.007$; Treatment: $F(1,18)=9.0$, $p=0.007$) impaired object location 1.5h after the sample trial (Figure 2A, B and C).

As AraC compromised locomotor activity, we chose not to test those animals on contextual fear conditioning. TMZ had a weak effect on fear memory ($t(17)=2$, $p=0.05$) (Figure 2D). However, animals that underwent to cranial irradiation showed fear memory impairment ($t(17)=4.9$, $p<0.0001$) (Figure 2E). To further analyze irradiation effect on memory, we tested irradiated animals on Morris water maze. Both groups, sham and irradiated, learned the hidden platform location by the end of the training days (Interaction: $F(3,54)=0.6$, $p=0.5$; Trial: $F(3,54)=27.3$, $p<0.0001$; Treatment: $F(1,18)=2.2$, $p=0.15$) (Figure 2F). Nevertheless, irradiated mice presented spatial memory deficit compared to sham animals ($t(18)=5.9$, $p<0.0001$) (Figure 2G).

3.4. Neurogenesis inhibition compromised long-term social recognition memory

We showed previously that enriched environment recovered social recognition memory impairment induced by social isolation (Monteiro et al., 2014) and also improved social recognition memory persistence in non-isolated animals (Pereira-

Caixeta et al., 2017). Both effects of enriched environment were proved to be neurogenesis-dependent. In the present study, we aimed to test whether constitutive neurogenesis, the one not boosted by enriched environment, would also play a role on social memory.

AraC treatment impaired long-term social memory (LTM) (Interaction: $F(1,11)=19.5$, $p=0.001$; Treatment: $F(1,11)=1.4$, $p=0.25$; Trial: $F(1,11)=1.9$, $p=0.18$) (Figure 3A). Likewise, TMZ treated animals presented LTM deficit (Interaction: $F(1,18)=23.6$, $p=0.0001$; Treatment: $F(1,18)=2.3$, $p=0.13$; Trial: $F(1,18)=2.5$, $p=0.12$) (Figure 3B). Finally, irradiation impaired LTM (Interaction: $F(1,25)=33.4$, $p<0.001$; Trial: $F(1,25)=3.5$, $p=0.07$; Treatment: $F(1,25)=27.2$, $p<0.0001$). Furthermore, irradiated animals explored the juvenile even more during the test (Figure 3C).

3.5. Granular cell layer of the olfactory bulb (OB) is more sensitive to AraC, TMZ and gamma irradiation

Social recognition relies on olfactory cues (Choleris et al. 2009; Stowers and Kuo, 2015; Yamamoto et al. 2017; Kavaliers and Choleris, 2017); and protein synthesis in the main olfactory bulb is required to social recognition memory consolidation (Pena et al., 2014). Furthermore, olfactory bulb is an important neurogenic area in mice (Díaz et al. 2017; Hanson et al. 2017).

Our results showed that AraC was effective in reducing cell proliferation ($t(18)=11$, $p<0.0001$), neurogenesis ($t(8)=7.9$, $p<0.0001$) and astrogenesis ($t(8)=10.6$, $p<0.0001$) in granular cell layer (Figure 4A). Similar results were found for mitral cell layer (proliferation: $t(18)=5.9$, $p<0.0001$; neurogenesis: $t(8)= 5$, $p<0.001$), except for

astrogenesis ($t(8)=1.34$, $p=0.21$) where no effect of AraC was observed (Figure 4B and C).

TMZ reduced proliferation ($t(18)=22$, $p<0.0001$), neurogenesis ($t(8)=7.1$, $p<0.0001$) and astrogenesis ($t(8)=10.2$, $p<0.0001$) in the granular cell layer (Figure 4D). However, in the mitral cell layer, TMZ was effective only in reducing neurogenesis ($t(8)=4.1$, $p<0.01$) (proliferation: $t(18)=1.9$, $p=0.07$; astrogenesis: $t(8)=1.9$, $p=0.09$) (Figure 4E and F).

Finally, gamma irradiation reduced proliferation ($t(18)=12.5$, $p<0.0001$), neurogenesis ($t(8)=6.4$, $p<0.0001$) and astrogenesis ($t(8)=4.5$, $p<0.01$) in the granular cell layer (Figure 4G) and also in mitral cell layer (proliferation: $t(18)10.5$, $p<0.0001$; neurogenesis: $t(8)=6.7$, $p<0.001$; astrogenesis: $t(8)=3.6$, $p<0.01$) (Figure 4H and I).

To test whether AraC, TMZ and gamma irradiation affected differently the number of $BrdU^+$ cells, we calculated the percentage inhibition for each method (compared to their respective control). We observed difference between groups in granular cell layer ($F(2,27)=14.2$, $p<0.0001$), with irradiation causing the lower percent of inhibition compared to AraC and TMZ (Figure 4J). However, when we analyzed the mitral cell layer we observed that TMZ was the less effective method ($F(2,27)=13.3$, $p<0.0001$) (Figure 4K). Neurogenesis in the granular cell layer presented higher levels of inhibition when animals were treated with AraC ($F(2,12)=11.7$, $p<0.001$) (Figure 4L), though no difference between groups was detected in the mitral cell layer ($F(2,12)=0.05$, $p=0.94$) (Figure 4M). Finally, astrogenesis was less inhibited by irradiation, compared to AraC and TMZ ($F(2,12)=13.8$, $p=0.0008$) (Figure 4N), while no difference between treatments was observed in the mitral cell layer ($F(2,12)=1.5$, $p=0.25$) (Figure 4O).

3.6. Neurogenesis inhibition did not alter the performance of mice in the spatial odor task.

Mitral cell layer is the first relay for sensory processing in the main olfactory bulb. As we observed distinct effects for each treatment in this layer, we decided to evaluate animals in the spatial odor task (SOT). SOT was recently described by our group and showed to be a paradigm highly dependent on olfactory-bulb-hippocampus coupling (Pena et al., 2017). No effect of AraC ($t(4)=1.08$, $p=0.34$) (Figure 5A), TMZ ($t(5)=0.57$, $p=0.58$) (Figure 5B) or irradiation ($t(4)=0.12$, $p=0.9$) (Figure 5C) was observed.

3.7. Dorsal and ventral hippocampus are differently affected by AraC, TMZ and gamma irradiation

It has been shown that AraC treatment decreases neurogenesis (Monteiro et al. 2014; Borg et al. 2014; Apkarian et al. 2016; Pereira-Caixeta et al. 2017). Here we showed that AraC decreased cell proliferation ($t(18)=20.2$, $p<0.0001$), neurogenesis ($t(8)=6.3$, $p<0.002$) and astrogenesis ($t(8)=13$, $p<0.0001$) in the dorsal hippocampus (Figure 6A and B). TMZ also reduced cell proliferation ($t(18)=20.2$, $p<0.0001$), neurogenesis ($t(8)=10.1$, $p<0.001$) and astrogenesis ($t(8)=7.4$, $p<0.001$) in the dorsal hippocampus (Figure 6C and D). Finally, gamma irradiation reduced cell proliferation ($t(18)=22.8$, $p<0.0001$), neurogenesis ($t(8)=13$, $p<0.0001$) and astrogenesis ($t(8)=11.4$, $p<0.0001$) in the dorsal hippocampus (Figure 6E and F). Thus, all methods used here, to inhibited neurogenesis, were effective in the dorsal hippocampus.

As we analyzed the olfactory bulb, we also compared the effect of inhibition methods in the dorsal hippocampus. Irradiation presented the higher inhibition magnitude for both proliferation ($F(2,27)=358.3$, $p<0.0001$) and neurogenesis ($F(2,12)=107.7$, $p<0.0001$), compared to AraC and TMZ (Figure 6G). In addition, a slightly difference between groups was found regarding astrogenesis inhibition ($F(2,12)=4$, $p=0.04$). AraC inhibited more the astrogenesis compared to TMZ (Figure 6I).

In the ventral hippocampus, AraC inhibited cell proliferation ($t(18)=16.9$, $p<0.0001$), neurogenesis ($t(8)=6.3$, $p=0.0002$) and astrogenesis ($t(8)=13$, $p<0.0001$) (Figure 7A and B), as well as TMZ (cell proliferation: $t(18)=10.1$, $p<0.001$; neurogenesis: $t(8)=3.7$, $p<0.001$; astrogenesis: $t(8)=2.7$, $p<0.05$) (Figure 7C and D) and gamma irradiation (cell proliferation: $t(18)=18.5$, $p<0.0001$; neurogenesis: $t(8)=5.4$, $p<0.001$; astrogenesis: $t(8)=2.9$, $p<0.05$) (Figure 7E and F).

Regarding the treatment-dependent effect on the percentage inhibition in the ventral hippocampus, we found a difference on proliferation ($F(2,27)=45.21$, $p<0.0001$) (Figure 7G), with AraC showing the higher level of inhibition. No difference between groups was found for neurogenesis ($F(2,12)=2.0$, $p=0.17$) (Figure 7H) and astrogenesis ($F(2,12)=2.23$, $p=0.14$) (Figure 7I) inhibition.

3.8. Social recognition memory performance decreases as the neurogenesis inhibition enhances in the dentate gyrus of the dorsal hippocampus

In an attempt to further explore the contribution of ventral and dorsal hippocampus, as well as the olfactory bulb (OB), to social recognition memory, we

performed a linear regression between social recognition index (Figure 3) and cell proliferation inhibition in each area. Values of social recognition index lower than 0.5 indicate better performance.

In AraC-treated animals, we found a weak, though significant correlation in the dorsal (R^2=0.66, p=0.04), but not in the ventral hippocampus (R^2=0.61, p=0.66) or granular cell layer from OB (R^2=0.22, p=0.34) (Figure 8A).

More consistent results were observed for TMZ. There was a significant correlation in the dorsal hippocampus (R^2=0.72, p=0.007), though, once again, no correlation was observed in the ventral hippocampus (R^2=0.02, p=0.71) and the OB (R^2=0.18, p=0.28) (Figure 8B).

Interestingly, inhibition of proliferation induced by gamma irradiation was correlated with social recognition memory performance in all analyzed areas: dorsal hippocampus (R^2=0.74, p=0.001), ventral hippocampus (R^2=0.88, p<0.0001) and OB (R^2=0.84, p=0.0002) (Figure 8C).

4. Discussion

We showed that decreasing constitutive neurogenesis in the olfactory bulb and the hippocampus, using three distinct tools, compromised social recognition memory. Those results reinforce the idea that social recognition memory, similar to other hippocampus-dependent memories, is particularly sensitive to neurogenesis levels (Monteiro et al. 2014; Garrett et al. 2015; Pereira-Caixeta et al. 2017).

Researches have taking advantage of multiple tools to modify neurogenesis and infer about the functional role of newborn neurons. Inducible transgenic mice with

reduction on neurogenesis showed no deficit on working memory, though social discrimination was compromised (Garrett at al. 2015). Using a distinct target to genetically decrease neurogenesis, a study found no effect on social behavior of adult mice, only in juvenile mice (Wei et al. 2011). In addition, D2 KO mice have less DCX positive cells in the hippocampus and no memory deficits, tested in distinct paradigms (Jaholkowski et al. 2009). These contradictory effects on memory, caused by gene-target strategies that alter neurogenesis, can be avoided by using a combination of other approaches (Breton-Provencher et al. 2009; Castilla-Ortega et al. 2016; Jiang et al. 2018; Ji et al. 2018). In fact, our results were consistent, since AraC, TMZ and gamma irradiation were effective on compromising long-term social recognition memory.

AraC, TMZ and gamma irradiation inhibited cell proliferation, neurogenesis and astrogenesis at different levels. AraC presented the broader effect. In other words, AraC induced a higher percentage inhibition of neurogenesis, astrogenesis and cell proliferation in the olfactory bulb, dorsal and ventral hippocampus, respectively. However, AraC diminished the weight gain along the treatment, and also decreased locomotor activity, corroborating others (Koros et al. 2007). However, we consider the effects of AraC on social recognition memory are neurogenesis related, rather than a consequence of locomotion impairment. If the locomotion was biasing the effect of AraC on social recognition memory, we should had observed a statistical difference on social exploration during training session between control and AraC groups, but we did not.

We have been using AraC to reduce neurogenesis and found no adverse effects on mice (Monteiro et al. 2014; Pereira-Caixeta et al. 2017). However, in our previous

studies, mice were kept in enriched environment during AraC treatment, which suggest a possible protective effect of the enriched environment against the side effects promoted by AraC. Additionally, our results differ from a previous study which found no side effects of AraC (Brenton-Provencher et al. 2009). However, animals were tested only 28 days after the treatment has finished, while here behavioral tests were conducted after 7 days of AraC infusion.

Although TMZ caused no significant side effects, as showed before (Garthe et al. 2009; Egeland et al. 2017), it showed the lower values of percentage inhibition, compared to AraC and gamma irradiation. Possibly, that is the reason why TMZ caused a weak effect on contextual fear conditioning. However, we also detected a deleterious effect of TMZ on object location memory, which is in accordance with others that used water maze to access spatial learning and memory (Garthe et al. 2009; Garthe et al. 2016).

One intriguing observation from our study was the higher values for locomotor activity in the control group for TMZ treatment, compared to other control groups. However, it is important to point out that the control groups are not the same. The control group for AraC treatment was subjected to a surgical procedure and received saline i.c.v. during 7 days before behavioral test. The control group for TMZ were animals that received 3 daily injections of 10% DMSO, 4 days before being tested. And finally, sham animals were exposed to all procedures, except the gamma irradiation, 30 days before behavioral evaluation. Therefore, our results reinforce the importance to use appropriate controls for studying effects of drugs on behavior.

Patients with metastatic melanoma, treated with TMZ, may present anxiety (Agarwala and Kirkwood, 2003). A recent study aimed to reproduce a standard chemotherapy for brain cancer, named 3 daily injections of TMZ during 6 weeks, with behavioral analysis performed 6 weeks after ending the treatment. In this study, they showed no effect of TMZ on plus maze (Egeland et al. 2017). We found an anxiogenic effect of TMZ, which was probably because we tested animals only 4 days after finishing TMZ treatment.

Gamma irradiation was the most effective method to reduce neurogenesis and cell proliferation in the dorsal hippocampus and to impair hippocampus-dependent memories tested in the object location test, contextual fear conditioning, Morris water maze and social recognition test. And most important, at the time of behavioral evaluation, irradiated mice were similar to control in terms of weight gain, locomotor activity and anxiety-like behavior. Cranial irradiation may impair (Son et al. 2016), increase (Kulgelman et al. 2016) or have no effect on fear memory (Clark et al. 2008), though this apparent contradiction is consequence of distinct experimental designs. Our results are more in consonance with Parihar and Limoli (2013) that showed a decrease in the total number, branches and length of dendrites in the granule cell layer of the hippocampus, after 30 days of 10Gy cranial irradiation in mice. In fact, we may suggest the synaptic plasticity impairment caused by irradiation (Parihar and Limoli, 2013) as a possible cause for the memory deficits we observed here. Accordingly, immature newborn neurons play an important role in the formation of memories, especially those of spatial nature (Deng et al. 2009).

Newborn neurons are integrated in the hippocampal dentate gyrus, which is a region that also receives multiple sensory inputs (Rolls, 1996). A loss in the number of newborn neurons could compromise their integration into the preexisting network, impacting the formation of hippocampus-dependent memories. Likewise, astrocytes may modulate synapses via its interactions with neurons (Pfrieger and Barres, 1997, Barker et al. 2008; Araque et al. 1999, Halassa et al. 2009, Perea et al. 2009). For instance, the presence of astrocytes may dictate excitability and synaptic activity of retinal neurons (Pfrieger and Barres, 1997, Barker et al. 2008). In our study, all treatments tested were efficient in reducing cell proliferation, neurogenesis and astrogenesis in the hippocampus, corroborating previous studies (Fike et al. 2007; Garthe et al. 2009; Monteiro et al. 2014). Therefore, we speculate that compromising the capacity to produce new neurons and astrocytes may impact on hippocampal synaptic plasticity, which could influence the memory formation.

In the olfactory bulb (OB), all three techniques were able to decrease cell proliferation, neurogenesis and astrogenesis in the granular layer. However, animals treated whit AraC, TMZ and gamma irradiation behaved similarly to control mice in the spatial olfactory task, known to recruit the OB (Pena et al., 2017). Olfaction can be analyzed by distinct paradigms, and each of them may access a particular aspect of the sense of smell. For instance, AraC decreased the production of new bulbar interneurons, though some, but not all, odor-associated tasks were impaired (Breton-Provencher et al., 2009). Using irradiation to reduce olfactory neurogenesis in female mice, an abnormal social interaction pattern with male, but not female conspecifics were observed (Feierstein et al., 2010). Accordantly the thresholds for detecting pure odorant

molecules and short-term olfactory memory were not affected by irradiation, though long-term olfactory memory was found to be sensitive to irradiation (Lazarini et al., 2009). Our results are limited concerning the effect of less OB neurogenesis on olfaction, because we tested only one paradigm. On the other hand, we cannot exclude an impact of decreasing OB neurogenesis on social recognition memory, since this type of memory seems to rely on the OB plasticity to be stored for 24h (Pena et al., 2014).

The longitudinal organization of the hippocampus has been shown to be functional. For instance, dorsal hippocampus executes cognitive functions, while the ventral hippocampus conveys emotional and affective-related functions (Fanselow and Dong, 2010; Strange et al., 2014). In an attempt to estimate the dorsal and ventral hippocampus contribution to social memory, we analyzed the hippocampus separately. In general, all three methods diminished BrdU, BrdU/NeuN and BrdU/GFAP positive cells in both dorsal and ventral hippocampus. However, comparison between the percent of inhibition revealed that hippocampal dorsoventral axis have distinct susceptibilities to AraC, TMZ and gamma irradiation. For example, inhibition of cell proliferation in the dorsal hippocampus was stronger (~60%) in response to irradiation, while in the ventral hippocampus, AraC was the most effective treatment (~50%). Furthermore, ventral hippocampus was particularly sensitive to cell proliferation inhibition, while in the dorsal portion of the hippocampus, neurogenesis and astrogenesis were also impacted in a method-dependent manner. Altogether, our results indicate a differential sensibility to neurogenesis inhibitors in ventral and dorsal hippocampus, and corroborate the notion that the hippocampus is a heterogeneous

structure, which not only functional, but also molecular (Fisher et al, 2016; Floriou-Servou et al., 2018) and cellular (Zhou et al., 2016) distinctions.

Social recognition memory seems to be particularly dependent on neurogenesis (Monteiro et al., 2014; Pereira-Caixeta et al., 2017). Thus, here, we tested whether animal's performance on social recognition test would be affected by the level of cell proliferation inhibition. We predicted that areas with higher contribution to social recognition memory would be more affected by neurogenesis inhibition. As long as the cell proliferation was inhibited in the dorsal hippocampus, the social recognition memory performance got worse. In other words, no matter which method was used to decrease $BrdU^+$ cells, as lower was their number, worse was the social recognition memory. In contrast, a positive correlation was only observed in ventral hippocampus and olfactory bulb, after irradiation. Based on our results we may speculate that neurogenesis in the dorsal hippocampus contributes more to social recognition memory, compared to ventral hippocampus and olfactory bulb.

Altogether, our results suggest irradiation as the best tool to inhibit neurogenesis an affect memory, without compromising the animal's general health and affective behavior. Our study showed for the first time that social recognition memory is consistently impaired when neurogenesis was reduced, independently of the tool used to decrease it.

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Legends to figures

Figure 1: Anti-mitotic drugs, but not gamma irradiation, caused side effects at the time of behavioral analysis. Timeline of the experimental design using **(A)** AraC, **(B)** TMZ and **(C)** gamma irradiation (7Gy) as agents to inhibit neurogenesis. **(D)** AraC treated animals gained less weight during the treatment compared to control animals. **($p<0.001$) and *** ($p<0.0001$) indicate difference between groups **(E)** TMZ did not affect the weight gain. **(F)** Gamma irradiation decreased the weight gain in the first 24h, probably related to the **(G)** inflammatory process observed 48h after irradiation using magnetic resonance imaging. **($p<0.001$) indicates difference from basal and 21 days (21d) **(H)** AraC, but not **(I)** TMZ or **(J)** gamma irradiation decreased locomotor activity in the open field. **($p<0.001$) indicates difference between groups. **(K)** TMZ treatment, but not **(L)** gamma irradiation, increased anxiety-like behaviors. *** ($p<0.0001$) indicates difference between groups.

Figure 2: Hippocampus-dependent memories are impaired by neurogenesis inhibitors. Object location memory was impaired in mice treated with **(A)** AraC, **(B)** TMZ and **(C)** gamma irradiation (7Gy). *($p<0.05$); **($p<0.001$) and ***($p<0.0001$) indicate difference from the hypothetical value of 0.5 in sample t-test analysis. Contextual fear memory was slightly affected by **(D)** TMZ, while **(E)** gamma irradiated animals presented a severe deficit in this memory. ***($p<0.0001$) indicates difference between groups. **(F)** Sham and irradiated mice learned to find the hidden platform in the Morris water maze. ****($p<0.00001$) indicates difference from the respective day 1. **(G)** Gamma irradiation impaired spatial memory during the probe test. ****($p<0.00001$) indicates difference between groups.

Figure 3: Long-term social recognition memory is severely affected by the inhibition of neurogenesis. All control animals explored less the familiar juvenile during the test (TT) compared to training (TR) session (*, ** and *** mean $p<0.05$; $p<0.001$ and $p<0.0001$, respectively). Gamma irradiated mice even explored more the familiar juvenile during the TT (*$p<0.05$).

Figure 4: AraC (**A**, **B** and **C**), TMZ (**D**, **E** and **F**) and gamma irradiation (7Gy) (**G**, **H** and **I)** inhibited cell proliferation ($BrdU^+$), neurogenesis ($BrdU/NeuN^+$) and astrogenesis ($BrdU/GFAP^+$) in the granular cell layer (A, D and G) and mitral cell layer (B, E and H) of the olfactory bulb. **$p<0.001$, *** $p<0.0001$ and **** $p<0.00001$ indicate difference from the respective control group. We also quantified the percent of inhibition in granular cell layer (J, L and N) and mitral cell layer (K, M and O). **(J)** In granular cell layer,

proliferation was less inhibited by gamma irradiation (7Gy) compared to AraC (***p<0.001) and TMZ (**p<0.01), though in **(K)** mitral cell layer, TMZ was the less effective, compared to AraC (**p<0.01) and 7Gy (****p<0.0001). **(L)** Neurogenesis was inhibited at higher levels by AraC (**p<0.01) in granular cell layer, while **(M)** no differences between treatments was observed in mitral cell layer. **(N)** Gamma irradiation inhibited less the astrogenesis compared to AraC and TMZ (*p<0.05 and **p<0.01, respectively). **(O)** Astrogenesesis in mitral cell layer was equally affected by the three methods.

Figure 5: Inhibiting neurogenesis with AraC **(A)**, TMZ **(B)** or gamma irradiation **(C)** produce similar behavior as their respective control group in the spatial olfactory task.

Figure 6: AraC (**A** and **B**), TMZ (**C** and **D**) and gamma irradiation (7Gy) (**E** and **F)** inhibited cell proliferation ($BrdU^+$), neurogenesis ($BrdU/NeuN^+$) and astrogenesis ($BrdU/GFAP^+$) in the dentate gyrus of dorsal hippocampus. *** p<0.0001 and **** p<0.00001 indicate difference from the respective control group. We also compared inhibition strategies by the percent of inhibition. **(G)** Cell proliferation and neurogenesis **(H)** presented higher levels of inhibition induced by gamma irradiation (7Gy) compared to AraC and TMZ (****p<0.0001). **(I)** AraC inhibited more astrogenesis compared to TMZ (*p<0.05).

Figure 7: AraC (**A** and **B**), TMZ (**C** and **D**) and gamma irradiation (7Gy) (**E** and **F)** inhibited cell proliferation ($BrdU^+$), neurogenesis ($BrdU/NeuN^+$) and astrogenesis ($BrdU/GFAP^+$) in the dentate gyrus of ventral hippocampus. *p<0.05, **p<0.01, *** p<0.0001 and **** p<0.0001 indicate difference from the respective control group. We also compared inhibition strategies by the percent of inhibition. **(G)** Cell proliferation was inhibited differently by AraC (*p<0.05, compared to 7Gy) and TMZ (****p<0.05, compared to AraC and 7Gy). **(H)** Neurogenesis and **(I)** astrogenesis were inhibited at similar levels by AraC, TMZ and 7Gy.

Figure 8: Correlation between percentage inhibition of proliferation and social recognition memory performance, expressed as social recognition index. Inhibition was induced by by **(A)** AraC, **(B)** TMZ or **(C)** Gamma Irradiation in dentate gyrus region of dorsal (Dorsal DG) and ventral (Ventral DG) hippocampus, as well as the granular cell layer from olfactory bulb. *, ** and *** indicate significant correlation.

Highlights

- Long-term social recognition memory relies on constitutive neurogenesis;
- AraC, TMZ and gamma irradiation inhibited cell proliferation, neurogenesis and astrogenesis at different levels;
- Dorsoventral axis of the hippocampus is distinctly susceptible to neurogenesis inhibition;
- Gamma irradiation was the method used to inhibit neurogenesis that cause less side effects;
- Cell proliferation inhibition in the dorsal hippocampus impacted animal's performance on social recognition test, independently of the method used to inhibit neurogenesis.