

Research Report

Age-related loss of recognition memory and its correlation with hippocampal and perirhinal cortex changes in female Sprague Dawley rats

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ABSTRACT

Ageing is associated with impaired performance in recognition memory, a process that consists of the discrimination of familiar and novel stimuli. Previous studies have shown the impact of ageing on object recognition memories. However, the early stages of memory impairment remain unknown. To fill this gap, we aimed at evaluating the ability of young (Y), middle-aged (MA), and senile (S) female Sprague-Dawley rats to retain 24 h long-term recognition memory. The MA cohort was included to characterise early memory deficits under two behavioural paradigms based on spontaneous location recognition (SLR) and spontaneous object recognition (SOR) tasks. In the SLR task, there was a markedly diminished novel discrimination capacity in the MA and S rats compared with the Y ones. In the SOR task, S rats evidenced a deterioration in novelty discrimination, while MA rats partially preserved the capacity to distinguish the new stimulus as compared with Y rats. Regarding early changes from MA to S rats, immunohistochemistry showed a marked decrease in the number and diameter of adult-born immature neurons in the Dentate Gyrus (DG) with a positive correlation with behavioural performance in the SLR task. Furthermore, we found a slight reduction in CA3 mature neurons and a decrease in the number of total microglia in the perirhinal cortex (Prh) in MA and S rats as compared with Y rats. As regards changes that were only observed in S rats, we found an increase in the number of total and reactive microglia in CA3 and a reduction in the number of total microglia in the DG. We conclude that spatial discrimination capacity could be affected earlier than feature discrimination capacity. We suggest that early depletion of neurogenesis in MA rats is involved in object location recognition deficits, whereas the disruption of microglial homeostasis in the Prh could be associated with object feature discrimination capacity.

1. Introduction

In humans and laboratory animals, ageing is known to affect episodic memory, the ability to recall unique events [17]. A subtype of episodic memory is called recognition memory, which is defined as the capacity to remember previously given information [61] and comprises familiarity and recalling information.

In rats, recognition memory is typically assessed using the nonmatch

to sample paradigm, which makes use of rats' innate predisposition to examine novel stimuli more thoroughly than familiar ones. The rat may be shown a sample stimulus, and then shown an identical sample stimulus with a novel lure, and it will spend more time investigating the novel lure. The impact of normal ageing on recognition and memory is multifaceted; the time interval between the sample and the choice phase is one of the factors. During long-term intervals (24–48 h), normal ageing impairs object recognition memory in rats, and decreases in

Abbreviations: Y, Young; MA, Middle-Aged; S, Senile; Prh, Perirhinal cortex; SLR, Spontaneous Location Recognition; SOR, Spontaneous Object Recognition; DG, Dentate Gyrus; CA3, *Cornus ammonis* 3; Dh, Dentate hilus; gcl, granular cell layer; sgz, subgranular zone; Spy-, *Stratum pyramidale*; Sl, *Stratum lucidum*; rf, rhinal fissure.

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novelty discrimination have been reported to occur at longer delay intervals [6,18,41,42,60]. When tested at short intervals (minutes-few hours), however, aged rats behave as if they are young [9]. Another item to take into account is the training protocol. Ageing was found to impair both object location and object feature recognition tasks when using relatively short training sessions [9,34]. Nonetheless, the high grade of reinforcement allows better performance in the choice phase in the aged mice [64]. One more point to consider is that recognition memory could be separated, as mentioned above, in object location and object features discrimination. Hence, there is a functional specialisation of structures within the medial temporal lobe “memory system”, as well as independence of age-related deficits in different cognitive domains, although it is well known that normal ageing processes affect several behaviours related to the medial temporal lobe. In this way, the hippocampus has a determined function – to make recollection assessments, which are related to context or spatial location [61] – and the Prh has a different function – to determine familiarity, which involves the recognition of previously presented objects [26] –. Memory consolidation in the object location discrimination seems to be hippocampus-dependent, while memory consolidation in the object features discrimination relies on different parts of the brain as Perirhinal cortex (Prh) [37].

Since object recognition has not been well characterised from middle-aged to very old rats, we submitted three cohorts of female Sprague-Dawley rats: young, late-middle-aged, and senile, submitted to Spontaneous location recognition (SLR) task and Spontaneous object recognition (SOR) task to study which cognitive domains were affected in each test. Including the late-middle-aged group let us determine which of those changes we observed in the senile group were taking place earlier. In order to correlate our behavioural data of SLR and SOR tasks with histopathological changes in the Dentate Gyrus-CA3 hippocampal subregions and the Prh, respectively, we also performed stereological quantification of neuroblast, neurons, and microglia.

2. Materials and methods

2.1. Animals

For the SLR and SOR experiments, Young (Y: 5 mo., $n = 25$), Middle-aged (MA: 18 mo., $n = 32$), and Senile (S: 28 mo., $n = 26$) female Sprague-Dawley (SD) rats were used.

To determine minimal distance to displaced objects for the SLR task and similar and dissimilar conditions for the SOR task, 6-month-old female Sprague-Dawley (SD) rats were used ($n = 18$).

Animals were housed in a temperature-controlled room (22 ± 3 °C) on a 12:12 h light/dark cycle with food and water available ad libitum. All experiments with animals were performed in accordance with the Institutional Animal Care and Use Committee (IACUC Protocol No P02-0-2017). Behavioural testing took place during the light phase (between 10:00 A.M. and 5:00 P.M.).

2.2. Behavioural tasks

In order to evaluate the effect of ageing on object recognition capabilities in female SD rats we performed two types of experiments. Animals were transferred to the testing room and acclimated for at least 1 h before habituation and testing. Each rat was habituated to the empty testing chamber (5 min) after being handled for 3 days.

2.2.1. Spontaneous location recognition (SLR) task

Rats were subdivided in three age groups: Y ($n = 12$), MA ($n = 15$), and S ($n = 13$). The testing chamber was a black circular medium density fibreboard chamber (90 cm diameter x 30 cm high). On the sample phase (10 min), rats were exposed to three identical objects (A1, A2, and A3) placed in equidistant locations, 40 cm apart. During the choice phase (5 min), two identical objects (A4 and A5), one of them in a novel location between and equidistant from the two previous locations

(displaced 20 cm from the original location), and the other one in its original location, were shown. Previously to the SLR task we set up the minimal distance that Y rats can discriminate. For this purpose, twenty-four hours after familiarisation with objects A1, A2, and A3 in the sample phase, 6-month-old rats were subdivided and tested on two distance categories in the choice phase (group A: 15 cm, $n = 4$; group B: 20 cm, $n = 4$).

2.2.2. Spontaneous object recognition (SOR) task

Rats were subdivided in three age groups: Y ($n = 12$), MA ($n = 15$), and S ($n = 13$). The testing chamber was a white foam board triangle device (each wall 60 cm long x 70 cm high). We employed the similar version of the task [31]. In the sample phase (10 min), two of the three objects (cans/bottles/lanterns) shared one feature (AB and BC). In the choice phase (5 min), a novel object was made of the two non-shared features of the objects presented in the sample phase (AC), and the familiar object was a copy of the third object (DE). To test the SOR task performances, we previously used low versus high overlap conditions (dissimilar versus similar) in 6-month-old rats (Group A dissimilar; $n = 6$; group B similar; $n = 4$). In the dissimilar condition rats were familiarised with object A and B during a 10 min sample phase. In the choice phase, a copy of object A was familiar and a novel object C was introduced during a 5 min choice phase.

In both experiments, the testing chamber and used objects were cleaned with 10 % ethanol before the next rat was tested.

Behaviour was recorded with a video tracking system. Frequency of object interactions and time spent exploring each object were analysed. The results were expressed as a discrimination ratio $[(t_{\text{novel}} - t_{\text{familiar}}) / t_{\text{total}}]$, where t_{novel} is the time that rats spent exploring the novel object/location, t_{familiar} is the time exploring the familiar object/location, and t_{total} is the total exploration time.

2.3. Brain processing

Animals were placed under deep anaesthesia and perfused with phosphate-buffered paraformaldehyde 4 % (pH 7.4, fixative). Brains were rapidly removed and stored in paraformaldehyde 4 % overnight (4 °C). Finally, brains were maintained in cryopreservative solution at -20 °C until use. Brains were coronally cut in 40 μm -thick sections with a vibratome (Leica Microsystems, Heerbrugg, Switzerland). For immunohistochemistry and image analysis, one every six serial sections was selected in order to obtain a set of non-contiguous serial sections of the dorsal hippocampus and Prh [39].

2.4. Immunohistochemistry

All immunohistochemical techniques were performed on free-floating sections. For each animal, separate sets of sections were immunohistochemically processed using anti-doublecortin (DCX) goat polyclonal antibody (marker of adult-born immature neurons; dilution 1:1000; c-18, Santa Cruz Biotech., Dallas, Texas), anti-neuronal nuclei (NeuN) mouse monoclonal antibody (marker of mature neurons; dilution 1:500; Millipore Cat# MAB377), and an anti-ionised calcium-binding adaptor molecule 1 (Iba-1) rabbit polyclonal antibody (marker of microglia; dilution 1: 1000; 016-20001; Wako Chemicals, Richmond, VA, USA). For detection, the Vectastain® Universal ABC kit (1:500, PK-6100, Vector Labs., Inc., Burlingame, CA, USA) employing 3, 3'-diaminobenzidine tetrahydro-chloride (DAB) as chromogen was used. After overnight incubation at 4 °C with the primary antibody, sections were incubated with biotinylated secondary antibody horse anti-goat (1:300, BA-9500, Vector Labs), horse anti-mouse (1:300, BA-2000, Vector Labs), or horse anti-rabbit (1:300, BA-1100, Vector Labs), as appropriate, for 120 min, rinsed and incubated with avidin-biotin-peroxidase complex (ABC Kit) for 90 min and then incubated with DAB. Sections were counterstained with Nissl method (0.5 % cresyl violet solution at 37 °C for 10 min) and mounted with Vectamount (Vector Labs) for

image analysis.

2.5. Image analysis

We applied double-blind criteria to randomly select rats from each group. To link the hippocampus with SLR, we analysed DCX, NeuN, and Iba1 immunohistochemistry (6 rats each). In the case of SOR, we associated NeuN and Iba1 immunohistochemistry changes in Prh (4 rats each). In each brain block, one out of six serial sections was selected to obtain a set of non-contiguous serial sections. For this task, an Olympus BX-51 microscope attached to an Olympus DP70 CCD video camera (Tokyo, Japan) was used. Cells were counted in four or five anatomically matched sections per animal. The total number of cells was estimated using a modified version of the optical dissector method [62]. Individual estimates of the total bilateral neuron number (N) for each region were calculated according to the following formula: $N = RQ\Sigma \times 1/ssf \times 1/asf \times 1/tsf$, where $RQ\Sigma$ is the sum of counted neurons, ssf is the section sampling fraction, asf is the area sampling fraction, and tsf is the thickness sampling fraction. In all cases, Image Pro Plus v5.1 software (IPP, Media Cybernetics) was used for the analysis.

2.5.1. Adult-born immature neuron analysis

Adult-born immature neurons of the DG were detected by DCX immunohistochemistry in the hippocampus. The cell number was assessed using a modified optical fractionator technique as previously described [33]. Due to the low number of DCX neurons in older rats, the entire subgranular zone and granular cell layer sections were used as asf . We did not use guard zones, making the hsf (height sampling fraction) equal to section thickness. The left and bottom borders were used as the exclusion borders. Estimates were based on counting DCX positive cell bodies as they came into focus multiplying by ssf . We also assessed the DCX positive cells diameter by using the manual measurement tool of the Image Pro Plus v5.1 after calibrating the image.

2.5.2. Mature neuron analysis

Mature neurons were detected as NeuN positive cells in the DG and CA3 *Stratum pyramidale* (Spy) regions of the hippocampus, as well as in the Prh. To analyse possible age-related changes, the NeuN immunoreactive area was determined as previously described by Zappa Villar et al. [67]. For this purpose, four sections per animal and three fields per section were sampled. In each field, NeuN positive cells were segmented using the colour cube method (Image Pro Plus v5.1). To calculate the immunoreactive area, the sum of individual areas was multiplied by the ssf .

2.5.3. Microglial cell analysis

Microglial cells were identified as Iba1 immunoreactive (Iba1ir) cells. These cells were counted in the hippocampus *Dentate hilus* (Dh) and *Stratum lucidum* (Sl) regions and in the Prh. The Iba1 cell number was estimated by a modified version of the optical dissector method [67]. In all analysed regions, four sections per animal were used, two fields per section, where a $250 \times 250 \mu\text{m}$ squared probe was superimposed over calibrated images. The left and bottom borders were used as the exclusion borders and cells inside the probe area were counted. Estimates were based on counting Iba1 positive cell bodies as they came into focus multiplying by $ssf \times asf$. For more details about DG and CA3 see [supplementary table 1](#).

In order to determine whether there was a neuroinflammatory phenotype, microglial cells were morphologically classified as Types I, II, III, IV, and V as previously described by Diz-Chaves [20]. Types I, II, and III were categorised as non-reactive microglia; Types IV and V were taken as reactive microglia.

2.5.4. Correlation between morphological changes and behavioural performance

To correlate hippocampal changes with SLR performance and Prh

changes with SOR performance, we applied double-blind criteria to randomly selected rats from each group.

2.6. Statistical analysis

Data were compiled and analysed with the SigmaPlot v.11 software (San Jose, CA, USA). Two-way repeated-measurements of ANOVA (Factors Age and Object as independent variables) were used to determine age-related preference for the objects in the sample phase. For the choice phase, one-way ANOVA was used to group comparisons (Factor age as independent variable) and one-sample t-test to evaluate discrimination capacity considering the difference between the mean of the sampled population and the hypothesised population mean is equal to zero (0). Stereological data were analysed with one-way ANOVA (Factor age as independent variable). In the case that data did not pass normality or equal variant criterion, Kruskal-Wallis ANOVA were made. Holm-Sidak and Dunn's post-hoc tests were used when appropriate.

Spearman rank order correlation was made to correlate brain changes with memory performance.

All data are presented as mean \pm SEM. Criteria for significant differences were set at the 95 % probability level.

3. Results

3.1. Spontaneous location recognition task

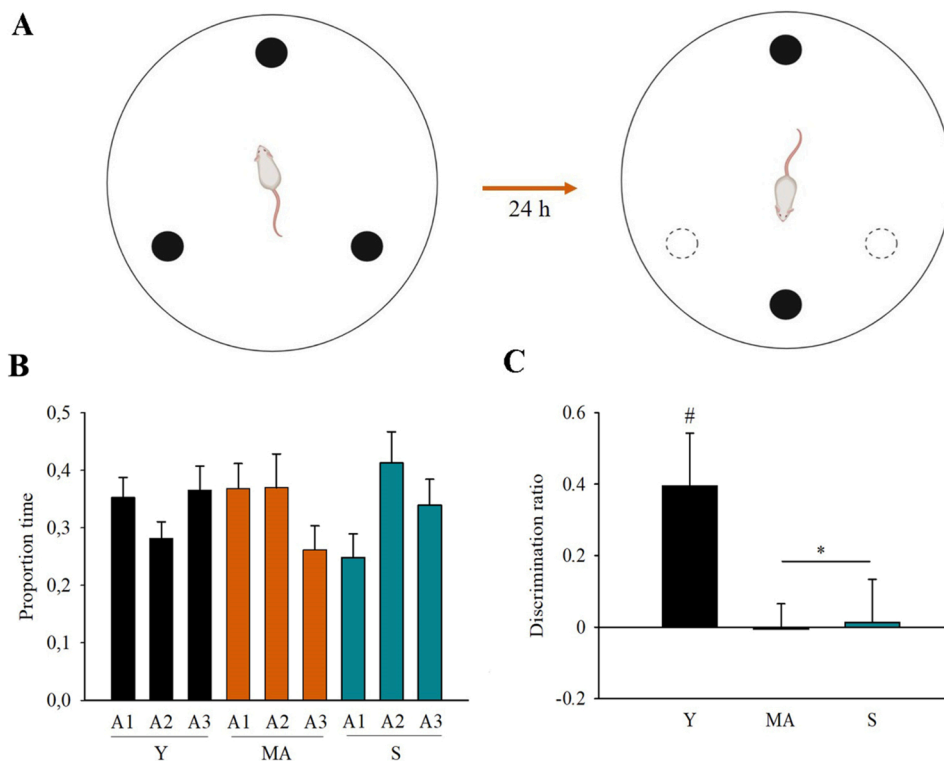
We analysed the fraction of time that rats spent exploring each of the objects in the sample phase. There was no significant difference in sampling exploration for each object among the groups (Two-way repeated-measures of ANOVA: Age factor: $F_{(2, 76)} = 3.2 \times 10^{-15}$; $P = 1$; Object factor: $F_{(2, 76)} = 0.32$; $P = 0.72$; Age x Object factor: $F_{(4, 76)} = 1.82$; $P = 0.13$) (Fig. 1B), so that the differences in the discrimination ratio cannot be explained by preferential exploration for any of the objects during this phase. For the choice phase, we expressed our results as a discrimination ratio $[(t_{\text{novel}} - t_{\text{familiar}})/t_{\text{total}}]$. The discrimination capacity was significantly reduced by age (One-way ANOVA: $F_{(2, 39)} = 3.85$; $P = 0.03$). *Post-hoc* comparisons revealed a significant deficit in performance in MA rats, and thus these changes are maintained in S rats (Holm-Sidak method; $P < 0.05$) (Fig. 1C). One-sample t-test indicated that only Y animals were able to discriminate the novel location ($T_{(11)} = 2.67$; $P = 0.02$), whereas the discrimination ratios of MA and S animals were not different from zero ($T_{(14)} = -0.07$; $P = 0.94$, and $T_{(12)} = 0.10$; $P = 0.92$, respectively). Additionally, we measured the exploratory activity during sample and choice phases and found no significant differences among the groups (Table 1).

3.2. Minimal distance setup for the SLR task

Both groups A and B explored equally all three objects during the 10 min sample trial (One-way repeated measures of ANOVA; Group A: $F_{(2,6)} = 0.70$, $P = 0.53$; Group B: $F_{(2,6)} = 1.66$, $P = 0.27$). During the choice phase, preference for the displaced object increased as a function of distance category (One-sample t-test; Group A: $T_{(6)} = 0.80$, $P = 0.45$; Group B: $T_{(6)} = -3.37$, $P = 0.015$) (Supplementary Fig. 1).

3.3. Spontaneous object recognition task

We assessed the same parameters as in the SLR task. There was no significant difference in sampling exploration among groups (Two-way repeated-measures of ANOVA: Age factor: $F_{(2,76)} = 1.65 \times 10^{-14}$; $P = 1$), but there was a different exploration frequency among objects (Two-way repeated-measures of ANOVA: Object factor: $F_{(2, 76)} = 20.25$; $P < 0.001$), and there is an interaction between age and object (Two-way repeated-measures ANOVA: Age x Object factor: $F_{(4, 76)} = 3.14$; $P = 0.02$). *Post-hoc* comparisons revealed that Y and MA rats showed an equal preference for objects AB and DE, but a minor preference for



object BC. S rats presented an equal preference for objects AB and BC, but a major preference for object DE (Holm-Sidak method; $P < 0.05$) (Fig. 2B).

In the choice phase we found that the performance was significantly affected by age (One-way ANOVA: $F(2, 36) = 13.79$; $P < 0.001$). *Post-hoc* comparisons showed statistically significant differences between Y and S rats ($T = 5.09$; $P < 0.001$) and between MA and S rats ($T = 3.75$; $P < 0.001$), but not between Y and MA rats (Holm Sidak test: $T = 1.59$; $P = 0.12$) (Fig. 2C). One-sample t-test indicated that only Y animals were able to discriminate the novel object ($T_{(12)} = 3.16$; $P = 0.008$), whereas the discrimination ratio of MA animals was not different from zero ($T_{(15)} = 1.51$; $P = 0.18$), and S animals discriminated negatively the novel object ($T_{(12)} = -3.44$; $P = 0.005$). Additionally, we measured the exploratory activity during sample and choice phases and found no significant differences among the groups (Table 1).

3.4. Dissimilar to similar objects features setup for the SOR task

Prior to run the SOR task in 6-month-old female rats, we tested discrimination capacity in a dissimilar to similar condition. In both conditions object discrimination of low and high similarity remained

Fig. 1. Spontaneous Location Recognition Task. Schematic representation of the SLR task (A). Proportion time of exploration for each of the locations during the sample phase of the SLR task. Rats spent an equal amount of time exploring each of the three locations during this phase (B). Discrimination ratios during the choice phase (C). Data are expressed as the mean \pm SEM. Discrimination ratios were significantly different at $*P < 0.05$ when compared with Y control group (Holm-Sidak post-hoc test), $\# P < 0.05$ with respect to zero (One-sample t-test); Y n = 12, MA n = 16, and S n = 13.

intact (Group A: One-sample t-test $T = 6.01$ (5), $P = 0.002$; Group B: $T = 13.12$ (2) ($P = 0.006$) (Supplementary Fig. 2)). Note that we discarded a rat in Group B because we did not complete the task.

3.5. Adult-born immature neuron analysis

The DCX immunohistochemistry revealed differences in the median values among the age groups (Kruskal-Wallis One-Way ANOVA on ranks: $H(2,12) = 9.5$; $P = 0.009$). *Post-hoc* comparison revealed a decrease in the DCX neuron population in the older groups (MA and S rats) (Dunn's method: $P < 0.05$) (Fig. 3D). We assessed the diameter of DCX cells, which was also affected by the age factor (One-way ANOVA: $F(2,12) = 8.67$; $P = 0.005$). *Post-hoc* comparison showed a reduction in the mean diameter in the older cohorts as compared with their young counterparts (Holm-Sidak method: $P < 0.05$) (Fig. 3D inset).

3.6. Mature neuron analysis

The NeuN immunoreactive area revealed significant differences between the older groups (MA and S) and the Y rats in CA3 Spy (Fig. 4D; Table 2; one-way ANOVA group factor $F(2, 13) = 9.38$; $P = 0.008$). *Post-hoc* comparisons showed a significant reduction of about 12 % MA and 18 % S of CA3 Spy as compared with Y counterparts (Holm-Sidak method: $T = 4.13$; $P = 0.003$ and $T = 3.06$; $P = 0.016$ for MA and O, respectively). In contrast, there were no changes in the principal granule neurons in the DG and Prh.

3.7. Microglial cell analysis

There were a significant changes between the groups in the total microglial cell number in the DG-CA3 hippocampal region as well as in the Prh cortex (One-way ANOVA group factor for DG: $F(2, 11) = 8$; $P = 0.001$; for CA3: $F(2,11) = 16.22$; $P = 0.001$; and for Prh: Kruskal-Wallis One-Way ANOVA on ranks: $H(2,7) = 6.3$; $P = 0.023$).

In the CA3, the quantification showed an increase of about 25 % in the number of microglial cells only in S rats as compared with the Y

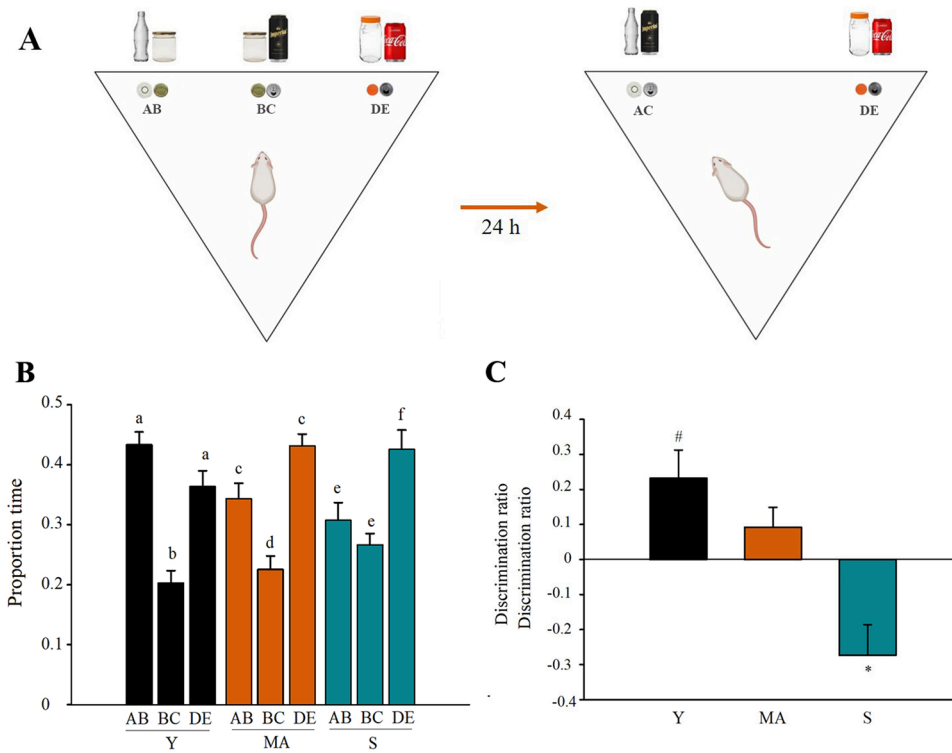


Fig. 2. Spontaneous Object Recognition Task. Schematic representation of the SOR task (A). Proportion time of exploration for each of the objects during the sample phase of the SOR task (B). There were differences in the time that rats spent exploring each of the three objects. Discrimination ratios during the choice phase (C). Data are expressed as the mean ± SEM. Equal preference for an object is represented by the same letter (a, c, and e) and difference preference is represented by a different letter (b, d, and f). Discrimination ratios were significantly different at * $P < 0.05$ when compared with Y control group (Holm-Sidak post-hoc test), # $P < 0.05$ to zero (One-sample t-test); Y n = 13, MA n = 16, and S n = 13.

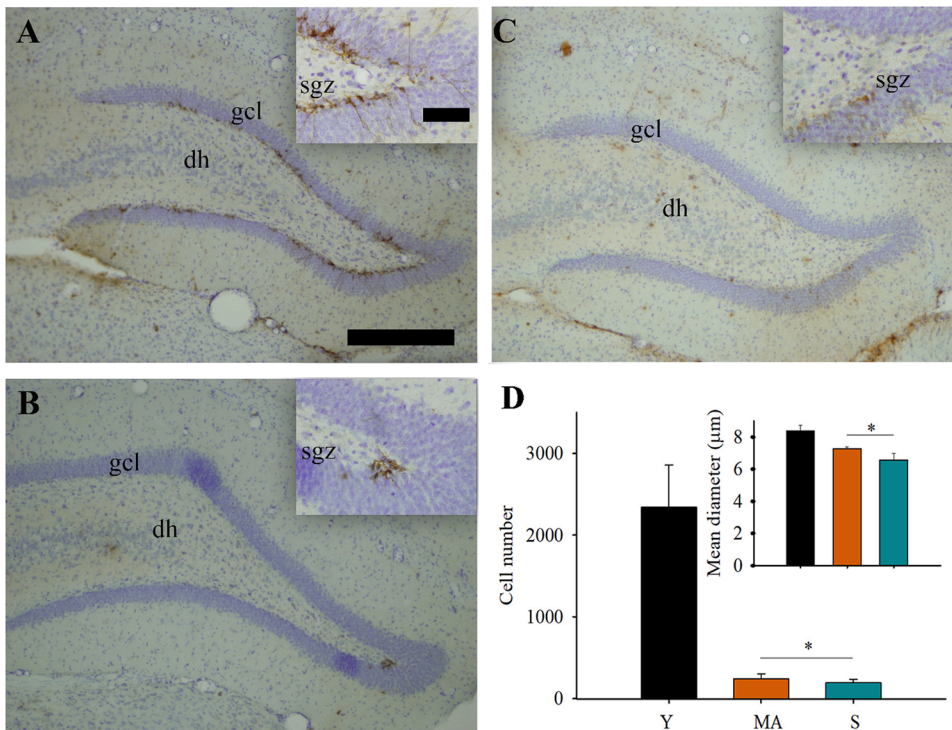


Fig. 3. Adult-born immature neurons number and mean diameter in the DG. Coronal sections of the DG in representative animals of each age group showing DCX positive neurons (A–C). Young group (A). Middle-Aged group (B). Senile group (C). Scale bar = 200 µm for panoramic images and 50 µm for inset. DCX cell numbers (D). Note the sharp age-related fall in DCX cells. Data are expressed as the mean ± SEM. Abbreviations: dh (dentate hilus); gcl (granular cell layer); sgz (subgranular zone). For cell number and mean diameter Dunn and Holm-Sidak post-hoc test was used for group comparisons respectively; * $P < 0.05$ of aged animals versus the corresponding Y controls. The number of Y, MA and S hippocampi assessed for DCX was 5 for all of the age groups.

group (Fig. 5D; Table 3). On the contrary, in the DG, quantification reveals a reduction of about 25 % in the number of the entire microglial cell population only in S rats (Supplementary Fig. 3), whereas in the Prh, the fall in the number of microglia affects early MA (about 20 %) and shows a precipitous fall towards old age in S rats, reaching about 30 % (Fig. 5D; Table 3).

In addition, microglial cells were classified as reactive and non-

reactive. We did not find significant differences between the groups in the number of reactive cells in the DG region (One-way ANOVA group factor $F(2, 11) = 0.76$; $P = 0.5$) or in the Prh (One-way ANOVA group factor $F(2, 14) = 0.31$; $P = 0.74$). However, we did find significant differences in the CA3 Stratum lucidum (Sl) region (One-way ANOVA group factor $F(2, 11) = 5.93$; $P = 0.023$). Group comparison revealed an increase in reactive microglial numbers only in S rats (Fig. 6D).

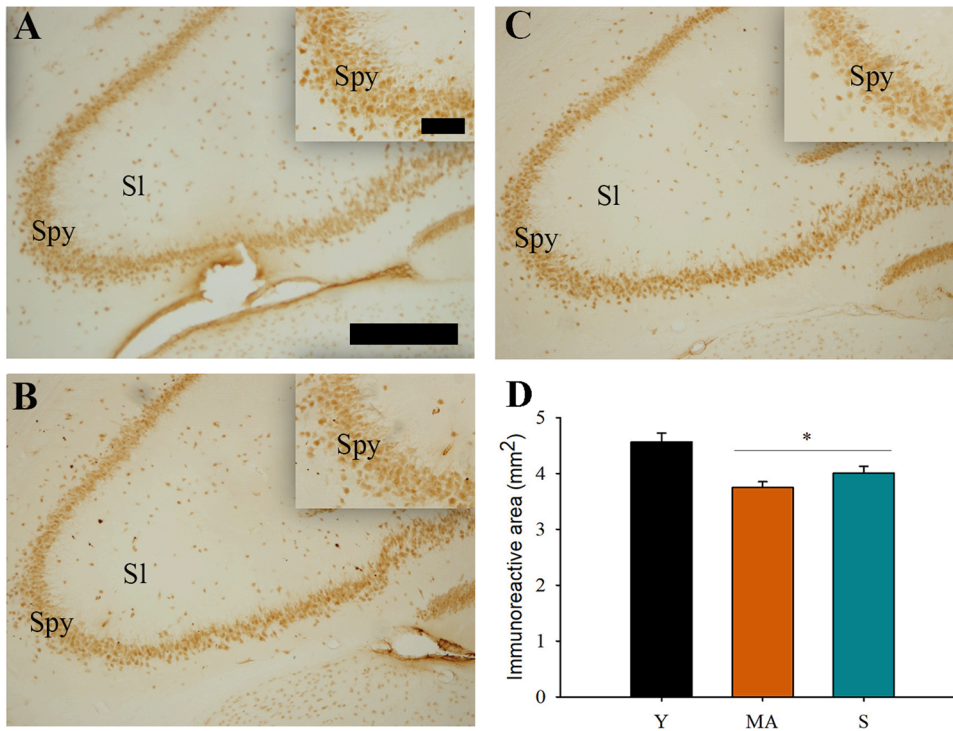


Fig. 4. Mature neuron immunoreactive area in the Stratum pyramidale CA3 region. Coronal sections of the CA3 in representative animals of each group showing NeuN positive cells (A–C). Young group (A). Middle-Aged group (B). Senile group (C). Scale bar= 200 μ m for panoramic images and 50 μ m for inset. NeuN immunoreactive area (D). Notice that the neuron immunoreactive area decreased in both MA and S rats. Abbreviation: Sl: stratum lucidum. Data are expressed as the mean \pm SEM. Comparisons were made versus the Y group data point (Holm-Sidak post-hoc test). * $P < 0.05$. Y n = 4; MA n = 3 and S n = 4.

Table 2
Neu-N immunoreactive area in DG, CA3 and Prh (Mean \pm SEM; mm²).

| | DG | CA3 | Prh |
|----|-------------------------|-------------------------|-------------------------|
| Y | 7 \pm 0.47 (N = 4) | 4.56 \pm 0.15 (N = 4) | 3.01 \pm 0.12 (N = 4) |
| MA | 7.19 \pm 0.03 (N = 3) | 3.74 \pm 0.10 (N = 3) | 2.65 \pm 0.33 (N = 2) |
| S | 6.61 \pm 0.29 (N = 4) | 4 \pm 0.12 (N = 4) | 2.84 \pm 0.09 (N = 5) |

However, the ratio between reactive and total microglia reveals no changes in S as compared to Y rats (Table 3).

3.8. Correlation between cellular changes and memory test

The correlations were calculated between cellular changes and behavioral measures obtained within each age group. The data corresponding to SLR discrimination ratio correlated positively with newborn immature neuron number (Spearman rank order correlation:

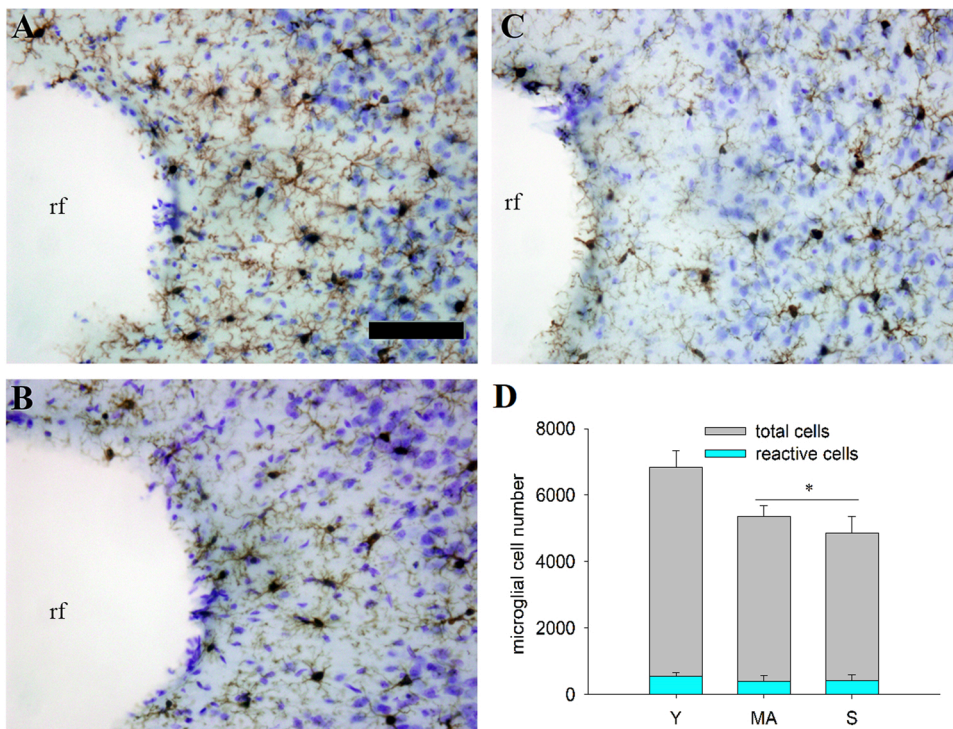


Fig. 5. Microglial cell number in the Prh. Coronal sections of the Prh in representative animals of each group showing Iba1 positive cells (A–C). Young group (A). Middle-Aged group (B). Senile group (C). Scale bar 100 μ m. The number of the total microglial cells decreased in the MA and S rats, whereas reactive microglia remained unchanged (D). Grey bars correspond to total microglia and cyan bars to reactive microglia. Abbreviation: rf: rhinal fissure. Data are expressed as the mean \pm SEM. Comparisons were made versus the Y group data point (Holm-Sidak post-hoc test). * $P < 0.05$. Y n = 3; MA n = 5 and S n = 6.

Table 3

Microglial cell number (Iba1) showed as total cells, reactive cells, and reactive/total ratio in DG, CA3 and Prh (Mean ± SEM, ratio, N = number of subjects).

| | DG | CA3 | Prh |
|----|--------------|--------------|--------------|
| Y | 3912 ± 272 | 2704 ± 78 | 6830 ± 497 |
| | 1566 ± 146 | 1054 ± 88 | 548 ± 120 |
| | 0.40 (N = 4) | 0.38 (N = 4) | 0.08 (N = 3) |
| MA | 3783 ± 88 | 2550 ± 229 | 5361 ± 323 |
| | 1314 ± 175 | 1093 ± 126 | 333 ± 136 |
| | 0.36 (N = 4) | 0.43 (N = 4) | 0.06 (N = 3) |
| S | 2898 ± 141 | 3667 ± 96 | 4861 ± 503 |
| | 1361 ± 138 | 1532 ± 110 | 292 ± 169 |
| | 0.45 (N = 4) | 0.42 (N = 4) | 0.08 (N = 4) |

$\rho_{(15)} = 0.629$; $p < 0.05$) and the new-born immature neuron diameter in the choice phase (Spearman rank order correlation: $\rho_{(15)} = 0.561$; $p < 0.05$) (Supplementary Fig. 4). Furthermore, we ran the correlation among a slight reduction in the immunoreactive area in CA3, increment of reactive microglia cell number in CA3 and discrimination ratio in SLR task. In corresponding to SOR task performance, we ran the correlation of the reduction of total microglia cell number in Prh. These data showed no significant relationships between any pair of variables (Table 4).

4. Discussion

The present study aims to characterise the effect of ageing on object recognition performance in Sprague-Dawley female rats. There are many reports on different rat strains and of different ages, but mostly on males. Our group has a great deal of experience studying the female Sprague-Dawley rat as an ageing model of spontaneous neuronal dysfunction in the hypothalamus and the substantia nigra [48,49]. More recently, we have reported differences in spatial memory performance between 26 and 32-month-old S rats and their correlation with hippocampal changes [33]. These results led us to visualise that functional changes in the brain accumulate progressively. Thus, to pave the way for the temporal window when mnemonic impairments become evident, the 18-month-old female rats were submitted to tests in order to track an early deficit in recognition memory. This cohort of 18-month-old female

rats (late middle age) was selected because early behavioural alterations are critical for detecting early changes for the starting of age-related cognitive decline previous to irreversible neuropathological changes. The S rats range from 24 to 32 months old, while MA rats range from 12 to 18 months old, depending on the strain and sex [54,52]. Comparisons between MA and S groups is essential to our understanding of the ageing process because developmental changes could take place before rats become older [15]. To test the impact of ageing on the ability to discriminate recognition memories, we designed two recognition tasks based on spontaneous recognition memory tasks, which are short, simple, and stress-free [65]. The rationale is that the hippocampus is more relevant in the context and spatial object recognition, while the Perirhinal cortex is important in the recognition of object features. Thus, we ran out two experiments to examine whether ageing has a differential impact on these temporal lobe structures.

In the first experiment, to increase the phase of encoding/consolidation and reduce the interference, we implemented a modified version of the SLR task [7]. In this case, three identical objects were shown, instead of two. Our results revealed that the MA and S rats had a significant deficit in the retention of spatial object recognition memory. Our findings are consistent with a study of SLR in aged C57BL / 6 J mice. At the challenging 20 cm distance, younger mice performed very well, whereas old mice performed close to chance level and very poorly compared to young mice [11]. This could be explained by the fact that MA and S rats would not encoding/consolidate the information 24 h after the object's presentation. Another explanation is that animals

Table 4

Spearman correlation between cellular number and memory test.

| | DG-DCX | CA3-NeuN | CA3-Iba1 | Prh-Iba1 |
|-----|-----------------------------------|------------------------------------|------------------------------------|------------------------------------|
| SLR | $\rho(15) = 0.63$; $p = 0.01$ | $\rho(11) = -0.01$; $p = 0.94$ | $\rho(12) = -0.19$; $p = 0.54$ | |
| SOR | | | | $\rho(15) = -0.09$; $p = 0.73$ |

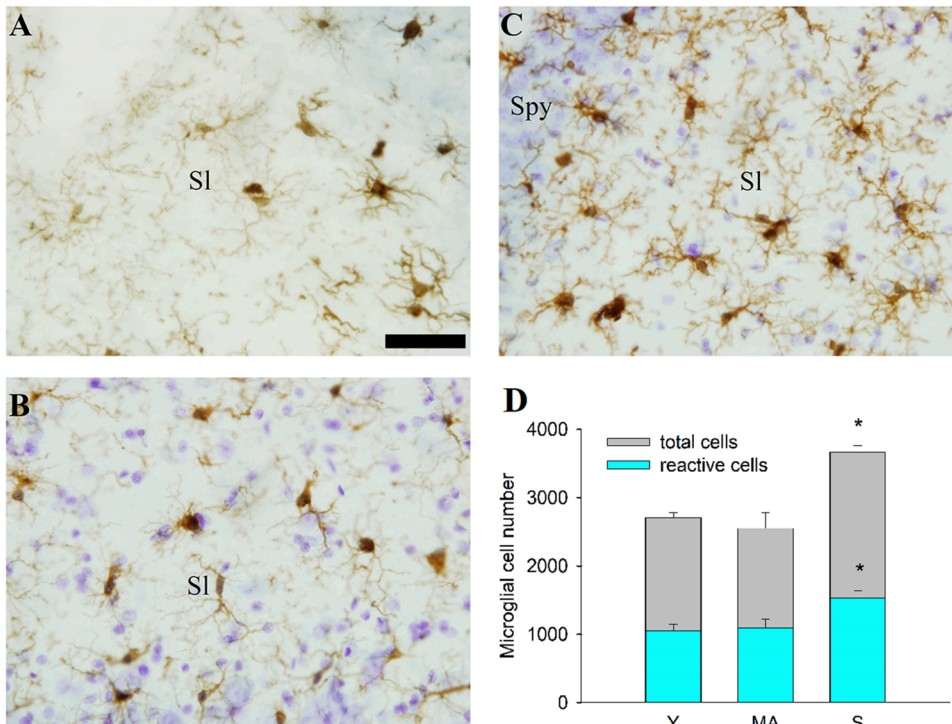


Fig. 6. Reactive microglial cell number in the CA3 Stratum Lucidum. Coronal sections of the CA3 in representative animals of each group showing Iba1 positive cells (A–C). Young group (A). Middle-Aged group (B). Senile group (C). Scale bar 50 μm. The both parameters, total and reactive microglial cell number increased only in S rats (D). Grey bars correspond to total microglia and cyan bars to reactive microglia. Data are expressed as the mean ± SEM. Group comparisons were made versus the Y group data point (Holm-Sidak post-hoc test). * $P < 0.05$. The number of Y, MA and S hippocampi assessed for Iba1 was 4 for all of the age groups.

would not distinguish the three equidistant locations at the time of encoding/consolidation in the sample phase. Therefore, in the choice phase, representations of new and familiar locations would be overlapped in a single representation in memory. This memory mechanism which is independent of the time of encoding/consolidation, is called pattern separation, a computational mechanism which the hippocampus uses to disambiguate similar inputs [14]. However, the fact that we did not test a low overlap condition in the both MA and S rats does not allow us to ensure that the deficit is related to separate overlapping stimulus as well as long-term stimulus retention [9].

In the SOR task, distinction of familiar object features and novel object features (resulting from the combination of objects that share visual features) is required. Taking into account the concepts applied in the SLR task, a modified version of the SOR task was used – the similar version of the task [31] –, consisting of a sample phase in which rats are exposed to three objects, where two objects are similar to each other (AB and BC) and the third one is dissimilar (DE). In the choice phase, the similarity between objects was manipulated by varying the number of shared features at the encoding phase, resulting in a novel object (AC). It is worth mentioning that Y and MA rats showed the same pattern of preference for objects AB and DE and a minor preference for the central object (BC). However, S rats from the beginning had a preference for the familiar object (DE), which is detrimental to similar objects' preferences (AB and BC). This behaviour was continued in the choice phase, where we observed that S rats not only impaired the ability to recognise the new object but also showed a negative preference index. That is, they presented a clear preference for the familiar object. This negative preference could be given by a poor encoding/consolidation and subsequently fail in the inputs long-term retention, indicating a recognition memory impairment. Regarding the similarity between stimulus, as mentioned before for SLR task, memory impairment could be related to pattern separation deficit. However, to confirm this hypothesis, it is extremely necessary to perform the dissimilar condition of the SOR in both MA and S rats, low similarity condition, to attribute a deficit in pattern separation instead of object recognition memory.

This finding is consistent with a study in ageing Fischer 344 x Brown Norway (F344 x BN) hybrid rat, in which the researchers used LEGO® blocks to perform the target-lure discrimination task demonstrating that aged rats were selectively impaired in trials with high target-lure similarity [28]. Interestingly, when comparing the preference for novel objects between MA and Y, they did not show significant differences, as observed in the SLR. However, MA did not have a significant preference for the novel object. This situation may be explained by intra-group variability. At that point, it suggests that the discrimination capacity of object features is partially retained in the late middle-aged (18-month-old), but some deeper investigation in the early middle-aged (12 months) would be needed to confirm this.

The Prh plays an important role in perceptual processing and, as noted, its lesion affects recognition memory [2,1]. While it is involved in object recognition once it is necessary to represent basic information about familiarity or novelty of an object, the hippocampus is involved in object memorisation by encoding information about the experiences related to the object. In the recognition memory formation, the dorsal hippocampus plays an important role, especially when spatial or contextual information is a relevant factor [24]. Although the hippocampus could not play a direct role in discriminating the different features of each object, it is fundamental as a novelty detector because of its role in comparing previously stored information with new incoming aspects of one particular situation [13]. Since ageing brings about a marked decline in the hippocampal function, it is likely that this decline constitutes a central factor in the recognition memory deficits observed in the present study. In the hippocampus, the DG is thought to be important for the storage of spatial memory relevant for pattern separation. A growing body of evidence supports the hypothesis that adult-born granule cells contribute to this process. Thus, the suppression of hippocampal adult neurogenesis in mice by X-ray irradiation alters

the animal's ability to distinguish subtle changes in spatial discrimination but not gross changes [14]. Furthermore, a dominant-negative strategy to inhibit the generation of new neurons in the DG of Long-Evans male rats affects discrimination of object locations when the load on pattern separation is high, but not when the load on pattern separation is low [8]. Conversely, genetically increased levels of adult neurogenesis improved spatial discrimination of two similar contexts [47]. We previously reported a decline in the number of adult-born immature neurons in the hippocampal DG in S rats [33,38]. In this work we described for the first time in the late MA female Sprague-Dawley rat that there is a sharp decrease in the number of adult-born immature neurons, a result that is in line with other studies [35,43]. The fact that neurogenesis decreases in MA rats at the same level observed in S rats suggests premature depletion of neurogenesis in the hippocampus. This state alters long-term recognition memory in a low-grade separation condition. Also, a decrease in the diameter of neuroblasts in the DG could be an additional hallmark of hippocampal ageing. Other authors report shrinkage of large neurons with consequently increasing numbers of small neurons in the ageing brain of healthy humans [57].

The implication of CA3 hippocampal subregion in the recognition memory impairment derives from pharmacological, functional imaging and electrophysiological in both humans and animals. Ibotenic acid lesion in the CA3 of Long Evans rats impairs object recognition in a study of pattern completion in the cheese board test [29]. MRI studies in old humans reveal that CA3 volume decrease correlates with object recognition accuracy impairment [19]. Additionally, hyperactivity in CA3 has been reported in humans [66], monkeys [58], and rats [63,46]. In another study in aged male F1 hybrid Fischer 344 Brown Norway rats (29- to 32-month-old), it was found that the up-regulation of A-type K⁺ channels in aged CA3 pyramidal neurons was associated with hyperactivity; thus, the alteration of neuronal signalling pathways may contribute to the accumulation of excitotoxicity and thereby promote cell death, which is more pronounced in pathological conditions such as Alzheimer's disease [53].

In this work, we examined the immunoreactive area of CA3 principal neurons labelled with NeuN, a nuclear antigen for mature neurons. Although other researchers reported no loss of relevant hippocampal neurons in ageing rats [45], we found a slight but significant reduction in the NeuN immunoreactive area in the CA3 region in MA and S rats. We hypothesize that this slight decrease in CA3 pyramidal neurons in older rats could be affecting the synaptic inputs from the entorhinal cortex, contributing to an impairment in recognition memory [4,22,66], but additional experiments would be implemented to confirm this hypothesis.

Lesion or chemical inactivation of several cortical regions impair recognition memory [12,3,25,27,32] suggesting that object recognition happens in many cortical regions. In fact, the disambiguation of object representations has been shown to require Prh [10,5].

To contribute to this issue, we proceeded to characterise age-related immunohistochemical changes in Prh. Using the same method of quantification for the characterisation of the hippocampus, we found that the NeuN immunoreactive area in Prh showed no change with age in rats, confirming previous data reporting no loss of Prh cortical neurons [44]. Other studies have found an opposite pattern of Prh activity between adult and aged exposure to odor stimulus, regarding c-Fos immunoreactivity [23].

As regards to microglia, there is a growing body of literature that has revealed its role in neural homeostasis [21,50]. In the central nervous system, microglia constitute the primary line of immune defense. Furthermore, they play regulatory and supportive roles in neuronal function by metabolising glutamate, providing nutritional support, and removing potentially toxic cell debris [16,36,59]. In this study, we observed an increase in the total and the reactive microglia in the CA3 hippocampal region only in the S group. It is important to mention that if there are no changes in the ratio between reactive and total microglia, then this increase in the reactive microglia could be a result of the total

microglial increase. We have previously described an increase in reactive microglia in another hippocampal region, the CA1 stratum radiatum [38]. In the case of DG we found a decrease in the total microglia only in the very old S rats and the reactive microglia remained unchanged. In a recently published work on MA and S male Fisher 344 rats, increased microglia numbers in MA and S rats were found in DG in contrast to a decrease in reactive microglia [30]. Additionally, it was found that microglial activation is amplified and prolonged in the aged hippocampus compared to the adult hippocampus, only affecting those regions surrounded by pyramidal neurons. Prolonged microglial activation leads to the release of pro-inflammatory cytokines that exacerbate neuroinflammation, contributing to neuronal loss and impairment of cognitive function [40,56].

To our knowledge, this is the first work reporting a morphometric analysis of microglia in the Prh of older rats. Our results evidenced a reduction in total microglial cells in the both elder cohorts, the MA and the S rats, suggesting a disruption in neuronal and microglial communication. In line with this, other groups have described a decreased number of microglia in the substantia nigra, striatum, and cerebral cortex of 18-month-old and 24-month-old male C57BL/6J mice [51]. Microglial cells have a self-renewing capacity and regenerate from their own cellular pool. Nonetheless, the mitotic activity of each cell is limited because of the shortening of telomeres, which happens during each cell division. Increasing evidence suggests that an ageing-associated microglial replicative senescence, which results in the degeneration and dystrophy of the microglia [55]. As suggested in Streit's work, age-dependent change or loss of microglial functions might be a possible explanation for the occurrence of neurodegenerative diseases in aged individuals.

Taken together, our major finding respecting object recognition memories suggests that ageing affects S rats and MA rats in both experiments. The fact that MA rats are impaired only in the SLR task but less affected in the SOR task could be explained by assuming that the Prh, which is required for the object feature discriminator node, is less affected by ageing than the hippocampus.

5. Conclusion

The present study is the first one to provide evidence regarding the effect of ageing on object recognition memories considering object location and features in female SD rats. Overall, these data provide important insight into age-related object recognition memory deficits in these rats and offer additional means for evaluating the impact of ageing on separate regions such as the DG-CA3 hippocampal circuitry and the Prh.

Credit authorship contribution statement

Gustavo R. Morel: Conception and design of study, analysis and/or interpretation of data, drafting the manuscript, revising the manuscript critically for important intellectual content, approval of the version of the manuscript to be published. **Martina Canatelli-Mallat:** Conception and design of study, acquisition of data, analysis and/or interpretation of data, drafting the manuscript, revising the manuscript critically for important intellectual content, approval of the version of the manuscript to be published. **Priscila Chiavellini:** Acquisition of data, analysis of data, approval of the version of the manuscript to be published. **Marianne Lehmann:** Acquisition of data, analysis of data, approval of the version of the manuscript to be published. **Rodolfo G. Goya:** Revising the manuscript critically for important intellectual content, approval of the version of the manuscript to be published.

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Disclosure of potential conflict of interest

We hereby declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.bbr.2022.114026](https://doi.org/10.1016/j.bbr.2022.114026).

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