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Environmental Enrichment Improves Behavior, Cognition, and Brain Functional Markers in Young Senescence-Accelerated Prone Mice (SAMP8)

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Abstract The environment in which organisms live can greatly influence their development. Consequently, environmental enrichment (EE) is progressively recognized as an important component in the improvement of brain function and development. It has been demonstrated that rodents raised under EE conditions exhibit favorable neuroanatomical effects that improve their learning, spatial memory, and behavioral performance. Here, by using senescence-accelerated prone mice (SAMP8) and these as a model of adverse genetic conditions for brain development, we determined the effect of EE by raising these mice during early life under favorable conditions. We found a better generalized performance of SAMP8 under EE in the results of four behavioral and learning tests. In addition, we demonstrated broad molecular correlation in the hippocampus by an increase in NeuN and Ki67 expression, as well as an increase in the expression of neurotrophic factors, such as pleiotrophin (PTN) and brain-derived neurotrophic factor (BDNF), with a parallel decrease in neurodegenerative markers such as GSK3, amyloid-beta precursor protein, and phosphorylated beta-catenin, and a reduction of SBDP120, Bax, GFAP, and interleukin-6 (IL-6), resulting in a neuroprotective panorama. Globally, it can be concluded that EE applied to SAMP8 at young ages resulted in epigenetic regulatory mechanisms that give rise to significant beneficial effects at the molecular, cellular, and behavioral levels during brain development, particularly in the hippocampus.

Keywords Neurodenegeration · Behavior · Learning · Enriched environment · Neurogenesis · Apoptosis · Inflammation · Hippocampus · Neurotrophin · Tau kinases · Aging · Cognition

Introduction

The suffering and distress of animals can produce physiological and behavioral changes that in turn are able to increase the variability in experimental data, or even worse, might invalidate the research. Therefore, the need to improve and enrich the environment in which the mice spend their lives represents an area of opportunity for increasing the quality of scientific research. The first studies that clearly demonstrated the neuroanatomical effects of environmental enrichment (EE) in rats were performed in the early 1960s [1]. Since that time, various reports have shown other neurological and behavioral effects in animals raised in cages by employing different elements of EE [2–6]

Currently, a large body of evidence demonstrated the beneficial effects of EE on the brains of rodents, including the

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following: hippocampal epigenetic modification [7] and increased neurogenesis; neuronal sprouting; cell size; and improvements in learning and memory [3–6]. Thus, these favorable responses strongly suggest an effect on the development of neuronal plasticity by means of EE, which in turn could play an important role in recovery after brain damage or neurodegenerative diseases, and also in their possible prevention of the latter [5–10].

At the behavioral level, exposing mice to EE can increase their exploratory activity in the open field test (OFT) [11]. EE consistently induces biochemical and structural changes in the cortex and hippocampus [12]. Additionally, EE increases neurogenesis and positively regulates the expression of neurotrophic factors, such as the brain-derived neurotrophic factor (BDNF) and the nerve growth factor (NGF) [11]. EE causes a significant change in the genetic expression of genes that are involved in neuronal structure, plasticity, and neurotransmission [12]. Furthermore, EE can affect cytokine production and glial cell function [13]. Taken together, evidence indicates that EE can modulate brain function by improving learning and memory and reducing memory loss in rodents. It has been proposed that cognitive stimulation increases neuronal connectivity in areas responsible for learning and memory, creating a cognitive reserve; thus, those animals growing under EE can better support brain damage and exhibit fewer signs of cognitive decline [11].

Epigenetic mechanisms, i.e., alterations of histone proteins and DNA, are essential for hippocampal synaptic plasticity and cognitive function diseases. Histone alterations and DNA methylation influence hippocampal memory formation, and growing evidence suggests that the regulation of these epigenetic processes by EE, stress, and/or hormones can participate in memory function. In addition to histone modifications, DNA methylation also plays a major role in regulating hippocampal memory consolidation [14–17]. DNA methylation generally decreases transcriptional access to DNA, although the functional effects of this gene silencing depend on the genes that are altered. As indicated previously, NMDAR activity drives regulation of *LSD1*, and its activation reduces *LSD1* [18] transcription, indicating a greater extent of DNA methylation and correlation with its cofactor *CoREST*, which participates in the activation complex of histone methylation by *LSD1*.

During aging, continuous deterrence of brain capabilities presents; therefore, understanding the mechanisms involved, as well as the analysis of possible mechanisms of prevention and/or of recovery of behavioral, memory, and knowledge competences, is of unquestionable relevance. In general, it is accepted that the mechanism promoting neurogenesis could be important in preventing neuronal loss, hence age- or disease-related cognitive impairment [19]. To explore the possible beneficial effects of EE on brain performance under adverse genetic conditions, we employ here senescence-accelerated prone mice, SAMP8, a well-characterized model for studying brain aging and neurodegeneration [20, 21]. These mice present signs of accelerated aging in several organic systems, such as the skin, skeletal muscle, eyes, vessels, and brain [20, 22, 23]. Brain-related disturbances include cognitive and behavioral alterations, which are accompanied by molecular features typical of Alzheimer disease (AD), such as increased oxidative stress (OS), increased tau phosphorylation, and overproduction of amyloid-beta protein [23–27]. Consequently, under these adverse genetic and developmental conditions, we tested the influence of EE on the early life stage of these mice.

Methods

Animals and Enriched Environmental Experimental Design

Male SAMP8 mice (n=25) 3 months of age were used, with free access to food and water, under standard temperature conditions (22±2 °C) and 12-h:12-h light-dark cycles (300 lx/0 lx).

The animals were maintained until day 21 with their mothers, and afterward were separated into cages at up to 3 months of age. Fifteen SAMP8 were used for the environmental enrichment (EE) group (EE-SAMP8), and 10 were maintained under standard conditions as control mice (Ct-SAMP8). Plastic tubes (20 cm long and 2.5 cm in diameter) were placed in EE cardboard house cages, in addition to plastic dolls or toys, which were added, extracted, or changed each week (Supplementary Fig. 1). Studies were performed in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona.

Behavioral and Cognitive Experiments

Open Field

The open field (OF) apparatus was constructed of white plywood (50×50×25 cm). Red lines were drawn on dividing the floor into 25-cm squares. Behavior was scored with SMART ver. 3.0 software, and each trial was recorded for later analysis, utilizing a camera fixed on the ceiling at a height of 2.1 m situated above the apparatus. Mice were placed at the center, or at one of the four corners, of the open field and allowed to explore the apparatus for 5 min. After the 5-min test, the mice were returned to their home cages, and the open field was cleaned with 70 % ethyl alcohol and allowed to dry between tests. To assess the animals' habituation process to the novelty of the arena, the mice were exposed to the apparatus for 5 min

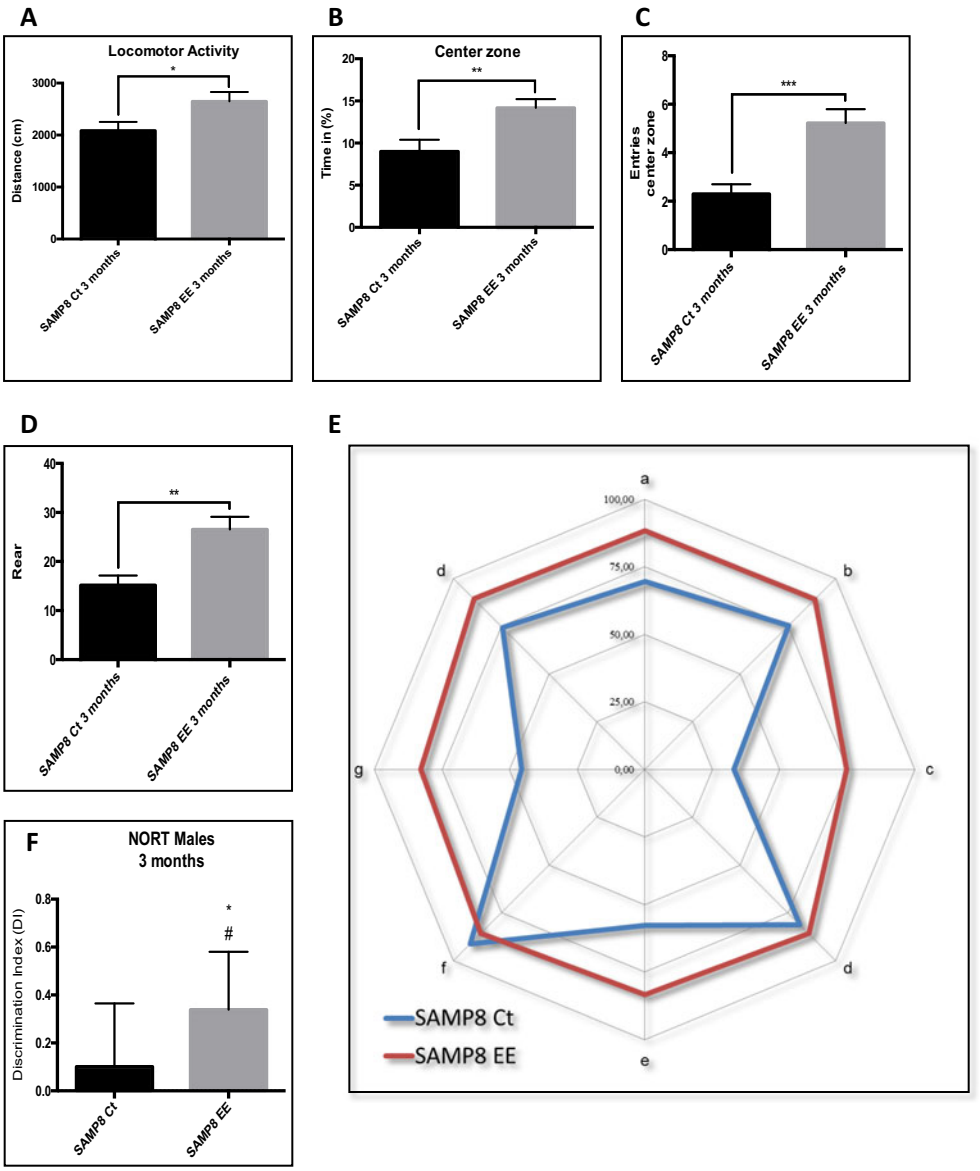

Fig. 1 Effects of environment enhancement (*EE*) treatments on the open field test (*OFT*) and the novel object recognition test (*NORT*) test. In the OFT, increases in locomotor activity (**a**), anxiety-like behavior (**b**, **c**), and rearing (**d**) activity were determined in environmental enhancement-senescence accelerated prone mice (*EE-SAMP8*). Polygonal graph (**e**) presented complete parameters obtained by the open field (*OF*) procedure, normalized to 100: total distance (*cm*), *a*, total entries; *b*, entries into zone center; *c*, entries into zone periphery; *d*, Center (%); *e*, distance in zone periphery (*cm*); *f*, distance in zone center (*cm*); *g*, NORT results showed a significant increase in distance delivered by the EE-SAMP8 group, both at the baseline level and to control-SAMP8 (*Ct-SAMP8*) mice (**f**). Values are mean±standard error of the mean (*SEM*); n=5–6. Statistics: *P<0.05; **p<0.01, and ***p<0.001 compared with Ct-SAMP8; #P<0.05 vs baseline value

on 2 consecutive days. The behaviors scored included line crossing, center entries, center stay duration, rearing, defecation, and urination [28, 29]. Each animal was then given a score for total locomotor activity, which was calculated as the sum of line crosses and number of rearings.

Novel Object Recognition Test (NORT)

The protocol employed was a modification of Ennaceur and Delacour [30, 31]. In brief, mice were placed in a 90°, two-arm, 25-cm-long, 20-cm-high, 5-cm-wide black maze. The walls could be lifted off for easy cleaning. Light intensity in the middle of the field was 30 lx. The objects to be discriminated were made of plastic and were chosen not to frighten the mice, and objects with parts that could be bitten were avoided. Before performing the test, the mice were individually habituated to the apparatus for 10 min during 3 days. On day 4, the animals were submitted to a 10-min acquisition trial (first trial), during which they were placed in the maze in the presence of two identical, novel objects (A+A or B+B) at the end of each arm. A 10-min retention trial (second trial) was carried out 2 h later. During this second trial, objects A and B were placed in the maze and the behavior of the mice recorded with a camera. Time that the mice explored the new object (TN) and time that the mice explored the old object (TO) were measured. A discrimination index (DI) was defined as (TN−TO)/(TN+TO). In order to avoid object preference biases, objects A and B were counterbalanced so that one half of the animals in each experimental group were exposed first to object A and then to object B, whereas the remaining half saw object B first and then object A. The maze

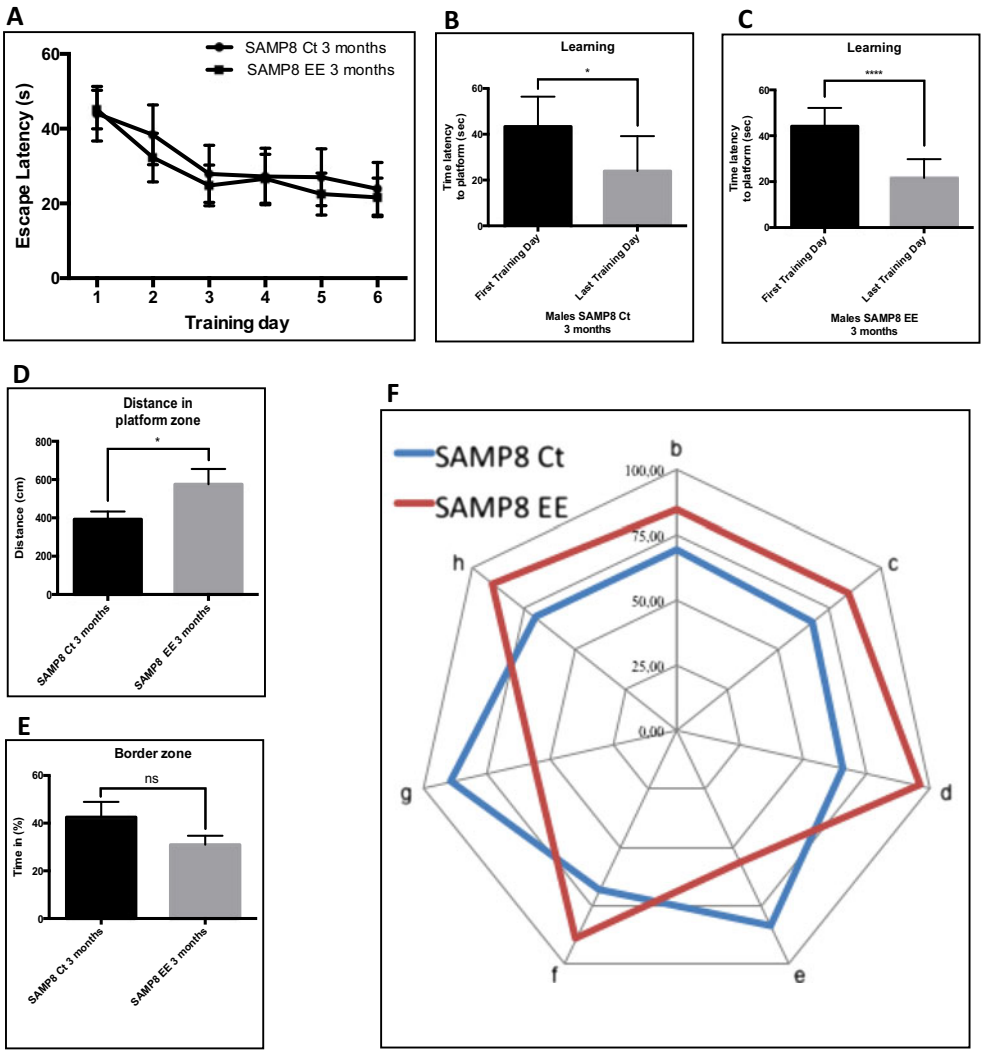

Fig. 2 Enriched environment (*EE*) ameliorated the spatial memory acquisition and retention of senescence-accelerated prone mice (*SAMP8*) mice assessed in the morris water maze (*MWM*) test. Escape latency time to reach the visible platform during cue learning day (**a**–**c**) was similar in control (*Ct*)- and environmental enhanced-senescence-accelerated prone mouse (*EE-SAMP8*) groups. Distance (*D*) covered swimming in the platform quadrant of the pool when the platform was removed to test learning retention, which was improved by EE (**d**) (see text for details). Polygonal graph (**e**) depicts complete parameters obtained by the MWM test and normalized to 100 as follows: latency (sec), *b*; time in platform quadrant (sec), *c*; distance, *d*; border time (%), *e*; platform quadrant time (%), *f*; border platform time, *g*; distance (%), *h*; and platform quadrant entry, *i*. Values are mean±standard error of the mean (*SEM*), $n=5$; $^{*}P<0.05$; $^{***}p$ 0.001 compared with Ct-SAMP8

and the objects were cleaned with 96° ethanol after each test in order to eliminate olfactory cues.

Morris Water Maze Test

An open circular pool (100 cm in diameter, 50 cm in height) was filled halfway with water [32], and water temperature was maintained at 22 °C±1. Two principal perpendicular axes were defined; thus, the water surface was divided into four quadrants (NE, SE, SW, and NW), and five starting points were set (NE, E, SE, S, and SW). Four visual clues were placed on the walls of the tank (N, E, S, and W). Nontoxic white latex paint was added to make the water opaque, and a white escape platform was submerged 1 cm below the water level (approximately in the middle of one of the quadrants).

The animals' swimming paths were recorded by a video camera mounted above the center of the pool, and the data were analyzed with SMART ver. 3.0 software. The learning phase consisted of 6 days of trials for each mouse. The animals were submitted to five trials each day starting from the position set (in random order) and without a resting phase between each trial and the subsequent one. At each trial, the mouse was placed gently into the water, facing the wall of the pool, and allowed to swim for 60 s. If not able to locate the platform in this period of time, the mouse was guided to the platform by the investigator. Animals were left on the platform each time for 30 s in order to allow spatial orientation.

The parameters measured were latency time in finding the platform, time spent in each quadrant, and distance swum for each trial; the mean was calculated for each trial day. A memory test was performed at the end of the learning days, in which the platform was removed and the time spent by each mouse in each quadrant was measured.

Three-Chamber Social Test (TCST)

The TCST allows the studying of cognition and general sociability (time spent with rodents) and social novelty (time spent with a novel intruder in contrast with a familiar one) [33]. In brief, the TCST identifies mice with changes in sociability and

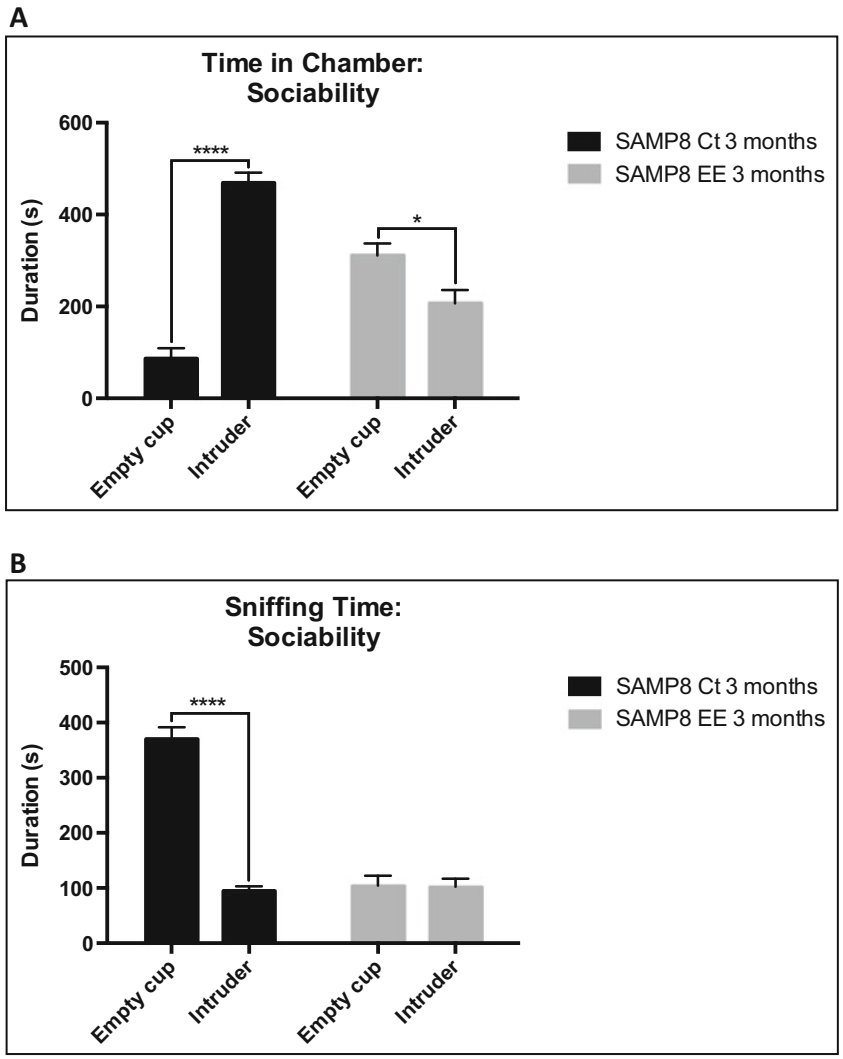

Fig. 3 Effects of enriched environment (*EE*) on the three-chamber test. EE reduced time in intruder cup, anxiety (**a**), and aggressiveness behavior present in senescence-accelerated prone mice (*SAMP8*), and demonstrated a decrease in sniffing time (**b**). Values are mean±standard error of the mean (SEM), $n=5$. ***$P<0.001$ compared with empty cup

social novelty. Testing took place in two sessions within a three-chambered box (15×15×20 cm) with openings among the chambers. After a 5-min period of adaptation to the empty box, the mice encounter a never-before-met intruder under one pencil cup and an empty pencil cup in the "sociability" session. The subject then encounters the first intruder, as well as a second, never-before-met intruder under another pencil cup in the "social novelty" session. Time spent sniffing each pencil cup, time spent in each chamber, and number of entries into each chamber were measured.

Immunodetection Experiments

After the behavioral test, the animals were intracardially perfused with a 0.9 % NaCl solution after being anesthetized with 80 mg/kg of sodium pentobarbital. The brains were dissected and separated sagitally into two hemispheres, frozen in liquid N^2, maintained at −80 °C, and defrosted on ice immediately prior to homogenization procedures. For the Western blot (WB) experiment, aliquots of homogenized hippocampus containing 20–30 μg of protein per sample were used. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5–18 %) and transferred onto polyvinyldiene difluoride (PVDF) membranes (Millipore). The membranes were blocked in 5 % nonfat milk in Tris-buffered saline (TBS) solution containing 0.1 % Tween 20 (TBS-T) for 1 h at room temperature, followed by overnight incubation at 4 °C with the primary antibodies listed in Table 1 (Supplementary files). The membranes were then washed and incubated with secondary antibodies for 1 h at room temperature. Immunoreactive protein was viewed with the chemiluminescence-based ChemiLucent™ detection kit, following the manufacturer's protocol (ECL kit; Millipore), and digital images were acquired using a ChemiDoc XRS+ System (BioRad). Semiquantitative analyses were conducted using ImageLab software (BioRad), and the results were expressed in arbitrary units (AU). Protein loading was

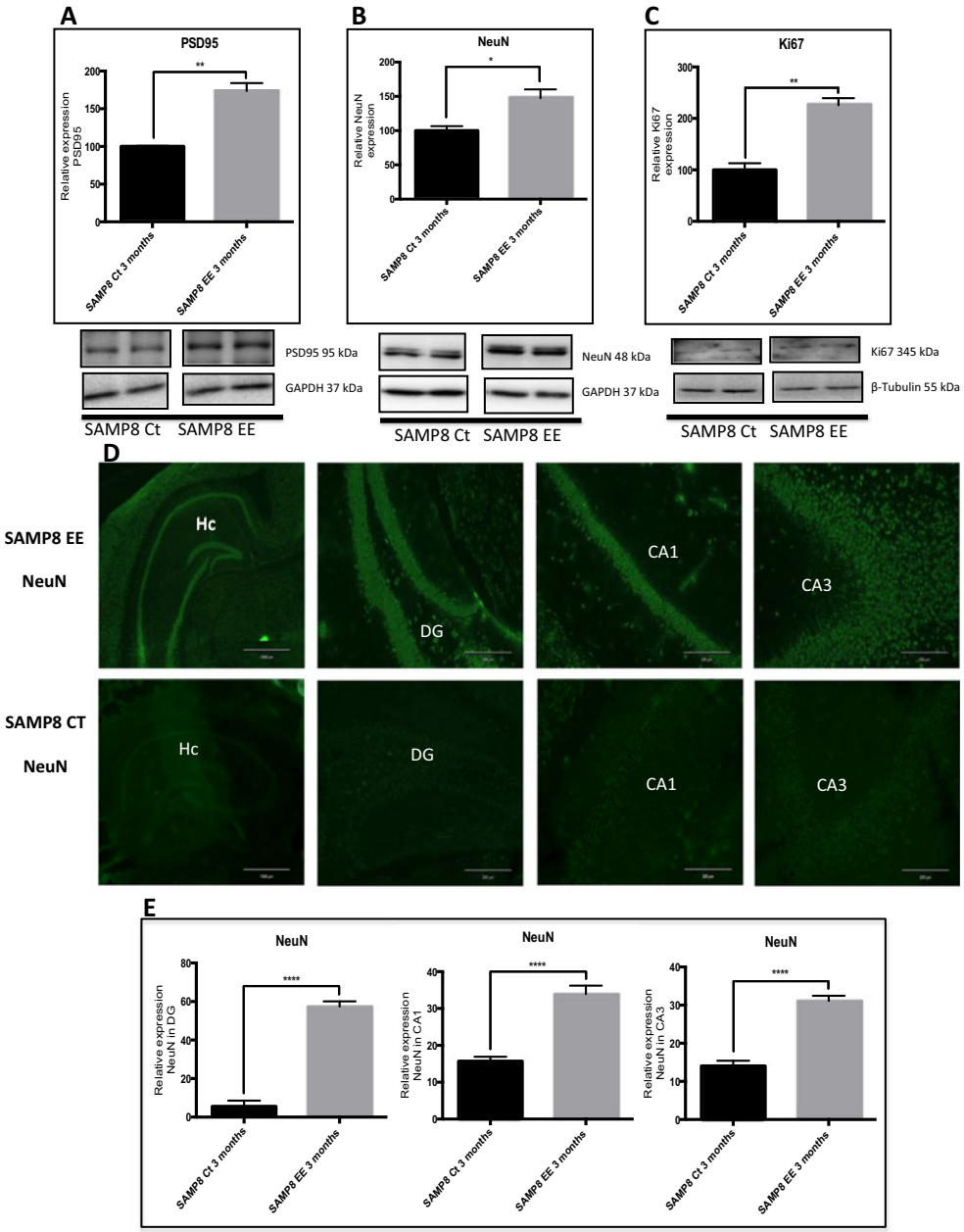

Fig. 4 Representative Western blot (*WB*) for PSD95 (**a**), NeuN (**b**), and Ki67 (**c**) in senescence-accelerated prone mice (*SAMP8*). Values in *bar graphs* are adjusted to 100 % for protein levels of control SAMP8 (*Ct-SAMP8*). Representative images, immunohistochemical assay (**d**), and bar chart quantification (**e**) of NeuN. *Bars* represent mean± standard error of the mean (*SEM*), $n=5$. *Hc* hippocampus; *GD* dentate gyrus. *Scale bar* for immunohistochemical images is 200 μm. *$P<0.05$; **$p<0.01$; ***$p<0.001$ vs Ct-SAMP8

routinely monitored by phenol red staining of the membrane or by GADPH immunodetection.

For immunohistochemical studies, the frozen brains were embedded in OCT Cryostat Embedding Compound (Tissue-Tek, Torrance, CA, USA), cut into 20-μm-thick sections on a cryostat (Leyca Microsystems, Germany) at −20 °C, and placed on slides. After 3 h of drying time at room temperature, the slices were fixed with acetone at 4 °C for 10 min, allowed to dry overnight, and finally stored at −20 °C until their further staining. For the staining procedure, the brain sections were first rehydrated by 5-min incubation in phosphate-buffered solution (PBS). Afterward, the blocking/permeabilization step was performed (20 min in PBS 1 % bovine serum albumin (BSA)+1 % Triton). Following two, 5-min washings in PBS, the slices were incubated overnight at 4 °C with the primary antibodies (see Table 1 for dilutions). Two further washings were carried out prior to incubation with the fluorescent secondary antibody (1 h at room temperature, see table for dilutions). Finally, before mounting with Fluoromount-G™ (EMS, Hatfield, NJ, USA), nuclear staining was performed with Hoechst 2 μg/mL for 5 min at room temperature. Slides were allowed to dry overnight after mounting, and image acquisition was performed with a fluorescence laser microscope (Olympus BX41; Germany).

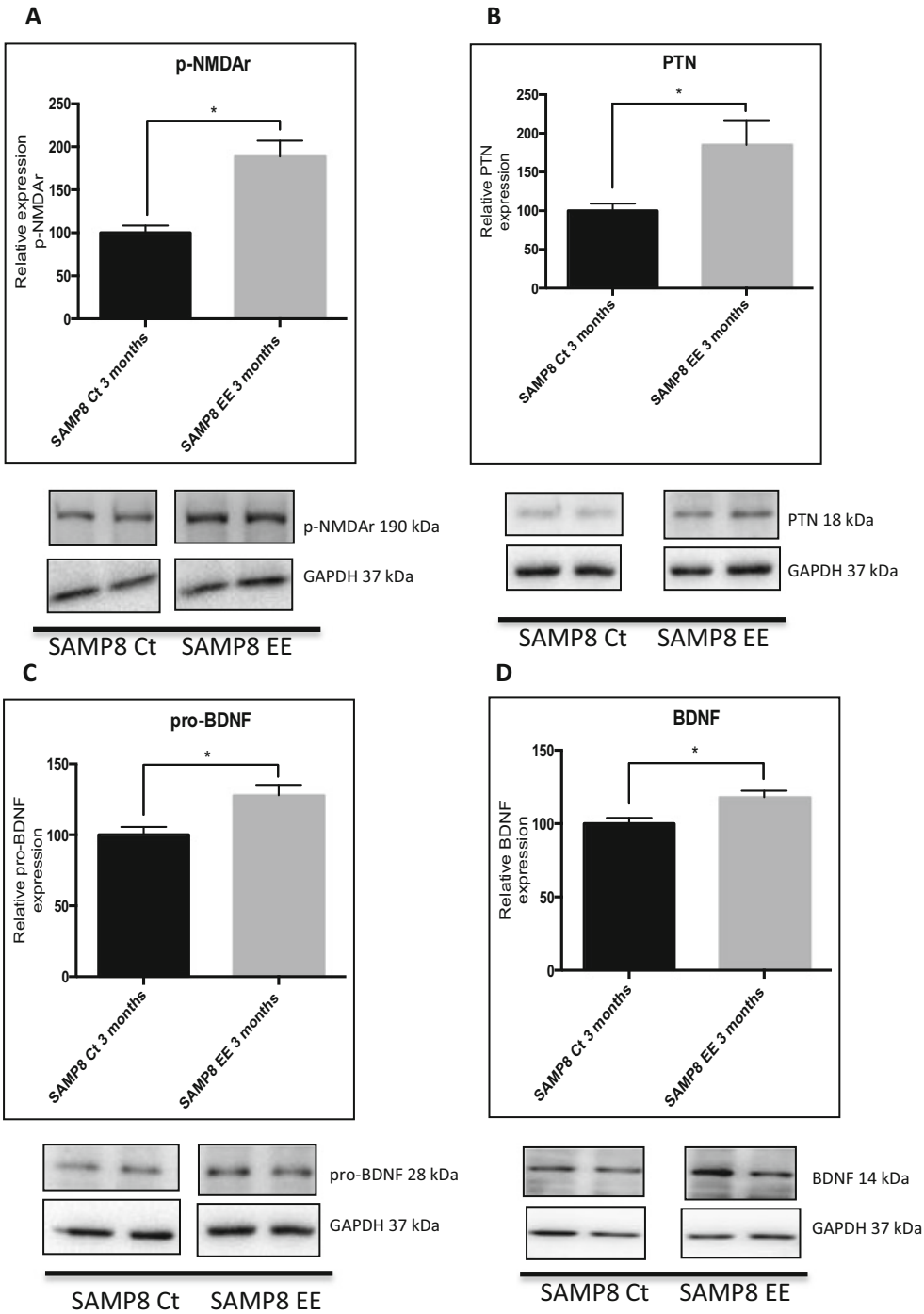

Fig. 5 Representative Western blot (*WB*) of the effect of EE on the p-N-methyl-D-aspartate (*p-NMDA*) receptor (**a**), pleiotrophin (*PTN*) (**b**), pro-brain-derived neurotrophic factor (*pro-BDNF*) (**c**), and BDNF (**d**). Increased levels for the protein-of-interest were found in enhanced environment-senescence-accelerated prone mice (*EE-SAMP8*). *Bars* represent mean± standard error of the mean (*SEM*), $n=5$. Values are adjusted to 100 % for levels of control-senescence-accelerated prone mice (Ct-SAMP8). *$P<0.05$; **$p<0.01$ vs. Ct-SAMP8

RNA Extraction and Gene Expression Determination

Total RNA isolation was carried out by means of Trizol reagent following the manufacturer's instructions. RNA content in the samples was measured at 260 nm, and the purity of the samples was determined by the A260/280 ratio in a NanoDrop™ ND-1000 (Thermo Scientific). Samples were also tested in an Aglient 2100B Bioanalyzer (Agilent Technologies) to determine the RNA integrity number. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows: 2 μg of messenger RNA (mRNA) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was used to quantify the mRNA expression of inflammatory genes interleukin 6 (*IL-6*) and tumor necrosis factor-alpha (*TNF-α*), epigenetically modulated genes *LSD1* and *CoREST*, and neurotrophin gene *BDNF*.

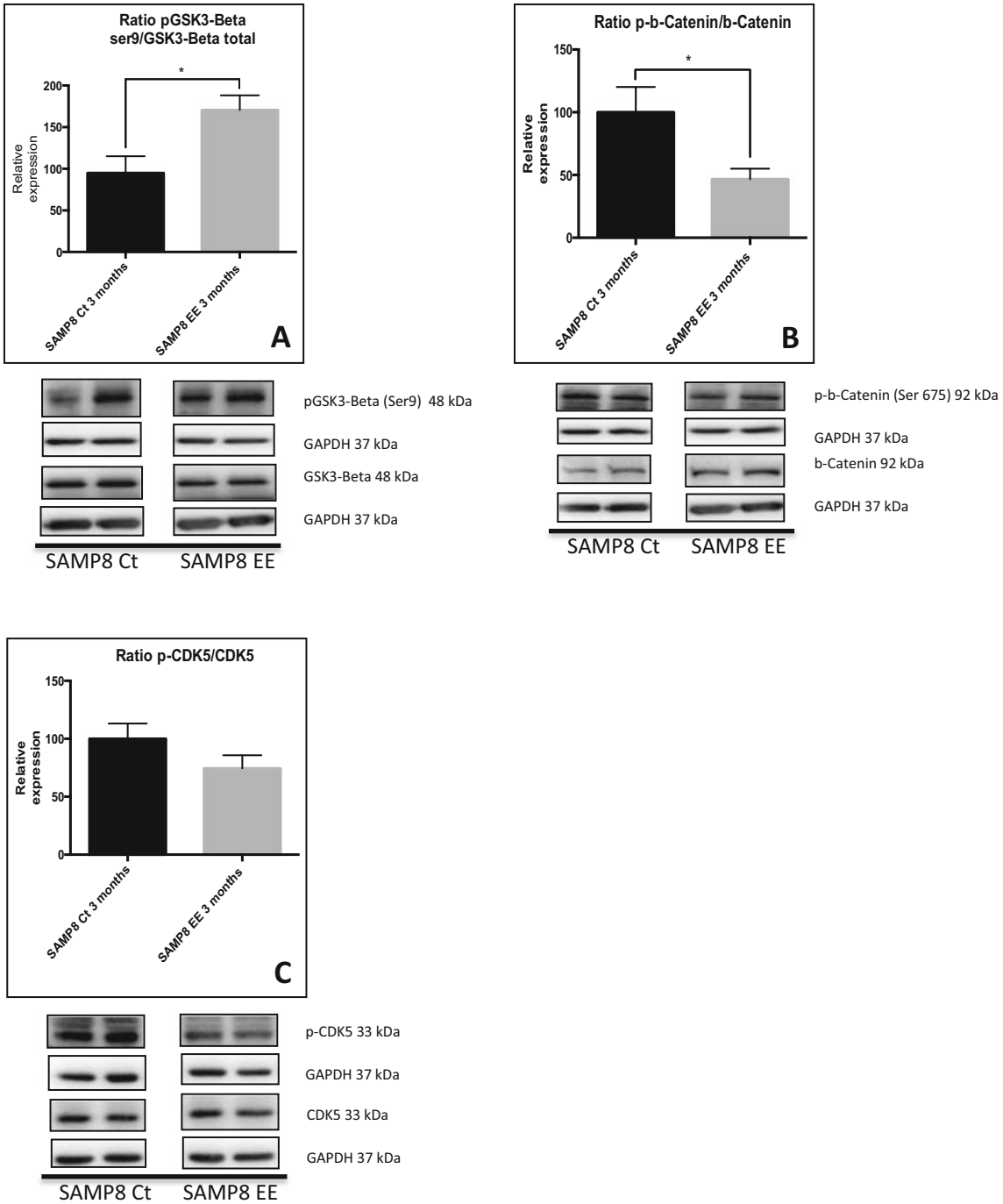

Fig. 6 Levels of GSK3β (**a**), β catenin (**b**), CDK5 (**c**), JNK (**d**, **e**), total Tau, and pTau Ser404 (**f**, **g**). *Bars* represent mean±standard error of the mean (*SEM*) for $n=5$, and values are adjusted to 100 % for levels of the control-senescence-accelerated prone mouse (Ct-SAMP8) group. $^{*}P<0.05$; $^{**}p<0.01$ vs Ct-SAMP8

Normalization of expression levels was performed with actin. The primers were as follows: for *IL-6*, forward 5′-ATCCAGTTGCCTTCTTGGGACTGA-3′ and reverse TAAGCCTCCGACTTGTGAAGTGGT; for *TNF-α*, forward 5′-TCGGGGTGATCGGTCCCCAA-3′ and reverse 5′-TGGTTTGCTACGACGTGGGCT-3′; for *LSD1*, forward 5′-AAGCCAGGGATCGAGTAGGT-3′ and reverse 5′-GGAACAGCTTGTCCATTGGC-3′; for *CoREST*, forward 5′-CCAGAGCATGAAGCAGACCA-3′ and reverse 5′-CACCTCTGCAATAGCCCCAA-3′; for *BDNF*, forward 5′-GGGAAATCTCCTGAGCCGAG-3′ and reverse 5′-AGCTTTCTCAACGCCTGTCA-3′; for amyloid beta A4 precursor (PreAPP), forward AGGACTGACCAC TCGACCAG and reverse CTTCCGAGATCTCTTCCG TCT, and for actin, 5′-CAACGAGCGGTTCCGAT-3′ and reverse 5′-GCCACAGGATTCCATACCCA-3′.

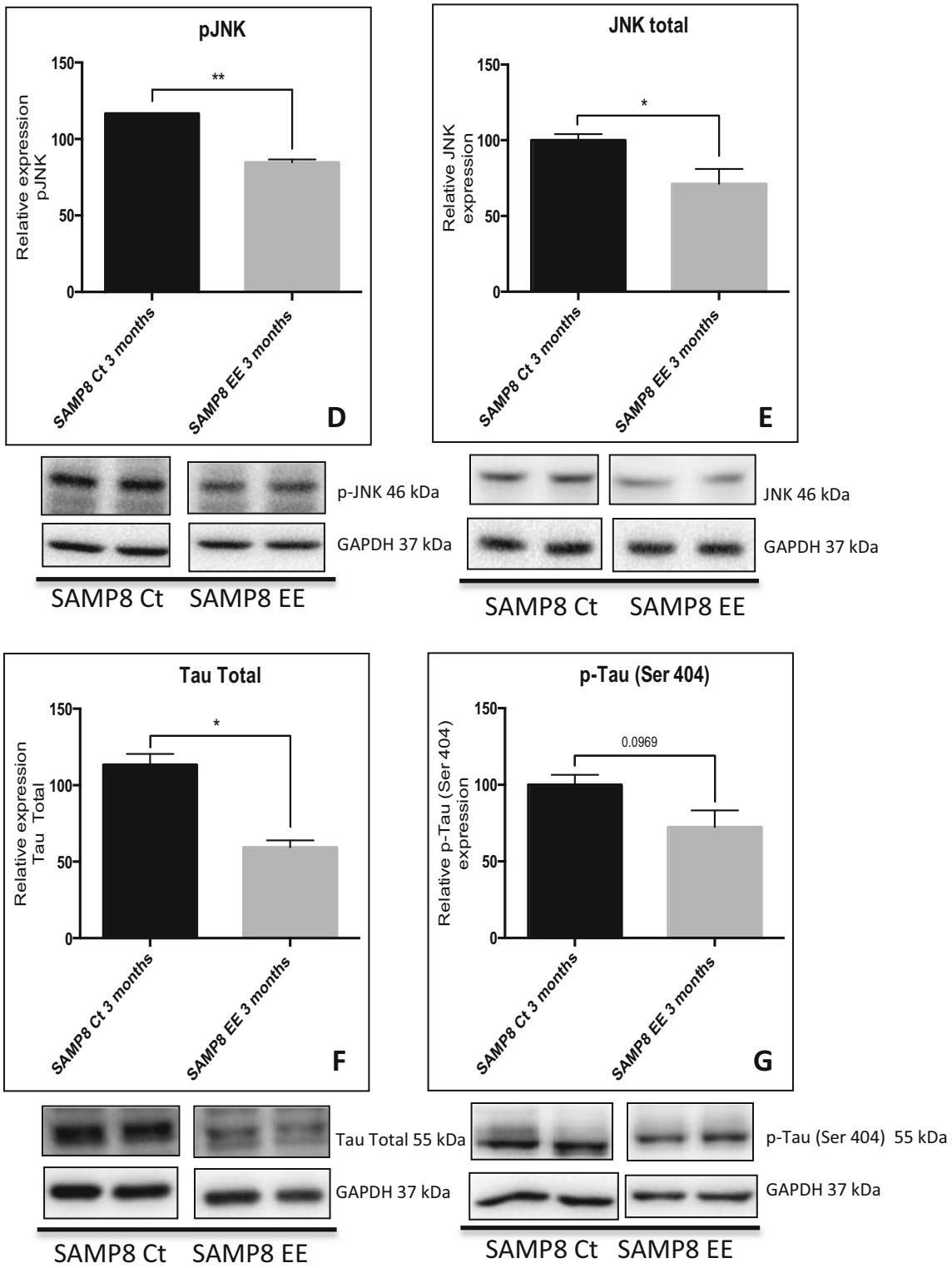

Fig. 6 (continued)

SYBER Green real-time PCR was performed on the Step One Plus Detection System (Applied Biosystems) Employing the SYBR Green PCR Master Mix (Applied Biosystems). Each reaction mixture contained 7.5 μL of complementary DNA (cDNA), whose concentration was 2 μg, 0.75 μL of each primer (whose concentration was 100 nM), and 7.5 μL of SYBR Green PCR Master Mix (2×).

Data were analyzed utilizing the comparative cycle threshold (Ct) method (ΔΔCt) where the actin transcript level was

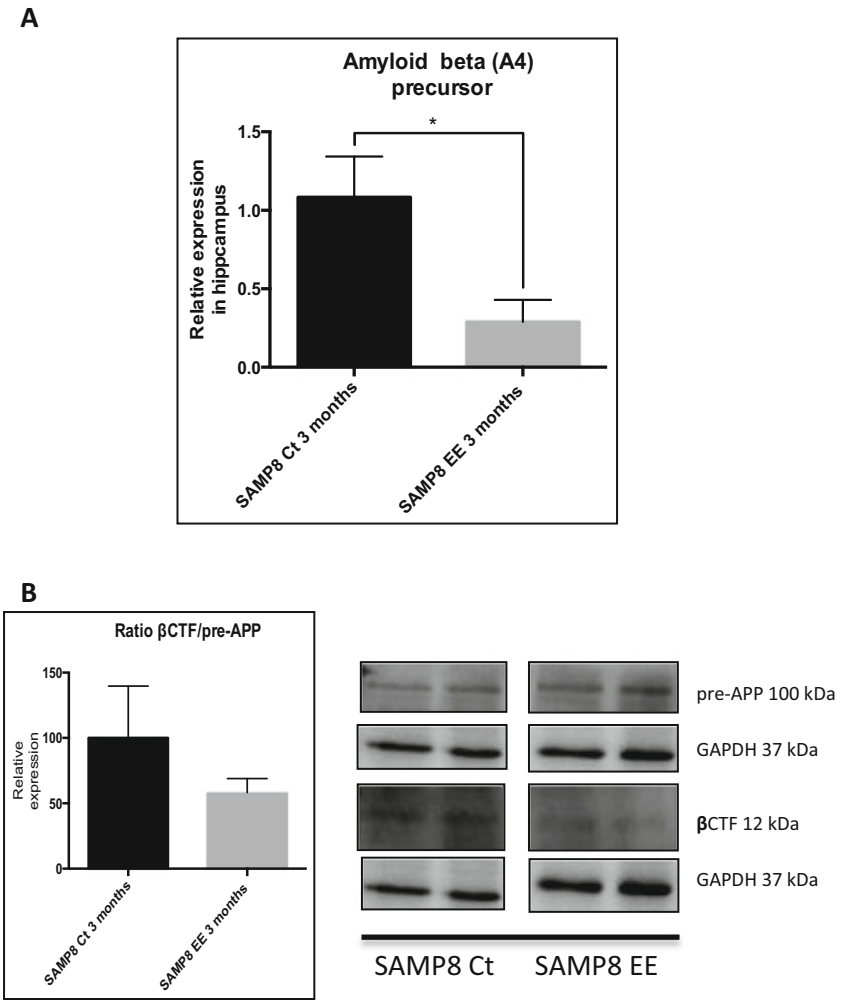

Fig. 7 pAPP (*A4*) gene expression (**a**) and β-CTF/APP ratio on Western blot (*WB*) analysis (**b**). *Bars* represent mean ±standard error of the mean (SEM) for $n=5$, and values are adjusted to 100 % for the control-senescence-accelerated prone mice (Ct-SAMP8 group levels. *$P<0.05$ vs. Ct-SAMP8

used to normalize differences in sample loading and preparation. Each sample ($n=5$–6) was analyzed in triplicate, and the results represented the n-fold difference of transcript levels among different samples.

Data Analysis

Data are expressed as the mean±standard error of the mean (SEM) from at least 5–6 samples. Data analysis was conducted using GraphPad Prism ver. 5 statistical software. Means were compared with the two-tailed, unpaired Student's *t* test. Statistical significance was considered when *p* values were <0.05.

Results

Cognitive and Motivational Behavior Analysis

Results obtained in the open field test (OFT) indicated that the EE-SAMP8 group exhibited a significant increase in locomotor activity, with a higher number of entries and time spent in the open field (OF) central zone (Fig. 1b, c), and increased rearings (Fig. 1d), as compared with Ct-SAMP8. The polygonal graph (Fig. 1e) clearly depicts differences between two experimental groups by the graphing of all eight OF parameters, measured as the relative maximal values obtained in individual measurements, indicating better performance in tests for the EE-SAMP8 group (see figure legend for details). All results obtained in the OFT are listed in Table 2 (Supplementary files). In addition, the novel object recognition test (NORT) test demonstrated that EE-SAMP8 mice exhibited significantly improved memory capabilities (Fig. 1f), obtaining highest DI and baseline values, with statistically significant differences.

The results obtained in spatial learning acquisition and retention in the MWM test are illustrated in Fig. 2. Although SAMP8 and both the control and the EE groups were able to learn over the trial days (Fig. 2a–c), final acquisition of the EE-SAMP8 group was slightly better than that of the control group, although not reaching statistical significance. In the removal test, EE intervention improved the spatial retention of learning, demonstrating a significant preference for the platform zone, measured by

distance into this target zone (Fig. 2d) for EE-SAMP8 in reference to control animals, and time in the border zone (Fig. 2e), while number of entries, latency, or time spent in platform quadrant did not present significant differences (Fig. 2f).

TCST demonstrated that SAMP8 animals exhibited higher dominance behavior, aggressiveness, and anxiety. It was determined that control animals spent more time in the intruder chamber than in the empty one, but spent less time in sniffing the new individual (Fig. 3). EE-SAMP8 did not demonstrate differences in sniffing time or in time spent in the intruder chamber with regard to the empty case, indicating reduced anxiety, aggressiveness, and sociability.

EE Improved Neuronal Marker Levels in SAMP8 Hippocampus

In accordance with NORT results, we showed that EE induced the expression of several neural growth markers, such as an increase in PSD-95 protein, a marker of synapsis (Fig. 4a), as well as an increase in NeuN, suggesting a higher number of neuronal cells (Fig. 4b). This increase in NeuN by WB corresponds to significantly higher immunocytochemically determined NeuN staining in CA1, CA3, and dentate gyrus (DG) in EE-SAMP8 hippocampus (Fig. 4d, e). In addition, EE increased Ki67 protein levels in SAMP8 (Fig. 4c). Furthermore, p-N-methyl-D-aspartate (pNMDA) receptor protein levels were higher in EE-SAMP8 compared with Ct-SAMP8 (Fig. 5a), additionally suggesting better neuronal functionality. Moreover, EE induced an increase in neurotrophic factors, such as pleiotrophin (PTN) protein levels (Fig. 5b), as well as in BDNF and in proBDNF (Fig. 5c, d).

EE Influenced Kinase Pathways and preAPP Processing

Significant increases in GSK3 (ser9) (the inactive form of this kinase) protein levels were found (Fig. 6a). This result was accompanied by a significant decrease in phosphorylated beta-catenin, one of the main GSK3-beta substrates (Fig. 6b). Additionally, CDK5 activity was slightly reduced, although it did not reach statistical significance (Fig. 6c). Furthermore, a significant reduction in total JNK and p-JNK levels was determined in EE-SAMP8 in reference to Ct-SAMP8, but p-JNK/JNK calculated ratios did not exhibit significant changes between both conditions (Fig. 6d, e). Because GSK3 beta (Ser 9) demonstrated diminution, tau phosphorylation was also

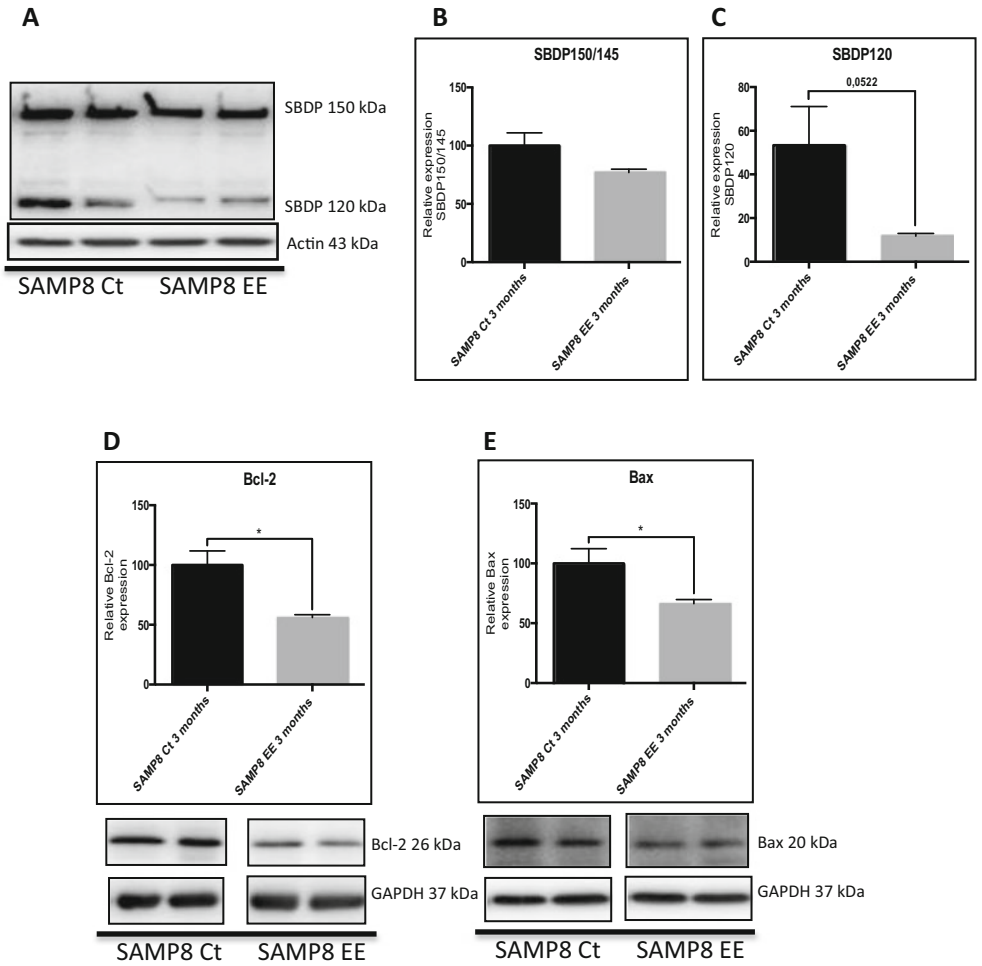

Fig. 8 Representative Western blot (*WB*) for alpha-spectrin (**a**), and quantification of SBDP 150/145 (**b**) and SBDP12 (**c**) fragments. Bcl-2 (**d**) and Bax (**e**) levels after enhanced environment (*EE*) intervention. *Bar*s represent mean±standard error of the mean (*SEM*), and values are adjusted to 100 % for levels of control-senescence-accelerated prone mice (Ct-SAMP8). **P*<0.05 vs Ct-SAMP8

determined. EE produced a significant decrease in total tau protein and a nonsignificant diminution in p-Tau Ser404; no changes in p-Tau/Tau protein occurred (Fig. 6f, g). Additionally, preAPP (*A4*) gene expression was significantly reduced in EE-SAMP8 (Fig. 7a), and β-CTF (the fragment delivered by β-secretase) was diminished (Fig. 7b).

EE Reduced Caspase-3 Activation and Inflammatory Markers in SAMP8

Caspase and calpain activities were investigated by measuring the alpha-spectrin protein. The 150-kDa spectrin breakdown product (SBDP150) is generated by calpain activation, whereas caspase-3 produces 120 kDa SBD as a product (SBDP120). EE induced a reduction of SBDP120, indicating preventive caspase-3 activity by EE in SAMP8, but no changes in SBDP150 were found (Fig. 8a). Bax and Bcl-2, apoptotic proteins belonging to the intrinsic caspase cascade, were also investigated. Bax protein levels were significantly diminished (Fig. 8e), as were antiapoptotic Bcl-2 protein levels (Fig. 8d).

Neuroimmunological responsive parameters were determined through GFAP protein expression and *IL-6* or *TNF-α* genic expression. GFAP expression was reduced by EE in hippocampal CA3, but not in CA1 and DG (Fig. 9b). In reference to *IL-6* results, a lesser expression of this proinflammatory cytokine was determined in the cortex and hippocampus (Fig. 10a, b). No significant changes in tumor nuclear factor alpha (TNF-alpha) were detected (data not shown).

Finally, *LSD1* and its cofactor, *CoREST*, were studied. Results showed a significant diminution in lysine demethylase 1 (LSD1), and also its cofactor, *CoREST*, in EE-SAMP8 mice with respect to the control group (Fig. 10c, d).

Discussion

The environment in which the mouse lives can greatly influence its development; therefore, the housing needs of any laboratory animal must be considered. Consequently, whenever animals are used in laboratories where experimental results

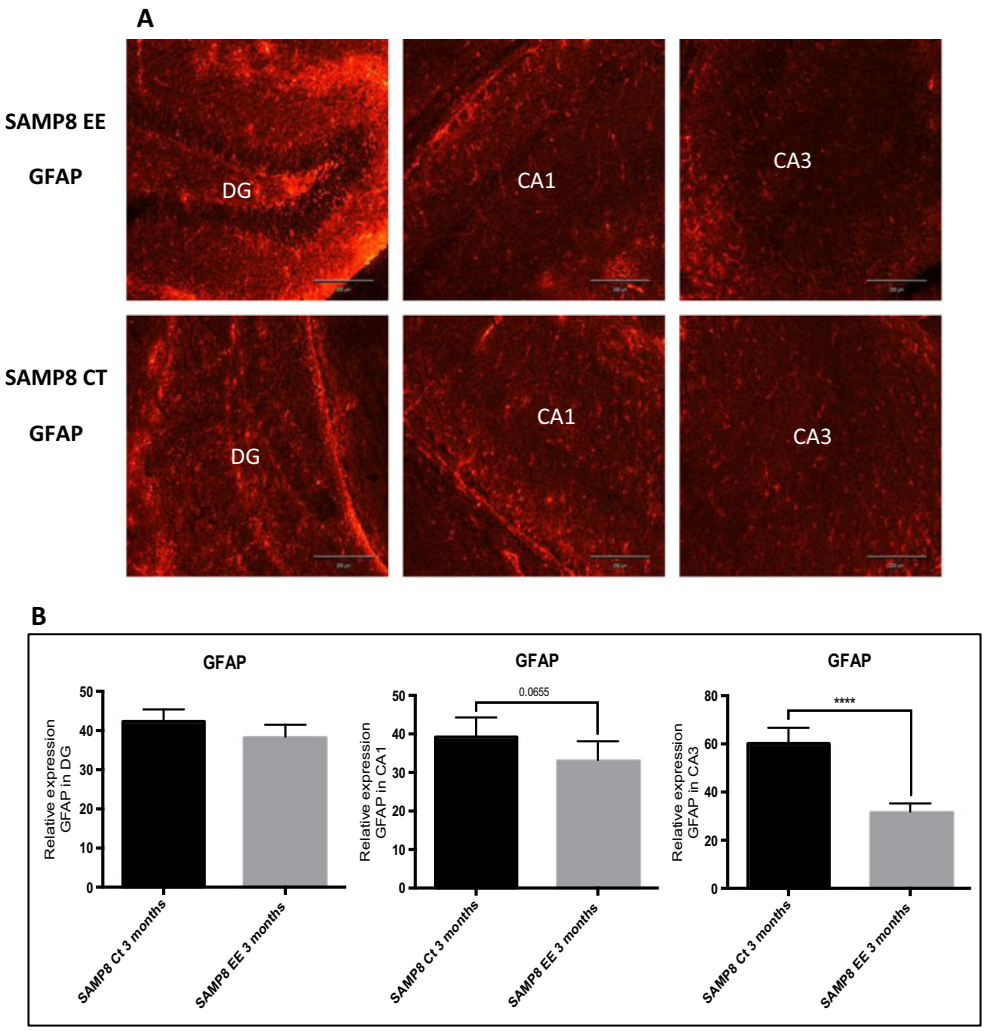

Fig. 9 Representative images for GFAP immunostaining (**a**) and quantification on the bar chart (**e**). *Bars* represent mean±standard error of the mean (*SEM*), $n=5$. *Hc* hippocampus; *DG* dentate gyrus. *Scale bar* for immunohistochemical images is 200 μm. ****$P<0.0001$ vs control-senescence-accelerated prone mice (*Ct-SAMP8*)

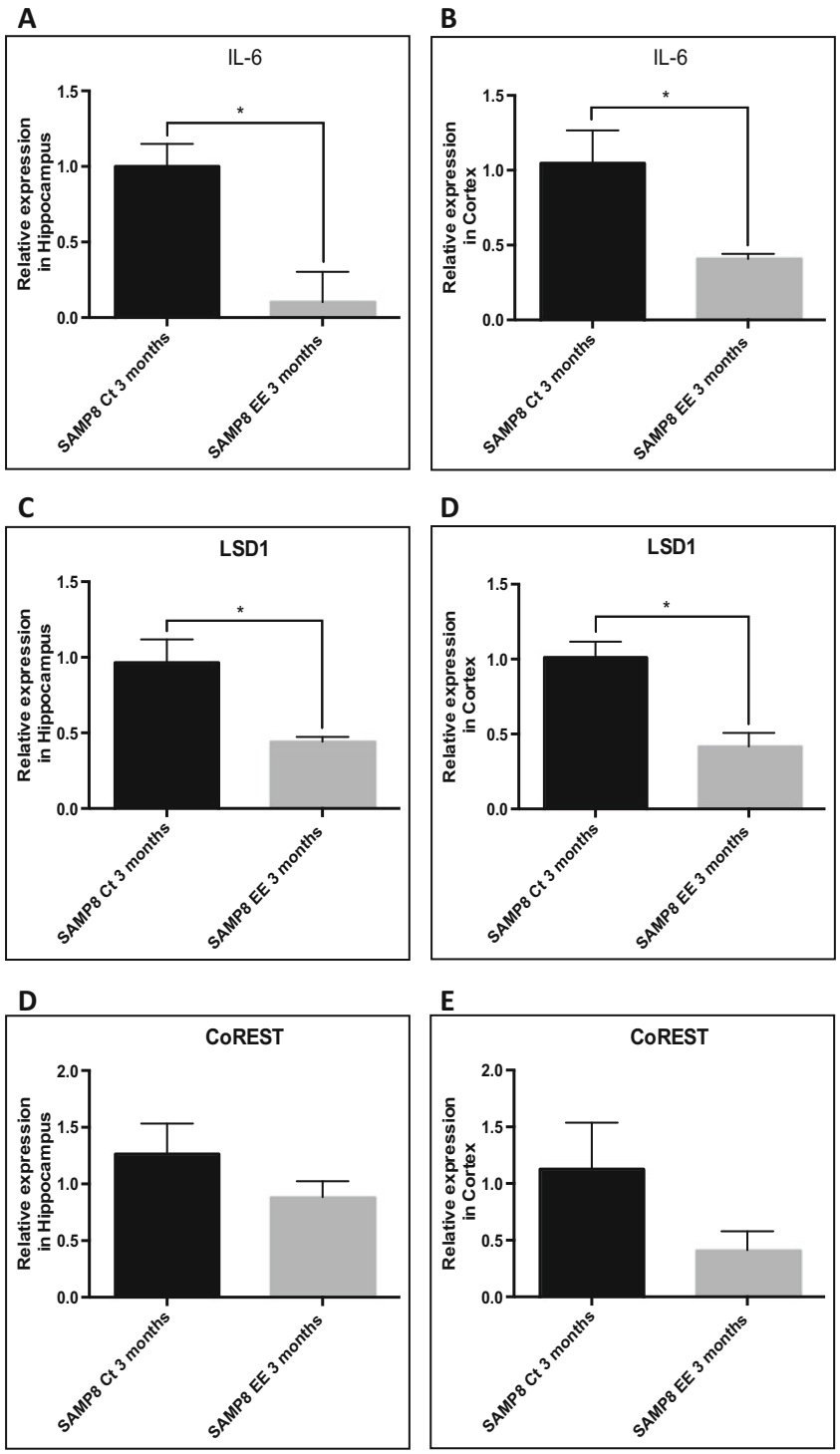

Fig. 10 Proinflammatory gene expression and epigenetic response in senescence-accelerated prone mouse (*SAMP8*) hippocampus and cortex after enhanced environment (*EE*) intervention. Interleukin 6 (*IL-6*) (**a**–**b**), *LDS1* (**c**–**d**), and *CoREST* (**e**–**f**). Gene expression levels were determined by real-time PCR. Mean±standard error of the mean (*SEM*) from five independent experiments performed in triplicate are represented. **P*<0.05 vs control-senescence-accelerated prone mice (*Ct-SAMP8*)

are to be obtained, it is important to reduce any of their discomfort or pain. Conceptually, environmental enrichment (EE) is the improvement of the physical medium or social environment of an animal. This concept is increasingly recognized as an important component in the refinement of the housing of research animals, and its use can be considered good laboratory practice [34]. At the neurobiological level, if EE is present before adulthood (8–10 weeks of age in rodents), it could give rise to additional effects on brain development compared with those obtained if EE is present at a later stage [5].

It was demonstrated that mice that have access to new objects and accessories develop major visuospatial attention and also improvement in spatial memory when subjected to the morris water maze (MWM) test; this could comprise an effect

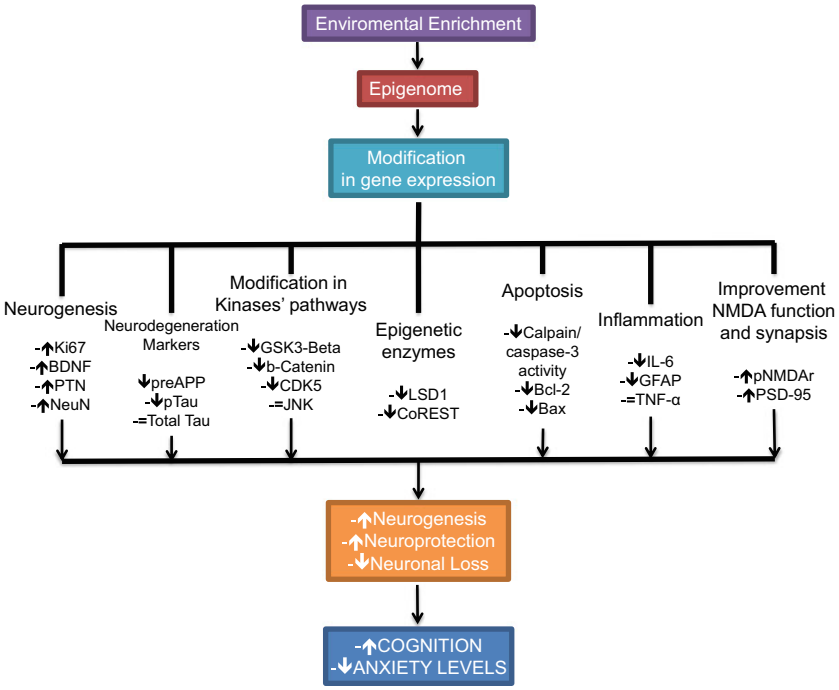

Fig. 11 Representative flow chart of molecular mechanisms modified in senescence-accelerated prone mice (*SAMP8*) after enhanced environment (*EE*)

involved in increasing the integrity of the hippocampal and neurotrophin levels therein [13]. EE also increases behavioral performance when memory is evaluated by NORT [35, 36].

SAMP8 has been studied as a nontransgenic murine model for accelerated senescence and late-onset AD [20–23, 37]. These mice exhibited cognitive and emotional disturbances from young ages, probably due to brain pathological hallmarks such as OS, inflammation, and activated neuronal death pathways, mainly affecting cortex and hippocampus. Molecular changes at young ages are based on cerebral and cognitive dysfunction in SAMP8 [38].

Here, we established that young SAMP8 raised under EE exhibit beneficial changes in behavior and cognitive parameters. The behavioral tests applied demonstrated an increase in locomotion and reduced anxiety in EE-SAMP8, leading mice to be quieter and more sociable. Cognitive improvement was also demonstrated.

Because abnormal expression levels and altered NMDA receptor function have been implicated in numerous pathological conditions [39], the increase in pNMDA in EE-SAMP8 compared with Ct-SAMP8 suggests that EE induced an improvement in NMDA functioning. Additionally, our results showed an increase in EE-SAMP8 of PSD-95, a protein involved with NMDA receptor signaling [40], suggesting an increase in neuronal synapses, thus reinforcing the beneficial effects of EE on SAMP8.

Consistent with the behavioral results, the EE-SAMP8 group exhibited a significant difference related with hippocampal integrity compared with the Ct-SAMP8 group, measured by greater NeuN immunostaining in the CA1 area. NeuN is at the core of the majority of neural cells during development, is expressed in postmitotic neurons from early stages of differentiation, and its expression persists in the adult [41]. The EE neurogenic effect was also supported by a significant increase in Ki67, a protein strictly associated with cell proliferation [42].

As mentioned previously, EE induced neurotrophic factor expression. PTN is an extracellular matrix protein that promotes neurite outgrowth [43] and that provides neuroprotection [44]. Additionally, PTN has been involved in neuronal plasticity during learning and also during recovery after lesion or in neuropathological situation functioning as a neuromodulatory peptide, particularly in the hippocampus [45]. The increase in PTN and the pNMDA receptor protein level explain best task performance of the MWM, a robust and reliable test that is strongly correlated with hippocampal synaptic plasticity and NMDA receptor function [46].

Moreover, in line with published results [11, 47], we observed an increase of BDNF in the EE-SAMP8 compared with the Ct-SAMP8 group. BDNF promotes neuronal differentiation, survival during early development, adult neurogenesis, and neural plasticity [48], comprising the main neurotrophic factor involved in AD [47]. Furthermore, it was reported that neurotrophins such as BDNF are involved in depression, aggressiveness, or anxiety; therefore, increased levels of BDNF or PTN in hippocampus of EE-SAMP8 abrogated for EE beneficial effects, based on the positive locomotive and motivational behavior results presented in this work, contributed to hippocampal neuron preservation and rendered these neurons less susceptible to harmful factors [11].

Spectrin, a neurocytoskeletal structural protein, is a substrate for caspases and calpains; its cleavage is related with changes in cellular morphology that are produced in cells

undergoing apoptosis [49]. Proapoptotic proteins such as Bax activate caspases that mediate cell destruction through many pathways. Here, we found reduced Bax protein levels in EE-SAMP8, this correlating with a diminution in caspase-3 activity. We previously demonstrated increased caspase-3 activity and apoptotic pathway in 10-month-old SAMP8 [50], reinforcing the importance of developing strategies to stop or to slow apoptosis in order to minimize cognitive impairment and neuronal loss during the aging process.

As mentioned previously, tau protein is modified in different ways, including tau kinases such as GSK3, CDK5, or JNK [51]. We found a significant decrease in the GSK3 beta-activated form, but only a marginal decrease in CDK5 expression and in slight changes in JNK in EE-SAMP8. Despite these results in tau kinases, a diminution in p-Tau (Ser404) was demonstrated in the hippocampus. The APP protein is cleaved by secretases α and β, leading to different molecular weight fragments, with β-CTF fragment or C99 formed from the cleavage of APP by β-secretase, participating in the amyloidogenic route [52]. EE-SAMP8 reduced preAPP (*A4*) gene expression in hippocampus and a downward trend in β-CTF; thus, it could deliver a diminution in βA.

IL-6 is involved in the pathogenesis of neurodegenerative diseases such as AD [53]. In the nervous system, *IL-6* mainly occurs in activated glia, such as astrocytes and microglial cells. Our results demonstrated a significant decrease in *IL-6* genic expression in the EE-SAMP8 compared with the Ct-SAMP8 group, suggesting a diminution in inflammatory processes. A lower degree of neuroinflammation after implementation of the EE strategy was also demonstrated by a significant decrease in GFAP expression levels in the EE-SAMP8 CA3 region of the hippocampus compared with Ct-SAMP8.

Changes in *CoREST* and *LSD1* under EE conditions could be the initial and key step related with DNA transcription in the molecular stream developed in this strain by EE, ameliorating cognition and other harmful factors gated to neurodegeneration, described as neuronal loss and apoptosis prevention, downregulated kinase activity, and reduction in OS [54].

From the ethical point of view, EE ameliorated SAMP8 care and can be taken into account in colony breeding. At the behavioral level, EE improved learning and reduced memory loss in mice. In addition, EE increased exploratory activity and reduced emotionality and fearfulness levels. At the molecular and histological levels, neuroprotective activity can be summarized by the increase of NeuN, PTN, and BDNF, giving rise to a neurogenic condition, as well as by the reduction of GSK3, CDK5, JNK, and APP production and calpain and caspase apoptotic pathways, rendering a moderate decrease in inflammatory activity (Fig. 11).

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