

Manuscript Number: EXG-15-438R2

Title: Behaviour and cognitive changes correlated with hippocampal neuroinflammation and neuronal markers in female SAMP8, a model of accelerated senescence

Article Type: Research Paper

Section/Category: Neuroscience

Keywords: Aging, neurodegeneration, behaviour, learning, cognition, inflammation, oxidative stress

Corresponding Author: Dr. Mercè Pallàs Lliberia,

Corresponding Author's Institution: Universitat de Barcelona

First Author: Christian Griñan-Ferré

Order of Authors: Christian Griñan-Ferré; Verónica Palomera-Ávalos; Dolors Puigoriol-Illamola; Antoni Camins; David Porquet; Virginia Plá; Fernando Aguado; Mercè Pallàs Lliberia

Abstract: Senescence accelerated mice P8 (SAMP8) is a phenotypic model of age, characterized by deficits in memory and altered behaviour. Here determined the effect of age in SAMP8, compared with the resistant strain, SAMR1, in behaviour and learning parameters linking these disturbances with oxidative stress environment. We found impairment in emotional behaviour with regard to fear and anxiety in young SAMP8 vs. age-mated SAMR1. Differences were attenuated with age. In contrast, learning capabilities are worse in SAMP8, both in young and aged animals, with regard to SAMR1. These waves in behaviour and cognition were correlated with an excess of Oxidative stress (OS) in SAMP8 at younger ages that diminished with age. In this manner, we found changes in the hippocampal expression of ALDH2, IL-6, HMOX1, COX2, CXCL10, iNOS, and MCP-1 with an altered amyloidogenic pathway by increasing the Amyloid beta precursor protein (APP) and BACE1, and reduced ADAM10 expression; in addition, astrogliosis and neuronal markers decreased. Moreover, Superoxide dismutase 1 (SOD1) and Nuclear factor-kappa beta (NF- κ B) expression and protein levels were higher in younger SAMP8 than in SAMR1. In conclusion, the accelerated senescence process present in SAMP8 can be linked with an initial deregulation in redox homeostasis, named neuroinflammation, by inducing molecular changes that lead to neuroinflammation and the neurodegenerative process. These changes are reflected in the emotional and cognitive behaviour of SAMP8 that differs from that of SAMR1 and that highlighted the importance of earlier oxidative processes in the onset of neurodegeneration.

Barcelona, 16th, March, 2016

Dear Sirs,

We would like to re submit the manuscript entitled **Behaviour and cognitive changes correlated with hippocampal neuroinflammaging and neuronal markers in female SAMP8, a model of accelerated senescence** by C. Griñan-Ferré to Experimental Gerontology.

We have addressed referees comments and an English-speaking person have revised language. We hope that the manuscript will be ready for publication in this forma

Sincerely yours

Prof. Dr. Mercè Pallàs
Professor of Pharmacology
Faculty of Pharmacy
University of Barcelona, Spain

Ms. Ref. No.: EXG-15-438R1

Title: Behaviour and cognitive changes correlated with hippocampal neuroinflammation and neuronal markers in female SAMP8, a model of accelerated senescence Experimental Gerontology

Point by point answer to reviewers

Reviewer #2: The authors have done a nice job in revising this manuscript and addressing the critiques of the reviewers overall.

However, the paper appears to be too long, particularly the introduction and conclusion.

Additionally, the paper would benefit from having a native English speaker edit the wording to improve clarity.

Thus, I would recommend an additional revision at this time to improve the overall quality of the final paper.

We thank for the commentaries of the reviewer, and we resubmit the manuscripts to a mother tongue service for further grammatical correctness

Reviewer #5: The revised version of the paper looks much better. The systematic is improved and it is overall easier to read and follow. I want to address some minor points:

-First sentence of "Experimental Procedures": There is something wrong with the use of parentheses; SAMR1 have to exclude or SAMP8 have to include with them.

-Page 8 - line 2, page 11 - line 49, page 12 - line 7: Will these tables not be published. Why the term "supplementary data"?

-Again, page 12 - line 7: I think Table '3' is meant.

-Page 14 - line 38; page 15 - line 19: Table 4, not 1.

-Page 13 - line 51: Necrosis or nuclear factor?

-Page 17 - line 7: that or than?

-Legends of Figures 3 and 5: the definition for CA1 and CA3 is missing.

We go through the manuscript correcting several issues indicated by the reviewer.

Supplementary data (both Tables and figures) will be published as a supplemental material as it allowed by Exp. Biogerontology

Highlights

In SAMP8 a phenotypic model of senescence behavior and learning parameters are linked with early oxidative stress environment.

SAMP8 showed emotional behavior disturbances beside cognitive impairment compared with SAMR1.

Emotional behavior and molecular differences between strain were attenuated with age but not learning capabilities

SAMP8 senescence process is linked with an earlier oxidative stress imbalance, inducing molecular changes that lead to neuroinflammation and the neurodegenerative process.

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Behaviour and cognitive changes correlated with hippocampal neuroinflammation and neuronal markers in female SAMP8, a model of accelerated senescence.

Christian Griñan-Ferré¹, Verónica Palomera-Ávalos¹, Dolors Puigoriol-Illamola¹, Antoni Camins¹, David Porquet¹, Virginia Plá², Fernando Aguado² and Mercè Pallàs¹

¹Department of Pharmacology and Therapeutic Chemistry. Institut of Neuroscience. University of Barcelona, Avda. Joan XXIII, 27, 08028 Barcelona, Spain

²Department of Cellular Biology and Institute of Biomedicine (IBUB), University of Barcelona, Avda. Diagonal 646, 08028 Barcelona, Spain

Corresponding author: Mercè Pallàs, PhD, Unitat de Farmacologia, Farmacognòsia i Terapèutic, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII 27, 08028 Barcelona, Spain.

Phone; +343934024531

E-mail: pallas@ub.edu

Keywords: Aging, neurodegeneration, behaviour, learning, cognition, inflammation, oxidative stress.

Abstract

Senescence accelerated mice P8 (SAMP8) is a phenotypic model of age, characterized by deficits in memory and altered behaviour. Here determined the effect of age in SAMP8, compared with the resistant strain, SAMR1, in behaviour and learning parameters linking these disturbances with oxidative stress environment. We found impairment in emotional behaviour with regard to fear and anxiety in young SAMP8 vs. age-mated SAMR1. Differences were attenuated with age. In contrast, learning capabilities are worse in SAMP8, both in young and aged animals, with regard to SAMR1. These waves in behaviour and cognition were correlated with an excess of Oxidative stress (OS) in SAMP8 at younger ages that diminished with age. In this manner, we found changes in the hippocampal expression of *ALDH2*, *IL-6*, *HMOX1*, *COX2*, *CXCL10*, *iNOS*, and *MCP-1* with an altered amyloidogenic pathway by increasing the Amyloid beta precursor protein (APP) and BACE1, and reduced *ADAM10* expression; in addition, astrogliosis and neuronal markers decreased. Moreover, Superoxide dismutase 1 (SOD1) and Nuclear factor-kappa beta (NF- κ B) expression and protein levels were higher in younger SAMP8 than in SAMR1. In conclusion, the accelerated senescence process present in SAMP8 can be linked with an initial deregulation in redox homeostasis, named neuroinflammaging, by inducing molecular changes that lead to neuroinflammation and the neurodegenerative process. These changes are reflected in the emotional and cognitive behaviour of SAMP8 that differs from that of SAMR1 and that highlighted the importance of earlier oxidative processes in the onset of neurodegeneration.

Introduction

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3 With the increase in life expectancy and aging, age-related cognitive
4 impairments are becoming one of the most important issues for human health.
5 Aging is a multifaceted process characterized by an intricate and irreversible
6 accumulation of physiological changes, and is associated with an increase in
7 transcriptional noise, aberrant production, and the maturation of many
8 messenger RNA (mRNA) (López-Otín et al., 2013). Understanding the
9 magnitude and physiological significance of earlier oxidative processes on
10 cognitive and behavioural changes and their relationship with aging processes
11 or pathological settings comprises a frontier to be crossed in order to
12 prevent/treat neurodegenerative disorders (Valko et al., 2007; Bilici et al., 2001).
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21 The brain is especially sensitive to oxidative damage and possesses a relatively
22 modest antioxidant defence (Ng et al., 2008; Halliwell et al., 2006). Oxidative
23 stress (OS) has been reported as important in the pathophysiology of a number
24 of age-related diseases, including Alzheimer disease (AD). AD is characterized
25 by the presence of three pathological hallmarks: synapse loss; extracellular
26 Senile plaques (SP), and intracellular Neurofibrillary tangles (NFT). Signs of AD
27 include accelerated memory loss and alterations of mood, reason, judgment,
28 and language. Both extracellular amyloid plaques and NFT are found in the
29 post-mortem brains of patients with AD in the cortex, hippocampus, and
30 amygdala, structures implicated in learning, memory, and emotional processes.
31 The major component of SP is Amyloid beta-peptide (A β), shown to induce OS.
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42 The majority of research on AD has focused on the disease's molecular and
43 neuropathological features and on the characteristic cognitive deficits
44 associated with the disorder. Although cognitive deficits are related with the
45 disorder, non-cognitive symptoms are becoming increasingly important due to
46 their prevalence and the dysfunctions that they generate. These non-cognitive
47 symptoms, commonly referred to as "Behavioural and psychological symptoms
48 of dementia" (BPSD), include abnormal motor behaviour, depression, fear,
49 anxiety, and personality disorders (such as aggression and irritability). In this
50 context, behavioural abnormalities such as neophobia, seizures, and increased
51 aggression or locomotor activity have often been described in AD mouse
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1 models (García-Mesa et al., 2011) or in the Senescence-accelerated mouse
2 prone 8 (SAMP8) model (Griñan-Ferré et al., 2015), together with alterations in
3 basal circadian activity.
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6 The role of OS in psychiatric and neurological disorders, including anxiety, has
7 been the focus of many investigations (Andreotti et al., 2013; Millan et al., 2012;
8 Bouayed et al., 2009; Bouayed & Bohn, 2010; Bouayed et al., 2010; Gibson et
9 al., 2012). Anxiety is a normal emotional response, but when it is inappropriate,
10 it constitutes a disorder (Gross et al., 2004; Weinberger, 2001). Studies in both
11 humans and animals have demonstrated a strong correlation between anxiety
12 and OS. It is noteworthy that several studies demonstrated that inflammatory
13 cytokines increased after OS (Anderson et al., 2013; Casadesús et al., 2002;
14 Ye et al., 1999; Terao A, et al., 2002). Mice expressing high cytokine levels
15 present enhancement of anxiety behaviour (O'Donovan et al., 2010), and the
16 overexpression of Interleukin 6 (IL-6) or Tumor necrosis factor alpha (TNF- α)
17 leads to an anxiogenic phenotype (Patki et al., 2013). Therefore, in general,
18 results regarding the role of inflammation in anxiety disorders suggest a relation
19 between these two conditions.
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32 The main objective of this work was to delve deeper into the evolution of
33 neuroinflammation and its correlation with cognitive and behavioural
34 parameters, including the molecular and cellular changes associated with age
35 and neurodegenerative processes in SAMP8, a well-characterized model for
36 studying brain aging and neurodegeneration.
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45 **Experimental Procedures**

46 Female SAMR1 ($n = 28$) and SAMP8 ($n = 28$), 2 and 9 months of age,
47 respectively, were used. These animals had free access to food and water and
48 were maintained under standard temperature conditions ($22 \pm 2^\circ\text{C}$) and
49 12h:12h light-dark cycles (300 lux/0 lux).
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56 Studies were performed in accordance with the Institutional Guidelines for the
57 Care and Use of Laboratory Animals established by the Ethical Committee for
58 Animal Experimentation at the University of Barcelona.
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3 **Behavioural and cognitive experiments**
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8 **Elevated Plus Maze**
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10 The Elevated Plus Maze (EPM) apparatus was constructed of dark and white
11 plywood (30 × 5 × 15 cm). Behaviour was scored with SMART ver. 3.0
12 software, and each trial was recorded for later analysis, utilizing a camera fixed
13 to the ceiling at height of 2.1 m and situated above the apparatus. The two
14 closed arms were darkened with cardboard to block out the light. The arms
15 radiated from a central platform (5 × 5 cm). To initiate the test session, the mice
16 were placed on the central platform, facing an open arm, and allowed to explore
17 the apparatus for 5 min. After the 5-min test, the mice were returned to their
18 home cages, and the EPM apparatus was cleaned with 70% ethyl alcohol and
19 allowed to dry between tests. Parameters recorded included time spent on open
20 arms, time spent on closed arms, time spent at the centre, rearing, freezing,
21 defecation, and urination.
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36 **Open Field**
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38 The Open Field (OF) apparatus was constructed of white plywood (50 × 50 × 25
39 cm). Red lines were drawn to divide the floor into 25-cm squares. Behaviour
40 was scored with SMART ver. 3.0 software, and each trial was recorded for later
41 analysis, utilizing a camera fixed to the ceiling at a height of 2.1 m and situated
42 above the apparatus. Mice were placed at the centre, or at one of the four
43 corners, of the open field and allowed to explore the apparatus for 5 min. After
44 the 5-min test, the mice were returned to their home cages, and the open field
45 was cleaned with 70% ethyl alcohol and allowed to dry between tests.
46 Behaviours scored included Locomotor Activity, Centre Stay Duration,
47 Periphery Stay Duration, Freezing, Rearing, Defecation, and Urination. Each
48 animal was then given a score for total locomotor activity, which was calculated
49 as the sum of line crosses and number of rearing's.
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Novel Object Recognition Test (NORT)

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 lux. 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 behaviour of the mice was 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 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, and water temperature was maintained at a temperature of 22°C \pm 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). Non-toxic 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).

1 The animals' swimming paths were recorded by a video camera mounted above
2 the centre of the pool, and the data were analysed with SMART ver. 3.0
3 software. The learning phase consisted of 6 days of trials for each mouse. The
4 animals were submitted to five trials each day starting from the positions set (in
5 random order) and without a resting phase between each trial and the
6 subsequent one. At each trial, the mouse was placed gently into the water,
7 facing the wall of the pool, and allowed to swim for 60 sec. If not able to locate
8 the platform in this time, the mouse was guided to the platform by the
9 investigator. Animals were left on the platform each time for 30 sec in order to
10 allow spatial orientation.
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19 The parameters measured were latency time in finding the platform, time spent
20 in each quadrant, and distance swum for each trial; the mean was calculated for
21 each trial day. A memory test was performed at the end of the learning days, in
22 which the platform was removed and the time spent by each mouse in each
23 quadrant was measured.
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31 **Immunodetection experiments and quantification**

32 After the behavioural test, the animals were intracardially perfused with a 4%
33 paraformaldehyde in 0.9% NaCl solution after being anesthetized with 80 mg/kg
34 of sodium pentobarbital. Brains were dissected and separated sagittally into two
35 hemispheres, frozen in liquid N₂, maintained at -80°C, and defrosted on ice
36 immediately prior to homogenization procedures. For the Western blot (WB)
37 experiment, aliquots of homogenized hippocampus containing 20–30 µg of
38 protein per sample were used. The protein samples were separated by Sodium
39 dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) (5–18%) and
40 transferred onto Polyvinylidene difluoride (PVDF) membranes (Millipore). The
41 membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS)
42 solution containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature,
43 followed by overnight incubation at 4°C with the primary antibodies listed in
44 Table 1 (Supplementary data). The membranes were then washed and
45 incubated with secondary antibodies for 1 h at room temperature.
46 Immunoreactive protein was viewed with the chemiluminescence-based
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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 routinely monitored by phenol red staining of the membrane or by Glyceraldehyde-3-phosphate dehydrogenase (GADPH) immunodetection.

For immunohistochemical studies, the frozen brains were embedded in OCT Cryostat Embedding Compound (Tissue-Tek, Torrance, CA, USA), cut into 20- μ m-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/permeabilisation 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 Supplementary Table 1). Two further washings were carried out prior to incubation with the fluorescent secondary antibody (1 h at room temperature, see Table 1 for dilutions). Finally, before mounting with Fluoromount-G™ (EMS, Hatfield, NJ, USA), nuclear staining was performed with Hoechst 2 $\mu\text{g}/\text{mL}$ 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). At least four images from 4 different individuals by group were analysed with ImageJ/Fiji software available online from the National Institutes of Health).

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 Agilent 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 aldehyde dehydrogenase 2 (*ALDH2*), inflammatory genes Interleukin 6 (*IL-6*), heme oxygenase (decycling) 1 (*HMOX1*), cyclooxygenase 2 (*COX2*), C-X-C motif chemokine 10 (*CXCL10*), inducible nitric oxide synthase (*iNOS*), monocyte chemotactic protein 1 (*MCP-1*), Matrix metalloproteinase 9 (*MMP9*), amyloid beta A4 precursor (*PreAPP*), β-secretase 1 (*BACE1*) and Disintegrin and Metalloproteinase 10 (*ADAM10*). Normalization of expression levels was performed with actin for SYBER Green and TATA-binding protein (*Tbp*) for TaqMan. The primers were as follows: for *ALDH2*, forward 5'-GCAGGCGTACACAGAAGTGA-3' and reverse 5'-TGAGCTTCATCCCCTACCCA-3'; for *IL-6*, forward 5'-ATCCAGTTGCCTTCTTGGGACTGA-3' and reverse TAAGCCTCCGACTTGTGAAGTGGT; for *HMOX1*, (Mm00516005_m1), for *COX2*, forward 5'-TGACCCCAAGGCTCAAATA-3' and reverse 5'-CCCAGGTCCTCGCTTATGATC-3', for *CXCL10*, forward 5'-GGTAGTCCTAATTGCCCTTGG-3' and reverse 5'-TTGTCTCAGGACCATGGCTTG-3', for *iNOS*, forward 5'-GGCAGCCTGTGAGACCTTTG-3' and reverse 5'-GAAGCGTTTCGGGATCTGAA-3', for *MCP-1*, forward 5'-CCCCTCACCTGCTGCTACT-3' and reverse 5'-TCTGGACCCATTCTTCTTG-3', for *MMP9*, forward 5'-CTTCTCTGGACGTCAAATGTG and reverse 5'-AGAAGAATTTGCCATGGCAG-3', for *PreAPP*, forward 5'-AGGACTGACCACTCGACCAG-3' and reverse 5'-CTTCCGAGATCTCTTCCGTCT-3', for *BACE1*, forward 5'-AAGCTGCCGTCAAGTCCATC-3' and reverse 5'-GCGGAAGGACTGATTGGTGA-3', for *ADAM10*, forward 5'-GGGAAGAAATGCAAGCTGAA-3' and reverse 5'-CTGTACAGCAGGGTCCTTGAC-3', for actin, forward 5'-CAACGAGCGTTCCGAT-3' and reverse 5'-GCCACAGGTTCCATACCCA-3' and *Tbp*, (Mm00446971_m1).

1 For SYBER Green, real-time PCR was performed on the Step One Plus
2 Detection System (Applied Biosystems) employing the SYBR Green PCR
3 Master Mix (Applied Biosystems). Each reaction mixture contained 7.5 μ L of
4 complementary DNA (cDNA), whose concentration was 2 μ g, 0.75 μ L of each
5 primer (whose concentration was 100 nM), and 7.5 μ L of SYBR Green PCR
6 Master Mix (2X) and for TaqMan gene expression assays (Applied Biosystems),
7 each 20 μ L of TaqMan reaction, 9 μ L cDNA (18ng) was mixed with 1 μ L 20x
8 probe of TaqMan Gene Expression Assays and 10 μ L of 2X TaqMan Universal
9 PCR Master Mix.
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17 Data were analysed utilizing the comparative Cycle threshold (Ct) method
18 ($\Delta\Delta$ Ct) where the actin transcript level was used to normalize differences in
19 sample loading and preparation. Each sample ($n = 4-5$) was analysed in
20 triplicate, and the results represented the n -fold difference of transcript levels
21 among different samples.
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30 **Data analysis**

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32 Data are expressed as the mean \pm Standard error of the mean (SEM) from at
33 least 4–5 samples. Data analysis was conducted using GraphPad Prism ver. 6
34 statistical software. Means were compared with two-way Analysis of variance
35 (ANOVA) and post hoc analysis. Comparisons between groups were performed
36 by unpaired Student's t test for independent samples. Statistical significance
37 was considered when p values were <0.05 . Statistical outliers were performed
38 out with Grubs' test and were removed from analysis.
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46 In addition, partial correlation controlling for group were calculated using SPSS+
47 21.00, between the variables of interest (see figure legend for details).
48 Spearman's partial correlation coefficients between each possible pair of
49 behavioural or neuronal markers were calculated with p -value adjustment for all
50 to eliminate false positives correlations.
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59 **Results**

Motivational Behaviour Analysis in Female SAMR1 and SAMP8 with age

Elevated Plus Maze (EPM) was used to determine anxiety levels in these models and the age evolution for the two strains. The specific anxiety value obtained with this paradigm, time spent closed arms, showed no changes with strain or age (Supplementary figure 1A); however, SAMP8 demonstrated a longer time spent in EPM open arms (Fig. 1A). There are strain differences in centre time occupation, but not between ages (significant differences for strain, $F(1, 28) = 11.35$, $p = 0.0022$, and age $F(1, 28) = 0.02883$, $p = 0.8664$). In EPM, level of freezing was significant elevated in young SAMP8, but there is a diminution in old SAMP8 (significant differences for age $F(1, 28) = 6.674$, $p < 0.01$, and strain $F(1, 28) = 8.600$, $p < 0.006$) (Fig. 1B). In contrast, rear behaviour presented a diminution in old SAMR1 and SAMP8 with respect to young ones, and lower levels of rears when SAMP8 was compared with age-matched SAMR1 (significant differences in age, $F(1, 28) = 34.53$, $p < 0.0001$ and strain $F(1, 28) = 16.30$, $p = 0.0004$) (Fig. 1C). For defecation, strain differences were found: [$F(1, 28) = 3.047$, $p < 0.075$] (Fig. 1D). Results obtained in the Elevated Plus Maze test (EPM) indicate that the young SAMP8 group exhibited changes in fear-anxiety-like behaviour. Additional parameters and statistical scores obtained in the EPM are listed in Table 2 (Supplementary data).

Results obtained in the OFT indicated that the young SAMP8 group exhibited a significant increase in locomotor activity [age, $F(1, 28) = 5.170$, $p < 0.0308$, and strain, $F(1, 28) = 6.777$, $p < 0.0146$], with a higher number of rears and higher freezing behaviour than age-matched R1, although this latter item did not reach significance (Figs. 2A–2C). In addition, young SAMP8 spent less time in the central zone [strain, $F(1, 28) = 30.06$, $p \leq 0.0014$, and age, $F(1, 28) = 12.56$, $p < 0.0001$], although reduced defecation events (Figs. 2D and 2E) as compared with young SAMR1 [strain, $F(1, 28) = 3.407$, $p = 0.0755$]. Additional parameters obtained in the OFT are listed in Table 3 (Supplementary data). Results for 9-months old SAMP8 exhibited a clear behaviour change with regard to young SAMP8 and in reference to SAMR1 animals. Old SAMP8 reduced locomotor activity in reference to young SAMR1 and SAMP8 ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 2A) Defecations and rear events decreased with respect to

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young SAMP8 ($p > 0.05$ (Figs. 2B and 2E). Time in centre zone and freezing did not change with age.

Results indicated a higher level of stress in young SAMP8 vs. young SAMR1, while at later ages the behaviour was similar in the two strains.

Novel Object Recognition Test (NORT) and Morris Water Maze (MWM) analysis in Female SAMR1 and SAMP8 with age

In reference to learning tests, the NORT demonstrated that SAMP8 mice exhibited impaired memory capabilities that reached significance at 9 months in comparison with age-matched SAMR1 obtaining lowest Discrimination Index (DI) [on two-way ANOVA, analysis demonstrated significant differences for age, $F(1, 28) = 8.782$, $p = 0.0061$, and for strain, $F(1, 28) = 19.17$, $p = 0.0002$] (Fig. 2F).

The results obtained in spatial-learning acquisition and retention in the MWM test illustrated that all mouse groups were able to learn over the trial days, although SAMP8 mice exhibited a slow learning progress measured as latency to escape from the platform (Fig. 1E). Final acquisition of the SAMP8 group was worse than that of the SAMR1 at both ages studied ($p < 0.01$ vs. age-matched R1). The removal test demonstrated that SAMP8 remained less time in (%) in the platform quadrant than age-matched SAMR1, and also demonstrated a lower number of entries and distance swum in the platform zone (Figs. 1F–H). The distance swum by SAMP8 inside the MWM tank exhibited a circular border swim profile without any orientation or preference for the platform area. In contrast, 2-months SAMR1 showed clear directionality to the platform quadrant at both ages tested (Supplementary figure 2).

Neuroinflammation and OS Markers in Female SAMR1 and SAMP8 Hippocampus

Neuroimmunological responsive parameters were determined through Glial fibrillary acidic protein (GFAP) expression and *IL-6* genic expression. GFAP

1 expression was significant higher (in hippocampal CA3, CA1, and Dentate
2 gyrus. DG) in young SAMP8 than in young SAMR1 (Figs. 3A and 3B-D), and
3 only statistically significant differences were observed in the DG in SAMR1 old
4 mice (For statistics details see figure notation). In reference to inflammation
5 mediator results, higher expression of *IL-6*, *CXCL10*, *ALDH2*, *HMOX1*, *MCP-1*,
6 *COX2*, *iNOS*, and *MMP9* were determined in young SAMP8 hippocampus than
7 in young SAMR1 (Figs. 4A–H). Expression of *IL-6* and *MCP-1* were significantly
8 lower in old SAMP8 than in young mice, reaching values nearest to those of old
9 SAMR1, although remaining significantly higher. *HMOX1*, *CXCL10*, and *iNOS*
10 expression levels were maintained higher in old SAMP8 compared with old
11 SAMR1, whereas *COX2* levels increased in the old SAMR1 strain. *ALDH2*
12 expression was diminished in SAMP8 at all ages studied, indicating a lower
13 capability for responding to OS than SAMR1. No significant changes in *MMP9*
14 were detected.
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26 Additionally, protein levels of 4-Hydroxynonenal (4-HNE) and Superoxide
27 dismutase 1 (SOD1) were studied. Results showed significantly higher levels of
28 these OS markers in SAMP8 with regard to SAMR1 at any ages of the studied
29 (for statistics details see figure notations) (Figs. 4I and 4L). Conversely, the p65
30 active fraction of Nuclear factor-kappa beta (NF- κ B) was found at the same
31 levels in young mice (SAMP8 and SAMR1) and decreased in old SAMP8
32 ($p < 0.05$) (Figs. 4I and 4J).
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42 **Neurodegeneration and AD parameters in Female SAMR1 and SAMP8** 43 **Hippocampus** 44

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46 NeuN, Bax protein, tau phosphorylation, and the Amyloid precursor protein
47 (APP) pathway were studied as indicative of the neurodegeneration process.
48 Immunohistochemical staining indicated lower NeuN hippocampal levels in
49 SAMP8 at the ages studied vs. SAMR1 (Figs. 5A and 5B-D). Increased Bax
50 protein expression was found in SAMP8 vs. SAMR1 both at 2 and at 9 months
51 of age (Figs. 4I and 4M). In reference to tau post-transcriptional modification,
52 phosphorylation in Ser199 and Ser396 were studied. Results showed an
53 increase in tau hyperphosphorylation in SAMP8 compared with SAMR1 [pTau
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1 Ser 199, Strain: $F(1, 8) = 43.36, p = 0.0002$; Age: $F(1, 8) = 17.14, p = 0.0033$;
2 pTau Ser396, Age: $F(1, 8) = 28.59, p = 0.0007$] (Figs. 6A–C). APP pathway
3 was found also increased in SAMP8; in this respect, SAMP8 presented a
4 significant diminution in *ADAM10* gene expression compared with SAMR1 at all
5 ages studied, but an increase in APP gene expression and higher BACE1
6 expression at younger ages in SAMP8 with significant increase in sAPP β and
7 BACE1 protein levels, accompanied by reduced sAPP α (For statistic details see
8 figure notations) (Figs. 10D-K).

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15 Finally, partial correlation analysis determined the robust relationship among the
16 cognitive, behaviour, OS, inflammaging, neurodegenerative and AD parameters
17 evaluated through the work in young and old SAMP8 and SAMR1 (Table 4 and
18 Fig. 7).

22 Discussion

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29 It has been described that with age, OS increases, and this oxidative
30 environment plays a nuclear role in the senescence process. The focus of the
31 work was to explore the possible effects of OS on ageing and their correlation
32 with emotional disorders, such as anxiety and cognitive decline, and we
33 employed herein SAMP8, a well-characterized model for studying brain aging
34 and neurodegeneration. SAMP8 mice present signs of accelerated aging in
35 several organic systems, such as skin, skeletal muscle, eyes, vessels, and
36 brain, display a short life span (10.2 months) compared with control strain
37 SAMR1 mice (Nomura et al., 1999; Morley et al., 2012a). SAMP8 has been
38 studied as a non-transgenic murine model for accelerated senescence and late-
39 onset AD (Pallàs et al., 2008). These mice exhibited cognitive and emotional
40 disturbances from young ages, probably due to brain pathological hallmarks
41 such as OS, inflammation, and activated neuronal death pathways, mainly
42 affecting the brain's cortex and hippocampus (Takeda, 2010).

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49 Here, we established that young female SAMP8 had raised neurodegenerative
50 and cognitive/emotional disturbances induced by an oxidant environment that
51 fostered changes in the molecular markers of inflammation, gliosis, tau
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1 hyperphosphorylation, and Bax (Fig. 7 and Table 4). The majority of these
2 desynchronized parameters normalized with aged SAMR1, indicating that these
3 adverse conditions at young ages occur based in accelerated senescence in
4 this mouse model of aging.
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9 The behavioural tests applied demonstrated that young SAMP8 presented, on
10 the whole, anxiety and restless, fearful behaviour, with higher locomotion and
11 rears, avoiding the OF centre zone. This anxious behaviour reduced with age,
12 resembling that of old SAMR1. As expected, cognitive impairment occurs earlier
13 in female SAMP8 than in SAMR1. Behaviour and cognitive changes in SAMP8
14 in comparison with SAMR1 were demonstrated in males but, to our knowledge,
15 this is the first time that these have been demonstrated in female SAMP8.
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23 Inflammaging, in brain “neuroinflamm-aging”, is a current concept that involves
24 changes in molecular, biochemical, and cellular changes or processes that are
25 implicated in senescence. Moreover, growing evidence suggests that the brain
26 tissues of patients with AD are characterized early by greater OS. This process
27 is included in whole, increased OS, altered expression in OS mediators, gliosis,
28 and neurodegeneration. For instance, numerous studies indicate that
29 hemeoxygenase is a major cell-adaptive responder to stress (Sanli et al., 2014).
30 Because altered inflammatory marker levels are described in SAMP8, here we
31 screened some of the most important gene expression proteins, such as
32 cytokines *HMOX1*, *CXCL10*, *COX2*, and *iNOS*, or protein levels of SOD1, of a
33 NF- κ B complex member (p65), and of 4-HNE, a by-product of protein oxidation
34 implicated in the oxidative process and that subsequently participates in
35 neuroinflammation.
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49 The results obtained here exhibited higher gene expression of *IL-6*, *HMOX1*,
50 *CXCL10*, *COX2*, and *iNOS* in 2-month-old SAMP8 vs. SAMR1, demonstrating
51 that neuroinflammation is present at early ages in SAMP8, rendering the
52 oxidative environment that would occur at the starting point of the senescence
53 process in these mice. Moreover, consistent with OS parameters and
54 behavioural results, the SAMP8 group exhibited a significant difference related
55 with hippocampal integrity compared with SAMR1, measured by the loss in
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1 NeuN immunostaining in the hippocampus. NeuN is localized at the core of the
2 majority of neural cells during development, is expressed in post-mitotic
3 neurons from early stages of differentiation, and its expression persists in the
4 adult (Mullen et al., 1992). These results are in agreement with a number of
5 reports in which insufficient neurogenesis is demonstrated in SAMP8 to
6 compensate for neuronal loss during aging and neurodegeneration (Gang et al.,
7 2011; Díaz-Moreno et al., 2013; Griñan-Ferré et al., 2015).
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14 Additionally, our results demonstrated an increase in Bax protein and in the
15 level of hyperphosphorylated tau forms, clearly indicative that the
16 neurodegenerative process has been initiated at these young ages in SAMP8.
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21 Neuronal altered markers were reflected in impairment in the learning
22 capabilities shown in MWM and NORT, and also in the emotional disturbances
23 described in young SAMP8 (Griñan-Ferré et al., 2015), thus reinforcing the key
24 role of the initial oxidative process in the SAMP8 senescence phenotype.
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30 Increases in tau phosphorylation were reported in 5-month-old SAMP8 males
31 (Canudas et al., 2005) and a time-dependent accumulation of
32 hyperphosphorylated tau in SAMP8 males (Casadesús et al., 2012). It is
33 noteworthy that here we demonstrated the increase in p-Tau (Ser199) and p-
34 Tau (ser396) in the hippocampus as early as 2 months in SAMP8. Tau
35 phosphorylation increases have been linked with OS (Castellani et al., 2008;
36 Moreira et al., 2010) and also with SAMP8 (Casadesús et al., 2012). Some
37 authors claim a compensatory role of phospho tau driven by cells against OS
38 that serves a protective function (Bonda et al., 2011). In control-strain SAMR1,
39 tau did not increase its phosphorylated state and indeed did increase in aged
40 mice, indicating that OS is lower in the SAMR1 brain; thus, this possible
41 compensatory effect is not required. Lower OS in SAMR1 was demonstrated
42 because levels of oxidative markers such as *MCP-1*, *IL-6*, *iNOS*, *CXCL10*, and
43 *HMOX1* did not change with age in SAMR1, and NF- κ B is indeed lower in aged
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58 Alteration in APP processing is also postulated with an earlier onset of AD. OS-
59 induced BACE activity and sAPP β levels were suppressed by gamma (γ -
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secretase inhibitors (Jo et al., 2010). Activities of both enzymes were greater in
brain-tissue samples from patients with AD, and protein levels of BACE1 were
elevated in 3xTg-AD mice, thus the OS-induced expression of BACE1 resulting
in excessive A β production in AD (Jo et al., 2010). In 2-month-old SAMP8, in
addition to an increase in *APP* gene expression, a misbalance in the APP
amyloidogenic pathway was demonstrated with increases in BACE1 and
reduced *ADAM10* gene and protein expression; both are secretases implicated
in APP processing and the generation of amyloid beta. It is noteworthy that OS
is also implicated in the enzymatic activity of secretases (Mouton-Liger et al.,
2012), and the pattern exhibited by young female SAMP8 demonstrated that
increased OS gave rise to disturbances in APP processing similar to those
involved in the occurrence of AD when ageing SAMP8 and SAMR1 presented
similar APP pathway processing but, as noted, this appeared earlier in SAMP8,
correlating with behavioural disturbances and cognitive deficits.

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IL-6 is involved in the pathogenesis of neurodegenerative diseases such as AD
(Quintanilla et al., 2004). In the nervous system, *IL-6* mainly occurs in activated
glia, such as astrocytes and microglial cells. Our results demonstrated a
significant increase in *IL-6* genic expression in the SAMP8 compared with the
SAMR1 group, suggesting an alteration in inflammatory processes gated to the
SAMP8 strain that impairs SAMR1 on ageing. A higher degree of
neuroinflammation in young SAMP8 correlated with earlier cognitive impairment
and the initial neurodegenerative process in these mice. The more normalized
or equal inflammation levels in aged SAMP8 when compared with SAMR1,
denoting accelerated senescence in the SAMP8 strain, is correlated with
inflammaging and the neuroinflammation process at earlier ages.

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In this respect, the SAMP8 model fits as a useful tool to study earlier
inflammaging changes and neurodegenerative processes at late ages.
Therefore, young SAMP8 can be used to study the initial and key step of the
senescence process that leads, in elderly SAMP8, to cognitive impairment and
neurodegeneration.

60 Author Contributions

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CG-F and VP-A performed genomic and proteomic experimental procedures, VP-A and DP performed behavioural and cognitive procedures. CG-F and AC evaluated the results. FA and MP are seniors scientists who designed the experiments and wrote the manuscript after elaborate discussion by all of the authors.

Acknowledgements

We thank Maggie Brunner, M.A., for reviewing the language and style of the manuscript, and Dr. Antonio Miñarro for his help in statistical analysis. This study was supported by Spanish MINECO, and the European Regional Development Fund (SAF-2012-39852) to MP and BFU2013-48822-R to FA. CG-F, VP-A, AC, and MP belong to CIBERNED and 2014SGR 525.

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Figure legends

Figure 1. Results of Elevated plus maze (EPM) and Morris water maze (MWM) in SAMR1 and SAMP8 at 2 and 9 months. For EPM test: Time spent in open arms (A), freezing (B), rears (C) and defecations (D). Results of spatial learning and memory: Escape latency time to reach the hidden platform during training days (E), percentage time spent in platform zone during 60 s probe trial of MWM test (F), number of entries in platform zone during 60 s probe trial of MWM test (G), trail distance in platform zone during 60 s probe trial of MWM test (H). Data represented as observed mean \pm Standard error of the mean (SEM); ($n = 14$ for each group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 2. Results of Open field test in SAMR1 and SAMP8 at 2 and 9 months. Locomotor activity (A), rears (B), freezing (C), time spent in centre zone (D), defecations (E). Results of Discrimination index of Novel Object recognition testes (NORT) (F). Data represented as observed mean \pm Standard error of the mean (SEM) ($n = 14$ for each group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.000$.

Figure 3. Representative images for GFAP immunostaining (A) and quantification on the bar chart (B-D) in SAMR1 and SAMP8 at 2 and 9 months. Bars represent mean \pm Standard error of the mean (SEM); ($n = 4$ for each group). *** $p < 0.001$; **** $p < 0.0001$. CA: *Cornu Amonis*, DG: *Dentate Gyrus*. Scale bar for immunohistochemical images is 200 μm .

Figure 4. Pro-inflammatory and oxidative stress gene expression in SAMR1 and SAMP8 at 2 and 9 months for *IL-6* (A), *MCP-1* (B), *HMOX1* (C), *CXCL10* (D), *iNOS* (E), *COX2* (F), *ALDH2* (G), *MMP9* (H). Gene expression levels were determined by real-time PCR. Representative Western blot for NF- κ B, SOD1, 4-HNE and Bax (I), and quantifications (J-M). Mean \pm Standard error of the mean (SEM) from five independent experiments performed in triplicate are represented. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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Figure 5. Representative images for NeuN immunostaining (A) and quantification on the bar chart (B-D) in SAMR1 and SAMP8 at 2 and 9 months. Bars represent mean \pm Standard error of the mean (SEM); ($n = 4$ for each group; CA: *Cornu Amonis*; DG: *Dentate Gyrus*. Scale bar for immunohistochemical images is 200 μm ; *** $p < 0.001$; **** $p < 0.0001$.

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Figure 6. Representative Western blot for p-Tau (Ser199), p-Tau (Ser396) and Tau total (A), sAPP α , sAPP β and BACE1 (D), and quantifications (B-C, E-G). Beta-amyloid pathway gene expression in SAMR1 and SAMP8 at 2 and 9 months for *Amyloid Beta (A4) precursor* (H), *ADAM10* (I) and *BACE1* (K). Gene expression levels were determined by real-time PCR. Mean \pm Standard error of the mean (SEM) from five independent experiments performed in triplicate are represented. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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Figure 7. Behavioral and neuronal markers hierarchical network of the SAMR1 and SAMP8 at two ages ($n=56$) obtained by using yEd graph editor (v. 3.14.4). Each node represents one behavioral or neuronal marker and each edge between two nodes represents the partial correlation. Colors represent the different variables and node dimensions represent Behavioral and neuronal markers hierarchical network of the SAMR1 and SAMP8 at two ages the number of correlations. Solid black line represents positive correlation and dotted red line represents negative correlation.

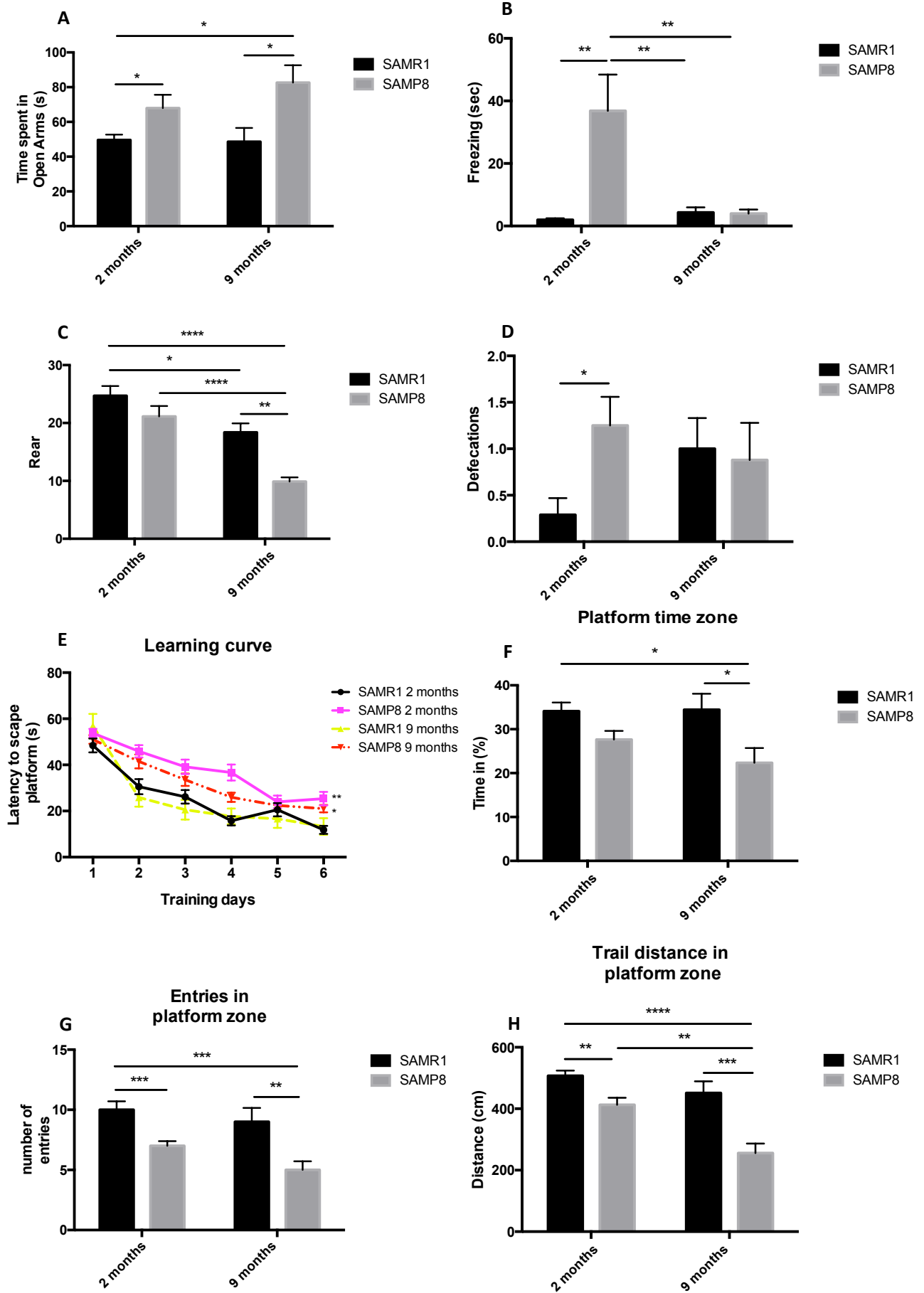
Table 4(s)

Table 4: Partial correlation controlling for group coefficients between selected variables included in the study.

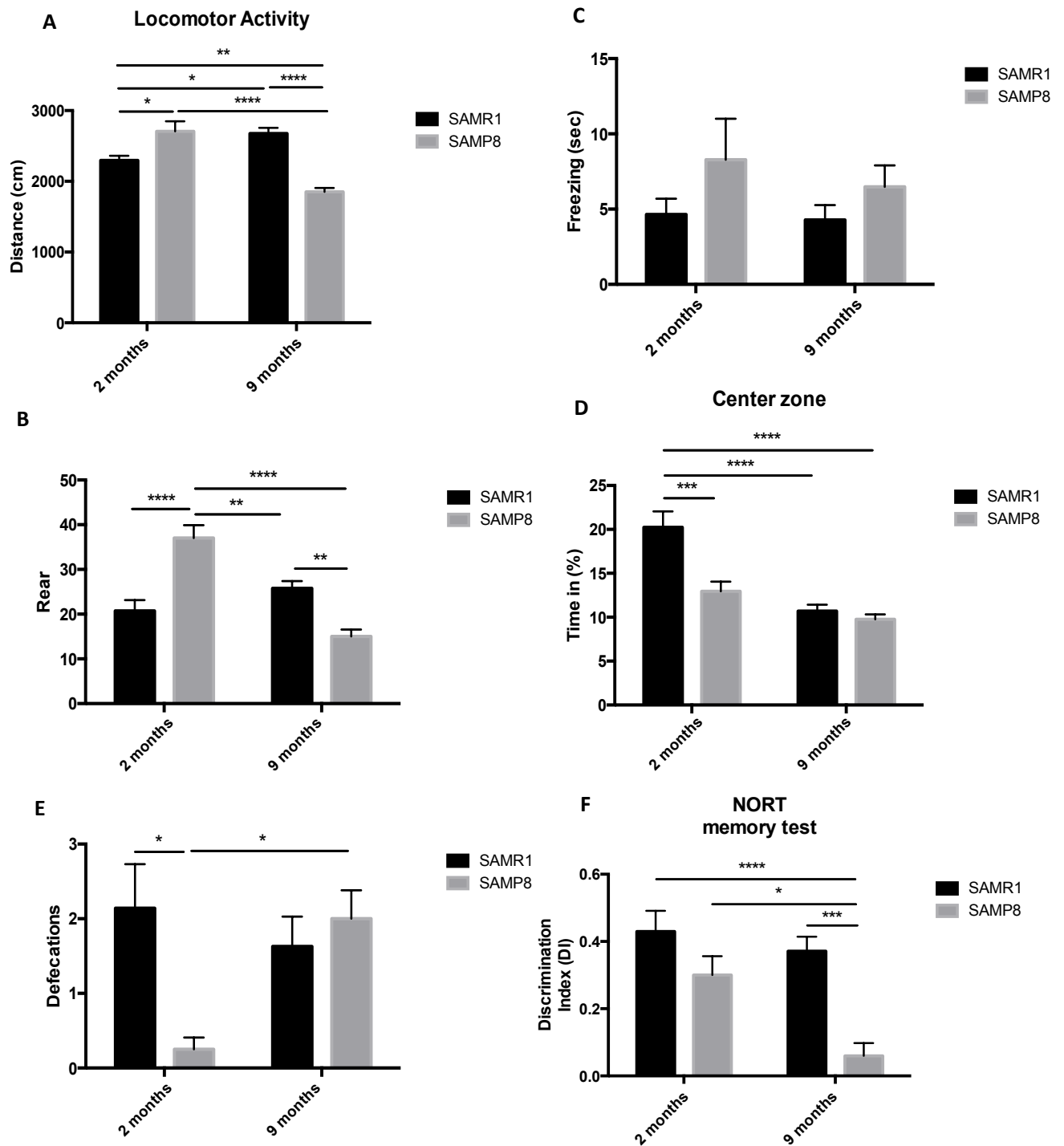
	DI	Platform time (%) MWM	Rears (n) OF	Freezing (sec) EPM	IL-6	CXCL10	COX2	iNOS	ALDH2	HMOX1	MCP-1	ADAM10	Amyloid precursor r (A4)	SOD1	Bax	4-HNE	BACE1	Ratio pTau199	Ratio pTau396	NF-KB	sAPP α	sAPP β	GFAP	NeuN		
DI	1	0,435^{**}	0,164	0,027	-0,277	-0,249	0,05	-0,626^{**}	-0,068	-0,479	-0,153	0,298	-0,223	-0,563^{**}	-0,439^{**}	-0,312	-0,182	-0,313	-0,455^{**}	-0,076	0,12	-0,677^{***}	0,064	0,714^{***}		
Platform time (%) MWM		1	0,135	-0,003	-0,264	0,03	0,283	-0,564^{**}	-0,169	-0,583^{**}	-0,244	0,581^{**}	-0,353	-0,747^{***}	-0,512^{**}	-0,494^{**}	-0,196	-0,408	-0,477^{***}	-0,027	0,265	-0,76^{***}	0,011	0,814^{***}		
Rears (n) OF			1	0,677^{***}	0,682^{**}	-0,423	0,127	0,243	0,159	0,297	0,749^{**}	-0,353	0,067	-0,332	-0,009	-0,669^{***}	-0,79^{***}	-0,733^{***}	-0,636^{**}	0,693^{***}	-0,067	-0,522^{**}	0,716^{***}	0,534^{**}		
Freezing (sec) EPM				1	0,632^{**}	-0,693^{**}	0,119	0,315	0,166	0,509	0,718^{**}	-0,594^{**}	0,405	-0,359	0,069	-0,6^{**}	-0,634^{***}	-0,437^{**}	-0,302	0,57^{**}	-0,261	-0,235	0,691^{***}	0,341		
IL-6					1	-0,308	-0,428	0,514^{**}	0,452	0,66^{**}	0,843^{***}	-0,636^{**}	0,309	0,108	0,447	-0,692^{***}	-0,856^{***}	-0,464	-0,188	0,862^{***}	-0,669^{***}	-0,038	0,671^{***}	-0,02		
CXCL10						1	0,011	0,054	-0,437	-0,425	-0,54^{**}	0,346	-0,408	0,236	0,027	0,314	0,405	0,033	0,399	-0,439	0,285	0,361	-0,543^{**}	-0,329		
COX2							1	-0,238	-0,399	-0,339	-0,171	0,383	0,123	-0,137	-0,102	0,447	0,251	-0,084	-0,252	-0,135	0,74^{**}	-0,448	-0,048	0,493		
iNOS								1	0,449	0,803	0,392	-0,389	0,275	0,346	0,62^{**}	-0,422	-0,485	-0,341	0,451	0,686^{**}	-0,549^{**}	0,128	0,32	-0,228		
ALDH2									1	0,673^{**}	0,324	-0,302	0,554^{**}	0,315	0,468	-0,06	-0,262	0,078	0,119	0,6^{**}	-0,574^{**}	0,221	0,178	-0,217		
HMOX1										1	0,592^{**}	-0,478	0,392	0,168	0,473	-0,554^{**}	-0,573^{**}	-0,124	0,327	0,814^{***}	-0,763^{***}	-0,007	0,402	-0,223		
MCP-1											1	-0,581^{**}	0,413	-0,138	0,286	-0,781^{***}	-0,917^{***}	-0,603^{***}	-0,403	0,85^{***}	-0,436	-0,375	0,854	0,317		
ADAM10												1	-0,378	0,267	-0,213	0,563^{**}	0,657^{**}	0,312	0,145	-0,564^{**}	0,598^{**}	-0,201	-0,568^{**}	0,128		
Amyloid precursor r (A4)													1	0,149	0,646^{**}	-0,105	-0,332	-0,099	0,007	0,561^{**}	-0,284	0,058	0,353	0,07		
SOD1														1	0,608^{**}	0,317	0,151	0,008	0,334	0,216	-0,104	0,182	-0,109	-0,091		
Bax															1	-0,111	-0,43^{**}	-0,271	0,149	0,579^{**}	-0,447^{**}	0,199	0,34	-0,053		
4-HNE																1	0,764^{***}	0,302	0,063	-0,487^{**}	0,654^{**}	-0,055	-0,575^{**}	0,108		
BACE1																	1	0,66^{**}	0,377	-0,795^{***}	0,559^{**}	0,137	-0,88^{***}	-0,196		
Ratio pTau199																		1	0,611^{**}	-0,456^{**}	-0,085	0,441^{**}	-0,782^{**}	-0,616^{**}		
Ratio pTau396																			1	-0,168	-0,39	0,601^{**}	-0,559^{**}	-0,679^{***}		
NF-KB																				1	-0,566^{**}	0,013	0,705^{***}	0,047		
sAPP α																					1	-0,468^{**}	-0,298	0,518^{**}		
sAPP β																							1	-0,298^{**}	-0,646^{**}	
GFAP																									1	
NeuN																										1

The values used to calculate Partial correlation controlling for group coefficients were behavioral parameters (showed in Figures 1E, 2C, 3 and 4B); protein levels (showed in Figures 7B-E, 9B-C and 10B-D); gene expression (showed in Figures 6A-H and 10E-G) and Immunocytochemistry (showed in Figures 5B and 8B). Correlation (2-tailed) is significant * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$; (-) Negative covariation of two variables.

Griñán-Ferré et al, Figure 1.

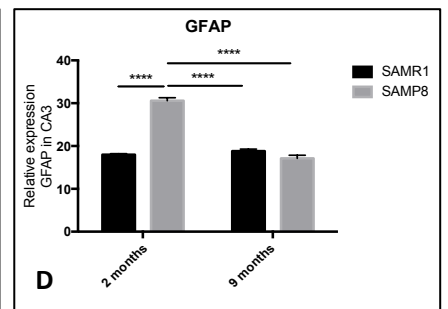
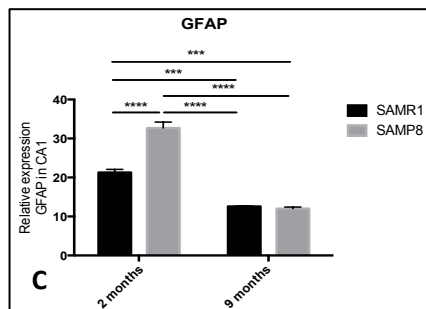
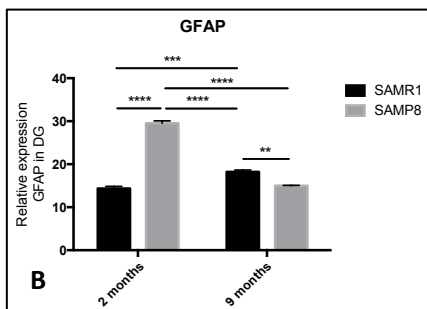
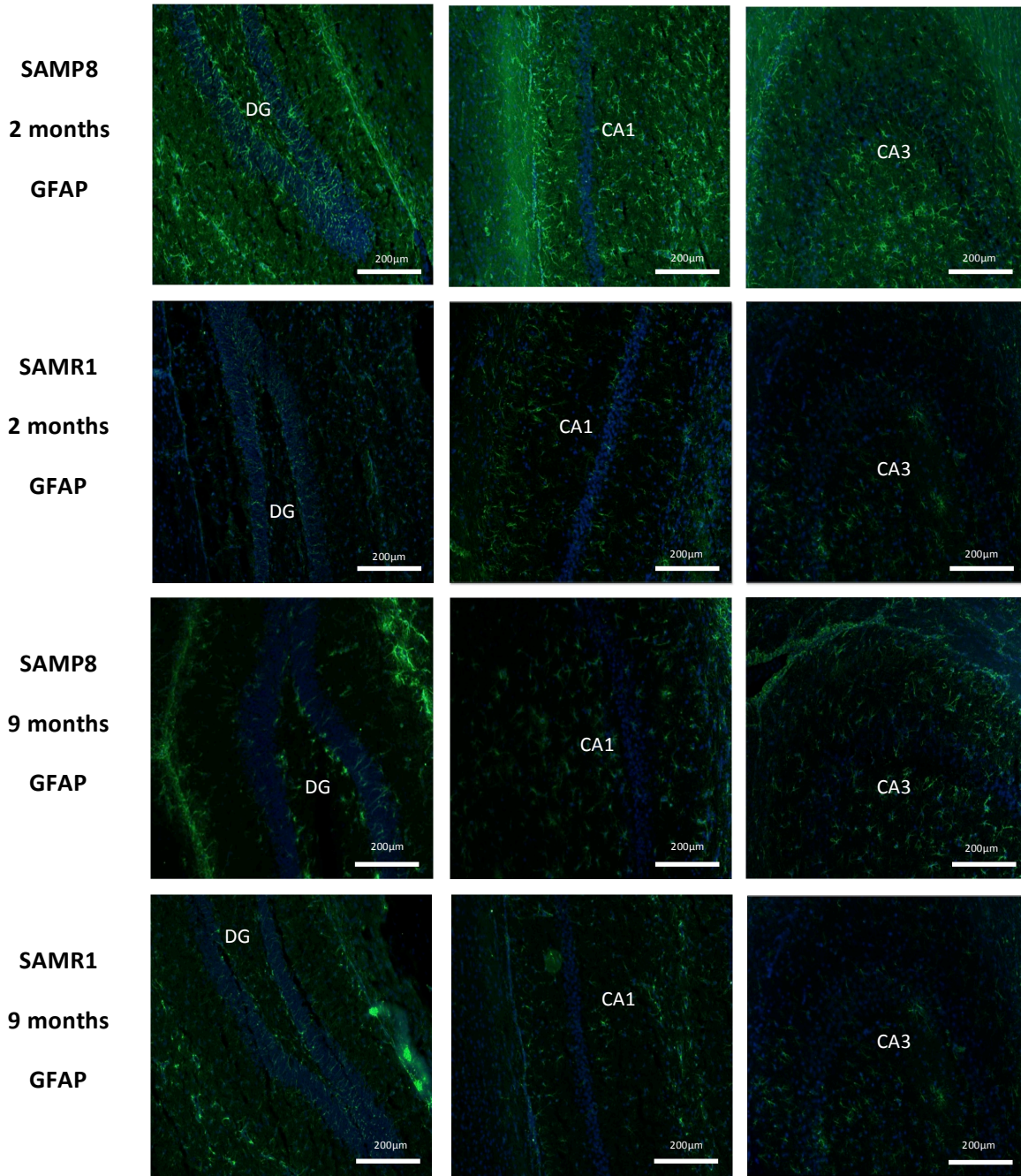


Griñán-Ferré et al, Fig 2.

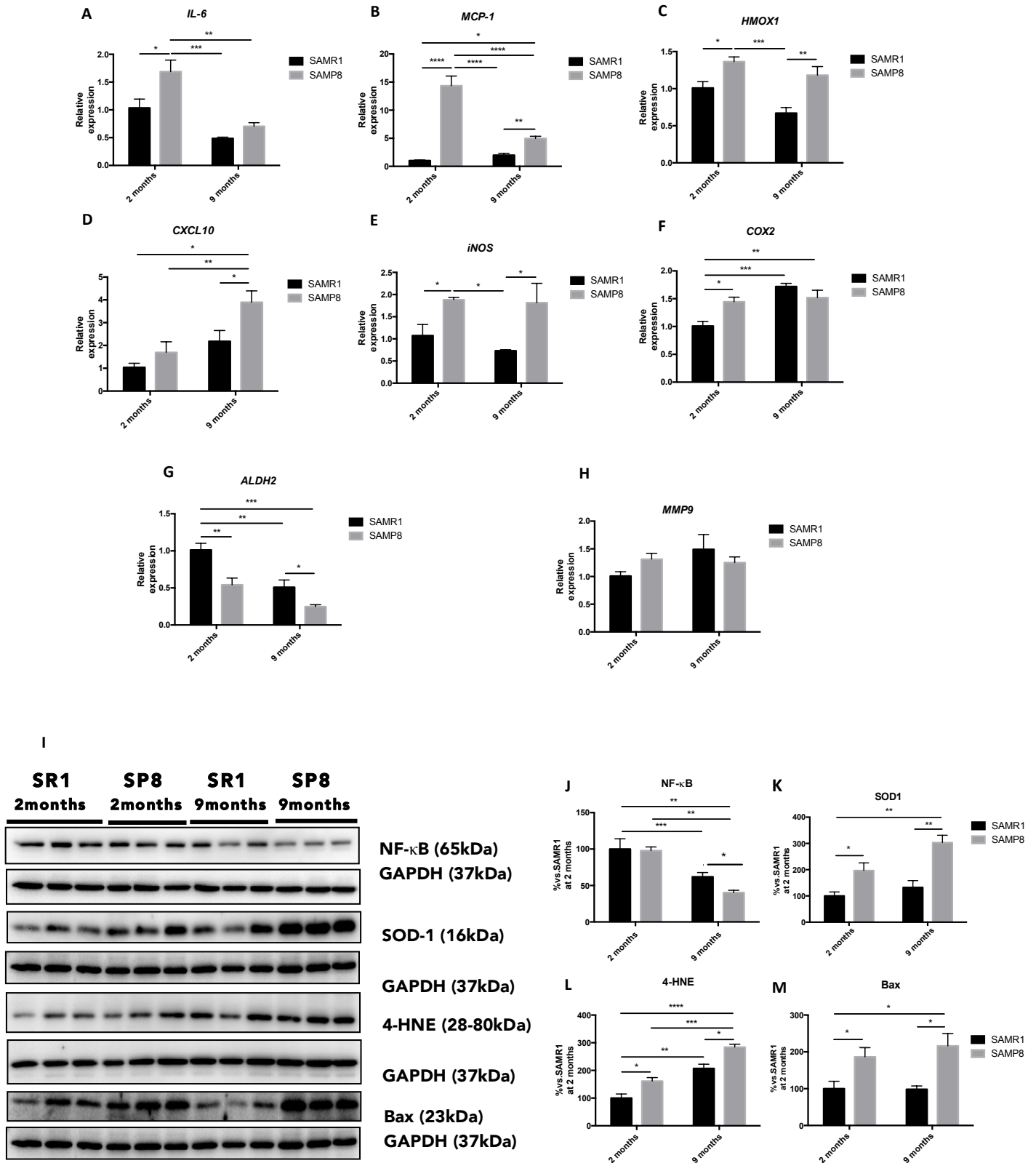


Griñán-Ferré et al, Figura 3.

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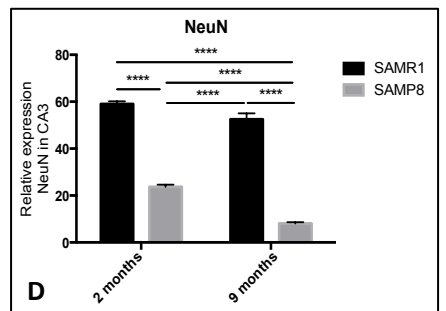
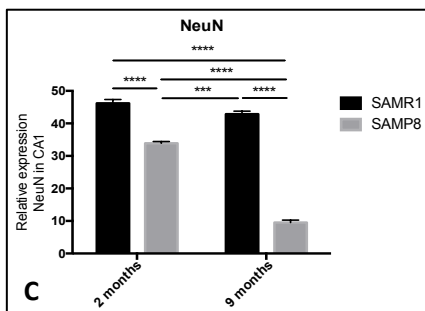
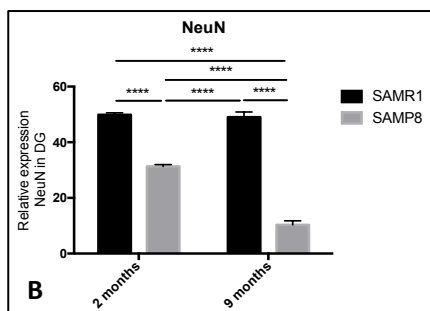
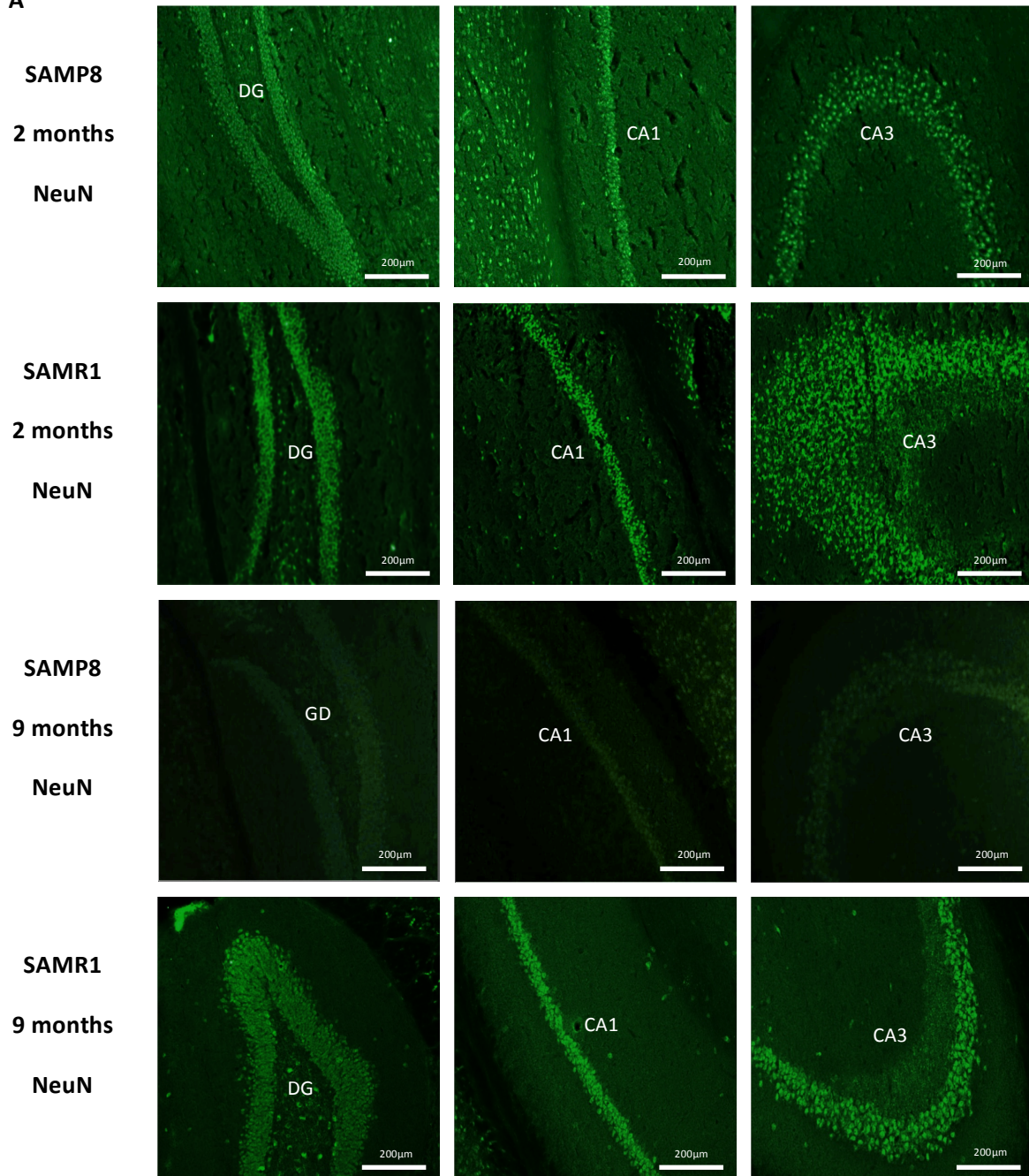


Griñán-Ferré et al, Fig 4.

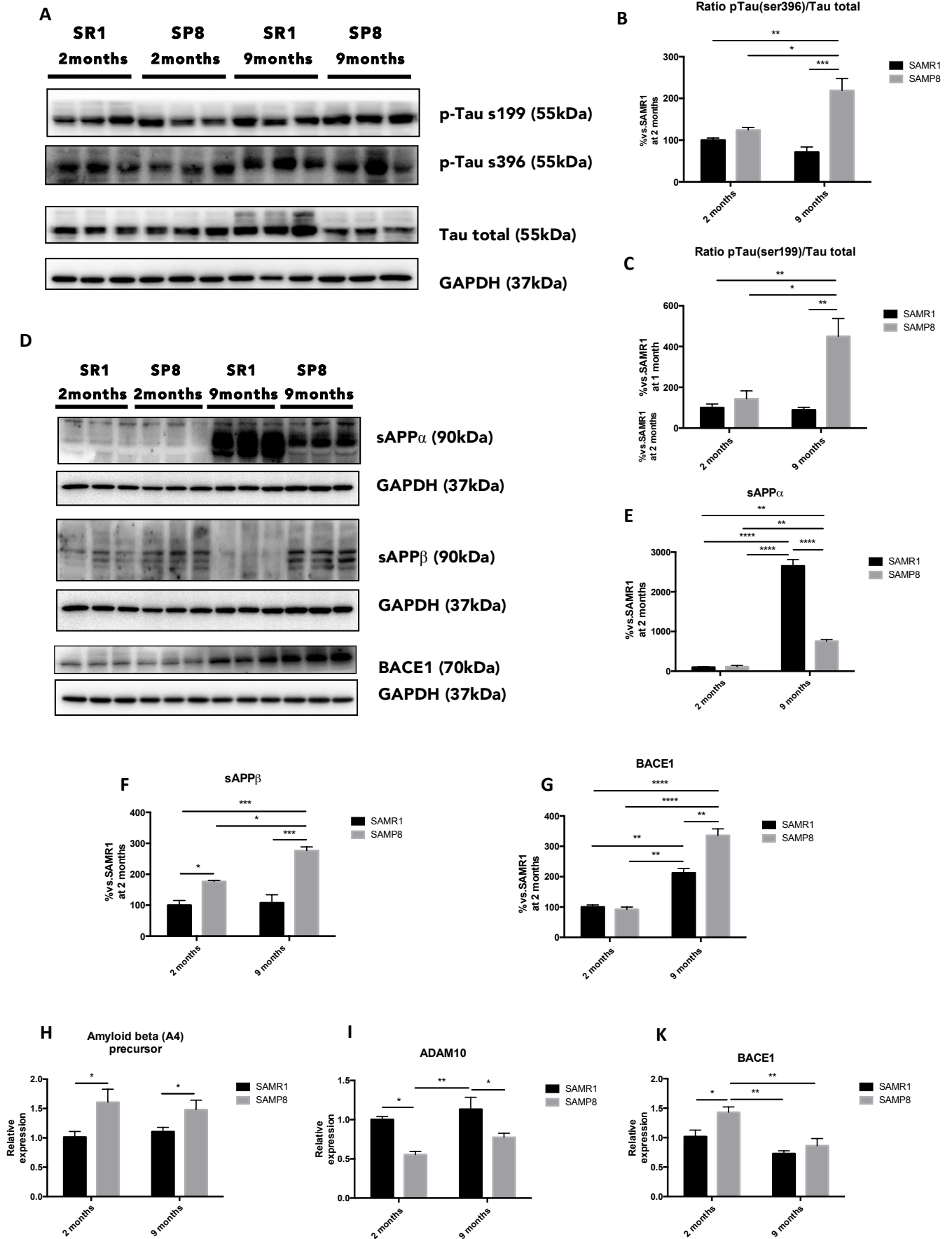


Griñán-Ferré et al., Figura 5.

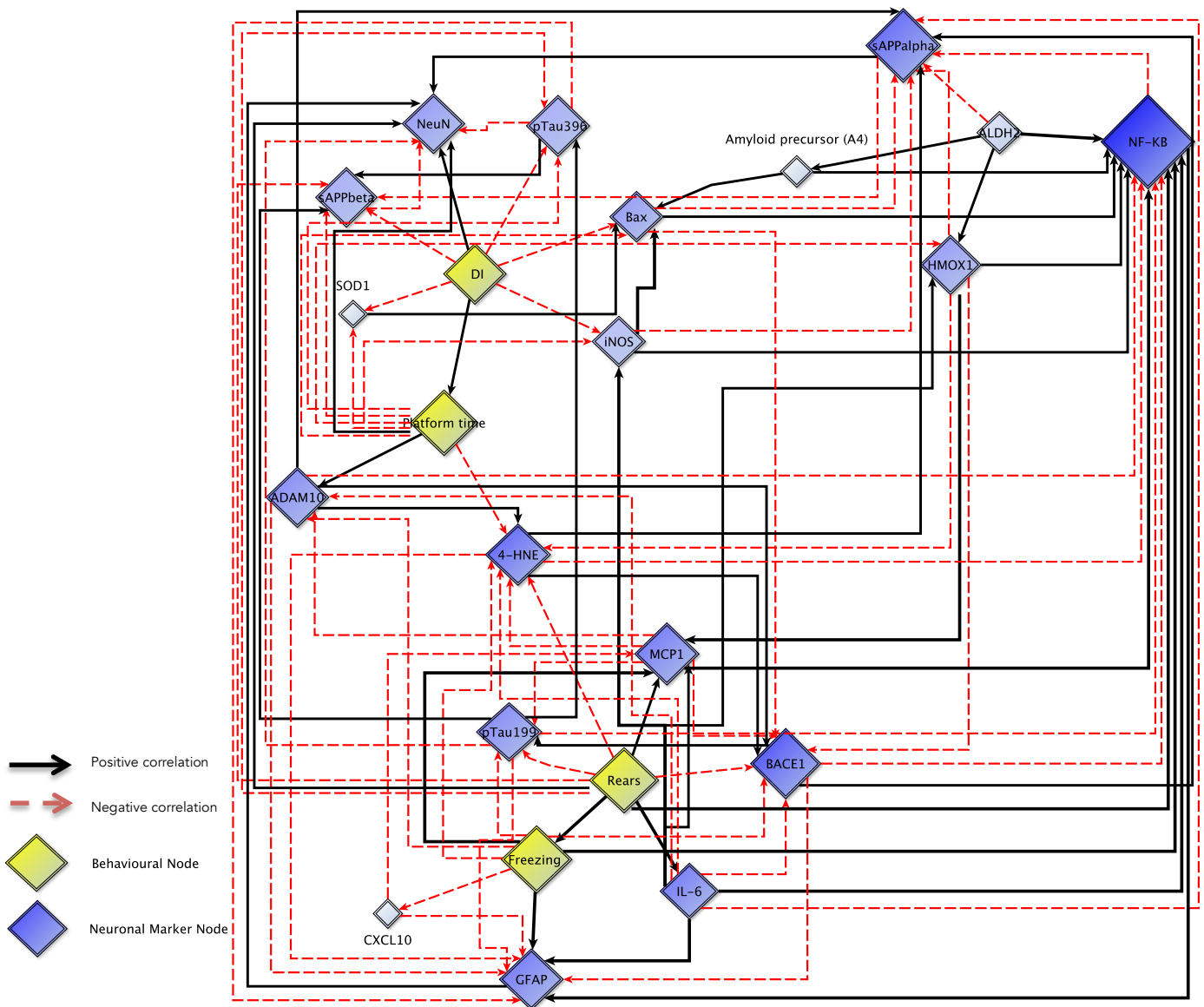
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Griñán-Ferré et al, Figure 6



Griñán-Ferré et al., Figure 7.



The network was calculated using yEd graph editor version 3.14.4.

Table 1. Antibodies used in Western blot and Immunohistochemical studies.

Antibody	Host	Source/Catalog	WB dilution	ICH dilution
Bax	Rabbit	Cell Signaling/#2772	1:1000	
BACE1	Rabbit	Cell Signaling/D10E5	1:1000	
4-HNE	Rabbit	Abcam/ab46545	1:1000	
SOD1	Sheep	Calbiochem/574597	1:1000	
sAPPα	Rabbit	Covance/SIG-39139-005	1:1000	
sAPPβ	Rabbit	Covance/SIG-39138-050	1:1000	
p65	Rabbit	Cell Signaling/D14E12	1:1000	
GAPDH	Mouse	Millipore/MAB374	1:2000	
NeuN	Mouse	Millipore/MAB377	1:1000	1:100
GFAP	Mouse	Abcam/ab48050-100		1:400
p-Tau s396	Rabbit	Invitrogen/44752G	1:1000	
Tau total	Goat	Santa cruz/sc-1995	1:1000	
p-Tau s199	Rabbit	Invitrogen /44734G	1:1000	
Alexa Fluor 546 donkey anti-rabbit IgG A		Molecular probes/ AF488:A21202		1:400
Alexa Fluor 488 donkey anti-mouse IgG A Alexa		Molecular probes/AF555:A31572		1:250
Donkey-anti-goat HRP conjugated		Santa Cruz Biotech/ sc-2020	1:3000	
Goat-anti-mouse HRP conjugated		Biorad/# 170-5047	1:2000	
Goat-anti-rabbit HRP conjugated		Cell Signaling/# 7074	1:2000	

Table 2. Parameters measured in the Elevated Plus Maze Test (EPM). Results are expressed as mean \pm Standard error of the mean (SEM). *p <0.05; **p <0.05; ***p<0.001 vs SAMR1, 2 months. #p <0.05; ##p <0.001 vs SAMP8, 2 months. \$p<0.05 vs SAMR1, 9 months.

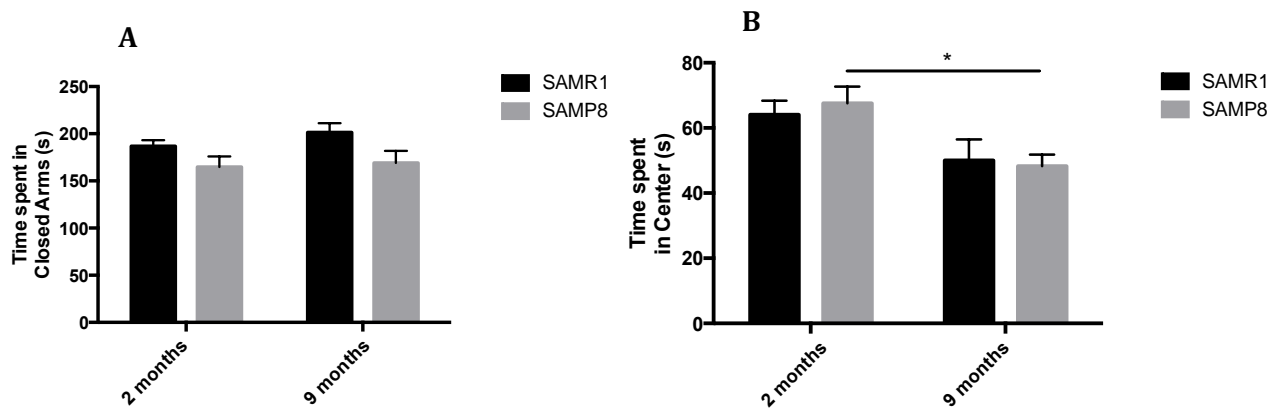
	SAMR1 2 months	SAMP8 2 months	SAMR1 9 months	SAMP8 9 months
Time in zone- Center (sec), a	64.12 \pm 4.24	67.56 \pm 5.11	50.05 \pm 6.42	48.29 \pm 3.55 [#]
Time in zone- Open Arms (sec), b	49.64 \pm 3.06	67.97 \pm 7.69 [*]	48.64 \pm 7.86	82.65 \pm 9.98 ^{*,§}
Time in zone- Closed Arms (sec), c	186.53 \pm 6.40	164.63 \pm 11.36	201.25 \pm 9.91	169.02 \pm 12.66
Freezing (sec), d	1.98 \pm 0.47 ^{##}	36.86 \pm 11.58 ^{**}	4.33 \pm 1.64 ^{##}	3.99 \pm 1.28 ^{##}
Rearings (n), e	24.71 \pm 1.67	21.13 \pm 1.80	18.38 \pm 1.55 [*]	9.88 \pm 0.72 ^{****,####,\$\$}
Defecations (n), f	0.29 \pm 0.18	1.25 \pm 0.31 [*]	1.00 \pm 0.33	0.88 \pm 0.40 [#]
Urinations (n), g	0.00 \pm 0.00	0.00 \pm 0.00	0.38 \pm 0.18	0.38 \pm 0.18

Table 3. Parameters measured in the Open Field Test (OFT). Results are expressed as a mean \pm Standard error of the mean (SEM). * $p < 0.05$; ** $p < 0.05$; *** $p < 0.001$ vs SAMR1, 2 months. # $p < 0.05$; ### $p < 0.001$ vs SAMP8, 2 months. \$ $p < 0.05$ vs SAMR1, 9 months.

	SAMR1 2 months	SAMP8 2 months	SAMR1 9 months	SAMP8 9 months
Total Distance (cm), a	2,297.92 \pm 63.72*	2,706.36 \pm 140.98*	2,676.39 \pm 80.67	1,850.20 \pm 56.41**, ###, \$\$\$
Distance in Zone Center (cm), b	466.26 \pm 46.25	355.16 \pm 41.60	286.86 \pm 22.51	181.21 \pm 13.27
Entries into Zone Periphery, c	1,831.08 \pm 60.32	2,351.20 \pm 112.87	2,389.53 \pm 71.85	1,669.00 \pm 48.91
Center (%), d	20.23 \pm 1.82	12.95 \pm 1.10***	10.70 \pm 0.73****	9.75 \pm 0.58****
Periphery (%), e	79.77 \pm 1.82	87.05 \pm 1.10	89.31 \pm 0.73	90.25 \pm 0.58
Freezing (sec), f	4.64 \pm 1.05	8.28 \pm 2.72	4.28 \pm 0.99	6.48 \pm 1.42
Rearings (n), g	20.71 \pm 2.44	37.00 \pm 2.91****	25.75 \pm 1.64	15.00 \pm 1.58####, \$\$
Defecations (n), h	2.14 \pm 0.59	0.25 \pm 0.16*	1.63 \pm 0.40	2.00 \pm 0.38\$
Urinations (n), i	0.29 \pm 0.18	0.13 \pm 0.13	0.50 \pm 0.29	0.75 \pm 0.25

Supplementary 1. Results of Elevated plus maze in SAMR1 and SAMP8 at 2 and 9 months. Time spent in closed arms (A), time spent in centre (B). Data represented as observed mean \pm Standard error of the mean (SEM); ($n = 14$ for each group). * $p < 0.05$.

Griñán-Ferré et al., Supplementary 1.



Supplementary 2. Representative swimming paths in SAMR1 and SAMP8 at 2 and 9 months during 60 s probe trial of MWM.

Griñán-Ferré et al., Supplementary 2.

