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Long-Term Exercise Modulates Hippocampal Gene Expression in Senescent Female Mice

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Abstract. The senescence-accelerated SAMP8 mouse is considered a useful non-transgenic model for studying aspects of progressive cognitive decline and Alzheimer's disease (AD). Using SAMR1 mice as controls, here we explored the effects of 6 months of voluntary wheel running in 10-month-old female SAMP8 mice. Exercise in SAMP8 mice improved phenotypic features associated with premature aging (i.e., skin color and body tremor) and enhanced vascularization and BDNF gene expression in the hippocampus compared with controls. With the aim of identifying genes involved in brain aging responsive to long-term exercise, we performed whole genome microarray studies in hippocampus from sedentary SAMP8 (P8sed), SAMR1 (R1sed), and exercised SAMP8 (P8run) mice. The genes differentially expressed in P8sed versus R1sed were considered as putative aging markers (i) and those differentially expressed in P8run versus P8sed were considered as genes modulated by exercise (ii). Genes differentially expressed in both comparisons (i and ii) were considered as putative aging genes responsive to physical exercise. We identified 34 genes which met both criteria. Gene ontology analysis revealed that they are mainly involved in functions related to extracellular matrix maintenance. Selected genes were validated by real-time quantitative PCR assays, i.e., collagen type 1 alpha 1 (col1a1), collagen type 1 alpha 2 (col1a2), fibromodulin (fmod), prostaglandin D(2) synthase (ptgds), and aldehyde dehydrogenase (Aldh1a2). As a whole, our study suggests that exercise training during adulthood may prevent or delay gene expression alterations and processes associated with hippocampal aging in at-risk subjects.

Keywords: Aging, Alzheimer's disease, brain, exercise, gene, long-term, mice, microarrays, SAMP8, voluntary

INTRODUCTION

It is now well accepted that sedentarism is a risk factor for cardiovascular and metabolic diseases and for premature mortality. Indeed, the World Health Organization has stressed the relevance of an active lifestyle to prevent chronic diseases and promote healthy aging [1]. The regular practice of moderate physical exercise enhances cardiovascular health [2–5], decreases the impact of risk factors affecting metabolic and brain health [6–11], and protects cognitive and psychological functions [12–15]. There is increasing evidence of the beneficial effects of aerobic physical exercise on synapse and general brain

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function, at both young and old ages and in both healthy and pathological states [15, 16]. In particular, several studies using transgenic mouse models of Alzheimer's disease (AD) have demonstrated the protective effects of this healthy lifestyle against neurodegeneration [17]. The rodent cortex and hippocampus are particularly sensitive to exercise-induced modulation of genes involved in neuronal plasticity and activity [18–21]. Although transcriptional responsiveness varies over the lifetime, many brain genes still seem to be responsive to exercise in old animals and in animal models for neurodegenerative diseases [22–24]. The precise molecular pathways that sustain these processes are unknown but recent data have described that epigenetic modifications seem to be involved in the mechanisms of cognitive and stress resistance improvement associated with physical exercise [17].

The senescence-accelerated P8 (SAMP8) mouse model of aging displays many features known to occur early in the pathogenesis of chronic diseases. The SAMP8 strain was initially selected from AKR/J mice for its spontaneous differential phenotypic characteristics indicative of an accelerated aging process. SAM resistant mice (SAMR1), with a similar genetic background to SAMP8, show normal aging characteristics and have been extensively used as an appropriate control model [25]. SAMP8 mice suffer increased oxidative stress, vascular dysfunction, overproduction of amyloid-β protein precursor (AβPP) and amyloid-β (Aβ) protein, cholinergic deficits in the forebrain, increased tau phosphorylation, and cognitive and behavioral alterations [26–28]. Therefore, the SAMP8 mouse is considered a useful model to study certain aspects of cognitive decline and AD.

Here we show that a long-term voluntary exercise intervention in adult SAMP8 mice improved external signs of phenotypic decline (i.e., skin color, coat appearance, body tremor) and enhanced vascularization and BDNF gene expression in the hippocampus. Through whole genome microarray analyses, we identified hippocampal genes that were altered in sedentary SAMP8 and reached control levels in response to exercise, therefore representing putative aging genes responsive to chronic exercise practice.

MATERIALS AND METHODS

Animal care

All experimental procedures were approved by the Ethics Committee of the University Autonomous of Barcelona (Comissió Ètica d'Experimentació Animal i Humana, CEEAH, UAB), following the 'Principles of laboratory animal care', and were carried out in accordance with the European Communities Council Directive (86/609/EEC).

SAMP8 and SAMR1 female mice were provided by El Parc Tecnològic (Barcelona, Spain). They were maintained under standard conditions (temperature $23 \pm 1^{\circ}$C, humidity 50–60%, 12 : 12-h light-dark cycle, lights on at 7 : 00 a.m.), with food (A04, Harlan, Spain) and tap water available *ad libitum* throughout the study. Body weight (g) was measured weekly. Sentinels from the facility were tested regularly to ensure the facility was virus- and pathogen-free.

Voluntary exercise paradigm

The running wheels (ENV-044 Mouse Low-Profile Wireless Running Wheel, Med Associates Inc.; 15.5 cm circumference; 25° from horizontal plane) were located in the animal colony room inside cages of 19 cm high × 27 cm wide × 40 cm deep. Wheel-running activity was monitored through a wireless transmitter system by using a Hub (13.70×15.25 cm^2) located in the same animal colony room. Revolutions were monitored continuously although voluntary activity occurred primarily during the dark phase.

Four-month-old mice were randomly divided into four groups (R1 sedentary, R1 running, P8 sedentary, and P8 running ($n = 14$/group) and housed 2–3 mice per cage in plastic Macrolon colony boxes (15 cm high × 27 cm wide × 27 cm deep) with a sawdust floor. For 6 months, three alternate days a week (Monday–Wednesday–Friday or Tuesday–Thursday–Saturday), mice were individually accommodated in large cages containing only sawdust (sedentary groups) or sawdust with a running wheel available *ad libitum* for voluntary exercise (exercise groups)

Modified SHIRPA test

The day before sacrifice, the degree of senescence was evaluated according to a modified SHIRPA Test [29]. The SHIRPA is a comprehensive assessment during which a total of nine separate measurements are recorded for each animal ($n = 14$/group). The mice were first weighed, and evaluated for body position (0, active; 1, inactive or hyperactive), skin color (0, pink; 1, bleached or deep red), coat appearance (0, tidy; 1, irregularities), body tremor (0, absent; 1, present), eyes closure (0, open; 1, one closed; 2, both closed), lordokyphosis of the spine (0, absent;

1, present), and lacrimation (0, absent; 1, present). The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 15.0). The non-parametric Mann-Whitney *U* test was applied to analyze the data. Values are expressed as mean ± standard error of the mean.

Brain processing and tissue harvesting

Animals were decapitated and brains were dissected on ice to obtain hippocampi which were immediately frozen and stored at –80°C for further analysis ($n = 7$/group).

For the immunohistochemistry studies, the animals were transcardially perfused with saline buffer ($n = 7$/group). Brains were dissected, fixed in methacarn (60% methanol, 30% acetic acid, 10% chloroform), dehydrated, and embedded in paraffin wax. Microtome coronal sections of 20 μm were made and brain slices containing the central zone of the hippocampus (bregma –2.30 approximately) were selected for immunostaining.

Total RNA extraction and DNA microarray analysis

Total RNA was extracted using *mir*Vana™ RNA Isolation Kit (Applied Biosystems) following the manufacturer's instructions. The yield, purity, and quality of RNA were determined spectrophotometrically (NanoDrop, USA) and using the Bioanalyzer 2100 capillary electrophoresis. RNAs with 260/280 ratios and RIN higher than 1.9 and 7.5, respectively, were selected. Gene expression analysis in hippocampus from 10-month-old sedentary SAMR1 (R1sed), sedentary SAMP8 (P8sed), and exercised SAMP8 mice (P8run) were carried out with 4×44 K whole mouse genome microarrays (Agilent; $n = 5$/group). Total RNA (500 ng) was labeled with Cy3 dye using the Quick Amp Labeling Kit, One-Color from Agilent. Fluorescently labeled samples were hybridized to the microarray slide with 60-mer probes. Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Axon 4000B scanner. GenPix Pro 6.0 extraction software was used for the detection of signals.

Raw microarray data were deposited with GEO Accession Numbers GSE36571. The R Project for Statistical Computing (http://www.r-project.org/) and Perl Programming Language (http://www.perl.org/) were used to analyze the data considering the following analysis steps: Quality control of raw data (Imageplots, MAplots, Boxplots, Density Plots, Spike-Mix, Quality Filters, Pearson Cor. coef, HCL and PCA); Data filtering; Background correction and normalization; Quality control of normalized data. Background correction and normalization were done with Bioconductor's limma package using the method's "minimum" and "quantile" respectively [30].

Table 1
List of primers and probe sets used for real time q-PCR analysis. TaqMan FAM-labeled specific probes, Applied Biosystems (ABI)

Target	Reference (ABI)	Ref Seq ID
Aldh1a2	Mm00501306_m1	NM_009022.4
Bdnf	Mm01334042_m1	NM_001048142.1
Col1a1	Mm00801666_g1	NM_007742.3
Col1a2	Mm00483888_m1	NM_007743.2
Fmod	Mm00491215_m1	NM_021355.3
Ptgds	Mm01330616_g1	NM_008963.2
Tbp	Mm00446971_m1	NM_013684.3
TrkB	Mm00435422_m1	NM_001025074.1, NM_008745.2
Vegfa	Mm01281449_m1	NM_001025250.3, NM_001110266.1, NM_001110267.1, NM_009505.4

The Venn diagram was generated from the lists of differentially expressed genes showing a fold change greater than 1.5 ($\log2 > |0.5849|$) and a p value <0.05 in the comparison "P8sed vs. R1sed" (putative aging genes) and in the comparison "P8run vs. P8sed" (putative exercise-modulated genes). The Database for Annotation Visualization and Integrated Discovery (DAVID v6.7) bioinformatics program and the Gene Ontology (GO) database were used for functional pathway enrichment analysis of the genes grouped in the intersection of the Venn diagram. GO categories with p values and Benjamini adjustment <0.05 were considered statistically significant.

To validate the microarray data, real-time quantitative PCR was performed on the same RNA samples.

Real-time quantitative PCR

Random-primed cDNA synthesis was performed at 37°C starting with 0.3 μg of RNA, using the High Capacity cDNA Archive kit (Applied Biosystems). Gene expression was measured in an ABI Prism 7900HT Real Time PCR system using TaqMan FAM-labeled specific probes (Applied Biosystems, Table 1). Results were normalized to Tbp gene expression. Transcript variant 4 (NM_001048142.1) corresponding to exon V of BDNF gene was analyzed.

Immunohistochemistry

Slides with brain sections were deparaffinized in xylene, rehydrated in a graded series of ethanol solutions (100, 90, and 70%) and finally washed twice with PBS for 5 min. Then, brain sections were blocked and permeabilized with PBS containing 1% bovine serum albumin and 0.1% Triton-X-100 (Sigma-Aldrich) for 20 min. After two 5-min washes in PBS, slides were incubated with the primary antibody, rabbit anti-laminin (Sigma, St. Louis, Missouri, USA; dilution 1 : 100) overnight at 4°C. They were then washed again and incubated for 1 h at room temperature in the dark with the secondary antibody, AlexaFluor 546 donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA; dilution 1 : 250). Finally, slides were washed, mounted using Prolong Gold (Invitrogen) anti-fade medium, allowed to dry overnight at room temperature and stored at 4°C. Image acquisition was performed with a fluorescence laser microscope (BX41, Olympus, Germany). To quantify the area of the blood vessels (VA), images were analyzed with the open-source Fiji software. Briefly, in order to exclude incidental variations, CA1 picture from each animal ($n = 4$, 13–15 slices/group) were taken, the images were binarized (vessels = black, background = white), a region of interest was set to include all the CA1 seen in the image and the positive black area was quantified. The positive black area corresponds to the positive laminin staining and is equivalent to the amount of vessel surface in the studied region.

RESULTS

Long-term exercise performance induces phenotypic improvements in old SAMP8 mice

We explored the effects of 6 months of voluntary wheel running in SAMP8 mice which were 10-month-old at the end of the training. Consistent with their accelerated aging phenotype, during the intervention SAMP8 mice displayed less exercise than SAMR1 control mice (Fig. 1A) and the mean number of wheel revolutions/week for the complete intervention was significantly lower in SAMP8 mice compared with SAMR1 controls (SAMR1 = 74296 ± 5590; SAMP8 = 56964 ± 5186; Fig. 1B).

Taking into account the advanced age of the SAMP8 mice upon completion of the long-term intervention, we analyzed the impact of the training on a set of phenotypic traits using a modified SHIRPA test, which evaluates different aging traits (i.e., skin color, coat

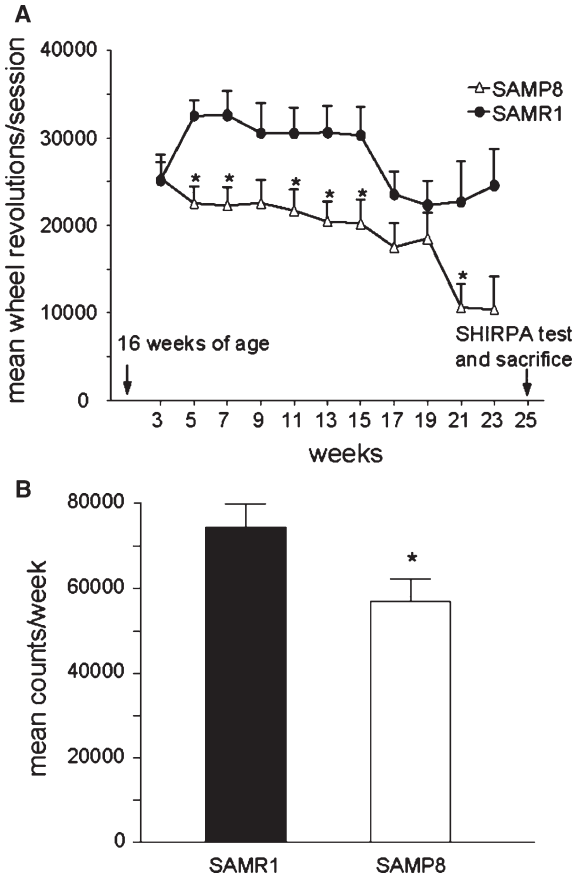

Fig. 1. Long-term exercise performance in SAMP8 and SAMR1 mice. Wheel-running activity was continuously monitored through a wireless transmitter during the 6-month intervention. A) Mean number of wheel revolutions per session was calculated as the total wheel revolutions per week/numbers of sessions of the week (ANOVA: $F(1,26) = 7.868$ $^*p < 0.01$). B) Mean number of wheel revolutions per week was calculated as the mean total wheel revolutions/total number of weeks ($n = 14$/group; $^*p < 0.05$ versus R1 run group).

appearance, body tremor, lacrimation, and others). Mean scores for lordokyphosis of the spine (hump), body tremor, and skin color were significantly higher in sedentary SAMP8 (P8sed) mice compared with sedentary SAMR1 controls (R1sed). While the lordokyphosis of the spine was not responsive to the intervention, exercise practice abolished the tremor signs in SAMP8 mice and beneficially influenced skin color aging signs in both groups (Fig. 2).

Another positive effect of the 6-month exercise intervention was the significant hippocampal revascularization in exercised SAMR1 and SAMP8 mice (Fig. 3A–E). Microscopic visualization of immunostained brain sections with laminin showed reduced blood vessel area in P8sed mice compared with R1sed animals (Fig. 3 C versus A). Both groups of exercised

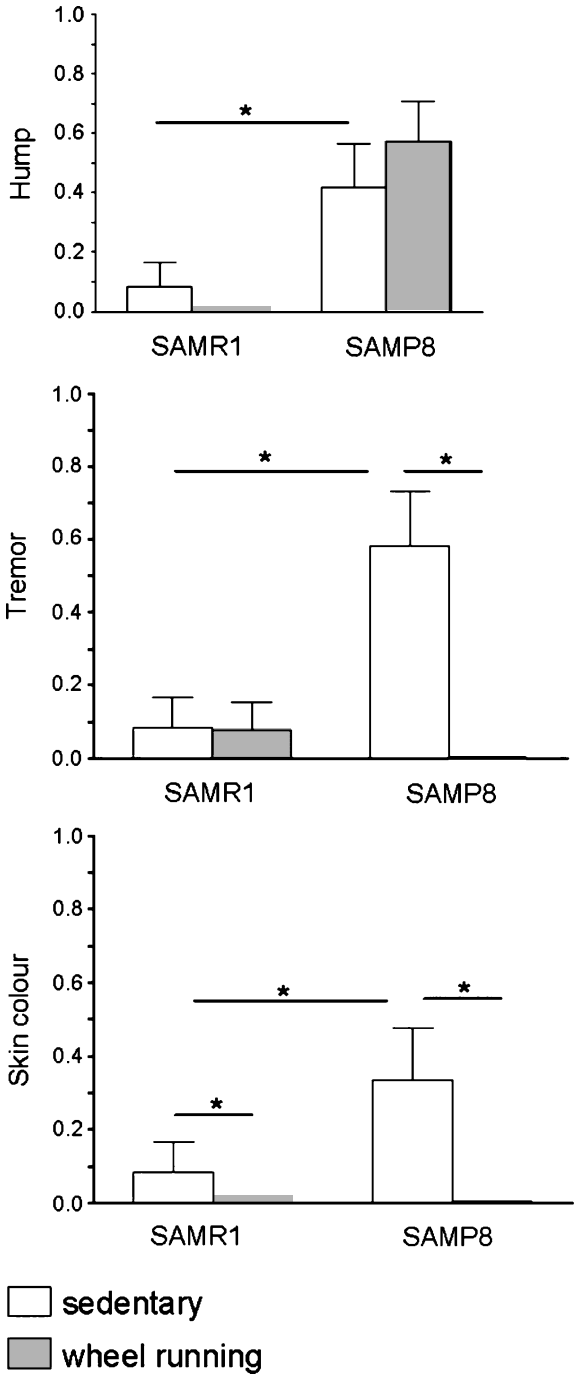

Fig. 2. Exercise delays aging traits in old SAMP8 mice. Mice were evaluated for aging-sensitive traits using the modified SHIRPA test. Higher scores correspond to more advanced age phenotypes. Traits showing significant basal differences between groups included: lordokyphosis of the spine (0, absent; 1, present), body tremor (0, absent; 1, present), and skin color (0, pink; 1, bleached or deep red). Total score was recorded as the sum of the scores for each of the categories (*$p < 0.05$, $n = 14$/group; non-parametric Mann-Whitney U test).

mice exhibited enhanced vascularization compared with their respective age-matched sedentary controls (Fig. 3B versus A, and D versus C). Remarkably, the area of blood vessels in P8run mice was significantly higher than in R1sed mice and reached levels undistinguishable from those observed in R1run mice (Fig. 3D versus A and B, respectively) The effects of exercise on revascularization seem to be unrelated to VEGF expression which was significantly decreased in the SAMP8 mice but was not modified by the intervention in any group (Fig. 3F).

Upregulation of Bdnf gene expression in hippocampus from old SAMP8 mice after long-term exercise training

Bdnf upregulation is emerging as one of the most reproducible molecular outcomes of physical exercise in the rodent hippocampus [17]. Before performing the microarray studies, we further confirmed the efficacy of the long-term exercise intervention in old SAMP8 mice by examining Bdnf gene expression as well as the expression of its receptor TrkB. Both genes were found underexpressed in P8sed compared with R1sed mice (Fig. 4). After the exercise intervention, Bdnf gene expression was significantly upregulated in P8run mice (Fig. 4A). In contrast, TrkB gene expression showed no improvement after the exercise training (Fig. 4B). No effect of exercise was observed on Bdnf or TrkB genes in SAMR1 mice.

Whole genome microarray analysis in hippocampus of old SAMP8 mice after long-term exercise training

With the aim of identifying genes involved in brain aging and modulated by long-term voluntary exercise, we performed whole genome microarray studies in the hippocampus from sedentary SAMP8 (P8sed), sedentary SAMR1 (R1sed). and exercised SAMP8 (P8run) mice. We compared "P8sed vs. R1sed" in order to detect putative aging markers (A) and "P8run vs. P8sed" to detect genes modulated by exercise (B). For both comparisons, only genes differentially expressed with a fold change ≥ 1.5 and a p value <0.05 were included. Genes differentially expressed in A and B were integrated in a Venn diagram which yielded 34 genes in the intersection (Fig. 5). The overlap between the lists A and B was significant as revealed by Fisher's exact test (p-value <3 E-16). These genes therefore met the criteria for putative aging genes modulated by exercise (Table 2).

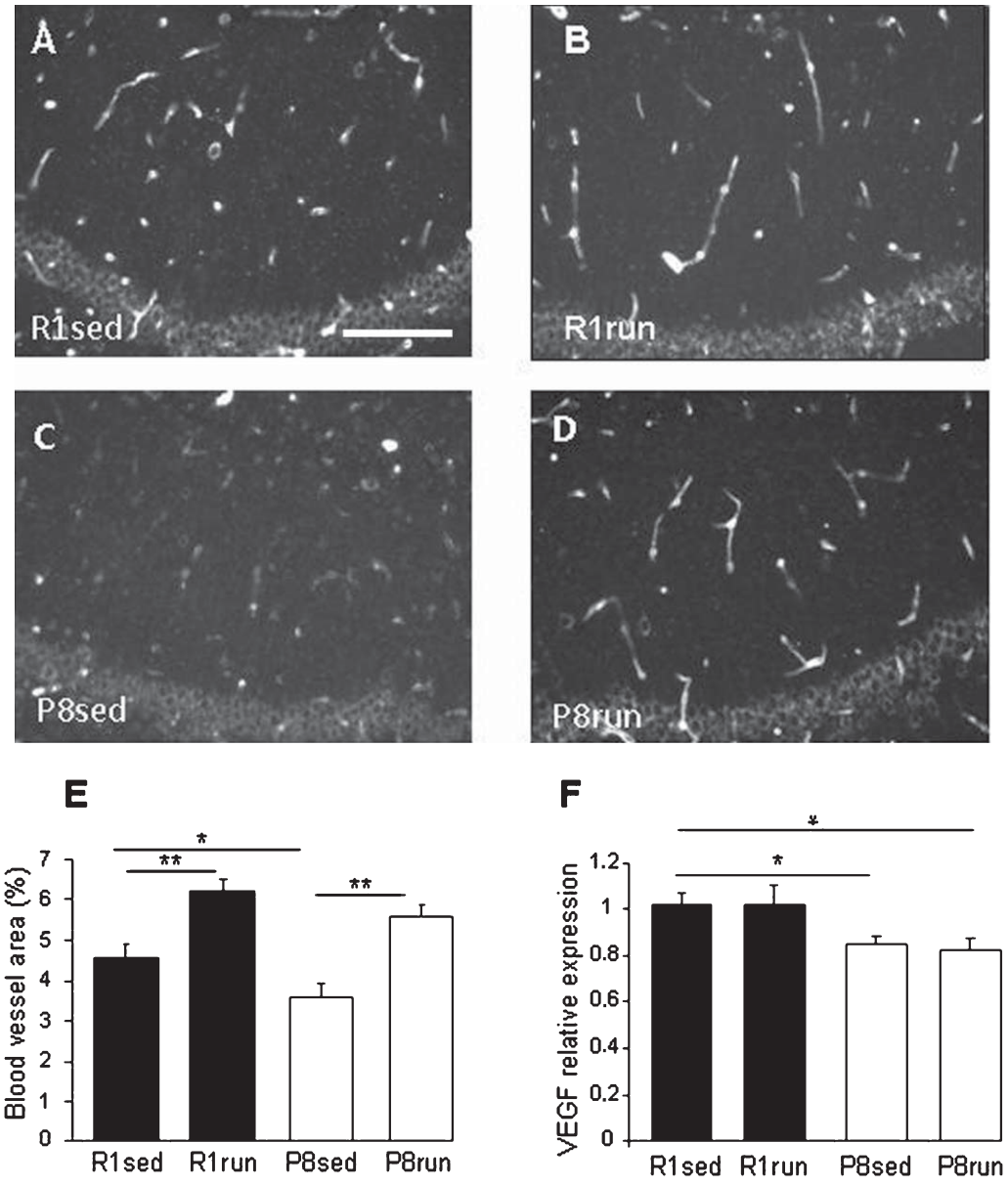

Fig. 3. Effects of 6 months of voluntary exercise on hippocampal vascularization in old SAMP8 mice. Hippocampal sections were incubated with the primary antibody rabbit anti-laminin (red) in sedentary SAMR1 (A, R1sed), SAMR1 (B, R1run), sedentary SAMP8 (C, P8sed), and exercised SAMP8 (D, P8run). Representative images are shown, scale bar: 100 μm in all panels. E) Area of the blood vessels (VA) was quantified with the open-source Fiji software. F) Vegf gene expression was measured by real time quantitative PCR. *$p < 0.05$ 2 tail t-test; ** $p < 0.005$ 2 tail t-test ($n = 4$, 13–15 slices/group).

Pathway enrichment analysis of the genes listed in Table 2 was performed using the DAVID program and the Gene Ontology (GO) database. GO categories with p values and Benjamini adjustment <0.05 were considered statistically significant (Table 3A). We found that these genes were mainly clustered in functions associated with extracellular matrix maintenance. The reported functions in the central nervous system of the genes included in the GO categories are listed in Table 3B [31–43].

Moreover, in accordance with the literature (see Discussion), functionally relevant genes identified through the microarray analysis were validated by real-time quantitative PCR assays, i.e., collagen type 1 alpha

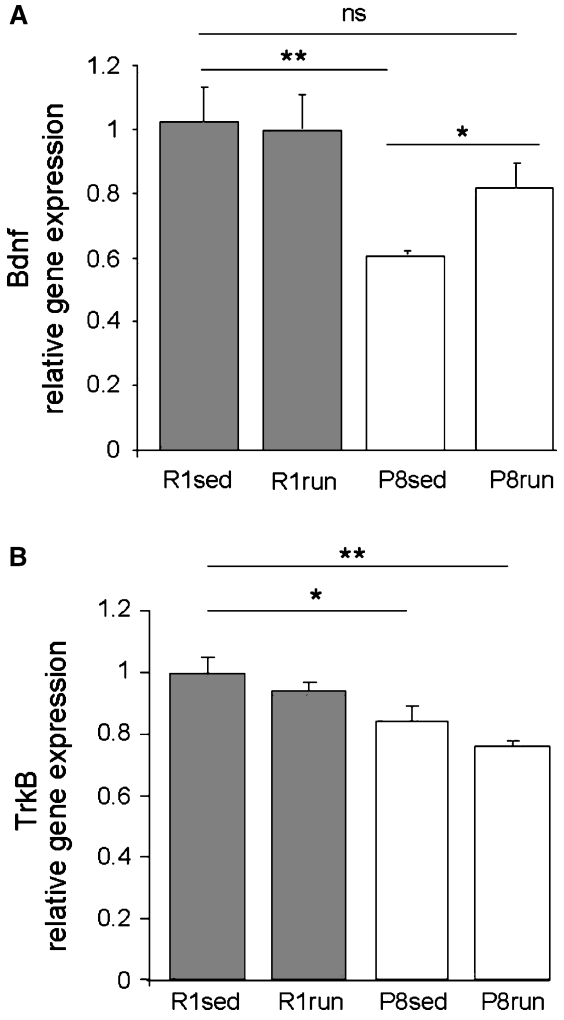

Fig. 4. Long-term voluntary exercise improves hippocampal Bdnf gene expression in old SAMP8 mice. Bdnf (A) and TrkB (B) gene expression was measured by real time quantitative PCR. Sed, sedentary mice; run, exercised mice (*$p < 0.05$; **$p < 0.01$, 2-tail t-test; $n = 5$/group).

1 (col1a1), collagen type 1 alpha 2 (col1a2), fibromodulin (fmod), prostaglandin D(2) synthase (ptgds), and aldehyde dehydrogenase (Aldh1a2) (Fig. 6). The solute carrier family 6 (GABA transporter) member 13 SLC6A13 and the Solute carrier family 47, member 1 (Scl47a1) followed the tendencies observed in the microarray studies but differences between groups did not reach statistical significance when checked by real time PCR (data not shown).

DISCUSSION

Here we studied the effect of long-term voluntary exercise in a spontaneous mouse model of accelerated

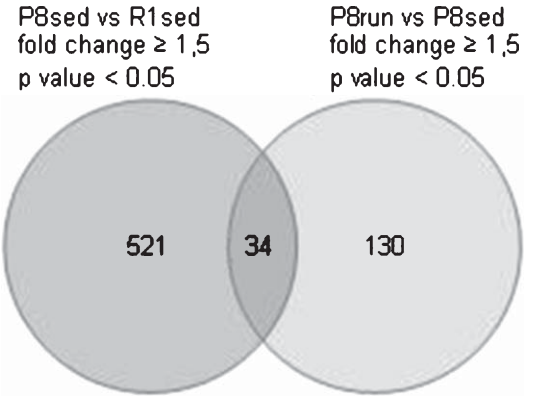

Fig. 5. Putative aging genes modulated by long-term voluntary exercise in hippocampus. Genes from the comparisons "P8sed vs. R1sed" (left circle) and "P8run vs. P8sed" (right circle) with both fold changes greater than 1.5 ($\log2 > |0.5849|$) and p value <0.05 were considered to be differentially expressed and were included in a Venn diagram. The intersection of circles corresponds to putative aging genes modulated by physical exercise.

aging, the SAMP8 mice. Previous reports have shown the SAMP8 mouse model is a suitable non-transgenic model for the study of many aspects of aging and AD [44, 45]. In the literature there is little information on the effects of exercise in female rodents although previous studies have described gender-dependent neuro-immuno-endocrine responses to physical training in AD mouse models [46, 47]. Moreover, marked sex differences have been described in the patterns of voluntary exercise in mice, females running faster and longer than males [48]. In this context, our data expand current knowledge on the molecular impact of physical exercise on brain aging in female mice.

The characteristics of the exercise protocol used here were selected (i) to analyze the effects of exercise as a continuous practice during adulthood and (ii) to avoid the deleterious effects of long-term isolation on cognitive impairment or cardiac autonomic dysregulation [49]. Indeed, upon completion of the long-term intervention, SAMP8 mice were at the final stage of their lives (10-month-old), considering that their median survival time is 40% shorter than that of SAMR1 mice (median life: 9.7 months and 16.3 months, SAMP8 and SAMR1, respectively; [28]). To avoid isolation, mice were housed in groups of 2–3 and individually placed 3 alternate days per week in a cage with a running wheel for voluntary exercise. In these conditions we found that 6 months of voluntary training on a running wheel improved aging traits such as skin color and body tremor of SAMP8 mice. Moreover, the intervention increased vascularization and modulated gene expression in the hippocampus.

Table 2
Putative aging genes sensitive to physical exercise in hippocampus. List of the differentially expressed genes from the comparisons "P8sed vs. R1sed" and "P8run vs. P8sed" with both fold changes greater than 1.5 (log2 > |0.5849|) and p value <0.05 found in the intersection of the Venn diagram (Fig. 5). These genes represent putative aging genes sensitive to exercise

Gene symbol	Gene name	Primary Accession	P8FS vs R1FS		P8FR vs P8FS	
			Fold Change	p-val	Fold Change	p-val
Htr2b	5-hydroxytryptamine (serotonin) receptor 2B	NM_ 008311	3,9	0.009	–4,0	0.008
Olfr470	olfactory receptor 470	NM_146425	3,9	0.000	–2,7	0.002
Fyb	FYN binding protein	AK038161	3,2	0.015	–2,7	0.038
Aldh1a2	aldehyde dehydrogenase family 1, subfamily A2	NM_009022	3,0	0.000	–1,7	0.017
Defb1	defensin beta 1	NM_007843	2,9	0.005	–2,8	0.006
Col1a1	collagen, type I, alpha 1	NM_007742	2,6	0.000	–1,5	0.014
Slc6a13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	NM_144512	2,5	0.000	–1,6	0.035
Fmod	fibromodulin	NM_021355	2,4	0.000	–1,6	0.038
Tnn	tenascin N	NM_177839	2,4	0.003	–2,0	0.016
Ms4a4b	membrane-spanning 4-domains, subfamily A, member 4B	NM_021718	2,3	0.000	–2,2	0.000
Pygl	liver glycogen phosphorylase	AK083075	2,3	0.001	–1,9	0.011
A230056P14Rik	RIKEN cDNA A230056P14 gene	NM_001033396	2,3	0.004	–2,0	0.017
Tbx15	T-box 15	NM_009323	2,2	0.018	–2,3	0.015
Fus	fusion, derived from t(12;16) malignant liposarcoma (human)	AK038226	2,0	0.003	–2,0	0.004
Slc47a1	solute carrier family 47, member 1	NM_026183	2,0	0.000	–1,5	0.009
Col1a2	collagen, type I, alpha 2	NM_007743	2,0	0.000	–1,6	0.005
Fbxw14	F-box and WD-40 domain protein 14	NM_015793	1,9	0.043	–2,0	0.038
Ogn	osteoglycin	NM_008760	1,9	0.003	–1,9	0.002
Setd7	SET domain containing (lysine methyltransferase) 7	NM_080793	1,9	0.026	–1,8	0.041
Mgp	matrix Gla protein	NM_008597	1,9	0.000	–1,5	0.006
Spp1	secreted phosphoprotein 1	NM_009263	1,8	0.017	–1,7	0.024
6430548G04	hypothetical protein 6430548G04	AK032445	1,7	0.007	–1,7	0.007
Gpr126	G protein-coupled receptor 126	NM_001002268	1,7	0.033	–1,7	0.030
Gpr182	G protein-coupled receptor 182	NM_007412	1,6	0.002	–1,5	0.005
Ptgds	prostaglandin D2 synthase (brain)	NM_008963	1,5	0.045	–1,7	0.015
Pard3	par-3 (partitioning defective 3) homolog (C. elegans)	NM_001013581	–1,5	0.000	1,4	0.020
Nup205	nucleoporin 205	AK041272	–1,8	0.039	1,9	0.020
Gsbs	G substrate	AF071562	–1,8	0.030	2,4	0.002
Cftr	cystic fibrosis transmembrane conductance regulator homolog	NM_021050	–2,2	0.006	1,7	0.008
Olfr1099	olfactory receptor 1099	NM_146768	–2,4	0.005	2,5	0.004
Tnks2	tankyrase, TRF1-interacting ankyrin-related ADP ribose polymerase 2	XM_001000101	–1,5	0.018	–1,5	0.012
Sp4	trans-acting transcription factor 4	NM_009239	–1,5	0.010	–1,5	0.013
Prmt1	protein arginine N-methyltransferase 1	NM_019830	–1,6	0.031	–1,8	0.004
Metrn	meteorin, glial cell differentiation regulator	NM_133719	–2,0	0.006	–2,3	0.001

It has recently been shown that in response to exercise the brain of middle-aged female mice appears to retain significant vascular plasticity [50]. Therefore, we used the blood vessel area as a readout to test the effectiveness of the exercise training. We observed that exercise training enhanced hippocampal vascularization both in SAMR1 and SAMP8 mice, suggesting that the intervention was effective. We also found a decreased blood vessel area in SAMP8 compared with SAMR1 mice. This result is consistent with other reports suggesting that vascularity may be reduced in the AD brain. It has been shown that Aβ peptides may have profound anti-angiogenic effects *in vitro* and *in vivo* [51] and that the overexpression of AβPP in the vasculature may oppose angiogenesis [52]. However, the data in the literature regarding angiogenesis and AD are contradictory: for example, angiogenesis has been observed in AD-affected brain regions [53].

We also observed an upregulation in Bdnf gene expression in P8run mice, further supporting the notion that Bdnf upregulation is one of the most reproducible molecular outcomes of physical exercise in the rodent

Table 3
Functional analysis of aging genes modulated by physical exercise in hippocampus. A) The Database for Annotation Visualization and Integrated Discovery (DAVID v6.7) bioinformatics program was used for functional annotation analysis of genes presented in Table 2. Only gene ontology categories presenting both p value < 0.05 and Benjamini adjustment < 0.05 are shown. Genes in Table 2 that did not reverse their expression pattern in response to exercise were not included in the DAVID analysis (last four genes in bold in Table 2). B) Reported role in the central nervous system of the genes included in the significant GO categories

A

Category	Benjamini p-value	Genes
GO:0031012~ extracellular matrix	0.034	FMOD, OGN, COL1A2, TNN, COL1A1
GO:0005584~ collagen type I	0.041	COL1A2, COL1A1
GO:0005576~ extracellular region	0.041	FMOD, OGN, PTGDS, COL1A2, MGP, TNN, COL1A1, DEFB1, SPP1
GO:0005578~ proteinaceous extracellular matrix	0.044	FMOD, OGN, COL1A2, TNN, COL1A1

B

Gene symbol	Primary accession	Gene name and reported role in CNS	References
Fmod	NM_021355	Fibromodulin. Regulation of collagen type I and type II and extracellular matrix organization. Downregulation in amygdala in an induced-depression mice model. Association with tumor progression and brain invasion in glioblastomas	[31, 32]
Ogn	NM_008760	Osteoglycin. Induction of bone formation in conjunction with TGFb. Downregulation in amygdala in an induced-depression mice model	[31]
Ptgds	NM_008963	Prostaglandin D2 synthase. Neuromodulator and trophic factor in the central nervous system. Increment in cerebrospinal fluid (CSF) concentration in ageing. Up-regulated in central nervous diseases like attention deficit hyperactivity disorder (ADHD) and A	[33–35]
Col1a2	NM_007743	Collagen, type I, alpha 2 (fibrillar forming collagen). Increased in Abeta deposits in AD brains	[36]
Mgp	NM_008597	Matrix gla protein (MGP). Present in neurons (mainly expressed in the peripheral nervous system). Regulator of bone morphogenetic proteins (BMPs) in the vascular system. Increased levels associated with neuron vulnerability	[37]
Tnn	NM_177839	Tenascin N. Predominantly expressed in hippocampus adult neurons. Involvement in neurite outgrowth and cell migration. Possible contribution in synaptic modulation	[38]
Col1a1	NM_007742	Collagen, type I, alpha 1. Increased in Abeta deposits in AD brains	[36]
Defb1	NM_007843	Defensin beta 1. Cationic peptide expressed in CNS. Immunomodulator of adaptative and innate immune system. Hypothesised to be involved in CNS chronic disease-related neuropathological changes	[39,40]
Spp1	NM_009263	Secreted phosphoprotein 1. Extracellular matrix glycophosphopotein. Roles in cell adhesion, chemotaxis, immune regulation , tumor cell metastasis and protection against apoptosis. Proinflammatory and subsequent neuroprotective roles in acute neuroinflamma	[41–43]

hippocampus (reviewed in [17]). Indeed, the upregulation of Bdnf gene in response to exercise was also recently reported in younger (2-month-old) male SAMP8 mice after four months of wheel running [54].

Once we confirmed the efficacy of the intervention, the objective of the microarray analysis was to identify novel putative genes involved in hippocampus aging which could be beneficially influenced by long-term exercise practice.

In the last five years, several microarray studies have been performed in the SAMP8 mouse [1, 54–58]. Two of them are methodological studies which cannot be

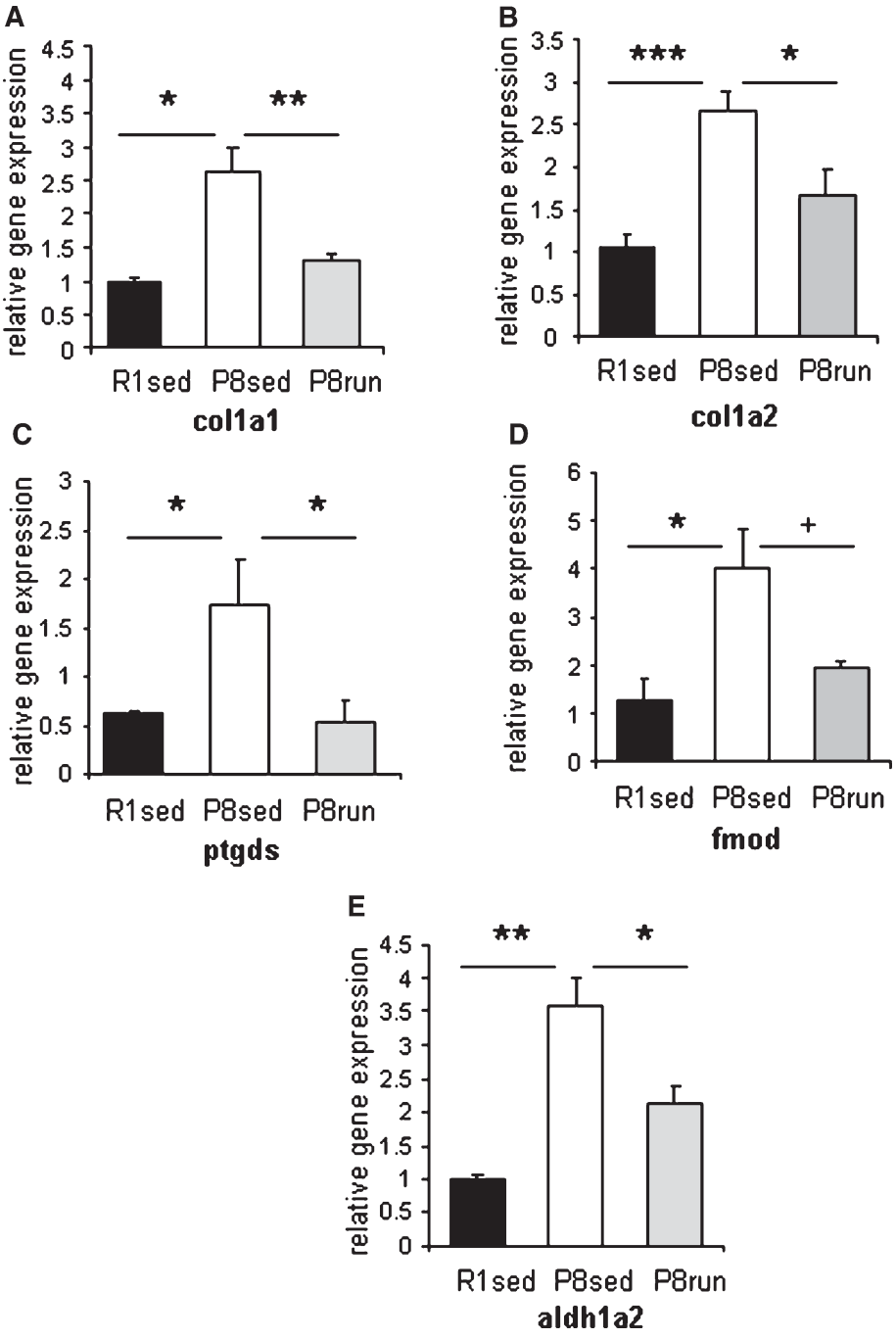

Fig. 6. Validation by Q-PCR of microarray-selected hippocampal aging genes modulated by long-term physical exercise. Genes from Table 2 were validated by real-time quantitative PCR in sedentary SAMP8 (P8sed), exercised SAMP8 mice (P8run), and sedentary SAMR1 (R1sed) mice. A) Col1a1, collagen, type I, alpha 1; B) collagen type 1 alpha 2 (col1a2); C) Ptgds, prostaglandin D2 synthase; (D) fibromodulin (fmod); and E) aldehyde dehydrogenase (Aldh1a2). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{+}p = 0.06$; 2-tail t-test; $n = 5$/group).

related to our results [56, 59]. Two other studies did not include SAMR1 mice as control and so we cannot compare their data with our findings [55, 58].

Sakurai et al. [54] identified in young (2-month-old) male SAMP8 mice an increase in the expression of the leucine zipper transcription factor-like protein

1 (Lztfl1) levels after four months of wheel running. However, in 12-old-month mice, we did not observe any effect of exercise in the expression of Lztfl1 in SAMP8 mice. The study by Cheng et al. [57] analyzed microarray expression profiles in the hippocampus of 12-month-old male SAMP8 and SAMR1 mice. In agreement with our study in females, they detected the down-regulation of Dusp12 (Dual specificity protein phosphatase 12) in the SAMP8 hippocampus. Recent evidence suggests that this enzyme plays a role in cell survival and ribosome biogenesis [60]. We did not detect any change in Dusp12 gene expression in response to exercise. Finally, using microarray analysis, Casadesús et al. [61] compared the expression of 62 genes involved in different signaling pathways such as oxidative stress, mitochondrial-related apoptosis, and cell cycle, in the hippocampus of 3-, 6-, and 9-months-old male SAMP8 and SAMR1 mice, and found no differences between strains at any of the ages analyzed. Of these 62 genes, we found that, compared with SAMR1 mice, female SAMP8 mice presented a down-regulation in Park2 gene (Park2 is an E3 ubiquitin ligase that regulates systemic lipid metabolism and is defective in early onset Parkinson's disease patients [62]) and an upregulation of the Gfra1 gene (Gfra1 gene encodes the glial cell line-derived neurotrophic factor family receptor α-1, GFR α-1, which has been reported to be beneficial for the survival of hippocampal neurons [63]). However, the expression levels of these genes were not modulated by exercise.

Regarding proteomic studies using SAMP8 mice, Zhu et al. [64] analyzed hippocampus and cortex of 5- and 15-month-old male SAMP8 and SAMR1 mice. In agreement with their findings, we found a down-regulation Uchl3 (Ubiquitin carboxyl-terminal hydrolase isozyme L3) and a down-regulation of Hebp1 in female SAMP8 hippocampus compared with SAMR1 mice, although exercise did not modified their expression levels. Interestingly, another proteomic study reported a down regulation of Uchl3 in the SAMP8 hippocampus at six months [65], strongly suggesting that lower expression of Uchl3 is a distinctive feature of the SAMP8 hippocampus and may be associated with the pathological phenotype of these mice.

Differences in the profiling platforms used, in the animal sex and age, and/or in the intervention designs are some of the factors that could explain the low agreements between our study and the cited reports. Moreover, the limitations of the microarray technique are highlighted by the observation that in response to exercise we have found an upregulation of BDNF gene expression by real time PCR but not by microarray analysis.

In this study, we found 34 genes in hippocampus that met our criteria for aging genes sensitive to physical exercise. Gene ontology analysis revealed that long-term physical exercise modulated pathways mainly involved in extracellular matrix (ECM) maintenance in hippocampus. Interestingly, the ECM not only provides physical support for neuronal function and survival but also plays a crucial homeostatic role [66] and changes in ECM components have been described as pathological features of AD [66, 67]. In this context, our data suggest that physical exercise may counteract at least in part such processes in early-stage AD.

Although the relevance of microarray findings will require further characterization, based on the previous literature some inferences about their functional significance can be made. Our microarray studies in hippocampus showed increased expression of the collagen type 1 alpha 1 (col1a1) and alpha 2 (col1a2) genes in SAMP8 mice compared with controls and a decrease in their expression levels in response to exercise. These effects were confirmed by quantitative real-time PCR. Interestingly, the 3xTg-AD transgenic mouse model presents a 20% increase in brain collagen content compared with controls and a significant vascular volume reduction in the hippocampus, but not in the frontal cortex or cerebellum [36]. These results are of particular interest since AβPP can physically interact with many different collagen and extracellular matrix components and because other forms of collagen (e.g., XXV and XVIII) have been found in amyloid plaques [68, 69]. Another gene beneficially modulated by exercise in SAMP8 hippocampus was the prostaglandin D(2) synthase (Pgds). Pgds is expressed in astrocytes and oligodendrocytes [70] and has been proposed as a biomarker of aging in cerebrospinal fluid [33]. Using real-time PCR, we also validated the beneficial effect of exercise on fibromodulin gene expression (Fmod) in SAMP8 mice. Fmod is a proteoglycan component involved in the regulation and assembly of extracellular matrix organization. It has been associated with brain tumor progression in glioblastomas [32] and there is growing evidence that keratan sulfate proteoglycans enhance Aβ aggregation and senile plaques formation in the pathogenesis of AD [71]. Finally, exercise also restored gene expression of the aldehyde dehydrogenase Aldh1a2 in the SAMP8 hippocampus. Aldh1a2 is the rate-limiting retinoic acid-synthesizing enzyme and it has been reported that both decreased [72] and increased [73] retinoic acid levels in the hippocampus lead to

impaired neurogenesis and performance in cognitive tasks.

In summary, our study identifies new candidate aging genes in the hippocampus which are sensitive to physical exercise, and suggests that an active lifestyle during adulthood may promote a healthier aging in at-risk populations by preventing or reversing senescent signs in the brain.

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