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Mouse Model of Familial Alzheimer's Disease Induced by Carbamylated-Erythropoietin is Accompanied by Modulation of Synaptic Genes

Mercedes Armand-Ugón[a,*], Ester Aso[a], Jesús Moreno[a,b], Miquel Riera-Codina[c], Alex Sánchez[d,e], Esteban Vegas[d] and Isidre Ferrer[a,b]
[a]*Institut de Neuropatologia, IDIBELL-Hospital Universitari de Bellvitge, Universitat de Barcelona, L'Hospitalet de Llobregat, Spain*
[b]*CIBERNED (Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas), Spain*
[c]*Departament de Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain*
[d]*Departament d'Estadística, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain*
[e]*Unitat d'Estadistica i Bioinformàtica, Institut de Recerca de l'Hospital Universitari de Vall d'Hebron, Barcelona, Spain*

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Abstract. Neuroprotection of erythropoietin (EPO) following long-term administration is hampered by the associated undesirable effects on hematopoiesis and body weight. For this reason, we tested carbamylated-EPO (CEPO), which has no effect on erythropoiesis, and compared it with EPO in the AβPP/PS1 mouse model of familial Alzheimer's disease. Groups of 5-month-old wild type (WT) and transgenic mice received chronic treatment consisting of CEPO (2,500 or 5,000 UI/kg) or EPO (2,500 UI/kg) 3 days/week for 4 weeks. Memory at the end of treatment was assessed with the object recognition test. Microarray analysis and quantitative-PCR were used for gene expression studies. No alterations in erythropoiesis were observed in CEPO-treated WT and AβPP/PS1 transgenic mice. EPO and CEPO improved memory in AβPP/PS1 animals. However, only EPO decreased amyloid-β (Aβ) plaque burden and soluble $A\beta_{40.}$ Microarray analysis of gene expression revealed a limited number of common genes modulated by EPO and CEPO. CEPO but not EPO significantly increased gene expression of dopamine receptors 1 and 2, and adenosine receptor 2a, and significantly down-regulated adrenergic receptor α1D and gastrin releasing peptide. CEPO treatment resulted in higher protein levels of dopamine receptors 1 and 2 in WT and AβPP/PS1 animals, whereas the adenosine receptor 2a was reduced in WT animals. The present results suggest that the improved behavior observed in AβPP/PS1 transgenic mice after CEPO treatment may be mediated, at least in part, by the observed modulation of the expression of molecules involved in neurotransmission.

Keywords: AβPP/PS1 transgenic mice, Alzheimer's disease, amyloid-β, carbamylated erythropoietin, erythropoietin, neurotransmitter receptors

*Correspondence to: Mercedes Armand-Ugón, Institut de Neuropatologia-IDIBELL, Universitat de Barcelona, Pavelló de Goven 4a, 4141, C/Feixa Llarga s/n, 08907–L'Hospitalet de Llobregat, Spain. Tel.: +34 934039994; Fax: +34 932607503; E-mail: marmand@idibell.cat.

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INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone with a well-known role in erythroid precursors. This effect is triggered by hypoxia and is transduced through binding of EPO to a homodimer of its receptor [1]. Cytoprotective functions for EPO have also been described and these extend to a variety of cell types including nervous tissue. The neuroprotective effects of EPO have been described in acute experimental brain injuries [2], and in experimental models of multiple sclerosis [3], amyotrophic lateral sclerosis [4], and Parkinson's disease [5]. Clinical improvement has also been reported in patients suffering from depression and schizophrenia [6,7]. An equivalent to the mammalian neuroprotective EPO system has been described in insects showing its relevance and conservation during evolution [8]. It is believed that EPO exerts its neuroprotective effects through interaction with a heteromeric receptor composed of two EPO receptors and one common β-subunit (cβR or CD131) [9,10]. Despite some controversy about the expression of EPO receptor in the brain [11], the receptor for EPO has been described as being expressed in neurons, glia, and endothelial cells [12,13].

Side effects of chronic EPO administration related to cardiovascular risk factors [14,15] and thrombocytopenia [16] reduce the clinical applicability of EPO as a neuroprotective agent [17]. This has prompted the search for molecules that do not have erythropoietic activity but conserve protective functions. The carbamylated form of EPO (CEPO) is a class of these molecules. Seven lysine residues of EPO are carbamylated, a reaction naturally occurring during bloodstream circulation [3,18,19]. CEPO and EPO have been proven to cross the blood-brain barrier. CEPO has been assayed in certain settings without inducing changes in blood cell parameters [19,20].

EPO improves memory in a transgenic mouse model of Alzheimer's disease (AD) (Tg2576) and this effect is associated with amyloid-β (Aβ) clearance [21]. In addition, its effect on erythroid precursors and endothelial tissue can potentiate beneficial outcome by improving blood supply and circulatory conditions. However, the applicability of EPO in AD is limited considering the appearance of undesirable side effects linked to long-term treatment in chronic disease. Therefore, the present study explores the effects of chronic EPO and CEPO in AβPP/PS1 mice in order to evaluate the relevance of the specific features of each molecule. Treatment effectiveness was measured in terms of memory improvement, and mechanisms involved were assessed by analyzing Aβ, by performing mRNA microarrays, and by using quantitative PCR and protein quantification in western blots.

MATERIALS AND METHODS

Animals

Male AβPP/PS1 transgenic mice and corresponding wild-type (C57BL/6J) animals were obtained from Jackson Laboratory (USA). The AβPP/PS1 model expresses a chimeric mouse/human APP (Mo/HuAPP695swe: APP Swedish mutation) and a mutant human presenilin 1 (PS1-dE9), both directed to central nervous system neurons [22]. Animals were maintained under standard animal housing conditions in a 12 h dark–light cycle with free access to food and water. Treatment started at the age of 5 months. All the procedures concerning animal handling were conducted according to ethical guidelines (European Communities Council Directive 86/609/EEC) and were approved by the local ethics committee.

Treatment

The carbamylated EPO molecule CEPO [19] was obtained from Lundbeck (Denmark). EPO-β (NeoRecormon, Roche, Germany) and CEPO were diluted in isotonic solution (0.9% NaCl). Mice received intraperitoneal (IP) volumes of 10 ml/kg containing doses of 2,500 UI/kg of EPO, 2,500 UI/kg of CEPO, or 5,000 UI/kg of CEPO. A group of animals received an equivalent volume (10 ml/kg) of vehicle (0.9% NaCl). Doses were administered 3 days/week for 4 weeks. Treatment is abbreviated as follows: Veh (for vehicle), CE25 and CE50 (for treatment with 2,500 or 5,000 UI/kg of CEPO), and E25 (for treatment with 2,500 UI/kg of EPO). Body weight was measured each administration day.

Memory test

Memory was assessed with the novel object recognition test. The test was performed in a V-maze (Panlab, Barcelona, Spain) in which one object is placed at the end of each arm. The V-maze consists of two corridors (30 cm long × 4.5 cm wide, and 15 cm high walls) set at a 90° angle [23]. The test is given during two sessions (training and test) that start by placing the animals on the vertex of the maze and in which they are allowed to freely explore the maze for 9 min. In the training sessions, the two objects are identical

whereas in the test session one of the objects is replaced by a different object. The ability of the animal to remember the familiar object is an indicator of the memory of the animal and is evaluated by calculating the Discrimination Index (DI). The DI was calculated with the values recorded during the test session, based on the time (T) that the animal spent exploring the novel object (T_N) versus the familiar object (T_F) : $DI = (T_N\text{-}T_F)/(T_N + T_F)$. DI values of zero indicate that the animal had no preference for exploring one or the other object, while positive values are given when the animal spent more time exploring the novel object, indicating recognition of the familiar one. Only animals that reached the minimum of 5 s of object exploring time were included for data analysis and further study. This threshold was previously established with our colony as it allows discriminating between WT and AβPP/PS1 memory phenotypes.

Tissue preparation

After the memory test, animals were deeply anaesthetized by intraperitoneal (0.2 mL/10 g body weight) with a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg).

Blood was extracted by intracardiac puncture and collected in Vacuette® Blood collection tubes, K3 EDTA. Then animals were perfused with cold 0.1 M phosphate buffer (PB) with a peristaltic pump at 19 mL/minute until blood was cleared from the blood vessels. Brains were removed and divided into two hemispheres. The left hemisphere was dissected into different brain regions, immediately frozen on dry ice and stored at −80ºC until needed. The right hemisphere was fixed by immersion in 4% paraformaldehyde (PFA) for 24 h after which PFA was replaced by PBS 1x. Tissue was then dehydrated by sequential immersion in ethanol at 70%, 96%, and 100%, and xylene, before being embedded in paraffin blocks. Sections 4-μm-thick were obtained with a Leica microtome for immunohistochemical study.

Blood analysis

Blood parameters were acquired with a veterinary analyzer (MEK-6318, Nihon Kohden). Reticulocyte count was determined by cytometry after Thiazole Orange (TO) (Sigma, USA) staining of 5 μl blood. Briefly, blood was washed in 1 mL DPBS 1x (2.7 KCl; 1.5 mM KH_2PO_4; 8.1 mM Na_2HPO_4; 138 mM NaCl), centrifuged, fixed for 10 min in 500 μl solution of 1% glutaraldehyde (Merck) and 0.003% SDS (Sigma) in DPBS 1x, washed in 1 ml DPBS1x, centrifuged and then stained in the dark for 30 min at RT in 1 ml of staining solution containing 0.5 μg/ml TO, 0.02% NaN_3 (Merck, Germany) and 2 mM EDTA in DPBS 1x. All centrifugation steps were 5 min and 800 *g*. After washing with DPBS1x, pellet was resuspended in 1 mL DPBS1x. 1/10 dilution was used for cytometry analysis. A total of 10^5 events of the cell population gate were acquired in a FACS Calibur cytometer (Becton Dickinson). The F1 negative population was determined with an unstained sample, and TO+reticulocyte population was quantified. Reticulocyte index was calculated with the reticulocyte count (RC) and hematocrite count (HTC) and the value of normal HTC (NHTC) of 45%: $RI = RC \times HTC/NHTC$.

Evaluation of Aβ burden and Aβ soluble forms

Extracellular Aβ deposition was quantified with Aβ immunostaining. Three non-overlapping paraffin coronal sections (separated by at least a 40 μm gap) from each animal were prepared backwards from Bregma (−0.70 to −1.7 mm). Although this 1 mm spanning of the brain sections may seem broad, there are no differences between treatment groups regarding the areas analyzed for amyloid burden as all distances from Bregma are represented in each group. Paraffin sections were de-waxed, treated for 3 min with formic acid, boiled for 20 min in citrate buffer (8.2 mM sodium citrate and 2 mM citric acid; pH 6), blocked in PBS 1x with 3% serum normal horse for 1 h at room temperature (RT), and stained with the Aβ antibody clone 6F/3D (Dako, Denmank) overnight at 4ºC. Staining was visualized after biotin-streptavidin labelling of primary antibody with the Universal LSAB™+Kit/HRP (Dako, Denmank) and subsequent incubations with diaminobenzidine (Sigma, USA) and H_2O_2.

The quantification of the cortical area occupied by the Aβ plaques was done using the analysis tool available in the Adobe Photoshop CS5-Extended version. The cerebral cortex area analyzed covered the motor and somatosensory cortices, granular, dysgranular, and agranular insular cortices, ectorhinal and perirhinal cortices, piriform cortex and amygdaloid nucleus. The cortex was outlined manually and its area quantified (A_{Cx}). Plaques were automatically selected by color and the selection was manually corrected. The sum of plaque areas (A_P) was automatically quantified by the software. The Aβ burden was obtained by calculating

$(A_P / A_{Cx}) \times 100$ and the % Aβ burden values were normalized to the control group (Veh-treated) of animals of each treatment set.

Soluble non-fibrillar forms of Aβ in cortical tissue homogenate from nine AβPP/PS1 animals per treatment group were quantified with $A\beta_{40}$ and $A\beta_{42}$ ELISA kits (Invitrogen, USA) following the manufacturer's instructions. Tissue was homogenized in 4 brain volumes (4 μl/mg) of chilled Tris Buffered Saline (TBS; 140 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, 5 mM EDTA, pH 7.4) and complete EDTA-free Mini protease inhibitor (Roche, Germany). Tissue was homogenized with a Polytron (Kinematica, Switzerland) and then centrifuged at 100,000 *g* for 1 h at 4ºC. Supernatant protein content was quantified with BCA kit (Thermo scientific).

RNA microarray studies

RNA from frozen neocortex samples of treated mice was extracted according to the manufacturer's instructions (Rneasy Lipid Tissue Mini Kit, Qiagen® GmbH, Hilden, Germany). RNA quality was checked with the Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) and the RNA concentration was evaluated using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). A total of 16 samples (6 AβPP/PS1-Veh, 5 AβPP/PS1-CE50, and 5 AβPP/PS1-E25) were sent to the Unit of High Technology at the Vall d'Hebron Research Institute (Barcelona, Spain) for microarray hybridization with the Affimetrix GeneChip® Mouse Gene 1.0 ST Array (Santa Clara, CA, USA). Microarrays were pre-processed with a three (+1) step on the probe values to turn them into comparable gene-level expression values: background correction (RMA), normalization (Quantiles), summarization (Median Polish) and transcript-level summarization (Average). Non-specific filtering was applied to remove controls, low signal genes and low variability genes. This pre-processing left 6,419 genes for further study.

The selection of differentially expressed genes between conditions was based on a linear model analysis with empirical Bayes moderation of the variance estimates following the methodology developed by Smyth [24]. The analysis yields standard test statistics such as fold changes and (moderated)-*t* or *p*–values which can be used to rank the genes from most to least differentially expressed. Functional annotation and biological term enrichment analysis were done using the DAVID database [25,26].

Quantitative PCR

1 μg of total RNA from brain neocortex was reverse-transcribed to cDNA with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Transcript level quantification of a particular gene was performed in duplicate reactions with gene-specific TaqMan® probes and the TaqMan Universal PCR Master Mix (Applied Biosystems). The following TaqMan probes were used: Mm00802075_m1 (Adora2a), Mm01328600_m1 (Adra1d), Mm01353211_m1 (Drd1a), Mm00438545_m1 (Drd2), Mm00432887_m1 (Drd3), Mm00456650_m1 (Egr2), Mm00612977_m1 (Grp), Mm01193520_m1 (Htr1d) and Mm01230885_m1 (Oprk1). Housekeeping gene probes were Mm00507627_m1 (Aars), Mm01545399_m1 (Hprt), and Mm00460040_m1 (Xpnpep1) [27]. QPCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System. Ct values were obtained by performing a relative quantification run using the threshold value calculated automatically by the SDS 2.2.2 software. Gene expression was calculated with the double delta CT (ΔΔCT) method using vehicle-treated WT samples as reference group.

Western blotting

Five cerebral cortex samples per genotype and treatment were analyzed with western blotting. Proteins were extracted from frozen tissue with RIPA buffer (50 mM Tris-HCl, pH8.0; 150 mM NaCl, 1% Nonidet P-40; 0.5% Na-Deoxycholate; 0.1% SDS; 1 mM PMSF; 1 mM Na_3OV_4) supplemented with protease and phosphatase inhibitors (Roche, Germany), and quantified with the Bradford reagent (Sigma, USA) using bovine serum albumin (BSA) standard. Protein samples were mixed with loading sample buffer (final concentrations: 62.5 mM Tris, pH 6.8; 2% SDS; 10% glycerol and 0.0025% bromophenol blue) and heated at 95ºC for 5 min. 30 μg of proteins was separated by electrophoresis in SDS-PAGE gels with 10% acrylamide and transferred to nitrocellulose membranes (0.2 μm pore, BioRad). Membranes were incubated with blocking buffer TBS-Tween (TBS-T; 10 mM Tris; 140 mM NaCl and 1% Tween) with 5% non-fatty milk for 1 h at RT. Membranes were incubated overnight at 4°C with primary antibodies (or for 1 h at RT for β-Actin): mouse anti-adenosine receptor A2a (A2A) (Millipore), rabbit anti-dopamine receptor 2 (DRD2), goat anti-dopamine receptor 1 (DRD1) (Frontier Institute), and mouse anti-β-actin (Sigma). Primary antibodies were diluted 1:1,000 (A2A, DRD1,

and DRD2) or 1:30,000 (β-Actin) in TBS-T with 0.05% NaN_3 (Merck) and 5% non-fatty milk (A2A) or 3% BSA (Sigma, USA) (DRD1, DRD2, and β-Actin). Membranes were washed with TBS-T and incubated for 1 h with their corresponding HRP-conjugated IgG secondary antibody (Dako, Denmark) diluted 1:1,000 in blocking buffer. Immunoreactivity was visualized by incubating the membranes with chemiluminescence reagent (ECL, Amersham) and exposing Amersham Hyperfilm ECL (GE Healthcare Lifesciences). Blot quantification of each band (A2A, 50 kDa; DRD1 75 kDa, DRD2 100 kDa and β-Actin 42 kDa) was performed with Totallab software (TL100 v.2006b). Bands were normalized to β-actin levels.

Statistical analysis

Memory test and histological analysis were performed blind to genotype and/or treatment. Grubbs' test or ESD method (extreme studentized deviate) was applied to determine significant outlier values. Statistical significance of the data was evaluated with either Student's *t*-test or one-way ANOVA, followed by Dunnett's post-test or two-way ANOVA, in turn followed by Bonferroni post-tests. Data were analyzed and plotted with the GraphPad Prism 5.01 software. In all experiments the significance level was set at $p < 0.05$.

RESULTS

Effect of treatment on body weight and blood parameters

WT and AβPP/PS1 animals were treated with Vehicle, EPO (2,500 UI/kg), or CEPO (2,500 UI/ kg or 5,000 UI/ kg), hereafter referred to as Veh, E25, CE25, and CE50, respectively. Given the chronicity of the treatment, we analyzed whether body weight changes occurred due to the treatment. Body weight control during treatment showed that in WT animals, EPO accounted for a slight but significant decrease in the area under the curve (AUC) calculated with the % values of the initial weight ($F_{3,139} = 3.63$; $p < 0.05$) (Fig. 1A). No weight loss was found in AβPP/PS1-treated animals with EPO and CEPO.

Given the erythropoietic properties of EPO, blood parameters were controlled and differences between treatments were analyzed. Blood from treated animals was collected and analyzed after performance of the memory test. Mean values for each treatment group are presented in Table 1. Two-way ANOVA analysis indicated that treatment accounted for the

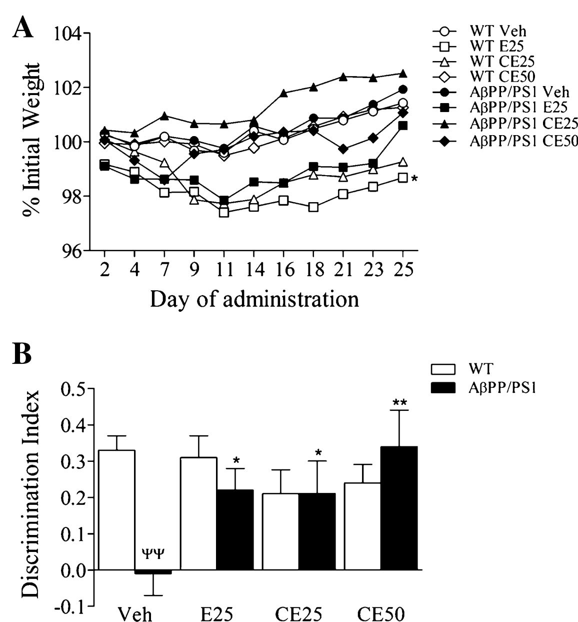

Fig. 1. A) Body weight during treatment is represented as the % initial weight recorded for each administration day. Only WT animals had a reduced weight when treated with EPO, as determined with two-way ANOVA and Bonferroni post-test of the area under the curve calculated for each animal. The mean % weight of each group at every administration day is represented. Deviations from the mean are not represented for the sake of clarity. B) Discrimination index of WT and AβPP/PS1 mice after treatment with Vehicle (Veh), EPO (E25), or CEPO (CE25 and CE50). The AβPP/PS1-Veh group shows memory defect compared to the WT-Veh group (Ψ symbol). We observe that AβPP/PS1 animals show improved memory performance compared to the Veh-treated AβPP/PS1 group after E25, CE25, and CE50 treatment (significance indicated by *). Mean ± SEM are represented. E25: animals treated with doses of 2500 UI/kg; CE25 and CE50: animals treated with doses of 2,500 UI or 5,000 UI/kg, respectively. $N_{WT\text{-}Veh} = 14$; $N_{A\beta PP/PS1\text{-}Veh} = 15$; $N_{WT\text{-}E25} = 16$; $N_{A\beta PP/PS1\text{-}E25} = 12$; $N_{WT\text{-}CE25} = 14$; $N_{A\beta PP/PS1\text{-}CE25} = 12$; $N_{WT\text{-}CE50} = 16$; $N_{A\beta PP/PS1\text{-}CE50} = 9$. Ψ = Genotype effect; * = Treatment effect compared to Veh group; *$p < 0.05$, ΨΨ/**$p < 0.01$. Two-way ANOVA followed by Bonferroni post-test.

significant differences in variance observed and that there was no interaction with genotype. Compared to vehicle-treated animals, EPO induced similar changes in the levels of red blood cells (RBC) ($F_{3,59} = 11.7$; $p < 0.0001$) and HTC, hemoglobin (HB) ($F_{3,59} = 15.10$; $p < 0.0001$), RC ($F_{3,27} = 32.24$; $p < 0.0001$), and reticulotyte index (RI) ($F_{3,27} = 22.73$; $p < 0.0001$) in both WT and AβPP/PS1 genotypes. Bonferroni post-test indicated that RBC, HTC, and HB were all significantly increased between 1.26 and 1.29-fold ($p < 0.001$), and RC and RI were reduced by 82% and 86% in WT ($p < 0.001$) and by 81% and 77% in AβPP/PS1 ($p < 0.001$) mice, respectively. PLT

Table 1
Blood parameters of animals after EPO or CEPO treatment

	Wild type				AβPP/PS1			
	Veh	E25	CE25	CE50	Veh	E25	CE25	CE50
WBC	2.0 ± 0.3	3.5 ± 0.9	1.9 ± 0.3	3.1 ± 0.6	3.1 ± 0.8	4.2 ± 0.6	2.6 ± 0.6	2.1 ± 0.6
RBC	9.0 ± 0.2	$11.5 \pm 0.4^{***}$	9.3 ± 0.2	9.7 ± 0.2	8.8 ± 0.4	$11.0 \pm 0.9^{***}$	9.4 ± 0.3	9.3 ± 0.2
HB	13.6 ± 0.3	$17.5 \pm 0.6^{***}$	14.0 ± 0.3	14.5 ± 0.4	13.1 ± 0.5	$17.0 \pm 1.2^{***}$	13.8 ± 0.5	13.4 ± 0.5
HTC	41.4 ± 0.7	$53.5 \pm 1.7^{***}$	42.8 ± 0.8	44.1 ± 1.1	40.9 ± 1.3	$52.3 \pm 3.6^{***}$	42.8 ± 1.0	42.4 ± 1.4
MCV	46.3 ± 0.6	46.6 ± 1.0	$45.9 \pm 08.$	45.6 ± 0.6	46.8 ± 0.7	47.7 ± 1.3	45.5 ± 0.5	45.7 ± 0.4
MCH	15.2 ± 0.2	15.2 ± 0.3	15.1 ± 0.3	14.9 ± 0.2	15.0 ± 0.2	15.4 ± 0.4	14.6 ± 0.3	14.4 ± 0.3
MCHC	32.9 ± 0.3	32.5 ± 0.2	32.7 ± 0.4	32.8 ± 0.4	32.0 ± 0.5	32.4 ± 0.3	32.1 ± 0.7	31.7 ± 0.5
PLT	1110.2 ± 64.0	$683.2 \pm 94.3^{**}$	971.6 ± 93.2	1040.3 ± 99.3	1112.0 ± 66.9	$724.0 \pm 86.4^{*}$	816.6 ± 195.7	788.5 ± 130.2
RC	3.2 ± 0.2	$0.6 \pm 0.2^{***}$	3.2 ± 7	3.0 ± 0.2	3.0 ± 0.2	$0.4 \pm 0.2^{***}$	3.2 ± 0.5	2.3 ± 0.2
RI	3.1 ± 0.2	$0.7 \pm 0.2^{***}$	3.2 ± 0.8	2.9 ± 0.2	2.9 ± 0.2	$0.6 \pm 0.2^{***}$	3.1 ± 0.5	2.2 ± 0.2

Mean values ± SEM of white blood cells (WBC; $10^3/\mu L$), red blood cells (RBC; $10^6/\mu L$), hemoglobin (HB; g/dL), hematocrit (HTC; %), mean corpuscular volume (MCV; fL), mean corpuscular hemoglobin (MCH; pg), mean corpuscular hemoglobin concentration (MCHC; g/dL), platelets (PLT; $10^3/\mu L$), reticulocyte count (RC; %), and reticulocyte index (RI). Two-way ANOVA followed by Bonferroni post-test; Significance compared to matching genotype Veh-treated group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

was significantly reduced ($F_{3,27} = 5.25$; $p = 0.003$), by 35%, in the WT-E25 ($p < 0.01$), and by 38% in AβPP/PS1-E25 ($p < 0.05$). However, no modifications in any of these parameters were observed in WT or AβPP/PS1 animals after either CEPO treatment (CE25 or CE50) (Table 1). No modifications in the levels of WBC ($F_{3,57} = 2.69$; $p = 0.055$), MCV ($F_{3,57} = 1.19$; $p = 0.321$), MCH ($F_{3,58} = 1.60$; $p = 0.199$), and MCHC ($F_{3,58} = 0.13$; $p = 0.943$) were observed among the treated groups.

EPO and CEPO improve memory of AβPP/PS1 mice

At the end of the treatment, we tested the effect of EPO and CEPO on the cognitive status of the AβPP/PS1 mice. The memory of each group was assessed with the novel object recognition test, and subsequently the discrimination index (DI) was calculated. As expected, we observed reduced memory in the transgenic mice, and vehicle-treated WT and AβPP/PS1 animals showed a DI of 0.33 ± 0.04 and -0.01 ± 0.06, respectively. Two-way ANOVA analysis indicated that there was interaction between treatment and genotype ($F_{3,105} = 4.172$, $p = 0.0078$). Bonferroni post-test indicated that chronic treatment with EPO ($p < 0.05$) and both doses of CEPO (CE25, $p < 0.05$; and CE50 $p < 0.01$) significantly improved memory of AβPP/PS1 animals to levels similar to WT animals; yet EPO and CEPO had no effect on the DI of WT animals (Fig. 1B).

EPO and CEPO effects on brain Aβ

Cognitive deficit in the AβPP/PS1 mice arising after the processing of Aβ is measurable. Soluble forms of $A\beta_{40}$ and $A\beta_{42}$ can be detected and gradually increase from the age of 3 to 6 months. Aβ deposition is also readily detectable in this region from 3 months of age and the plaque burden increases during mouse lifetime [28]. In order to learn whether the memory improvement observed after EPO and CEPO treatments was linked to a modulation of Aβ processing, we evaluated these two parameters in treated AβPP/PS1 groups.

Aβ burden was quantified by immunohistochemistry in three non-overlapping coronal slices per animal. The area covered by plaques in the cerebral cortex was calculated with respect to the total cerebral cortex area (see Materials and Methods section). Mean Aβ burden of AβPP/PS1-veh mice was 1.3%. This value was reduced by 20% after treatment with EPO (One-way ANOVA, $F_{3,66} = 3.36$; $p < 0.05$). In contrast, CEPO did not produce any significant effect on Aβ burden (Fig. 2A). Sections from WT samples were all negative for Aβ immunoreactivity, as expected.

The TBS-soluble fraction of cortical tissue was extracted and the amounts of $A\beta_{40}$ and $A\beta_{42}$ were quantified by ELISA. Samples from WT animals were negative for soluble $A\beta_{40}$ and $A\beta_{42}$. Compared to the vehicle-treated AβPP/PS1 group, one-way ANOVA showed that soluble $A\beta_{40}$ was reduced by EPO by 59% (Dunnett's post-test; $p < 0.05$). The levels of $A\beta_{42}$ showed a trend toward decrease in AβPP/PS1 animals treated with EPO (55% lower) and with CEPO when administered at the highest dose (46%

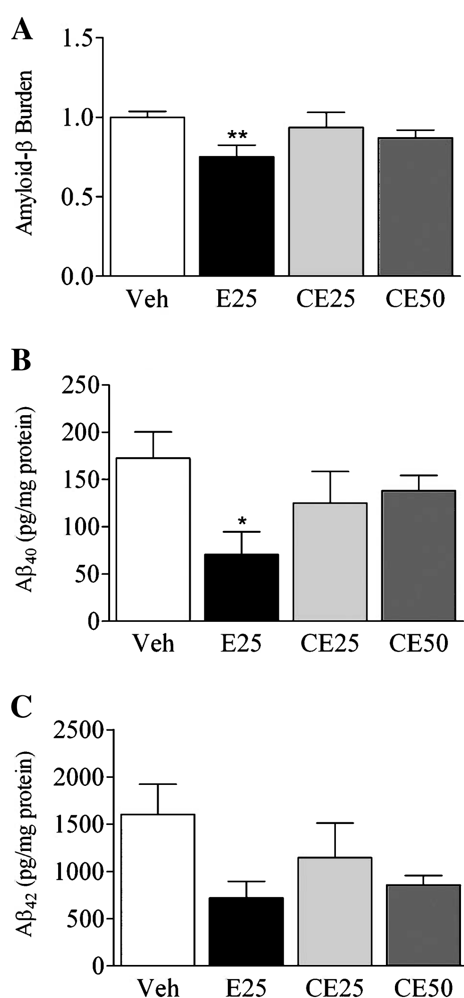

Fig. 2. Amyloid-β in AβPP/PS1 mice after treatment with vehicle (Veh), EPO (E25), or CEPO (CE25 and CE50). A) For each animal, fibrillar Aβ burden in plaques was quantified with immunohistochemistry. Three non-overlapping coronal sections of the cerebral cortex were analyzed. AβPP/PS1 animals treated with EPO had lower burden than vehicle-treated AβPP/PS1 mice (* $p < 0.05$; One-way ANOVA and Dunnett's post-test). Quantification of soluble Aβ forms: B) $A\beta_{40}$ and C) $A\beta_{42}$. EPO treatment reduced the levels of the soluble $A\beta_{40}$ form compared to the control treatment (Veh). (* $p < 0.05$; One-way ANOVA and Dunnett's post-test). Veh: vehicle-treated animals; E25: animals treated with doses of 2,500 UI/kg; CE25 and CE50: animals treated with doses of 2,500 UI or 5,000 UI/kg, respectively. Mean ± SEM values are represented.

lower) (Fig. 2B-C). No statistically significant differences were detected when comparing EPO and CEPO treatments.

RNA microarray evidence that CEPO and EPO have different signaling pathways

Our analysis of Aβ in the neocortex of treated mice indicated that EPO could modify the Aβ processing, whereas CEPO had little or no effect, thus indicating that EPO and CEPO may act on different targets. Therefore, we decided to search for molecular routes to differentiate the two treatments by gene expression using mRNA microarray. RNA was extracted from the neocortex, as this is the brain region most abundant in Aβ plaques which start developing at the age of 3 months [28]. We performed gene expression microarray analysis of samples from AβPP/PS1-Veh ($n = 6$), AβPP/PS1-E25 ($n = 5$), and AβPP/PS1-CE50 ($n = 5$). Gene expression profile in the AβPP/PS1-vehicle group showed high individual variation. There were 423 genes with significant ($p < 0.05$) changes in expression between vehicle and CEPO or EPO-treated groups (Fig. 3A), and log2 fold changes (log2 FC) ranged between -1.59 and 0.95 when comparing E25 with Veh and -1.70 and 0.50 when comparing CE50 with Veh. Only 1.42% (6 genes) of these genes were shared by EPO and CEPO (Fig. 3B).

Then we performed a comparative study of EPO and CEPO treatments in AβPP/PS1 mice. Using a cut-off value of $p < 0.05$, 514 genes had higher and 576 had lower expression in EPO-treated animals compared to CEPO-treated animals (Supplementary Table 1). In order to find out which genes best differentiated each treatment, we searched for the genes with log2 FC higher than 1 or lower than -1 (equivalent to a doubled or halved expression of the gene in CE50 with respect to E25 treatment, respectively) (Fig. 3 C). This comparison yielded a list of 50 genes (Table 2) that were submitted to the DAVID database.

Functional annotations and clustering rendered a list of 39 enriched terms (Table 3). The functional analyses indicated that differences between EPO and CEPO were related to dopamine, synaptic transmission, and neuropeptide signaling, among others. Fifteen genes were selected for the 20 most enriched functional terms including 7 neurotransmitter receptor genes: adenosine A2a receptor, Adora2a; adrenergic receptor alpha 1d (Adra1d); dopamine (DA) receptors Drd1a, Drd2 and Drd3; 5-hydroxytryptamine (serotonin) receptor 1D, Htr1d and opioid receptor kappa 1, Oprk1. Microarray data revealed higher transcript levels of all three DA receptors in the animals treated with CE50 compared to animals that received EPO. The -fold changes ranged from 2.69 to 4.02 (Moderated-t, $p < 0.05$, Table 2). The remaining eight non-neurotransmitter receptor genes were related to neuropeptides and amidation, and contained genes for proenkephalin (Penk), tachykinin precursor 1 (Tac1), tachykinin receptor 1 (Tacr1), vasoactive intestinal peptide (Vip), and gastrin releasing peptide (Grp). Two genes related to retinoic acid

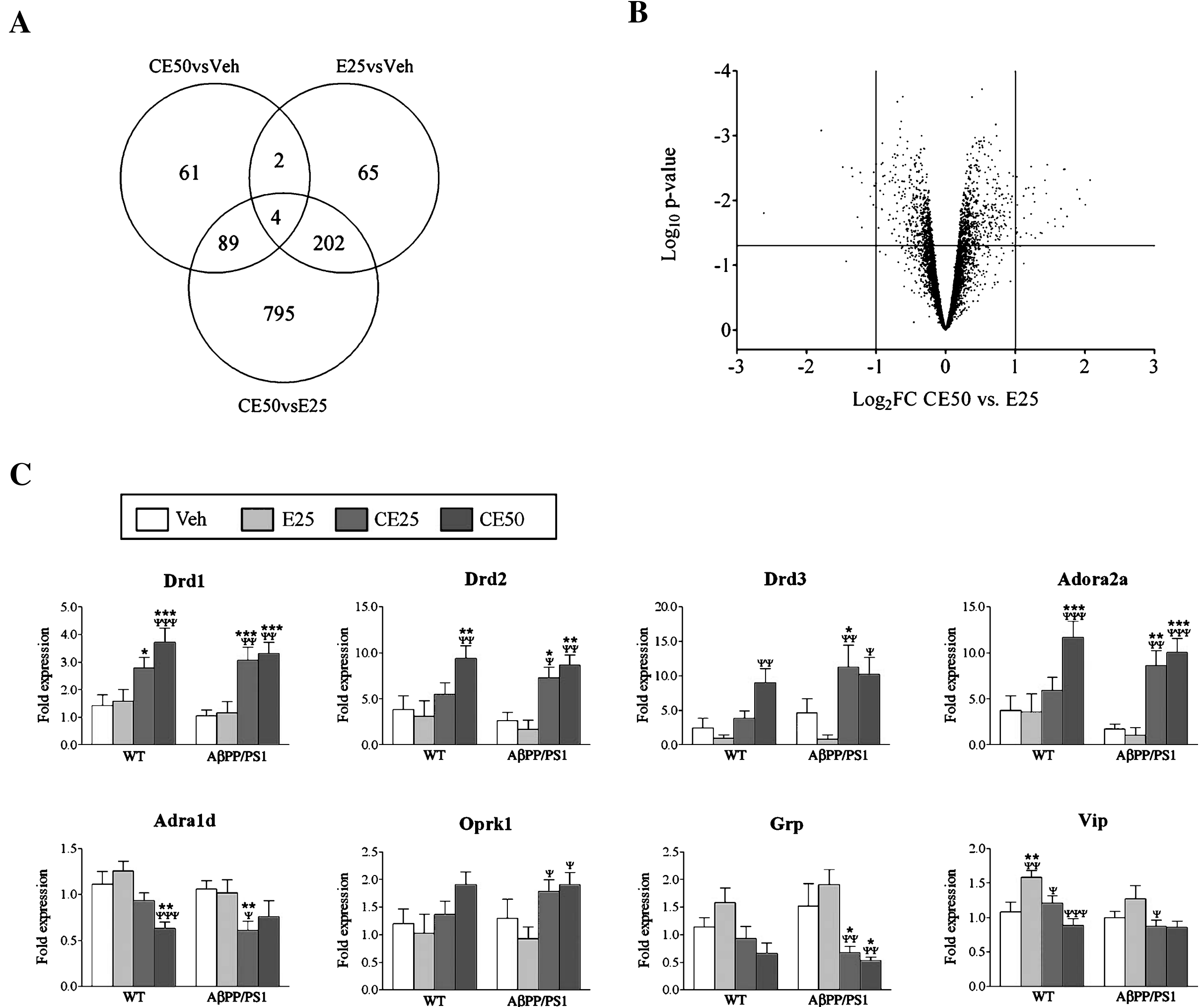

Fig. 3. RNA microarray results and subsequent validation by Q-PCR. The microarray was performed and analyzed with samples of AβPP/PS1 mice from three treatment groups (Veh, E25, and CE50). A) Venn diagram showing the distribution of genes with different expression ($p < 0.05$) in the comparisons of CE50 and E25 versus Veh and between CE50 and E25 treatments. B) Volcano plot with the Log10 of the p-value and the Log2 fold change values calculated by comparing the expression of all the genes between CE50 and E25 treatments. Lines highlight the cut-off values ($p < 0.05$ and Log2FC below -1 and above 1) used to select for the genes that best differentiate the treatments, which are located in both the upper right and upper left quadrants. C) Microarray data validation. Expression of selected genes in the brain cortex of treated WT and AβPP/PS1 mice was determined with Q-PCR on individual samples. Genes: Drd1, Drd2 and Drd3a (Dopamine receptors); Adora2a (Adenosine receptor a2a); Adra1d (Adrenergic receptor a1d); Oprk1 (Opioid receptor κ1); Grp (Gastrin releasing peptide) and Vip (Vasoactive intestinal peptide). Each bar represents the mean ± SEM of 11–16 samples, * = significant with respect to Veh group; Ψ = significant with respect to E25 group; */Ψ$p < 0.05$; **/ΨΨ$p < 0.01$. Two-way ANOVA followed by Bonferroni post-test.

signaling were classified in the Zinc finger term; these genes were retinoic acid receptor beta (Rarb) and retinoid X receptor gamma (rxrg). The early growth response 2 gene (Egr2) clustered with other genes in 15 out of the 39 terms related to synaptic plasticity, cell differentiation, and learning and memory.

Given the role of inflammation [28,29] in this mouse model, we checked whether inflammation-related terms were selected among CEPO and EPO treatments, and vehicle. No gene cluster related the effect of CEPO or EPO treatments with a modulation of inflammation. We also checked whether the olfactory system was modulated by treatment as we had recently described changes in the expression of some genes in the neocortex of the AβPP/PS1 mice compared to WT animals [30]. EPO but not CEPO showed a down-modulation of the Gnal (guanine nucleotide binding protein α-activation activity polypeptide, olfactory

Table 2
List of genes with highest expression-fold changes in the comparison of CEPO and EPO

Symbols A	Gene name	Entrez	ID	Log2 FC	*p*-value
Serpina9	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9	71907	10402422	2.08	0.005
Drd2	dopamine receptor 2	13489	10585169	2.01	0.012
Adora2a	adenosine A2a receptor	11540	10364030	1.93	0.009
Sh3rf2	SH3 domain containing ring finger 2	269016	10455299	1.89	0.007
Gpr149	G protein-coupled receptor 149	229357	10498441	1.74	0.018
Abi3bp	ABI gene family, member 3 (NESH) binding protein	320712	10436304	1.71	0.003
Rgs9	regulator of G-protein signaling 9	19739	10392415	1.70	0.003
Drd3	dopamine receptor 3	13490	10435793	1.69	0.025
Rxrg	retinoid X receptor gamma	20183	10351430	1.66	0.013
Slc10a4	solute carrier family 10 (sodium/bile acid cotransporter family), member 4	231290	10522388	1.57	0.025
Ndst4	similar to N-deacetylase/N-sulfotransferase 4; N-deacetylase/N-sulfotransferase (heparin glucosaminyl) 4	64580	10495878	1.57	0.019
Tac1	tachykinin 1	21333	10536363	1.47	0.022
Htr1d	5-hydroxytryptamine (serotonin) receptor 1D	15552	10509238	1.46	0.003
Gpr6	G protein-coupled receptor 6	140741	10368780	1.45	0.008
Drd1a	dopamine receptor D1A	13488	10409319	1.43	0.005
Isl1	ISL1 transcription factor, LIM/homeodomain	16392	10412335	1.38	0.032
Slc10a4/Gm5868	solute carrier family 10 (sodium/bile acid cotransporter family), member 4	231290	10530499	1.36	0.031
Slc5a7	solute carrier family 5 (choline transporter), member 7	63993	10451838	1.29	0.038
Gpr88	G-protein coupled receptor 88	64378	10501622	1.28	0.010
Oprk1	opioid receptor, kappa 1	18387	10344653	1.27	0.006
Rarb	retinoic acid receptor, beta	218772	10417713	1.26	0.008
Lrrc10b	predicted gene 705	278795	10465963	1.23	0.034
Penk	preproenkephalin	18619	10511363	1.23	0.016
Cdhr1	protocadherin 21	170677	10419015	1.23	0.003
Fam40b	family with sequence similarity 40, member B	320609	10536949	1.19	0.006
Tcfap2d	transcription factor AP-2, delta	226896	10345007	1.16	0.032
Tacr1	tachykinin receptor 1	21336	10539244	1.15	0.007
Epyc//Gm10754	ENSMUSG00000074776	13516	10372106	1.13	0.019
Ppp1r1b	protein phosphatase 1, regulatory (inhibitor) subunit 1B	19049	10380862	1.12	0.009
Car12	carbonic anyhydrase 12	76459	10586591	1.08	0.037
Ptpro	protein tyrosine phosphatase, non-receptor type 15; protein tyrosine phosphatase, receptor type, O	19277	10542414	1.03	0.012
Mir376b	ENSMUSG00000076043	723934	10398408	1.03	0.031
Pde10a	phosphodiesterase 10A	23984	10441680	1.02	0.004
Crabp1	cellular retinoic acid binding protein I	12903	10585438	1.01	0.033
Dchs2	similar to dachsous 2 isoform 2; predicted gene 6731	229459	10492774	1.01	0.026
Cbln1	cerebellin 1 precursor protein; similar to precerebellin-1	12404	10580469	−1.00	0.003
Fos	FBJ osteosarcoma oncogene	14281	10397346	−1.00	0.005
Glt8d2//Tdg	glycosyltransferase 8 domain containing 2	74782	10371296	−1.03	0.006
Vip	vasoactive intestinal polypeptide	22353	10367582	−1.03	0.004
Adra1d	adrenergic receptor, alpha 1d	11550	10487886	−1.04	0.012
Tshz2	teashirt zinc finger family member 2	228911	10478928	−1.08	0.009
Satb2	special AT-rich sequence binding protein 2	212712	10354777	−1.20	0.026
Egr2	early growth response 2	13654	10363735	−1.20	0.005
Npas4	neuronal PAS domain protein 4	225872	10464905	−1.22	0.004
Neurod6	neurogenic differentiation 6	11922	10544936	−1.26	0.018
Rnf39	ring finger protein 39	386454	10445061	−1.34	0.004
Fezf2	Fez family zinc finger 2	54713	10417620	−1.35	0.003
Akr1c18	aldo-keto reductase family 1, member C18	105349	10407435	−1.48	0.003
Grp	gastrin releasing peptide	225642	10456353	−1.79	0.001
Baiap2l1	BAI1-associated protein 2-like 1	66898	10535559	−2.61	0.016

Genes with significant ($p < 0.05$) difference in expression when comparing the expression in AβPP/PS1 mice treated with CE50 with respect to E25. The log2 FC cut-off values were −1 and +1.

Table 3
Functional annotation for genes differently modulated between CEPO and EPO

Category	Term	p-value	Fold enrichment
BP	Response to amphetamine	<0.0001	100.7
SP	G protein-coupled receptor	<0.0001	35.6
MF	Amine receptor activity	<0.0001	31.3
IP	Dopamine receptor	0.0001	236.8
BP	Multicellular organismal response to stress	0.0001	40.8
BP	Regulation of synaptic plasticity	0.0003	31.5
BP	Transmission of nerve impulse	0.0003	10
BP	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.0003	5.9
BP	Positive regulation of transcription from RNA polymerase II promoter	0.0003	7.4
BP	Regulation of catecholamine secretion	0.0004	94.4
BP	Regulation of synaptic transmission, glutamatergic	0.0004	94.4
MF	Amine binding	0.0007	21.8
BP	Negative regulation of blood pressure	0.0008	70.8
BP	Forebrain development	0.0009	11.3
BP	Neuron projection morphogenesis	0.001	10.7
BP	Synaptic transmission	0.0011	10.6
UP	Cytoplasmic	0.0011	2.3
BP	Response to endogenous stimulus	0.0012	10.3
UP	DNA-binding	0.0015	3.2
BP	Learning or memory	0.0015	17
SP	Neuropeptide	0.0024	40.6
BP	Blood circulation	0.0029	13.6
BP	Circulatory system process	0.0029	13.6
BP	Activation of adenylate cyclase activity by G-protein signaling pathway	0.003	35.4
SP	Amidation	0.0042	30.4
BP	Ion homeostasis	0.0066	6.4
BP	Glial cell differentiation	0.0068	23.6
BP	Axonogenesis	0.0084	9.3
SP	Zinc finger	0.011	18.4
BP	Telencephalon development	0.0147	15.7
BP	Neuropeptide signaling pathway	0.0171	14.5
BP	Response to drug	0.0215	12.9
BP	Cell migration	0.0235	6.3
CC	Axon	0.0253	11.7
BP	Rhythmic process	0.0263	11.6
BP	Axon guidance	0.0263	11.6
BP	Negative regulation of multicellular organismal process	0.0273	11.3
BP	Cellular ion homeostasis	0.0292	5.8
BP	Regulation of neurogenesis	0.0452	8.6

Genes with differences in expression of at least two-fold when comparing microarray data from EPO and CEPO treatments (cutoff $p < 0.05$) were analyzed with DAVID. The functional annotation clustering tool was used with default settings and highest classification stringency. Only terms from GeneOntology (BP, biological process; MF, molecular function; CC, cellular component), Interpro (IP) and SP_PIR_KEYWORDS (SP) are included.

type) gene (log2 FC of −0.57, $p = 0.022$) (Supplementary Table 1). Although the function of this system in non-olfactory tissue is still unknown, this gene has been found to be upregulated in AβPP/PS1 mice at the age of 3 months followed by a return to WT levels at the age of 6 months [30].

Microarray validation

We selected genes to validate the microarray data by performing quantitative PCR on individual samples obtained from treated animals. Gene expression was evaluated in both WT and AβPP/PS1-treated animals that had received Veh, CE25, CE50, and E25 treatment. Given the greater variability in the control group as analyzed by microarray, we increased the sample size for this validation step in order to boost the statistical power of the ANOVA test. QPCR was performed to validate the mRNA expression of neurotransmitter receptor and neuropeptide genes. These were seven neurotransmitter receptors (Adora2a, Adra1d, Drd1a, Drd2, Drd3, Htr1d, and Oprk1) and two neuropeptides (Grp and Vip). We also evaluated the expression of transcription factor Egr2, as it also was differently

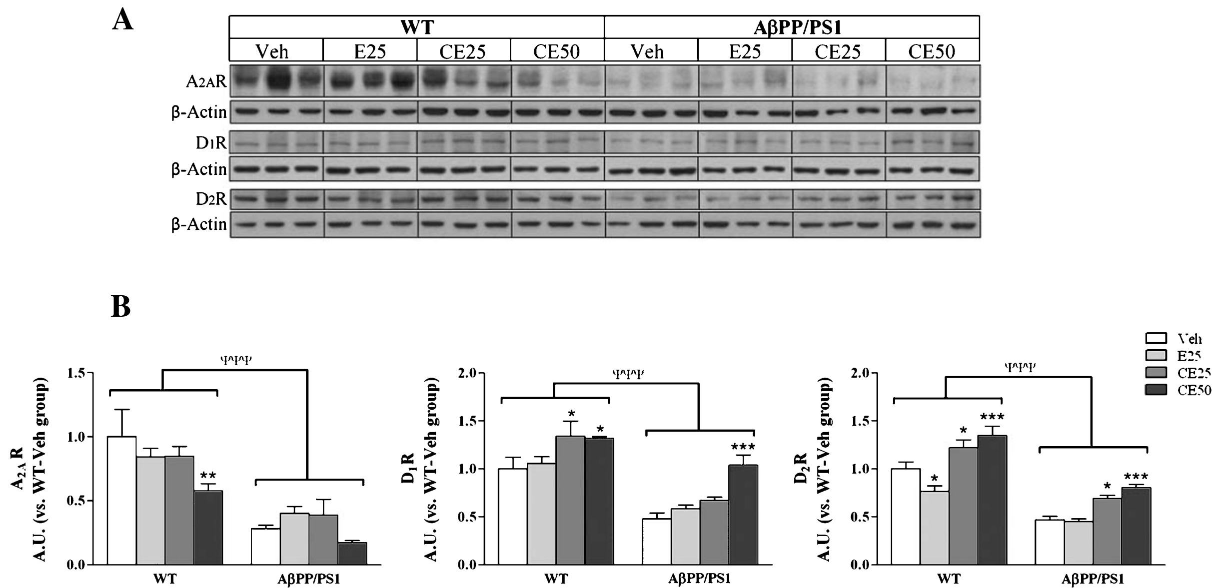

Fig. 4. Analysis of the protein levels of the neurotransmitter receptors $A_{2A}R$, D_1R, and D_2R. A) Western blots of the three antibodies and the loading control (β-actin). Three representative samples per genotype and group are shown. B) Bar diagram of the quantification of the blots. Two-way ANOVA analysis followed by Bonferroni post-test indicated that there was a genotype effect (Ψ) with protein levels always lower in the AβPP/PS1 mice compared to the WT mice. $A_{2A}R$ levels were reduced after CE50 treatment in the WT mice. D_1R and D_2R protein levels were increased with CEPO treatment in both genotype groups. D_2R was decreased by EPO in the WT mice. Bars represent the mean ± SEM of five samples per group and genotype, from two-three independent blots. To join data from different blots, values were calculated with respect to the mean of the WT-Veh group; A.U. arbitrary units. *$p < 0.05$; **$p < 0.01$; and ***/ΨΨΨ$p < 0.001$, as indicated by two-way ANOVA and Bonferroni post-test.

modulated by EPO and CEPO (Table 2); Egr2 has previously been related to acute EPO signaling after experimental stroke *in vitro* and *in vivo* [31]. All the data from the QPCR assays are shown in Fig. 3C.

Considering crossed comparisons between WT and AβPP/PP1 mice treated with vehicle, EPO, CE25, and CE50, two-way ANOVA analysis indicated that treatment accounted for differences in the expression of Drd1a ($F_{3,80} = 11.37$; $p < 0.0001$), Drd2 ($F_{3,80} = 7.8$; $p = 0.0001$), Drd3 ($F_{3,66} = 6.98$; $p = 0.0004$), Adora2a ($F_{3,78} = 9.27$; $p < 0.0001$), Adra1d ($F_{3,76} = 8.23$; $p < 0.0001$), Oprk1 ($F_{3,66} = 3.74$; $p = 0.0152$), Grp ($F_{3,75} = 7.75$, $p < 0.0001$), and Vip ($F_{3.75} = 7.95$, $p < 0.0001$). Under our experimental conditions, Htr1d expression was at the limit of detection and the analysis by QPCR could not be completed. Regarding Egr2, qPCR did not validate the differences observed in the microarray ($F_{3.72} = 0.95$; $p = 0.419$) (not shown in Fig. 3C).

Comparted to VEH-treated animals, Bonferroni post-test showed that CEPO (CE25 and CE50) significantly increased the expression of Drd1 in WT (CE25 $p < 0.05$; CE50 $p < 0.001$) and AβPP/PS1 (CE25 $p < 0.05$; CE50 $p < 0.05$) mice whereas Drd2 and Adora2a were significantly increased only with CE50 in WT (Drd2 $p < 0.01$; Adora2a $p < 0.01$) but not in AβPP/PS1 transgenic mice. CEPO significantly decreased the expression of Adra1d in WT (at doses of CE50; $p < 0.001$) and AβPP/PS1 (at doses of CE25; $p < 0.05$), and Grp at CE25 ($p < 0.01$) and CE50 ($p < 0.01$) doses in AβPP/PS1 but not in WT animals (Fig. 3C). Additionally, EPO had no effect on the analyzed genes excepting a significant increase of Vip in WT ($p < 0.01$) but not in AβPP/PS1 transgenic mice (Fig. 3C). For the purpose of validation only, additional significant differences (labelled with Ψ) between EPO and CEPO values are indicated in the same figure. Details of the statistical analysis of Q-PCR data are summarized in Supplementary Table 2.

Protein quantification of A_{2A}, D_1 and D_2 neurotransmitter receptors

The results of RNA expression indicated that CEPO treatment induces changes in the expression of certain neurotransmitter receptor genes, most significantly the genes coding for the adenosine receptor a2a ($A_{2A}R$) and the dopamine receptors 1 and 2 (D_1R and D_2R). In order to learn whether this change in expression

was translated to different protein levels, western blot analysis of these three receptors was performed in the different groups of animals (Fig. 4).

Protein levels of all three receptors were lower in the AβPP/PS1 mice compared to the WT mice as indicated by two-way ANOVA analysis of the blots of $A_{2A}R$ ($F_{1,32} = 53.10$; $p < 0.0001$), D_1R ($F_{1,32} = 61.82$; $p < 0.0001$), and D_2R ($F_{1,32} = 123.21$; $p < 0.0001$). If we compare the mean expression of the WT-Veh and AβPP/PS1-VEH groups, the protein expression levels of $A_{2A}R$, D_1R and D_2R were 72.0%, 51.9% and 53.3% lower, respectively. There was a statistically significant treatment effect on the levels of $A_{2A}R$ ($F_{3,32} = 3.30$; $p = 0.0326$), D_1R ($F_{3,32} = 10.04$; $p < 0.0001$), and D_2R ($F_{3,32} = 23.70$; $p < 0.0001$). Bonferrroni post-test revealed that $A_{2A}R$ levels were down-modulated in the WT animals by CE50 treatment ($p < 0.01$), reduced by 42.2%. On the other hand, D_1R levels were increased by 34.1% and 31.8% in the WT animals after CE25 and CE50 treatment, respectively ($p < 0.05$). In the AβPP/PS1-CE50 group, the levels of D_1R were 55.6% higher than the AβPP/PS1-Veh ($p < 0.001$), reaching levels similar to those for WT-Veh. CEPO also induced an increment of D2R levels in both WT and AβPP/PS1 mice. Compared to their Veh-treated counterparts, CE25 and CE50 increased D_2R by 21.2% ($p < 0.05$) and 34.7% ($p < 0.001$) in the WT mice, and by 48.3% ($p < 0.05$) and 72.2% ($p < 0.001$) in the AβPP/PS1 mice, respectively. The only significant effect of EPO compared to Veh-treated animals was detected in the levels of D_2R in the WT mice, which showed 23.3% lower ($p < 0.05$) D_2R levels. Details of the statistical analysis of western blot data are summarized in Supplementary Table 3.

DISCUSSION

The present results show that CEPO and EPO improve the memory of AβPP/PS1 transgenic mice. As expected, blood parameters related to red blood cell production RBC, HTC, HB, and RI are modified after treatment with EPO. We also observed a decrease in the platelet cell population by EPO, which has been previously reported as related to stem-cell competition between erythroid and platelet precursors [16]. Reduced body weight by EPO was observed in WT mice, an observation which is in accordance with previous studies showing reduced body weight by EPO in models of obesity [32], and in C57Black/C transgenic mice expressing EPOs that reduce lipid mass by increasing muscle fat oxidation and upregulating genes related to thermogenesis [33]. In contrast, the present study demonstrates that CEPO has no deleterious effects on body weight and blood parameters in WT and AβPP/PS1 transgenic mice. Therefore, CEPO stands a better chance of being used as a putative therapeutic agent administered during long periods.

Interestingly, EPO decreases fibrillar amyloid burden (plaques) and soluble $A\beta_{40}$ in brain of transgenic mice. Similar effects of EPO on Aβ levels have also been observed in the Tg2576 mouse model of AD [21]. However, CEPO has no effect on Aβ plaque burden or Aβ soluble Aβ. These novel findings suggest that the effects of EPO and CEPO on behavior have different molecular substrates.

RNA microarray assays followed by PCR validation were carried out to identify putative targets of CEPO. Microarray data reveal a limited number of common genes modulated by EPO and CEPO, suggesting divergent signaling routes. These different mechanisms of action may be due to different molecular properties of the primary compounds and to different receptors (or conformation of the same receptors) for EPO and CEPO [9,10]. Previous studies on the brain transcriptome of the AβPP/PS1 mouse described age-related changes associated with immune pathways [29] and the olfactory receptor system [30]. Yet our microarray data from AβPP/PS1-treated mice did not indicate that CEPO or EPO had major effects on the expression of genes related to immune processes. In contrast, the expression of certain neurotransmitter receptors appeared to be upregulated by CEPO. Moreover, after validating the selected genes, most of the observed treatment-associated changes regarding neurotransmitter receptors were similarly present in WT and AβPP/PS1 mice.

Significant upregulation of the transcript of dopamine receptors Drd1a, Drd2 and Drd3, and adenosine receptor 2a, Adora2a, together with significant down-regulation of the adrenergic receptor α1D (Adra1d), are discriminate CEPO and EPO treatments; the effects of CEPO have no similar correspondence in animals treated with EPO. Therefore, Drd1a, Drd2, Drd3, Adora2a, and Adra1d are identified for the first time as primary brain targets of CEPO.

Western blotting reveals lower protein levels of all the receptors analyzed in AβPP/PS1 mice when compared to controls, which is in agreement with earlier observations showing reduced protein translation and altered synaptic protein levels in this mouse model [34]. Moreover, western blotting demonstrates that CEPO reduces this difference regarding the levels of D_1R and D_2R. In contrast, increased RNA

levels of Adora2a are not linked to increased $A_{2A}R$ protein levels. Neurotransmitter receptors can heteromerize in a variety of combinations [35] leading to modifications of the intracellular signal elicited by ligand binding. Adenosine or dopamine binding to a $A_{2A}R$-D_2R heteromer results in the intracellular antagonism of the other ligand [36]. Furthermore, D_1R and D_2R heteromers have been described as modulating the calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) in the striatum, and a role has been suggested in synaptic plasticity [37]. In this line, it would be interesting to learn whether the observed down-regulation of $A_{2A}R$ and upregulation of D_1R and D_2R after CEPO treatment affects heteromer arrangements.

Compared to Veh-treated AβPP/PS1 animals, Adra1d and Grp were two other genes that were both down-modulated by CEPO. Mice lacking Adra1d have been associated with lower locomotor responses to behavioral activation [38], and upregulation of Adra1d transcripts have been recorded in the dentate gyrus in response to stress [39]. On the other hand, Grp has been related to fear-associated memory [40] but also with opposite effects on neurogenesis and neuronal development, as low GRP peptide levels are associated with increased neurogenesis but also with low neuronal development [41]. It is, however, too soon to speculate about the possible effects of Adra1d and Grp down-regulation in the present setting.

The present observations support the value of CEPO as a protective agent in the prevention of memory loss in AD-related models, and they may have practical implications in the treatment of AD. The beneficial effect can be related to modulation of neurotransmission. This would agree with the concept of different receptors for each, and *in vitro* and *in vivo* studies that have observed differences between CEPO and EPO [9,10,20]. Furthermore, the present results indicate that CEPO is not merely an equivalent of EPO without erythropoietic properties, but rather a different compound with different molecular targets.

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-141389.

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