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CEPO-Fc (An EPO Derivative) Protects Hippocampus Against Aβ-induced Memory Deterioration: A Behavioral and Molecular Study in a Rat Model of Aβ Toxicity

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Abstract—Alzheimer's disease (AD) is a debilitating neurodegenerative disease, characterized by extracellular deposition of senile plaques, mostly amyloid β-protein (Aβ) and neuronal loss. The neuroprotective effects of erythropoietin (EPO) have been reported in some models of neurodegenerative disease, but because of its hematopoietic side effects, its derivatives lacking hematopoietic bioactivity is recommended. In this study, the neuroprotective effects of carbamylated erythropoietin-Fc (CEPO-Fc) against beta amyloid-induced memory deficit were evaluated. Adult male Wistar rats weighing 250–300 g were bilaterally cannulated into CA1. $A\beta_{25-35}$ was administered intrahippocampally for 4 consecutive days (5 μg/2.5 μL/each side/day). CEPO-Fc (500 or 5000 IU) was injected intraperitoneally during days 4–9. Learning and memory performance of rats was assessed on days 10–13 using Morris Water Maze, then hippocampi were isolated and the amount of activated forms of hippocampal MAPKs' subfamily, Akt/GSK-3β and MMP-2 were analyzed using Western blot. From the behavioral results, it was revealed that CEPO-Fc treatment in both 500 and 5000 IU significantly reversed Aβ-induced learning and memory deterioration. From the molecular analysis, an increment of MAPKs and MMP-2 activity and an imbalance in Akt/GSK-3β signaling after $A\beta_{25-35}$ administration was observed. CEPO-Fc treatment prevented the elevation of hippocampal of P38, ERK, MMP-2 activity and also Akt/GSK-3β signaling impairment induced by $A\beta_{25-35}$ but it had no effect on JNK. It seems that CEPO-Fc prevents Aβ-induced learning and memory deterioration, and also modulates hippocampal MAPKs, Akt/GSK-3β and MMP-2 activity. This study suggests that CEPO-Fc can be considered as a potential therapeutic strategy for memory deficits like AD.

Key words: Alzheimer's disease, CEPO-Fc, MAPKs, Akt/GSK-3β, MMP-2.

INTRODUCTION

Erythropoietin (EPO) is a 34-KDa cytokine primarily identified as a hematopoietic stimulus factor which is produced in the kidney, liver and spleen, and also the brain (Sasaki, 2003; Genc et al., 2004). In addition to hematopoietic activity, various *in-vivo* (Genc et al., 2001; Bianchi et al., 2004; Castaneda-Arellano et al., 2014) and *in-vitro* studies (Montero et al., 2007; Li et al., 2008) have demonstrated neuroprotective effects for EPO through binding to erythropoietin receptors (EPORs). However, EPO-induced side effects on the hematopoietic system and erythropoiesis have raised

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Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; BBB, blood–brain barrier; CEPO-Fc, carbamylated erythropoietin-Fc; EPO, erythropoietin; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MAPKs, Mitogen-activated protein kinases.

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concerns about the clinical applications of EPO. Moreover, it has a relatively short half-life. Accordingly, the development of neuroprotective EPO-like compounds lacking hematopoietic activity with a longer half-life can open new therapeutic potentials in the treatment of neurodegenerative diseases. One of these compositions is the carbamylated form of EPO-Fc fusion protein known as CEPO-Fc which has a prolonged half-life and can cross the blood–brain barrier (BBB) (Leist et al., 2004; Sirén et al., 2009). This prolonged half-life is obtained by adding a constant region of an immunoglobulin (Fc). Additionally, the profound alteration of protein conformation and avoidance of undesirable side effects on the hematopoietic system by the process of carbamylation is well documented. In accordance, CEPO does not bind to the classical EPOR but rather exerts its own protective effects through the EPOR/βcR (β common receptor) heteroreceptor. In agreement with previous findings that CEPO does its signaling through a different receptor from EPOR, Sturm and colleagues (2010) demonstrated that CEPO also affects human monocytic Leukemia THP-1 cells that lack EPOR (Sturm et al., 2010). Furthermore, Armand-Ug'on and colleagues (2014) reported that improved memory observed in AβPP/PS1 transgenic mice after CEPO treatment can be attributed to the modulation of synaptic genes and neurotransmission system in a different way from EPO (Armand-Ugón et al., 2015). Several other studies have also elucidated the neuroprotective effects of CEPO in neurodegenerative diseases models in both *in-vitro* (Montero et al., 2007; Wang et al., 2007) and *in-vivo* conditions (Leconte et al., 2011; Tayra et al., 2013). These findings support the value of CEPO as a protective element in preventing memory defects in neurodegenerative disease models like AD.

Numerous studies suggest that the impairment in intracellular signaling molecules is associated with AD-related learning and memory disruption. Mitogen-activated protein kinases (MAPKs) are a family of serine-threonine kinases which are categorized into at least three families: extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 MAPK (Zhang and Liu, 2002; Jin et al., 2006). Several studies have demonstrated the alterations in the activity of MAPKs in AD and it is assumed that these kinases contribute to the development of AD pathology (Li et al., 2003; Colombo et al., 2009; Ghasemi et al., 2014b). Furthermore, some evidences have also shown that MAPKs' signaling pathway relates to EPO activity. Activation of EPO receptor has been shown to recruit and modulate several protein kinases, including MAPKs (Hernandez et al., 2017). In this regard, Chong et al. (2002) have reviewed some *in vitro* studies showing the involvement of MAPKs in EPO induced proliferation and differentiation in an erythroid cell line. However, they mentioned that EPO-triggered modulation of JNK and P38 might be cell specific (Chong et al., 2002). Different studies have reported that, by activating ERK1/2, EPO has some neural protective effects (Kilic et al., 2005a; 2005b). Nevertheless, in a rat model of global cerebral ischemia, Zhang et al. (2006) showed that EPO treatment has no significant effect on JNK activity, although a transient increment in ERK phosphorylation was observed. Therefore, they suggested that ERK activation plays a minor role in EPO-mediated neuroprotection of CA1 neurons (Zhang et al., 2006). Howbeit, the interplay between MAPKs and CEPO-Fc is not obviously clear.

Other signaling molecules involved in learning and memory are Serine–threonine kinase B (PKB), also known as Akt, and glycogen synthase kinase-3β (GSK-3β). AD familial mutations cause a down regulation in PI3K/Akt pathway (Ryder et al., 2004). In addition, presenilin mutations also cause the sensitization of neurons and neuronal apoptosis, which is related to a decrease in Akt activity (Baki et al., 2004). On the other hand, GSK-3β activation is involved in memory deterioration, and its inhibition restores this deficiency (Ponce-Lopez et al., 2011; Moosavi et al., 2014). GSK-3β is also reported to be involved in synaptic plasticity such that its inactivation is critical for LTP induction (Hooper et al., 2007; Peineau et al., 2007; Zhu et al., 2007). Moreover, some studies support the role of Akt/GSK-3β pathway activity in the protective effects by EPO. For instance, it has been reported that EPO protects against cerebral ischemia by activation of Akt/GSK-3β pathway (Kilic et al., 2005a; Zhang et al., 2006), as well as, EPO attenuates Aβ-induced cognitive deficits through the regulation of GSK-3β (Li et al., 2015). On the other hand, in acute ischemia/reperfusion injury, CEPO could have protect myocardium through an Akt-dependent mechanism and inhibition of the PI3K/Akt signaling pathway eliminated the cardioprotection mediated by CEPO (Xu et al., 2009).

Matrix metalloproteinases (MMPs) are Zn^{2+}- and Ca^{2+}-dependent endopeptidases. Enhanced expression of MMPs in the brain tissue of patients diagnosed with AD after death indicates that MMPs play serious role in the pathogenesis of AD (Wang et al., 2014). MMP-2 is likely a major MMP directly associated with Aβ in the brain. Furthermore, impaired function of MMP-2 would affect the processing of $A\beta_{1-40/42}$ *in vivo* and *in vitro* conditions, since the elimination of MMP-2 leads to more accumulation of Aβ (Miners et al., 2008). Some limited evidences have also shown that EPO exerts cellular protection through MMPs modulation. In addition, Sifringer et al. (2009) reported that EPO attenuates hyperoxia-mediated cell death by decreasing MMP-2 activity (Sifringer et al., 2009a). However, it has been revealed that treatment of endothelial cells with rhEPO significantly increases secretion of MMP2, which in turn promotes the migration of neural progenitor cells (Wang et al., 2006). However, the role played by MMPs in the effects of CEPO-Fc is remained to be elucidated.

Considering the deteriorating effects of Aβ on learning and memory and neuroprotective effect of CEPO, this study investigated the possibility of CEPO-Fc to restore Aβ-induced spatial memory deficit in a rat model of AD. In addition, as the molecular effects of CEPO-Fc remained unclear, alterations in the activity of hippocampal Akt/GSK-3β, MAPKs and MMP-2 were assessed after treatment with $A\beta_{25-35}$ and/or CEPO-Fc.

EXPERIMENTAL PROCEDURES

Animals

In this study, adult male Wistar rats weighing 250–350 g were used. The animals were obtained from Laboratory Animal Center, Shahid Beheshti University of Medical Sciences. They were kept in Plexiglas cages in groups of 2–3 per cage at room temperature (25 ± 2 °C) under standard 12–12 h light–dark cycle (lights on at 07:00) with free access to laboratory chow and tap water. The experimental protocols were approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.SM. REC.1394.78) and all experiments were performed in accordance with the guide for the care and use of laboratory animals (National Institutes of Health Publication No. 80–23, revised 1996).

MATERIALS

Amyloid β-Protein 25–35 ($A\beta_{25-35}$) (A4559) was purchased from Sigma; Carbamylated Erythropoietin-Fc (CEPO-Fc) was prepared in our lab (Vienna, Austria). Western blot antibodies including phospho-Akt(Ser473), (4060); Akt(4685); phospho-GSK-3β (Ser9), (5558); GSK-3β (9315); phospho-p38 (9211); p38 (9212); phospho-ERK (4377); ERK (4695); phospho-JNK (4671); JNK (9258); MMP-2 (4022); Beta-Actin (4970) and secondary HRP-conjugated (7074) were purchased from Cell Signaling Technology Company. Amersham ECL select (RPN2235) reagent kit was purchased from GE healthcare. Protease and phosphatase inhibitor cocktail was purchased from Pierce. PVDF membrane was purchased from Millipore. Salts were obtained from Merck.

Drugs' preparation and administration

$A\beta_{25-35}$ peptide was dissolved in sterile distilled water (vehicle) at a concentration of 2 μg/μl and was stored in −20 °C. Based on previous studies (Maurice et al., 2013; Ghasemi et al., 2014b) aggregation of $A\beta_{25-35}$ was done by in-vitro incubation at 37 °C for 4 days. The CEPO-Fc molecule used for this study is a fusion protein comprising two rhEPO molecules connected with the Fc domain of a human antibody IgG1. Manufacture and biochemical characterization have been described previously (Schriebl et al., 2006). The fusion protein was then carbamylated until no erythropoietic potency remained. CEPO-Fc was dissolved in phosphate-buffered saline (PBS) at a concentration of 1.91 mg/ml or 2.3×10^5 IU (main stock). The required concentrations for injection were prepared by diluting the main stock.

For intra-hippocampal injection of Aβ or its vehicle, a 5-μl Hamilton syringe was used. The syringe was connected to the injection needle (30 gauge) through a short piece of polyethylene tube and the injection needle was inserted 0.5 mm beyond the tip of guide cannula. Intra-hippocampal injection of Aβ (5 μg/2.5 μl each side/day) or its vehicle (equal volume each side/day) was done four times during four consecutive starting at the day of surgery (days 0–3). During all microinjections, the rats were allowed to move around freely in their box. All microinjections were performed in speed 0.5 μl/min and the injection needle was left in place for additional 2 min to allow the solution completely diffuse from the tip and minimize drug backflow in the cannula. CEPO-Fc 5000 IU/kg (or 42 μg/kg) and 500 IU/kg (or 4.2 μg/kg) or equal volume of its vehicle (PBS) was administered intraperitoneally (IP) during succeeding 6 days (days 4–9). Ten days after surgery (day 10), behavioral Morris Water Maze test (MWM) was carried out to assay rat's spatial learning and memory.

Surgery

For anesthetizing of rats (n = 9–11 per each group) i.p. injection of mixed ketamine (100 mg/kg) and xylazine (10 mg/kg) was used. The animals were fixed into a stereotaxic frame as it was done previously (Ghasemi et al., 2014b), stainless steel guide cannula (23 gauge) were implanted bilaterally into the dorsal hippocampus (AP: −3.8, ML: ±2.2, DV: −2.7) according to Paxinos brain atlas. The cannula was held by acrylic cement and anchored to stainless steel screws that were fixed to the skull.

Behavioral test

Morris water maze apparatus. The water maze was a black circular pool (150 cm in diameter and 60 cm in height) which was filled with water to a depth of 25 cm. The temperature of water was kept at 20 ± 1 °C. Four distinct geographic areas (four equal quadrants) were designed in the maze and release points were considered at each quadrant as 1, 2, 3 and 4 zones. A hidden circular platform (11 cm in diameter), was placed in the center of the 1st quadrant, underwater 1.5 cm below the surface of the water. Visual cues at different locations around the maze (i.e. computer, book shelves, and posters) were fixed during all days of test. The animal's motion was recorded by a CCD camera mounted above the center of the maze and sent to the computer. The path of animals swimming was automatically recorded by a computerized system (Noldus EthoVision, 7.1 versions) and then several parameters including latency to find the platform, traveled distance and the swimming speed were analyzed by software.

Procedure. In order to evaluate spatial learning and memory, MWM test was performed. Animals are trained according to a protocol including 4 days of training session (Ghasemi et al., 2014b). In the first 3 days, a hidden platform (about 1.5 cm below water surface) was placed in one fixed location within the pool. Each learning session was consisted of 4 trials with 4 different starting locations. In each trail, animals were released into the water from one of the 4 different starting zones, and allowed to swim and find the hidden platform. The rats' behavior was recorded for 90 s. After finding and standing on the platform, the animals were allowed to stay there for 20 s until the start of the next trial. In situations that animals failed to find the platform in 90 s, they were guided to the platform by an examiner. On day 4, the probe trial (retention test) was performed; the hidden platform was removed from the tank and each animal was released into the water from the opposite point of the target zone, then allowed to swim for 60 s and the time spent in the target quadrant was recorded. After probe trial, for assessment of rat motivation, visual ability and sensory–motor coordination, a visible platform test was done. So, a visible platform covered by aluminum foil, was put in another area above the surface of water and 4 trials similar to hidden sessions was done and the latency to find the visible platform was recorded.

Tissue preparation

For decapitation of animals and extraction of their hippocampi, after completion of behavioral test in the last day, they were anesthetized by CO_2 inhalation immediately and their hippocampi were isolated on ice and transferred to liquid nitrogen for 24 h and then stored at −80 °C until molecular analysis.

Western blot analysis

The hippocampi were homogenized on ice in cold RIPA lysis buffer (50 mM Tris–HCl, pH8.0; 150 mM NaCl; 1% Triton X-100; 0.5% Na-Deoxycholate; 0.1% SDS (sodium dodecyl sulfate)) supplemented with protease and phosphatase inhibitors cocktail. The lysates were centrifuged at 14,000 rpm for 30 min at 4 °C to remove debris. Lowry method using bovine serum albumin (BSA) standard was used to quantify the protein content of samples. Protein samples were mixed with loading sample buffer and heated at 100 °C for 5 min. Samples with equal amounts of protein (50 μg) were then separated by 12% polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were incubated with blocking buffer (5% BSA) 1 h in room temperature. Then, membranes were probed overnight at 4 °C with primary antibodies (Akt, GSK-3β, MAPKs, MMP-2 and β-actin). After washing with TBS-T, the membranes were incubated for 1:30 h with horseradish-peroxidase-conjugated anti-rabbit antibody. For detection of Beta-Actin and corresponding total forms of phosphorylated proteins, the membranes were stripped with stripping buffer (containing Tris-base 1 M, PH6.8, SDS 10% and 2-Mercaptoethanol 0.4%) at 54 °C for 25 min. In this way, the previous bands were completely removed from the stripped blots. Immunoreactivity was visualized by incubating the blots with ECL select kit. At the end, the radiographic films were scanned and blot quantification of protein bands' density was calculated by Image-J software.

Statistical analysis

Data analysis was performed using SPSS Statistics 21 and GraghPad Prism 7.01. Data obtained from training days were analyzed by a three-way repeated measure followed by post hoc Bonferroni's test and a two-way analysis of variance (ANOVA) followed by post hoc Tukey's test was used for data of retention day and also data of molecular tests. All results have been shown as mean ± S.E.M. In all statistical comparisons, $P < 0.05$ is considered as a significant difference.

RESULTS

CEPO-Fc prevented $A\beta_{25-35}$-mediated learning and memory spatial impairment

In order to assess the animal's spatial learning and memory following vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration, Morris water maze test was employed. Learning patterns of animals in all groups demonstrated a negative linear correlation between escape latency and training days, however, the performance of animals receiving $A\beta_{25-35}$ is weaker than other groups. A three-way ANOVA repeated measure analysis revealed a significant interaction of day × Aβ×CEPO-Fc (F (4, 104) = 2.821, P = 0.029) as well as main effect of day (F (2, 104) = 100.872, $P < 0.001$). On the other hand, no significant interaction of day × Aβ (F (2, 104) = 0.727, P = 0.486) and day × CEPO-Fc (F (4, 104) = 1.672, P = 0.162) was seen. The following Post hoc analysis by Bonferroni's test revealed that escape latency in $A\beta_{25-35}$ receiving group is significantly increased than vehicle receiving group on days 1 and 2 ($P < 0.001$), indicating Aβ-induced deterioration in $A\beta_{25-35}$ group. As it is evident in Fig. 1, CEPO-Fc treatment in both doses of 5000 IU and 500 IU ($P < 0.001$) could reverse this deficit in day 1. Although Aβ + CEPO-Fc-receiving groups did not show a significant difference with $A\beta_{25-35}$-treated group in days 2 and 3, there was no significant difference between control and Aβ + CEPO-Fc (both doses of 5000 IU and 500 IU)-treated groups in these days, indicating a protective role for CEPO-Fc against $A\beta_{25-35}$ toxicity.

The traveled distance results has been shown in Fig. 2. A three-way ANOVA repeated measure analysis revealed a significant main effect of day (F (2, 104) = 55.17, $P < 0.001$), but no significant interaction of day × Aβ×CEPO-Fc (F (4, 104) = 1.13, P = 0.346), day × Aβ (F (2, 104) = 0.016, P = 0.984) and day × CEPO-Fc (F (4, 104) = 2.401, P = 0.055). Post hoc Bonferroni's test revealed that the traveled distance in $A\beta_{25-35}$-treated group is significantly increased comparing with the vehicle-receiving group on day 1 ($P < 0.001$) and day 2 ($P < 0.01$). Treatment with CEPO-Fc in both doses 5000 IU (day 1: $P < 0.001$ and day 2: $P < 0.05$) and 500 IU (day 1: $P < 0.001$ and day 2: $P < 0.05$) nullified Aβ-induced deterioration. Collectively, these results demonstrated that CEPO-Fc restores learning and memory impairment mediated by $A\beta_{25-35}$.

CEPO-Fc prevented $A\beta_{25-35}$-mediated memory retention impairment

The time spent in target area on probe day (day 4) is shown in Fig. 3. A two-way ANOVA followed by Tukey's test showed that treatment with CEPO-Fc in $A\beta_{25-35}$ receiving group could significantly increase the time

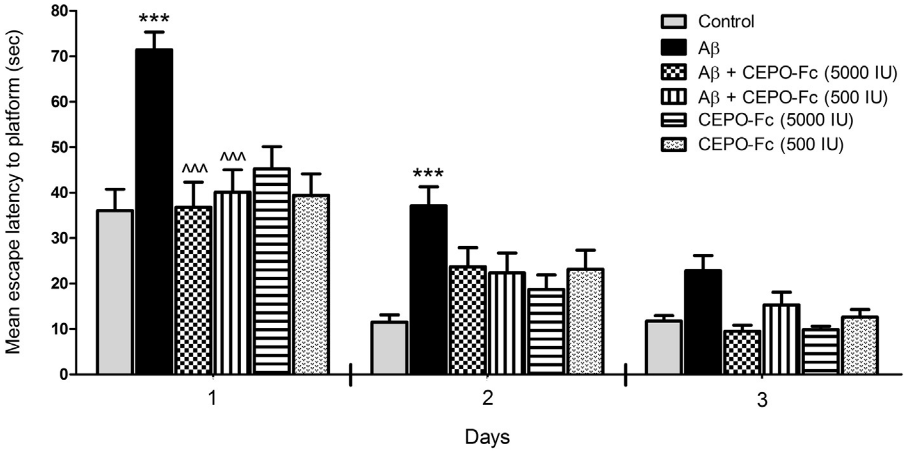

Fig. 1. The effect of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration on water maze performance. The escape latency to reach the invisible platform during training days: This figure demonstrates that escape latency is longer in $A\beta_{25-35}$-treated group, while CEPO-Fc in both of doses 5000 IU and 500 IU reserved this deficit. Data are represented as mean ± SEM. $^{***}P < 0.001$ represents the difference between $A\beta_{25-35}$- and vehicle-treated groups. $^{\hat{}\hat{}\hat{}}P < 0.001$ represents the difference between $A\beta_{25-35}$- and other treated groups.

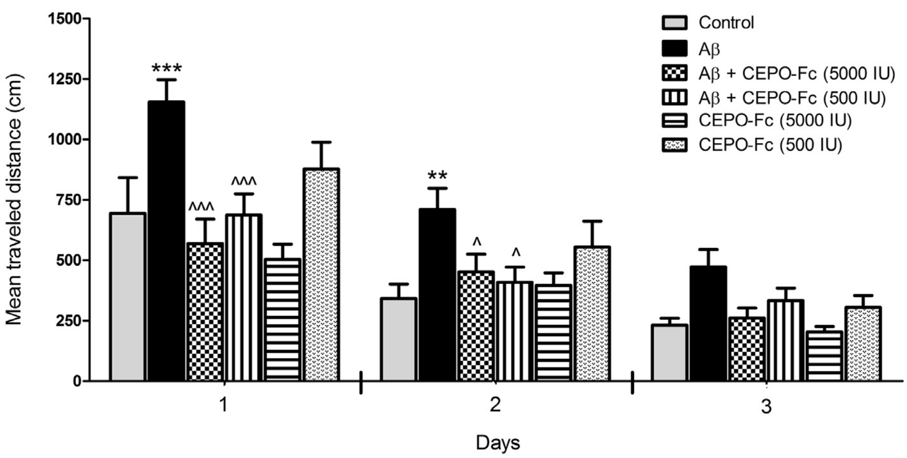

Fig. 2. The effect of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration on traveled distance. The traveled distance to reach the hidden platform during days 1–3 of training: This figure shows that traveled distance is greater in $A\beta_{25-35}$-receiving group but in CEPO-Fc-treated animals this parameter is significantly decreased. Data are represented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ represent the difference between $A\beta_{25-35}$- and vehicle-treated groups. $^{\hat{}}P < 0.05$ and $^{\hat{}\hat{}\hat{}}P < 0.001$ represent the difference between $A\beta_{25-35}$- and other treated groups.

spent in target area (Aβ main effect: F (1, 52) = 5.93, P = 0.0184); CEPO-Fc main effect: F (2, 52) = 7.261, P = 0.0017); Aβ×CEPO-Fc interaction effect: (F (2, 52) = 6, P = 0.0045)). Post hoc analysis by Tukey's test displayed that the time spent in target area was decreased significantly in $A\beta_{25-35}$-treated group ($P < 0.01$) while both 5000 IU ($P < 0.01$) and 500 IU ($P < 0.001$) doses of CEPO-Fc significantly prevented Aβ-induced amnesia.

$A\beta_{25-35}$ and/or CEPO-Fc treatment did not affect swimming speed and vision/motivation functions of animals

In order to assess the possible effect of drugs on motor performance swimming speed of animals, their swimming speed was assessed (Fig. 4A). A two-way ANOVA followed by Tukey's test showed that treatment with $A\beta_{25-35}$ and/or CEPO-Fc did not affect swimming speed (Aβ main effect: F (1, 50) = 2.401, P = 0.1275); CEPO-Fc main effect: F (2, 50) = 1.901, P = 0.1601); Aβ×CEPO-Fc interaction effect: (F (2, 50) = 0.4472, P = 0.6420)). Moreover, to evaluate sensory-motor coordination, vision and motivation of animals, a visible platform test was performed on day 4 after probe trial. The effects of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc on escape latency to the visible platform is depicted in Fig. 4B. A two-way ANOVA followed by Tukey's test did not show significant differences between $A\beta_{25-35}$ and/or CEPO-Fc-treated groups (Aβ main effect: F (1, 50) = 0.5555, P = 0.4596); CEPO-Fc main effect: F (2, 50) = 0.4909, P = 0.6150); Aβ×CEPO-Fc interaction effect: (F (2, 50) = 0.6018, P = 0.5518)). These data indicate that observed effects of $A\beta_{25-35}$ and/or CEPO-Fc treatment on learning and memory are not bring about by swimming speed or visual/motor dysfunction.

Akt/GSK-3β alterations mediated by $A\beta_{25-35}$ and/or CEPO-Fc

Western blot studies was done to investigate hippocampal phosphorylated-Akt (P.Akt), phosphorylated-GSK-3β (P. GSK-3β), phosphorylated-P38 (P.P38), phosphorylated-ERK1/2 (P.ERK1/2), phosphorylated-JNK (P.JNK), and MMP-2 proteins. In the case of antibodies that recognize two bands corresponding to different proteins (ERK, JNK and MMP-2), the summation of the two bands is quantified for analysis.

Antibodies against phosphorylated Akt(Ser473) and total Akt (T.Akt), P.GSK-3β and total GSK-3β (T.GSK-3β) detected bands at 60 kDa and 46 kDa respectively. A two-way ANOVA followed by Tukey's test showed that CEPO-Fc treatment could increase Akt decrement induced by $A\beta_{25-35}$ (Aβ main effect: F (1, 18) = 4.82, P = 0.0415); CEPO-Fc main effect: F (2, 18) = 3.319, P = 0.0593); Aβ×CEPO-Fc interaction effect: (F (2, 18) = 10.51, P = 0.0009)). Post hoc analysis by Tukey's test displayed that $A\beta_{25-35}$ administration decreased

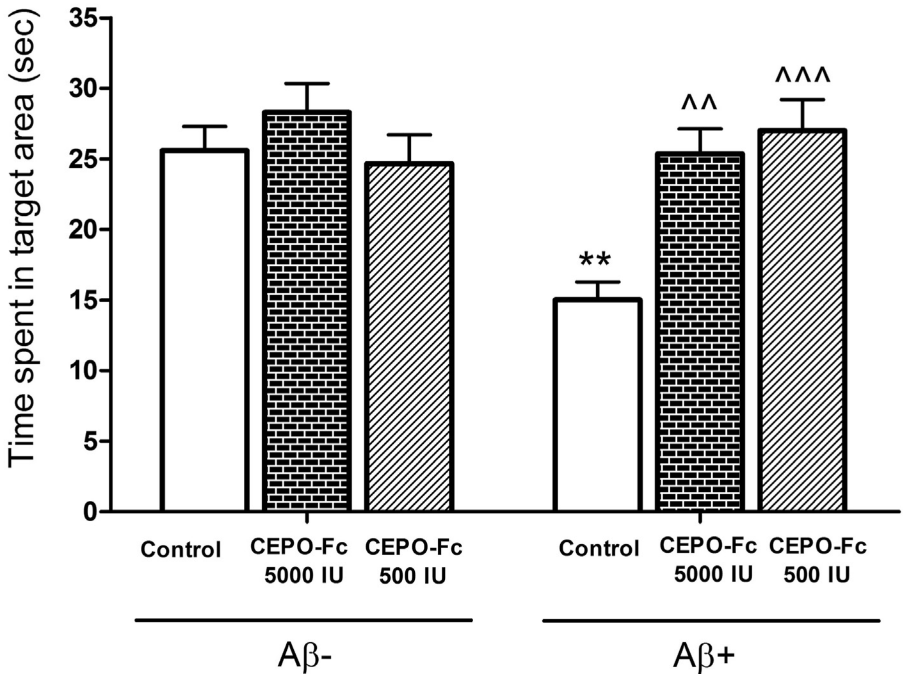

Fig. 3. The effect of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration on ability of memory in retention day. Total time that animals spent in target zone. These data reveal that $A\beta_{25-35}$ deteriorated animal's memory, while CEPO-Fc in both doses restored Aβ-induced impairment. Data are represented as mean ± SEM. $^{**}P < 0.01$ represents the difference between $A\beta_{25-35}$- and vehicle-treated groups. $^{\hat{}\hat{}}P < 0.01$ and $^{\hat{}\hat{}\hat{}}P < 0.001$ represent the difference between $A\beta_{25-35}$- and other treated groups.

hippocampal P.Akt/T.Akt ratio ($P < 0.01$) while CEPO-Fc treatment in both doses 5000 IU ($P < 0.05$) and 500 IU ($P < 0.01$) reversed Aβ-induced decrement of Akt. In addition, a two-way ANOVA followed by Tukey's test showed that CEPO-Fc treatment could reverse GSK-3β decrement induced by $A\beta_{25-35}$ (Aβ main effect: F (1, 18) = 1.482, $P = 0.2392$); CEPO-Fc main effect: F (2, 18) = 8.159, $P = 0.0030$); Aβ×CEPO-Fc interaction effect: (F (2, 18) = 8.311, $P = 0.0028$)). Post hoc analysis by Tukey's test displayed that $A\beta_{25-35}$ administration decreased hippocampal P.GSK-3β/T.GSK-3β ratio ($P < 0.01$) while CEPO-Fc treatment in both doses 5000 IU ($P < 0.001$) and 500 IU ($P < 0.05$) reversed this alteration. These findings are illustrated in Figs. 5 and 6.

MAPKs' alterations mediated by $A\beta_{25-35}$ and/or CEPO-Fc

Western blot results of hippocampal MAPKs are shown in Figs. 7–9. The antibodies against phosphorylated (active form) and total P38 (T.P38) detected a band at 43 kDa (Fig. 7). A two-way ANOVA followed by Tukey's test showed that CEPO-Fc treatment could decrease P38 increment induced by $A\beta_{25-35}$ (Aβ main effect: F (1, 18) = 11.65, $P = 0.0031$); CEPO-Fc main effect: F (2, 18) = 2.774, $P = 0.0891$); Aβ×CEPO-Fc interaction effect: (F (2, 18) = 6.615, $P = 0.0070$)). Post hoc analysis by Tukey's test displayed that $A\beta_{25-35}$ administration increased hippocampal P.P38/T.P38 ratio ($P < 0.01$) while CEPO-Fc treatment in both doses reversed Aβ-induced enhancement of P38 ($P < 0.05$). Furthermore, antibodies against phosphorylated and total ERK (T.ERK) detected two bands at 42 and 44 kDa (Fig. 8). A two-way ANOVA followed by Tukey's test showed that CEPO-Fc treatment could decrease ERK1/2 increment induced by $A\beta_{25-35}$ (Aβ main effect: F (1, 18) = 6.355, $P = 0.0214$);

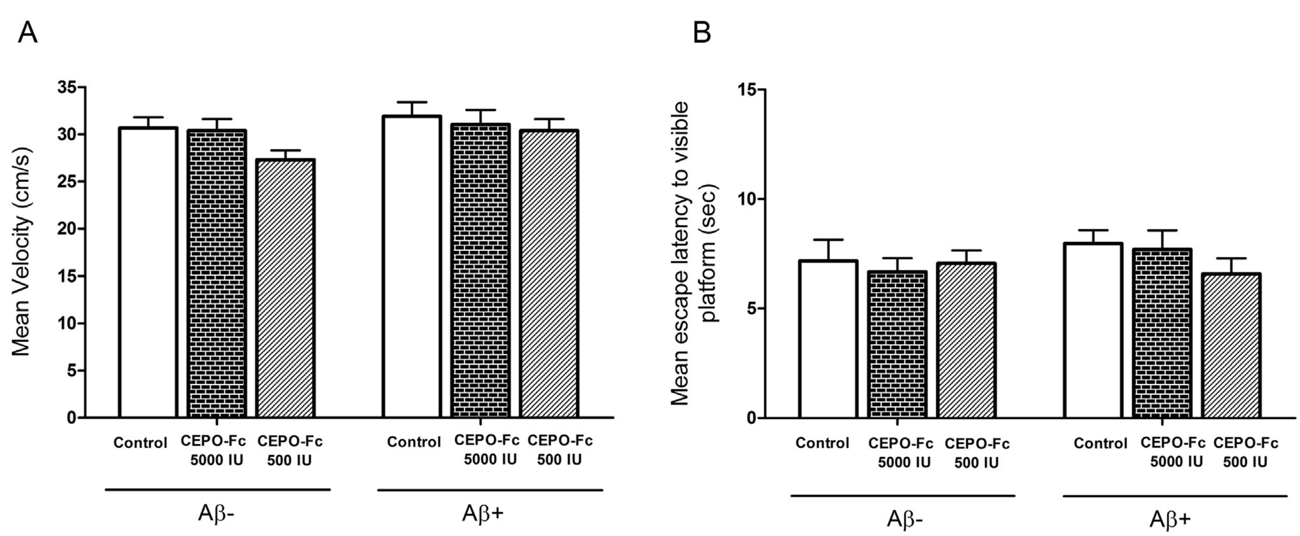

Fig. 4. The effect of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration on mean swimming speed (A) and animal's performance in visible platform test (B). These results show no significant difference between groups. Data are represented as mean ± SEM.

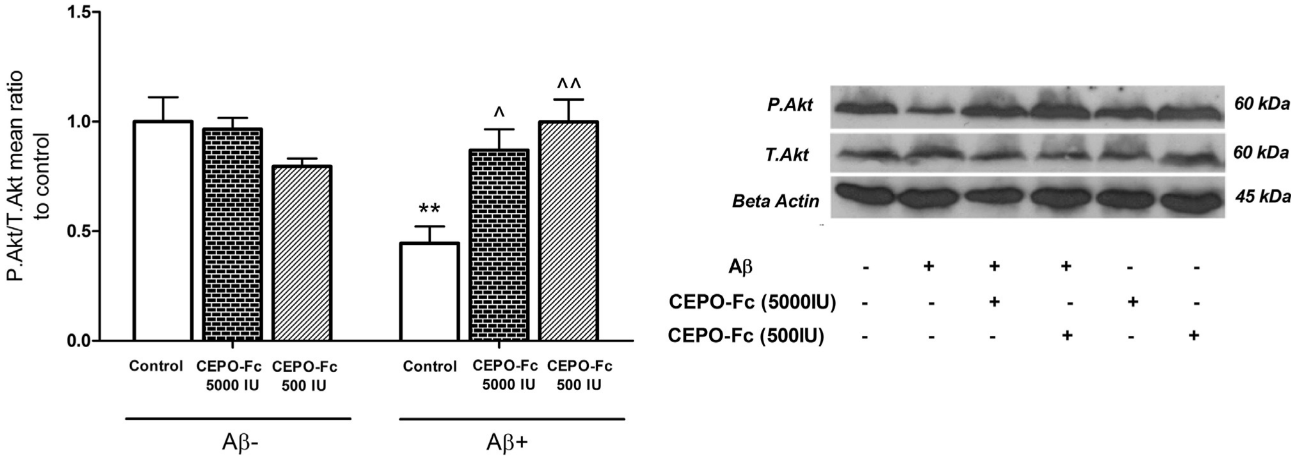

Fig. 5. Western blot analysis showing the effects of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration on hippocampal P.Akt/T.Akt ratio. Western immunoblots were probed with antibodies against phosphorylated and total Akt and Beta Actin. Data are represented as mean ± SEM. $^{**}P < 0.01$ represents the difference between $A\beta_{25-35}$- and vehicle-treated groups. $^{\wedge}P < 0.05$ and $^{\wedge\wedge}P < 0.01$ represent the difference between animals received $A\beta_{25-35}$- and other treated groups.

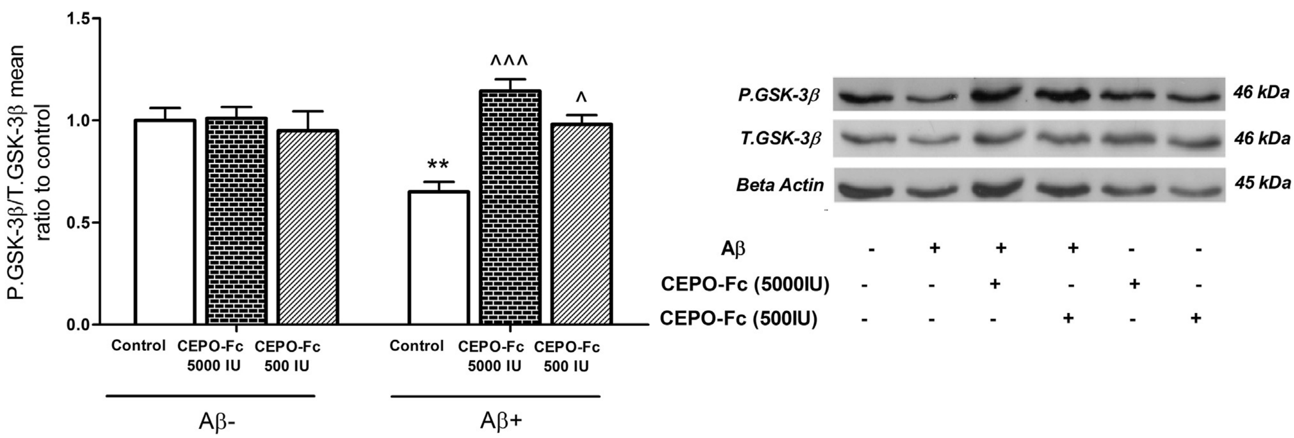

Fig. 6. Western blot analysis showing the effects of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration on P.GSK-3β/T.GSK-3β ratio in the hippocampi of rats. Western immunoblots were probed with antibodies against phosphorylated and total GSK-3β and Beta Actin. Data are represented as mean ± SEM. $^{**}P < 0.01$ represents the difference between $A\beta_{25-35}$- and vehicle-treated groups. $^{\wedge}P < 0.05$ and $^{\wedge\wedge\wedge}P < 0.001$ represent the difference between animals received $A\beta_{25-35}$- and other treated groups.

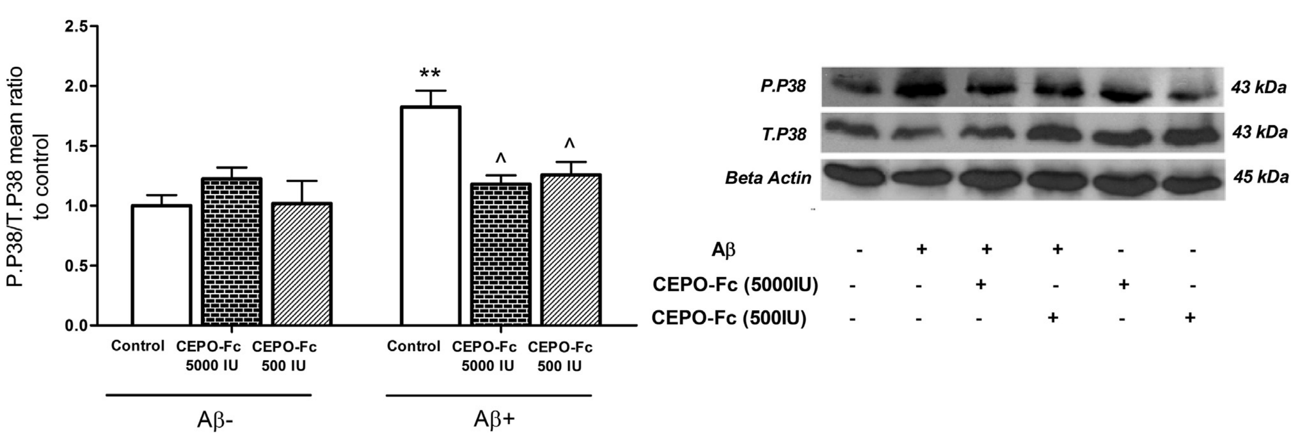

Fig. 7. Western blot analysis showing the effects of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc on P.P38/T.P38 ratio in the hippocampi of rats. Western immunoblots were probed with antibodies against phosphorylated and total P38 and Beta Actin. Data are represented as mean ± SEM. $^{**}P < 0.01$ represents the difference between $A\beta_{25-35}$- and vehicle-treated groups. $^{\wedge}P < 0.05$ represents the difference between animals received $A\beta_{25-35}$- and other treated groups.

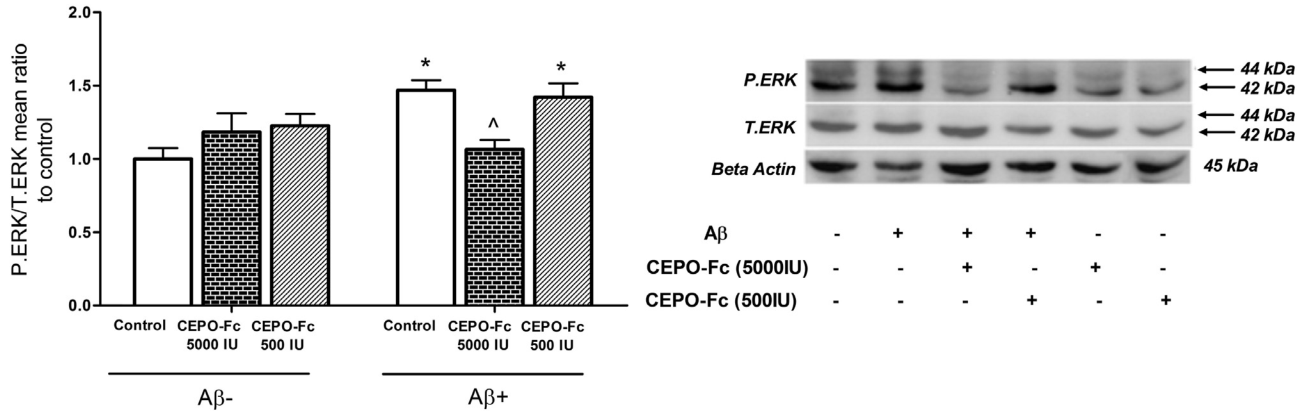

Fig. 8. Western blot analysis showing the effects of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc on P.ERK/T.ERK ratio in the hippocampi of rats. Western immunoblots were probed with antibodies against phosphorylated and total ERK and Beta Actin. Data are represented as mean ± SEM. $^{*}P < 0.05$ represents the difference between vehicle treated group and the others. $^{\wedge}P < 0.05$ represents the difference between animals received $A\beta_{25-35}$ and animals which received $A\beta_{25-35}$ + CEPO-Fc (5000 IU).

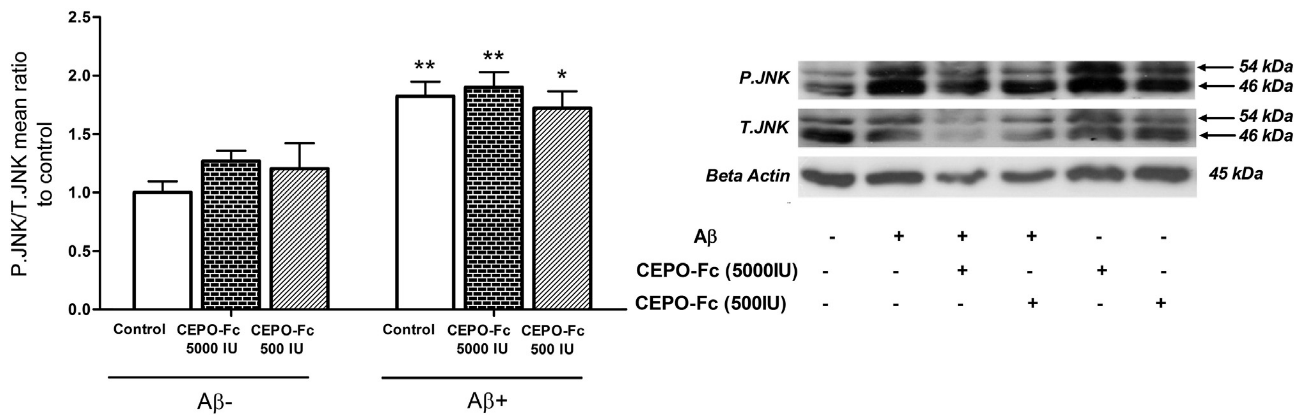

Fig. 9. Western blot analysis showing the effects of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration on hippocampal P.JNK/T.JNK ratio. Western immunoblots were probed with antibodies against phosphorylated and total JNK and Beta Actin. Data are represented as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ represent the difference between vehicle-treated group and the others.

CEPO-Fc main effect: F (2, 18) = 2.587, P = 0.1029); Aβ×CEPO-Fc interaction effect: (F (2, 18) = 5.562, P = 0.0132)). Post hoc analysis by Tukey's test displayed that P.ERK/T.ERK is increased after $A\beta_{25-35}$ treatment ($P < 0.05$) while CEPO-Fc treatment in dose 5000 IU, but not 500 IU, reversed ERK1/2 increment induced by $A\beta_{25-35}$ ($P < 0.05$). These findings suggest that spatial memory disruption observed after $A\beta_{25-35}$ administration is paralleled with increased activity of P38 and ERK1/2 in the hippocampus and their corrections after CEPO-Fc treatment might have a role in its memory improving effect.

Fig. 9 depicts the results of western blot analysis of hippocampal JNK. Antibodies against phosphorylated and total JNK (T.JNK) detected two bands at 46 and 54 kDa. A two-way ANOVA followed by Tukey's test showed that CEPO-Fc treatment could not reversed JNK increment induced by $A\beta_{25-35}$ (Aβ main effect: F (1, 18) = 33.1, $P < 0.0001$); CEPO-Fc main effect: F (2, 18) = 0.8052, P = 0.4625); Aβ×CEPO-Fc interaction effect: (F (2, 18) = 0.6158, P = 0.5512)). Post hoc by Tukey's test showed although $A\beta_{25-35}$ administration enhanced JNK phosphorylation significantly ($P < 0.01$), CEPO-Fc treatment could not reversed this increment in JNK phosphorylation. This result implies that the neuroprotective effect of CEPO-Fc might not be related to its effect on JNK.

MMP-2 alterations mediated by $A\beta_{25-35}$ and/or CEPO-Fc

The results showing the amount of MMP-2 in the hippocampi of the animals are depicted in Fig. 10. The antibody against MMP-2 detected two bands at 72 (Pro-MMP2) and 64 (Active-MMP2) kDa. Beta actin antibody (a band at 45 kDa) was used as an internal control. A two-way ANOVA followed by Tukey's test showed that CEPO-Fc treatment could reversed MMP-2 increment induced by $A\beta_{25-35}$ (Aβ main effect: F (1, 18) = 1.038, P = 0.3219); CEPO-Fc main effect: F (2, 18) = 6.671, P = 0.0068); Aβ×CEPO-Fc interaction effect: (F (2, 18) = 8.941, P = 0.002)). Post hoc analysis by Tukey's test represented that $A\beta_{25-35}$ significantly increased the expression of MMP-2 ($P < 0.01$) while treatment with

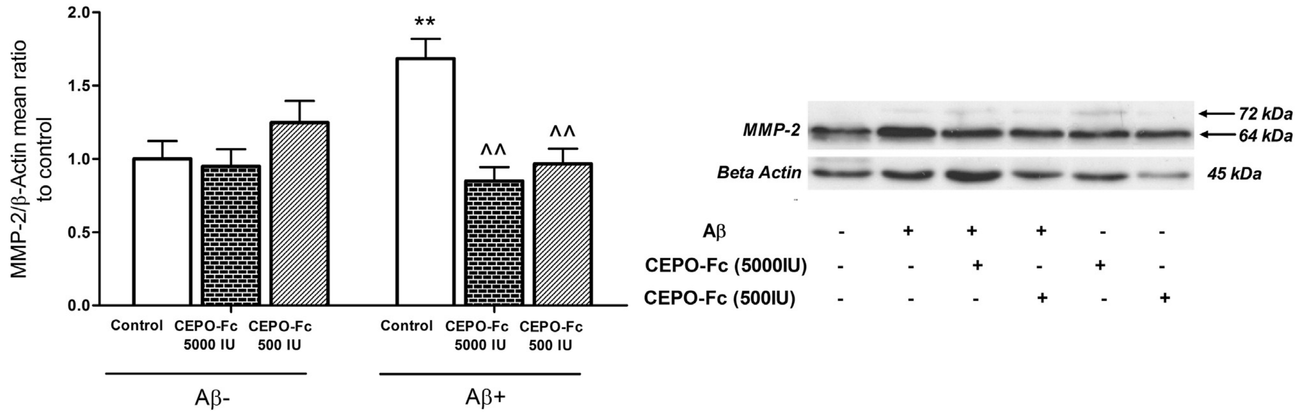

Fig. 10. Western blot analysis showing the effects of vehicle, $A\beta_{25-35}$ and/or CEPO-Fc administration on hippocampal MMP-2 protein activity. Western immunoblots were probed with antibodies against MMP-2 and Beta Actin. Data are represented as mean ± SEM. $^{**}P < 0.01$ represents the difference between $A\beta_{25-35}$- and vehicle-treated groups. $^{\wedge\wedge}P < 0.01$ represents the difference between animals received $A\beta_{25-35}$- and other treated groups.

CEPO-Fc in both doses reversed this Aβ-induced enhancement of MMP-2 levels ($P < 0.01$). This result displays that neuroprotection mediated by CEPO-Fc against $A\beta_{25-35}$ is accompanied with hippocampal MMP-2 decline.

DISCUSSION

This study aimed to evaluate the neuroprotective effects of i.p. administration of CEPO-Fc against $A\beta_{25-35}$-induced learning and memory impairment. The doses of $A\beta_{25-35}$ and CEPO-Fc were selected according to the previous studies (Ghasemi et al., 2014a; 2014b; Armand-Ugón et al., 2015). The findings revealed that although escape latency and traveled distance of the animals to reach the hidden platform was increased by $A\beta_{25-35}$, i.p. administration of CEPO-Fc in doses 5000 IU and 500 IU prevented such a disturbance. Additionally, retention test result (probe trial) showed that the time spent in the target quadrant in CEPO-Fc-treated groups was significantly greater than $A\beta_{25-35}$-receiving group. The results of visible platform test and swimming speed demonstrated that the observed behavioral effects were not affected by swimming speed or visual/motor abnormality. Furthermore, there was no CEPO-Fc dose–effect in almost any test. According to the results of this study, as well as an *in vitro* study that we are doing in our lab, we concluded that a plateau in the neuroprotective effects profile of CEPO-Fc might exist. Therefore, it is probable that selected doses in this work might be in this neuroprotective range. In agreement with this theory, in our *in vitro* studies, we observed that while low doses of CEPO-Fc were not protective, middle doses were protective in a plateau form (dose independent) and then higher doses were toxic. Therefore, it is possible that some similar pattern exist in our *in vivo* model. The protective effects of CEPO-Fc against memory deficit, as seen in the present study, are consistent with the previous evidence concerning cognition and memory improvement induced by CEPO-Fc. These effects were achieved through modulation of synaptic genes and neurotransmitter system in a mouse model of familial AD (Armand-Ugón et al., 2015). Furthermore, memory improvement induced by Neuro-EPO in an APP_{Swe} transgenic mouse model has also been reported (Rodriguez Cruz et al., 2017). It was reported that repeated administration of CEPO-enhanced spatial and non-spatial recognition memory in adult healthy mice which was accompanied by an increase in the number of NeuN/BrdU double-labeled cells in the dentate gyrus of the hippocampus (Leconte et al., 2011). It has been demonstrated that CEPO can cross BBB (Leist et al., 2004; Bar-Or and Thomas, 2011; Bouzat et al., 2011). Since the hippocampus is considered as the most important structure involved in spatial learning and memory, and repeated injection of $A\beta_{25-35}$ was done locally into the hippocampus, it seems that hippocampus is one of the direct targets for CEPO-Fc. Wang and colleagues (2007) reported that CEPO promotes neural progenitor cell proliferation and differentiation into neurons (Wang et al., 2007). As hippocampal neurogenesis is a key process of memory formation (Kempermann et al., 2015), it seems that protective effects of CEPO-Fc against $A\beta_{25-35}$ may be achieved via promoting neurogenesis.

The results revealed that $A\beta_{25-35}$ decreased phosphorylation of Akt (as an indicator for PI3K/Akt activity) and GSK-3β. The PI3K/Akt signaling pathway was activated by a wide range of stimulants and was conveyed via phosphorylating different residue of Akt protein such as Ser473. This pathway therefore plays a regulating role in diverse biological processes like proliferation and cell survival (Lawlor and Alessi, 2001; Brazil et al., 2004). In addition, several lines of evidences have shown that PI3K/Akt signaling pathway actively participates in memory acquisition, consolidation and retrieval (Chen et al., 2005b; Sui et al., 2008). Some studies, supporting the correlation between Akt signaling disruption and AD, have revealed that activation of PI3K/Akt pathway plays a protective role against Aβ neuronal toxicity *in vitro* (Martín et al., 2001; Kong et al., 2013; Ghasemi et al., 2015) and *in vivo* (Stein and Johnson, 2002; Cui et al., 2013). In this regard, it has been documented in some experiments that inhibition and/or diminution of PI3K/Akt signaling impairs passive avoidance learning (Barros et al., 2001)), learning associated with fear (Lin et al., 2001; Chen et al., 2005a) and hippocampal learning and memory (Moosavi et al., 2012).

One of the most important target molecules of Akt is GSK-3β. Activated Akt directly phosphorylates GSK-3β at Ser-9, consequently inhibiting its activity. It is developing that GSK-3β is a strategic signaling molecule that induces neurodegeneration and memory formation deficits in AD. It has been suggested that enhanced GSK-3β signaling activity might result in impairments in hippocampal memory formation (Giese, 2009). Although numerous studies have reported that GSK-3β signaling contributes in learning and memory impairment (Hoppe et al., 2013; Hui et al., 2018), Kimura et al. (2008) showed that the genetic reduction and pharmacological inhibition of GSK-3β impaired reconsolidation of hippocampus-dependent place memory. They suggested that Memory reconsolidation in adult hippocampus requires GSK-3β activation (Kimura et al., 2008). Nevertheless, accumulating evidence have proved that GSK-3β over-activity plays an important role in the pathology of AD by preventing LTP induction, memory impairment, Tau hyperphosphorylation, APP processing, increased production of Aβ, microglia-dependent inflammation, and neuronal death. This involvement led scientist to propose ''GSK-3β hypothesis in AD" (Balaraman et al., 2006; Peineau et al., 2007). Additionally, GSK-3β is involved in main stages of apoptotic signaling pathways (Beurel and Jope, 2006). In accordance, the results of this present study also demonstrated that $A\beta_{25-35}$ inhibited Akt and disinhibited GSk-3β activity (as shown by decreased Ser9 phosphorylation). Treatment with CEPO-Fc prevented these changes mediated by $A\beta_{25-35}$ in hippocampal Akt/GSk-3β signaling. In support of the present findings, following the activation of EPOR, PI3K signaling pathway is activated (Hernandez et al., 2017; Castillo et al., 2018). In addition, it has been demonstrated that EPO treatment activates Akt/GSK-3β pathway and has protective effects (van der Kooij et al., 2008). Therefore, it is a possibility that, similar to EPO, CEPO-Fc exerts its neuroprotective effects via restoring Akt/GSK-3β pathway.

The present results indicated that, while $A\beta_{25-35}$-induced memory loss was associated with an increment inP38, JNK and ERK, chronic treatment with CEPO-Fc restored P38 and ERK hyperactivity with no effect on JNK activation. Several studies believe that MAPKs cascade is necessary for consolidation of the resultant learning (Atkins et al., 1998) and synaptic plasticity and memory (reviewed by Sweatt, 2004). There was an in vitro study claiming that P38 inhibition diminished neprilysin expression and caused Aβ accumulation (Yamamoto et al., 2013). However, since this result was obtained from an isolated condition in cell culture, it can hardly be extrapolated to in vivo condition. Some others have revealed the interplay between MAPKs activity and development of AD pathology (Li et al., 2003; Colombo et al., 2009). In accordance with the findings of this study, other reports have highlighted the role of P38 activation in $A\beta_{25-35}$-induced neurotoxicity, LTP damage, cognitive deficit and memory disturbances (Zhu et al., 2005; Origlia et al., 2008; Canas et al., 2009; Huang et al., 2017; Lee and Kim, 2017). Moreover, Dai et al. (2016) revealed that inhibition of p38 in hippocampus led to the improvement of cognitive function and hippocampal synaptic plasticity (Dai et al., 2016). Thus, regarding the results of the current study, it seems that increased level of P38 might be related to the development of memory disturbance. Besides, diminution of P38 level following treatment with CEPO-Fc suggests that the improving effects of CEPO-Fc could be achieved at least partly through P38. Moreover, there exist several reports suggesting the involvement of ERK activation in cell death (Stanciu et al., 2000; Lesuisse and Martin, 2002). It has been reported that Aβ-induced increasing or decreasing in ERK activity in turn involves the neural cell death or survival (Townsend et al., 2007; Frasca et al., 2008). However, recent studies have suggested that persistent ERK activation is accompanied by memory impairment and apoptosis (Stanciu et al., 2000; Ghasemi et al., 2014b) while transient ERK activation might be protective and useful in memory formation (Zhuang and Schnellmann, 2006). Moreover, Mazzucchelli et al. (2002) demonstrated a critical regulatory role for ERK1 in striatum. They reported that knockout of ERK1 enhanced synaptic plasticity and learning and memory mediated by striatum (Mazzucchelli et al., 2002). One of the ways by which ERK activation promotes cell death is the suppression of survival signaling pathways. For instance, an *in vitro* model of primary cultures of mouse renal proximal tubular cells (MK-PT) demonstrated that withdrawal of all survival factors from MK-PT cells caused a progressive enhancement in ERK activity. This increment was associated with a gradual diminution in phosphorylated Akt activity; and exposure of cells with ERK inhibitors prevented Akt reduction and cell death (Sinha et al., 2004). Therefore, in the proposed model, it seems that besides nullifying P38 activity, CEPO-Fc affects ERK hyperactivity.

The present study demonstrated that while $A\beta_{25-35}$ increased hippocampal MMP-2 expression, CEPO-Fc treatment prevented this elevation. MMPs are Zn^{2+}- and Ca^{2+}-dependent endopeptidases that play a key role in restoring the extracellular environment via cell surface constituents, neurotransmitter receptors, and cleavage moderating of extracellular matrix proteins (Wang et al., 2014). Some documents suggest that MMP-2 contributes in memory formation. For example, separate studies have reported that inhibition or reduction in MMP-2 causes memory impairment in conditioned place preference test (Natarajan et al., 2013) and passive avoidance learning (Moosavi et al., 2018). Conversely, the increased level of MMP-2 in some other studies suggests a destructive role for MMP-2 in AD pathology. In parallel, it has been reported that MMP-2 is not only induced by Aβ, but also contributes in its decomposition (Fujimoto et al., 2008; Merlo and Sortino, 2012). On the other hand, oligomeric Aβ can enhance MMP-2 expression by inducing the expression of pro-inflammatory cytokines (Li et al., 2011; Du et al., 2012). This increased

MMP-2 level was significantly reduced by application of ERK and JNK inhibitors (Du et al., 2012). Also, some studies have reported that inhibition of MEK-1/2, but not P38, suppressed MMP-2 mRNA and protein (Nagai et al., 2009). These findings are in agreement with the present results that $A\beta_{25-35}$ increased MMP-2, ERK and JNK activity in a parallel way. A number of studies indicating the modulatory effect of EPO or its derivatives on the MMP-2 exist. For instance, Sifringer et al. reported that modulation of MMP-2 is involved in rEPO (recombinant EPO)-mediated reduction in hyperoxia-induced cell death (Sifringer et al., 2009b). The use of PI3K/Akt and ERK1/2 selective inhibitors significantly reduced the rhEPO-induced MMP-2 secretion, suggesting that MMP-2 activity could be affected by PI3K/Akt and ERK signaling (Wang et al., 2006). Therefore, based on previous studies and the present study, it could be inferred that after increasing of MMP-2 activity by Aβ in the hippocampus (directly or indirectly via increment of MAPKs or decrement of Akt/GSK-3β activity), treatment with CEPO-Fc may directly cause an attenuated activation of MMP-2 and/or indirectly through alteration in MAPKs and/or Akt/GSK-3β signaling. Then, the effect of CEPO-Fc on MMP-2 activity, in addition to its effect on MAPKs and Akt/GSK-3β signaling, might contribute to its anti-amnesic effect.

CONCLUSION

This study revealed for the first time that CEPO-Fc protects against Aβ-induced memory impairment in rat. Additionally, CEPO-Fc reversed the effect of $A\beta_{25-35}$ on hippocampal ERK, p38, Akt/GSk-3β and MMP-2 alterations. Although further studies are required to elucidate the neuroprotective mechanisms of CEPO-Fc, the present observations support the fact that CEPO-Fc could be considered as a potential protective agent for AD-related models of learning and memory loss and also for developing new neuroprotective strategies in human treatments.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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