

## RESEARCH PAPER

# Activating mitochondrial function and haemoglobin expression with EH-201, an inducer of erythropoietin in neuronal cells, reverses memory impairment

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### Received

28 February 2015

### Revised

29 June 2015

### Accepted

2 July 2015

## BACKGROUND AND PURPOSE

Memory impairment can be progressive in neurodegenerative diseases, and physiological ageing or brain injury, mitochondrial dysfunction and oxidative stress are critical components of these issues. An early clinical study has demonstrated cognitive improvement during erythropoietin treatment in patients with chronic renal failure. As erythropoietin cannot freely cross the blood–brain barrier, we tested EH-201 (2,3,5,4'-tetrahydroxystilbene-2-O- $\beta$ -d-glucoside, also known as TSG), a low MW inducer of erythropoietin, for its therapeutic effects on memory impairment in models of neurodegenerative diseases, physiological ageing or brain injury.

## EXPERIMENTAL APPROACH

The effects of EH-201 were investigated in astrocytes and PC12 neuronal-like cells. *In vivo*, we used sleep-deprived (SD) mice as a stress model, amyloid- $\beta$  (A $\beta$ )-injected mice as a physiological ageing model and kainic acid (KA)-injected mice as a brain damage model to assess the therapeutic effects of EH-201.

## KEY RESULTS

EH-201 induced expression of erythropoietin, PPAR- $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) and haemoglobin in astrocytes and PC12 neuronal-like cells. *In vivo*, EH-201 treatment restored memory impairment, as assessed by the passive avoidance test, in SD, A $\beta$  and KA mouse models. In the hippocampus of mice given EH-201 in their diet, levels of erythropoietin, PGC-1 $\alpha$  and haemoglobin were increased

## CONCLUSIONS AND IMPLICATIONS

The induction of endogenous erythropoietin in neuronal cells by inducers such as EH-201 might be a therapeutic strategy for memory impairment in neurodegenerative disease, physiological ageing or traumatic brain injury.

## Abbreviations

A $\beta$ , amyloid- $\beta$ ; APP, amyloid precursor protein; EH-201 (TSG, THSG, PMSG); 2,3,5,4'-tetrahydroxystilbene-2-O- $\beta$ -d-glucoside; EPO, erythropoietin; rhEPO, recombinant human EPO; HO-1, haem oxygenase-1; KA, kainic acid; PGC-1 $\alpha$ , PPAR- $\gamma$  coactivator 1 $\alpha$ ; ROS, reactive oxygen species; SD, sleep-deprived

## Tables of Links

TARGETS
<b>Enzymes</b>
HO-1, haem oxygenase-1

LIGANDS
EPO, erythropoietin
KA, kainic acid

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

## Introduction

Memory impairment can be progressive in neurodegenerative diseases and physiological ageing, or immediate in brain injury. Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), are a heterogeneous group of disorders characterized by a progressive and selective loss of memory (Huber and Paulson, 1987; Walsh and Selkoe, 2004; Weintraub *et al.*, 2004). Memory also declines with advancing age (Craik and Rose, 2012) or after traumatic brain injury (Vakil, 2005). Regardless of its heterogeneity, mitochondrial dysfunction and oxidative stress are critical components, common to these conditions (Cui *et al.*, 2006; Manczak *et al.*, 2006; Schapira, 2008). Sleep has been implicated in the plastic cerebral changes that underlie learning and memory (Maquet, 2001). Sleep deprivation (SD) also results in memory loss (Chee and Chuah, 2008) and impaired mitochondrial activity (Andreazza *et al.*, 2010). Memory loss is accompanied by the accumulation of oxidative damage to lipids, proteins and nucleic acids and by mitochondrial decay, all of which can disrupt neuronal function in ageing and disease (Harman, 1992). Increased brain oxidative stress is widely associated with cognitive impairment caused by normal ageing and neurodegenerative diseases (Silva *et al.*, 2004). Patients with dementias, such as AD, often have nocturnally disrupted sleep (Ismail *et al.*, 2009). Diverse evidence suggests that amyloid- $\beta$  (A $\beta$ ) peptides have a causal role in the pathogenesis of these diseases (Palop and Mucke, 2010). Mitochondria are a direct site of A $\beta$  accumulation in AD neurons, and mitochondrial dysfunction is a hallmark of AD (Manczak *et al.*, 2006; Pagani and Eckert, 2011). Haemoglobin is the main oxygen transporter in erythrocytes. Recently, haemoglobin was unexpectedly found in many non-hematopoietic cells, and it is possible that it facilitates tissue oxygen transport or increases cellular oxygenation and therefore provides an intrinsic protective mechanism against hypoxic/ischaemic injury (Nishi *et al.*, 2008; Biagioli *et al.*, 2009; Tezel *et al.*, 2010).

Recent studies implicate excitotoxicity in a variety of neuropathological conditions. Excitotoxicity is involved in a variety of neurodegenerative diseases such as AD, PD and HD

(Mehta *et al.*, 2013). Kainic acid (KA), an analogue of excitotoxic glutamate, is 30-fold more potent as a neurotoxin than glutamate, and KA-mediated excitotoxicity is used as a model for neurodegeneration. Administration of KA can induce a Ca<sup>2+</sup> influx, production of reactive oxygen species (ROS), mitochondrial dysfunction and apoptosis in the neurons of the brain (Wang *et al.*, 2005; Zhang and Zhu, 2011).

Erythropoietin (EPO), originally characterized as a hematopoietic cytokine produced in the kidney (Goldwasser, 1975), has been shown to have many neuroprotective effects in the CNS (van der Kooij *et al.*, 2008). Both EPO and the EPO receptor are expressed in neurons and astrocytes, and EPO is produced primarily by astrocytes in the brain (Juul *et al.*, 1999). Recombinant human EPO (rhEPO) is widely used to enhance erythropoiesis in patients with anaemia and has recently been found to have many non-hematopoietic beneficial effects, including cardioprotection (Parsa *et al.*, 2003) and neuroprotection (Digicaylioglu and Lipton, 2001). An early clinical study has demonstrated cognitive improvement during EPO treatment among patients with chronic renal failure (Pickett *et al.*, 1999). Recently, studies have shown that high-dose EPO treatments improve hippocampal plasticity and cognitive performance in patients suffering from neuropsychiatric diseases (Ehrenreich *et al.*, 2007). However, rhEPO, given in low doses to treat anaemia or in high doses for tissue protection, increased mortality (Lund *et al.*, 2014). In patients with ischaemic stroke, administration of only three high doses of rhEPO caused an almost twofold increase in the overall death rate (Ehrenreich *et al.*, 2009). Therefore, the development of an inducer of this tissue-protective, endogenous EPO signalling which lacks the adverse side effects of systemic exogenous EPO is necessary.

Radix *Polygoni multiflori* is a traditional Chinese medicine for the treatment of anaemia, liver disease and other diseases commonly associated with ageing (Huang *et al.*, 2007). 2,3,5,4'-Tetrahydroxystilbene-2-O- $\beta$ -d-glucoside (TSG) is one of the active compounds extracted from the root of *Radix Polygoni multiflori* and this compound improved learning memory in both the AD model of amyloid precursor protein (APP) transgenic mice and aged rats, and was protective against cerebral ischaemic injury both *in vivo* and *in vitro* (Zhang *et al.*, 2006; Wang *et al.*, 2007). Recently, TSG has been

shown to exhibit a protective effect against APP<sup>+</sup>-induced cell death (Sun *et al.*, 2011). Although reports demonstrate that TSG has neuroprotective effects in neurodegenerative disorders, which are attributed to its antioxidant and free radical-scavenging properties, the pharmacological mechanism underlying these effects of TSG remains unknown. We have referred to TSG in an earlier study (Hsu *et al.*, 2013) and here as EH-201. In the earlier study we showed EH-201 to be a potent inducer of EPO in non-haematopoietic cells. Also EH-201 showed a therapeutic effect, instead of only a preventive effect, after cisplatin-induced nephropathy or doxorubicin-induced cardiomyopathy was established in mice (Hsu *et al.*, 2013). In the present study, we investigated the hypothesis that EPO may play a pivotal role in the treatment of the induced impairment of hippocampal learning and memory, by modulating downstream mitochondrial regulator expression as well as inducing haemoglobin, in neuronal cells. The clinical use of EPO targeting the CNS has been limited by its lack of permeation through the blood–brain barrier (BBB) (Lieutaud *et al.*, 2008). This means that, in order to attain neuroprotective levels in the CNS, systemic high-dose rhEPO would have to be given, with the consequent unacceptable side effects (Juul *et al.*, 2004).

As an alternative, we used EH-201 to induce EPO in neuronal cells and found EPO-mediated activation of mitochondrial function through PPAR- $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) and haemoglobin- $\beta$  expression in these cells. We also were able to show, *in vivo*, recovery of memory impairment in SD, A $\beta$  and KA mouse models after treatment with dietary EH-201. Therefore, our results suggest that the induction of EPO in neuronal cells might be a therapeutic strategy for memory impairment in neurodegenerative diseases or physiological ageing.

## Methods

### Animals

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques, if available. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 110 animals were used in the experiments described here.

Eight to 12-week-old specific pathogen-free C57BL/6J mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The animals were housed five to six per cage at a constant temperature of 22  $\pm$  2°C, with a 12 h light–dark cycle. Rodent chow (PMI, Brentwood, MO, USA) and water were provided *ad libitum*.

### Cell culture

Astrocyte-enriched cultures were prepared from 1-day-old C57BL/6J mice obtained from the Animal Center at the National Yang-Ming University. Briefly, cortical tissue was digested with trypsin, and the resulting dissociated cells were

suspended in DMEM containing 10% FBS (Life Technologies, Grand Island, NY, USA) and incubated in a 10 cm dish for 7–8 days before use (Chou *et al.*, 2003). Cells prepared by this method consisted of approximately 90–95% astrocytes as determined by immunochemical staining with an antibody against glial fibrillary acidic protein, a specific marker for astrocytes. Rat pheochromocytoma (PC12; PATCC, Manassas, VA, USA) cells were maintained in RPMI 1640 medium containing 5% FBS and 10% horse serum. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### RNA isolation and real-time quantitative PCR

Total RNA was extracted using the RNA-Bee™ reagent (Tel-Test, Friendswood, TX, USA) and reverse transcribed by AMV-RT (Promega, Madison, WI, USA). EPO, PGC-1 $\alpha$ , haemoglobin- $\beta$ , haem oxygenase-1 (HO-1) and GAPDH mRNA expression was quantified by real-time PCR with an ABI Prism 7500 Sequence Detection System using SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA). All data were normalized to GAPDH. The relative expression levels were calculated by the 2<sup>- $\Delta\Delta$ CT</sup> method. All primer sequences are listed in Supporting Information Table S1.

### Western blot

Cell and brain tissue lysates were prepared using a radioimmunoprecipitation assay lysis buffer. Approximately 20  $\mu$ g of protein was loaded, and Western blot analysis was performed using antibodies against EPO, PGC-1 $\alpha$ , haemoglobin- $\beta$  (Santa Cruz, CA, USA) and GAPDH (Abcam, Cambridge, UK). A HRP-conjugated anti-IgG secondary antibody was used for enhanced chemiluminescence detection (Amersham, Buckinghamshire, UK).

### Succinate dehydrogenase assay

Astrocytes or PC12 cells were plated at 10<sup>4</sup> cells per well in 96-well plates. Twenty-four hours later, the cells were incubated with or without EPO or EH-201-containing media (100  $\mu$ l per well) for 48 h. Succinate dehydrogenase activity was determined by an MTT reduction assay. The activity was normalized to the cellular protein level with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA), and changes in absorbance were measured using a microplate reader (PerkinElmer Life Sciences Wallac Victor2; PerkinElmer, Waltham, MA, USA). Activity was expressed relative to the control condition.

### Intracellular ROS generation

Astrocytes and PC12 cells were treated with EPO or EH-201 for 24 h. The culture medium was replaced with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and cells were incubated for 6 h (astrocytes) or 30 min (PC12 cells). ROS production in cells was then measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA). Cells were incubated with 50  $\mu$ M DCFH-DA at 37°C for 30 min, and fluorescence intensity was measured using a fluorescence microplate reader (PerkinElmer Life Sciences Wallac Victor2).

### H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in astrocytes and neuron-like PC12 cells

Astrocytes and PC12 cells were treated with EPO or EH-201 for 24 h. Astrocyte culture medium was replaced with 500  $\mu$ M

H<sub>2</sub>O<sub>2</sub>, and the cells were incubated for 6 h. PC12 cell culture medium was replaced with 250 µM H<sub>2</sub>O<sub>2</sub>, and the cells were incubated for 4 h. Cell viability was determined by the exclusion of Trypan blue as assessed by light microscopy.

### SD procedure

The method of SD has been previously described (Vecsey *et al.*, 2009). Briefly, 40 C57BL/6J male mice (12 weeks of age) were housed on a 12/12 h light/dark schedule with lights on at 0600 h and were handled for 7 days. The mice were trained to complete the passive avoidance test, and the animals that did not master this task were excluded from the experiments. Because short-term SD is a quickly reversible model of memory impairment, the qualified mice (29/40) were randomly divided into five groups: control ( $n = 5$ ) and SD-only groups ( $n = 6$ ) were fed *ad libitum* with a chow diet, SD + EH-201 groups were fed *ad libitum* with a chow diet containing different doses of EH-201 (50, 100 or 200 mg·kg<sup>-1</sup> per day,  $n = 6$  for each group) for 3 days before SD; preparation of the EH-201 diet is described later. The average food intake was 4 g chow per day for each mouse (average body weight was 25 g). The SD and SD + EH-201 groups of mice were sleep-deprived in their home cages for 5 h by gentle handling beginning at 0600 h, and control mice were left undisturbed (non-SD mice). After completing all behavioural tests, the mice were killed by i.p. injection of a lethal dose of sodium pentobarbital. The brains were removed and the hippocampus was isolated rapidly for RNA isolation and Western blot analysis.

### i.c.v. injection

i.c.v. injection has been described in a previous study (Park *et al.*, 2011). Briefly, C57BL/6J female mice (12 weeks of age) were anaesthetized by i.p. injection of sodium pentobarbital (50 mg·kg<sup>-1</sup>) and placed on a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The depth of anaesthesia was monitored by assessing the reaction to toe pinch and skin pinch tests and also by determining jaw tone. Each mouse was injected with KA or Aβ<sub>25-35</sub> at 1 mm lateral to the midline, 0.5 mm posterior to the bregma and 3.5 mm deep using a stereotaxic injector (KD Scientific, Holliston, MA, USA) in a total volume of 3 or 5 µL at a rate of 0.5 µL·min<sup>-1</sup>. The sham-operated groups were injected with PBS.

### KA injection model

Thirty-four C57BL/6J female mice (12 weeks of age) were subjected to KA injection ( $n = 28$ ), and the control group was injected with PBS ( $n = 6$ ). Briefly, KA (0.15 µg per 3 µL PBS; Sigma-Aldrich, St. Louis, MO, USA) was injected into the brain (i.c.v.) and induced epileptiform seizures (seizure score 3–4) (Racine, 1972). The KA-injected mice were randomly divided into four groups. Mice were fed *ad libitum* with a chow diet or a chow diet containing different concentrations of EH-201 (50, 100 or 200 mg·kg<sup>-1</sup>·day<sup>-1</sup>,  $n = 7$  for each group) beginning on day 6 after KA-induced memory impairment. KA intoxication resulted in long-term damage in the brain, which means a longer recovery period is required for this model, to evaluate the effect of EH-201. Learning and memory tests were performed on day 13 and day 20 after the operation. After 2 weeks of EH-201

treatment and completing all behavioural tests, the mice were killed by i.p. injection of a lethal dose of sodium pentobarbital. The brains were removed and the hippocampus was isolated rapidly for RNA isolation and Western blot analysis.

### Aβ<sub>25-35</sub> injection model

Thirty-six C57BL/6J male mice (8 weeks of age) were subjected to Aβ<sub>25-35</sub> injection ( $n = 31$ ), and the control group was injected with PBS ( $n = 5$ ). Briefly, Aβ<sub>25-35</sub> (AnaSpec, Fremont, CA, USA) was dissolved in sterile saline at a concentration of 2 µg·µL<sup>-1</sup> and aggregated by incubation at 37°C for 4 days before use (Maurice *et al.*, 1996). Aβ<sub>25-35</sub> (10 µg per 5 µL PBS) was injected into the brain (i.c.v.) to induce memory loss. After PBS or Aβ<sub>25-35</sub> injection, the mice were trained in the passive avoidance test and 7/31 mice were excluded from the experiments. The 24 qualified Aβ<sub>25-35</sub>-injected mice were randomly divided into four groups. The Aβ<sub>25-35</sub>-injected mice were fed *ad libitum* with a chow diet or a chow diet containing different concentrations of EH-201 (50, 100 or 200 mg·kg<sup>-1</sup>·day<sup>-1</sup>,  $n = 6$  for each group) beginning on day 10 after Aβ<sub>25-35</sub>-induced memory impairment. Aβ<sub>25-35</sub> intoxication resulted in long-term damage in the brain, which means longer recovery period is required for this model to evaluate the effect of EH-201. Learning and memory tests were performed on day 17 and day 24 after the operation. After 2 weeks of EH-201 treatment and completing all behavioural tests, the mice were killed by i.p. injection of a lethal dose of sodium pentobarbital. The brains were removed and the hippocampus was isolated rapidly for RNA isolation and Western blot analysis.

### Passive avoidance task

Passive avoidance experiments were conducted as previously described with minor modifications (Jarvik and Kopp, 1967). A two-way shuttle box with a guillotine door placed between the modular testing chambers was employed. One chamber was illuminated with a 40 W bulb, whereas the other chamber remained dark. In the training session, the animals were individually placed in the illuminated chamber that faced away from the guillotine door. When the animal entered the darkened chamber, the door was silently lowered and a 0.5 mA foot shock was applied for 2 s through the grid floor. In the test sessions, the animals were again placed in the illuminated chamber, but no foot shock was applied. Latency to step through was recorded in each session.

### Data analysis

All results are expressed as the mean and SD. The significance of differences of means between more than two groups was determined using one-way ANOVA followed by Tukey's *post hoc* test. Student's *t*-test was employed for the statistical comparison of paired samples. A *P*-value of <0.05 was considered statistically significant.

**Materials.** EH-201 was purified from *Polygonum multiflorum* Thunb., as described earlier (Hsu *et al.*, 2013) to 99.7% purity. Chow diet containing EH201 was made in our laboratory. Briefly, we crushed Purina chow diet and then mixed it with

solutions of EH-201 to provide the appropriate content of EH-201, and reformed to the format of original Purina chow diet by lyophilization. EPO was supplied by Roche (Mannheim, Germany).

## Results

### *EPO stimulated expression of the mitochondrial regulator PGC-1 $\alpha$ and increased mitochondrial activity in primary astrocytes and neuron-like PC12 cells*

Because EPO has been reported to activate mitochondrial biogenesis (Carraway *et al.*, 2010) and haemoglobin expression (Tezel *et al.*, 2010) in non-haematopoietic cells, we assessed the effects of EPO on primary astrocytes and PC12 cells. Real-time PCR analysis showed that rhEPO treatment for 24 h increased mRNA expression of the mitochondrial regulator PGC-1 $\alpha$  in primary astrocytes (Figure 1A) and PC12 cells (Figure 1C). rhEPO also induced mitochondrial activity in both astrocytes (Figure 1B) and PC12 cells (Figure 1D).

### *EH-201 is a neuronal EPO inducer that elevated the expression of the mitochondrial regulator PGC-1 $\alpha$ and increased mitochondrial activity in primary astrocytes and neuron-like PC12 cells*

We tested the ability of EH-201 to induce EPO expression. After treatment for 24 h, EH-201 increased EPO mRNA expression in primary astrocytes and PC12 cells (Figure 1E and G). The intracellular EPO protein expression in astrocytes and PC12 cells was up-regulated during EH-201 treatment (Figure 1F and H). Next, we examined whether EH-201 increased mitochondrial activity through EPO-induced signalling. After treatment for 24 h, EH-201 induced PGC-1 $\alpha$  mRNA and protein expression in primary astrocytes (Figure 1I and J) and PC12 cells (Figure 1L). EH-201 also induced mitochondrial activity in both astrocytes (Figure 1K) and PC12 cells (Figure 1M). Furthermore, EPO neutralizing antibody treatment abolished the PGC-1 $\alpha$  mRNA and protein expression induced by EH-201 (Figure 1I and J). The results showed that PGC-1 $\alpha$  expression was induced by EH-201 via EPO-mediated signalling.

### *EH-201 elevated the expression of haemoglobin- $\beta$ in primary astrocytes and neuron-like PC12 cells*

Because PGC-1 $\alpha$  and haemoglobin are known to be mitochondrial regulators (St-Pierre *et al.*, 2006; Biagioli *et al.*, 2009), we analysed which form of haemoglobin was regulated by EPO or EH-201. Real-time PCR analysis showed that rhEPO treatment for 24 h increased haemoglobin- $\beta$  mRNA expression in primary astrocytes (Figure 2A) and PC12 cells (Figure 2D). After EH-201 treatment for 24 h, real-time PCR revealed that EH-201 elevated the expression of haemoglobin- $\beta$  mRNA in astrocytes (Figure 2B) and PC12 cells (Figure 2E). Haemoglobin- $\alpha$  expression, however, was not significantly changed (data not shown). Furthermore,

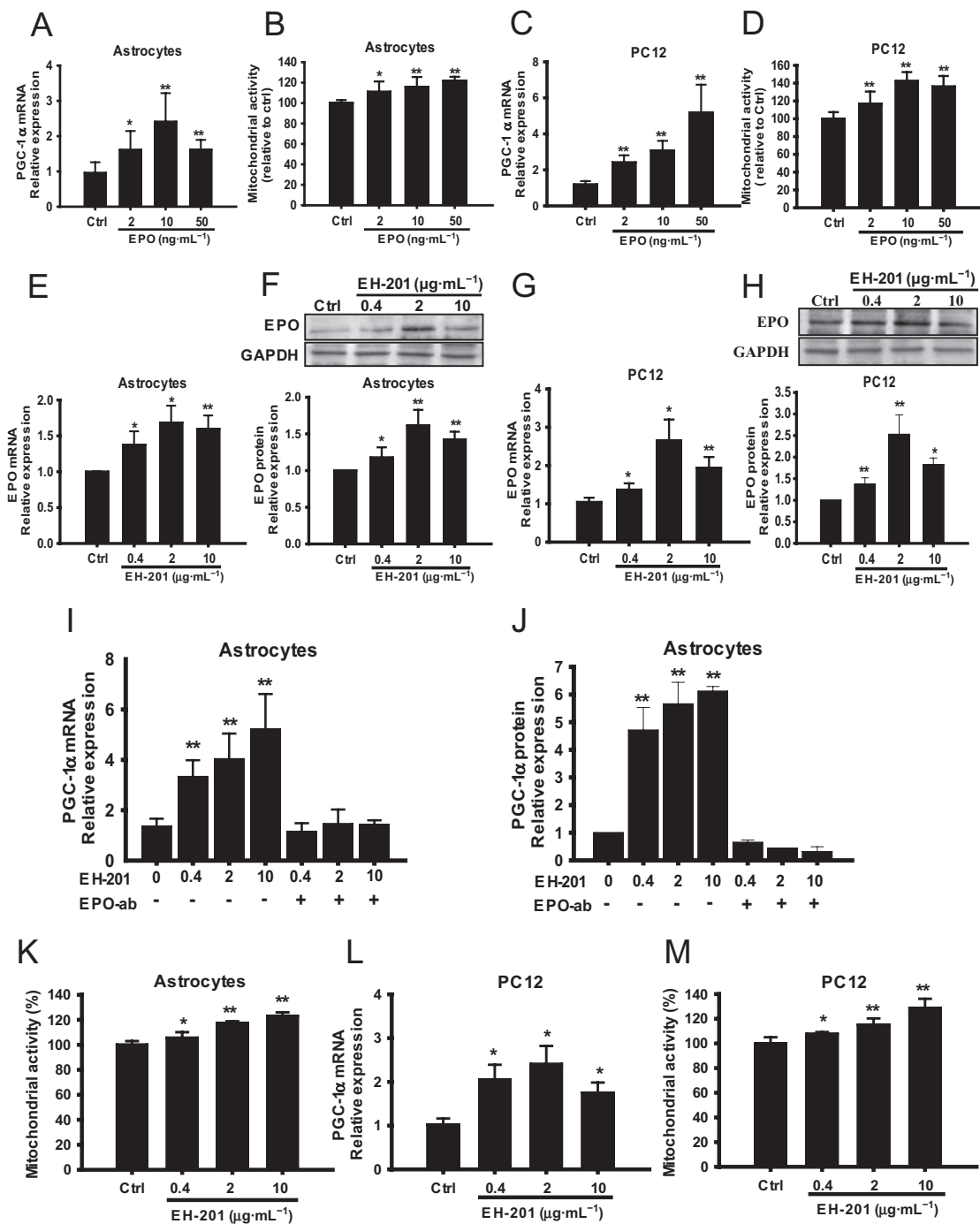
EPO neutralizing antibody treatment abolished the haemoglobin- $\beta$  mRNA expression induced by EH-201 (Figure 2B) in astrocytes. Additionally, we observed that EH-201 increased expression of all three forms of haemoglobin protein in astrocytes (Figure 2C) and PC12 cells (Figure 2F). The results showed that haemoglobin induction by EH-201 occurred via EPO-mediated signalling.

### *EPO is required for EH-201-mediated increased mitochondrial activity and attenuation of oxidative stress in primary astrocytes and neuron-like PC12 cells*

We evaluated whether the increased mitochondrial activity and the reduction in H<sub>2</sub>O<sub>2</sub>-induced ROS generation and cytotoxicity following treatment with EH-201 in astrocytes and PC12 cells were dependent on EPO. Real-time PCR analysis showed that rhEPO or EH-201 treatment for 24 h increased mRNA expression of the antioxidant gene HO-1 in primary astrocytes (Figure 3A and D) and PC12 cells (Figure 4A and D). Next, we estimated ROS generation in cultured cells after exposure to H<sub>2</sub>O<sub>2</sub> using an oxidative probe, CM-H<sub>2</sub>DCFDA. rhEPO or EH-201 treatment decreased intracellular ROS in astrocytes (Figure 3B and E) and PC12 cells (Figure 4B and E). rhEPO or EH-201 also decreased cell toxicity in cells exposed to H<sub>2</sub>O<sub>2</sub> in astrocytes (Figure 3C and F) and PC12 cells (Figure 4C and F). The reduction of ROS generation induced by H<sub>2</sub>O<sub>2</sub> in astrocytes and PC12 cells treated with EH-201 was abolished when cells were co-incubated with an anti-EPO antibody (Figure 3G, 4G). The anti-EPO antibody also inhibited the EH-201-mediated reduction in H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity (Figure 3H, 4H). The increased mitochondrial activity observed with EH-201 treatment was blocked in the presence of an anti-EPO antibody, as measured by an MTT assay (Figure 3I, 4I). These results show that EPO is required for the increased mitochondrial activity and attenuation of oxidative stress induced by EH-201.

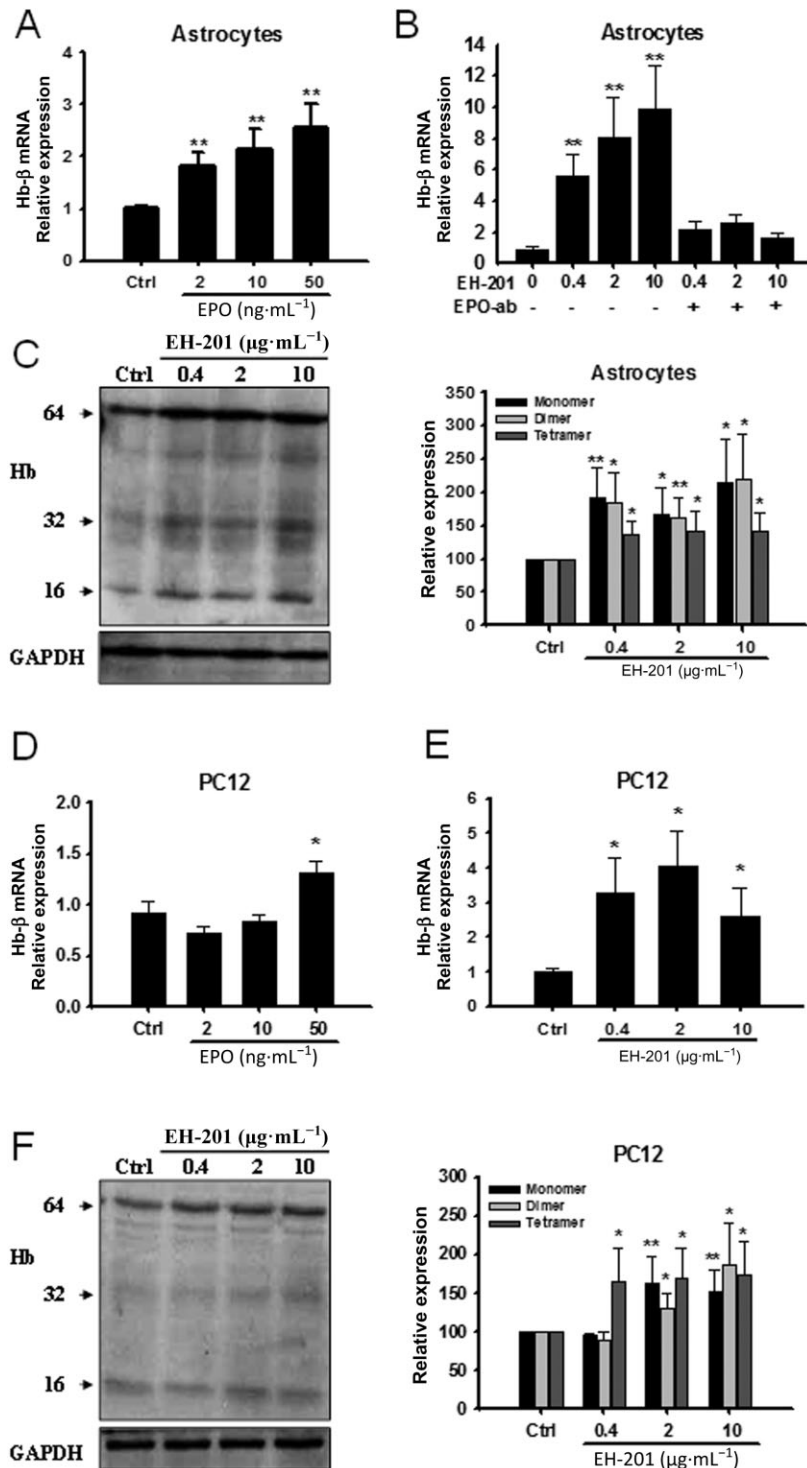
### *Effects of EH-201 on memory impairment induced by SD*

We next evaluated the neuroprotective effect of EH-201 on memory using the model of SD mice. The experimental procedure is outlined in Figure 5A. We analysed EPO expression in the hippocampus from each treated animal. Real-time PCR and Western blotting showed an increase in EPO expression in animals fed with a diet containing EH-201 (Figure 5B and D). Haemoglobin- $\beta$ , HO-1 and PGC-1 $\alpha$  mRNA expression in the hippocampus were also analysed by real-time PCR (Figure 5E). We further evaluated the mitochondrial succinate dehydrogenase activity using an MTT assay (Figure 5F). In the passive avoidance test, mice fed EH-201 for 3 days did not exhibit a difference in the ability to learn (Figure 5G). However, there was a significant improvement in memory performance of EH-201-fed mice after SD in the passive avoidance test (Figure 5H). Interestingly, the gene expression changes and the increased mitochondrial activity in the hippocampi from mice fed with EH-201, especially at doses of 100 and 200 mg·kg<sup>-1</sup>, correlated with the passive avoidance test.



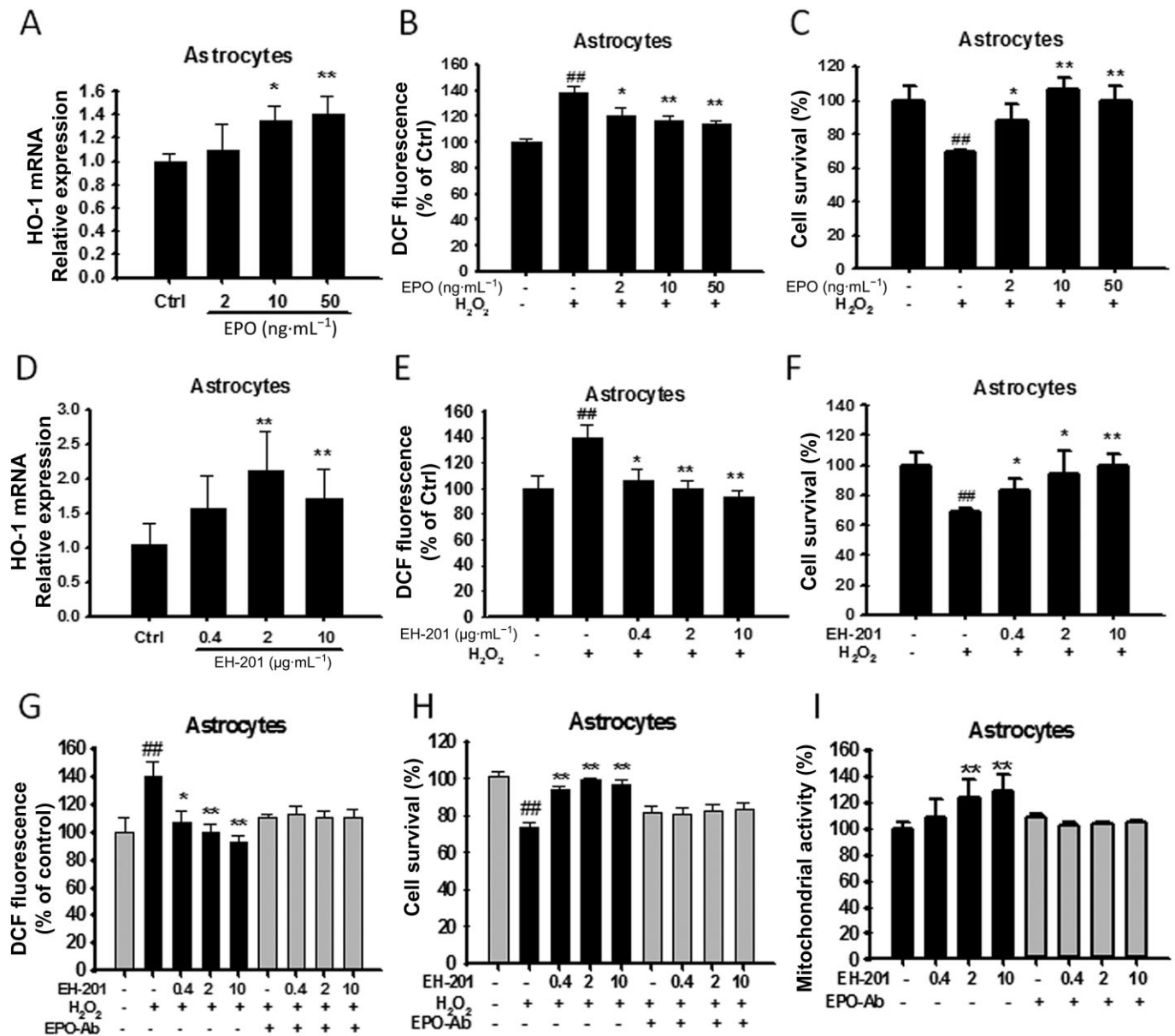
**Figure 1**

EPO and EPO inducer EH-201 stimulate mitochondrial activity in primary astrocytes and neuron-like PC12 cells. (A, C) EPO treatment for 24 h increased PGC-1 $\alpha$  mRNA expression in primary astrocytes and PC12 cells. The expression of GAPDH was used as an internal control. The results are expressed as the relative index of untreated controls (means  $\pm$  SD) from at least three independent measurements. \* $P$  < 0.05, \*\* $P$  < 0.01; significantly different from control; one-way ANOVA followed by Tukey's multiple comparison test. (B, D, K, M) Mitochondrial activity was determined by the succinate dehydrogenase activity of astrocytes and PC12 cells that were treated with rhEPO or EH-201 for 24 h using an MTT reduction assay and is expressed relative to the respective control conditions. The values indicate the means  $\pm$  SD ( $n$  = 8), \* $P$  < 0.05, \*\* $P$  < 0.01 compared with untreated controls; Student's  $t$ -test. (E, F) EH-201 treatment for 24 h increased EPO mRNA and protein expression in astrocytes and (G, H) PC12 cells. (I, J) EH-201-treated primary astrocytes with or without the neutralizing EPO antibody (nEPO-ab, 1  $\mu$ g·mL<sup>-1</sup>) were analysed for PGC-1 $\alpha$  mRNA and protein expression. (L) EH-201 treatment for 24 h increased PGC-1 $\alpha$  mRNA expression in PC12 cells. The expression of GAPDH was used as an internal control. The results are expressed as the relative index of untreated controls means  $\pm$  SD from at least three independent measurements. \* $P$  < 0.05, \*\* $P$  < 0.01, significantly different from control; by one-way ANOVA followed by Tukey's multiple comparison test.



## Figure 2

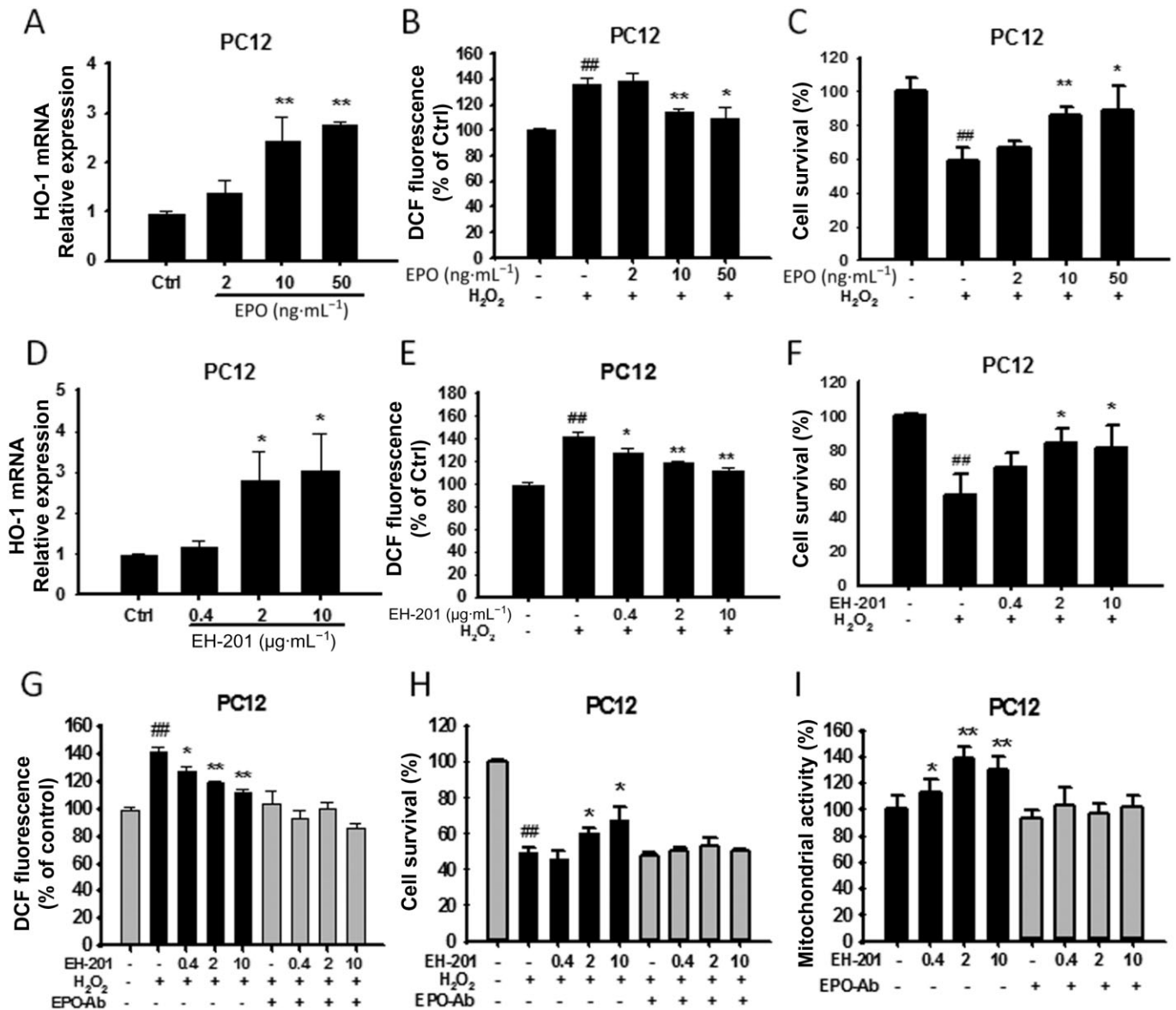
The induction of haemoglobin expression in primary astrocytes and neuron-like PC12 cells by EH-201 is mediated by EPO. (A, D) rhEPO treatment for 24 h increased haemoglobin-β mRNA expression in astrocytes and PC12 cells. (B) EH-201-treated primary astrocytes with or without the neutralizing EPO antibody (nEPO-ab, 1 μg·mL<sup>-1</sup>) were analysed for haemoglobin-β expression. (E) EH-201 treatment for 24 h increased haemoglobin-β mRNA expression in PC12 cells. The expression of GAPDH was used as an internal control. The results are expressed as the relative index of untreated controls means ± SD from at least three independent measurements. \**P* < 0.05, \*\**P* < 0.01; significantly different from control; one-way ANOVA followed by Tukey's multiple comparison test. (C, F) Different forms of haemoglobin (monomer: 16 kD, dimer: 32 kD, tetramer: 64 kD) expression identified by an haemoglobin-β Ab in primary astrocytes and PC12 cells treated with EH-201. The results are expressed as the relative expression of untreated controls ± SD from at least three independent measurements. \**P* < 0.05, \*\**P* < 0.01, significantly different from control; Student's *t*-test.



**Figure 3**

EH-201 stimulated antioxidant gene (HO-1) expression, decreased intracellular ROS and attenuated H<sub>2</sub>O<sub>2</sub>-induced cell toxicity in primary astrocytes. (A, D) EPO or EH-201 treatment for 24 h increased HO-1 mRNA expression in primary astrocytes. The expression of GAPDH was used as an internal control. The results are expressed as the relative index of untreated controls means ± SD of at least three independent measurements. \**P* < 0.05, \*\**P* < 0.01; compared with untreated controls; one-way ANOVA followed by Tukey's multiple comparison test. (B, E) Astrocytes treated with EPO or EH-201 for 24 h were exposed to 100 μM H<sub>2</sub>O<sub>2</sub> for 6 h. Intracellular ROS formation was measured using a DCFH-DA assay. The graph shows the results in relative percentage of fluorescence units to the control. The values indicate the means ± SD (*n* = 8). (C, F) Astrocytes treated with EPO or EH-201 for 24 h were exposed to 500 μM H<sub>2</sub>O<sub>2</sub> for 6 h. Cell survival was analysed with Trypan blue staining. The values indicate the means ± SD (*n* = 3). (G) Co-incubation of EH-201 with an anti-EPO antibody for 24 h resulted in the loss of the EH-201-mediated reduced ROS generation induced by H<sub>2</sub>O<sub>2</sub>, as assessed by a DCFH-DA assay (*n* = 8), and (H) reduced H<sub>2</sub>O<sub>2</sub>-mediated cytotoxicity, as assessed by Trypan blue staining (*n* = 3). The values indicate the means ± SD, ##*P* < 0.01 compared with untreated controls; \**P* < 0.05, \*\**P* < 0.01, compared with H<sub>2</sub>O<sub>2</sub> controls. (I) Co-incubation of EH-201 with an anti-EPO antibody resulted in the loss of the EH-201-induced increase in succinate dehydrogenase activity, as assessed by an MTT reduction assay. The values indicate the means ± SD (*n* = 8), \**P* < 0.05, \*\**P* < 0.01 compared with controls; Student's *t*-test.





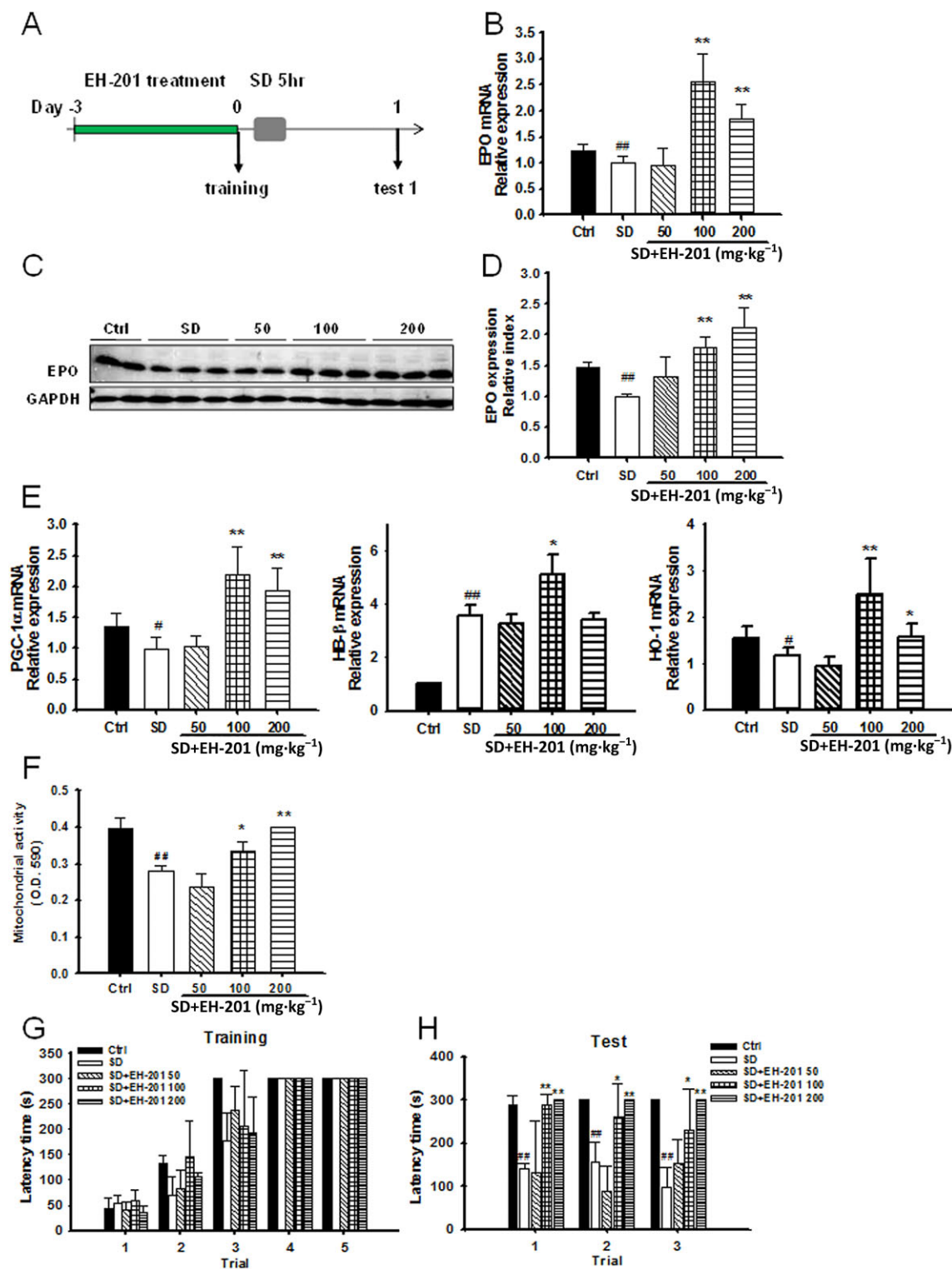
**Figure 4**

EH-201 stimulated antioxidant gene (HO-1) expression, decreased intracellular ROS and attenuated H<sub>2</sub>O<sub>2</sub>-induced cell toxicity in neuron-like PC12 cells. (A, D) EPO or EH-201 treatment for 24 h increased HO-1 mRNA in PC12 cells. The expression of GAPDH was used as an internal control. The results are expressed as the relative index of untreated controls  $\pm$  SD of at least three independent measurements. \* $P$  < 0.05, \*\* $P$  < 0.01, compared with untreated controls; one-way ANOVA followed by Tukey's multiple comparison test. (B, E) PC12 cells treated with EPO or EH-201 for 24 h were exposed to 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 6 h. Intracellular ROS formation was measured using a DCFH-DA assay. The graph shows results in relative percentage of fluorescence units to the control. The values indicate the means  $\pm$  SD ( $n$  = 8). (C, F) PC12 cells treated with EPO or EH-201 for 24 h were exposed to 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 6 h. Cell survival was analysed with Trypan blue staining. The values indicate the means  $\pm$  SD ( $n$  = 3). (G) Co-incubation of EH-201 with an anti-EPO antibody for 24 h resulted in the loss of the EH-201-mediated reduction in ROS generation induced by H<sub>2</sub>O<sub>2</sub>, as assessed by a DCFH-DA assay ( $n$  = 8), and (H) reduced H<sub>2</sub>O<sub>2</sub>-mediated cytotoxicity, as assessed by Trypan blue staining ( $n$  = 3). The values indicate the means  $\pm$  SD, ## $P$  < 0.01 compared with untreated controls; \* $P$  < 0.05, \*\* $P$  < 0.01, compared with H<sub>2</sub>O<sub>2</sub> controls. (I) Co-incubation of EH-201 with an anti-EPO antibody resulted in the loss of the EH-201-induced increase in succinate dehydrogenase activity, as assessed by an MTT reduction assay. The values indicate the means  $\pm$  SD ( $n$  = 8), \* $P$  < 0.05, \*\* $P$  < 0.01, compared with control; Student's  $t$ -test.

### EH-201 improved KA-induced memory impairment in the behavioural test

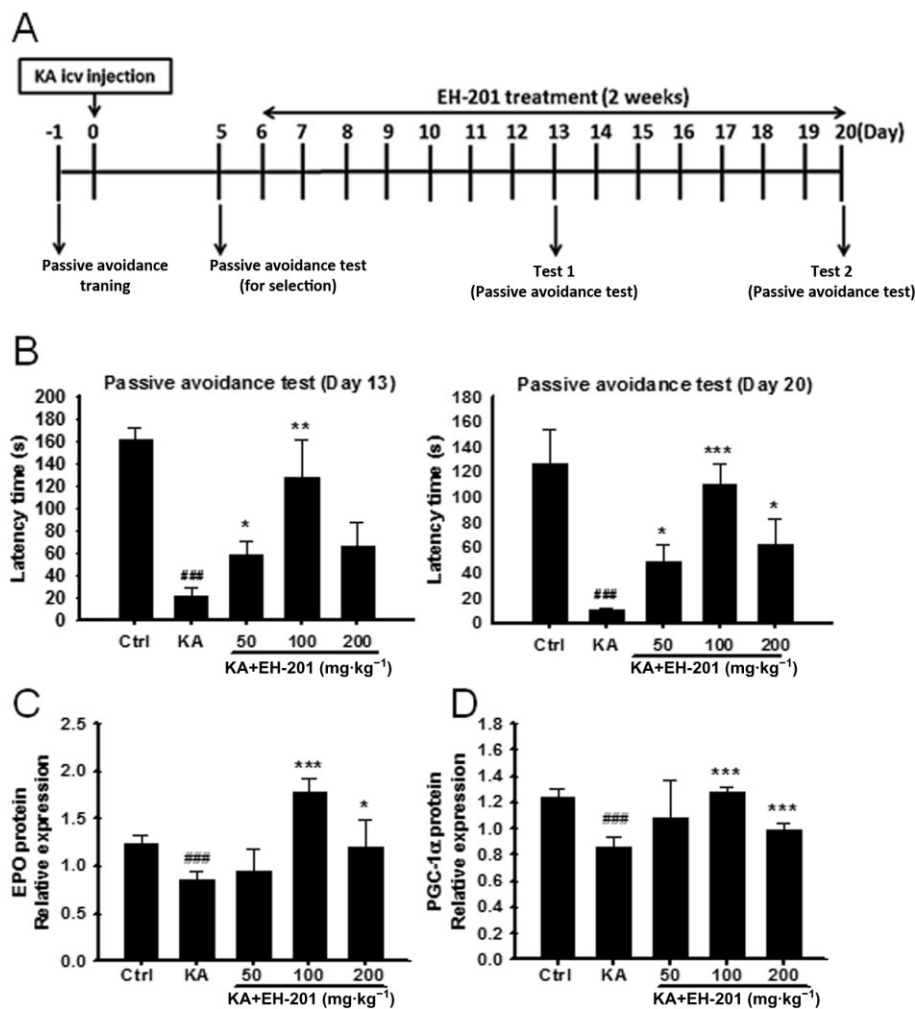
Memory loss was induced in KA-injected mice. The experimental procedure is outlined in Figure 6A. Injection of KA (0.15  $\mu$ g per mouse) in mice induced epileptiform seizures

(scoring 3–4). There was a significant improvement in the memory performance of mice in the passive avoidance test after EH-201 diet for 1 week (day 13) and 2 weeks (day 20) (Figure 6B). EH-201 also elevated the protein level of EPO and PGC-1 $\alpha$  expression in the hippocampus (Figure 6C and D).



**Figure 5**

Effects of EH-201 on SD-induced memory impairment. (A) Procedure of EH-201 treatment in SD mice. (B) Real-time PCR and (C, D) Western blot analysis of EPO expression in mouse hippocampus from each group. (E) Real-time PCR analysis of PGC-1 $\alpha$ , haemoglobin- $\beta$  and HO-1 expression levels in mouse hippocampus ( $n = 6$ ). (F) The MTT assay was used as a marker for mitochondrial activity. The values depict mitochondrial function after SD of untreated control mice and EH-201-treated mice. (G) Acquisition of step-through passive avoidance during five successive training trials in mice treated with or without EH-201. EH-201 treatment did not affect learning ability in mice. (H) Acquisition of step-through passive avoidance during three successive testing trials in mice treated with or without EH-201. The values indicate the means  $\pm$  SD, # $P < 0.05$ , ## $P < 0.01$ , compared with the control: \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the SD group; one-way ANOVA followed by Tukey's multiple comparison test.



## Figure 6

EH-201 ameliorated KA-induced memory impairment. (A) Experimental procedure of KA injection and EH-201 treatment. After KA-induced memory impairment, mice were fed a EH-201 diet on days 6–20. (B) The memory performance of KA-injected mice after EH-201 administration for 1 week (day 13) and 2 weeks (day 20) was measured using a passive avoidance task analysis. ( $n = 6–7$ ). (C, D) EH-201 improved the protein expression of EPO and PGC-1 $\alpha$  in the hippocampus ( $n = 5$ ). The values indicate the means  $\pm$  SEM, ### $P < 0.001$  compared with the control group; \* $P < 0.05$ , \*\* $P < 0.05$  and \*\*\* $P < 0.001$  compared with the KA-injected only group; Student's  $t$ -test.

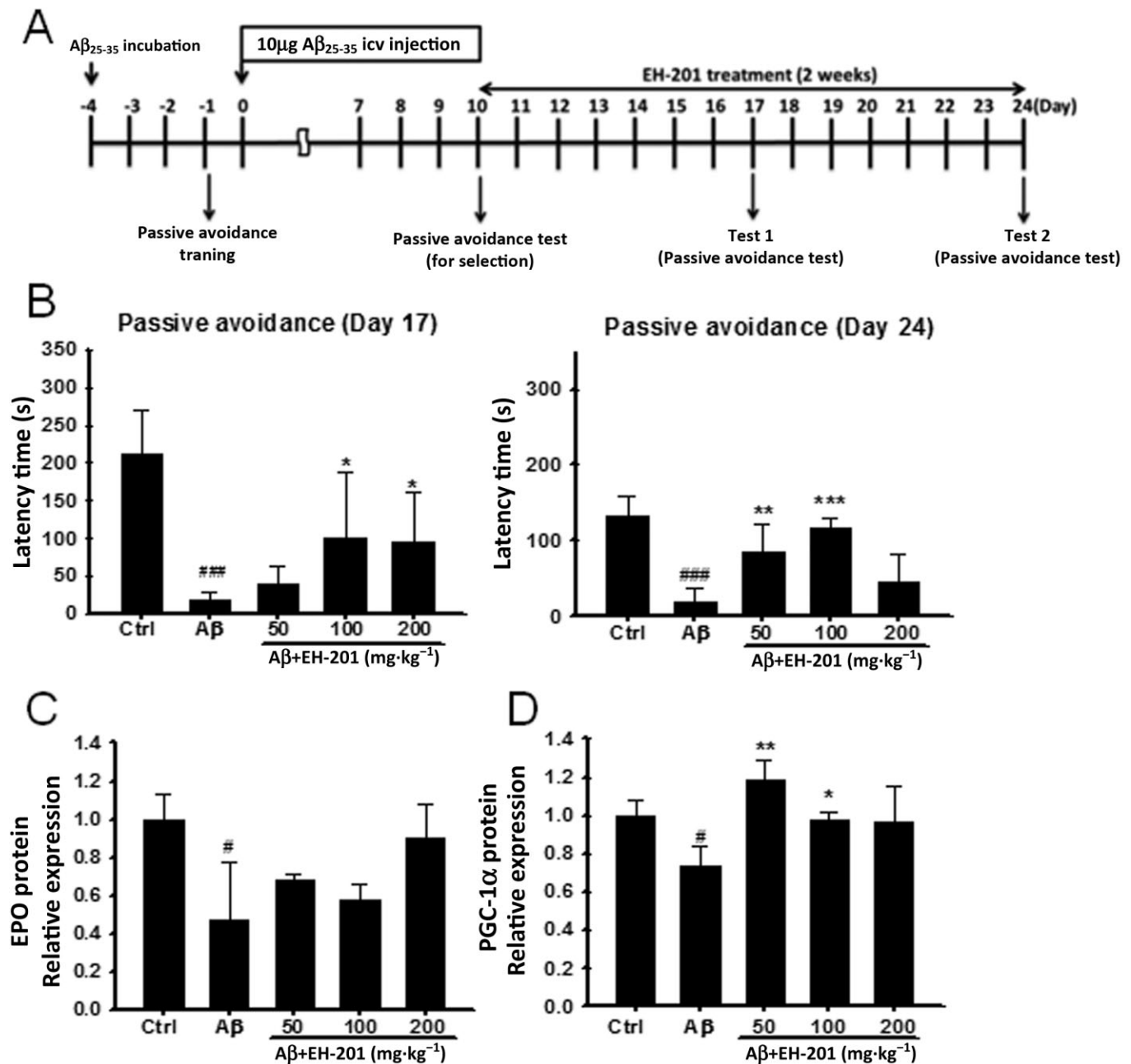
EH-201 improved memory performance after i.c.v. injection of KA in mice.

### *EH-201 improved $A\beta_{25-35}$ -induced memory impairment in the behavioural test*

Memory loss was induced in mice injected with  $A\beta_{25-35}$ . The experimental procedure is outlined in Figure 7A. There was a significant improvement in the memory performance of mice in the passive avoidance test after feeding the EH-201 diet for 1 week (day 17) and 2 weeks (day 24) (Figure 7B). EH-201 also elevated the level of EPO and PGC-1 $\alpha$  protein expression in the hippocampus (Figure 7C and D). EH-201 improved memory performance after i.c.v. injection of  $A\beta_{25-35}$  in mice.

## Discussion and conclusions

rhEPO has recently emerged as a highly attractive candidate for neuroprotection in both schizophrenia (Ehrenreich *et al.*, 2007) and stroke patients (Ehrenreich *et al.*, 2002). However, a significant challenge for future clinical use of EPO in CNS diseases is that EPO cannot easily cross the BBB (Lieutaud *et al.*, 2008). The use of relatively high doses of exogenous rhEPO has the potential to increase thrombosis and the viscosity of the blood with potentially fatal results (Shaskey and Green, 2000). The discovery that EPO and its receptor are expressed in the brain (Juul *et al.*, 1999) has led to the suggestion that EPO exerts a direct effect on the CNS, independent of haematopoiesis. Exogenous administration of EPO in



**Figure 7**

EH-201 improved memory performance after i.c.v. injection of  $A\beta_{25-35}$  in mice. (A) Experimental procedure of  $A\beta_{25-35}$  injection and EH-201 treatment. After  $A\beta_{25-35}$ -induced memory impairment, mice were fed EH-201 on days 10–24. (B) The memory performance of  $A\beta_{25-35}$ -injected mice after EH-201 administration for 1 week (day 17) and 2 weeks (day 24) was measured using a passive avoidance task analysis ( $n = 5-6$ ). (C, D) EH-201 improved the protein expression of EPO and PGC-1 $\alpha$  in the hippocampus ( $n = 4-5$ ). The values indicate the means  $\pm$  SEM, # $P < 0.05$ , compared with the control group; \* $P < 0.05$ , \*\* $P < 0.05$ , \*\*\* $P < 0.001$ , compared with the  $A\beta_{25-35}$ -injected only group; Student's  $t$ -test.

rodents has been shown to rescue hippocampal CA1 neurons from lethal ischaemic damage and to prevent ischaemia-induced learning disabilities (Sakanaka *et al.*, 1998) as well as providing neuroprotective effects against glutamate neurotoxicity (Morishita *et al.*, 1997) and traumatic brain (Cherian *et al.*, 2007) and spinal cord injury (Celik *et al.*, 2002). EPO treatment enhances hippocampal long-term potentiation by modulating the plasticity, synaptic connectivity and activity

of memory-related neuronal networks (Adamcio *et al.*, 2008) and improves the operant conditioning stability of cognitive performance in healthy mice (El-Kordi *et al.*, 2009). However, it has been reported that hippocampal neurons with EPO receptors respond to EPO in a limited concentration range and that high concentrations of EPO induced a rapid down-regulation of EPO receptors, resulting in a failure to transmit EPO-mediated signals to the neurons (Sakanaka *et al.*, 1998).

EPO treatment has been shown to activate cardiac mitochondrial biogenesis through up-regulation of PGC-1 $\alpha$  (Carraway *et al.*, 2010), which is a transcriptional co-activator that has been identified as an inducible regulator of mitochondrial biogenesis and function (Lin *et al.*, 2005). Haemoglobin is the main oxygen carrier in erythrocytes. Unexpectedly, haemoglobin has been found in non-haematopoietic cells, such as neurons and glial cells, which may facilitate tissue oxygen transport or increase cellular oxygenation, thus linking haemoglobin expression and mitochondrial activity (Biagioli *et al.*, 2009). The monomer/dimer form of haemoglobin has a higher affinity for oxygen than the conventional tetrameric form (Marden *et al.*, 1995; Richter *et al.*, 2009). Thus, monomeric/dimeric haemoglobin expression may play an active role in facilitating oxygen extraction from capillaries as well as improving the inter- and intra-cellular oxygen transport that are associated with mitochondrial biogenesis to improve tissue oxygenation and energy production. A study also showed that hypoxia-induced, glial EPO-dependent expression of glial haemoglobin aided in free radical scavenging and NO detoxification; this intrinsic protective mechanism against hypoxic/oxidative injury may have important implications in glaucomatous neurodegeneration (Tezel *et al.*, 2010). Haemoglobin can also protect different cell types from H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and associated cell damage (Widmer *et al.*, 2010). However, the neuroprotective doses of peripherally administered rhEPO are much higher than those needed for stimulation of haematopoiesis, and have been demonstrated to increase morbidity and mortality (Ehrenreich *et al.*, 2009; Lund *et al.*, 2014).

In our previous study, EH-201 acted as an EPO inducer via enhancement of EPO and EPO receptor binding to activate mitochondrial function and haemoglobin expression in non-hematopoietic cells, such as cardiomyocytes, liver cells, kidney cells and bone marrow cells (Hsu *et al.*, 2013). In this study, we have suggested that EH-201 is a potent inducer of endogenous EPO in the brain that regulates mitochondrial biogenesis and haemoglobin expression in neuronal cells. Our results showed memory recovery in A $\beta_{25-35}$ -induced AD mice, KA-damaged mice and SD mice, after treatment with dietary EH-201, compared with the mice fed the control diet. There was a significant increase in the expression of EPO and its downstream genes in the EH-201-treated group in the SD model. Mitochondrial succinate dehydrogenase activity in the hippocampus was also improved in the EH-201-fed group. Also, EH-201 elevated the expression of EPO, resulting in PGC-1 $\alpha$  expression and haemoglobin expression in the hippocampus of the A $\beta_{25-35}$ -induced AD mice and the KA-injected excitotoxicity model. In addition, HO-1, an antioxidant protein, the expression of which is induced by EPO, attenuated inflammatory or oxidative stress in the mouse brain (Chen *et al.*, 2010; Sifringer *et al.*, 2010). Our results showed that EH-201, an EPO inducer, provided neuroprotective effects, most likely through the up-regulation of HO-1 in primary astrocytes and neuron-like PC12 cells, and thus leads to clearance of ROS and reduced neurotoxicity in the H<sub>2</sub>O<sub>2</sub>-induced cell stress model.

According to pharmacokinetics studies performed *in vivo* (Lv *et al.*, 2011; Zhao *et al.*, 2013), EH-201 (TSG in these reports) was absorbed rapidly and distributed freely the heart,

kidney, liver and lung. Small amounts were retained in the brain, but these correlated with the effective concentrations. EH-201 was then excreted quickly after oral administration. Lv and co-workers performed a pharmacokinetic study to quantify EH-201 (PM-SG in this report) in mouse plasma after oral administration of 100 mg·kg<sup>-1</sup>, and their results showed a the maximum concentration ( $C_{max}$ ) and time to reach maximum concentration ( $T_{max}$ ) of 29.6  $\mu$ g·mL<sup>-1</sup> and 60 min respectively. Additionally, Zhao and co-workers have also performed pharmacokinetics and tissue distribution studies of EH-201 (TSG) in rats following a single oral (100 mg·kg<sup>-1</sup>) dose, and their results showed that brain contained about 33  $\mu$ g EH-201 per g tissue after 30 min of oral administration (100 mg·kg<sup>-1</sup>). Furthermore, Zhang *et al.* had detected the presence of EH-201 in both blood plasma and CSF in rabbits after administration by gavage, showing that that EH-201 could go through the BBB (Zhang *et al.*, 2006). Our *in vitro* studies showed that EH-201 in concentrations of 0.4–2.0  $\mu$ g·mL<sup>-1</sup> induced EPO expression in neuronal cells. Such concentrations might reasonably be attained *in vivo* in the CNS and provide a direct effect of EH-201 in the CNS. Therefore, it is highly likely that EH-201, a low MW compound, did cross the BBB and was effective in our three mouse models of memory impairment. Furthermore, EH-201 (THSG) improved blood flow and ameliorated vascular senescence in spontaneously hypertensive rats (SHRs) (Han *et al.*, 2012), without affecting blood pressure and body weight, after treatment with 50 mg·kg<sup>-1</sup>·day<sup>-1</sup> for 14 weeks. Moreover, we have data (obtained by another laboratory) showing a normal haematocrit after 3 months of dosing with EH-201, at up to 2.5 g·kg<sup>-1</sup> (data not shown). Thus, many of the toxic effects associated with exogenous rhEPO may be avoided by the use of and inducer such as EH-201.

Any new strategies to increase EPO expression in the brain will greatly broaden the clinical applications of EPO for the treatment of neurological diseases. The development of a stabilizer of the hypoxia-inducible factor (HIF) or an inhibitor of the HIF-specific prolyl hydroxylase (HIF-PH) has the potential to achieve this effect (Hsieh *et al.*, 2007). However, HIF transcription factors regulate a wide range of biological processes, and intermittent HIF activation over prolonged periods of time may lead to profound changes in cellular metabolism, growth and differentiation. Consequently, pharmacological activation of HIF could result in serious side effects (Haase, 2010). The long-term inactivation of HIF-PH and the subsequent long-term activation of HIF has been shown to contribute to the pathogenesis of ischaemic cardiomyopathy (Moslehi *et al.*, 2010). Moreover, in contrast to a HIF stabilizer that stimulates a high level of erythropoiesis, EH-201 treatment increased the numbers of red blood cells but these were still within the physiological range and involved a mechanism that did not produce HIF- $\alpha$  activation (Hsu *et al.*, 2013).

Millions of people commonly suffer from insufficient sleep, which is a problem of considerable health, social and economical importance (Vgontzas and Kales, 1999). Clinical data have shown that SD causes deficits in several forms of learning and memory (Miyata *et al.*, 2010) and increases in hypothalamic and thalamic oxidative stress levels were found in SD rats (D'Almeida *et al.*, 1998). The accumulation of free radicals may further cause oxidative damage to mitochondria

that leads to more ROS generation, which can disrupt neuronal function, as neurons are critically dependent on mitochondrial function to establish membrane excitability and to execute the complex processes of neurotransmission and plasticity (Kann and Kovacs, 2007). The loss of cognitive function is one of the problems that many people face as they age. Patients with AD or other types of dementia are also subject to losses in cognitive function. Current drugs for AD target cholinergic and glutamatergic neurotransmission, thus improving clinical symptoms, although their therapeutic efficacy is still debated (Mangialasche *et al.*, 2010).

Our results showed that EPO can modulate downstream mitochondrial biogenesis and haemoglobin induction in neuronal cells. EH-201 induced EPO in neuronal cells, and its EPO-mediated activation of mitochondrial function through PGC-1 $\alpha$ , haemoglobin- $\beta$  and HO-1 expression in neuronal cells improved mitochondrial energy generation, cellular oxygenation and reduces oxidative stress, leading to the recovery of memory impairment in SD-, A $\beta$ - and KA-induced disease mouse models. Therefore, our results suggest that the induction of endogenous EPO in neuronal cells might be a rational therapeutic strategy for memory impairment in neurodegenerative diseases, physiological ageing or traumatic brain injury.

## Acknowledgements

We are grateful for a grant from the Ministry of Education, Republic of China (Taiwan), Aim for the Top University Plan and Ministry of Science and Technology, grant MOST 103-2320-B-010-016.

## Author contributions

L.-Y. H. isolated the EH-201 by EPO inducer-based fractionation bioassay and also wrote carefully for this manuscript. P.-L. H. made the figures beautiful. L.-W. C. was responsible for the part of the SD animal study of this manuscript. W.-Z. T. was responsible for the part of the A $\beta$  and KA animal study of this manuscript. K.-T. H. evaluate the memory lost and recovery in our laboratory. C.-L. W. made great contribution at the recrystallization of EH-201 at the purity of 99.67%. R.-T. W. made major modification in this manuscript.

## Conflict of interest

The authors certify that they have no actual or potential conflicts of interest regarding the research reported in this paper.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13248>

**Table S1.** Sequences of the specific gene used for Q-PCR.