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# Erythropoietin improves object placement recognition memory in a time dependent manner in both, uninjured animals and fimbria-fornix-lesioned male rats

W. Almaguer-Melian[a], D. Mercerón-Martinez[b], S. Delgado-Ocaña[a], E. Alberti-Amador[a], R. Gonzalez-Gómez[b], Jorge A. Bergado[a,c,*]

[a] Centro Internacional de Restauración Neurológica (CIREN), Habana 11300, Cuba
[b] Centro de Neurociencias de Cuba, 11300 Habana, Cuba
[c] Universidad del Sinú "Elías Bechara Zainún", Montería, Colombia

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ABSTRACT

An increasing number of reports sustain a possible role of erythropoietin (EPO) as neuroprotective agent. In two previous articles we have evaluated EPO as plasticity promoting agent, and to contribute the restoration of brain function affected by acquired damage. We have shown that EPO is able to induce an increased synaptic efficacy in vivo along with a plasticity promoting effect. In the Morris water maze EPO administration to fimbria-fornix lesioned male rats induces a significant improvement of their spatial memory, affected by the lesion. Singularly, EPO was only effective when administered shortly after training (10 min) but not after several hours (5 h), suggesting a specific EPO effect on time dependent plasticity process. In the present paper we have expanded this line of evidence using a low stress paradigm of object placement recognition in lesioned and healthy male rats. The memory trace in this model is short-lasting; animals could recognize the change in object position when tested 24 h after, but not 48 or 72 h after the acquisition session. EPO administration 10 min after acquisition significantly prolongs retention to, at least, 72 h in healthy rats. No effect was seen if EPO was administered 5 h after training, suggesting a specific EPO modulatory effect on the consolidation process. Remarkably, early EPO treatment to fimbria fornix lesioned animals reverts the memory deficit caused by the lesion. An increased expression of the plasticity related gene *arc*, was also confirmed in the hippocampus and the prefrontal cortex, that is likely to be involved in the behavioral improvement observed.

## 1. Introduction

Erythropoietin (EPO) is a glycoprotein with a well-established role stimulating the production of erythrocytes in the bone marrow. EPO is produced mainly by the kidney in response to hypoxia forming a negative feed-back loop. The therapeutic use of EPO to relieve anemia caused by renal failure renewed the interest in this cytokine. By the end of the XXth century it was discovered that EPO receptors were expressed in many tissues, including the brain (Marti et al., 1997; Marti et al., 1996). This discovery was rapidly followed by experimental studies in animal models of brain hypoperfusion (Sakanaka et al., 1998). Additionally, erythropoietin attenuates axonal injury after middle cerebral artery occlusion in mice (Wang et al., 2017), reduced infarct size by 30.0% (95% CI: 21.3 to 38.8) and neurobehavioral outcome by 39.8% (Jerndal et al., 2010) sustaining a neuroprotective effect of EPO. In addition, EPO treatment for three weeks before a learning task and electrophysiological study improves both, hippocampal dependent memory and in vitro long-term synaptic potentiation (LTP) (Adamcio et al., 2008). For a comprehensive review of EPO see Jelkmann (2007).

We have recently shown that EPO administration shortly after training can not only prevent or reduce neural damage, but also promotes neural recovery from installed damage to the nervous system in fimbria-fornix (FF) lesioned rats trained in the Morris water maze. Daily post-training injections of EPO significantly improve the acquisition of this spatial memory task. Interestingly, this effect was time-dependent; EPO was only effective when applied 10 min after training, but not 5 h later, suggesting a specific EPO modulatory effect on time dependent plasticity process (Almaguer-Melian et al., 2015).

The water maze is a highly stressful paradigm (Harrison et al., 2009), opening the question of whether the observed memory enhancing effects were due to a peripheral interaction of EPO and stress

* Corresponding author at: UNIVERSIDAD DEL SINÚ "Elías Bechara Zainúm", Cra. 1w No. 38-153 Barrio Juan XXIII, Montería, Córdoba, Colombia.
*E-mail addresses:* william@neuro.ciren.cu (W. Almaguer-Melian), alberti@neuro.ciren.cu (E. Alberti-Amador), jorge.bergado@infomed.sld.cu (J.A. Bergado).

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hormones. Therefore, we have now tested EPO (injected shortly after the training) in a low stress visual recognition memory model, both in healthy and FF-lesioned rats. Since we also study EPO effects on memory duration in healthy animals, is possible to clarify if EPO modulates memory consolidation processes. An increase in memory duration in both healthy and lesioned animals is evidence of an EPO modulatory effect on time dependent plasticity processes, which sustain memory and neural functional recovery. Positive results will reinforce the potential use of EPO in Restorative Neurology.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats, eight weeks old (280–320 g body weight at the beginning) obtained from the Cuban National Center for Laboratory Animals (CENPALAB) were used. Animals were maintained under controlled environmental conditions (23 °C, 50% humidity, 12 h light-dark cycle) in translucent plastic cages (5 animals per cage). Access to food and water was free during the whole time. Efforts were made to reduce pain and discomfort, according to the internationals ethical norms for the use of laboratory animals, the Cuban regulations published by CENPALAB, and the internal regulations of CIREN. The day before beginning the experiments, the animals were gently handled by the experimenter during about 2 h to habituate them and reduce handling stress.

### 2.2. Object placement recognition memory test

The memory test used in these experiments was an object placement recognition test in healthy (10 experimental groups) and lesioned rats (4 experimental groups). This test uses the natural tendency of rats to explore novel environmental conditions and is relatively less stressful. It was performed in three consecutive trials. In the first day (habituation), the animals were placed in the center of an empty open field box (50 × 50 cm) painted in light blue illuminated by a 40 W light bulb placed 1 m above the floor, facing a cue (a bold black capital A, 650 dpi) attached at the north wall of the open field. The animals were allowed to freely explore the empty box during 5 min, in order to habituate to the box. As can be seen in Fig. 1A (Control group, N = 26), the exploratory activity of the animals (number of entries in the area along the walls) diminished with the time in the box, indicating their habituation to this novel environment. The *sec*ond trial (acquisition) was performed 24 h later. Animals were again placed in the center of the open field, but this time there was two identical objects (white plastic bottles filled with water) positioned 10 cm away from the north wall and the east and west walls respectively, with a distance of 30 cm between them (see inset Fig. 1B, N = 14). The time used by the animals exploring each object was measured (in sec) by two experimenters (blind to the treatment) with time watches by direct observation. Each experimenter recorded time exploration from one object. The minimal exploration time required during the training trial was 5 s, however no animal needed to be rejected because of this. Since the experimenters are close to open field apparatus they could be a potential spatial cue for the test animal as well. Therefore, the experimenters always occupied the same position. The third and final trial (recognition) was performed 24, 48 or 72 h after the second trial in different animal groups for each time. In this trial, the object placed near the east wall was moved 30 cm to the south wall, and rotated 45° along its vertical axis introducing a novelty element in its position within the box. The time devoted to explore each of the objects (constant and displaced) was measured (Fig. 1D). Exploration time was measured when the rat stayed with the head close and in front of one of the objects or with its fore limbs placed on the object. Habituation, training and object placement recognition memory test were carried out between 09:00–12:30 h to minimize circadian influences.

### 2.3. The exploration time for each object was transformed in percentage of the total exploration time

To asses for possible differential object relevance or positional influences on the results, we have evaluated the exploration time in two independent groups before the beginning of the experiments. As can be seen in Fig. 1B and C (N = 14), both objects were explored equally, independently of their position or any other non-adverted cues. To eliminate the possible influence of odor cues left by a previous animal, the box and the objects were thoroughly cleaned after each animal with an alcohol soaked cloth.

For statistical analysis, we compared the percentage of time dedicated by the animals to explore the displaced object, compared to the percentage of time devoted to the same object in the training session. The N for control groups to test memory duration was: 24 h (N = 14), 48 h (N = 12) or 72 h (N = 10).

### 2.4. Erythropoietin administration

Human recombinant erythropoietin (EPO) were obtained from the Cuban Center for Molecular Immunology (ior©EPOCIM) as well as the vehicle (Placebo EPOCIM) used as placebo treatment. A single of 10,000 UI/kg dose of EPO, or an equal volume (1 ml/kg) of placebo, was injected i.p. 10 min or 5 h after the end of the second trial (acquisition). The N for all evaluated groups at 48 h was N = 10; meanwhile, rats evaluated at 72 h: EPO-10 min, N = 11; EPO-5h, N = 11 and Placebo group, N = 12.

### 2.5. Fimbria-fornix lesion

To test the possible effects of EPO in lesioned brains, a transection of the fimbria-fornix fibers was performed in male rats groups. Surgery was carried out under chloral hydrate narcosis (420 mg/kg, i.p) according to the procedure described elsewhere (23). The animals were mounted on a stereotactic frame (David Kopf Inst., Saint Louis, MO, USA), the skin was incised and the skull exposed. A bilateral window was opened in the skull at coordinates AP: 1.4 mm, ML: 0.5 to 5.2 mm. A reduced #11 surgical blade was lowered at 15° (with respect to the vertical direction) and a bilateral knife transection was carried out at the coordinates AP:1.4 mm, ML: 0.8 to 3.1 mm, and DV: 5.0 mm (24). Lesions were performed bilaterally to prevent recovery by sprouting of contralateral fibers. In sham operated animals all procedures were carried out identically, but no blades were inserted and no transection performed (false operated, FO). Lesioned and false operated rats were evaluated in the object placement recognition memory task a week after surgery.

The N for fimbria-fornix evaluated groups in object placement recognition memory were: FF, N = 11; FO, N = 6; FF-EPO, N = 8 y FF-5h-EPO, N = 10.

In order to validate the lesion procedure after finishing the experiments we carried out a conventional stain for the enzyme acetyl cholinesterase in two randomly selected animals from each group of lesioned animals. The results showed a complete cholinergic denervation of the whole hippocampal formation bilaterally in full agreement with those shown in previous reports (Almaguer-Melian et al., 2015; Merceron-Martinez et al., 2016; Merceron-Martinez et al., 2013) (AChE, data not shown).

### 2.6. Measuring arc expression

To assess the involvement of plasticity related genes in the effects of EPO on recognition memory we have measured the expression of the *arc*-gene in a random sample of five rats from each of the following groups: naïve untrained animals; trained untreated animals, trained placebo-treated animals 10 min or 5 h after training; and trained EPO-treated animals 10 min of 5 h after training (acquisition trial). The