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Authors

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McWhirt, Joshua
Sathyanesan, Monica
et al.

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Carbamoylated erythropoietin produces antidepressant-like effects in male and female mice

Dayalan Sampath[1], **Joshua McWhirt**[2], **Monica Sathyanesan**[1], **Samuel S Newton**[1,*]

[1]Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, SD 57069; Sioux Falls VA Healthcare System, Sioux Falls, SD 57105

[2]Division of Basic Medical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, SD 57069

Abstract

Major depressive disorder and related illnesses are globally prevalent, with a significant risk for suicidality if untreated. Antidepressant drugs that are currently prescribed do not benefit 30% of treated individuals. Furthermore, there is a delay of 3 or more weeks before a reduction in symptoms. Results from preclinical studies have indicated an important role for trophic factors in regulating behavior. Erythropoietin (Epo), which is widely prescribed for anemia, has been shown to produce robust neurotrophic actions in the CNS. Although Epo's antidepressant activity has been successfully demonstrated in multiple clinical trials, the inherent ability to elevate RBC counts and other hematological parameters preclude its development as a mainstream CNS drug. A chemically engineered derivative, carbamoylated Epo (Cepo) has no hematological activity, but retains the neurotrophic actions of Epo. Cepo is therefore an attractive candidate to be tested as an antidepressant.

Objective: To evaluate the antidepressant properties of Cepo in established antidepressant-responsive rodent behavioral assays.

Methods: Adult male and female BALB/c mice were used for this study. Cepo (30 μgrams/ kg BWT) or vehicle (PBS) was administered intraperitoneally for 4 days before the test of novelty induced hypophagia and subsequently at five hours before testing in forced swim test (FST), tail suspension test (TST) and open field test (OFT). To obtain mechanistic insight we examined the phosphorylation of the transcription factor cAMP response element binding protein (CREB).

Results: Administration of Cepo at 30 μgrams/ kg BWT, for 4 days produced significant reduction in latency to consume a palatable drink in a novel environment in male and female mice.

*Corresponding author. Samuel.Sathyanesan@usd.edu.

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Ethical statement:
Affirmation: We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Male BALB/c mice had a significant reduction in immobility in both tail suspension and forced swim tests, and female mice exhibited lower immobility in the forced swim test.

Keywords

Carbamoylated erythropoietin; behavioral despair; antidepressant mechanism of action; phosphorylated CREB

Introduction

Major Depressive disorder is a widely prevalent and debilitating mental health illness with about 20.6% of people suffering from it (Hasin et al., 2018). Despite substantial gains in increasing disease awareness and treatment initiatives, currently prescribed antidepressant drugs are effective in only 60% of treated individuals. The most widely used drugs continue to be those that were designed to elevate synaptic monoamine levels. Although there has been significant progress in understanding depression neurobiology, such as the important role of neurotrophic factors in the antidepressant activity (Duman et al., 1997, translating these findings to the clinic has been slow. The challenges include development of neurotrophic molecules as drugs, penetration across the blood brain barrier, and unknown clinical safety profile {Wong, 2010 #1667). In this context, it is interesting to note that erythropoietin (Epo) which is widely prescribed to treat anemia, has emerged as a clinically relevant neurotrophic molecule (Miskowiak et al., 2012). Epo's known safety profile has facilitated multiple clinical trials testing it for antidepressant activity (Miskowiak et al., 2007; Miskowiak et al., 2014; Miskowiak et al., 2015). While the results are promising, the potent erythropoeitic activity inherent to Epo is likely to hamper its development as a viable antidepressant drug (Coleman et al., 2006; Ehrenreich et al., 2009).

The discovery that Epo can be chemically modified via an in vitro post-translational modification, carbamoylation, to render it non-erythropoeitic enabled the field to address the hematological side effect limitations of Epo (Leist et al., 2004). Carbamoylated Epo (Cepo) retains neurotrophic and neuroprotective activity but is devoid of erythropoeitic activity. Cepo has been tested in rodent behavioral models after chronic 28 day administration, implicating that the behavioral effects are coupled with an increase in neurogenesis (Leconte et al., 2011). We recently showed that Cepo produces cognitive improvement after just 4 doses, and that the gene expression changes persist for over 2 weeks (Sathyanesan et al., 2018). We therefore asked whether Cepo could also produce antidepressant effects after a similar short-term administration in a manner that would be consistent with neurotrophic activity but neurogenesis independent. As the incidence of depression in the female population is significantly higher than in males, we tested the antidepressant-like properties of Cepo in male and female BALBc mice using established antidepressant-responsive behavioral assays.

Materials and Methods

Mice

Male and female BALB/c mice were procured from Envigo. The mice were 8-9 weeks old on delivery to the vivarium. The institutional animal care use committee- University of South Dakota, approved all experimental procedures. The mice were quarantined for a week after its arrival, and before beginning the experiments. Each mouse was optimally handled and habituated to the research setting, researcher, and the housing colony. All experiments were conducted as represented in the schema (Fig 1).

Drug dosing

After 3 days of handling, carbamoylated erythropoietin was administered at the dose of 30 micrograms/ Kg BW/day for 4 days. The experimenter was blinded from the drug/ veh and its dosing constitution.

Estrous assay in female mice

Female mice were periodically assayed for their estrous stages specifically on the days of behavioral experiments, to understand if the female mice in different estrous stages have differential impact of the drug treatment response in general, and with reference to the depressive behavior. The lavage of exfoliates from the vaginal opening was collected with distilled water and left to dry on probe-on plus (Fisher) slides. The cells were then stained with cresyl violet for microscopic analysis (McLean et al., 2012). The stained sections were cover slipped and observed under bright field microscopy for morphological classification of the cells to determine stage of estrous. Based on the vaginal cell morphology, the mice were classified to be either of the four stages, namely estrous, pro-estrous, metestrous, and diestrous. These stages were decided based on cell types, cornified epithelial cells, nucleated squamous epithelial cells, and leucocytes including neutrophils (Fig. 6 a–e).

Novelty induced hypophagia

On the last day of drug administration, mice were trained in their colony room for the Novelty induced hypophagia test (NIH) (Dulawa and Hen, 2005). During the training session, mice were trained to drink sweetened milk (carnation-condensed sweet milk-Nestle) in calibrated 25ml pipettes. The training was provided for thirty minutes each for three days, before testing in the home cage, followed by testing in the novel cage during the next two days. The quantity of milk consumed and the latency to start drinking the milk was evaluated both in the home Cage (50 lux light) and novel cage with cage lid removed (500 lux).

Tail suspension test (TST)

After one-day interval of testing in NIH, mice were tested in the tail suspension test (TST) (Cryan et al., 2005). The mice were suspended in a rectangular chamber with the front-end open for viewing and recording with a digital camera. The length of the tape used to suspend was kept constant at 15 centimeters throughout the experiment. The mobility of the mice were video recorded for 7 minutes and analyzed offline with a behavior analysis software

(Ethovision 14XT-Noldus-Virginia) for indices of immobility. Only the last 6 minutes of the video are considered for quantification.

Forced swim test

Behavioral despair was tested using the Porsolt test (Bourin et al., 1998) , widely termed as forced swim test. Mice were tested in pairs in separate chambers (20.2 cm diameter and 25.5 cm height), and the entire session was recorded with a camera for offline analysis of immobility. The experiment was conducted for 15 minutes and the last 13 minutes of the data were used for further analysis. The time spent immobile by each mouse during the 13-minute session was quantified. The water temperature was maintained at 27 degrees.

Open field test

At the end of FST, mice were taken in quadruplets equally from the veh and Cepo treated group and placed in an open field (n=4) and recorded for 10 minutes. The procedure was repeated the next day, five hours after the administration of Cepo / Veh. The difference in the locomotor activity between the baseline (1^{st} day) and after Cepo administration (2^{nd} day) were quantified and analyzed.

Automated behavior tracking

The behavioral responses measured in various assays were recorded and quantified using Ethovision XT (14) software from Noldus. The behaviors were recorded at the rate of 30 frames/ second and analyzed offline for the indices of locomotion and immobility. The validation was performed by an experimenter blinded about the groups and the different treatments. The detection and threshold settings were kept constant for all the batches of mice.

Immunohistochemistry

Male and female BALB/c mice were injected with four doses of Cepo in 4 days and sacrificed 5 hours after the last dose. Brains were removed and frozen on dry ice. Immunohistochemical analysis was performed as previously described (23). Briefly, 16 μm coronal, cryocut sections were incubated overnight at 4°C with primary antibody (pCREB, Abcam 32096, 1:250) in antibody solution. Antibodies were used as per manufacturer's instructions and specificity was tested using incubation in antibody solutions lacking primary antibody. Following primary antibody incubation, slides were rinsed in PBS and then incubated in fluorescent secondary antibody (Alexa-594, 1:500) for 2h at room temperature. Slides were then rinsed in PBS and coverslipped using VectaMount (Vector Labs). Sections were viewed and images captured using a Nikon Eclipse Ni microscope equipped with a DS-Qi1 monochrome, cooled digital camera and NIS-AR 4.20 Elements imaging software. Sections from Cepo and vehicle-treated mice were captured using identical exposure settings.

Statistical analysis

The latency to drink the palatable milk was analyzed with Wilcoxon's signed paired-rank test, since the data is not normally distributed. The immobility in the tail suspension test and

the forced swim test were analyzed with the Mann Whitney test, performed using GraphPad Prism version 8.00 for Windows, GraphPad Software, La Jolla, California USA, www.graphpad.com

Results

Novelty induced hypophagia

Initial testing in the home cage for the latency to drink milk and the quantity of milk consumed did not show a difference. On subsequent exposure in the novel cage, there was significantly reduced latency to drink from the sipper tube among the Cepo treated mice (Fig. 2a), P=0.0047. Wilcoxon matched pair signed rank test was used. N=36 each in vehicle and Cepo treated groups of mice. Similarly, among the female BALB/c mice the Cepo treated group took significantly less time to drink the palatable milk in the novel environment (Fig. 2b), P=0.0004; N=32 in each group. We observed these results consistently from 3 cohorts of male mice and two groups of female mice in both Cepo and vehicle treated groups.

Tests of behavioral despair

Tests of behavioral despair in male and female mice. Fig 3a, shows the time male mice remained immobile in the tail suspension test 5 hours after administration of the 5^{th} dose of Cepo. The Cepo treated mice moved significantly as a measure of escape, in contrast to the vehicle treated controls, which showed greater immobility. P= 0.029; n=18 in controls and 19 in Cepo treated mice. Mann Whitney's test was performed. However, the female mice did not move much in comparison to their male counterparts of both groups (Fig 3b). Instead, they adopted a somewhat tonic posture and exhibited extended immobility. Female mice were then tested in the forced swim test. The Cepo treated mice spent significantly less time immobile compared to the vehicle treated controls (Fig 3c). P= 0.002 in male mice, n=16 in each group. Similarly, female mice treated with Cepo spent significantly less time immobile during the forced swim test. P= 0.0046; n= 13 in each group (Figure 3d).

Open field test

Male and female Cepo treated mice had comparable locomotor behavior as vehicle groups in 10 minutes of open field activity at day 1 (baseline) and day 2 (tested 5 hours after the injection of Cepo / veh (Fig 4a–d), P >0.05; n=12 in each groups of male and female mice. Additional OFT data pertaining to distance traveled and time spent in the center showed no statistical significance between vehicle and Cepo treated groups in males and female (Supplementary Fig.S1A and B).

Behavioral performance based on estrous staging

Based on the stage of estrous on the days of testing (Fig. 6), female mice were grouped into four categories namely, proestrous, estrous, metestrous and diestrous. The estrous stage exhibited a trend towards reduction of latency to drink the palatable milk in the anxiogenic and novel environment (Fig. 5a). The low animal numbers in the individual estrous stages precluded statistical analysis (Fig. 5a, b).

Immuno Histochemistry of phosphorylated CREB

Immuno histochemical analysis and quantification of phospho CREB expression in the hippocampus showed a modest but significant increase in the DG region, of both male and female BALB/c mice. Representative images of both Cepo and veh treated mice are shown (Fig 7 a, b and c). P</= 0.05; (Fig 7 e, and f). In addition, an increase in pCREB stained cells was observed in the hilus region of the hippocampus.

Discussion

In this study, administration of carbamoylated erythropoietin improved the performance of BALB/c mice in established antidepressant-responsive behavioral assays. These specific effects were observed with intraperitoneal administration of seven doses of Cepo, distributed over 16 days of experimentation. We conducted the experiments in BALB/c mice as we wanted to test Cepo's effects in a stress-susceptible strain. A substantial body of evidence has shown that BALB/c mice are more vulnerable to the deleterious effects of stress in comparison to C57 mice due to increased corticosterone, enhanced emotionality, reduced locomotion, and poor maternal care for the newborn litters (Crawley et al., 1997; Flint and Tinkle, 2001). Cepo treated mice were not significantly different from controls in the open field task, indicating that the reduction in latency to approach the familiar palatable drink in NIH, and reduced immobility in TST and FST tests are due to a specific antidepressant-like effects.

It is well known that the lifetime prevalence of major depressive disorder is higher in females (26.1%), than males (14.7%) (Hasin et al., 2018). In preclinical studies, male animals are predominantly used in tests of novel antidepressants. We observed antidepressant effects of Cepo in both male and female mice. It is interesting to note that in the test for novelty-induced hypophagia (NIH), females exhibited a much-lower latency compared with their vehicle treated controls. The effect size was also much higher than that for male BALB/c mice treated with Cepo. Enhanced response of female mice to Cepo could be due to the synergistic effects of naturally expressed estrous hormones. We were however unable to demonstrate any specific estrous stage dependent effects in either NIH or FST because the representation of animal numbers in the individual estrous stage groups precluded statistical analysis.

Enhanced performance in the novelty-induced hypophagia (NIH) with a short dosing regimen indicates that Cepo can decrease latency of antidepressant effects

A major limitation of conventional antidepressants is the latency of several weeks before patients experience a reduction in symptoms. Preclinical studies indicate that this delay in behavioral effects of antidepressants is likely due to a requirement for alteration in intracellular signaling, activation of transcription factors and elevation in levels of neurotrophic factors (Duman et al., 1997). BDNF produced antidepressant response within few days of intra hippocampal infusion in rodent brain, demonstrating the role of trophic factors in producing faster behavioral effects (Shirayama et al., 2002). Interestingly, ketamine produces antidepressant action by release of trophic factors such as BDNF and VEGF (Autry et al., 2011; Deyama et al., 2019). Clinical studies have reported that Epo is

capable of producing behavioral effects as early as 3 days after administration (Miskowiak et al., 2008). Testing Epo for a comparable early onset of behavioral response in preclinical tests is somewhat challenging. Conventional and widely used antidepressant-responsive assays such as the forced swim test (FST) and tail suspension (TST) are not ideal when the dosing regimen involves multiple doses, as these tests are sensitive to a single acute dose. In this context, the novelty-induced hypophagia assay is useful as it responds only to a chronic dosing regimen (Dulawa and Hen, 2005). In this study, we were able to demonstrate the effect of Cepo in the NIH test after only four doses (1 dose/ day). Administration of four doses of Cepo (30 micrograms/ Kg BWT) significantly reduced the latency to approach the palatable drink in the novel environment. Three male and two female cohorts of BALB/c mice were used to demonstrate this effect and all the batches of mice showed the same behavioral response.

Neurotrophic effects of Cepo

Previous studies have indicated that Epo and Cepo produce behavioral effects by a neurogenesis-dependent mechanism, necessitating chronic drug administration over 35 days (Leconte et al., 2011). Our results showing a robust effect in the NIH test with just four doses, indicates that Cepo is capable of producing neurogenesis-independent behavioral effects with a shorter dosing regimen. However, our results do not preclude a role for neurogenesis in Cepo's behavioral effects, as there could be further improvement in behavioral response with continued dosing and an increase in neurogenesis. In this context it is useful to note that although clinical studies have reported effects as early as 3 days, there is continued increase in improvement when the once weekly doses are extended for 8 weeks in treatment-resistant depression (Miskowiak et al., 2014). Interestingly, the effects remain significant at 14 weeks, 7 weeks after the last Epo dose (Miskowiak et al., 2014).

Multifunctional trophic factors such as Epo exhibit not only neurogenic effects but also neurotrophic and angiogenic actions (Newton et al., 2013). We previously showed that Epo produces antidepressant-like effects in both mice and rats, elevating well-known neurotrophic factors such as BDNF, VGF and neuritin (Girgenti et al., 2009). Recently, we showed that Cepo also elevates the expression of neurotrophic factors in the hippocampus (Sathyanesan et al., 2018). Furthermore, bioinformatics analysis of Cepo's gene profile from our recent unbiased, genome-wide transcriptome study in neuronal cells indicated that Cepo activates the cAMP response element binding protein (CREB) pathway (Tiwari et al., 2019). This was unexpected, as the cAMP signaling cascade is not conventionally included in the canonical pathways regulated by Epo. However, CREB is well known as a crucial transcription factor that regulates neurotrophic factor-induced gene expression (Finkbeiner et al., 1997). The CREB pathway has also been strongly implicated in depression and antidepressant activity by a substantial body of evidence (Blendy, 2006), including elegant transgenic mouse studies employing dominant negative mutant CREB mice (Newton et al., 2002).

Cepo enhanced the expression of phosphorylated CREB specifically in the hippocampal dentate gyrus of male and female mice. Previous studies of Epo and Cepo-induced neurotrophic gene expression have also reported the strongest effects in the DG (Girgenti et

al., 2009; Sathyanesan et al., 2018). Interestingly, human imaging studies have shown that Epo is able to reverse left hippocampal volume loss in treatment resistant depression (Miskowiak et al., 2015). Although focused studies are needed to determine the mechanism involved in the hippocampal action of Epo and Cepo, it is tempting to speculate that Epo receptor density could be the underlying reason. Recent, precise volumetric analysis in depressed patients reported specific reduction in hippocampal subfields (Roddy et al., 2019), providing a neurobiological basis for testing trophic factor-based therapies in depression.

In order to further develop the translational utility of Cepo it will be important in future studies to investigate its ability to rescue behavioral deficits produced by chronic stress models. It would also be of significant interest to obtain mechanistic insight into Cepo's action in the brain by selective knockdown of Epo receptors in the hippocampus. This can shed light on the role of signaling via Epo receptors in the modulation of behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights:

This study demonstrates that a chemically engineered non-erythropoietic derivative of erythropoietin, carbamoylated erythropoietin, produces antidepressant-like effects in both male and female mice. It acts faster than prescription antidepressant drugs in the novelty induced hypophagia test.

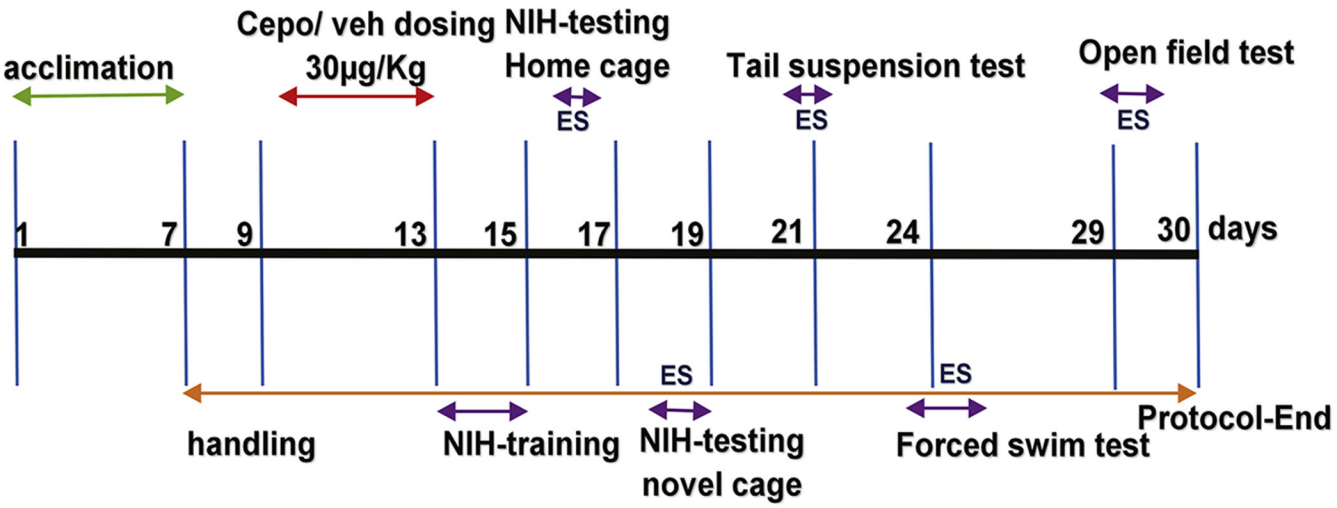

Fig 1. Schema of the experimental protocol

Experimental scheme and assays employed in the study. Green arrows- Acclimation time for the mice to adapt to the environment in vivarium kept in holding room and quarantined for a week from experimenter handling. Red arrows- days and extent of the Cepo dosing. Purple arrows- days of behavior experimentation and orange arrow correspond to the days of handling. ES-estrous staging conducted after completion of each behavior assays with NIH, TST, and OFT, and before conducting FST; NIH- novelty induced hypophagia.

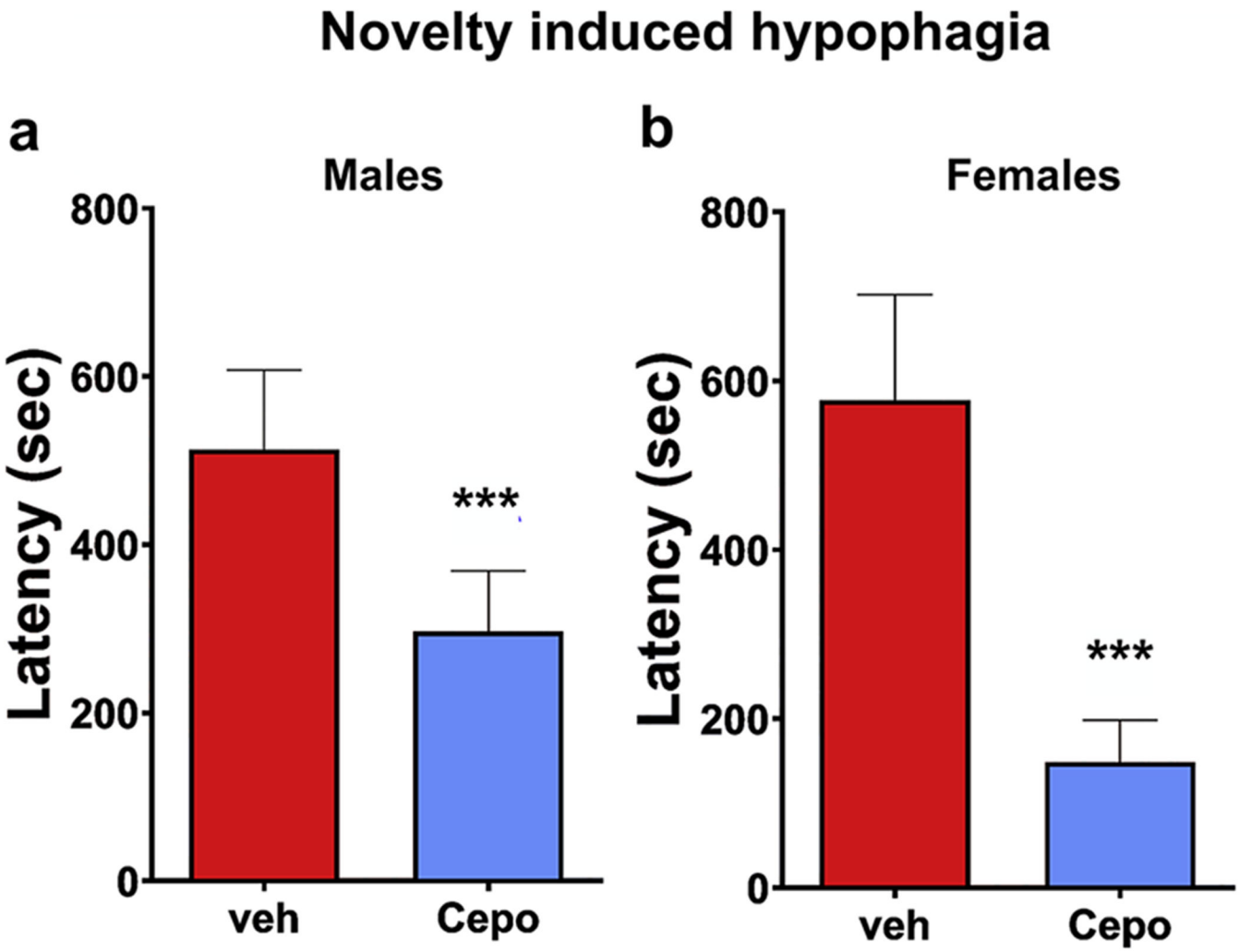

Fig 2. Effect of Cepo in Novelty-induced hypophagia

Four days of Cepo treatment significantly reduced the latency to drink the palatable milk in anxiogenic environment. **a**) Novelty induced hypophagia in males, *p= 0.0004, N=32 in vehicle and Cepo treated groups. The y-axis represents latency to drink the milk expressed in seconds. Error bars are +/− SEM. **b**) NIH in female BALB/c mice, p=0.0001, N=17 in each group. Wilcoxon matched-pairs signed rank test.

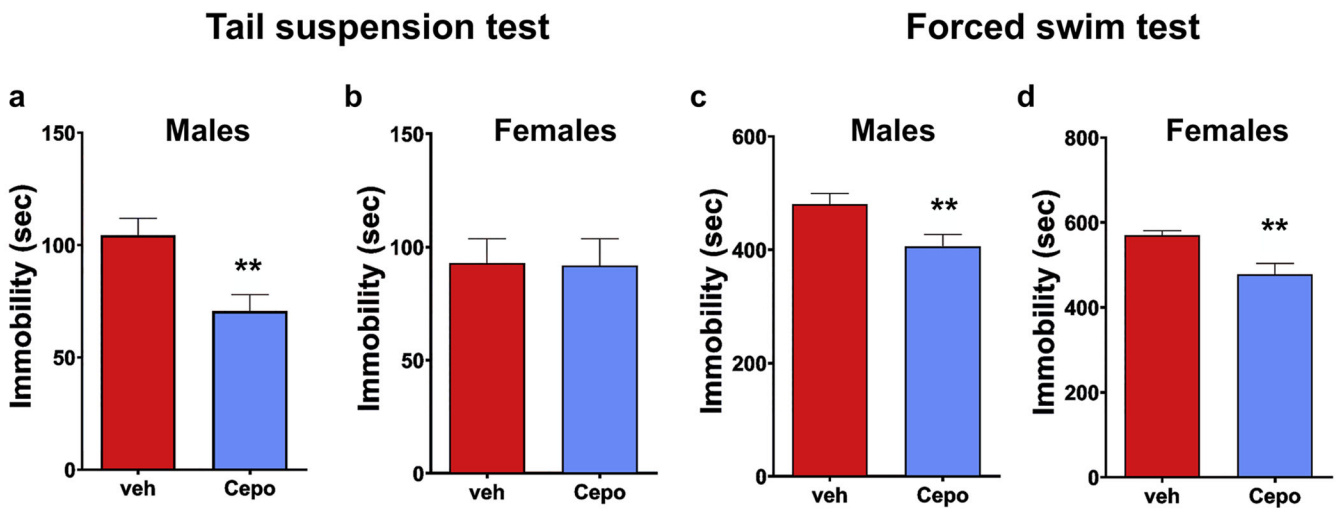

Fig 3. Tests of behavioral despair

a) Tail suspension in male mice. Vehicle treated mice were significantly more immobile than Cepo treated mice, *p= 0.029, n=18 in veh group, 19 in Cepo treated group. Mann-Whitney test. The y- axis denotes time spent immobile in seconds, **b**) Tail suspension in female mice, p > 0.05, N= 23 in veh and 22 in Cepo treated groups of mice, **c**) Forced swim test in males, *p=0.0017, n=16 in each group, **d**) Forced swim test in female group, *p=0.0046, n=13 in each group. Wilcoxon's matched pair signed rank test.

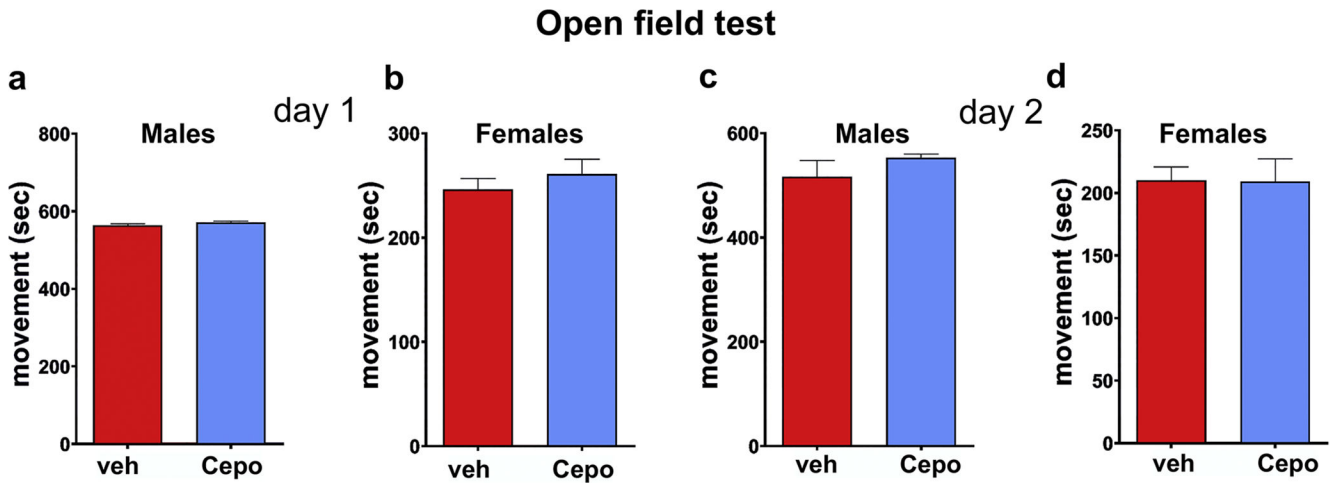

Fig 4. Open field test

a) Open field test in males on day 1 and **b**) Females. **c**) Day 2, 5 hours after drug injection, open field test in males **d**) Females. P> 0.05, n= 12 in all groups of Cepo and vehicle treated male and female mice. Wilcoxon's matched pair rank test.

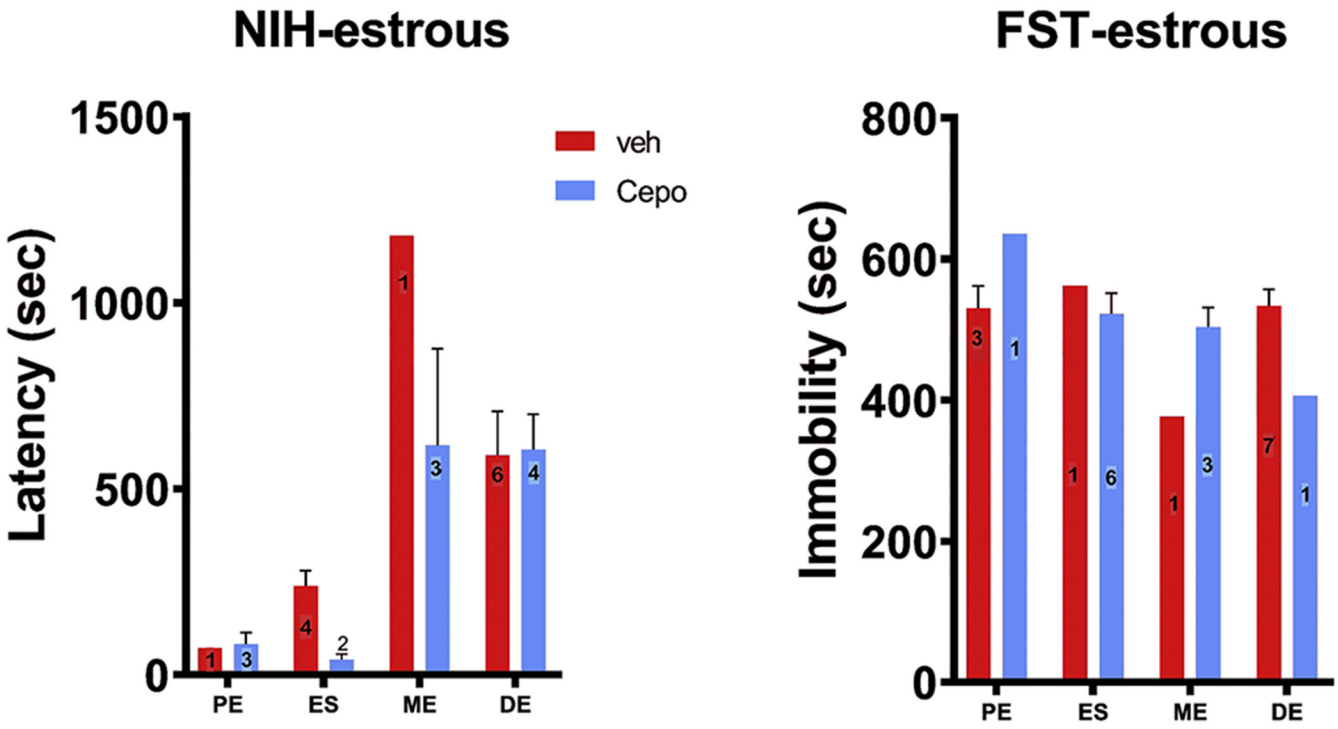

Fig 5. The effect of estrous stage on behavioral performance

a) Latency to drink palatable milk in novel and anxious environment vs stage of estrous. b) Immobility in forced swim test vs stage of estrous.

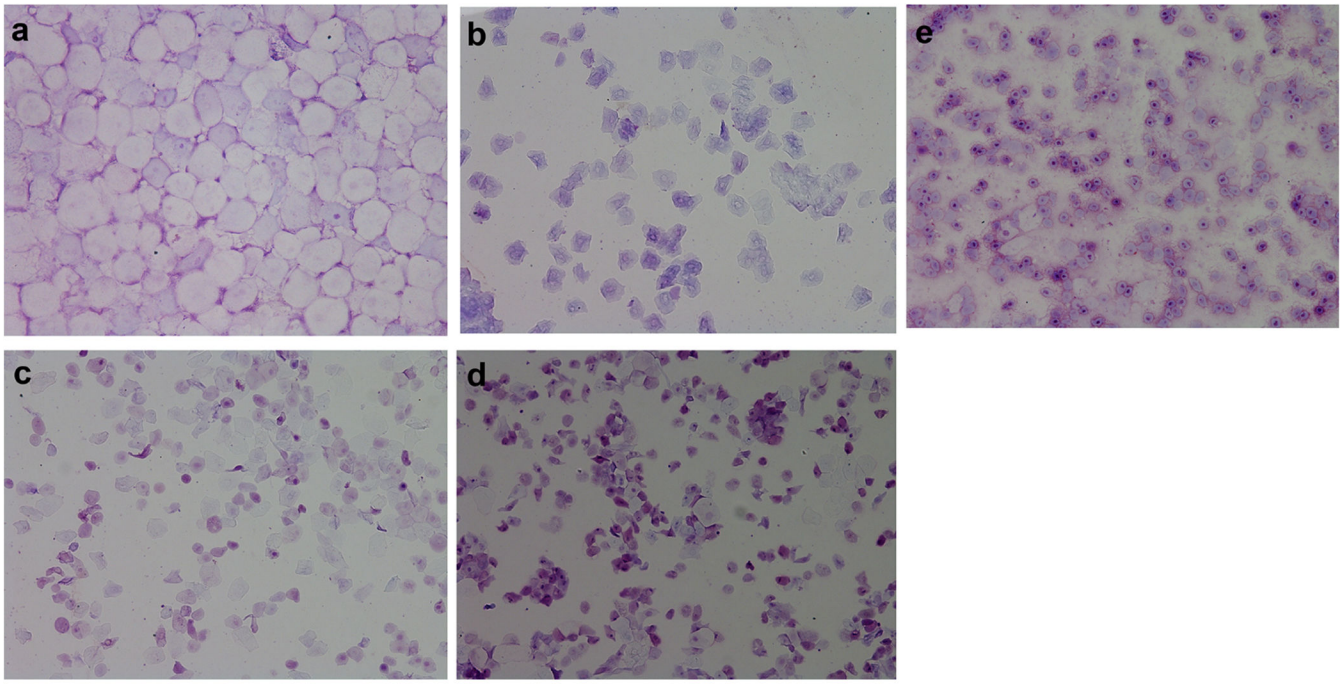

Figure 6. Estrous assay with four stages of the mouse estrous cycle.

a) Proestrous stage with large oval shaped epithelial cells, b) Estrous stage with significantly larger cornified epithelial cells. c) Metestrous stage with a combination of cornified epithelial cells, smaller neutrophils of irregular shape with large nucleus. d) Diestrous stage with numerous small neutrophils. e) Transitional stage from Diestrous to proestrous with neutrophils slowly transitioning to large nucleated epithelial cells.

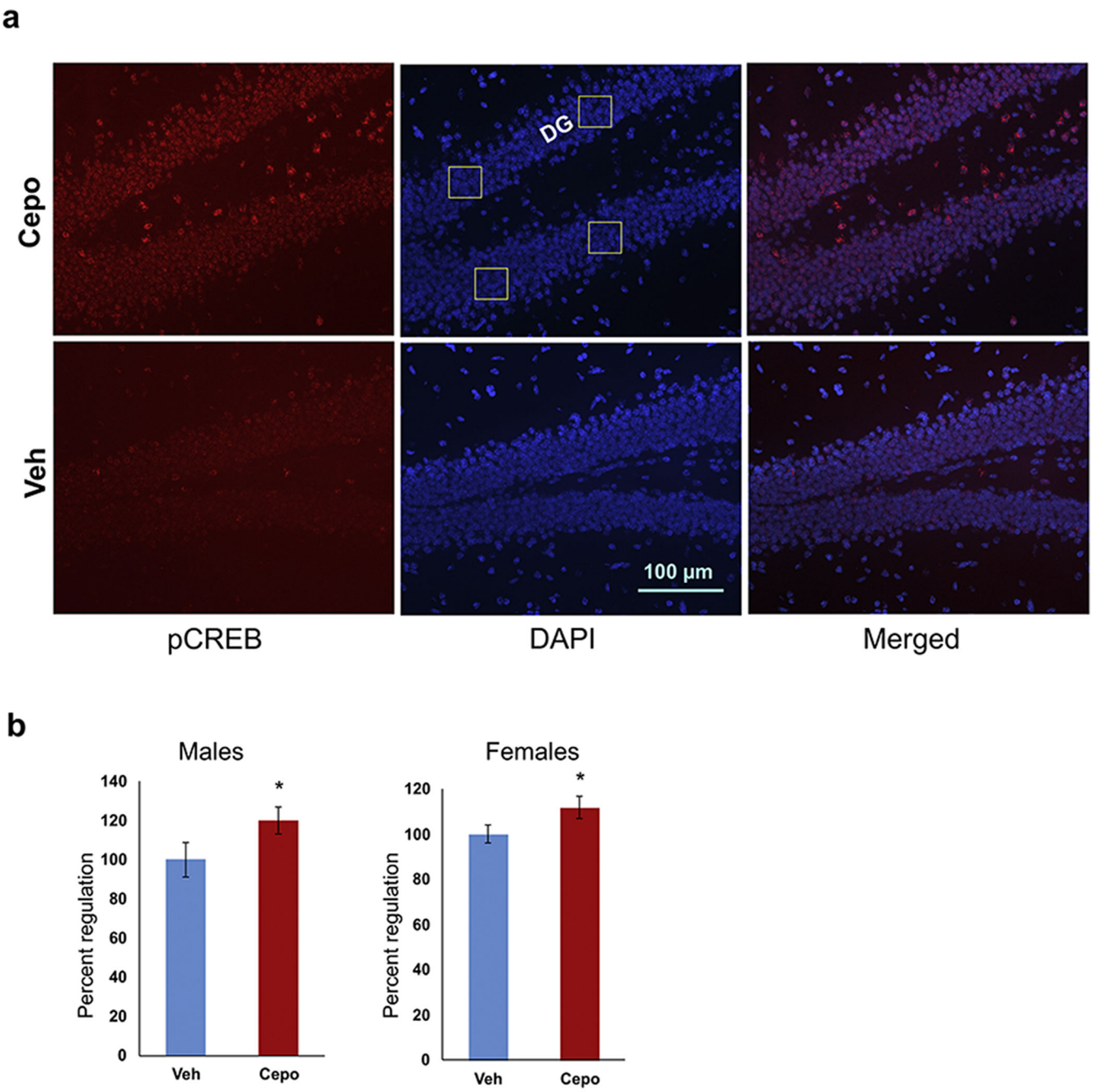

Figure 7. Immunohistochemical analysis of pCREB expression.

a) The expression of pCREB is shown in the hippocampal dentate gyrus (DG) of Cepo - treated (top row) and vehicle treated (bottom row). Square boxes represent region of interest areas used for quantitation. b) Bar graph of relative quantitation in Nikon NIS software (N=5). Error bars are ± SEM; *$p < 0.05$.