A comparative analysis of erythropoietin and carbamoylated erythropoietin-induced proteome profiles

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Abstract
In recent years erythropoietin (EPO) has emerged as a useful neuroprotective and neurotrophic molecule that produces antidepressant and cognitive enhancing effects in psychiatric disorders.

However, EPO robustly induces erythropoiesis and elevates red blood cell counts. Chronic administration is therefore likely to increase blood viscosity and produce adverse effects in non-anemic populations. Carbamyolated erythropoietin (CEPO), a chemically engineered modification of EPO, is non-erythropoietic but retains the neurotrophic and neurotrophic activity of EPO. Blood profile analysis after EPO and CEPO administration showed that CEPO has no effect on red blood cell or platelet counts.

We conducted an unbiased, quantitative, mass spectrometry-based proteomics study to comparatively investigate EPO and CEPO-induced protein profiles in neuronal phenotype PC12 cells. Bioinformatics enrichment analysis of the protein expression profiles revealed the upregulation of protein functions related to memory formation such as synaptic plasticity, long term potentiation (LTP), neurotransmitter transport, synaptic vesicle priming, and dendritic spine development. The regulated proteins, with roles in LTP and synaptic plasticity, include Neudesin, Chromogranin b, Cortactin, Elongation initiation factor 3a and Proteasome 26s subunit, ATPase. We examined the expression of a subset of regulated proteins by immunohistochemical analysis in mouse brain. The results of our study sheds light on potential mechanisms whereby EPO and CEPO produce cognitive enhancing effects in clinical and preclinical studies.

Introduction:
Erythropoietin (EPO) is a 165 amino acid glycoprotein with well-known roles in red blood cell production in the body. Besides erythropoiesis, EPO also acts as an important neurotrophic molecule in brain development. After brain injury, the levels of EPO have been shown to increase in the brain, where it functions as a neuroprotectant (1). Due to its neurotrophic and neuroprotective effects, EPO has been extensively tested in both preclinical and clinical CNS studies. Treatment-resistant depressed patients treated with EPO in a double-blind, randomized clinical trial reported improvement in depression scores and cognition (2). EPO clinical trials conducted in combination with brain imaging reported a positive correlation between memory improvement and reversal of brain matter loss in
specific hippocampal subregions of depressed patients (3). These studies indicate that EPO has antidepressant and cognitive enhancing effects.

Despite the promising results in psychiatric disorders, it is important to note that EPO has potent erythropoietic activity. Chronic administration to non-anemic patients can lead to increased blood viscosity and harmful vascular complications. A chemically engineered modification of EPO, Carbamoylated Erythropoietin (CEPO) has no erythropoietic effects (4) and produces comparable neurotrophic effects and behavioral effects as EPO (5, 6). Behavioral studies in mice treated with EPO and CEPO found improved performance in spatial and recognition memory tests which indicates that both molecules can improve cognition (7). However, the molecular mechanism involved in their cognitive actions is unknown. The goal of this study was to conduct an unbiased, comparative analysis of EPO and CEPO-induced protein regulation to obtain molecular insight into their mechanism of action. We utilized neuronal phenotype PC-12 cells that have been used extensively to investigate EPO signal transduction (8-10). EPO and CEPO treated tissue homogenates were subjected to label-free, quantitative proteome analysis. The proteomics data was then subjected to bioinformatics analysis to mine the data for signaling pathways, relationships and interactions with neurobiological significance. We performed secondary validation of the data using western blot and immunohistochemical analysis.

Materials And Methods:
Carbamoylation of EPO

Erythropoietin was purchased from Prospec Bio (Israel) and carbamoylated in 1 mg aliquots as previously reported (5, 11). Briefly, EPO was deprotonated in a high pH (pH = 8.9) borate buffer and then exposed to potassium cyanate for 16hr at 36 °C. CEPO was exhaustively dialyzed for 6hr against PBS. CEPO concentration was determined using the Qubit protein assay (ThermoFisher). CEPO purity was verified by silver staining after electrophoretic gel analysis.

Cell Culture

Rat pheochromocytoma cells (PC-12 cells) were obtained from American Type Culture Collection (ATCC). The cells were grown and cultured as mentioned previously with some modifications (12). The cells were grown in suspension in RPMI-1640 (ATCC) with 10% heat inactivated horse serum, 5% fetal
bovine serum (Gibco) at 37 °C and 5% CO₂. To differentiate the cells into neuronal cells PC-12 cells were plated in collagen coated dishes (Corning) and were grown in RPMI-1640 with NGF (100 ng/ml, Alomone Labs) and 1% Horse Inactivated serum (Gibco). The cells were grown for 10 days and the medium was changed every 2 days. Neuronal morphology and robust neurite outgrowth were confirmed by microscopy. NGF was removed overnight before the day of experiment. PC-12 cells were treated with EPO and CEPO 100 ng/ml for 5 hr. Vehicle-treated (PBS) cells were used as control.

Label-free quantitative proteome analysis

100 µg of protein per sample from six biological replicates per group was taken and detergent was removed by chloroform/methanol extraction, and the protein pellet was re-suspended in 100 mM ammonium bicarbonate and digested with MS-grade trypsin (Pierce) overnight at 37°C with. Peptides cleaned with PepClean C18 spin columns (Thermo) were re-suspended in 2% acetonitrile (ACN) and 0.1% formic acid (FA) and 500 ng of each sample was loaded onto trap column Acclaim PepMap 100 75 µm x 2 cm C18 LC Columns (Thermo Scientific™) at flow rate of 4 µl/min then separated with a Thermo RSLC Ultimate 3000 (Thermo Scientific™) on a Thermo Easy-Spray PepMap RSLC C18 75 µm x 50 cm C-182 µm column (Thermo Scientific™) with a step gradient of 4–25% solvent B (0.1% FA in 80% ACN) from 10-130 min and 25–45% solvent B for 130–145 min at 300 nL/min and 50°C with a 180 min total run time. Eluted peptides were analyzed by a Thermo Orbitrap Fusion Lumos Tribrid (Thermo Scientific™) mass spectrometer in a data dependent acquisition mode. A survey full scan MS (from m/z 350–1800) was acquired in the Orbitrap with a resolution of 120,000. The AGC target for MS1 was set as 4 × 10⁵ and ion filling time set as 100 ms. The most intense ions with charge state 2–6 were isolated in 3 s cycle and fragmented using HCD fragmentation with 35% normalized collision energy and detected at a mass resolution of 30,000 at 200 m/z. The AGC target for MS/MS was set as 5 × 10⁴ and ion filling time set 60 ms dynamic exclusion was set for 30 s with a 10 ppm mass window. Protein identification was performed by searching MS/MS data against the swiss-prot human protein database downloaded on Feb 13, 2019 using the in-house mascot 2.6.2 (Matrix Science) search engine. The search was set up for full tryptic peptides with a maximum of two missed cleavage sites.
Acetylation of protein N-terminus and oxidized methionine were included as variable modifications and carboxamidomethylation of cysteine was set as fixed modification. The precursor mass tolerance threshold was set 10 ppm for and maximum fragment mass error was 0.02 Da. The significance threshold of the ion score was calculated based on a false discovery rate of ≤ 1%. Qualitative analysis was performed using progenesis QI proteomics 4.1 (Nonlinear Dynamics).

Bioinformatics and Statistical analysis
Perseus software (version 1.6.6.0, Max Planck Institute of Biochemistry, Martinsried, Germany) was used to perform bioinformatic and statistical analysis (13). The normalized LFQ intensities were log2 transformed. Proteins with at least 70% valid values in each group were analyzed. Missing values imputation of protein intensities were performed from a normal distribution (width: 0.3, down shift: 1.8). In order to estimate the variabilities between biological replicates correlation analyses was performed. Column correlation heat map was drawn based on the Pearson correlation coefficients value obtained between biological replicates. Multiscatter plot was drawn based on Pearson correlation coefficients obtained from average LFQ intensity of each treatment group. In order to estimate the variabilities between biological replicates of the treatment sample a Principal Component Analysis (PCA) plot was generated using protein LFQ values as variables. PCA was done on logarithmized values without imputation. Multiple- sample test (one-way ANOVA), controlled by Permutation based FDR threshold of 0.05, was used to identify the significant differences in the protein among Control, EPO and CEPO. The logarithmized intensities values of significant proteins from ANOVA after z-score normalization were used for Hierarchical clustering using Euclidean distances. The resulting heat map can be interpreted based on color intensity. For enrichment analysis, Fisher exact test was computed on GO terms of significant proteins. Student’s T- test was performed for statistical analysis, and statistical filters were set with a p value of 0.05 to detect differential protein ratios between two samples. All those proteins that showed a fold-change of at least ± 1.3 and satisfied p ≤ 0.05 were considered differentially expressed were depicted in Volcano plot.

The proteomics data were analyzed using Ingenuity Pathway Analysis software (IPA, Qiagen) to find
the signaling pathways upregulated in the study.

Animals

Adult male Sprague-Dawley rats (n = 6 per group, mass 220-240 gm; Envigo) were pair-housed according to treatment group (Vehicle, EPO and CEPO) for the duration of the experiments. Rats were maintained on a standard 12hr light-dark cycle with free access to food and water. All procedures were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approval by the USD Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used. Rats received single daily i.p. injections of either vehicle (PBS), EPO or CEPO (30 µg/kg) for 4 consecutive days (5, 11). Five hours after the last dose animals were decapitated according to American Veterinary Medical Association guidelines. Brain samples were hemisected, one half of the brain was used to dissect out the hippocampus and other half was frozen on dry ice and then kept at -80°C for further use.

Blood analysis

Adult C57BL/6J mice (n = 6) were administered EPO, CEPO (30 µg/kg/day, i.p.) or vehicle (PBS) for a total of 10 doses over 12 days. Trunk blood was collected in Sarstedt lithium heparin tubes (CD300LH). Whole blood samples were analyzed using an IDEXX LaserCyte Dx Hematology Analyzer using the appropriate species-specific settings. Analyses was performed by trained laboratory technicians using two levels of control material.

Western Blot Analysis

Western blot analysis was used to quantify changes in phospho-signaling proteins (14). The hippocampus samples were homogenized in the RIPA buffer with the complete protease inhibitor cocktail (ThermoFisher). Homogenates (30 µg) were mixed with Laemmli sample buffer and then resolved by SDS–PAGE using 5-14% at 60V for 30 min followed by 90V for 2hrs. Proteins were blotted to nitrocellulose membranes, which were blocked by 1% BSA in Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.4, 0.9% NaCl) containing 0.1% Tween 20 (TBS-T) for 1hr at room temperature, and then probed overnight at 4 °C with primary antibodies diluted in TBS-T. Primary antibodies were Phospho AKT (ser473) (Cell Sig, 193H12, 1:1,000 dilution), Phospho-p44/42 MAPK (Erk1/2) (Cell Sig, 4370S, 1:1,000 dilution ) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling, 5174S,
1:1,000 dilution) which was used as a loading control. Membranes were rinsed and incubated with secondary antibodies for 1 hr. at room temperature. Secondary antibody AF680 goat anti rabbit (ThermoFisher, A21109, 1:1,000 dilution) was used to visualize the detected proteins by the Odyssey Infrared Imaging System. Semi-quantitative analysis was performed by Image Studio Lite 5.2.5. ANOVA was calculated using GraphPad-Prism. A difference was considered as significant when the p-value was less than 0.05 (p < 0.05). Data were reported as mean ± SEM.

Immunohistochemistry
Immunohistochemical analysis on hemisected rat brains was performed as previously described (15). Briefly, 16 µm coronal, cryocut sections were incubated overnight at 4°C with primary antibody (Grb2, Santa Cruz Biotechnology, 1:200; Cortactin, Thermofisher, 1:500; Pleiotrophin, 1:100) 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, Alexa-488 1:500) for 2 h at room temperature. Slides were then rinsed in PBS and coverslips secured 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 EPO, CEPO and vehicle (PBS)-treated rat brain sections were captured using identical exposure settings.

Results
Hematological analysis
Several hematological parameters were measured after EPO and CEPO administration (Table 1). EPO strongly elevated several of the measured values while CEPO was comparable to control values. EPO increased red blood cell (RBC) counts by 60% and drastically increased reticulocyte number which was 9 times higher than control. EPO also doubled platelet counts whereas platelet counts in the blood of CEPO-treated animals was similar to control.

Exploratory analysis of the LFQ data for EPO and CEPO treated neuronal cell culture
A total of 2216 proteins were identified, and 2121 proteins were quantified with at least 70 percent
valid values in each group. The reproducibility of the biological replicates was assessed by the Column correlation heatmap (Fig. 1A). The hierarchical cluster for the column correlation was derived by the Pearson correlation coefficient (PCC) values determined based on Label-Free Quantitation (LFQ) intensities (supplementary Table S1). The heatmap shows a very high correlation between the replicates of the same treatment. These data indicate that the sample replicates had a high degree of reproducibility. The Multi-scatter plot was derived from the PCC for average LFQ intensity values for the control, EPO and CEPO groups (Fig. 1B). The PCC values were above 0.92 for all the groups. The PCC value between EPO and CEPO average LFQ intensity (0.946) was higher than the PCC value between CEPO and control average LFQ intensity (0.925). This suggests a higher similarity in the proteomic pattern between EPO and CEPO as compared to CEPO and Control. All quantified proteins were explored by Principal Component Analysis (PCA) that displayed three different clusters according to their abundance variation (Fig. 1C). Principal component 2 which consists of 25.1% of the total variation and Principal component 3 which consists of 8.4% of variation leads to separation of control, EPO and CEPO samples into different principal components. The closed clustering of samples between these groups, suggest high consistency between each sample of control, EPO and CEPO.

Enrichment analysis of the LFQ data for EPO and CEPO treated neuronal cell culture

There were 605 significant proteins out of 2121 proteins after ANOVA analysis (supplementary Table S2). The Hierarchical clustering analysis (HCA) of the significant proteins was carried out to identify the protein groups with a similar expression pattern (Fig. 2A). The biological replicates of each treatment condition represent the column header. The cluster analysis also shows different regulation patterns for different groups of proteins based on treatment. The expression of proteins is either up/down regulated or remain unchanged. The HCA grouped all the significant proteins into seven main clusters. Cluster 1 consists of 21 proteins, cluster 2 consists of 13 proteins, cluster 3 consists of 184 proteins, cluster 4 consists of 9 proteins, cluster 5 consists of 9 proteins, cluster 6 consists of 201 proteins, and cluster 7 consists of 168 proteins. The profile plot for cluster 2 indicates protein group that is upregulated with CEPO treatment, cluster 7 indicates protein group that is upregulated with
EPO treatment and cluster 3 indicates protein group that is upregulated with both EPO and CEPO (Fig. 2B). We did enrichment analysis to find significant physiological functions regulated by these proteins. Using Fisher’s exact test, the enrichment analysis of different protein clusters for gene ontologies and pathways was done (Fig. 2C and Supplementary table S3). We found that cluster 2 was enriched for the function related to CNS myelination, astrocyte development, regulation of neurogenesis, axon development and activating transcription factor binding. Cluster 7 was enriched for the function related to the regulation of developmental growth, endothelial cell proliferation, positive regulation of LTP, ionotropic glutamate receptor binding and positive regulation of axon extension. Cluster 3 was enriched for function related to the regulation of neurogenesis, neurotransmitter transport, regulation of synaptic plasticity, memory and neurotrophic signaling.

The signaling pathways upregulated in EPO and CEPO treated neuronal cell culture

Ingenuity Pathway Analysis (IPA) was used to find the canonical signaling pathways that were significantly upregulated in EPO vs control and CEPO vs control (Fig. 3A and 3B, Supplementary table S4 and S5). There are many canonical signaling pathways related to memory formation that are significantly upregulated. There was significant increase in ERK/MAPK signaling with both EPO (-log p-value = 7.08) and CEPO (-log p-value = 7.08). There was significant increase in CREB signaling with both EPO (-log p-value = 3.26) and CEPO (-log p-value = 3.26). There was significant increase in Synaptic Long-term Potentiation signaling with both EPO (-log p-value = 7.36) and CEPO (-log p-value = 7.36). There was significant increase in synaptogenesis signaling with both EPO (-log p-value = 9.10) and CEPO (-log p-value = 9.10) EPO and CEPO treated rat hippocampal samples were used for western blot studies to further confirm for activation of PI3/AKT signaling and ERK/MAPK signaling results from the IPA analysis. We found a significant increase in phospho-AKT signaling molecule both in EPO and CEPO treated rats (Fig. 4A and 4B.). Also, we found a significant increase in phospho-ERK1/2 signaling molecule both in EPO and CEPO treated rats. (Fig. 4C and 4D).

Differentially expressed proteins in EPO and CEPO treated neuronal cell culture

EPO vs control 101 proteins out of 2121 proteins showed significant p-values after applying Student’s T-test. The significant proteins were plotted for the p-values and T-test difference. The T-test
difference was set as $\pm 0.379$ to get significant differentially expressed proteins with $\pm 1.3$-fold change. 77 proteins demonstrated $\geq 1.3$-fold increase expression whereas 11 proteins demonstrated $\leq -1.3$-fold decreased expression (Fig. 5A). For CEPO vs control, 209 proteins out of 2121 proteins showed significant p-values after applying Student’s T-test. The significant proteins were plotted for the p-values and T-test difference. The T-test difference was set as $\pm 0.379$ to get differentially expressed proteins with $\pm 1.3$-fold change. 63 proteins demonstrated $\geq 1.3$-fold increase expression whereas 126 proteins demonstrated $\leq -1.3$-fold decreased expression (Fig. 5B). Among the differentially expressed proteins, synaptic proteins such as Nenf, Chgb, Ctn, Camk1, Eif3a, Rplp2, Psmc3 were upregulated that have a role in synaptic plasticity and cognition (Supplementary table S6 and S7).

**Immunohistochemical analysis in brain tissue**

Qualitative analysis of in vivo, brain expression of EPO and CEPO-induced proteins was performed by immunohistochemical analysis using commercially available antibodies (Fig. 6). Although hippocampal sections were used, we examined the entire section for differential protein expression between the 3 experimental groups. Brain subregions exhibiting the highest differential regulation are shown. Growth factor receptor bound 2 (Grb2) expression was increased by both EPO and CEPO administration, specifically in the dentate gyrus (Fig. 6A). Cortactin was elevated only by CEPO and was most noticeable in the dentate gyrus molecular layer (Fig. 6B). Pleiotrophin expression was detected at low levels in cortical vasculature and was elevated only by EPO (Fig. 6C). Vascular cell phenotype was determined by morphology of staining (dotted ovals, Fig. 6C).

**Discussion**

The neuroprotective and neurotrophic actions of EPO have made it a useful molecule to investigate in clinical studies of neuropsychiatric disorders. As an FDA approved biologic drug that is widely prescribed to treat anemia, the safety profile is well documented. However, the potential for adverse hematological effects with chronic dosing is a major limitation for its use as a CNS drug. CEPO is devoid of erythropoietic activity and helps address this key limitation. Our results also show that CEPO had no effect on platelet counts, whereas EPO sharply elevated it. EPO’s effect on reticulocyte
number (9x higher than control) is rather striking. As with other hematological parameters, CEPO’s effect on reticulocyte number was comparable to controls. The apparent lack of hematopoietic cascade activation raises interesting questions regarding CEPO’s mechanisms of action in mediating behavioral effects which are comparable to EPO (6, 16). Gene expression studies have shown that EPO (16) and CEPO (11) share an overlap in the neurotrophic factors that they induce in the hippocampus, such as BDNF, VGF and neuritin. These three neurotrophic molecules have been previously shown to independently produce antidepressant-like and cognitive enhancing effects in rodent models (17–22).

Our comparative analysis of EPO and CEPO-induced protein expression profiles provides additional insight into their potential mechanisms of action. Both ligands elevated the expression of neurotrophic and neurogenic proteins. Interestingly, more classes of trophic factor molecules were induced by EPO than CEPO. Trophic factors such as Transforming growth factor beta (TGF b), Myotrophin and Neudesin were elevated only by EPO. This suggests that CEPO has a more limited trophic role in comparison to EPO which is known to be pleiotrophic. It is likely that this is due to differential activation of intracellular signal transduction cascades by EPO and CEPO.

Both molecules comparably induced MAPK and Akt in the rat hippocampus. These cascades could be involved in their behavioral effects as these kinase pathways have been strongly implicated in antidepressant-like activity (23, 24). The elevation of Growth factor receptor-bound 2 (Grb2), a crucial adaptor molecule that links growth factor receptors to intracellular signaling, provides additional support for the overlap in trophic factor signaling pathways induced by CEPO and EPO. It is tempting to speculate that CEPO (6) recapitulates EPO’s antidepressant (2, 3) effects by virtue of activating trophic signaling pathways but is non-erythropoietic because it does not induce the canonical Jak-STAT hematopoietic cascade. While the results from this study indicate an overlap in trophic pathways, we did not find evidence indicating selective activation of the hematopoietic pathway by EPO. Previous work that carefully examined the differences in hematopoietic signaling molecules induced by wildtype EPO and a non-erythropoietic mutant EPO, reported differences that were subtle and dynamic (25). Our studies, conducted at a single timepoint, were likely unable to capture these
Bioinformatics pathway analysis revealed the enrichment of CEPO-induced proteins with functions related to neurogenesis, synaptic plasticity, neurotransmitter transport, synaptic vesicle priming, LTP and dendritic spine development. Upregulated proteins included Nenf, Chgb, Ctttn, Camk1, Eif3a, Rplp2 and Psmc3. A neurotrophic molecule, Neudesin neurotrophic factor (Nenf), regulates hippocampal neurogenesis. Nenf knockout mice display a decrease in cell proliferation and newborn neurons in the sub granular zone (SGZ) of the hippocampus (26). A secretory protein present in synaptic vesicles, Chromogranin b (Chgb), promotes neurotransmitter release and differentiation of hippocampal neuronal precursor cells (27, 28). An F-actin binding protein, Cortactin (Ctttn), is present in dendritic spines in the hippocampus. During the synaptic activity, it causes changes in spine shape and size by interacting with actin filaments and supporting the induction of LTP. Additionally, Ctttn interacts with PSD-95, causing an increase in spine density and facilitates LTP and synaptic plasticity (29, 30). CEPO induced Cortactin specifically in the molecular layer of the dentate gyrus, which could indicate that CEPO’s actions prominently involve the hippocampus. Long term memory formation occurs due to an increase in synaptic strength and is facilitated by new protein synthesis. The key components of protein synthesis are elongation factors such as Eif3a that aid in the protein synthesis initiation step and ribosomal subunits (Rplp2) involved in protein translation (31–33).

The upregulation of ubiquitination and proteasomal proteins such as Psmc3 by both EPO and CEPO can seem counterintuitive. However, it is useful to note that synaptic plasticity causes rapid protein synthesis as well as protein degradation. Ubiquitination and proteasomal pathways play important roles in controlled protein degradation. Interestingly, inhibiting these degradative pathways can cause a reduction in potentiation and LTP formation (34, 35). Overall, the EPO and CEPO-induced protein expression profiles provide mechanistic insight into their behavioral actions, particularly the cognitive effects that have been reported in preclinical and clinical studies. Our study was focused on global protein expression changes that are essentially downstream from receptor activation and did not capture alterations that are transient and dynamic. In future studies aimed at understanding CEPO’s lack of hematopoietic effects it will be useful to focus on posttranslational modifications that regulate dynamic changes.
signal transduction. It is widely thought that CEPO signals via a betacommon receptor and EPO receptor heteromer rather than the EPO receptor dimer employed by EPO. Further studies are needed to understand this important ligand-receptor interaction and how it affects cellular signaling. The possibility of additional receptors and adaptor molecules should also be considered. A global phosphoproteome approach shortly after receptor activation has the potential to shed light on differential signaling pathway activation by EPO and CEPO.

Declarations

Ethics approval and consent to participate

Animal studies were approved by the University of South Dakota IACUC and firmly adhered to NIH guidelines.

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Author’s contributions

NT performed the western blot analysis, IPA and Perseus analysis and wrote the manuscript. MS performed the immunohistochemical analysis and was responsible for the hematological analysis. VK conducted the mass spectrometry analysis. SSN designed the study, interpreted the data and wrote the manuscript.

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Table
Due to technical limitations, Table 1 is provided in the Supplementary Files section.

Caption:
Table 1. Comparative analysis of hematological parameters. Whole blood from EPO and CEPO treated mice were analyzed using an IDEXX hematology analyzer. Mice were administered 10 doses of EPO or CEPO (30 µg/kg/day) in PBS. Data shown are mean values from N = 6.

Figures
Figure 1

Exploratory analysis of the LFQ data for EPO and CEPO treated neuronal cell culture. A. Hierarchical clustering of all samples based on Pearson Correlation Coefficients. Correlation values were color coded from blue to red, corresponding to lower or higher values B. Multi Scatter plot of Average LFQ values for Control, EPO and CEPO treated samples based on Pearson Correlation Coefficients. All Pearson Correlation Coefficient values were higher than 0.920 C. Principal component analysis (PCA) of the LFQ intensities obtained from the control, EPO and CEPO treated samples.
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Enrichment analysis of the LFQ data for EPO and CEPO treated neuronal cell culture. A. Heatmap of the significant proteins expressed in CEPO, EPO and Control samples. Hierarchical clustering was performed for rows, where rows represent one protein and column represent biological replicates. Significant proteins were calculated with multi-sample ANOVA test with a permutation-based cutoff of 0.05 was applied on the logarithmic intensities. Intensities values were color coded from green to red, corresponding to downregulation or upregulation. B. Profile plot for three selected clusters showing distinct behavior with respect to different treatments: Cluster 2 strongly expressed in CEPO treatment; Cluster 7 strongly expressed in EPO treatment; Cluster 3 strongly expressed in EPO and CEPO both. C. Enrichment analysis of protein annotations shows functional categories enriched in the three selected clusters 2, 7, 3. The enriched terms, the corresponding enrichment factor and p-value are shown.
Figure 2

Enrichment analysis of the LFQ data for EPO and CEPO treated neuronal cell culture. A. Heatmap of the significant proteins expressed in CEPO, EPO and Control samples. Hierarchical clustering was performed for rows, where rows represent one protein and column represent biological replicates. Significant proteins were calculated with multi-sample ANOVA test with a permutation-based cutoff of 0.05 was applied on the logarithmic intensities. Intensities values were color coded from green to red, corresponding to downregulation or upregulation. B. Profile plot for three selected clusters showing distinct behavior with respect to different treatments: Cluster 2 strongly expressed in CEPO treatment; Cluster 7 strongly expressed in EPO treatment; Cluster 3 strongly expressed in EPO and CEPO both. C. Enrichment analysis of protein annotations shows functional categories enriched in the three selected clusters 2, 7, 3. The enriched terms, the corresponding enrichment factor and p-value are shown.
Figure 3

The signaling pathways upregulated in EPO and CEPO treated neuronal cell culture. A) EPO vs Control. B) CEPO vs Control. The canonical pathways are represented on the x axis. The y
axis represents the significance scores as \(-\log p\)-value. The threshold line in dark green indicates the significance \((p<0.05)\) cutoff. The height of the bar shows the level of significance.
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Figure 4

EPO and CEPO treatment upregulate pAKT and pERK signaling in neuronal cell culture A. Western blot B. Graphical representation of western blot results showing increase in phosphorylated-AKT after 4 days of EPO and CEPO (30µg/kg) treatment in Sprague dawley rats. C. Western blot D. Graphical representation of western results showing increase in phosphorylated-ERK1/2 after 4 days of EPO and CEPO (30µg/kg) treatment in Sprague dawley rats. The data were reported as mean (±SEM) and P values <0.05 were considered as significant.
EPO and CEPO treatment upregulate pAKT and pERK signaling in neuronal cell culture A.

Western blot B. Graphical representation of western blot results showing increase in phosphorylated-AKT after 4 days of EPO and CEPO (30µg/kg) treatment in Sprague dawley rats. C. Western blot D. Graphical representation of western results showing increase in phosphorylated-ERK1/2 after 4 days of EPO and CEPO (30µg/kg) treatment in Sprague dawley rats. The data were reported as mean (±SEM) and P values <0.05 were considered as significant.
Differentially expressed proteins in EPO and CEPO treated neuronal cell culture. A. Volcano plot showing differentially expressed proteins between EPO vs control. The -log (P-value) is plotted against the Student T-test difference of EPO and control. The downregulated proteins in EPO are on the left and significant ones are labeled in blue; the upregulated proteins are on the right and significant ones are labeled in red. B. Volcano plot showing differentially expressed proteins between CEPO vs control. The -log (P-value) is plotted against the Student T-test difference of CEPO and control. The downregulated proteins in CEPO are on the left and significant ones are labeled in blue; the upregulated proteins are on the right and significant ones are labeled in red.
Figure 5
Differentially expressed proteins in EPO and CEPO treated neuronal cell culture. A. Volcano plot showing differentially expressed proteins between EPO vs control. The -log (P-value) is plotted against the Student T-test difference of EPO and control. The downregulated proteins in EPO are on the left and significant ones are labeled in blue; the upregulated proteins are on the right and significant ones are labeled in red. B. Volcano plot showing differentially expressed proteins between CEPO vs control. The -log (P-value) is plotted against the Student T-test difference of CEPO and control. The downregulated proteins in CEPO are on the left and significant ones are labeled in blue; the upregulated proteins are on the right and significant ones are labeled in red.
Figure 6

Immunohistochemical analysis of protein expression in rat brain. Rats were administered
EPO or CEPO for 4 days (30 µg/kg/day). Cryocut hippocampal brain sections were processed for immunochemical detection of 3 proteins in the 3 experimental groups, EPO, CEPO and Control (PBS). Representative images are shown from an N = 4. A - Growth factor bound 2 (Grb2), B – Cortactin and C – Pleiotrophin. Dotted ovals indicate vasculature in the cortex.

DG – dentate gyrus, DGml – dentate gyrus molecular layer, Ctx – cortex.
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Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

Table S7 (CEPOvsCt_Sig-1.3fold).xlsx
Table S2 (Anova analysis).xlsx
Table S4(EPOvsCont).xlsx
Table S5(CEPOvsCont).xlsx
Table S6(EPOvsCt_Sig-1.3fold).xlsx
Table S7 (CEPOvsCt_Sig-1.3fold).xlsx
Table S2 (Anova analysis).xlsx
Table S4(EPOvsCont).xlsx
Table S5(CEPOvsCont).xlsx
Table S6(EPOvsCt_Sig-1.3fold).xlsx
Table S1 (Pearson correlation coefficient values ).xlsx
Table 1.pdf
Table S3 (Enrichment analysis).xlsx
Table S1 (Pearson correlation coefficient values).xlsx
Table 1.pdf
Table S3 (Enrichment analysis).xlsx