STUDY OF ERYTHROPOIETIN AND CRYOMELT MN EFFECT ON EXPRESSION OF NEUROTROPHIC BDNF, NGF FACTORS

Abstract

The regulation of expression of neurotrophins and their receptors in the brain is currently considered as one of the most promising ways to affect some pathological CNS conditions which result in neurons' degeneration and death.

At the same time, some of the known to-date compounds that are capable of regulating the neurotrophins' expression in the brain, such as anti-depressants, possess certain negative adverse effects. Such undesirable effects restrict clinical use of the above compounds.

The study investigated the activity of drugs, named respectively Erythropoietin N1 and Erythropoietin N2, in regulating the expression of the neurotrophic factors BDNF and NGF both in vitro and in vivo.

Erythropoietin N1 is a modified form of human recombinant erythropoietin with the addition (1: 1) of the drug Cryomelt MN (5 mg / ml).

The Erythropoietin N2 is medicinal form of human recombinant erythropoietin with a brand name "Erythrostim".

It has been shown that in cultured rat neuroglial cortex cells the drug at a 3 nM concentration caused statistically significant (2 – 5-fold) stimulation of both BDNF and NGF expression after 1 and 4 hours from drug injection, as compared to control non-stimulated cells. The highest stimulation of the BDNF neurotrophic factor expression was found 4 hours after Erythropoietin N1 injection; it reached 340% compared to the control.

The effect of Erythropoetin N1 and Erythropoetin N2 on the expression of the BDNF and NGF factors in the rat's cerebral hemisphere cortex and hippocampus is demonstrated *in vivo*, 1 hour after the intra-peritoneal and intranasal introduction of 10000 units of EPO per 1 kg of the rat's weight. For Erythropoetin N1 and Erythropoetin N2 it is proved that in the dose of 10000 units per 1 kg of the weight the statistically significant expression stimulation of both BDNF and NGF factors takes place 2-3 times as in the rat's hippocampus, so the cerebral hemisphere cortex, at intranasal introduction. As for the hippocampus being especially important at neuro-degenerative diseases, a significant enhancement of both BDNF and NGF factors expression was noted only at intranasal introduction. For Erythropoetin N1 and Erythropoetin N2 it was proved that in 1 hour after intraperitoneal introduction of 10000 units per 1 kg of the rat's weight, no alteration of either BDNF, or NGF factors expression in hippocampus was detected.

Since BDNF possesses high neuroprotective and neurotrophic activities, it would be possible to create a pharmaceutical drug, taking Erythropoietin N1 as a base that could be active against demyelinating diseases at intranasal introduction.

Introduction

The neutrophins are a family of the neural tissue regulatory proteins that are synthesized by neurons and neuroglial cells. They promote differentiation and maintain viability and functioning of peripheral and central neurons by using autocrine and paracrine regulatory mechanisms. The neurotrophins regulate neuronal differentiation, induce dendrite ramification (arborization) and axone growth (sprouting) towards target cells. In the mature nervous system the neurotrophins regulate both short-term synaptic transmission and long-term potential transmission, thus partaking in execution of nervous system plasticity that is required for its normal functioning.

The mature active BDNF forms are represented by stable homodimers with molecular mass of about 28 kD. They support growth of spinal sensor neurons as well as survival and growth of motor neurons and sensor, ganglion, dophaminergic, cholinergic and GAMK-ergic neurons. The BDNF is primarily produced by the neuroglial cells of cortex and medulla as well as by the Schwann cells associated with peripheral motor neurons.

Materials and Methods

Materials and Reagents

We used the following reagents: L-glutamin, MEM, F12, DMEM, fetal bovine serum (ICN), sucrose, BSA, SDS, EDTA, NaOH, Na2CO3, Na2HPO4, NaCl, PPO, POPOP, benzamidine, PMSF, BDNF, Tris, insulin, transferrin, progesteron, putrescine, Na2SeO3, trifluoroacetic acid, heptafluorbutanoic acid, trichloroacetic acid, acetonitryl, polyethylenimine, D-glucose, L-glutamine, aprotinine, leupeptine (Sigma, Sigma-Aldrich), CuSO4, paraformaldehyde, Ca,Na-tartrate, hydrochloric acid, toluene (Reachim), CaCl2, Folin reagent (Merck), triton X-100 (“Ferak, Berlin”). The tissue culture plasticware was from Nunc and Costar companies. The manufacturers of other used materials and reagents are indicated in the relevant sections.

Erythropoetin N1- is a form of human recombinant EPO with low sialic acid characteristics that doesn’t contain human albumin. Its injection form is produced on the basis of patient for EPO stabilized solution based on 10% reopolyglukin solution. Form of production - 1 dose (1ml) shall be comprise 200 mcg, equivalent to 20 000 ME per 1 dose. Concentration of EPO (№1) used for the purpose of the given study shall be 0,2 mg ml in 10% reopolyglukin solution.

Erythropoetin N2- is a dosage form having trade name- Erythrostim. It contains highly refined (99, 5%) human recombinant EPO, human serum albumin, isotonic citrate buffer solution. Form of production - 1 dose (1ml) 20 000 ME in ampoules or flacks. Initial EPO №2 concentration decreased EPO №1 concentration by 10 times. 15 ml of EPO №2 dosage form was freeze dried, lysed by 1.5 ml of distilled water and diffused with the help of saline. As a result EPO№2 solution was produced having concentration equal to 0,2 mg/ml in saline containing about 10% serum human albumin.

Primary neuroglia cell culture

Primary neuroglia cell culture was obtained by a standard method (Cole, 1989). The Sprague-Dowly rats aged 1-3 days were asphyxiated by a 15 minute exposure to CO2 and submerged in 80% ethanol for one minute. All subsequent operations were carried out in aseptic conditions at 4-70C. Isolated brains were used for membranes-free cortex tissue isolation in Hanks salts buffer. The isolated tissues were washed once with Hanks salts buffer and transferred to the MEM/F12 (1:1) containing 20% fetal bovine serum and 2 мM L-glutamine. The tissue was mechanically dissociated into single cells. The cell suspension obtained was washed once with cell culture medium by centrifugation at 200 x g. Cells were plated at 200 000 cells/cm2 in poly-L-lysin-treated 75 cm2 tissue culture flasks and cultured in 5% CO2-incubator (5% CO2 and 95% air) at 370C in MEM/F12 supplemented with 15% fetal bovine serum, 6 g/l D-glucose, 2 мM L-glutamine, 25 mg/l insuline, 100 mg/l transferrine, 20 nM progesteron, 100 nM putrescine and 30 nM sodium selenite, 100 μg/ml gentamycin. Fresh cell culture medium was added every 3-4 days. Cells were split at a ratio of 1:3 after they had reached the confluent state (the confluency time was 1.5 – 2 weeks).

Preparation of the brain sections for study in vivo

Males of Vistar line rats having weight of up to 200 g. were used for experiments. The animals were kept in standard conditions in vivarium with free access to water and food and 12-hour light cycle (light is switched on at 9 am, switched off at 9 pm). All the conditions required for minimizing stress were met. Each coop contained 4 animals representing both experimental and control groups. Study drugs were in dose of 10000 units per 1kg of animal's weight were intra- peritoneally and intra-nasally introduced in one-time regime in the volume of 100 mcl. The equivalent volume of saline was intra-peritoneally and intra-nasally introduced to the control rats. Each group contained 4 animals. In an hour after the drug introduction the rats were killed by the CO_2 asphyxia; immediately after that the studied brain sections were excreted (front cortex and hippocampus). The excreted tissue was cryocauterily frozen and stored at –80^{o}C.

Investigation of the Erythropoietin N1 and Erythropoietin N2 effect on the BDNF mRNA and NGF mRNA levels of expression

Total RNA isolation

To this end we used cells after the 3rd passage. Cells were seeded at a density of 100 000/cm2 in poly-lysine-treated 6-well clusters using the above cell culture medium. After cells have reached the confluent state the growth medium was changed to a serum-free medium (the one without fetal bovine serum). After 48 h incubation cells were fed with sterile solutions of tested compounds (0.04 ml per well, 3 nM final concentration, 3 parallel measurements per one experimental point). As a control, an equal volume of 0.9 % NaCl in water was used. After indicated time intervals the culture medium was aspirated, cells washed with cold phosphate-buffered saline and total RNA was isolated by the phenol-chlorophorm technique using YellowSolve Kit (Clonogen, Russia) according to manufacturer's instructions. The RNA purity and concentration was determined spectrophotometrically, and the RNA samples with ratio of A260/A280 >1.6 were used in the following experiments.

Reverse transcription and PCR

For reverse transcription 1 μg of total RNA was taken in the reaction mixture containing 8 U/ml of Moloney Murine Leukemia Virus (M-MLV)-reverse transcriptase, 10 мМ dithiothreitol, 800 μM dNTPs, random hexamer primers (20 μg/ml) and the first-strand buffer (50 мМ Трис-HCl, 75 мМ KCl, 3 мМ MgCl2) in 25 μl volume. The reaction was for 1 hr at 37oC followed by 10 minutes at 700C. The cDNA samples were stored at -200C.

The BDNF mRNA expression level was estimated using quantitative real-time PCR (Mx3000P system, Stratagene) using highly specific ds-DNA-binding dye SYBR green I. The reaction was carried out in 25 μl reaction volume containing either 2 μl cDNA of the sample, or the standard, or water (negative control), 250 μM dNTP mix (deoxynucleoside triphosphates), 2.5 мМ MgCl2, 15 мМ Трис-HCl (pH 8.8), 50 мМ KCl, 0,5% glycerol, 0.1% Tween 20, the intercalating dye SYBR Green I, 1 U of Taq DNA polymerase with enzyme activity-inhibited antibodies ("Syntol", Russia), and 10 pmols each of sense- and anti-sense primers ("Syntol", Russia, Table 1). The PCR conditions were as follows: start - 5 minutes at 950C, followed by 40 cycles including melting for 30 seconds at 950C, annealing for 30 seconds at 680C and elongation for 30 seconds at 720C, with fluorescence detection at the end of every elongation step.

Table 1

Target gene	Primers sequence (direct, reverse)
β-actin	5'-CTACAATGAGCTGCGTGTGGC-3' 5'-CAGGTCCAGACGCAGGATGGC-3'
BDNF	5'-AGCCTCCTCTGCTCTTTCTGCTGGA-3' 5'-CTTTTGTCTATGCCCCTGCAGCCTT-3'
NGF	5'-TCAGTGTGTGGGTTGGAGAT-3' 5'-AGCCTGTTTGTCGTCTGTTG-3'

For PCR product specificity validation, at the end of amplification the reactions were cooled to 60^{o}C, and 20 minutes later the melting curves were obtained by heating the reaction to 950^{o}C at a rate of 0.030C/sec with continuous fluorescence detection. For calibration curves generation, the cDNA sample mix was sequentially diluted with DNAse-free water to obtain a series of standard solutions with known relative concentration of the corresponding product. By using the manufacturer's software we detected the cycle number that corresponded to the maximal amplification rate, produced the calibrating curve that plotted the number of cycles against relative product concentration, and calculated the relative concentration in unknown sample with its subsequent normalization to the concentration of β-actin.

STATISTICAL ANALYSIS OF THE RESULTS

The results were preceded using Jandel Scientific SigmaPlot program. One-way ANOVA was used to asses reliability of group means differences. The Figure below shows the average group values group with the standard error of the mean value (Mean± SEM).

One-way Anova method was used to assess the reliability of the differences of the average group values. The Figure below shows the average group values with the standard error of mean value (Mean+_ SEM). The following indications of reliability levels are used: *- $p< 0.05$, **- $p< 0.01$, ***- $p< 0.001$.

RESULTS AND DISCUSSION

As a result of the given experiments the ability of Erythropoetin N1 and Erythropoetin N2 to control the best studied BDNF (brain–derived neurotrophic factor) and NGF (nerve growth factor) neurotrophins in some parts of cerebrum was tested. Real – time quantitive diverse transcription-PCR (diverse transcription products polymerase chain reaction) was used to test the ability of

Erythropoetin N1 and Erythropoetin N2 to impact BDNF and NGF expression at mRNA genes level in rat's hippocampus and cerebral hemisphere cortex. The effect of Erythropoetin N1 and Erythropoetin N2 on expression of the neurotrophic BDNF (Figures 1, 3) and NGF (Figures 2, 4) factors *in vivo* in the rat's hippocampus and cerebral hemispheres cortex in 1 hour after intra-peritoneal and intra-nasal introduction of 10000 EPO units per 1 kg of the weight is shown below.

EPO (№1 and 2) used for study was equal to 0,2 mg/ml in the presence of reopolyglukin polymer stabilizers (№1) and serum human albumin (№2) ten folds exceeding EPO drugs concentration. It is worth noticing that, while for intra-peritoneal introduction 100 mcl is considered to be appropriate, for intra-nasal introduction it several folds exceeds the optimal dose (20 mcl). Due to big volume of intra-nasal samples introduced, the major part of the drug was excreted from the rat's nostrils or was abdominally ingested. Thus, the efficient EPO intra-nasally introduced dose was much lower then that introduced intra-peritoneally. Figures 3 and 4 charactering neurotrophic BDNF and NGF factors expression *in vivo* in the rat's cortex in 1 hour after the drugs introduction show compatible effect both for intra-peritoneal and intra-nasal introduction. Significant enhance of neurotrophic BDNF and NGF factors expression on the background of reduction the sample volume being introduced intra-nasally can be suggested. Besides significant enhance of EPO penetration into the rat's brain on the background of polymer stabilizers concentration (reopolyglukin or serum human albumin) in the drugs can be suggested.

We think that it is worthwhile to compare the drugs activity in rat's brain *in vivo* (Figures 3,4) with the data regarding Erythropoetin N1 and Erythropoetin N2 drugs impact on neurotrophic BDNF and NGF factors expression in rat's incubated cerebral hemisphere cortex astrocytus *in vitro* (Figures 5,6). Erythropoetin N1 in experimental conditions *in vitro* at 1-hour incubation showed much less activity (Figure 5) while under experimental conditions *in vivo.* Erythropoetin N1 at intra-peritoneal introduction showed much more activity in the rat's cerebral cortex. At the intra-nasal introduction of Erythropoetin N1 activity in the rat's cerebral cortex was rather high but was reduced to some extent in regard to Erythropoetin N1 that can be explained by conditions impeding hematoencephalic barrier passed by the drugs.

The results regarding Erythropoetin N1 and Erythropoetin N2 drugs impact on rat's hippocampus *in vivo* (Figures 1,2) are of great interest for analyzing of molecular mechanisms ensuring EPO activity at demyelinizing diseases. For Erythropoetin N1 and Erythropoetin N2 it was shown that in the dose of 10000 units per 1 kg of the rat's weight at intra-nasal introduction statistically significant 2-3times stimulation of both BDNF and NGF factors expression in the rat's hippocampus and cerebral hemisphere cortex. As for the hippocampus being crucially important for neuro-degenerative

diseases, the significant expression enhancement of both BDNF and NGF factors, specially at intra-nasal introduction was shown (Figures 1, 2). For Erythropoetin N1 and Erythropoetin N2 it was shown that in 1 hour after intra-peritoneal introduction of 10 000 per 1 kg of rat's weight in hippocampus no alteration of both BDNF and NGF factors was detected. We think that it's reasonable to investigate Erythropoetin N1 and Erythropoetin N2 impact on neurotrophic BDNF and NGF factors expression in rat's incubated hippocampus astrocytes *in vitro*. It shall ensure separation of the drug's stimulating activity *in vivo* at EPO interaction with hippocampus cells from the drugs overall effect.

It can be suggested that the detected diversity of *in vivo* effects on the hippocampus and frontal cortex can be explained by different rate of the drug penetration into various parts of brain at intra-peritoneal and intra-nasal introduction, as well as by the nonspecific character of this organ. Accordingly this effect can be demonstrated in hippocampus at intra-peritoneal introduction at other time (later) or provided that other (large) EPO doses are introduced intra-peritoneally. We consider it reasonable to analyze the dose and timely aspects of Erythropoetin N1 activity in hippocampus *in vivo*.

Maintaining of the neuron viability is BDNF-dependant process conducted by neuroglia cells. It was suggested that BDNF and NGF expression induced by Erythropoetin N1 might be considered as one of the major molecular mechanisms ensuring Erythropoetin N1 therapeutic action. Taking into consideration that BDNF demonstrates high neuroprotective and neutrotrophic activities it's possible to develop pharmaceutical capable to exhibit its activity in patients with demyelinizing diseases and to stimulate neurotrophic BDNF and NGF factors expression at the intra-nasal introduction. Thus, it can be stated that there can be synthesized a pharmaceutical based on Erythropoetin N1 and capable to stimulate BDNF and NGF neurotrophic expression at the intra-nasal introduction. Besides, it can be suggested that this drug exhibits antidepressant activity as it's proved that direct BDNF injection in laboratory animals hippocampus induces antidepressant effect.

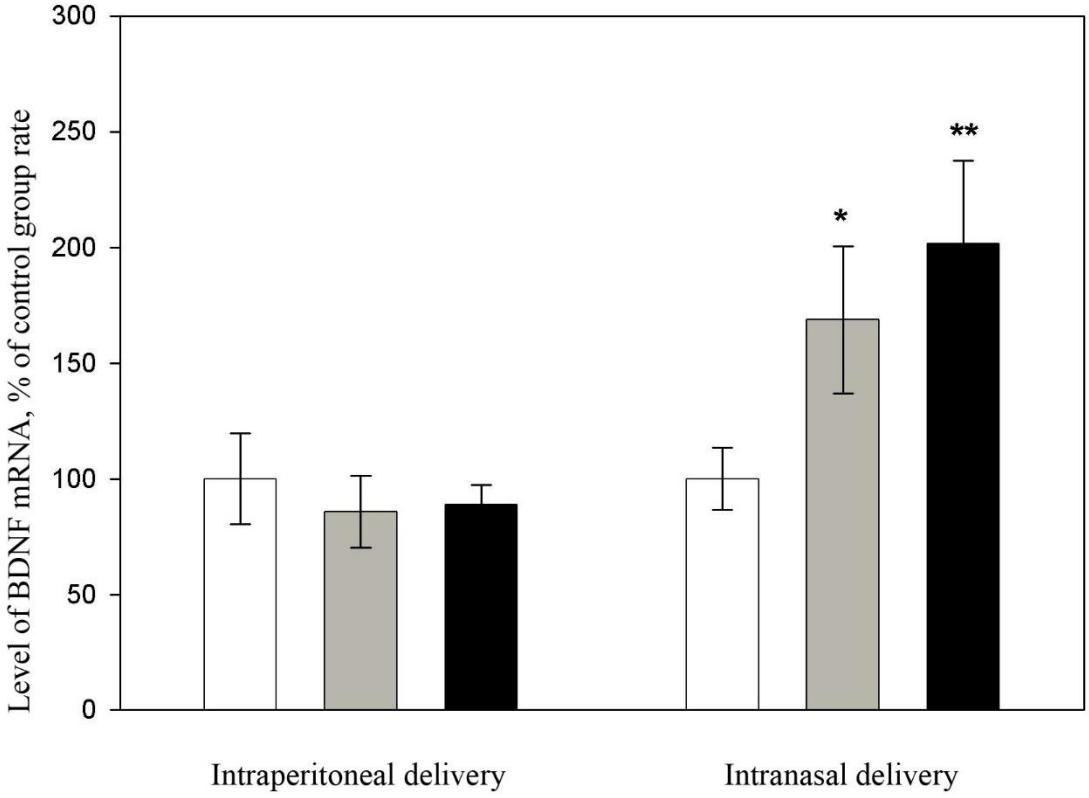

Figure 1. The effect of Erythropoetin N1 and Erythropoetin N2 on the expression of the neurotrophic BDNF factor *in vivo* in 1 hour after intra-peritoneal and intra-nasal introduction of 10000 EPO units per 1 kg of the weight in the rat's hippocampus.

(white column – control rate; gray column – Erythropoetin N1; black column – Erythropoetin N2 ratio *- $p < 0.05$, *- $p < 0.05$, **- $p < 0.01$).

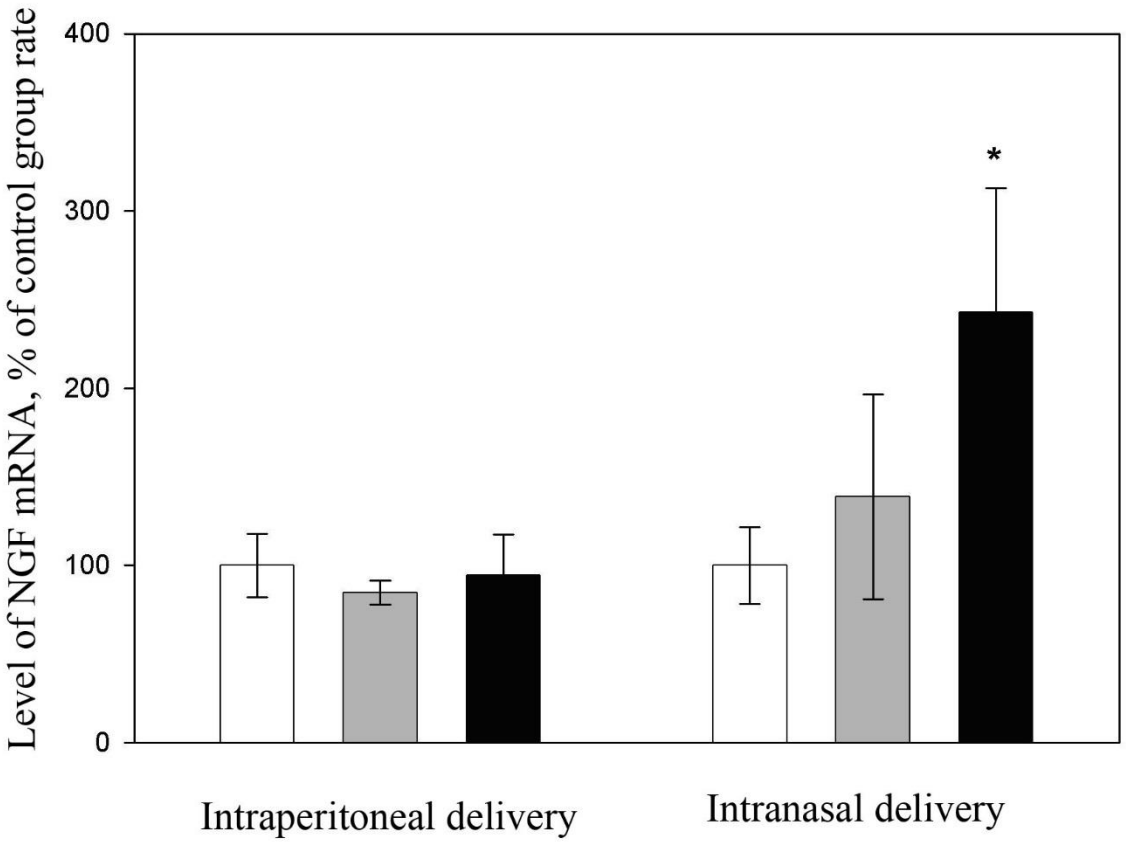

Figure 2. Effect of Erythropoetin N1 and Erythropoetin N2 drugs on neurotrophic NGF factor expression in vivo in 1 hour after the intra-peritoneal and intra-nasal introduction of 10000 EPO units per 1 kg of the weight in the rat's hippocampus.

(white column – control rate; gray column – Erythropoetin N1; black column – Erythropoetin N2 ratio *- $p< 0.05$, *- $p< 0.05$, **- $p< 0.01$).

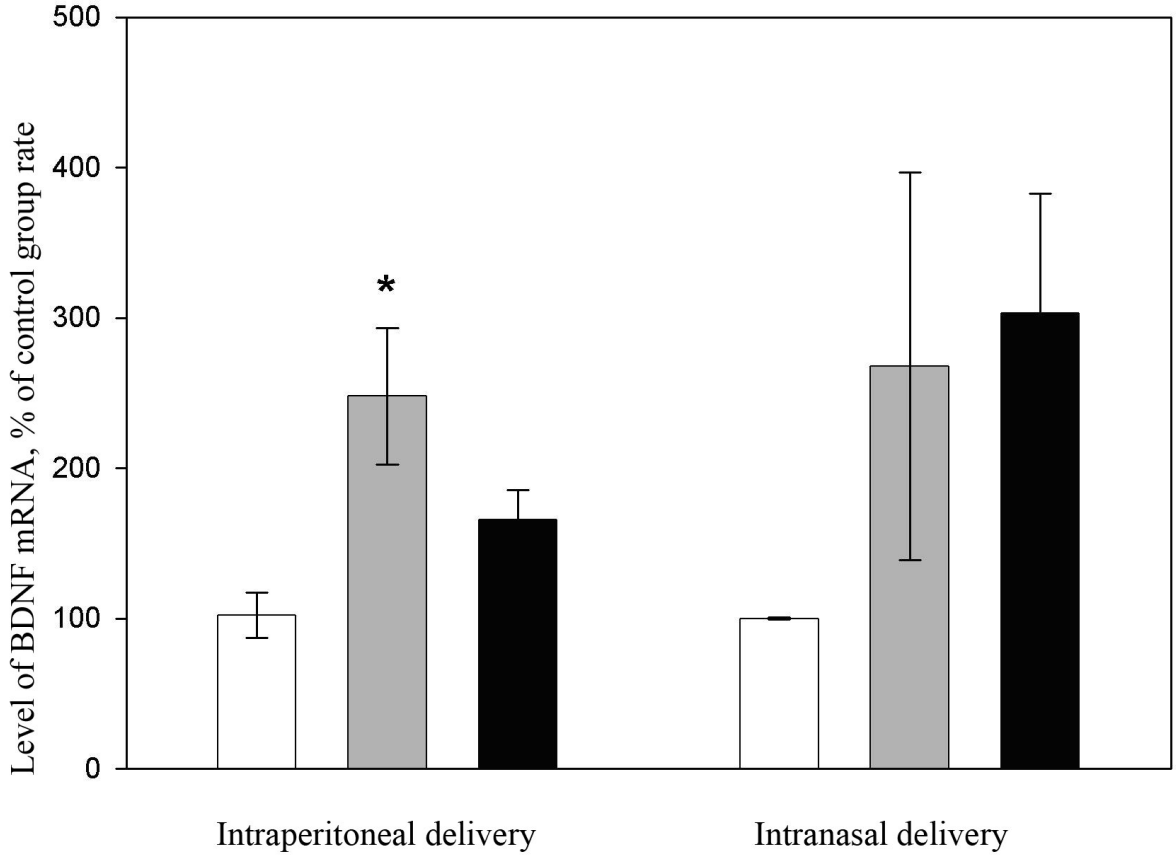

Figure 3. Effect of Erythropoetin N1 and Erythropoetin N2 on the expression of the neurotrophic BDNF factor in vivo in 1 hour after the intra-peritoneal and intra-nasal introduction of 10000 EPO units per 1 kg of the weight in the rat's cerebral hemispheres cortex.

(white column – control rate; gray column – Erythropoetin N1; black column – Erythropoetin N2 ratio *- $p < 0.05$, *- $p < 0.05$, **- $p < 0.01$).

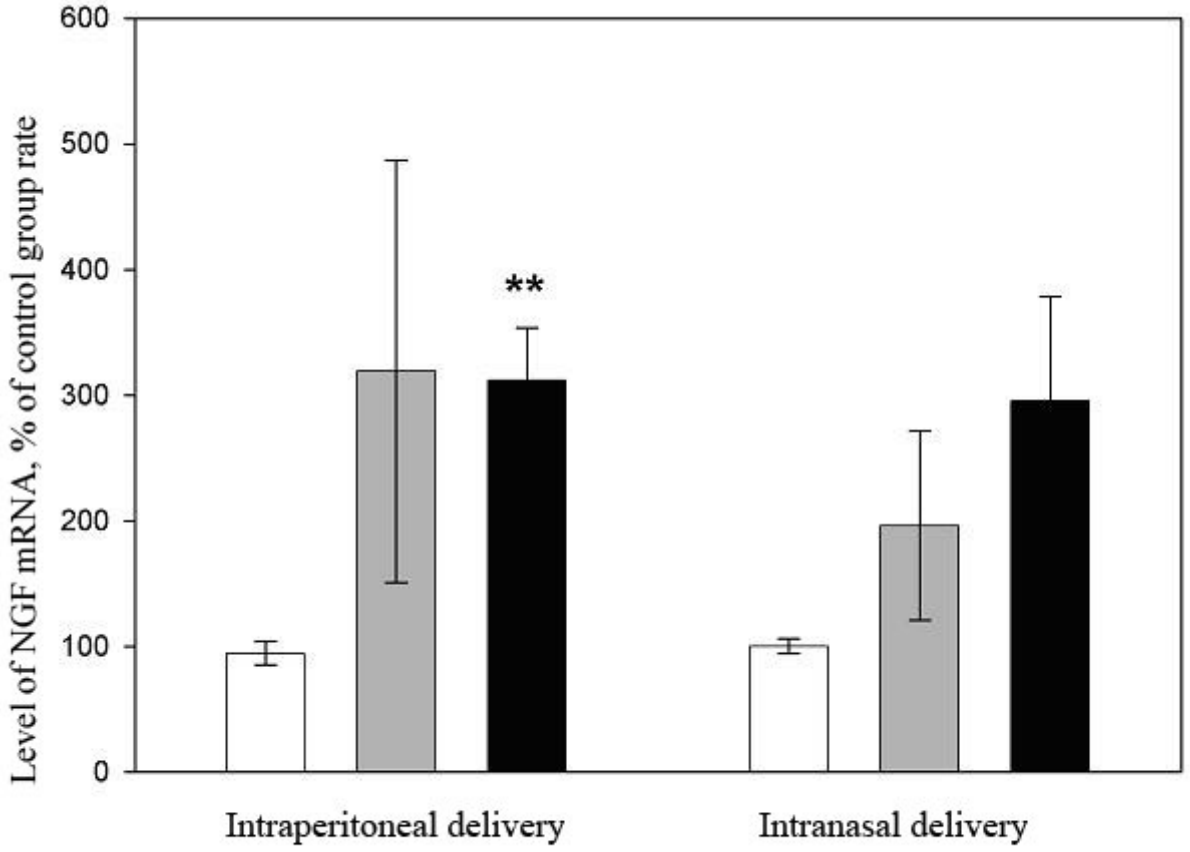

Figure 4. Effect of Erythropoetin N1 and Erythropoetin N2 on the expression of the neurotrophic NGF factor in vivo in 1 hour after intra-peritoneal and intra-nasal introduction of 10000 EPO units per 1 kg of the weight in the rat's cerebral hemispheres cortex.

(white column – control rate; gray column – Erythropoetin N1; black column – Erythropoetin N2 ratio *- $p < 0.05$, *).

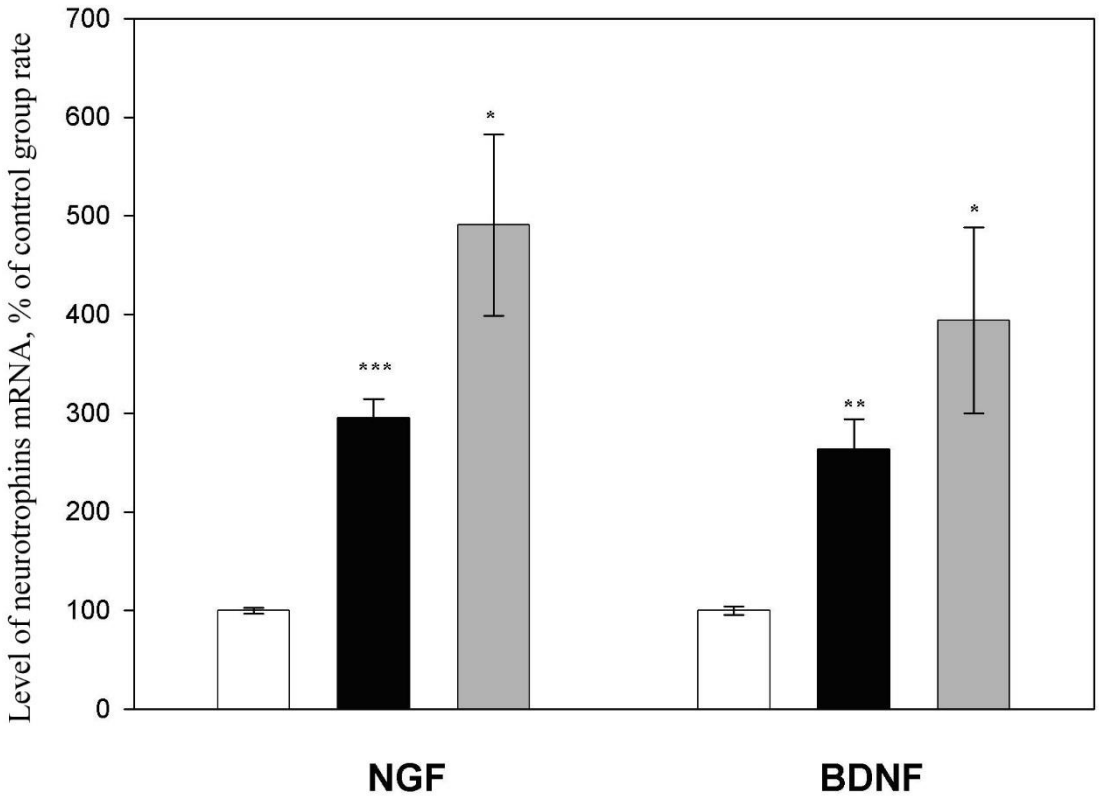

Figure 5. Effect of Erythropoetin N1 and Erythropoetin N2 in 3 nm dosing on neurotrophic BDNF, NGF factors expression in rat's cerebral hemisphere cortex astrocytus at 1 hour incubation (white column – control rate; black column – Erythropoetin N1; gray column – Erythropoetin N2; *- $p< 0.05$, **- $p< 0.01$, ***- $p< 0.001$).

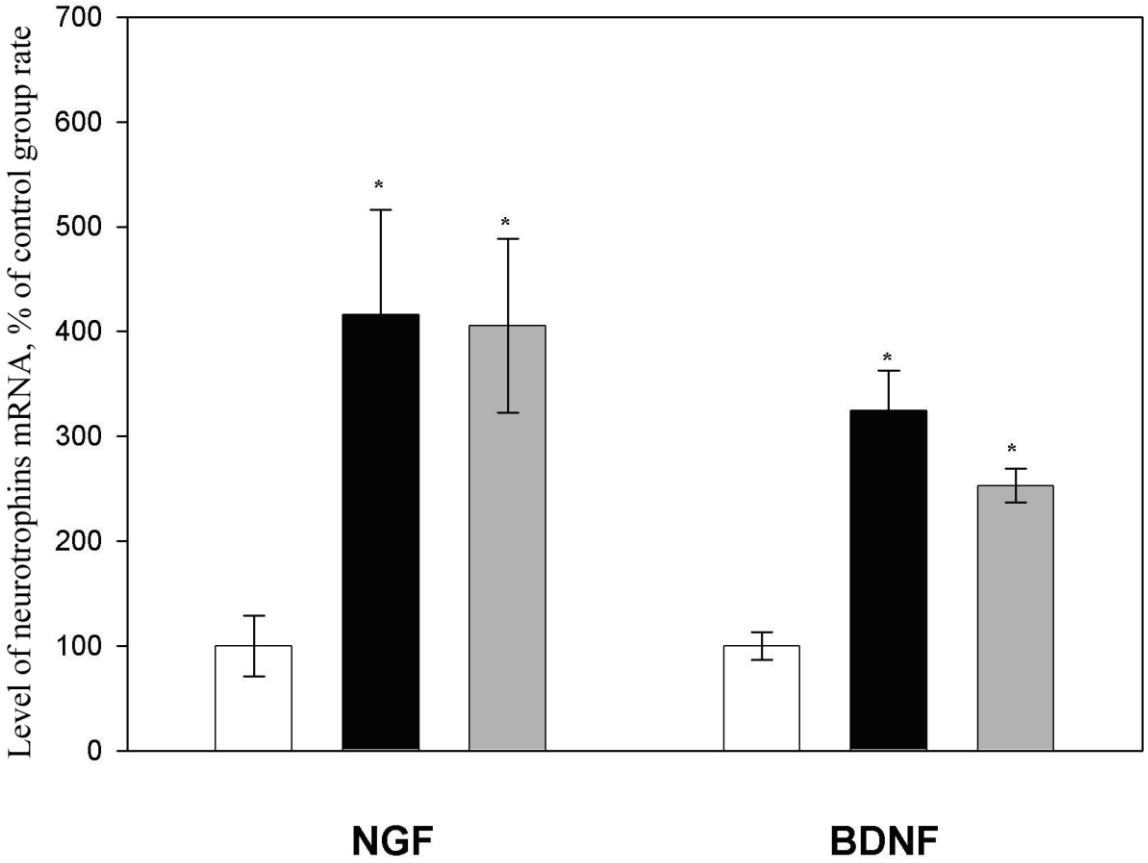

Figure 6. Effect of Erythropoetin N1 and Erythropoetin N2 in 3 nm dosing on neurotrophic BDNF, NGF factors expression in the rat's cerebral hemisphere cortex astrocytus at 4 hours incubation (white column – control rate; black column – Erythropoetin N1ratio; gray column – Erythropoetin N2; *- $p< 0.05$, **- $p< 0.01$, ***- $p< 0.001$).