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MORPHO-FUNCTIONAL PARAMETERS OF NEURONS IN THE SENSORIMOTOR CORTEX AND NEUROAPOPTOSIS UNDER CONDITIONS OF AN INDUCED EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN RATS AND A COURSE OF INTRANASAL GEL ADMINISTRATION CONTAINING N-PHENYLACETYL-L-PROLYLGLYCINE (NOOPEPT)

B. S. Burlaka[1], I. F. Bielenichev[1], S. G. Nosach[1], A. A. Nefedov[2]

Zaporizhzhya State Medical University[1]

SE «Dnipropetrovsk medical academy of Health Ministry of Ukraine»[2]

burlakabogdan@gmail.com

Burlaka B.S. https://orcid.org/0000-0003-4539-7331
Belenichev I.F. https://orcid.org/0000-0003-1273-5314
Nosach S. G. https://orcid.org/0000-0003-3844-9017
Nefedov O.O. https://orcid.org/0000-0002-5796-1852

Abstract

Purpose. The aim of the research is to evaluate a neuroprotective effect of the nasal dosage form containing Noopept (n-phenylacetyl-l-prolylglycine ethyl ester) by the degree of influence on morphofunctional parameters of neurons in the sensorimotor cortex of animals with experimental allergic encephalomyelitis.

Methods. The experiments were conducted on 260 outbred albino rats weighing 190-220 g which were obtained from the nursery of the State Institution «Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine». The quarantine (acclimatization period) for all animals lasted 14 days. An animal model of

experimental allergic encephalomyelitis (EAE) which has clinical manifestations and pathogenic mechanisms similar to multiple sclerosis was used for the study. There were five groups of animals in the experiment: 1) the intact group (10 rats); 2) the control group – non-treated subjects with EAE which received saline solution (20 rats); 3) the animals with EAE which received basic treatment – 3,4 mg / kg of Methylprednisolone (MP) intraperitoneally, administered slowly in saline solution with a volume of not more than 1/10 of the blood volume of a rat (20 rats); 4) the animals with EAE which received MP and a nasal gel containing n-phenylacetyl-l-prolylglycine at a dose of 10 mg / kg (20 rats); 5) the animals with EAE which received MP and Citicolinum (Ceraxon, «Ferrer Internacional S.A.», Spain) series D003U1, 500 mg / kg, intragastrically (20 rats). Morphometric studies were carried out with the help of Axioskop microscope (Ziess, Germany), a magnification of x40. The image of neurons in the area of the CA-1 zone of the hippocampus, obtained from the microscope using a highly sensitive video camera COHU-4922 (COCHU Inc., USA).

Results. It has been shown that the combination therapy with Methylprednisolone and an intranasal gel containing n-phenylacetyl-l-prolylglycine intensified neuroprotectivity in the animals with EAE. In particular, a neuronal density in rats with the experimental pathology, which received a combination of Methylprednisolone and a gel containing n-phenylacetyl-l-prolylglycine, increased by 14,7 ($p < 0,05$) and their area equaled in values with animals from the intact group; at the same time, the amount of RNA increased by 9,2%. In addition, in conditions of this experiment n-phenylacetyl-l-prolylglycine decreased density of apoptotic and destructive cells by 32,4% ($p < 0,05$) in relation to the control and the MP groups, and the amount of apoptotic neurons – by 44,6% ($p < 0,05$). Judging by effect on the neuronal density and the density of apoptotic and destructively altered neurons in the sensorimotor cortex of outbred albino rats with EAE, a combination of Methylprednisolone and a gel containing n-phenylacetyl-l-prolylglycine reliably exceeds the combined therapy of Methylprednisolone and Citicolinum and the MP monotherapy. A higher, according to some indicators, neuroprotective, nootropic, anxiolytic activity of a gel containing n-phenylacetyl-l-prolylglycine is due to its intranasal route of administration.

Conclusions: For the first time, we have obtained reliable data on the neuroprotective effect of the new dosage form containing n-phenylacetyl-l-prolylglycine (Noopept) – an intranasal gel – under conditions of an induced experimental multiple sclerosis. It has been shown that a course of intranasal administration of a gel containing n-phenylacetyl-l-prolylglycine (Noopept) at a dose of 10 mg / kg in combination with Methylprednisolone leads to a decrease in the death of neurons in the sensorimotor cortex of rats with EAE and an

increase in the amount of RNA in them. It has been shown that a course of intranasal administration of a gel containing n-phenylacetyl-l-prolylglycine (Noopept) at a dose of 10 mg / kg in combination with Methylprednisolone leads to neuroapoptosis inhibition – a decrease in the density of apoptotic and destructively altered neurons in the sensorimotor cortex of outbred albino rats with EAE. It has been found that judging by effect on the neuronal density and the density of apoptotic and destructively altered neurons in the sensorimotor cortex of the outbred albino rats with EAE, a combination of Methylprednisolone and a gel containing n-phenylacetyl-l-prolylglycine reliably exceeds the combined therapy of Methylprednisolone and Citicolinum and the MP monotherapy.

Key words: n-phenylacetyl-l-prolylglycine; neuroapoptosis; intranasal gel

INTRODUCTION

Recently, there has been a marked increase in the incidence of multiple sclerosis (MS) throughout the world. MS is a non-traumatic disease which often leads to disability and deterioration in the quality of life of the patients. To this day, the real causes of this pathology remain unknown. It is probably connected to a spurious gene interaction and environmental influences. There is a great possibility that such factors as low levels of vitamin D in serum, smoking, childhood obesity, infections – including the Epstein–Barr virus (EBV) – contribute to the disease development [1, 2, 3].

To date no universal medication for MS treatment has been created. The patient treatment is based on taking immunosuppressants and immunomodulators. A number of methods of treatment and disease modification have been developed with the purpose of reducing seizures and progression, aimed at the parameters of the inflammatory process. The use of monoclonal antibodies is considered as one of the new approaches to treatment of the pathology. The antibodies demonstrate activity in clinical trials in a primary progressive MS [4, 5]. The neuroprotection- and remyelination-oriented therapy directions deserve special attention. At present, the NMDA receptor antagonists, cholinomimetics, nootropics, drugs with neurotrophic effect and inhibitors of neuroapoptosis are being used as means of primary and secondary neuroprotective measures in MS. [5, 6]. N-phenylacetyl-l-prolylglycine (Noopept) is considered as a promising neuroprotective and nootropic agent, which has demonstrated good therapeutic efficacy in cerebrovascular disease and in neurodegenerative diseases [7]. When treating elderly patients with MS, it is especially important to make a right choice of dosage form and route of administration. We have developed a novel dosage form

containing N-phenylacetyl-l-prolylglycine (Noopept) – an intranasal gel which demonstrates advantages over reference drugs [8, 9].

The aim of the research is to evaluate a neuroprotective effect of the nasal dosage form containing Noopept (n-phenylacetyl-l-prolylglycine ethyl ester) by the degree of influence on morphofunctional parameters of neurons in the sensorimotor cortex of animals with experimental allergic encephalomyelitis.

MATERIALS AND METHODS

The experiments were conducted on 260 outbred albino rats weighing 190-220 g which were obtained from the nursery of the State Institution «Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine». The quarantine (acclimatization period) for all animals lasted 14 days. During the quarantine, each animal was examined daily (behavior and general condition); twice a day animals were observed in cages (morbidity and mortality). Before the beginning of the research, animals meeting the criteria for inclusion in the experiment were assigned to groups using a randomization method. Animals that did not meet the criteria were excluded from the study during quarantine. Cages with the animals were placed in separate rooms. The light conditions were the following: 12 hours of light and 12 hours of darkness. The temperature was maintained within 19-25°C, relative humidity – within 50-70%. Temperature and humidity indicators were recorded daily. The ventilation mode was set, providing about 15 room volumes per hour. Animals under the study were kept on same diet, in normal vivarium conditions. The animals were kept in standard cages – 5 rats in each cage. The diet included forage grain, bread and root vegetables (beets, carrots) [10].

An animal model of experimental allergic encephalomyelitis (EAE) which has clinical manifestations and pathogenic mechanisms similar to multiple sclerosis [11, 12, 13, 14] was used for the study.

The experimental allergic encephalomyelitis was induced by one-time subcutaneous inoculation of the encephalitogenic mixture (EGM) in Freund's Complete Adjuvant (FCA) at the rate of 100 mg of homologous spinal cord homogenate; 0,2 ml of FCA (a concentration of killed mycobacteria is 5 mg / ml) and 0,2 ml of saline per animal.

There were five groups of animals in the experiment:

1) the intact group (10 rats);

2) the control group – non-treated subjects with EAE which received saline solution (20 rats);

3) the animals with EAE which received basic treatment – 3,4 mg / kg of Methylprednisolone (MP) intraperitoneally, administered slowly in saline solution with a volume of not more than 1/10 of the blood volume of a rat (20 rats);

4) the animals with EAE which received MP and a nasal gel containing n-phenylacetyl-l-prolylglycine at a dose of 10 mg / kg (20 rats);

5) the animals with EAE which received MP and Citicolinum (Ceraxon, «Ferrer Internacional S.A.», Spain) series D003U1, 500 mg / kg, intragastrically (20 rats).

The medications were administered 2 days after the EAE induction: Methylprednisolone was administered over 7 days and a nasal gel containing n-phenylacetyl-l-prolylglycine and Citicolinum – over 14 days (a latent phase and an active phase till the end of the disease peak). Throughout the whole course, the rats from control and intact groups received saline intraperitoneally and intragastrically in similar volumes.

On the eighteenth day of the experiment, after pharmacological and physiological trials, the animals were withdrawn from the experiment under thiopental sodium anesthesia (40 mg / kg). The cerebrum of the experimental animals was fixed in Bouin's solution overnight and, after a standard histological tracing, the tissue was embedded in paraffin. To study the morphology of neurons, a rotary microtome was used. Sections 5 microns thick were made in the V-VI layer of the sensorimotor cortex. Sections of the hippocampus were dewaxed and stained with Einarson's gallocyanin-chrome alum stain for nucleic acid selectivity. Morphometric studies were carried out with the help of Axioskop microscope (Ziess, Germany), a magnification of x40. The image of neurons in the area of the CA-1 zone of the hippocampus, obtained from the microscope using a highly sensitive video camera COHU-4922 (COCHU Inc., USA), was introduced into the VIDAS Digital Image-Processing System, developed by Professor of the Chair of Pathophysiology of ZSMU, Doctor of Medicine A.V. Abramov. The image processing was performed in a semi-automatic mode.

The following parameters were determined:

- the density of neurons, apoptotic and destructively altered neurons (the number of cells per 1 mm^2 of the area of the cerebral cortex section),
- cellular composition in the region of IV-V cortex layers in percentage,
- area of bodies of neurons, apoptotic and destructively altered neurons (μm^2),
- the amount of RNA in neurons, apoptotic and destructively altered neurons (optical density, OD), which were calculated as the logarithm of ratio of the optical density of the cell body to the optical density of the intercellular substance,

- index of ratio of the number of surviving neurons to the number of apoptotic and destructively altered neurons.

Neurons demonstrating signs of karyopyknosis or cytolysis were considered degenerating. The density of the location of surviving and degenerating neurons, ratio of the number of intact neurons to the dying ones (a neurodegeneration index) and ratio of the density of surviving neurons when using the drug to the density of intact neurons in the control group (an improved survival index) were measured by software. As part of the dead neurons had been phagocytized by microglial cells before the histological study happened, the index of relative activity of microglia, equal to the quotient of dividing the difference in the density of surviving neurons by the difference in the density of degenerating neurons (the difference between the control group and the group receiving pharmacological drug), was evaluated separately. The value of the neurodegeneration index less than 1 indicated the prevalence of the number of dying neurons over the surviving ones; the index of improved survival and microglia activity more than 1 indicated a positive effect of the pharmacological drug, less than 1 – a negative effect. The functional state of surviving neurons was evaluated on the basis of changes in the area of the nuclei and nucleoli of neurons, the content of nucleic acids in them, the nuclear-cytoplasmic ratio, and the number of multinucleate cells.

Statistical processing of the results was conducted by means of the standard statistical package of the licensed program «STATISTICA® for Windows 6.0» (StatSoft Inc., № AXXR712D833214FAN5) and «SPSS 16.0», «Microsoft Excel 2016». Certain statistical procedures and algorithms were implemented as specially written macros for the corresponding programs. For all analysis types the differences at p-value <0,05 were considered statistically significant.

RESULTS

It has been found that the induced pathology resulted in damage to neurons in the sensorimotor cortex of the experimental animals. In particular, the rats from the group with EAE demonstrated a decrease in neuronal density by 19% ($p < 0,05$), which indicated the death of cells, and an increase in their area by 10%, which confirmed the development of their edema. In addition, under EAE conditions, a decrease in transcriptional processing in neurons in the sensorimotor cortex was recorded, as evidenced by a decrease in the RNA level by 21% ($p < 0.05$).

Table 1

Morpho-functional parameters of neurons in the sensorimotor cortex of the outbred albino rats with EAE, M ± m, n=10

The groups under experiment	*The neuronal density (neuron / mm^2)*	*Neuron area (μm^2)*	*The amount of RNA (OD)*
the intact group	1250,2 ± 25,5	83,0 ± 3,86	9,52 ± 0,33
EAE (the control group)	1006,7 ± 10,7	91,5 ± 3,93	7,45 ± 0,62
EAE + Methylprednisolone (MP)	1037,4 ± 6,8*	84,2 ± 2,73*	7,33 ± 0,44
EAE + MP + Citicolinum	1101,4 ± 7,4*^{2}	84,2 ± 2,11*	8,10 ± 0,42*^{2}
EAE +MP + n-phenylacetyl-l-prolylglycine	1154,1 ± 6,8*^{12}	84,7 ± 3,07*	8,14 ± 0,51*^{2}

*Note. * - $p \leq 0,05$ in relation to control indicators*
1 - $p \leq 0,05$ in relation to Citicolinum indicators
2 - $p \leq 0,05$ in relation to Methylprednisolone indicators

As the results demonstrate, the EAE induction has led to activation of neuroapoptosis. Thus, in the sensorimotor cortex of the animals with the experimental pathology an increase in density of apoptotic and destructive cells by 150% ($p < 0,05$) was observed (table 1). At the same time, the amount of apoptotic cells in the abovementioned cerebral part grew almost 5 times ($p < 0,05$). The administration of Methylprednisolone to the animals with EAE led to a reliably significant increase in the neuronal density in the sensorimotor cortex by 3% ($p < 0,05$) and a decrease in their area by 8% ($p < 0.05$), which indicated a direct neuroprotective effect of hormonal therapy. It should be noted that a Methylprednisolone administration in monotherapy affected neither functional neuron characteristics (the amount of RNA was practically unaltered), nor neuroapoptosis indicators.

It has been shown that the combination therapy with Methylprednisolone and Citicolinum intensified neuroprotectivity in the animals with EAE. In particular, a neuronal density in the rats with the experimental pathology, which received a combination of Methylprednisolone and Citicolinum, increased by 9,4% ($p < 0,05$) and their area equaled in values with animals from the intact group; at the same time, the amount of RNA increased by 8,7%. Judging by effect on some morpho-functional parameters, a combination of Methylprednisolone and Citicolinum reliably exceeds the MP monotherapy. In addition, in conditions of this experiment the density of apoptotic and destructive cells decreased by 19,5% ($p < 0,05$), and the amount of apoptotic neurons – by 42% ($p < 0,05$). Citicolinum is characterized by a pronounced mitoprotective effect which can be based on its

neuroprotective effect. In the works of professor I. F. Bielenichev et al. it has been shown that Citicolinum can maintain integrity of the inner mitochondrial membrane which is proved by restoring of its potential. Such mechanism is connected to the restoration of cardiolipin levels in the inner mitochondrial membrane. In addition, it has been revealed that Citicolinum regulates the level of reduced glutathione by indirect increase of the activity of glutathione-related enzymes (glutathione reductase and glutathione transferase).

Table 2

Density of apoptotic and destructively altered neuron cells in the sensorimotor cortex of outbred albino rats with EAE, M ± m, n=10

The groups under experiment	Density of apoptotic and destructively altered neuron cells per 1 mm^2	The amount of apoptotic cells, %
the intact group	59,4 ± 6,89	3,4 ± 0,96
EAE (the control group)	148,0 ± 16,4	15,7 ± 1,7
EAE + Methylprednisolone (MP)	141,6 ± 11,0	15,0 ± 1,0
EAE + MP + Citicolinum	119,2 ± 9,68*[2]	9,0 ± 1,0*[2]
EAE +MP + n-phenylacetyl-l-prolylglycine	100,1 ± 7,22*[1]	8,7 ± 0,7*[2]

*Note. * - $p \leq 0,05$ in relation to control indicators*
1 - $p \leq 0,05$ in relation to Citicolinum indicators
2 - $p \leq 0,05$ in relation to Methylprednisolone indicators

It has been shown that the combination therapy with Methylprednisolone and an intranasal gel containing n-phenylacetyl-l-prolylglycine intensified neuroprotectivity in the animals with EAE (table 2). In particular, a neuronal density in rats with the experimental pathology, which received a combination of Methylprednisolone and a gel containing n-phenylacetyl-l-prolylglycine, increased by 14,7 ($p < 0,05$) and their area equaled in values with animals from the intact group; at the same time, the amount of RNA increased by 9,2%. In addition, in conditions of this experiment n-phenylacetyl-l-prolylglycine decreased density of apoptotic and destructive cells by 32,4% ($p < 0,05$) in relation to the control and the MP groups, and the amount of apoptotic neurons – by 44,6% ($p < 0,05$). Judging by effect on the neuronal density and the density of apoptotic and destructively altered neurons in the sensorimotor cortex of outbred albino rats with EAE, a combination of Methylprednisolone and a gel containing n-phenylacetyl-l-prolylglycine reliably exceeds the combined therapy of Methylprednisolone and Citicolinum and the MP monotherapy. A higher, according to some

indicators, neuroprotective, nootropic, anxiolytic activity of a gel containing n-phenylacetyl-l-prolylglycine is due to its intranasal route of administration.

DISCUSSION

Intranasal administration ensures absorption of significant portion of substances into the blood, while smaller portion of substances enters directly into the brain via neurons in the olfactory tract using sensory nerves of perineural pathway. Then it spreads through the structures of the brain using mechanisms not related to blood flow. While there is no presystemic metabolism in the gastrointestinal tract and liver, the therapeutic effect is achieved more quickly, that is, there is a possibility of direct medicine entry into the brain. Mechanisms taking part in substance delivery from the nasal cavity to the brain have not been studied sufficiently, but for several: intracellular axonal pathway via the olfactory nerve, via the supporting epithelial cells, extra-neuronal pathway via the olfactory nerve. The peptides used are probably more characterized by extra-neuronal pathway via the olfactory nerve, through which short peptide molecules can enter the neural tissue directly in the intercellular space through the gap junctions between supporting cells and olfactory neurons. The mechanism of the neuroprotective action of n-phenylacetyl-l-prolylglycine (Noopept), in the form of an intranasal gel, is apparently due to the limitation of glutamate excitotoxicity, suppression of production of reactive oxygen species by neurochemical pathways of the neuron, as well as the effect on the expression of neurotrophic factors (NGF and BDNF) in the brain, cerebral cortex and hippocampus, as well as a decrease in proinflammatory cytokines - IL-1B, IL-6, TNF-a [6, 7, 15].

CONCLUSIONS:

1. For the first time, we have obtained reliable data on the neuroprotective effect of the new dosage form containing n-phenylacetyl-l-prolylglycine (Noopept) – an intranasal gel – under conditions of an induced experimental multiple sclerosis.

2. It has been shown that a course of intranasal administration of a gel containing n-phenylacetyl-l-prolylglycine (Noopept) at a dose of 10 mg / kg in combination with Methylprednisolone leads to a decrease in the death of neurons in the sensorimotor cortex of rats with EAE and an increase in the amount of RNA in them.

3. It has been shown that a course of intranasal administration of a gel containing n-phenylacetyl-l-prolylglycine (Noopept) at a dose of 10 mg / kg in combination with

Methylprednisolone leads to neuroapoptosis inhibition – a decrease in the density of apoptotic and destructively altered neurons in the sensorimotor cortex of outbred albino rats with EAE.

4. It has been found that judging by effect on the neuronal density and the density of apoptotic and destructively altered neurons in the sensorimotor cortex of the outbred albino rats with EAE, a combination of Methylprednisolone and a gel containing n-phenylacetyl-l-prolylglycine reliably exceeds the combined therapy of Methylprednisolone and Citicolinum and the MP monotherapy.

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