Application of the AAV-Syn-BDNF-EGFP Virus Vector as a Neuroprotective Agent in Modeling Hypoxia in vitro

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The aim of the study was to develop the AAV-Syn-BDNF-EGFP virus vector and study its effect on primary cultures in normal conditions and under hypoxia in vitro.

Materials and Methods. To produce the virus construct, AAV-Syn-EGFP, pDP5, DJ vector, and pHelper plasmids were used. The developed vector was tested on primary hippocampal cells obtained from C57BL/6 mouse embryos on the embryonic day 18 (E18). Infection of primary cultures with the developed virus construct was carried out on the 7th day of culture development in vitro (7 DIV).

The cell viability and spontaneous bioelectrical activity of the cultured cells were assessed on days 1, 3, and 7 after infection. On the 7th day after infection (14 DIV), spontaneous calcium activity of dissociated cultures was analyzed using functional calcium imaging. To evaluate the neuroprotective properties of the developed construct, in vitro hypoxia modeling was performed on the 14th day (14 DIV) of cultivation.

Results. The AAV-Syn-BDNF-EGFP adeno-associated virus vector carrying the sequence of the BDNF gene has been developed to increase this neurotrophin expression. Primary hippocampal cells infected by the vector were found to produce increased amounts of BDNF. The developed virus vector did not adversely affect the viability and functional activity of the neural networks in the primary hippocampal cultures. The BDNF overexpression enhanced the neuroprotective potential of cells subjected to oxygen deficiency.

Conclusion. The developed virus containing the BDNF gene sequence increases the endogenous BDNF expression by brain neurons, and thus reduces the death rate of nerve cells in the hypoxia model in vitro.

Key words: genetically engineered constructs; adeno-associated virus vectors; brain-derived neurotrophic factor; BDNF; cerebral ischemia; cerebral hypoxia; primary hippocampal cell cultures; neuroprotection.

Introduction

Ischemic stroke is characterized by severe damage to the central nervous system (CNS) after a short or prolonged occlusion of cerebral blood vessels. Currently, an active research into the effective strategies for the prevention and therapy of ischemic brain damage, and the ways to maintain the functional activity of neural networks are actively pursued. It should be noted that ischemic brain damage is a multifactorial disease. Hypoxia (oxygen deprivation), a decrease in the supply of energy substrates (glucose deprivation), and oxidative stress are the key detrimental factors of failed blood supply to the brain.

It has been shown [1–4] that an effective approach to correcting the ischemic brain damage is the use of the brain-derived neurotrophic factor (BDNF). This factor represents the family of neurotrophins — a key element in the brain embryogenesis and the subsequent postnatal period [5, 6]. BDNF is involved in the
modulation of synaptic transmission in the hippocampus and certain parts of the cerebral cortex [7–10]. Numerous studies indicate that exogenous neurotrophin increases the resistance of nerve cells to ischemic damage both in vitro and in vivo [11–14]. BDNF reduces neuronal death, limits the stroke volume, and accelerates neurological and cognitive recovery in post-ischemic animals [15, 16]. Another interesting feature of BDNF is its ability to preserve the neurons that are not part of neural networks in early ontogeny. Thanks to this effect of BDNF, in the post-ischemic period, those neurons that have lost their connections with other neurons remain alive.

One of the promising areas of today’s medical research is the development of biotechnological technologies that allow for delivery to the body of various neuroprotective compounds including virus vectors for gene therapy. This approach results in an increase in the level of the desired protein that lasts for a long time; this technology targets a strictly defined type of cells and allows one to avoid using a long-term drug therapy. Today the options of using virus vectors to deliver the BDNF gene to nerve cells in neurodegenerative diseases and neuro-traumas are actively studied. For example, Liu et al. [17] showed that the adeno-associated virus vector carrying the BDNF gene stimulates the growth of axons after a spinal cord injury. Others demonstrated favorable effects of the virus BDNF vector systems in retinal degeneration [18, 19], in Friedrich’s ataxia [20] and in Alzheimer’s disease [21]. However, there are reports showing difficulties of using such delivery methods. For example, in the work of Dekeyster et al. [22], no overexpression of BDNF was achieved after an attempted virus delivery in glaucoma.

Despite this, the initiation of BDNF expression with the help of a virus can be viewed as a new approach to the development of effective therapy of cerebral ischemic stroke. Such research is now at its initial stage worldwide. There are examples of a successful use of the modified AAV-BDNF virus [23]. When administered into the sub-ventricular zone of adult rats 14 days before an induced occlusion of the right middle cerebral artery, this treatment improved the recovery of the locomotor function after a stroke. This effect could be associated with an increased migration of neuro-progenitors in the brain of the experimental animals.

The aim of the study was to develop the AAV-Syn-BDNF-EGFP virus vector and study its effect on primary cultures of normal hippocampal cells and the cells under hypoxia in vitro. (The hypoxia model simulated the major factor of ischemia, i.e. ischemic hypoxia).

Materials and Methods

Plasmids. We utilized plasmids AAV-Syn-EGFP, pDP5, DJvector, and pHelper to develop the sought virus construct. The AAV-Syn-EGFP plasmid is derived from the bacterial pUC19 plasmid; it carries the sequences of the human synapsin (hSyn) promoter, the WPRE enhancer, and the SV40 polyA signal sequence flanked by the inverted terminal repeats (ITR) from the adeno-associated serotype 2 virus (AAV2). The other three plasmids (pDP5, DJvector, and pHelper) are helper plasmids required to assemble the adeno-associated virus vector that carries the Rep, Cap genes.

Isolation of total RNA. Isolation of total RNA from biological samples (mouse brain tissue) for subsequent production of cDNA encoding the BDNF protein was carried out according to the standard protocol using the Extract RNA reagent (Evrogen, Russia).

Polymerase chain reaction (PCR). A commercial kit of Phusion High-Fidelity PCR Kit containing Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, USA) was used to carry out the PCR for the generation of cloned fragments of the BDNF gene. The advantage of this polymerase is its high activity and accuracy. The reverse transcription PCR was performed using a MMLV reverse transcriptase (Evrogen, Russia).

Plasmid cloning. Purification of the resulting BDNF sequence was performed using the commercial Isolate II PCR and Gel Kit (Bioline, UK) according to the manufacturer’s protocol. For the construction of the plasmid, enzymes from Thermo Fisher Scientific (USA) and New England BioLabs (USA) were used. Restriction of the cloned BDNF fragment and AAV-Syn-EGFP-kid2 plasmid was performed with EcoRI and BamHI restriction enzymes to form sticky ends (Figure 1). A ligase mixture was further used to transform competent E. coli TopTEN cells.

Transformation of E. coli bacterial cells plasmid DNA, generation, and isolation of plasmid DNA. The competent cells of E. coli TopTEN were used for the transformation. The bacterial cells were kept on ice for 15–20 min, after which the plasmid cloned AAV-Syn-BDNF-EGFP was added in a volume of 1/10 of the cell volume and the mixture was incubated for 20 min on ice. The cells were then subjected to heat shock at 42°C for 45 s in a water bath and incubated on ice for 3 min.

After a 30–90-minute incubation at 37°C under continuous shaking (200–300 rpm) in a three-fold volume of the SOB medium for the cultivation of prokaryotic cells, the transformed cells were plated on Petri dishes containing LB medium (Luria-Bertani), agar and ampicillin (100 μg/ml). Cultivation on the plates was carried out at 37°C for 12–16 h, after which a part of the obtained bacterial colonies was further grown in liquid mini- and maxi-cultures. Then plasmid DNA was isolated using commercial sets of ISOLATE II Plasmid Mini Kit (Bioline, UK) and NucleoBond Xtra Midi/Maxi (MACHEREY-NAGEL, Germany) according to the manufacturer’s protocol.

Cultivation of the human embryonic kidney cell line HEK 293T and transfection. The conditions for the cultivation of the HEK 293T cell line were identical to those indicated in the culture certificate: DMEM; 10% bovine embryonic serum; 15 μg/ml gentamicin. The passage procedure: the cell removal with 0.25% trypsin
and 0.02% Versene (1:2–1:3), the seeding ratio of 1:2–1:3, and the optimal density (3.0–5.0) x 10^4 cells/cm². Cultivation was carried out in a CO₂ incubator at 37°C, 5% CO₂, 80% humidity.

The shuttle plasmid AAV-Syn-BDNF-EGFP and the helper plasmids pDP5, DJvector, pHelper were used for HEK transfection. The expression and helper plasmids were mixed in equimolar amounts (20 μg each) with the DMEM culture medium. A mixture of PEI/DMEM was added to the DNA/DMEM mixture and incubated for 15 min at room temperature, after which the volume of DMEM was adjusted to 15 ml. The culture medium of HEK 293T was replaced with a mixture of PEI/DNA/DMEM for 4–5 h at 37°C, 5% CO₂. After this, a complete replacement of the medium with DMEM containing 2% serum was performed and the cells were incubated for 3–5 days at 37°C, 5% CO₂.

**Isolation and purification of adeno-associated virus particles.** After transfection, the HEK 293T cells were removed from the substrate using phosphate buffered saline (PBS) containing no Ca²⁺, no Mg²⁺, then centrifuged at 1000 rpm for 5 min, and the precipitate dissolved in 2 ml of lysis buffer. The lysate was subjected to a freeze/thaw cycle (–80°C) three times. After the final thawing, the cell lysate was centrifuged at 2000 rpm for 5 min. Then, an enzymatic treatment with benzonase was performed to remove nucleotides and nucleic acids not protected by the virus capsid, centrifuged at 2000–2500 rpm for 5 min to sediment various protein granules and debris of large molecules, after which the supernatant was filtered through a 0.45 μm filter. Additional purification and concentration of the virus preparation were carried out using Amicon Ultra-15 columns (Millipore, UK).

**Cultivation of dissociated hippocampal cells.** The below studies were conducted on cultures of dissociated hippocampal cells obtained from E18 embryos of C57BL/6 mice. We followed the basic rules for the maintenance and care of experimental animals according to the Rules for the Work using Experimental Animals (Russia, 2010) and International Guiding Principles for Biomedical Research Involving Animals (CIOMS and ICLAS, 2012); the ethical principles established by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 2006). The permission to carry out experimental studies on animals was obtained from the Ethics Committee of the Lobachevsky State University of Nizhni Novgorod.

Enzymatic dissociation of embryonic hippocampal cells was carried out with a 0.25% trypsin solution (Gibco, USA). Culturing of primary cultures was performed in the Neurobasal™ medium (Thermo Fisher Scientific, USA) complexed with the B27 bioactive additive (Thermo Fisher Scientific, USA), L-glutamine (Thermo Fisher Scientific, USA), fetal calf serum (PanEko, Russia) according to the previously developed protocol [24] for 21 days on glasses coated with polyethyleneimine (Sigma, USA) to enhance the adhesion of cells to the surface. The initial density of the culture cells on the matrix was 9000 cells/mm². The cultures were maintained in a CO₂ incubator (MCO-18AIC, Sanyo, Japan) at 35.5°C and a gas mixture containing 5% CO₂.

**Immunocytochemistry.** Primary polyclonal chicken antibodies to BDNF and secondary polyclonal antibodies conjugated with a fluorescent label — Goat anti-Chicken IgY H&L (Alexa Fluor 647) (Abcam, USA) were used in the test. Cell cultures were fixed in a 4% solution of paraformaldehyde in PBS for 20 min at room temperature; a solution of 0.2% Triton X-100/PBS was used for cell permeabilization. It was performed the imaging of stained material using a Zeiss 510 NLO fluorescent confocal microscope (Carl Zeiss, Germany).

**Modeling of hypoxia (14 DIV) was performed by replacing the culture medium with a low oxygen medium for 10 min. Oxygen was removed by saturation of the culture medium with argon. The experiment was carried out in a sealed chamber, where air was also replaced by argon.

**Evaluation of cell viability in dissociated hippocampal cultures.** Cell viability was assessed on days 1, 3, and...
7 after the developed virus construct was added to the cells, and also day 7 after the hypoxia modeling. For this purpose, the number of cell nuclei stained with propidium iodide (Sigma, USA) (dead cells), and the nuclei stained with bis-benzimide (Sigma, USA) (all cells in culture) were counted. The cell death rate was calculated by the ratio of propidium iodide-positive to bis-benzimide-positive cells [24].

Functional calcium imaging. The metabolic activity of the hippocampal cells was evaluated with the help of calcium imaging, which reflects the changes in the concentration of cytoplasmic calcium. A specific calcium dye Oregon Green 488 BAPTA-1 acetoxymethyl ester (OGB-1 AM) (Invitrogen, USA) was used as a fluorescent probe. Staining was performed according to a standard protocol [25]. Fluorescence was recorded using a LSM 510 NLO laser scanning microscope (Carl Zeiss, Germany). The temporal images of the OGB-1 AM fluorescence field were recorded. The fluorescence was excited by an argon laser at a wavelength of 488 nm, and recorded using a light filter with a band of 500–530 nm. The change in fluorescence intensity (arbitrary units) of the selected vision field focused at the cell body was analyzed. Processing and analysis of calcium associated images were carried out with the help of the original software package Astroscanner (certificate of state registration of the computer program No.20114662670). The temporal function F (t) — the average OGB-1 AM fluorescence intensity — in the selected field versus time was also analyzed. The following parameters were taken into account: the duration (s), the frequency (number of calcium events/min), and the percentage of active cells in the culture [25].

Analysis of bioelectric activity. To study the changes in spontaneous bioelectric activity, multielectrode matrices MEA 60 (Multichannel Systems, Germany) and a set of Conductor™ software (Alpha Med Scientific, Japan) were used. Recording of the extracellular action potentials (7 DIV) was carried out before the addition of the virus construct; then the test was run daily for 7 days. The spikes (extracellular action potentials) were detected and recorded according to the following algorithm. A boundary of 8σ was chosen, where σ is the root-mean-square deviation. If the spontaneous peak exceeded the given threshold, the event was considered a spike; if the peak did not exceed the threshold it was recognized a noise and was not counted. The criterion of the “small network packet” was the appearance of spikes at least at four different matrix electrodes with an inter-spike interval within 100 ms. In data analysis, two spikes at least at four different matrix electrodes with an "small network packet" was the appearance of recognized a noise and was not counted. The criterion a spike; if the peak did not exceed the threshold it was exceeded the given threshold, the event was considered root-mean-square deviation. If the spontaneous peak were detected and recorded according to the following parameters were taken into account: the duration (s), the frequency (number of the "small network packet" was the appearance of recognized a noise and was not counted. The criterion a spike; if the peak did not exceed the threshold it was exceeded the given threshold, the event was considered root-mean-square deviation. If the spontaneous peak were detected and recorded according to the following parameters were taken into account: the duration (s), the frequency (number of calcium events/min), and the percentage of active cells in the culture [25].

Statistical analysis. The obtained data are presented as the mean ± standard error of the mean (M±SEM). The significance of the differences between the experimental groups was determined using the ANOVA package in SigmaPlot 11.0 (Systat Software Inc., USA). Differences were considered statistically significant at p<0.05.

Results

At the first stage of this study, we attempted to increase the expression of the neurotrophic factor BDNF in the neurons. To that end, the plasmid vector carrying the coding sequence of the BDNF gene was constructed and assembled using the molecular cloning techniques. The constructed plasmid vector served the essential basis for the virus. The following sequences were included in the plasmid vector (Figure 2): 1) the human promoter of the synapsin protein (hSyn), which is a strong promoter allowing the gene of interest expression only in neuronal cells; 2) the WPRE-enhancer (woodchuck hepatitis posttranscriptional regulatory element) — a regulatory element that significantly enhances the work of the hSyn synapase promoter; 3) the multilinker for the ORF (open reading frame) cloning of the inserted gene; 4) the EGFP gene, which can be cut out by appropriate restriction enzymes, if needed; 5) the SV40 polyA signal sequence flanked by ITR repeats — from the AAV of serotype 2; 6) a cassette of genes encoding the ampicillin resistance, which is necessary for the positive selection of the colonies that carry this plasmid (AmpR promoter and AmpR gene), and 7) a sequence corresponding to the nucleotide sequence encoding the BDNF functional protein.

In the study, the amplification of the neurotrophic factor BDNF was facilitated with the primer system mBDNF-EcoRI-fw and mBDNF-BamHI-rv (5’-ATTGAATTCATGGGCCACATGCTGTCC-3’ and 5’-AATGGAATCCACATCTCGCCTTTTATATGGCAGTG-3’), in which were integrated the restriction sites for subsequent restriction and ligation into the vector being constructed.

The temperature and reaction time were found out to allow for amplification of the oligonucleotide sequence of the neurotrophic factor BDNF. The initial denaturation was carried out at a temperature of 95°C for 1 min. Then, the working cycle of three procedures followed, repeating 30 times: 95°C — 15 s — denaturation, 65°C — 10 s — primer annealing, 72°C — 40 s — elongation. And after that, the final completion of the chains occurred at a temperature of 72°C for 7 min. As a result, the coding sequence for the neurotrophic factor BDNF was obtained. The constructed plasmid vector was generated from the AAV of serotype 2; 6) a cassette of genes encoding the ampicillin resistance, which is necessary for the positive selection of the colonies that carry this plasmid (AmpR promoter and AmpR gene), and 7) a sequence corresponding to the nucleotide sequence encoding the BDNF functional protein.

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The next stage of the work was the testing of the obtained virus vector with the primary hippocampal cultures. Using confocal microscopy we confirmed the expression of the green fluorescent EGFP protein in
cultures infected with the developed virus construct. To test the effectiveness of infection, immunocytochemical staining of cultures was performed using monoclonal antibody against BDNF (Figure 4). Notably, in cultures infected with the developed virus construct, the level of BDNF production was significantly higher than that in the intact cultures. This result implies that the developed virus construct effectively infects neuronal cells and stimulates the BDNF expression.

Since many viruses are known to kill neuronal cultures or impact the function of neural networks, the next stage of the study addressed the viability of primary hippocampal cell cultures; that was tested within 7 days after infection. It was found throughout the observation period that the number of dead cells in the experimental group of cultures did not differ from that in the intact controls, which was confirmed by the ANOVA comparison (Table 1).

To assess the effects of the virus construct on the neuronal network of the hippocampal cultures, the calcium-related cell function and the bioelectrical activity were assessed within 7 days upon the virus addition (experimental group) and compared with that of virus-free cultures.

Spontaneous bioelectrical activity was recorded immediately before the infection (7th day of cultivation (7 DIV)) and then daily for 7 days (up to 14 DIV). According to previously published data [27–29], this period is crucial for the formation of network connections in dissociated cultures in vitro. According to our results, the developed construct did not inhibit the spontaneous bioelectrical activity of the hippocampal cultures. In both intact and infected cultures, the spontaneous packet activity continued throughout the observation period; this pattern is characteristic for the in vitro neural networks at this stage of their development.

It was also found that upon the addition of AAV-Syn-BDNF-EGFP on day 7 of culture growth, the level of spontaneous bioelectrical activity did not significantly change, according to any of the measured parameters.
Table 1
The proportion of living cells (%) in the hippocampal cultures infected with the AAV-Syn-BDNF-EGFP virus (M±SEM)

<table>
<thead>
<tr>
<th>Days after the infection procedure</th>
<th>Intact cultures</th>
<th>Cultures infected with AAV-Syn-BDNF-EGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (8 DIV)</td>
<td>96.44±1.89</td>
<td>92.39±2.27</td>
</tr>
<tr>
<td>3 (10 DIV)</td>
<td>92.35±2.13</td>
<td>93.29±1.45</td>
</tr>
<tr>
<td>7 (14 DIV)</td>
<td>90.28±3.32</td>
<td>93.71±1.56</td>
</tr>
</tbody>
</table>

Note. The differences are statistically significant in comparison with intact cultures; ANOVA; p<0.05.
Figure 5. The raster graphs of spontaneous bioelectrical activity of the hippocampal cell culture in the post-hypoxic period:
(a) 1 day after infection (7 DIV); (b) 7 days after infection (14 DIV); left — intact culture; right — culture infected with the AAV-Syn-BDNF-EGFP virus

Table 2
Major parameters of spontaneous calcium activity on day 7 (14 DIV) after the culture was infected with the AAV-Syn-BDNF-EGFP virus (M±SEM)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intact cultures</th>
<th>Cultures infected with AAV-Syn-BDNF-EGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>The number of active cells (%)</td>
<td>73.54±10.23</td>
<td>83.10±11.55</td>
</tr>
<tr>
<td>Duration of Ca^{2+} oscillations (s)</td>
<td>7.80±0.55</td>
<td>7.60±0.82</td>
</tr>
<tr>
<td>Frequency of Ca^{2+} oscillations (oscillations per minute)</td>
<td>1.42±0.16</td>
<td>1.79±0.15</td>
</tr>
</tbody>
</table>
BDNF and the stimulation of intracellular BDNF expression (with the developed virus construct) helped prevent cell death in the post-hypoxic period. Thus, the proportion of living cells in the “hypoxia + BDNF” group was 81.6±4.1%, and the “hypoxia + AAV-Syn-BDNF-EGFP” group — 89.2±5.2% (Figure 6).

Discussion
It has been shown [1, 4, 6, 11] that BDNF has a pronounced neuroprotective effect against detrimental factors of ischemia, including hypoxia. The use of recombinant BDNF increases the resistance of nerve cells to the damaging factors of ischemia both in vitro [14, 24] and in vivo [2, 14, 30, 31]. These results rationalized the potential use of neurotrophic protectors in the clinical management of ischemic brain damage. There are studies confirming the role of BDNF in the CNS regeneration after the death of a significant part of functionally significant neurons [32]. However, systemic application of recombinant proteins causes just a temporary effect, which requires their permanent supply to the body. Therefore, targeted gene delivery to specific areas of the nervous system in the injury zone can be much more effective in the long-term treatment of CNS injuries [19, 20].

As a result of the present study, we have developed a virus construct containing the sequence of the BDNF gene that allows one to increase the expression of BDNF in brain neurons. In this report, we describe the functional capacity of this experimental product.

It is known that during cell cultivation, the newly formed neural networks undergo several characteristic changes in their morphology and functions [27–29]. Our in vitro study showed that the overexpression of BDNF had no significant effects on the bioelectrical or calcium-associated activities; it is, therefore, safe to use this approach to restore brain functions after ischemic damage. The developed virus preparation is not cytotoxic and does not negatively affect the hippocampal cell cultures.

The conducted experiments show that an increased expression of BDNF allows reducing the death of nerve cells caused by hypoxia in vitro. The demonstrated neuroprotective effect is comparable to the effect of the exogenously added recombinant BDNF. The obtained results provide the rationale for a further development of this method aiming to protect the brain against ischemic damage. The proposed approach may also find its use in the treatment of neurodegenerative diseases, where hypoxia plays a key role in the pathogenesis.

Conclusion
The adeno-associated virus vector AAV-Syn-BDNF-EGFP, carrying the sequence of the BDNF gene, has been designed to increase the expression of the BDNF neurotrophic factor in the brain. Primary cultures of mouse hippocampal cells infected with the developed vector increased their production of BDNF. The presence of this virus construct did not adversely affect the viability or functional activity of the neural networks formed in these cells cultures. The overexpression of BDNF increased the neuroprotective potential of cultured cells subjected to in vitro hypoxia.

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Conflict of interest. The authors declare they have no conflicts of interest that need to be reported.
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