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Optogenetic Stimulation Increases Level of Antiapoptotic Protein Bcl-xL in Neurons

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Abstract—The antiapoptotic protein Bcl-xL is associated with several neuroplastic processes such as formation of synapses, regulation of spontaneous and evoked synaptic responses, and release of neurotransmitters. Dependence of expression on activity of neurons is characteristic for many proteins participating in regulation of neuroplasticity. Whether such property is exhibited by the Bcl-xL protein was analyzed using *in vivo* optogenetic stimulation of hippocampal glutamatergic neurons expressing channelrhodopsin ChR2H134 under CAMKIIa promoter in the adeno-associated viral vector, followed by immunohistochemical determination of the level of Bcl-xL protein in these neurons and surrounding cells. Increase in the level of early response c-Fos protein following illumination with blue light was indicative of activation of these hippocampal neurons. The optogenetic activation of hippocampus resulted in a significant increase in the level of antiapoptotic protein Bcl-xL in the photosensitive neurons as well as in the surrounding cells. The dependence of the level of expression of Bcl-xL protein on the activity of neurons indicates that this protein possesses one more important property that is essential for participation in neuroplastic processes in the brain.

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Neurons and their networks can change their morphological and functional parameters in response to external stimuli, which is essential for the neural regulation of adaptive reactions of an organism to changing internal and external conditions. This most important property – neuroplasticity – is realized depending on the activity of brain cells and involves formation of new dendrites and spines, as well as synaptic contacts. Furthermore, the level of neuronal activity affects the efficiency of interneuron signal transmission via redistribution of receptor proteins in synaptic membranes and change in recycling of vesicles releasing neurotransmitters into the synaptic cleft. It was found that the antiapoptotic protein Bcl-xL was involved in some of these processes. It was established that in addition to its main function – protection of cells of all types from programmed cell death on the action of apoptogenic factors – it can increase the number, size, and activity of synapses, increasing the release of neurotransmitters, recycling of synaptic vesicles, as well as enhancing spontaneous and evoked synaptic responses [1-4]. These functions of Bcl-xL are characteristic for neuroplasticity-associated proteins that participate in the change in properties of neurons depending on their discharge activity. However, unlike the well-investigated products of the early response genes, which comprise most of this type of proteins [5-7], interrelation between the level of synthesis of the Bcl-xL protein and neuronal activity remains poorly explored. Investigation of such correlation is hindered by the fact that the expression of this protein is also controlled by many other stimuli associated, for example, with the protection of cells from death [8] or providing of energy sources under conditions of stress [9, 10]. One of the possible ways to solve this problem could be direct neuronal stimulation and evaluation of the expression of Bcl-xL protein in the stimulated neurons. The targeted activation of only marked neurons and the selective assessment of the level of the analyzed protein in them could be realized by the combined application of optogenetics and immunohistochemistry. In this study, we investigated the effect of optogenetic activation of hippocampal glutamatergic neurons on the level of the Bcl-xL protein in the neurons, assayed using immunohistochemical methods.

Abbreviations: AAV, adeno-associated viruses; ChR2 or ChR2H134, short and full designation of channelrhodopsin-2, respectively; PBS, phosphate buffered saline; PBST, PBS containing 0.1% Triton X-100; YFP and EGFP, yellow and enhanced green fluorescent proteins, respectively.

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MATERIALS AND METHODS

Viral vectors were constructed based on plasmids designed by the K. Deisseroth group and provided by Addgen (USA). Adeno-associated viruses (AAV) of the mixed 1-2 serotype were obtained by transfection of HEK29 cells by the mixture of plasmids for the virus assembly (pDP1 and pDP2), as well as a pAAV-CAMKIIa-ChR2H134-YFP plasmid carrying the sequence of the channelrhodopsin ChR2H134 and yellow fluorescent protein (YFP) using polyethyleneimine [11]. An AAV-CAMKIIa-EGFP vector encoding the green fluorescent protein similarly to the opto-vector, but not the photochannel, was used as a control. Virus particles were purified on heparin-Sepharose columns and concentrated using Millipore centrifuge concentrators to the titer of 10^{11}. The transgene expression in these vectors is under control of the CAMKIIa promoter, which is active only in glutamatergic neurons. Aliquots (5 µl) of viral vectors pAAV-CAMKIIa-ChR2H134-YFP and AAV-CAMKIIa-EGFP were injected into the brain lateral ventricles of 3-day-old rats from the control and test group under hypothermic anesthesia in a stereotaxic instrument as described previously [12, 13].

The effect of photostimulation on expression of the early response protein c-Fos and Bcl-xL protein in hippocampus was analyzed three weeks after injection of the vectors into the brain of the rats. For this purpose, animals from both groups that received injections with vector carrying the photochannel (ChR2) gene (test group) or only enhanced green fluorescent protein (EGFP) gene (control group) were anaesthetized with urethane and placed into a stereotaxic instrument. An optoprobe (A1 × 16–5mm–100–177–OA16LP; NeuroNexus Technologies, USA) was introduced into the CA1 field of the hippocampus through a small hole over the hippocampus, which allowed photostimulation of tissue (480 nm, 1.2 mV/mm^2, 20 pulses/s, 5 min, blue light source – photodiode; Thorlabs, USA) near the end of the optical fiber. Immunoreactivity of the c-Fos and Bcl-xL proteins in hippocampus was investigated 30 min after the completion of optical stimulation. For this purpose, the animals were anaesthetized with avertin and perfused transcardially with PBS containing 4% paraformaldehyde. Then the brain was post-fixed for 4 h in 4% paraformaldehyde, and 300-µm-thick brain slices were prepared with a vibratome (Microm, Germany).

Immunohistochemical protein identification was conducted using common procedures [7]. Slices were washed twice in PBS containing 0.1% Triton X-100 (PBST) for 15 min. Nonspecific binding was blocked by incubation of slices in 1.5% solution of bovine serum albumin in PBST for 2 h. Then the slices were incubated with primary antibodies (Cell Signaling, USA) using a 1 : 500 dilution overnight at 4°C. After that, slices were washed three times for 15 min in PBST and incubated for 4 h with secondary antibodies conjugated with Alexa Fluor 568 (Jackson Immunoresearch, USA) at dilution 1 : 500. Next the slices were washed, mounted on microscope slides, and embedded into medium with nuclear stain DAPI (4′,6-diamidino-2-phenylindole). The preparations were imaged using a LSM510META confocal microscope (Carl Zeiss, Germany) equipped with lasers with wavelengths 405, 488, and 533 nm. An image-processing program ZEN (Carl Zeiss) was used for image analysis. The total number of immunopositive cells for the target proteins (c-Fos or Bcl-xL) per 1 mm^2 was determined in 8-10 samples of each experimental group (test and control) both in the cells with fluorescent marker and without it. In addition, the number of cells expressing simultaneously one of the target proteins and the fluorescently labeled transgene ChR2 or EGFP was determined. The quantitative data were represented as mean value (M) ± standard deviation. The differences between the test and control groups in the total number of cells expressing the target protein as well and the number of cells expressing this protein simultaneously with the fluorescent expression marker of the AAV-vector were evaluated using Student's *t*-test, assuming statistically significant difference at $p < 0.05$.

RESULTS

Injection of the AAV-vectors carrying sequences encoding fluorescent proteins into the brain of neonatal rats resulted in the emergence of 35-40, on the average, fluorescently labeled cells per 1 mm^2 of hippocampus area illuminated with blue light 3 weeks after the procedure. The immunohistochemical staining of the early response protein c-Fos revealed its presence both in the photosensitive cells and in a small number of cells not containing the fluorescent label of the photosensitive channel ChR2 (Fig. 1). Illumination with blue light of the samples from the test group of animals, in which the vector encoding the ChR2 channel was injected, demonstrated that the total number of c-Fos-positive cells ($p < 0.03$), and especially the number of cells coexpressing c-Fos and the vector fluorescent marker ($p < 0.0001$) were significantly higher in comparison with similar preparations from the control group of animals that expressed only the EGFP fluorescent protein. The results of experiment presented in Fig. 1 indicates that the increase in the number of c-Fos-positive cells in the test group was achieved due to cells expressing this channel.

The immunohistochemical staining of the Bcl-xL protein also revealed its presence both in the photosensitive cells as well as in the cells not containing fluorescent label. The Bcl-xL protein determined via immunohistochemical staining in the preparations where some cells expressed vector with the ChR2 channel was also observed in many cells that were not labeled with the pho-

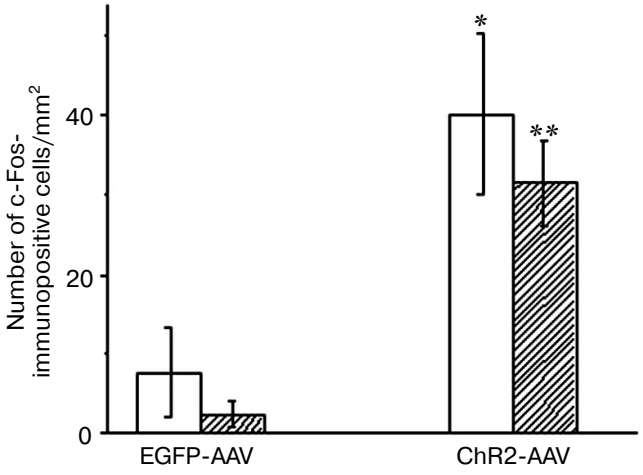

Fig. 1. Number of c-Fos-immunopositive cells on slides from animals injected with AAV-vectors encoding fluorescent protein (EGFP-AAV) or photosensitive ion channel (ChR2-AAV) after their illumination with blue light; empty bars – total number of c-Fos-positive cells; filled bars – number of c-Fos-positive cells coexpressing AAV-vector; * $p < 0.03$; ** $p < 0.0001$ in comparison with the respective indicators of the EGFP-AAV group.

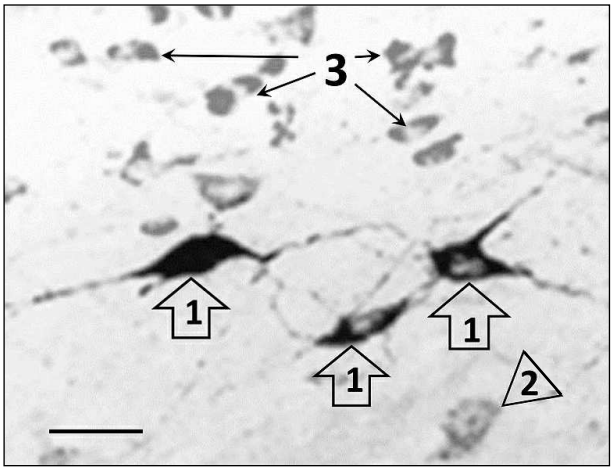

Fig. 2. Typical microphotograph of the Bcl-xL-immunopositive cells in the preparation treated with AAV-vector encoding photosensitive ion channel (ChR2-AAV) after illumination with blue light. 1) Cells coexpressing ChR2-AAV and Bcl-xL protein; 2) cell synthesizing only ChR2-AAV; 3) cells producing only Bcl-xL protein. Scale bar – 20 μm.

tosensitive vector (Fig. 2). In the samples from the test group of animals illuminated with blue light, the number of cells coexpressing the antiapoptotic protein and the vector fluorescent label ($p < 0.001$), as well as the total number of the Bcl-xL-positive cells ($p < 0.001$), were significantly higher than in the similar preparation from the control group of animals (Fig. 3).

DISCUSSION

The increase in expression of the early response protein c-Fos after stimulation of hippocampal glutamatergic neurons expressing the ChR2 photochannel under control of the CAMKIIa with blue light clearly indicates optogenetic activation of these neurons. The c-Fos protein is a transcription factor that is expressed in the neurons even without stimulation according to their spontaneous discharge activity [5, 6]. As shown in numerous studies, optogenetic stimulation of brain cells expressing channelrhodopsin increases the level of synthesis of this early response protein simultaneously with the increase in discharge activity of the neurons [14, 15].

In this work, we demonstrated that optogenetic stimulation of hippocampal glutamatergic neurons resulted in a significant increase in the level of production of the Bcl-xL antiapoptotic protein in the photosensitive neurons as well as in adjacent cells. This increase was observed less than one hour after the neuron stimulation onset and, thus, was in the time-frame for the induction of the early response proteins, for example c-Fos, which was found in many experiments including ours. The fast increase in the level of the Bcl-xL protein upon stimulation of neuronal activity together with its mentioned ability to increase the number, size, and activity of synapses, release of neurotransmitters, recycling of synaptic vesicles, as well as spontaneous and evoked synaptic responses [1-4] complement the spectrum of its properties characteristic for the proteins participating in neuroplasticity processes, changing the structure and properties of neurons during their activation. These properties can provide the basis of the

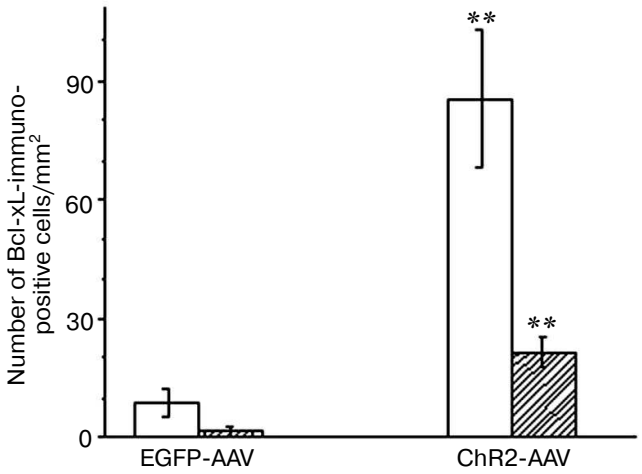

Fig. 3. Number of Bcl-xL-immunopositive cells on slides from animals injected with AAV-vectors encoding fluorescent protein (EGFP-AAV) or photosensitive ion channel (ChR2-AAV) after their illumination with blue light. Empty bars – total number of Bcl-xL positive cells; filled bars – number of Bcl-xL-positive cells coexpressing AAV-vector; ** $p < 0.001$ in comparison with the respective indicators of the EGFP-AAV group.

observed earlier association of the increased expression of this protein with psychoemotional and neurochemical stability under conditions of the short-term stress as well as under the action of antidepressants [16-19].

The pathway from depolarization of cell membrane caused by the release of positively charged ions during activation of the channelrhodopsin ChR2 by blue light [20] to the change in gene expression in the neuron is under active investigation, but it is still a poorly understood complex of intracellular events. The influx of calcium ions due to the discharge activity of the neuron and initiation of the Ca^{2+}-dependent kinase cascades followed by the stimulation of transcription factors by the kinases comprise the recognized main stages of this process [21]. For example, the transcription factor CREB participates in the regulation of c-Fos protein expression, which is dependent on neuronal activity [22]. Another participant of the rapid regulation of gene expression on the increase in the frequency of neuron firing is transcription factor NF-κB [23], which regulates the synthesis of the Bcl-xL protein together with c-Fos [24, 25]. However, it must be mentioned that participation of these transcription factors in the change in the expression level of Bcl-xL protein with increasing neuronal activity found in this work is only suggested and needs further experimental validation.

The fact that the light-mediated increase in Bcl-xL protein content in the non-light-sensitive cells was observed in preparations containing neurons expressing photosensitive channel is intriguing. This at first glance unexpected result can be explained by sensing of the increase in the light-sensitive neuronal activity induced by light by the adjacent cells. For example, astrocytes are sensitive to the activity of neurons located in their vicinity. Neuronal stimulation causes changes in the electrophysiological characteristics of astrocytes as well as of the concentration of Ca^{2+} in them during cultivation of these cells and brain slices *in vitro* [26]. The astrocytes in hippocampus and, in particular, in its CA1-field respond to the stimulation-induced changes of Ca^{2+} concentration in intracellular space by generation of the so-called "calcium wave", which spreads fast and thus involves additional cells that do not have direct contact with the initial stimulus into the area of its effect [27]. Considering that the first stage of activity-dependent induction of gene expression in the cell is the increase in concentration of Ca^{2+} in cytoplasm [21], it is quite possible that the "calcium wave" is a cause of the increase of the level of Bcl-xL protein in the cells lacking the photochannel.

Overall, the results of this study indicate that the expression of the Bcl-xL protein increases in the photosensitive hippocampal glutamatergic neurons very fast, by one order of magnitude in less than 1 h, during their optogenetic stimulation. The dependence of the expression level of the Bcl-xL protein on neuronal activity, which is first demonstrated in this work, supplemented the set of its properties as a participant of brain neuroplasticity processes.

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