UDC 53.047

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OPTOGENETIC APPROACH ALLOWS CONTROLLING MORPHOLOGICAL PARAMETERS OF DENDRITIC SPINES IN CORTICO-STRIATAL CO-CULTURE

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ОПТОГЕНЕТИЧЕСКИЙ ПОДХОД ДЛЯ КОНТРОЛЯ МОРФОЛОГИЧЕСКИХ ПАРАМЕТРОВ ДЕНДРИТНЫХ ШИПИКОВ В КОРТИКО-СТРИАТНОЙ КУЛЬТУРЕ

Optogenetics is a unique technique that allows controlling the physiological condition within single cell or specific cell population, including controlling individual neuron activity. In the present work optogenetics has been applied to control the morphology and density of synaptic connections between striatal and cortical neurons in mixed cortico-striatal co-culture system. Different methods for visualization of medium spiny neurons dendritic spines have been compared. Studying synaptic dysfunction is necessary for understanding both normal physiology of brain and pathological mechanisms in different neurodegenerative diseases. The described approach could be utilized in a numerous biomedical applications.

OPTOGENETICS, DENDRITIC SPINES, SYNAPTIC TRANSMISSION, NEURONAL CULTURE.

Оптогенетика – современный подход, позволяющий манипулировать физиологическим состоянием отдельных нейронов. В данной работе оптогенетический подход был применен для контроля морфологических параметров дендритных шипиков, образуемых между нейронами коры и стриатума в смешанной кортико-стриатной культуре. Также было проведено сравнение различных методов визуализации дендритных шипиков средних шипиковых нейронов стриатума в культуре. Изучение синаптических взаимодействий нейронов является важным для понимания как нормального функционирования нервной системы, так и понимания механизмов патологии. Данный подход может найти широкое применение в биомедицинских исследованиях.

ОПТОГЕНЕТИКА, ДЕНДРИТНЫЕ ШИПИКИ, СИНАПТИЧСКАЯ ПЕРЕДАЧА, КУЛЬТУРА НЕЙРОНОВ.

I. Introduction

All neurons communicate to each other through contacts called synapses. Synapses are sites of neuronal contacts that transmit chemical and electrical signals in the brain [1]. Total number of spines could be estimated as high as 10^{14}. Most synapses could be divided into two types. Inhibitory GABAergic (*gamma*-aminobutyric acid) synapses are the first. The second are excitatory glutamatergic synapses. Dendritic spines are small protrusions on dendritic shaft where excitatory synapses are located. Dendritic spines exhibit morphological changes after various physiological or pathological impacts. Spines shape demonstrates a high level of heterogeneity. Modification of this shape could be the morphological basis for synaptic plasticity. Synapses and dendritic spines are dynamic structures whose plasticity is considered to be the basis of learning and memory [2–4].

Several studies show that different spine shapes could have significantly different influ-

ence on brain function. Changing in the number of spines and morphology of individual spines recently has been connected to several neurological disorders. Different authors are discussing this connection at Alzheimer Disease [5, 6]. Recent paper [7] shows that multiple autism-linked genes lead to synapse elimination in autism mouse-model. Synaptic density is also connected to the progress of Parkinson disease [8] and different neuropsychiatric disorders e. g. schizophrenia and depression [9, 10]. Dendritic spine changes are also associated with normal aging [11]. However, in spite of numerous studies of structural changes of neurons and dendritic spines morphology during normal aging and pathological processes, the functional properties of these changes remain poorly understood. Synapse strength is closely correlated with dendritic spine morphology, and synaptic activity regulates spine shape and density during brain development, learning and aging.

Postsynaptic spines are usually classified into three groups according to their morphological properties. Mushroom spines have a large head and a fine neck; thin spines have a smaller head and a narrow neck; stubby spines have no obvious distinction between the head size and the attachment to the dendritic shaft.

Narrow necks observed in thin and mushroom spines serve for calcium compartmentalization and other second messenger molecules. Therefore, these types of spine could play a significant role in synaptic plasticity. The spine head of mushroom spines is much larger than in other spines, indicating that membrane dynamics and receptor turnover occur. Mushroom spines usually are suggested as memory storage sites. Taken together, these observations suggest that each spine shape is playing a particular role in neuronal function [12].

The study of synaptic connections is the key to understanding the functioning of neural circuits. At present, information on contacts between neurons is obtained mainly from electrical stimulation studies, which don't allow precise control of neuronal activity and also disturb nearby cells. However, recently developed optogenetics technique [13–15] allows modulating the electrical activity of neurons, modifying the strength of synaptic connections and functional analysis of the interaction between neurons.

Optogenetics is the method of studying cells by genetically introducing photosensitive components which could modify the properties of the cell in response to illumination with a certain wavelength light beam. This requires expression of specific proteins – opsins, which are light activated ion channels or pumps. Two of the most usually employed opsins are channelrhodopsin ChR2 [13] and halorodopsin NpHR [16]. ChR2 is a member of light-activated cation channels which allow photodepolarizing the membrane and activating the cells. Halorhodopsin, on the other hand, is a light-driven anionic pump selective for chloride ions, and it is used for photohyperpolarization and thus inactivation of cells. Upon delivery of the opsin gene using genetic engineering in a neuron, the light-sensitive channels appear on the plasma membrane, and the cell itself becomes photosensitive. The interesting fact is that these two opsins have maximum absorption spectrum at different wavelengths thereby allowing using them at the same time. During the exposure of blue light channelrodopsin opens (maximum absorption – 470 nm), which in turn causes the movement of positive ions into the cell, allowing the neuron membrane depolarization and generation of action potentials. When the orange light activates halorodopsin (absorption maximum – 580 nm), the neuronal membrane is hyperpolarizing, causing inhibition of the neuron. High temporal resolution of the optogenetics method allows precise regulation of synaptic events and is, therefore, an important tool to study interneuron connections. The most convenient model for such studies is an *in vitro* culture of neurons forming synaptic contacts *in vivo*, e. g. a mixed culture of neurons from the cortex and striatum.

The aim of this study was to adapt optogenetic approach to modify the strength of synaptic connections between cortical cells and medium spiny neurons (MSN) in the cortico-striatal mixed neuronal culture.

II. Methods

A. Neuronal Culture

In the present work mixed cortico-striatal cultures were prepared. Dissociated cultures

were established as previously described [17]. Briefly, striata and cortices of P0-P1 WT pups were dissected, digested with papain, dissociated and plated on poly-D-lysine (Sigma) coated 12 mm coverslips in Neurobasal-A medium (Invitrogen) supplemented with 2 % B27 (Gibco), 5 % FBS (Gibco), 1mM glutamine (Invitrogen) and kept at 37 °C in a 5 % CO_2 incubator. Cortices from a single brain were used to plate 9 wells, and striata from a single brain were used to plate 3 wells (1 : 3 cortical to MSN ratio; density $4 \cdot 10^5$ cells/well). Cultures were incubated for 14–21 days. For the optogenetics studies we used modified protocol allowing gene delivery in the specific neuronal population. On the first day dissociated culture of cortical neurons from neonatal mouse brain were prepared. Cultures were kept at 37 °C in 5 % CO_2 for one day. To achieve opsin expression in neurons, we used viral delivery system. On the second day of culturing, the cells were transfected using the lentiviral construct containing the channelrodopsin or halorodopsin gene fused with GFP. On the third day striatal intact neurons were plated on top of the cortical culture Thus, striatal and cortical neurons in the culture were differed by opsin gene expression that can be identified by expression of GFP in cortical neurons. The virus has previously been produced in HEK293T cell line according to the protocol. Viral delivery system allows achieving high levels of opsin expression in the cell culture several days later.

B. Immunochemistry

For immunocytochemical staining two primary antibodies were used: rabbit antibodies against neuronal marker MAP2 (Cell Signalling Technology), and mouse antibodies against the MSN marker DARPP32 (Cell Signalling Technology); secondary antibodies: anti- rabbit and mouse with fluorophores Alexa Fluor 488 and 593 (Invitrogen). Cells were fixed with 4 % paraformaldehyde and stained at 15 day *in vitro*. After staining cells were analyzed with the confocal microscope Zeiss LSM 700. For further morphology analysis, the sequence of micrographs of dentritic shafts and spines with an interval of 0.15 m in depth forming a three-dimensional image of neurons were obtained. Immersion lens with a 100-fold magnification was utilized, resolution of the image was 1024×1024 pixels. 3-dimensional images were obtained with Zeiss software ZEN 2011 Black edition.

C. Lucifer Yellow Dye Injection

Coverslips with mixed live neuronal cultures were placed in a Warner chamber of patch-clamp setup (Olympus IX71 microscope). During the experiment cells were placed in artificial cerebrospinal fluid containing the following (in mM): 85 NaCl, 24 $NaHCO_3$, 25 glucose, 2.5 KCl. The glass electrodes for dye injection were pulled from borosilicate glass capillary tubes (Sutter) using the Sutter Instruments P97. The electrode resistance ranged between 150 – 300 MΩ when filled with PBS. MSNs were selected visually under the microscope and patched with the electrodes filled with Lucifer yellow (L-12926, Invitrogen). Dye injection was performed by application of a constant negative current in the range of 500 – 800 pA for 10 min (Multiclamp 700B, Molecular Devices). Five neurons were injected in each coverslip. Neuron culture was then immediately fixed in 4 % PFA – sucrose. Injected neurons were analyzed by confocal imaging (Zeiss) with 100× lens magnification.

D. Transfection of Neurons

For morphology analysis of spines expression of dsTomato in neurons was achieved through calcium-phosphate transfection of plasmid encoding this protein. Transfection was performed as previously described at DIV 9 – 12 [18]. Neuron culture was incubated with Ca-containing solution of the plasmid (1 mg) until clearly visible precipitate was formed. Then the precipitate was being dissolved in acidified medium Neurobasal-A (Invitrogen) for 30 min and washed with culture medium without FBS. Transfected neurons were incubated for 14 – 19 days at 37 °C and 5 % CO_2, and used for morphology analysis 2 – 5 days after transfection.

E. Optical Stimulation

For optogenetic activation of opsins, pulses of light were generated. Coverslips with neuronal culture were placed under the microscope. Halogen arc lamp directly coupled to

the microscope light path was used as a source of light. Pulses of blue light (470 nm optical filter) and orange light (590 nm optical filter) were generated using Uniblitz shutter.

F. Morphological Analysis of Spines

Each image has a maximum resolution of 1024 × 1024 pixels. Approximately 10 neurons were analyzed for each coverslip. At least 10 coverslips from 4 – 5 cultures were utilized for each experiment. The secondary apical dendrites of medium spiny neurons were selected for taking images.

Morphological analysis was performed with Neuron Studio software [19], which automatically reconstructs a three-dimensional image of spines and distributes them into three groups (mushroom, thin, stubby) by several parameters. We used the following parameters: min stubby size – 50, non-stubby – 20, neck ratio – 1.1, thin ratio – 1.5, mushroom size – 0.35. The distribution density of spines was defined as the average number of spines per 10 um.

III. Results

Cortico-striatal co-culture model system was used as a simple and relevant *in vitro* model of synaptic connections between two populations of neurons. Study of changes in corticostriatal synaptic connections and dendritic spine morphology could be considered as signs of dysfunction.

Cultured neurons are the primary model system for studying the basic mechanisms regulating neuronal morphological structure and function.

A. MSN Spine Visualization Methods Comparison

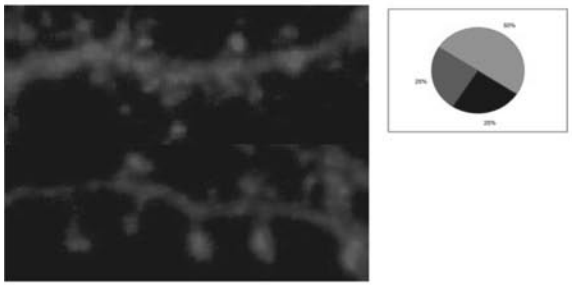
First objective of present work was establishing the most convenient method of spine analysis and quantification in medium spiny neurons in culture. The most common method of spine analysis is Goldgi staining. However, this technique does not allow seeing the differences between spine types. The main focus of this part was establishing a relevant method of spine shape analysis. For our study, we tested three other methods. These include immunocytochemistry, dsTomato transfection and dye injection into live neurons.

All these methods allow visualization of three types of dendritic spines and perform confocal 3-D analysis of images. During the immunochemistry procedure, cells were fixed at DIV 14–15 and then stained against DARPP-32. Tomato transfection was performed at DIV 9–12 with live cultured neurons. After transfection, cells were kept in the incubator until DIV 14–15 and then fixed. For dye injections live neurons were also utilized at DIV 14–15. During this experiment, cells were kept out of incubator in CSF for about 30 min and then immediately fixed.

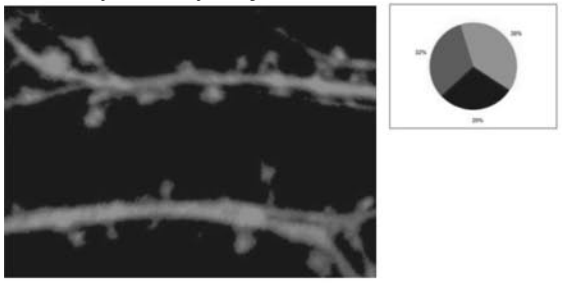
Spines of MSNs are very sensitive to any external impact. So, two methods performed with live neurons – transfection and dye injection – seriously affected both spine density and spine distribution between different spine types. Images were obtained 24 hours after tomato transfection. Spines were eliminated after transfection. Mean spine density in dsTomato transfected neurons was almost twice less than in immunostained culture. Despite the fact that this method is a standard for hippocampal neurons in culture, tomato transfection causes serious damage for MSNs spines and seems to be an absolutely inappropriate method for morphological analysis.

At the same time, the spine morphology in MSNs in culture was studied using Lucifer yellow injection technique. In Fig. 1 we compare the spine morphology data obtained by different methods in the same batch of cultures – immunocytochemistry and Lucifer yellow dye injections – using patch pipettes. MSNs suffer from dramatic decrease of mushroom spines after dye injection (10 min of injection). Analysis of spines after dye injections showed little decrease in the number of spines in culture compared to the culture immunostained against DARPP-32 protein. Also Lucifer yellow injection method shows altered ratio of spine types with mushroom spine decrease and thin and stubby spines increase. During the dye injection, neuron membrane becomes damaged. Additional stress for a cell during this procedure is a small negative current required for dying to spread out within the cell. However, this method affects spine density to a lesser degree than transfection. These results indicate that mushroom spines are transforming into other

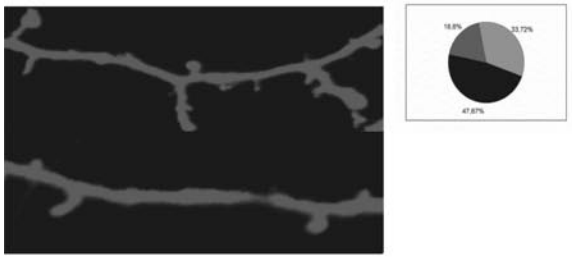
a) anti-DARPP32 immunostaining



b) Lucifer-yellow dye injection



c) Tomato transfection



d) Spine density

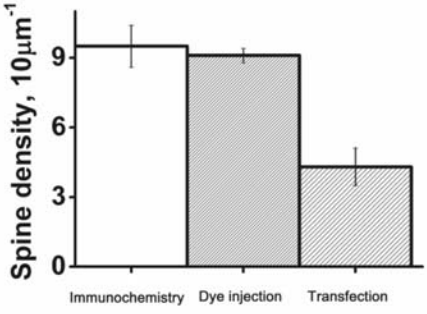


Fig. 1. Spine morphology of MSNs determined by immunocytochemistry (*a*), Lucifer yellow dye injection (*b*) and Tomato transfection (*c*). In the diagram: mushroom – light grey, thin – dark grey, stubby – black. Spine density analyzed after all methods (*d*). Error bars indicate standard deviation

types during these stressful conditions.

Overall, only the most nonperturbative method – immonocytochemistry – allows performing spine shape analysis with minimum impact on neuron health and dendritic spines.

B. Optogenetics Manipulation with Spine Morphology

Previously, we pointed out that cortical neurons in the culture immediately respond hyper- and depolarization after rhodopsins activation in orange and blue light, respectively [17]. MSN of the striatum has not expressed channelrodopsin. However, during activation of cortical neurons with blue light, hyperactivity was registered in MSNs.

Recorded activity of MSNs was completely blocked by addition of an inhibitor of AMPA receptor inhibitor DNQX. This indicates that nerve impulse transmission between MSNs and cortical neurons occurs through synaptic contacts.

One of the most important indicators of the strength and activity of synapses is their morphology, mainly – the head size of dendritic spines (this parameter is positively correlated with the amount of AMPA-receptors on postsynaptic terminal). It is known that the activation of synaptic connections between neurons increases the sizes of spines [20].

In a series of experiments we demonstrated the ability to change the strength of synaptic connections between neurons – dendritic spines – in response to exposition to the light of a certain wavelength. In this case, culture protocol was established as follows. On the first day dissociated culture of cortical neurons from neonatal mouse brain was prepared. On the second day the cells in the culture were transfected using the lentiviral construct containing halorodopsin gene fused with GFP. On the third day striatal intact neurons were plated on the top of the cortical culture. Thus halorodopsin expression was only confirmed in cortical neurons. GFP-labeling allows easy identification of cortical cells with fluorescent microscope.

For selective targeting the cells of interest we used whole-field illumination. Importantly, applying our updated protocol for gene delivery via lenti-viral systems, we were able to transfect only cortical neurons and the remains of striatal neurons unaffected and therefore insensitive either to blue or orange light.

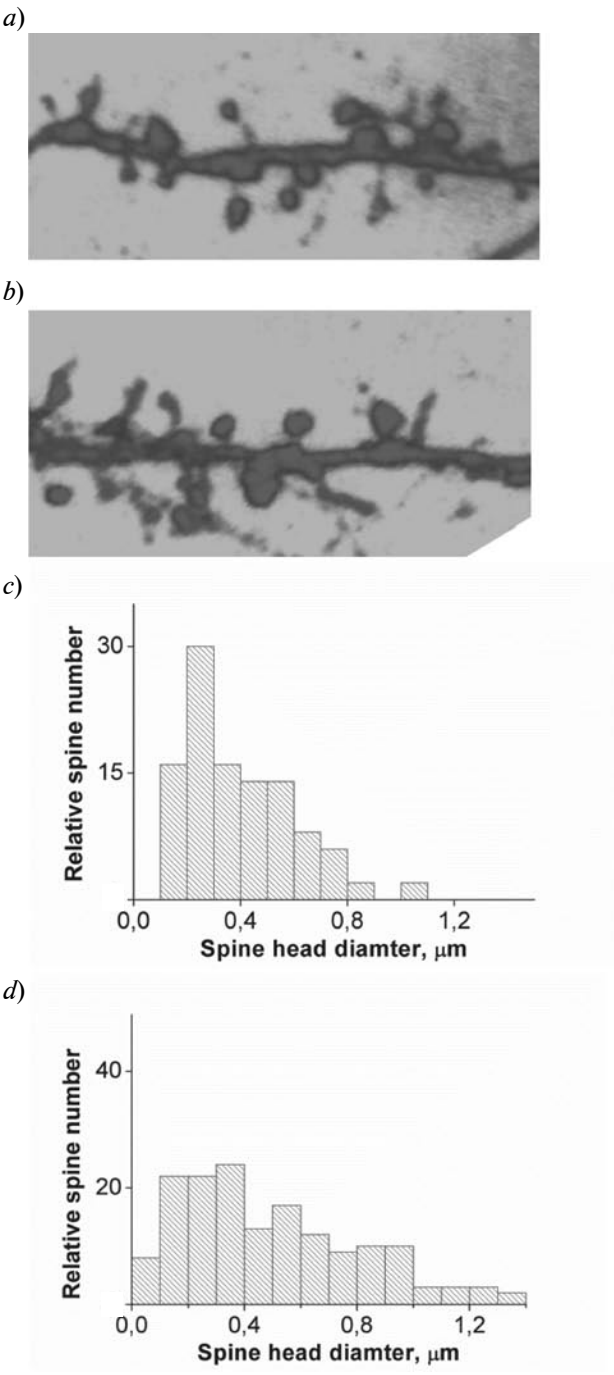


Fig. 2. Dendritic spine morphology of MSNs before (*a*) and after (*b*) optical activation of cortical neurons for 20 min at DIV 14; spine head distribution before (*c*) and after (*d*) blue light exposure

To analyze the functional significance of optogenetically induced activity of cortical neurons, quantitative evaluation of spine morphology changes at the postsynaptic side was performed. In a series of experiments we demonstrated the ability to change the synaptic connections between neurons and synapses when they were exposed to light of a certain wavelength. Dendritic spines of MSNs where most of the synapses are localized has been studied in a confocal microscope after immunocytochemical staining of neurons against striatum specific protein DARPP-32, so only medium spiny neurons were considered for spine quantification. Images were analyzed using the Neuron Studio.

It was found that the excitation of ChR2-expressing cortical neurons with a blue light for 20 minutes caused MSN spines increase in size (Fig. 2) indicating the benefit of physiologically significant impact on synaptic contacts with this method. Head diameter of mushroom spines was quantified, distribution of this parameter is presented in Fig. 2, *c*, *d*.

Thus, in this part of work we showed that in a mixed cortico-striatal coculture functional synaptic connections between cortical neurons and MSNs could be modulated by optogenetics.

In the next series of experiments mixed culture of neurons was exposed to orange light for over 45 min, then cells were immediately fixed in 4 % formaldehyde solution. Previously we demonstrated that during orange light irradiation electrical activity disappeared in both cortical neurons and MSN on the post-synaptic side [17].

We inhibited the activity of cortical neurons with optogenetics, using halorhodopsin. Transfection procedure was similar as described above. Only cortical neurons were infected with virus containing Halo-GFP plasmid. Orange light exposure silences cortical cells. As a result, MSNs demonstrate inhibiting activity. After 20 and 45 min of continuous orange light irradiation, cells were fixed and stained against DARPP-32 for spine analysis. Data is presented in Fig. 3. As it is shown here, MSN spines are sensitive to the long exposure. We observed a decrease of spine density in MSN only after 45 min. However, after 20 min MSN all spines survived and demonstrated no change by types. Activation of halorhodopsin did not inhibit all the activity of the cells. Several spikes could be observed under orange light. So we suppose

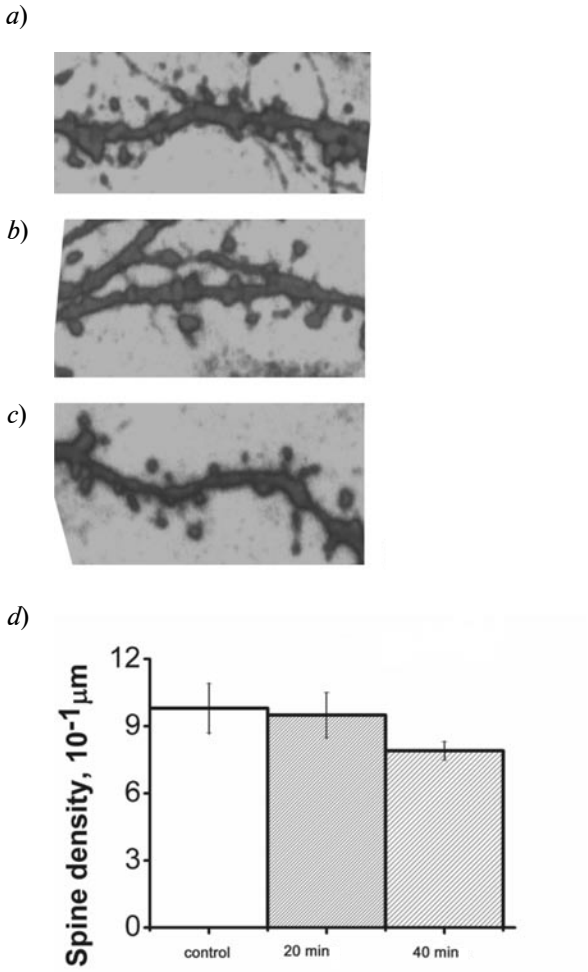


Fig. 3. MSN spines morphology (*a* – *c*) and spine density (*d*) before (*a*) and after (*b*, *c*)orange light exposure – 20 and 45 min. DIV 15. Error bars indicate standard deviation

that this residual activity from cortical cells is enough to maintain spines during short period of inhibition.

IV. Conclusion

Overall, we demonstrated an optical approach to controlling dendritic spine morphology using neurons expressing different opsins. This versatile method can be applied to studying the role of dendritic spines in live cultured neurons using widefield fluorescence or confocal microscopy. Cultures prepared from transgenic mouse models are primary model systems to study almost every neurodevelopmental and neurodegenerative disease. Future directions include optogenetic manipulation with dendritic spines in living animals modeling different diseases. This will require production of high-efficient LEDs and lasers in a clean room for material fabrication. While using these powerful light sources, it will be possible to activate or inhibit neurons directly in living brain. Thus, the precise speed and accuracy of optogenetics spine control, as compared to spine modification with different drugs, is absolutely necessary for studying the complicated mechanisms underlying normal and aberrant dendritic spine role in progress of neurological disorder as well as in normal aging.

This work was supported by grants FCKP Kadri («Научные и научно-педагогические кадры инновационной России» на 2009–2013 годы) –

№ 2012-1.1-12-000-1002-1101, соглашение №14. B37.21.0121 и 2012-1.2.2-12-000-1014-5674, соглашение № 14.B37.21.0219, and Russian Federation government grant (Грант государственной поддержки научных исследований, проводимых под руководством ведущих ученых в российских образовательных учреждениях высшего профессионального образования), № 11.G34.31.0056.

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