RESEARCH ARTICLE

Evaluation of the Functional Status of Signal Systems of Astrocytes by the Developed Reagent Kit Basedon Genetic Constructions (Experimental Study)

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Abstract

Objective: The objective of the study was to create a new type of therapy for neurodegenerative diseases; we have conducted a study with the goal to develop techniques based on functional and/or genetic constructions for the control of exocytosis based on a modification of the vesicles of brain astrocytes. **Materials and Methods:** For this purpose, we made specific transfection of astroglial cells by an experimental sample of the reagent kit developed by the authors. It represents genetic construction lentiviral vector (LVV) - glial fibrillary acidic protein (GFAP) -Case12 on the basis of a LVV. **Results and Discussions:** We can see results of introduction of the genetic construction into astroglial cells of adult Wistar rats, consisting in the activation of intracellular cascades. A comparison of the astrocytic response to ionomycin on organotypic cuts transfected with LVV-GFAP-Case12 was performed as well as on the organotypic cuts of the model of hepatic encephalopathy. This allowed us to objectively assess the astrocytic signaling systems, their overall functional status on modification of astrocytes of the cortex and brainstem in healthy rats *in vivo*, as well as in animal models of hepatic encephalopathy. **Conclusion:** In case of the model of hepatic encephalopathy, an amplitude of the astrocytic response to ionomycin was significantly reduced, which indicates pathological processes in the brain cells in this pathology, and also characterizes the developed genetic construction as an effective tool for assessing pathophysiological mechanisms occurring in the brain.

Key words: Astrocytes, astroglia, exocytosis, genetic constructions, reagents, targeted control, transfection

INTRODUCTION

At present, an incidence of neurodegenerative diseases, such as Alzheimer's and Parkinson's and amyotrophic lateral sclerosis, has become a global epidemic. According to the European Prevention of Alzheimer' Dementia, Alzheimer's disease affects more than 40 million people worldwide, and its prevalence is projected to double in the next 20 years.[1] Parkinson's disease is the second most important age-related neurodegenerative disorder in developed countries after Alzheimer's disease with the prevalence of over 1,900 per 100,000 people over the age of 80 years.[2] Moreover, neurodegenerative diseases are often accompanied by depression and dementia,[3] increasing the social burden of these diseases and the entire society.

In this regard, the study of the molecular mechanisms of these pathologies, as well as the development of new

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prevention and treatment methods based on the data obtained are becoming particularly relevant.

Astroglia is the main structural element of glia. It ensures normal functioning of neurons. Astrocytes provide the release of neuromodulating, neurotropic and neuroprotective factors. It has been shown that astrocytes secrete glutamate,[4] D-serine,[5] adenosine triphosphate (ATP),[6] gamma-aminobutyric acid, prostaglandins, and neuropeptides in a process called gliotransmission.[7] Molecular mechanisms of gliotransmission have not been sufficiently studied. However, Ca^{2+}-dependent vesicular exocytosis, as some researchers suggest, is one of the main mechanisms for releasing gliotransmitters.[8]

The severity of any pathological process localized in the brain directly depends on the functional activity of astrocytes. If there is a violation of the secretion of gliotransmitters by astrocytes, the death of neurons and the development of neurodegenerative processes are inevitable. Thus, qualitative-quantitative imbalance of gliotransmitter is one of the key pathophysiological mechanisms underlying neurodegenerative processes.[9,10]

The use of targeted gene therapy is one of the innovative therapeutic areas,[11-13] which can be aimed at modification of the activity of glial cells. The methods used in gene therapy can also be used to study the processes occurring in the brain, *in vivo*.

Gene transfer during gene therapy is performed using viral and non-viral vectors.[14] At the same time, LVVs and adenoviral vectors (AVV) are most widely used at the present time.[15,16]

The aim of this work is the development of techniques based on functional and/or genetic constructions for exocytosis control, based on a modification of the vesicles of the brain astrocytes to create a new type of therapy for neurodegenerative diseases. This paper presents the results of the project fragment consisting in testing experimental samples of genetic construction and a reagent kit for specific transfection of astroglial cell with the purpose of the targeted control of intracellular cascades and evaluation of signal systems of astrocytes as well as their general functional status on the background of modification of astrocytes of the cortex and the brainstem in healthy rats *in vivo*, as well as in animal models of hepatic encephalopathy.

MATERIALS AND METHODS

Evaluation of astrocyte signaling systems and their overall functional status in the modification of astrocytes of the cortex and brainstem in healthy rats *in vivo*, as well as in animal models of hepatic encephalopathy, was performed on organotypic brain cuts transfected *in vivo* by lentiviral vector (LVV)- glial fibrillary acidic protein (GFAP)-Case12 genetic construction.

Specific transfection of astroglial cells for the purpose of targeting intracellular cascades was carried out using three different reagent kits containing a genetic construction based on the LVV. The LVV was selected due to its stable expression in the brain cells of experimental animals. Specificity of transfection of astroglial cells of the brain is provided by the presence of an astrocyte selective GFAP promoter, and the possibility of targeting intracellular cascades by the presence of calcium indicator Case12.

Confirmation of the nature of the collected genetic constructions [Figure 1] was carried out by polymerase chain reaction (PCR) with primers specifically selected to the sequence of the LVV.

The results were visualized by gel electrophoresis in an agarose gel with intercalating dye [Figure 2].

In this design, an astrocyte-specific promoter, GFAP, provides selective transfection of astroglial cells, while Case12 calcium indicator provides the possibility of targeted control of intracellular signaling cascades.

The presence of specific marker proteins and determination of their number in the sequence of genetic construction was determined by PCR with primers specifically selected to nucleotide sequences of marker proteins - GFAP and fluorescent calcium-sensitive protein Case12.

The results were visualized by gel electrophoresis in an agarose gel with an intercalating dye [Figure 3].

The presence of differentiation in the transfected cells was confirmed by staining the cell culture using primary antibodies specific for the GFAP and then recording the fluorescent signal with a laser scanning confocal microscope, using DAPI staining as a control to visualize nuclei in all cells regardless of their type, i.e., astrocytes, neurons, oligodendrocytes, and microglia [Figure 4].

The lack of toxicity in the developed genetic construction was confirmed by carrying out MTT test (MTT-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which was a colorimetric assay for assessing metabolic activity of cells.

Chemical stimulation of signaling cascades during the introduction of genetic construction carrying the Ca^{2+}-sensitive protein was confirmed by adding activators to the pure culture of astroglial cells - ATP and isoproterenol.

Cascades of purine receptors P2X and P2Y, as well as β-adrenergic receptors, were considered as inducible cascades. The main method of visualizing the activation of purinergic receptors is the method of measuring the concentration of intracellular Ca^{2+}. To activate P2C-dependent Ca^{2+}-sensitive cascades, 10 μM 2-MeSATP was added to the experimental cell, while 10 μMa, b-mATP was added to activate P2X dependent Ca^{2+} cascades; to activate β-adrenergic dependent

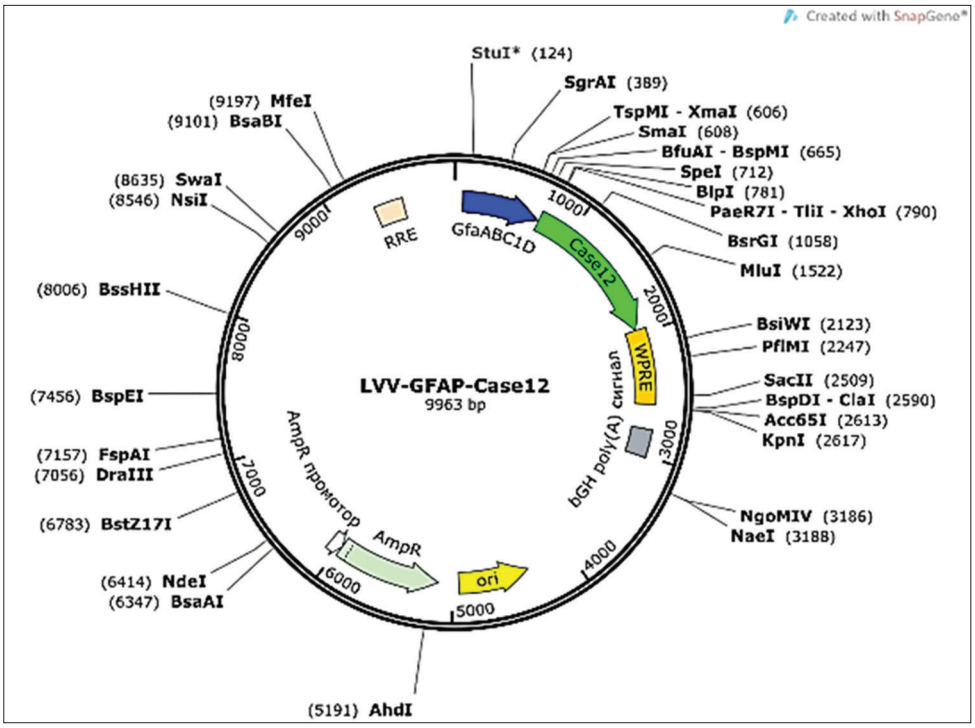

Figure 1: Map of the lentiviral vector - glial fibrillary acidic protein (GFAP)-Case12. GfaABC1D is a compact promoter of GFAP; Case12 - sequence that codes for green Ca^{2+}- sensitive fluorescent protein; WPRE - post-transcriptional control element of the marmot hepatitis virus; AmpR promoter - promoter that provides resistance to ampicillin; Ori - high-copy origin of replication of plasmids ColE1/pMB1/pBR322/pUC; BGH poly (A) signal - polyadenylation signal; RRE - Rev-sensitive element of HIV-1 that provides Rev-dependent export of mRNA from the nucleus to cytoplasm; 9363 bp- plasmid sequence with the length equal to 9363 nucleotides

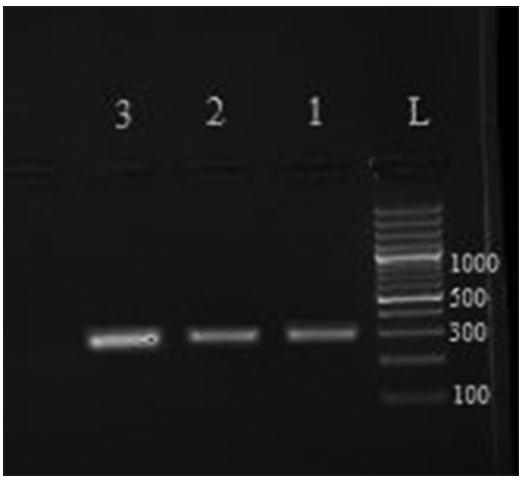

Figure 2: Result of electrophoretic separation of polymerase chain reaction products: Samples 1, 2, and 3 are nucleotide sequences obtained by amplification with the corresponding pair of primers to the lentiviral vector (LVV) fragment from the genetic construction of LVV - glial fibrillary acidic protein - Case12; L is marker of DNA length, bp

Ca^{2+} cascades, we added 50 μ isoproterenol and recorded changes in the level of fluorescence.

Registration of calcium dynamics on organotypic cuts was carried out after incubation for 2 days.

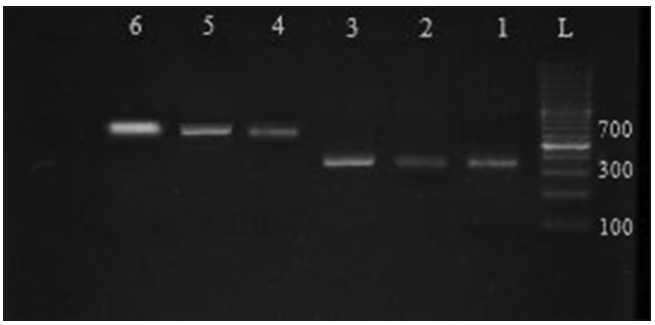

Figure 3: Result of electrophoretic separation of polymerase chain reaction products: Samples 1, 2, and 3 are nucleotide sequences obtained by amplification with the corresponding pair of primers to the fragment GfaABC1D; samples 4, 5, and 6 are nucleotide sequences obtained by amplification with the corresponding pair of primers to the Case12 fragment; L is marker of DNA length, bp

Detection of the green fluorescent signal on organotypic cuts was carried out using laser scanning confocal microscope LSM 780 ("Carl Zeiss," Germany). To assess the functional status of the cell, a 1 mM ionomycin solution (Life Technologies, USA) was prepared in 100% DMSO. When 1–10 μl of ionomycin solution was added, the change in fluorescence intensity was monitored in real time.

To simulate hepatic encephalopathy, paracetamol solution in propylene glycol was used (parenteral administration of the solution with evaluation of the indices at the day 3 of the experiment). Experimental animals were injected with 2 mL of paracetamol solution at the dose of 1 g/kg.

RESULTS

Deduction of the green fluorescent signal was carried out with 488 nm argon laser to stimulate the fluorescence (optimum for Case12 is 491 nm).

Preliminary processing of the data was carried out in ZEN software (Zeiss, Germany), using the function of isolating the regions of interest and measuring an average fluorescence intensity relative to the background.

Astrocytes on organotypic cuts transfected with LVV-GFAP-Case12 showed normal calcium dynamics when ionomycin was added [Figures 5 and 6]. In case of the model of hepatic encephalopathy, differences in calcium dynamics were observed [Figure 7]. In particular, an amplitude of the astrocytic response to ionomycin on organotypic cuts was significantly reduced, which indicated pathological processes in the brain cells.

During the study, stimulation of signaling cascades due to the introduction of the genetic construction was confirmed by detecting the change in the fluorescent signal by a laser scanning confocal microscope [Figures 8–10]. The effectiveness of targeted control of intracellular cascades was assessed by the change in fluorescence intensity of Case12 reporter protein in response to the addition of ATP gliotransmitter to the solution. The resulting data were processed in ImageJ using 3-D Hybrid Median Filter Plugin. The diameter of the transfected region and the dynamics of activation of transfected astrocytes were estimated.

DISCUSSION

In this study, astrocyte signaling systems and their overall functional status were assessed after the modification of astrocytes of the cortex and brainstem in healthy rats *in vivo*, as well as in animal models of hepatic encephalopathy. In the course of the study, we have found that calcium dynamics change in astrocytes during hepatic encephalopathy, in particular, in response to ionomycin, an amplitude of calcium response in astrocytes of organotypic cut is significantly reduced relative to the normal range. In addition, changes in the number and size of protoplasmic astrocytes are observed in acute hepatic encephalopathy.

The data obtained underscore the possible role of astroglial cells in the pathogenesis of neurodegenerative diseases,

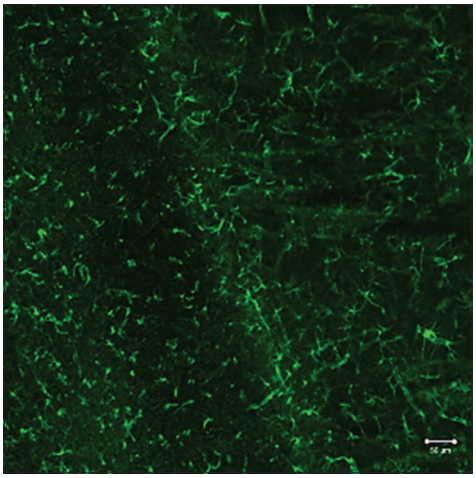

Figure 4: Astrocytes transfected with lentiviral vector - glial fibrillary acidic protein - Case12, on the brain cut

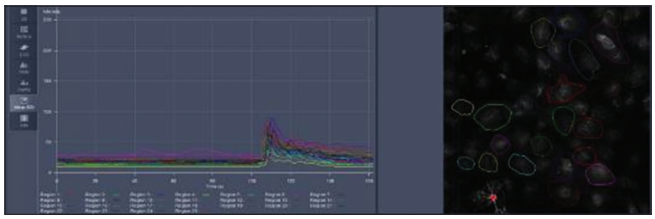

Figure 5: Assessment of the functional status of astrocytes transfected with lentiviral vector - glial fibrillary acidic protein - Case12 from the rat cerebral cortex. On the right side, in the photo of the cut, various regions of interest are highlighted in different colors. On the left side for each region there are measurements of the average fluorescence intensity relative to the background

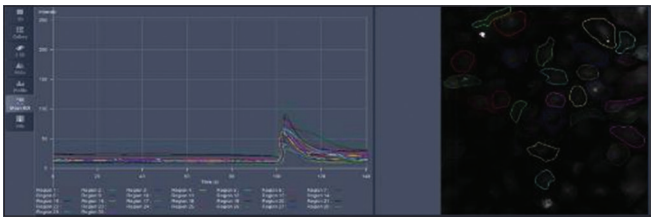

Figure 6: Evaluation of the functional status of astrocytes transfected with lentiviral vector - glial fibrillary acidic protein - Case12 from the rat brainstem. Insets in the figure on the right and on the left side are read in the same way as insets in Figure 9

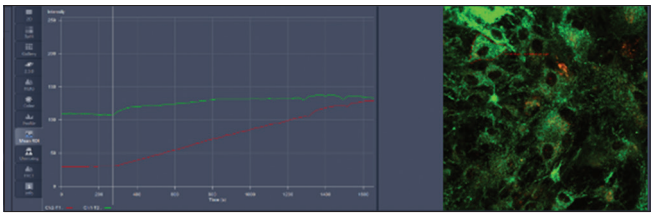

Figure 7: Evaluation of the functional status of astrocytes transfected with lentiviral vector - glial fibrillary acidic protein - Case12 in animal models of hepatic encephalopathy. We see a decrease in the amplitude and slowing of the rate of growth of the astrocytic response to ionomycin at the presented organotypic cut, as compared to the cuts without pathology

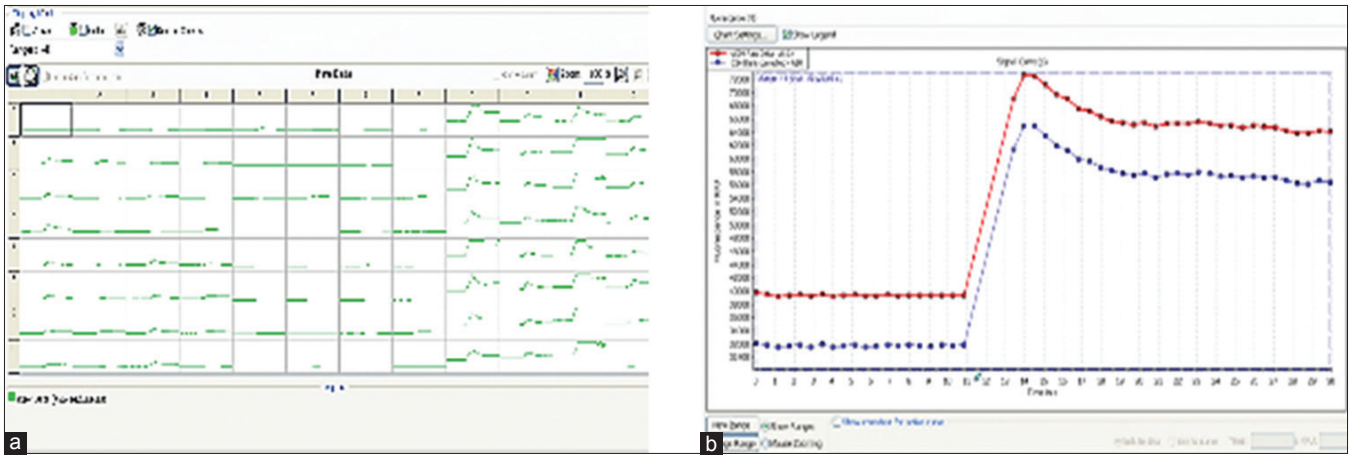

Figure 8: Study of calcium dynamics in astrocytes transfected with lentiviral vector - glial fibrillary acidic protein - Case12 genetic construction in response to the activation of purinergic P2Y receptors. (a) Matrix-graph represents a series of responses to the stimulus horizontally for a set of selected regions of interest and (b) the fragment A10 of the matrix chart on the left is highlighted – the 10th time series for the region of interest A

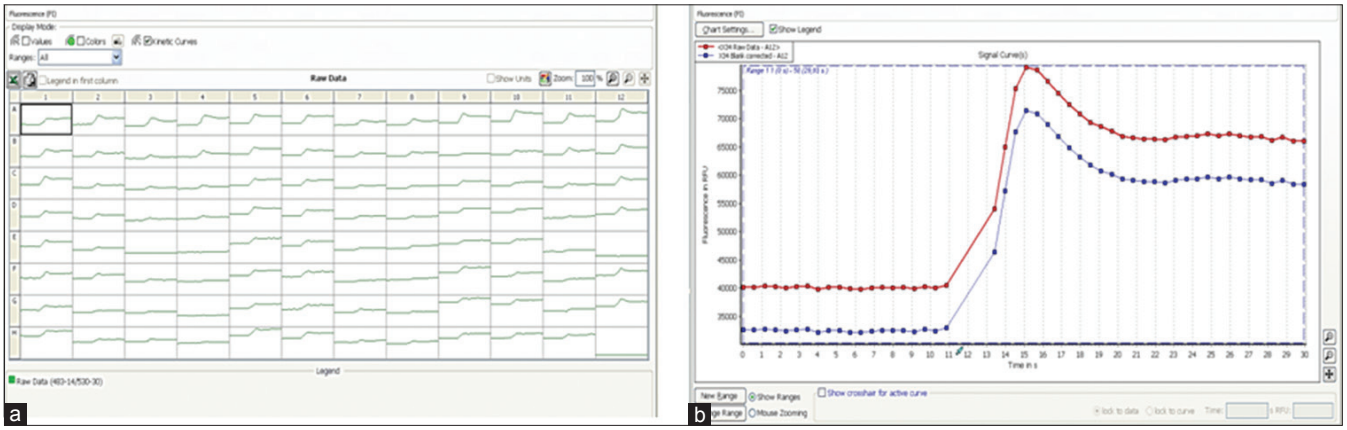

Figure 9: Study of calcium dynamics in astrocytes transfected with lentiviral vector - glial fibrillary acidic protein - Case12 genetic construction in response to the activation of purinergic receptors P2X (a) matrix response schedule, similar to Figure 5 (b) time series for A12 region

which indicates high prospectivity of studying astroglia for the purpose of developing targeted gene therapy based on the control of intracellular signaling cascades.

The effectiveness of gene therapy depends on the ability to deliver genetic material to target cells safely and purposefully, which can be carried out both outside the patient's body (at this case target cells are to be removed from the patient, transfected and returned back), and *in vivo* when the genetic vector is introduced into the cells of the patient, changing the activity of target cells directly in the patient's body. Transfer of genetic material into the patient's body is possible with the use of structures based on viruses or non-viral constructions. The use of virus-based vectors is, currently, the most common method used in gene therapy. Vectors created on the basis of various viruses can vary significantly in their properties (both transduction and transcription). Thus, the ability of viruses to express sufficient amounts of transgenic protein in the specific type of target cells, for example, in neurons or glial cells must be thoroughly tested *in vitro* before their introduction into the target brain region. However, the speed and quality of the method depend on the effectiveness of the viral penetration into the target cell, as well as on the activity of the promoter used for transgenic expression (transcriptional tropism). Some types of viral vectors, including alphaviruses, adeno-associated viruses (AAV), AVV, and LVV (of which AAV, LVV, and AAB are used most widely at the present time),[17-19] were tested as a means of delivering genes into brain cells.

Despite the fact that the ability of these vectors to introduce transgenes of brain neurons is known, it remains unclear whether these vectors are equally effective and whether the ratio of neuronal transduction to glial one is maintained in all brain regions. AVV and LVV have different advantages as research tools, which mutually complement each other. In our study, we used AVV and LVV. AVV can be rapidly amplified with a high yield, it may be convenient for *in vitro* experiments, and can also be used *in vivo*. With respect to LVVs, their advantages are the ability to transfer large

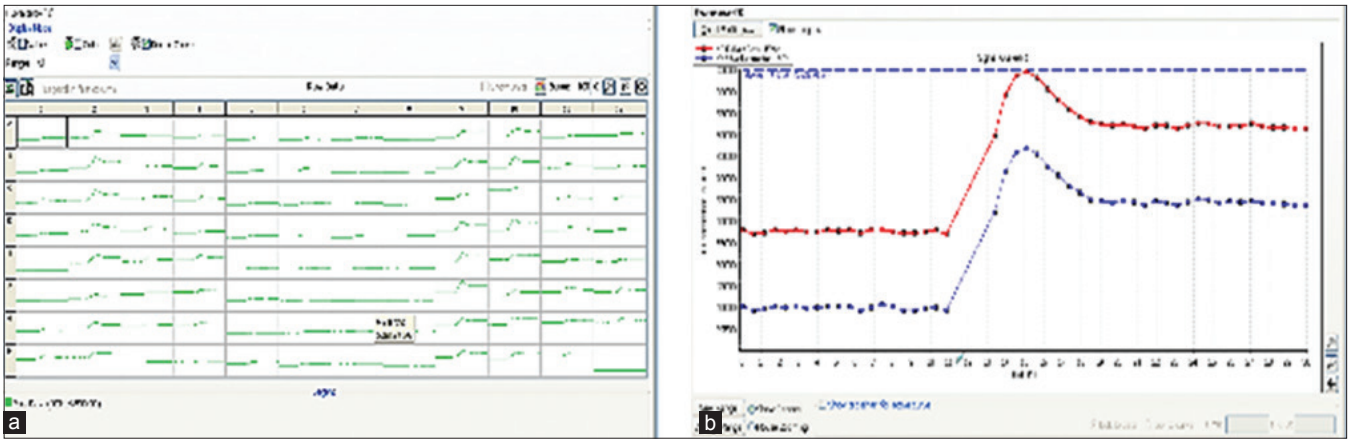

Figure 10: Study of calcium dynamics in astrocytes transfected with lentiviral vector - glial fibrillary acidic protein - Case12 genetic construction in response to the activation of β-adrenergic receptors (a) matrix response schedule, similar to Figure 5 (b) time series for region F09

transgenes[20] and stably transfect both dividing cells and cells at rest.[21-23] Such a wide tropism is provided by wrapping the virus of vesicular stomatitis.

LVVs are derived from HIV-1 and, due to their persistent expression in the brain, rats are the best choice when long-term and stable gene transfer is required *in vitro* and *in vivo*.

In the course of our study, we used self-developed genetic construction based on a lentivirus, its safety, and ability for specific trans figure of astroglial cells was shown. The results of the introduction of the genetic construction into astroglial cells of adult rats were presented, consisting in the activation of intracellular cascades.

The result of this study relates to the field of cellular and molecular biology, in particular, to the studies of lentiviral reporter vector systems for the analysis of intracellular signaling cascades in mammalian brain cells. Despite the fact that genetic constructions based on adenoviruses and AAV are widespread, lentiviral constructions have one important advantage - they successfully transfuse both dividing and non-dividing (after mitosis) cells. This is the key factor that affects stable and long-term expression of the targeted gene in brain cells. At the present time chemogenetic and optogenetic methods are widely used in neurobiological studies. The use of genetic constructions that allow the insertion of a specific gene into target cells, both *in vitro* and *in vivo*, is the highly technological and accurate method that helps to address a variety of neurobiological problems. However, this method, like any other, has not only advantages but also drawbacks. The limitations of virus-based vector systems include the lack of selectivity of cell transfection, or low efficiency of transfection of the cells being analyzed. At the same time, the genetic construction developed in our study should provide highly efficient and highly selective transfection of target cells (cell populations) and stable expression of the gene.

Within the framework of this project, a reagent kit has been created that allows successfully (i.e., efficiently and selectively) transfect astroglial cells in the brain of mammals to carry out subsequent monitoring of calcium dynamics. Due to this fact, it became possible to solve one of the most urgent problems of modern neuroscience methodically - to transfect glial cells of the brain with the aim of targeting intracellular cascades. Targeted control of intracellular calcium-dependent signaling cascades in astrocytes (carried out using the developed reagent kit) will be used in further studies of the functioning of mammalian brain, as well as in the development of the new methods for targeted manipulation of brain cells, including glial cells and neurons. Unlike neurons, glia remains largely unexplored, which is why glial cells are now the object of many advanced studies. The need for molecular instruments and new methods of investigation and manipulation of intracellular signaling cascades, as well as mechanisms of intercellular communication, is growing and constantly increasing. Thus, the genetic construction of the reagent kit developed within the framework of this project will find wide application both in further fundamental scientific and applied research, and in future studies when testing drugs and effectiveness of the treatment.

CONCLUSION

The field of application of the transfection protocol presented in the study and using lentivirus-based (LVV-GFAP-Case12) vector construction is fundamental scientific research in the field of neuroscience, in particular, studies of the molecular mechanisms of widespread neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. This protocol can also be used in the future to monitor the functional state of astroglial cells *in vivo* when testing the drugs being under development.

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