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The effect of valproic acid on the transcriptional activity of *Ngf* and *Bdnf* genes of *in vitro* cultured neurons under oxidative stress conditions

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Abstract – Brain-derived neurotrophic factor (BDNF) is a secretory molecule that promotes peripheral neurons synaptic transmission and plasticity by TrkB receptor activation. This is shown in cultured central nervous system (CNS) neurons, including hippocampal and cortical cholinergic, dopaminergic and serotonergic neurons. Hypotheses suggesting that BDNF may play a potential role in the pathophysiology of schizophrenia are based on the key role of BDNF in the synaptic plasticity and, consequently, regulation of cognitive functions. In the schizophrenia treatment valproic acid is used in complex combined therapy regimens. Treatment of schizophrenia patients with valproate increases the BDNF level. Since it is not yet clear whether the BDNF protein levels measured in serum samples and in the brain correlate, we investigated valproate effects on the cultured neurons *Bdnf* transcription level. The primary neuron-glia culture was obtained from the cerebellum of 8-9-day-old Wistar rats. Valproic acid was added to the neurons (at a concentration of 50 μg/ml), oxidative stress was stimulated by 40 μMof H_2O_2, and injury was caused by mechanical damage to the neuron culture. It was shown that valproic acid in 3-24 hours increases the transcriptional activity of the *Bdnf* and *Ngf* (nerve growth factor) genes 2–2.5-fold ($p<0.01$) and approximately 1.5-fold ($p<0.01$), respectively. Mechanical trauma, unlike oxidative stress, activates the transcriptional activity of the *Ngf* and *Bdnf* genes ($p<0.01$). However, under oxidative stress and mechanical damage to neurons, the effect of valproic acid on the *Ngf* and *Bdnf* genes expression was insignificant. Fluorescence microscopy analysis using specific antibodies to neurons (anti-Map-2) showed that in the presence of valproic acid, the number of neuronal processes and contacts between them significantly increased. Evidently, valproate addition to antipsychotics can be effective for the overall clinical response. Relatively little research has been done on the signaling pathways in neurons that are activated by the valproic acid. However, we have obtained evidence of activation of the *Ngf* and *Bdnf* genes transcription in cultured neurons *in vitro*. We also found that in the presence of valproic acid, the number of neuronal processes and contacts between them significantly increased. However, we have also found that the oxidative stress accompanying the schizophrenia can significantly reduce the valproic acid effect on the *Ngf* and *Bdnf* genes expression. The results of the study may be potentially useful for new schizophrenia therapy strategies development.

Keywords— BDNF, neurons, oxidative stress, valproic acid.

I. INTRODUCTION

Complex interactions between BDNF and neural activity may be key components in the control of cognitive functions in the mammalian brain disrupted in schizophrenia [1]. Schizophrenia is a multifactorial disease in which both genetic and environmental factors play a significant role. It is well known that physical and mental disorders can provoke psychotic behavior in (genetically) vulnerable patients [2]. These triggers can induce inflammatory changes associated with a decrease in neurotransmitter signaling and synaptic contacts, and increase oxidative stress level [3]. BDNF is a key synaptic plasticity regulator and, therefore, is considered extremely important for the cognitive functions preservation [4, 5]. It was shown that the level of BDNF and its TrkB receptor in the blood serum of patients with schizophrenia decreases [6]. Post-mortem studies have shown that treatment-resistant patients have significantly lower BDNF levels, especially in BDNF-rich brain structures such as the hippocampus [6]. A correlation

of schizophrenia with BDNF gene polymorphisms and with changes in BDNF mRNA levels has been reported [7]. It was found in the patients with the first schizophrenia episode that childhood trauma and stressful situations affected leukocytes BDNF mRNA levels [8]. BDNF levels were also negatively correlated with interleukin 6 (IL-6) expression, suggesting an inflammation-mediated BDNF expression decrease [8, 9].

BDNF signaling can be regulated or changed at the different levels. Valproic acid (VPA) is used in the schizophrenia complex combined treatment regimens both to reduce inflammation and to prevent the appearance of catatonic symptoms [9-12]. The use of valproates in the treatment of schizophrenia differs in different countries – for example, in 2018 in Hong Kong, 38.41% of women of childbearing age with bipolar disorder were prescribed valproates compared to 8.46% in the UK [11]. Treatment of schizophrenia patients with valproate has been shown to increase BDNF level [10]. However, it is still unclear whether BDNF protein levels measured in blood serum samples reflect the level of BDNF in the brain, since animal studies conducted so far yielded contradictory results [6].

We studied *Bdnf* gene transcription changes in neurons *in vitro* under valproic acid and oxidative stress that accompanies different diseases including schizophrenia. In addition, we analyzed *Ngf* gene transcriptional activity under valproic acid on cultured neurons under oxidative stress, since NGF plays an integral role, stimulating the proliferation, differentiation and development of the central nervous system neurons, and activates the PI3K, MAPK, PLC-γ and Ras signaling pathways in a similar way to BDNF [13].

II. MATERIALS AND METHODS

1. Cultivation of primary neuron-glia cell culture

In the present work we used *in vitro* method, studying VPA effects on the neuron-glia culture. Such approach has limitations as cells are grown generally outside their natural environment. Meanwhile, such methodology makes possible to carefully investigate gene expression in standard laboratory conditions. The primary neuron-glia culture was obtained from the cerebellum of 8-9-day-old Wistar rats. Research Centre for Medical Genetics (RCMG) ethics committee approval laboratory animals study (Protocol No. 14). The cerebellums tissue was lysed in a solution of 0.25% trypsin solution / Versen-EDTA solution (in equal volumes) for 15 minutes at 37°C. The supernatant was removed by centrifugation at 200 g for 30 seconds, the tissue was homogenized in a DMEM medium, after precipitation, the upper fraction was passed through a 70 microns filter (SPL Lifesciences), centrifuged at 200 g for 3 minutes, the cell precipitate was placed in a Neuromed medium (PanEco, RF), in which the cells were cultured. The number of cells from one cerebellum is approximately 15 million, the number of cells per well is 2.2 million. The experiment was carried out in 6-well plates with a pre-applied adhesive material: poly-D-lysine. Valproic acid was added to the neurons (at a concentration of 50 μg / ml), oxidative stress was created by 40 mM of H_2O_2, injury was caused by mechanical damage to the neuron culture. Before exposure to the damaging factors, the growth medium was replaced and the restoration of neurons was visually evaluated (AxioVert "CarlZeissMicroscopy" microscope, Germany). To confirm the presence of neurons in the culture, a fluorescent analysis was performed using specific neuronal antibodies (anti-Map-2, Fig. 1).

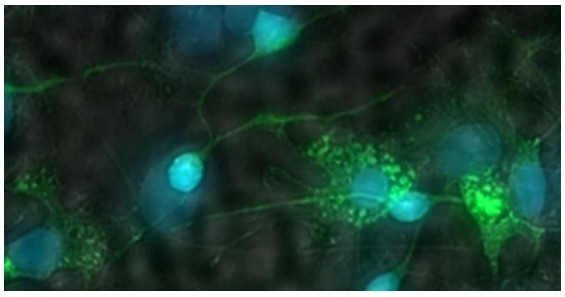

Fig. 1 Fluorescence microscopy. Staining of neurons of the primary neuron-glia culture of the rat cerebellum with anti-Map2 antibodies, followed by treatment with secondary antibodies conjugated with FITC (green). The nuclei are DAPI-colored (blue). Magnification x63.

The ability of VPA to activate the *Bdnf* and *Ngf* genes was analyzed in rat neurons.

2. Determination of the *Bdnf* and *Ngf* genes expression.

Total RNA was isolated with the RNeasy Mini kit (Qiagen, Germany) by the standard method and treated with

DNase I. The isolated RNA was stored at -80°C. The purity of the isolated RNA was determined spectrophotometrically on NanoDrop™ OneC (Thermo Scientific™, USA). The optimal values are greater than 1.8. If the sample had indicators below 1.8, additional cleaning was performed with RNA Clean & Concentrator – 5 kits (Zymo Research, USA). The concentration of the isolated RNA was determined using a Qubit 3.0 fluorimeter (Thermo Scientific™, USA). Reverse transcription was performed using "Silex" (Russia) reagents with random hexaprimers. For real-time polymerase chain reaction (PCR), an internal standard gene was selected from three genes most commonly used in the literature: *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *TBP* (a protein that binds the TATA sequence), *βACT* (*β-actin*). *TBP* was chosen as the gene least susceptible to change during exposure. PCR was performed with Synthol primers and SybrGreen intercalating dye (Invitrogen, USA) on a StepOnePlus device (Applied Byosystems, USA). Amplification reaction conditions: 95°C for 5 minutes, then 35 cycles in the mode: 96°C-20 seconds, 57°C-24 seconds, 72°C-24 seconds.

The level of gene expression was analyzed in several independent experiments; the results were processed in the PCR device software. The error was approximately 2%.

Tbp F: GCTCAGGGCTTGGCCTCCCC,
R: GCTGTCTTTGTTGCTCTTCC
Bdnf F: GTGTGGACCCTGAGTTCCAC,
R: CTGGGTAGGCCAAGTTGCCT
Ngf F: AAGGGGAGCGCATCGCTCTC
R: ATAGAAAGCTGCGTCCTTGG

3. Statistics methods

The reliability of the results obtained in the study was analyzed by statistical methods using the nonparametric Mann-Whitney criterion (U-criterion). Statistics was calculated using the standard software packages Statgraphics, Statistica 10 and SigmaStat.

III. RESULTS

The effect of valproic acid on *Bdnf* expression in the cells of the primary neuron-glia culture of the rat cerebellum was studied 3, 24 and 72 hours after its addition into the neuron culture medium. It was shown that 3 and 24 hours after the valproic acid addition to the primary neuron-glia culture, *Bdnf* expression increases 2.3-fold ($p < 0.01$) and 1.5-fold ($p < 0.01$), respectively (Fig. 2).

The schizophrenia is accompanied by oxidative stress. Oxidative stress in neuron-glia cell culture was modeled by hydrogen peroxide (40 μM) added into the cell culture medium for 30 min. Mechanical damage to neurons disrupts contacts between neurons to a greater extent, but is not accompanied by intense oxidative stress. Hydrogen peroxide in a dose 40 μM in the culture medium does not change *Bdnf* expression and valproic acid under oxidative stress causes only slight 1.5-fold ($p < 0.01$) *Bdnf* expression increase after 3 hours (Fig. 2). Both mechanical damage to neurons and the addition of valproic acid after mechanical culture damage increased *Bdnf* expression 2-2.5-fold ($p < 0.01$) after 3 to 24 hours. Addition of valproic acid in these conditions did not produce any significant effect (Fig.2).

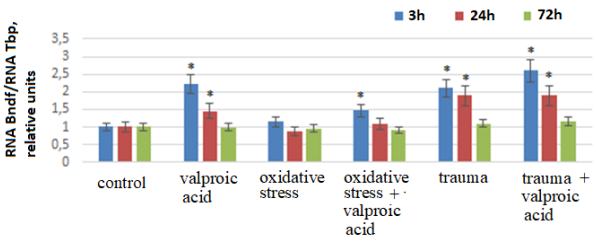

Fig. 2. *Bdnf* mRNA content in the cells. Bar chart reflects *Bdnf* expression in the cells of the primary neuron-glia culture of the rat cerebellum in 3, 24 and 72 hours. X-axis illustrate condition of experiment; control – the cells cultured without any action. *Bdnf* mRNA amount is the average value for three experiments relatively to the *TBP* standard gene expression. (*) data differ from the control according to the Mann-Whitney criterion; the differences are significant, $p < 0.01$.

Another factor necessary for the survival, growth of axons and dendrites, specification and formation of synapses of sympathetic and sensory neurons is NGF. The signaling pathways and processes that NGF triggers occur mainly in sympathetic and sensory neurons. NGF binds to the TrkA receptor leading to its phosphorylation. Then, similarly to BDNF effect, PI3K, MAPK, PLC - γ, Ras – signaling pathways in neuronal cells are activated [13].

We studied the valproic acid effect on the *Ngf* expression in the cells of the primary neuron-glia culture of the rat cerebellum 3, 24 and 72 hours after its addition into the neuron culture medium. It was shown that 3 and 24 hours after valproic acid addition to the primary neuron-glia culture, *Ngf* expression increases 1.6-fold ($p< 0.01$) and 1.5-fold ($p<0.01$), respectively (Fig. 3). Hydrogen peroxide in dose 40μM did not change *Ngf* gene expression. Valproic acid added under oxidative stress also did not change *Ngf* expression (Fig. 3). Mechanical damage to neurons significantly increased *Ngf* expression in 3 and 24 hours. *Ngf* expression in neurons 24 hours after mechanical damage and valproic acid addition also increased ($p< 0.01$). Valproic acid did not change neuronal *Ngf* expression 24 hours after mechanical injury (Fig. 3).

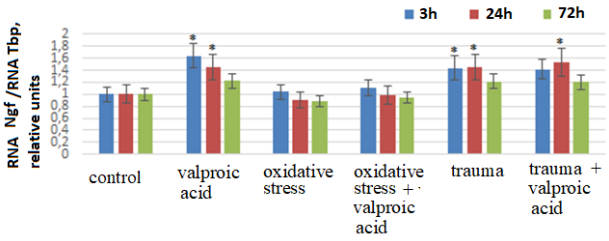

Fig. 3. *Ngf* mRNA content in the cells. Bar chart reflects the *Ngf* expression in the cells of the primary neuron-gliaculture of the rat cerebellum in 3, 24 and 72 hours. X-axis illustrate condition of experiment; control – the cells cultured without any action. *Ngf* mRNA amount is the average value for three experiments relatively to the *TBP* standard gene expression. (*) data differ from the control according to the Mann-Whitney criterion; the differences are significant, $p< 0.01$.

IV. DISSCUSSION

Therefore, we have shown that valproic acid increases *Ngf* and *Bdnf* expression in primary neuron-glia culture cells within 3-24 hours after addition to the neurons. The results of the present study show that mechanical trauma, unlike oxidative stress, activates *Ngf* and *Bdnf* transcriptional activity (Fig. 2, 3). Valproic acid effects on *Ngf* and *Bdnf* expression under oxidative stress and mechanical damage to neurons, was insignificant (Fig.2, 3).

Fluorescence analysis using specific neuronal antibodies (anti-Map-2) showed that in the presence of valproic acid, the number of neuronal processes and contacts between them significantly increased (Fig. 4). This confirms valproic acid positive effect on the neuroplasticity [4]. However, its effect on neurons is, possibly, more complex than simply the effect on the *Ngf* and *Bdnf* expression.

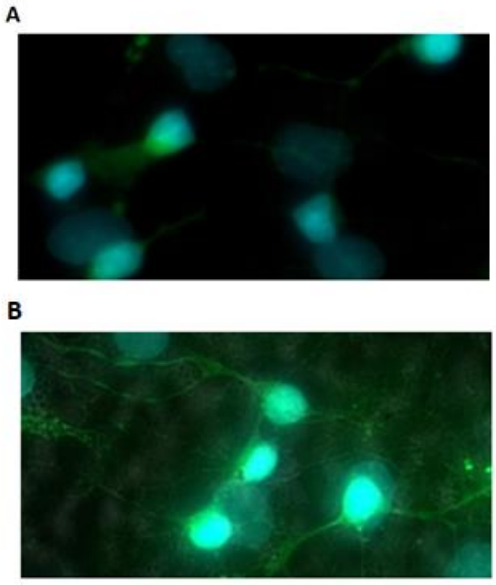

Fig. 4. Fluorescence microscopy. Staining of the primary neuron-glia culture of the rat cerebellum with anti-Map2 antibodies, followed by a treatment with secondary FITC-conjugated antibodies. (A) control, (B) 24 hours after cultivation with valproic acid. The nuclei were stained with DAPI. Magnification X63.

Possibly, valproic acid may affects BDNF binding with its receptor –TrkB, or *TrkB* gene expression, or pro-BDNF expression, or other signaling pathways in the cells [14]. Thus, it has been shown that valproate can inhibit glycogen synthase kinase 3 (GSK-3) and the sodium channels function [12]. GSK-3 can inhibit mTOR signaling and, thus, affect the transmission of BDNF signals [15]. Understanding of key signaling pathways genes transcriptional activity changes in cells is necessary for the reasonable drug therapy of different diseases, including schizophrenia.

V. CONCLUSION

There is a limited amount of evidence based on a number of studies that the addition of antipsychotics with valproate can be effective for the general therapeutic response, little research has been done on the signaling pathways in neurons that are activated by the action of valproic acid. However, we have obtained evidence of

activation of the *Ngf* and *Bdnf* genes transcription by VPA in cultured neurons *in vitro*. We also have found that in the presence of valproic acid, the number of neuronal processes and contacts between them significantly increased. The results of the present study show that valproic acid under oxidative stress conditions causes only slight *Bdnf* expression increase after 3 hours in comparison with valproic acid effect in control experiments. Therefore we conclude that oxidative stress accompanying the schizophrenia can significantly reduce the valproic acid effectiveness on the *Ngf* and *Bdnf* genes expression. Further studies of the valproic acid effect on the transcriptional activity of key signaling pathways genes in neurons under oxidative stress are needed. Also more research should be conducted to determine VPA effects using *in vivo* conditions to justify its effects in living mammalian brain and the oxidative stress role in schizophrenia patients' therapy approaches.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

REFERENCES

[1] A. Mohammadi, E. Rashidi, V.G. Amooeian, "Brain, blood, cerebrospinal fluid, and serum biomarkers in schizophrenia", Psychiatry Res, 265, 2018; pp. 25-38. doi: 10.1016/j.psychres.2018.04.036.

[2] M.E. Benros, P.R. Nielsen, M. Nordentoft et al., "Autoimmune diseases and severe infections as risk factors for schizophrenia: a 30-year population-based register study", Am J Psychiatry, 168, 2011, pp. 1303–1310. doi: 10.1176/appi.ajp.2011.11030516.

[3] G.M. Khandaker, L. Cousins, J. Deakin, et al., "Inflammation and immunity in schizophrenia: implications for pathophysiology and treatment" Lancet Psychiatry, 2, 2015, pp. 258–270. do 10.1016/S2215-0366(14)00122-9.

[4] G. Leal, D. Comprido, C.B. Duarte, "BDNF-induce local protein synthesis and synaptic plasticity' Neuropharmacology, 2014 Jan;76 Pt C:639-56. do 10.1016/j.neuropharm.2013.04.005. Epub 2013 Apr 16.

[5] Leal , C.R. Bramham , C.B. Duarte, "BDNF and Hippocampal Synaptic Plasticity", Vitam Horm, 2017;104:153-195. doi: 10.1016/bs.vh.2016.10.004. Epub 2016 Nov 29.

[6] B. Lima Giacobbo, J. Doorduin, H.C. Klein, RAJO Dierckx, E. Bromberg, EFJ de Vries, et al., "Brain-Derived Neurotrophic Factor in Brain Disorders: Focus on Neuroinflammation", Mol Neurobiol., 56, 2019, pp. 3295-3312. doi: 10.1007/s12035-018-1283-6.

[7] B. Lu, K. Lu B. Martinowich et al., "Cell biology of BDNF and its relevance to schizophrenia", Novartis Found Symp, 289, 2008, pp. 119-129; discussion 129-35, 193-5. doi: 10.1002/9780470751251.ch10.

[8] V. Mondelli, A. Cattaneo, M.B. Murri, et al., "Stress and inflammation reduce brain-derived neurotrophic factor expression in first-episode psychosis: a pathway to smaller hippocampal volume", J Clin Psychiatrym, 72, 2011, pp. 1677–1684. doi: 10.4088/JCP.10m06745.

[9] Y.K. Kim, H.G. Jung, A.M. Myint, et al., "Imbalance between pro-inflammatory and anti-inflammatory cytokines in bipolar disorder", J Affect Disord, 104, 2007, pp. 91–95. doi: 10.1016/j.jad.2007.02.018.

[10] C.A. Zarate, H.K. Manji, "Protein kinase C inhibitors: rationale for use and potential in the treatment of bipolar disorder", CNS Drugs, 23, 2009, pp. 569–582. doi: 10.2165/00023210-200923070-00003.

[11] V.W.S Ng, K.K.C. Man, L. Gao, E.W. Chan, E.H.M. Lee, J.F. Hayes, I.C.K. Wong, "Bipolar disorder prevalence and psychotropic medication utilisation in Hong Kong and the United Kingdom", Pharmacoepidemiol Drug Saf, 2021 doi: 10.1002/pds.5318.

[12] M. Saito, T. Takizawa, H. Miyaoka, "Factors associated with blood carnitine levels in adult epilepsy patients with chronic valproic acid therapy", Epilepsy Res, 175, 2021, 106697. doi: 10.1016/j.eplepsyres.2021.106697.

[13] M.C. Marlin, G. Li, "Biogenesis and function of the NGF/TrkA signaling endosome", Int Rev Cell Mol Biol, 314, pp. 239-257. doi: 10.1016/bs.ircmb.2014.10.002

[14] Dedoni S., Marras L., Olianas M.C., Ingianni A., Onali P.J., "Downregulation of TrkB Expression and Signaling by Valproic Acid and Other Histone Deacetylase Inhibitors", Parmacol Exp Ther., 2019 Sep;370(3):490-503. doi: 10.1124/jpet.119.258129

[15] T.D. Gould, J.A. Quiroz, J. Singh, et al, "Emerging experimental therapeutics for bipolar disorder: insights from the molecular and cellular actions of current mood stabilizers", Mol Psychiatry, 9, 2004, pp. 734–755. doi: 10.1038/sj.mp.4001518.