EFFECT OF CHRONIC EXOGENOUS STIMULATION OF NEUROTROPHIC FACTOR BDNF ON MITOCHONDRIA-ENDOPLASMATIC RETICULUM CONTACTS IN IMMATURE NEURONS

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Abstract. Mitochondria-endoplasmic reticulum contacts (MERC) are known as one of the key regulators of many cell functions. In particular, MERC effects on the mobility and morphology of mitochondria, exchange of calcium and lipids between organelles, and participates in the processes of autophagy and apoptosis that are crucial for neuronal development. MERC can influence the functioning of the neurotrophic factor BDNF through the activation of the Sigma-1 receptor. That points to the presence of feedback i.e. itself BDNF influence on the structural characteristics of MERC. At the same time, the effect of chronic stimulation of BDNF or blockade of TrkB receptors on an ability to form contacts between mitochondria and ER is different and depends on cellular compartment.

Keywords: brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase B (TrkB), mitochondria-endoplasmic reticulum contacts (MERC), mitochondria-associated membranes (MAM), endoplasmic reticulum (ER), primary hippocampal cultures.

List of Abbreviations

BDNF – brain-derived neurotrophic factor
TrkB – tropomyosin receptor kinase B
MERC – mitochondria-endoplasmic reticulum contacts
MAM – mitochondria-associated ER membranes
ER – endoplasmic reticulum
NMDA – N-Methyl-d-aspartic acid
Src – tyrosine-protein kinase CSK
D1R – dopamine (DA) D1 receptor
PLC – phospholipase C
DA – dopamine
DIV – day of culture development in vitro

Introduction

Despite the brain structure being formed before birth, its complete formation is determined by the postpartum state. External stimuli modulate the functional brain maturation and neurogenesis in adulthood (Sailor et al., 2017). The brain-derived neurotrophic factor (BDNF) via interaction with the tropomyosin receptor kinase B (TrkB) (Skaper, 2018) can affect the possibility of activation an intracellular signaling cascade, which in turn indirectly induce synaptic transmission and the formation of synaptic contacts, determining the structure of neural networks (Miter *et al.*, 2017).

Previous studies have demonstrated the existence of physical and functional connections between mitochondria and endoplasmic reticulum (ER) implemented through junctions such as mitochondria-endoplasmic reticulum contacts (MERC) or mitochondria-associated membranes (MAM) in case of an isolated fraction of these contacts (Giorgi *et al.*, 2015). MERC can influence the mobility and morphology of mitochondria, exchange of calcium and lipids between organelles and participates in the processes of autophagy and apoptosis. In addition, MERC, localized in neurons and astrocytes, are an indispensable participant in neurotransmission (Shirokova *et al.*, 2020). The Sigma-1 receptors (Sig-1R), a MAM component, is known to stimulate the release of BDNF from primary cortical astrocytes (Malik *et al.*, 2015) and also regulate the processing and transport of BDNF in nerve cells (Hayashi, 2015). According to a modern concept (Kourrich *et al.*, 2012), in development of neurological disorders, Sig-1R moves from MERC to other parts of the cell, and binds

to various ion channels, receptors or kinases (ex. NMDA, Src, D1R, PLC, DA transporter) modulating their activity. Since MERC is currently known to have a direct link to BDNF functioning via the Sigma-1R receptor, we aimed to determine whether chronic exogenous stimulation of BDNF affects the morphological state of the mitochondrial-endoplasmic contacts themselves.

Methods

Ethics statement. All experimental protocols were approved by the Bioethics Committee of Lobachevsky University and carried out in accordance to Act708n (23 08 2010) of the Russian Federation National Ministry of Public Health, which states the rules of laboratory practice for the care and use of laboratory animals, and the Council Directive 2010/63 EU of the European Parliament (September 22, 2010) on the protection of animals used for scientific purposes. C57BL / 6J mice were killed by cervical vertebra dislocation, and their embryos were then surgically removed and sacrificed by decapitation.

Experiment scheme. Recombinant human BDNF (1 ng/mL, Merck, GF301), a selective TrkB receptor blocker ANA-12 (1 μM, Sigma-Aldrich, SML0209) or their combination was added to the culture medium daily beginning on the third day of culture development in vitro (DIV) (Fig. 1a). The concentrations of substances were chosen according to our previous studies on spontaneous bioelectrical and calcium activity of primary neuronal cultures as well as characteristics of synaptic apparatus, the mitochondrion structure and its functional activity under chronic treatment with BDNF and ANA-12 (Mischenko *et al.*, 2019).

Cell culture. Hippocampal cells were obtained from embryos of C57BL/6J mice (day 18 of gestation) and cultured on coverslips (18 × × 18 mm) pretreated with polyethyleneimine solution (1 mg/mL, Sigma-Aldrich, P3143). Isolation and cultivation of primary hippocampal cultures were performed according to a previously developed protocol (Vedunova *et al.*, 2013). Initial density of cells was approximately 9,000 cells/mm^2. Cell viability was maintained under constant conditions of 35.5°C, 5% CO_2 and a humidified atmosphere in a cell culture incubator during 10 days.

Electron microscopy. Primary hippocampal cultures were fixed in 2.5% glutaraldehyde (Acros Organics, AC119980010) on DIV 10. The cultures were then washed three times with PBS and treated with 1% osmium tetroxide (Sigma-Aldrich, 20816-12-0) and 1.5% potassium ferrationide for 60 min. After additional washing steps, the samples were dehydrated in a series of ethanol solutions of increasing concentration (30–100%) followed by 100% acetone and then embedded in a mixture of acetone / EPON resin (50:50). The culture was ultimately embedded in EPON resin (Fluka, United States).

Morphometric analysis. Morphometric analysis of undamaged mitochondria with normal ultrastructure was performed using ImageJ software. The following parameters were assessed: (1) area occupied by mitochondria, (2) the mitochondrial shape calculated as the ratio of length to width of mitochondria, (3) the surface occupied by MERC, (4) the proportion of mitochondria in MERC, %, (5) the state of cristae. The observations were carried out in the neuron's body, axons and dendrites. Each experimental and control group included three cultures; 100 mitochondria were analyzed for each culture.

Statistical analysis. Statistical analyses were performed in Sigma Plot 11.0 software (Systat Software, Inc.) using the nonparametric Mann-Whitney test. Differences between groups were considered significant if the corresponding p-value was less than 0.05.

Results

Ultrastructural study of primary hippocampal cultures revealed a difference in the length of MERC and in the number of mitochondria involved in MERCs at different neuronal compartments (Fig. 1).

In the dendritic processes, the percentage of the mitochondrial surface occupied by contacts with the ER (19.24 ± 1.1%) was significantly higher compared to axonal mitochondria

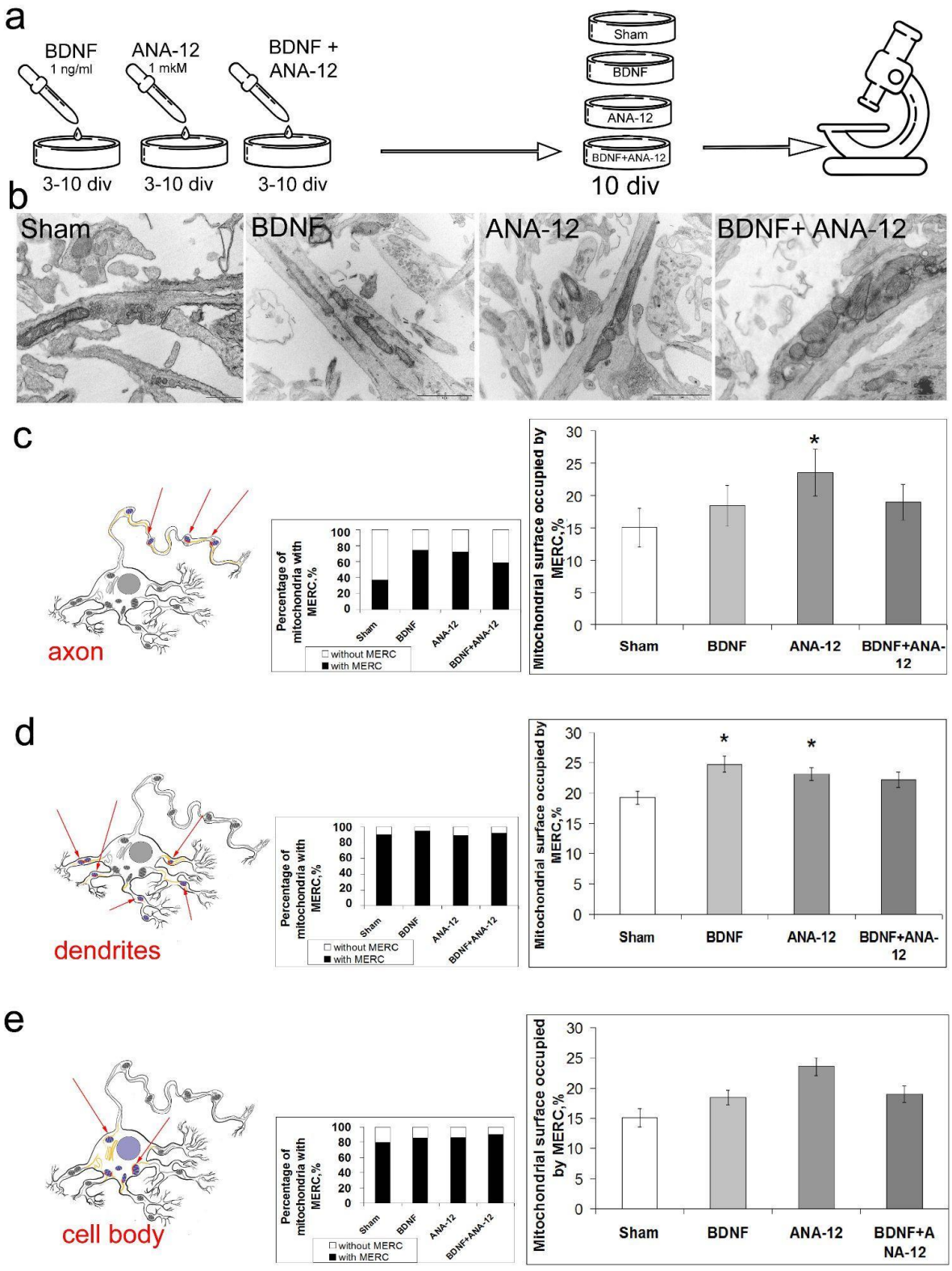

Fig. 1. Ultrastructural characteristics of mitochondria-endoplasmic contacts (MERC) of different parts of neurons (the percentage of mitochondria with MERC and the mitochondrial surface occupied by contact with the ER). a – experimental scheme; b – representative images of ultrastructural organization of the dendritic processes; c – MERC in the axons (highlighted in red); d – MERC in the dendrites (highlighted in red); e – MERC in the cell bodies (highlighted in red)

(15.06 ± 2.98%). In addition to the length of MERC, the proportion of axonal mitochondria interacted with the ER via MERC was 37.09%, whereas in dendrites this parameter amounted to 90.17% (Fig. 1d).

Daily application of the neurotrophic factor BDNF led to significant increase in the number of mitochondria involved in MERCs in axonal processes by DIV 10 (Control: 37.09%, BDNF 74.35%). The number of mitochondria interacted with MERC in other neuron's compartments did not change significantly. The mitochondria surface interacting with the ER in axons and cell bodies remains at the same level. Axonal mitochondria increase the surface that interacts with the ER. In general, the ultrastructural organization of neuronal mitochondria in the "BDNF" group did not have any peculiarities. However, a large number of ribosomes were observed in the cell bodies and dendritic processes, and osmiophilic granules were observed in the glial processes. (Fig. 1c, d, e).

Chronic blockade of the TrkB receptors in immature primary hippocampal cultures resulted in ultrastructural lesions of mitochondria and neuronal cells (Fig. 1b). The number of mitochondria interacting with the ER was increased and amounted to 71.91% of all axonal mitochondria. At the same time, the length of MERC of axonal mitochondria was significantly increased. On the other hand, in the dendrites, the number of such mitochondria did not change, but the length of contacts was significantly increased (Fig, 1).

In the "BDNF + Ana-12" group, a slight increase in the number of mitochondria involved in MERC was observed in the axons (Fig. 1). In the dendrites and cell bodies, a slight increase in the number of mitochondria interacting with the ER were shown. There were no significant differences in the length of contacts in all parts of the neuron.

Discussion

MERCs are necessary to maintain lipid and energy metabolism, as well as calcium ions transfer from the ER to mitochondria, which is important for mitochondrial integrity and bioenergetics (Bravo *et al.*, 2011). The morphology of mitochondria and their ability to form contacts with other organelles reflects changes in various cellular signaling cascades (Giacomello *et al.*, 2020.). It is currently known that an increase in the interacting surface of two organelles (mitochondria and the ER) occurs when some MAM components are affected, in particular, when increasing a protein tyrosine phosphatase interacting protein 51 (PTPIP51) (Gomez-Suaga *et al.*, 2017), decreasing the mitofusin-2 (Leal et al., 2016), at loss of E3 ubiquitin ligase (parkin) function (Bray *et al.*, 2018) and in various pathological conditions (Leal *et al.*, 2016). Here we showed that BDNF and ANA-12 - two substances opposite in action, but associated with the TrkB receptors activity, affect the contact sites between the ER and mitochondria. Thus, we revealed the ambiguity in the signaling pathways of the TrkB receptor system in neurons, affecting the functioning of MERC. We assume that the activation of different MERC functions depending on the conditions (group/compartment).

It was previously suggested that the width of MERC gap plays a crucial role for different functions on the cell (Giacomello and Pellegrini, 2016). Therefore, further studies on the correlation analysis of the length and width of the specific MERC gap should be carried out. In addition, we suggested that the MERC in the dendrites and axons of neurons has different functions (Shirokova *et al.*, 2020). In this case, changing in the MERC length in different compartments will lead to different effects on the neuronal network in general.

Many research groups use both BDNF and ANA-12 as a therapeutic molecules in different pathological states (Danelon *et al.*, 2016). Our previous studies have demonstrated that chronic application of ANA-12 leads to a significant decrease in the spontaneous bioelectrical activity of neural networks of primary hippocampal cultures by DIV 14 (Mishchenko *et al.*, 2019) that points to the destruction of neural networks after the beginning of TrkB blockade. The cause of the disruption of neuronal network's integrity

could be a consequence of the partial death of cell processes, cell energy disturbance or general morpho-functional instability of neurons. On DIV 10, the networks are still forming, and normally the neurons not involved in the network die, the number of glial cells increases, and electrical synapses replace by chemical synapses (Shirokova *et al.*, 2013). Thus, on DIV 10 it is already clear which neurons will be included in the network, so there is a high network stability in bioelectrical activity parameters, shown in our previous study (Mishchenko *et al.*, 2019). On the first stages of development, the BDNF-TrkB system is especially important for axons determining the neurite's fate: whether it will become an axon or a dendrite (Woo et al., 2019; Cheng *et al.*, 2011). It can be assumed that the chronic blockade of TrkB receptors at the early stage of primary cultures cultivation (starting at 3 DIV) delay the network's development, which is characterized by a decrease in the stability of spontaneous neural network activity and by a modulation of the frequency and the duration of intracellular calcium fluctuations in cells (Mishchenko *et al.*, 2019), typical of the earlier stages of ontogenesis in vitro (Shirokova *et al.*, 2013). At morphological level, the chronic application of ANA-12 leads to an increase in the MERC length which can cause either a positive effect (increased calcium signaling and ATP production, movement/fusion of mitochondria) and negative consequences (initiation of apoptotic signals) on cell physiology (Leal *et al.*, 2016).

The different effects observed in the neurites and cell bodies under chronic influence on TrkB receptors have several explanations. First, the neuronal soma and neurites have different molecular physiology, and, accordingly, different temporal patterns of reactions. Thus, neuronal death is often preceded by morpho-functional changes in neurites. In particular, the irregular distribution of TrkB isoforms in axons and dendrites precedes the neuronal death in status epilepticus (Danelon *et al.*, 2016). Second, in axons, TrkB receptors have predominantly intracellular localization, whereas in dendritic spines most of the pTrkB-ir is associated with the plasma membrane (Spencer-Segal *et al.*, 2011). At the same time, the quantitative ratio of TrkB receptors in the hippocampal axons and their endings, dendrites and dendritic spines, and glial cells are quite different (Spencer-Segal *et al.*, 2011). In this regard, the effect of the influence on BDNF-TrkB system should be evaluated depending on the cellular compartment.

An increase in the MERC length under chronic application of ANA-12 may indicate compensatory phenomena, since many mitochondria die and ATP synthesis must be increased via the effective calcium transfer between organelles (Lee *et al.*, 2019). In addition, a decrease in MERC initiates mitophagy (Böckler, 2014). For neurons, a more effective strategy for the survival of the entire network is not the destruction of damaged mitochondria, but the maintenance of a low level of mitophagy (Puri *et al.*, 2019). However, an increase in the length of MERC in dendrites in chronic blockade of TrkB receptors may be considered as an early signal for cell death, since too strong contact between two organelles can trigger apoptotic signals (Perkins & Ellisman, 2016). In addition, a question of how the alignment of the MERC length between the compartments observed in the "ANA-12" group will affect intracellular signaling in the neuron remains unresolved.

There are several assumptions regarding the effect of the chronic BDNF application on the length of contacts with ER in dendritic mitochondria rather than axonal ones. First, the used low concentration of BDNF may not be enough to activate TrkB receptors on endomembranes; in axonal processes and axonal buds, TrkB receptors are concentrated on the inner membranes. Therefore, we observed the effect of BDNF as an increase in the bioelectrical network parameters and an increase in metabolically active cells (Mishchenko *et al.*, 2019). To verify this hypothesis, it is necessary to study the excitability of individual neurons using the patch-clamp method or evoked potentials. In dendritic processes, as already stated, TrkB receptors are located in

the plasma membrane; therefore, the effect in dendrites is stronger. Notably, that in our previous studies we did not observe any catastrophic effects on overall mitochondrial morphology on 14 DIV (Mishchenko *et al.*, 2019). Moreover, the basal oxygen consumption rate of mitochondria and the activity of respiratory chain complex I and II in the cultures with chronic BDNF application are increased simultaneously with an increase in bioelectrical and metabolic activity of cells (Mishchenko *et al.*, 2019). Therefore, the observed increase in the MERC length may be considered as a positive function, that can increase the efficiency of calcium and lipid metabolism in nerve cells. However, this assumption needs to verify by a three-dimensional reconstruction, that helps to find correlations between the width and the length of a specific contact.

It is also worth noting that primary neuronal cultures contain both neurons, astrocytes, and microglia, which can also affect signaling pathways in chronic influence of BDNF and ANA-12. For instance, the presence of pTrkB in microglia (Spencer-Segal *et al.*, 2011) makes it another possible candidate in the regulation of BDNF signaling pathways and the observed effects in vitro.

Conclusion

This study demonstrates the effect of the neurotrophic factor BDNF on MERC in immature hippocampal neurons. The influence on the BDNF-TrkB system varies the response of mitochondria depending on their localization in the neuron. Whether the observed effect is induced by astrocytic influence or directly affects the neurons remains unclear and presents an exciting area for upcoming research.

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