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Research Article

Metal-containing taurine compounds protect rat's brain in reperfusion-induced injury

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Abstract

Introduction: The **study aim** was to explore a neuroprotective action of magnesium (LKhT-317) and zinc (LKhT-318) taurine salts on experimental models of reperfusion brain damage in rats and cell culture.

Materials and methods: The study was performed on male Sprague Dawley rats, and rat's hippocampal mixed neuroglial cell culture. Magnesium- (LKhT-317) and zinc-containing (LKhT-318) derivatives of taurine were studied. Reperfusion brain damage was induced 30 min after intraluminal cerebral middle artery occlusion. Severity of the injury was assessed by local blood flowmetry, neurological symptoms scaling and brain tissue staining. Levels of IL-1b, IL-10 and TNF-alpha in tissue were determined by qualitative ELISA. Caspase-3 and Bcl-2 expressions were detected by IHC. Neurons survival was assessed by cytochemistry. Cellular calcium responses were detected by fluorescent microscopy of Fura-2-containig cells.

Results and discussion: Metal-containing taurine derivatives – LKhT-317 and LKhT-318 – demonstrated a sufficient neuroprotective property in rats with a reperfusion-induced brain injury. Both derivatives effectively prevented severity of the animals' brain damage, motor deficiency, reduction of microvascular perfusion, and proinflammatory cytokines production. Magnesium-containing compound LKhT-317 was comparatively more effective than zinc-containing one. LKhT-317 possessed an anti-apoptotic action *in vivo*, and protected neurons from OGD-mediated cell death in mixed hippocampal culture. The aforementioned actions may be associated with an LKhT-317 inhibitory effect on NMDA-induced cellular Ca^{2+} response and, therefore, the anti-excitotoxic property of the compound.

Conclusion: Magnesium- and zinc-containing taurine derivatives may be considered as promising neuroprotectors in the reperfusion-induced brain injury.

Keywords

brain injury, metal-containing taurine derivatives, reperfusion, cerebral middle artery, Ca2+ response, hippocampal mixed cell culture, rats.

Introduction

Ischemic stroke (IS) remains one of the most deteriorating pathologies worldwide, especially impacting elderly people (Virani et al. 2020). About 85% of all cerebrovascular accidents are associated with IS, while only 15% of all cases are associated with brain hemorrhage (Harris et al. 2018).

Is usually the result of cerebral artery blockage due to *in situ* thrombosis or embolism. The pathological focus formation is characterized by cellular infiltration, disruption of the integrity of the blood-brain barrier, cellular regulation, cell death, followed by an inflammatory reaction and edema (Abbott et al. 2006; Brouns et al. 2011; Machinaga and Koyama 2015; Merali et al. 2017). Tissue perfusion decrease leads to the development of acute oxygen and nutrients with subsequent development of a necrotic core and ischemic zone (penumbra). Because of the potential viability of its cells, penumbra is the main target for novel and promising pharmacotherapeutic strategies (Schlaug et al. 1999; Guadagno et al. 2004; Tsai et al. 2014).

Current treatment strategies include recanalization of the blocked cerebral artery, either with the use of tissue plasminogen activators or by implementation of interventional endovascular methods. However, the restoration of cerebral blood flow entails, in addition to obviously positive consequences, the development of so-called reperfusion syndrome, a condition no less formidable than cerebral ischemia itself (Guadagno et al. 2004).

It has been shown that taurine, presented as a natural metabolite in mammalians, has a sufficient brain-protective action, the mechanism of which is associated with the key ways of brain acute ischemic and reperfusion injury pathogenesis. Therefore, the aim of our study is to explore the neuroprotective action of magnesium (LKhT-317) and zinc (LKhT-318) taurine salts on experimental models of reperfusion brain damage in rats and cell culture.

Materials and methods

All the study protocols were reviewed and approved by the Local Ethics Committee of Ogarev Mordovia State University (Minutes No. 10 of October 20, 2016). The experimental procedures met all the requirements of the Russian national regulations – *Principles of Good Laboratory Practice*.

Animals and cell cultures

In vivo experiments were performed on 360 male Wistar rats weighing 250-300 g, purchased from the Stolbovaya Nursery (Russian Scientific Center for Biomedical Technologies, Russia). The experimental groups included up to 20 animals because of the necessity of time-dependent three-stage withdrawal of rats 3, 24 and 48 hours after middle cerebral artery (MCA) recanalization. All the invasive manipulations were accompanied by mandatory pain-control procedures. Intravenous anesthesia was used (urethane 800 mg/kg of rat weight). The animals were withdrawn from the experiment under ether anesthesia by an air embolism.

In vitro experiments were carried out on mixed neuroglial cell culture. The culture was prepared from the hippocampus of newborns (1-3 days after birth) of linear Sprague Dawley rats.

Substances and drugs

Two original taurine compounds, magnesium bis-2-acetaminoethanesulfonoate (laboratory name LKhT-317) and zinc 2-aminoethanesulfonoate (laboratory name LKhT-318) were studied as substances with purity no less than 98.95%. The structures of the molecules are shown in Fig. 1.

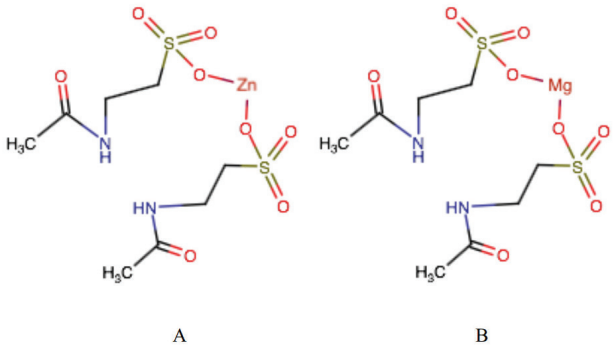

Figure 1. Chemical structures of Zinc bis-2-acetaminoethanesulfonoate (**A**) and Magnesiumbis-2-acetaminoethanesulfonoate (**B**).

Compounds LKhT-317 and LKhT-318 were synthesized in the Department of Chemistry, Technology of Synthetic Medicines and Analytical Control of All-Union Research Center for Safety of Biological Active Substances. Both substances are white microcrystalline powder with a slight odor. Zinc salt is less soluble in water than magnesium one, and requires additional heating to dissolve. After dissolution, both substances form stable solutions.

We used Nimodipine in the form of a substance (Nimodipine, purity of 98.85%, CAS Number: 66085-59-4, manufactured by Merck, SIGMA-Aldrich, Germany) as a reference pharmacological agent in the animal part of the study.

All the studied substances after being dissolved in 0.9% saline were administered intravenously in 1.0 ml volume in tail vein. We practiced two therapeutic regimens as follows: 10 minutes before MCA occlusion or 10 minutes before MCA reperfusion. The compounds were introduced in doses related to 2.5 and 5.0% of LD_{50} index (Turovsky et al. 2018).

In the *in vitro* part of the study LKhT-317 and LKhT-318 were added into the cultivation medium or, when registering cells' calcium response, were mixed with the dialysis solution in concentration ranged from 5 μM to 10 mM.

Experimental methods

The brain-protective activity of the compounds was assessed using the model of intraluminal occlusion/reperfusion

of MCA in rats. Animals' brain blood flow was assessed by laser magnetic flowmetry (BIOPAC 160, USA) 3, 24 and 48 hours after MCA recanalization. At the same time rats' behavior and neurological symptoms were assessed using the scale proposed by Huang et al. (1994). The volume of the brain necrotic core was determined by the triphenyl tetrazolium chloride (TTC) staining method. Tissue pH, ATP concentration and the intensity of edema were recorded. TNF-alpha, IL-10, and IL-1beta were determined in the reperfused brain regions using quantitative ELISA, a StatFax 4200 automatic reader (USA) and rat antibodies (CUSABIO BIOTECH, Inc., China). An antiapoptotic activity was evaluated by immunohistochemical (IHC) detection of caspase-3 and Bcl-2 expression levels in rats' brain 4 μm sections.

A double-wave fluorescent probe Fura-2 was used to determine Ca^{2+}concentration ($[Ca^{2+}]_i$) in the cytoplasm of hippocampal cells. After recording the calcium responses, the cells were fixed, stained with antibodies against glutamate decarboxylase (GAD65/67) and calcium-binding proteins – parvalbumin (PV) or calbindin (CB), and then the fluorescent image of the cells was recorded on an inverted confocal microscope (Gaidin et al. 2020). The number of viable and dead cells was determined by staining with propidium iodide (PI, 1 μg / ml).

Statistical processing of the obtained results was carried out using conventional statistics methods by means of BioStatsoftware for Microsoft Windows, SPSS, and PC iMac Retina (USA).

Results and discussion

We evaluated the size of reperfusion-induced brain damage by TTC-staining method. The volume of the injured rats' ipsilateral hemisphere was assessed 3, 24 and 48 hours after the restoration of cerebral circulation (Fig. 2). Both compounds were more effective when administered before MCA occlusion. At the same time, 25 mg/kg intravenous LKhT-317 was more effective than zinc-containing taurine derivative (LKhT-318). The brain-protective property of LKhT-317 was comparable with that of the reference selective calcium antagonist nimodipine.

Restoration of cerebral perfusion plays a pivotal prognostic role for brain damage outcomes. However, as we have repeatedly pointed out, brain reperfusion itself frequently causes a disastrous impact on ischemic brain. At the same time, the volumetric index of brain tissue perfusion, measured at a fixed point and evaluated over time, can be a reliable indicator of the viability of damaged tissues. We evaluated rats' cerebral tissue perfusion by laser flowmetric method, using a BIOPAC-160 system. It turned out that both compounds effectively maintained tissue microvascular perfusion in tissues neighboring the injured ones. It has statistical significance for all the experimental groups. Moreover, compound LKhT-317 after 24 and 48 hours of observation was comparable to nimodipine in its ability to restore cerebral perfusion at the lesion focus. Both substances were superior to the zinc-containing derivative LKhT-318.We also demonstrated inverse correlation between stagnation of cerebral tissue perfusion and brain damage volume (Table 1).

We then assessed the severity of neurological disorders of rats with a reperfusion-induced brain injury. Animals' extremity weakness and motor discoordina-

Table 1. Correlations Between Cerebral Tissue Perfusion and Brain Injury Volume in Rats with Experimental MCA Reperfusion (M ± SD)

Goup	Brain necrosis size, Δ	Perfusion volume, Δ	r, p
Control group	21 ± 1.8	-43 ± 4.2	-0.99; 0.001
LKhT-318 before MCAo	15 ± 2.2	-25 ± 3.7	-0.93; 0.001
LKhT-318 before MCAr	11 ± 1.9	-24 ± 2.7	-0.97; 0.001
LKhT-317 before MCAo	12 ± 2.3	-22 ± 2.4	-0.97; 0.001
LKhT-317 before MCAr	18 ± 3.1	-25 ± 3.3	-0.90; 0.01
Nimodipine before MCAo	10 ± 1.5	-21 ± 2.9	-0.97; 0.001
Nimodipine before MCAr	17 ± 2.6	-21 ± 4.1	-0.88; 0.02

Note: MCAo – middle cerebral artery occlusion; MCAr – middle cerebral artery reperfusion.

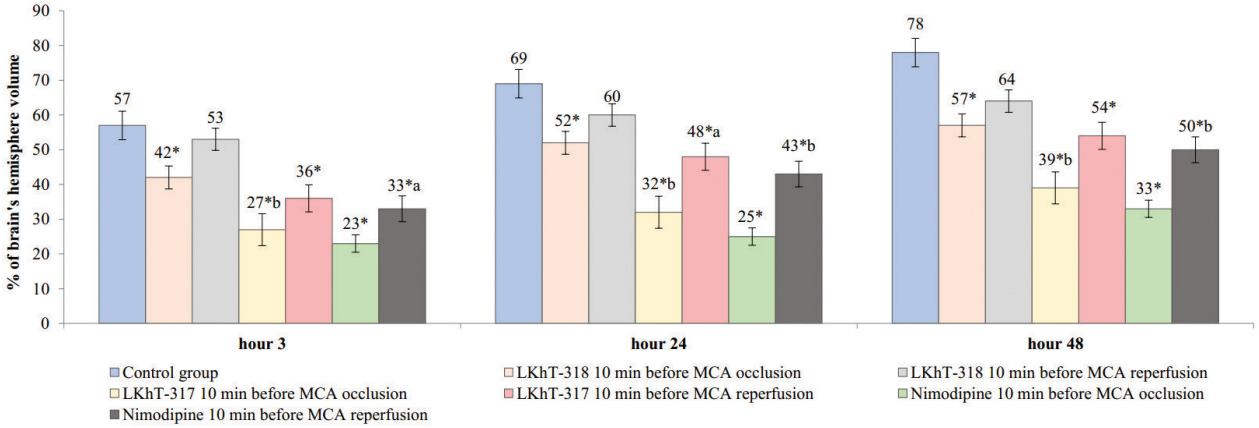

Figure 2. Volume of experimental rats' brain damage 3, 24 and 48 hours after MCA reperfusion (n = 5 in each group). **Note:** MCA – middle cerebral artery; * – $p < 0.05$ when compared with control group; [b] – $p < 0.05$ when compared with LKhT-318 (*ANOVA*, *Tukey's criterion*); [a] – $p < 0.05$ when compared with the same compound injected before MCA occlusion (*Wilcoxon's criterion*).

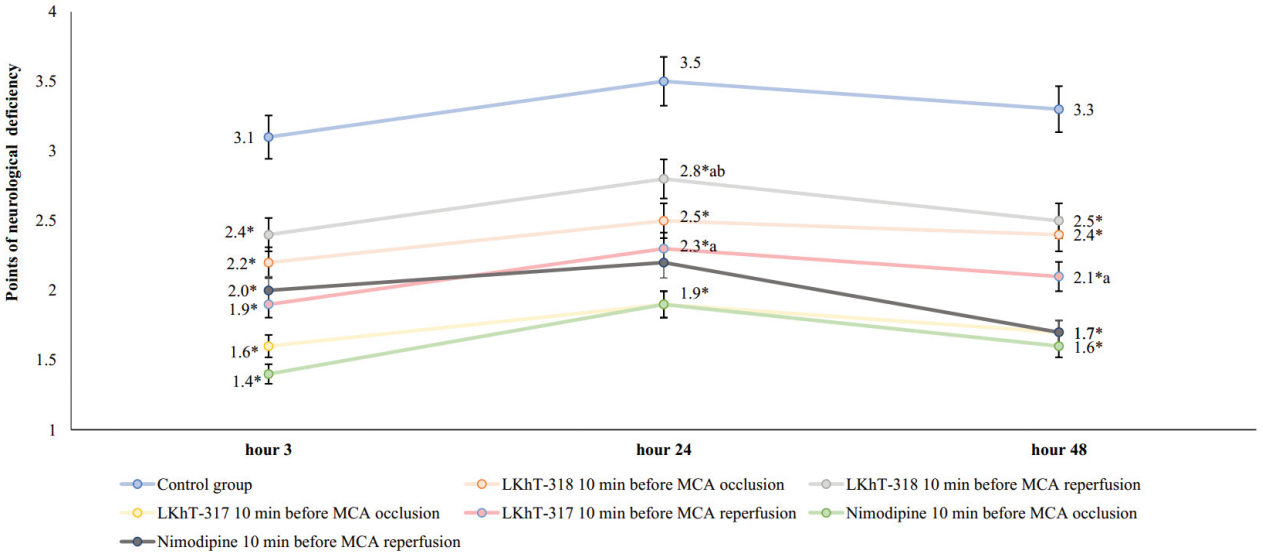

Figure 3. Neurological deficiency of rats with experimental MCA reperfusion assessed in accordance with Huang's scale. **Note:** * – $p < 0.05$ when compared with control group; [b] – $p < 0.05$ when compared with LKhT-318 (*ANOVA*, *Tukey's criterion*); [a] – $p < 0.05$ when compared with the same compound injected before MCA occlusion (*Wilcoxon's criterion*).

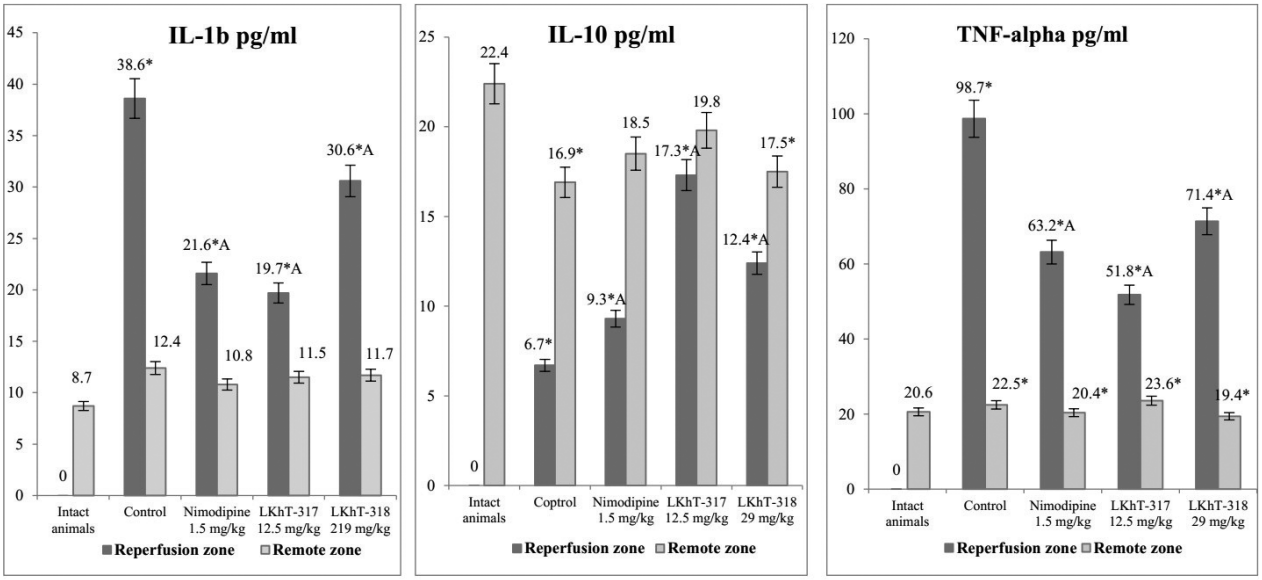

Figure 4. Levels of IL-1b, IL-10, and TNF-alpha in cerebral tissues of rats 48 hours after MCA reperfusion ($n = 5$ in each group). **Note:** * – $p < 0.05$ when compared with intact rats; A – $p < 0.05$ when compared with control group (*ANOVA*, *Dannet's criterion*).

tion were evaluated using a four-point scale by Huang et al. (1994). 6, 24 and 48 hours after MCA reperfusion (Fig. 3). Both compounds effectively curbed the motor deficiency onset and dynamics. It should be emphasized that magnesium-containing compound was more effective in comparison with the zinc-substituted derivative at all points of evaluation ($p < 0.05$). The neuroprotective action of the compounds depended on an experimental regimen, and was significantly higher when introduced before MCA occlusion.

A deteriorating role of inflammation in brain ischemic and reperfusion injury progression was profoundly discussed in (Puig et al. 2018). Violation of the blood-brain barrier integrity, migration of neutrophils and macrophages to the focus of the ischemic nucleus and the area of the penumbra, active production of cytokines and chemokines are the main factors of propelling the cerebral inflammatory reaction (Puig et al. 2018). TNF-alpha, IL-1beta, and IL-6 induce the inflammatory response during the sequential cascade reaction resulting in infiltration and edema. Another cytokine, IL-10, acts as a natural anti-inflammatory molecule. Using qualitative ELISA, we found out that intravenous administration of metal-containing taurine derivatives inhibited the inflammatory reaction outbreak in rats with MCA post-ischemic reperfusion, since they limited the growth of proinflammatory cytokines and inhibited the suppression of IL-10 production (Fig. 4).

Magnesium-containing taurine compound inhibited caspase-3 expression and simultaneously 3.5 times induced Bcl-2 expression in comparison with reperfusion-damaged brain tissue of control animals. Hence, 12.5 mg/kg of LKhT-317 had the anti-apoptotic property. We then assessed cells survival in mixed neural and glial hippocampal cell cultures. Before beginning the experiment, cell death was observed in 11% of the population. Oxygen-glucose deprivation (OGD) increased cell death to 68%, whereas addition of 1 mM of LKhT-317 along with the development of OGD decreased the percentage of perished cells to 21% (p = 0.002).At the same time, the network morphology, in general, remained unchanged (Fig. 5).

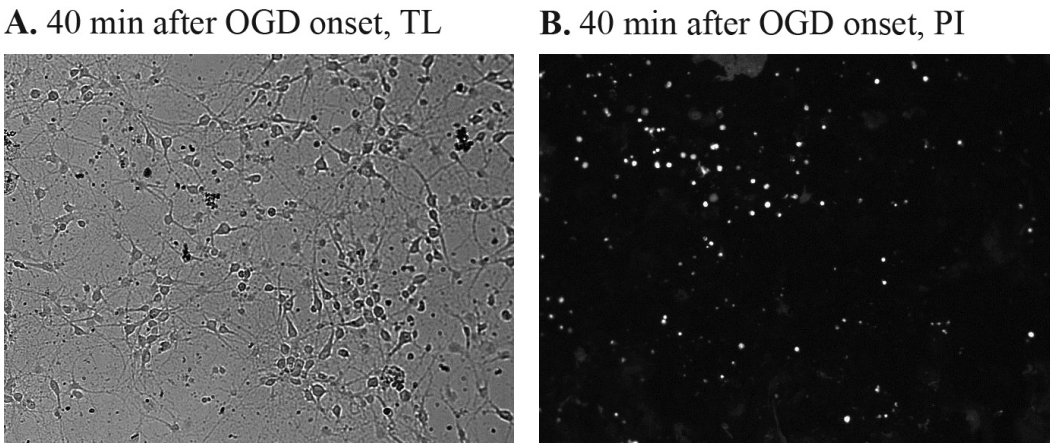

Figure 5. Cell survival in mixed hippocampal culture under OGD condition: A – cellular net image. **Note: A** – Transmitted light image of hippocampal cell culture 40 min after OGD onset, 1 мMLKhT-317; **B** – Image of cell culture in the Propidium Iodide channel 40 min after OGD onset, 1 мMLKhT-317.

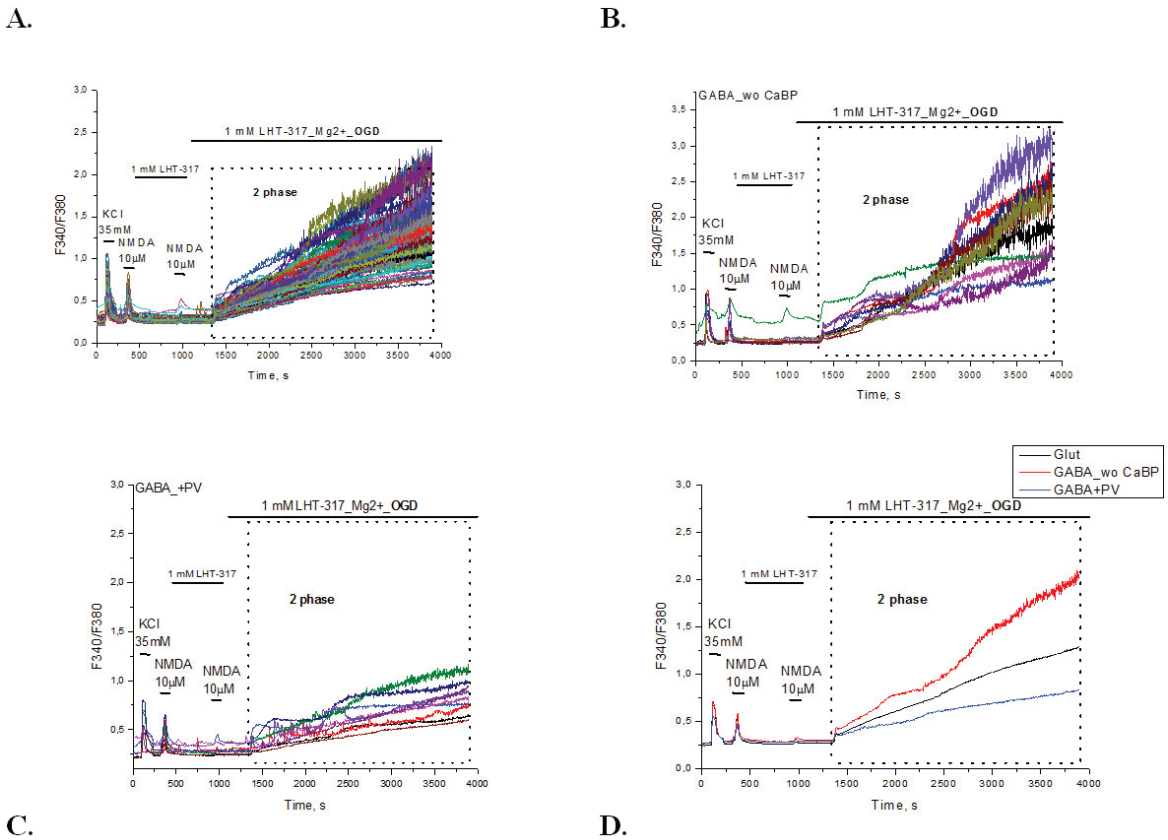

Figure 6. Ischemic-like condition impacts Ca^{2+} responses in glutamatergic and GABA-ergic neurons in mixed hippocampal culture (40 min after OGD outbreak): **A** – 1 mM of LKhT-317 inhibits Ca^{2+}-signals of glutamatergic neurons (n = 53) induced by 10 μM of NMDA; OGD, and magnesium-free medium; **B** – effect of 1 mM of LKhT-317 on Ca^{2+}-signalsof GABA-ergic PV-free neurons (n = 61) induced by 10 μM of NMDA; OGD, and magnesium-free medium; **C** – effect of 1 mM of LKhT-317 on Ca^{2+}-signals of GABA-ergic PV-expressing neurons (n = 47) induced by 10 μM of NMDA; OGD, and magnesium-free medium; **D** – Comparison of average 10 μM of NMDA-induced Ca^{2+} responses of glutamatergic, and GABA-ergic neurons maintained in presence of 10 mM of LKhT-317; OGD, and magnesium-free medium.

LKhT-317 at 1 mM concentration completely inhibited cellular Ca^{2+} responses by 10 μM NMDA in all types of neurons in all the experiments (Fig. 6). Moreover, the inhibitory effect completely suppressed the first phase of OGD-induced $[Ca^{2+}]_i$ growth in both glutamatergic and GABA-ergic neurons (with and without parvalbumin (PV)). In addition, LKhT-317 at the aforementioned concentration significantly limited Ca^{2+}entry into the cytosol of all types of hippocampal neurons during the second phase of OGD, typically characterized by a global Ca^{2+}increase (Turovsky et al. 2018).

Thus, metal-containing taurine derivatives LKhT-317 and LKhT-318 demonstrated a sufficient neuroprotective property in rats with a reperfusion-induced brain injury. Both derivatives effectively prevented severity of the animals' brain damage, motor deficiency, reduction of microvascular perfusion and proinflammatory cytokines production. Magnesium-containing compound LKhT-317 was comparatively more effective than a zinc-containing one. LKhT-317 possessed an anti-apoptotic action *in vivo*, and protected neurons from OGD-mediated cell death in mixed hippocampal culture. The aforementioned actions may be associated with the LKhT-317 inhibitory effect on NMDA-induced cellular Ca^{2+} response and, therefore the compound anti-excitotoxic property.

Conclusion

According to the obtained data, the following conclusions were made:

1. Magnesium-containing taurine derivative LKhT-317 at the concentration of 12.5 mg/kg and zinc-containing compound LKhT-318 at the concentration of 29 mg/kg when intravenously administered curb the area of rats' brain reperfusion-induced damage, maintain blood flow in injured tissue and limits the severity of motor deficiency.
2. The compounds decrease the tissue level of proinflammatory cytokines IL-1b and TNF-alpha, while anti-inflammatory IL-10 tends to remain in the damaged and remote brain zones.
3. LKhT-317 inhibits caspase-3 expression and stimulate Bcl-2 expression in the penumbra within 48 hours after middle cerebral artery reperfusion, therefore the compound has an anti-apoptotic property.
4. LKhT-317 at 1 mM concentration protects neurons' survival in mixed hippocampal cell culture within 40 hours after OGD outbreak.
5. Magnesium-containing taurine derivative LKhT-317 inhibits Ca^{2+} entry through activated NMDA receptors of glutamatergic and GABA-ergic neurons in cell culture under OGD condition.

Conflicts of interest

The authors have no conflict of interest to declare.

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