THE ROLE OF CALCIUM-BINDING PROTEINS IN GABAERGIC NEURONS REACTIONS TO THE EXCITOTOXIC EFFECT OF GLUTAMATE

M.V. Turovskaya[1,2*]

[1] Lobachevsky State University of Nizhny Novgorod, 23 Gagarin Ave., Nizhny Novgorod, 603022, Russia;
[2] Institute of Cell Biophysics, Russian Academy of Sciences, Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences", 3 Institutskaya St., Pushchino, Moscow region, 142290, Russia.

* Corresponding author: m_turovskaya@mail.ru

Abstract. Using immunocytochemistry and fluorescence microscopy, it was shown that the expression of the calcium-binding proteins parvalbumin, calbindin and calretinin in GABAergic neurons limited the increase in the level of cytosolic calcium ([Ca2+]i) during the excitotoxic effect of glutamate (GluTox). Under conditions of repeated episodes of hypoxia, Ca2+ oscillations were generated in GABAergic neurons, and the expression of calcium-binding proteins determined the amplitude of hypoxia-induced Ca2+ impulses. Expression of parvalbumin during hypoxia was most effective in suppressing the amplitude of Ca2+ signals. With GluTox, irreversible depolarization of the mitochondria of GABAergic neurons occurred, which lacked calcium-binding proteins, while the expression of parvalbumin, calbindin or calretinin contributed to the preservation of mitochondrial polarization and maintenance of their functioning under the influence of glutamate. At the same time, parvalbumin also turned out to be the most effective calcium-binding protein. As a result, restrictions on the level of [Ca2+]i during GluTox by calcium-binding proteins in GABAergic neurons led to suppression of the production of reactive oxygen species by mitochondria on the one hand, and on the other hand, calcium-binding proteins were able to protect GABAergic neurons from hyperproduction of nitric oxide. Thus, calcium-binding proteins were not only a marker of subtypes of GABAergic neurons, but also determined their physiological parameters under stressor conditions, which can be used to identify the subtype of GABAergic neurons by fluorescent signals of ROS production, nitric oxide, or the kinetics of Ca2+ signals.

Keywords: neurons, GABAergic neurons, calcium binding proteins, glutamate toxicity.

List of Abbreviations

PV – parvalbumin
CB – calbindin
CR – calretinin
ROS – reactive oxygen species
NO – nitric oxide
GluTox – glutamate toxicity
CaBP – calcium binding proteins
[Ca2+]i – intracellular Ca2+ concentration

Introduction

In the hippocampus, neurons are heterogeneous cells and are divided into two main types – pyramidal (glutamatergic) neurons, secreting glutamate and performing an excitatory function, and interneurons, secreting γ-aminobutyric acid (GABA) and performing an inhibitory function. There is also a minor population of spiny interneurons that are capable of secreting both glutamate and GABA. The vast majority of neurons are pyramidal neurons, and interneurons make up about 15% of neurons (McCormick, 1992; Attili *et al.*, 2022). In contrast to glutamatergic neurons, GABAergic neurons are divided into subpopulations, both by morphology and electrophysiological activity, and by the type of calcium-binding protein (CaBP) expressed. Moreover, the calcium-binding proteins calbindin, calretinin, and parvalbumin are specific markers of GABAergic interneurons (Fish *et al.*, 2018; Bogus-Nowakowska *et al.*, 2022).

Ca2+ ions mediate numerous intracellular processes, and to maintain Ca2+ homeostasis in neurons, there are a large number of Ca2+ transport systems and compartmentalization methods (Bagur *et al.*, 2017; Costas-Ferreira *et al.*, 2021). However, in addition to these mechanisms, calcium-binding proteins are an additional way to regulate cytosolic calcium concentrations in GABAergic neurons (Schwaller,

2020). Calbindin (CB), calretinin (CR) and parvalbumin (PV) are EF-hand motif proteins (Phillips *et al.*, 1999) and have different affinities for Ca2+ in the concentration range from 10-9 to 10-6 M (Kd for CR and CB it is 0.3–0.5 μM, and for PV – 0.01–0.1 μM). All of these CaBPs are mobile calcium buffers that can transport Ca2+ into the endoplasmic reticulum and mitochondria, promoting the diffusion of Ca2+ ions in the cytoplasm and the propagation of Ca2+ signals (Schwaller, 2010; Berridge *et al.*, 2000).

Calbindin is not only a Ca2+ buffer, but also an intracellular transporter of Ca2+ ions. In addition, this CaBP also performs a signaling function by binding to Ran-binding protein (Lutz *et al.*, 2003), caspase-3 (Bellido *et al.*, 2000), 3'5'-cyclic nucleotide phosphodiesterase (Reisner *et al.*, 1992), plasma membrane ATPase (Morgan *et al.*, 1986) and the α subunit of the L-type Ca2+ channel (Akhter *et al.*, 2007). It has been established that a defect in the structure of calbindin accompanies the development of Alzheimer's, Parkinson's, Down syndrome, epilepsy, and also contributes to neurodegeneration during ischemia (Heizmann, 1993).

Calretinin functions as a buffer and Ca2+ transporter, and is also involved in the phosphorylation of a number of intracellular proteins (Heizmann, 1993). Calretinin has been shown to have a protective effect against glutamate toxicity and ischemia (D'Orlando *et al.*, 2001; Turovsky *et al.*, 2018). Calretinin knockout mice are characterized by impaired long-term potentiation in the hippocampus (Schurmans *et al.*, 1997) and increased excitability, correlating with the generation of fast action potentials in such GABAergic neurons (Gall *et al.*, 2003).

Parvalbumin is expressed in fast GABAergic neurons of the hippocampus, neocortex, and cerebellum (Heizmann & Braun, 1992). The connection of PV with the generation of fast action potentials is explained by the activation of Ca2+-dependent small-conductance K+ channels by this protein (Orduz *et al.*, 2013). Defects in the PV structure accompany the following pathologies: Alzheimer's disease, epilepsy, ischemia, Down syndrome, meningioma, neurofibromatosis (Heizmann, 1993).

Interestingly, the removal of any of the CaBPs by knockout was not compensated for by increased expression of another of the 240 known calcium-binding proteins. In PV knockout, none of the other calcium buffers (CB-D28k, CB-D9k, or CR) are expressed in the brain (Schwaller *et al.*, 2010), resulting in defects in intracellular signaling. This effect may be explained by the fact that neurons expressing a certain Ca2+ buffer have permanently inactivated promoters of other CaBPs or have special properties (affinity, kinetics, cooperativity, mobility) of each CaBP (Schwaller, 2009).

Thus, the purpose of this study was to study the effects of the expression of various types of calcium-binding proteins on the signaling of GABAergic neurons during the excitotoxic effect of glutamate. In addition, this work attempts to determine the subtype of GABAergic neurons based on their Ca2+ signals, mitochondrial depolarization, and the production of reactive oxygen species and nitric oxide.

Material and Methods

Hippocampal cell culture

A mixed neuroglial cell culture of the hippocampus was obtained from newborn (P1–3) Spraque Dawley rats. After decapitation, the hippocampus was removed and transferred to cold Hanks solution. The tissue was minced with scissors, placed in Versene solution with the addition of 0.2% trypsin and incubated for 10 min at 37 °C on a thermoshaker at 600 rpm. Enzyme-treated tissue pieces were washed three times with neurobasal medium, then carefully pipetted and centrifuged (2 min at 300 g). Next, the supernatant was removed, and the cells were resuspended in neurobasal medium supplemented with glutamine (0.5 mM), Supplement B27 (2%), and gentamicin (15 μg/ml). The suspension was added to glass cylinders with ground ends with an internal diameter of 6 mm, standing on round coverslips with a diameter of 25 mm (VWR International), coated with polyethyleneimine, and placed in 35 mm Petri dishes (Greiner). 100 μl of cell suspension was added to each cylinder and left for 2 hours

for attachment in a CO2 incubator at 37 °C. After this, the cylinders were removed, and the volume of the culture medium was adjusted to 1.5 ml. Every 3 days, 2/3 of the volume of the culture medium was replaced with fresh one. Experiments were carried out on cultures aged 10–13 days (10–13 DIV).

Modeling the excitotoxic effect of glutamate (GluTox) in vitro

To create conditions for the acute (within 40 min) excitotoxic effect of glutamate (GluTox), 100 μM glutamate was added to the cells in a magnesium-free medium containing 20 μM glycine. The effects of GluTox were assessed by the shape and amplitude of Ca2+ signals of neurons.

Creation of short-term episodes of hypoxia-reoxygenation in vitro

To create short-term episodes of hypoxia, oxygen was displaced from Hanks' incubation solution containing 10 mM HEPES by purging the solution with an inert gas argon in a special vacuum system, which was mounted on an experimental chamber with cells located on the stage of a fluorescent microscope. The experiment consisted of three short-term episodes of hypoxia, each of which was followed by 10 minutes of reoxygenation. The effects of hypoxia episodes on neurons were assessed by the amplitude and frequency of Ca2+ oscillations.

Immunocytochemical method for identifying GABAergic neurons and comparing them with neuroimaging results

An immunocytochemical method was used to identify GABAergic neurons (Fig. 1). The cover glass with the culture was mounted in the experimental chamber and a grid was applied on the bottom side of the glass using a thin indelible marker for further detection of the area of interest with cells on different microscopes and comparison of intravital imaging and culture data after staining with antibodies. Next, the chamber was mounted on the stage of an inverted microscope, one of the squares of the drawn grid was selected, and fluorescence was recorded in the selected area. After the experiments, cells were photographed in phase contrast, numbered, fixed, and analyzed immunocytochemically with antibodies against glutamate decarboxylase (GAD65/67) and calcium-binding proteins (parvalbumin, calbindin, and calretinin). For this purpose, the following solutions were used: phosphate-buffered saline (PBS), pH 7.4; 4% parapharmaldehyde solution in PBS; 1% and 10% solution of donkey serum in PBS. Cells were fixed in 4% parapharmamaldehyde for 20 minutes and then washed three times for 5 minutes with cold PBS and permeabilized in 0.1% Triton X-100 in PBS for 15 minutes. To inhibit sites nonspecific antibody binding, cells were incubated for 30 minutes in 10% donkey serum at room temperature, then incubated with primary rabbit-antibodies for 12 hours at 4 °C (1:500 dilution in 1% donkey serum), washed in PBS 3 times for 5 minutes and incubated with secondary antibodies conjugated to a fluorescent tag. Antibody fluorescence was recorded on a Leica TCS SP5 confocal microscope equipped with a He-Ne laser. Images of Antibody fluorescence and intravital imaging were compared using ImageJ software. This approach allowed us to compare changes in the dynamics of [Ca2+]i, ROS, mitochondrial potential and nitric oxide in GAD 65/67-positive (GABAergic) neurons containing calcium-binding proteins.

Fluorescence measurements

To record the level of calcium in the cytoplasm of cells ([Ca2+]i), we used a Cell Observer image analysis system (Carl Zeiss, Germany) based on an Axiovert 200M inverted microscope equipped with an AxioCam HSm monochrome CCD camera and a Ludl MAC5000 high-speed excitation filter changing system. A Plan-Neofluar 10×/0.3 lens was used. An HBO 103W/2 mercury lamp illuminator was used as a fluorescence excitation source. To excite and record Fura-2 fluorescence, we used a set of Filter set 21HE (Carl Zeiss, Germany) with excitation filters BP340/30 and BP387/15, a beam splitter FT409, and an emission filter BP510/90. To measure fluorescence, a round cover glass with a cell culture was mounted in a special measure-

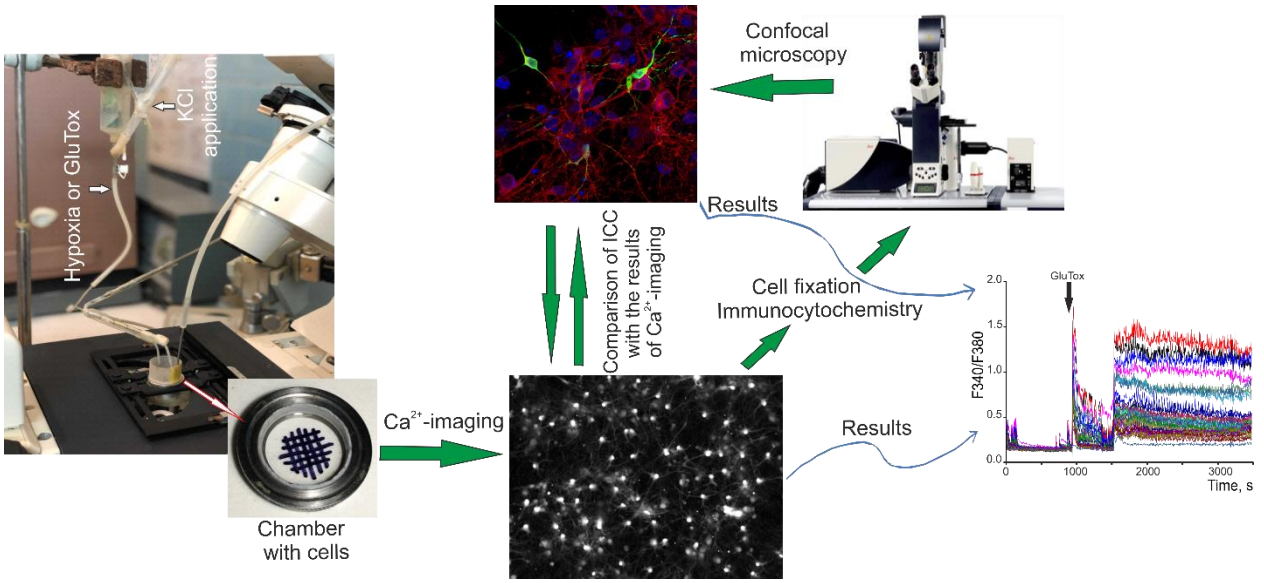

Fig. 1. Scheme of the original setup for creating hypoxia/GluTox in vitro and a method for comparing the results of neuroimaging with data from immunocytochemical staining of cell cultures. Hippocampal cells were grown on round coverslips and mounted in an experimental chamber mounted on the stage of an inverted fluorescence microscope. A hypoxic or GluTox medium supply system was connected to the chamber. After recording the dynamics of [Ca2+]i, ROS or NO, the cells were fixed and stained with specific antibodies. Next, the chamber with cells was transferred to an inverted confocal microscope and, using a grid, an area with cells was found in which the physiological parameters of the cells were measured. The resulting confocal images were compared with a series of images obtained using a fluorescence microscope

ing chamber. The volume of medium in the chamber was 0.5 ml. Reagents were added and washed by replacing the medium tenfold using a system providing perfusion at a rate of 15 ml/min. Measurements were carried out at 28 °C. Series of images were acquired at intervals of 1 frame every 3 s. To identify neurons, the experiment included a short-term (20 s) test addition of 35 mM KCl. The resulting time series of two-channel images (at excitation light wavelengths of 340 and 380 nm) were processed in the ImageJ program with the Time series analyzer software module. The amplitude of calcium responses of single cells was measured, expressed as the ratio of Fura-2 fluorescence signals at excitation of 340 and 380 nm. Origin 8.5 was used for plotting and statistical processing. Results are presented as single cell signals generally representative of the GABAergic neuron subtype shown or as the average cell signal per field of view ± standard deviation (SD).

Nitric oxide (NO) production was measured using a fluorescent probe DAF-FM diacetate (Molecular Probes, USA). Cells were loaded with 5 μM DAF-FM for 40 min at 37 °C. After loading, the cells were additionally incubated in Hanks balanced salt solution (HBSS) for 20 min to complete the deesterification of the dyes. DAF-FM fluorescence was excited using a BP 475/40 filter. The emission of DAF-FM was recorded at 530 ± 25 nm. Collected 8-bit time-lapse images were analyzed using the ImageJ software with Time Series Analyzer and RatioPlus plugins. The experimentally obtained curves for NO were smoothed to decrease the effect of noises. The curve ΔF/F0 characterizes the fluorescence intensity of benzotriazole, a product of nitrosylation of DAF-FM accumulated in the cells.

ROS production in mitochondria was determined using the MitoSox Red probe (incubation for 15 minutes at a final concentration of 5 μM at 37 °C). After incubation with probes, cells were washed three times with Hanks' solution. The dynamics of ROS formation were recorded using an image analysis system based on a Leica DMI6000B inverted microscope.

A set of BP510/84 filters was used to excite and record MitoSox fluorescence. Lens Leica HC PL APO 20/0.7 IMM. Light source Leica EL6000 with high pressure mercury lamp HBO 103 W/2.

To record mitochondrial membrane potential (ΔΨm), cells were loaded with rhodamine 123 (Rh123; 1 μg/ml) for 15 minutes. The oxidative phosphorylation uncoupler FCCP (1 μM) was added at the end of the experiments to induce complete depolarization of the mitochondrial membrane and subsequent calibration of the Rh123 fluorescence signal. Rh123 fluorescence was excited using a xenon lamp and monochromator (Cairn Research) at 490 nm and detected at 515 nm.

Image analysis and statistical data processing

The resulting time series of images were processed in the ImageJ program with the Time series analyzer plugin. In this case, the amplitude of signals from single cells was measured, expressed in units of probe fluorescence. Results were presented as single cell signals or the average cell signal per field of view ± standard error (SE). Origin 8.5 (Microcal Software Inc., Northampton, MA) and Prism 5 (GraphPad Software, La Jolla, CA) were used for plotting and statistical processing. The significance of differences between experimental groups was determined using Student's t-test, and within groups - Student's t-test. Differences were considered significant: *** at $p \leq 0.001$, ** at $p < 0.01$, * at $p < 0.05$, n/s – differences are not significant.

Results

Ca2+ signals of GABAergic neurons of the hippocampus when exposed to an excitotoxic concentration of glutamate (GluTox) or hypoxia, depending on the type of calcium-binding protein

Modeling the excitotoxic effect of glutamate in the form of application of 100 μM glutamate in a magnesium-free medium with the addition of 20 μM glycine (GluTox) resulted in biphasic Ca2+ signals in hippocampal neurons (Fig. 2A). The first phase of the Ca2+ response to GluTox is a rapid increase in [Ca2+]i followed by pumping to an almost basic level, and the second phase is an irreversible increase in [Ca2+]i and reaching a new stationary level of [Ca2+]i. As can be seen, the Ca2+ responses of neurons to GluTox are individual and differ in the level of increase in [Ca2+]i in the second phase of the Ca2+ response (Fig. 2A). Immunocytochemical staining of cell cultures with antibodies against GAD65/67 and calcium-binding proteins and comparison with the results of Ca2+ imaging showed that this difference in the responses of hippocampal neurons to GluTox is associated with the presence of buffer concentrations of CaBP in the cytosol of GABAergic neurons (Fig. 2B). GABAergic neurons in which none of the studied CaBPs were detected are characterized by increased spontaneous Ca2+ activity and the highest amplitude of the increase in [Ca2+]i in both the first and second phases of GluTox-induced signals (Fig. 2B - red curve). GABAergic neurons in which calcium-binding proteins were detected - calbindin (CB+) and calretinin (CR+) are characterized by less pronounced spontaneous Ca2+ activity, a significantly smaller increase in [Ca2+]i during GluTox and a recovery of [Ca2+]i to the basal level after the first phase GluTox response (Fig. 2B). The differences between CB+ and CR+ GABAergic neurons in the amplitude and shape of Ca2+ signals on GluTox were not significantly different. Neurons containing parvalbumin as a CaBP showed a trend similar to other calcium-binding proteins in the form of GluTox signals, but the level of [Ca2+]i during the second phase of GluTox-induced Ca2+ signaling was even lower (Fig. 2B – black curve) .

An increase in glutamate concentration occurs in the brain under hypoxic conditions. Repeated episodes of hypoxia caused the generation of Ca2+ oscillations in GABAergic neurons (Fig. 3A). In panels B-E, GABAergic neurons are separately constructed depending on the calcium binding protein expressed in them (Fig. 3B-E). It turned out that in GABAergic neurons without CaBP (Fig. 2B) or in which calbindin (Fig. 3C) and calretinin (Fig. 3D) are expressed, Ca2+ oscillations during hypoxia occurred at an increased level of [Ca2+]i, while

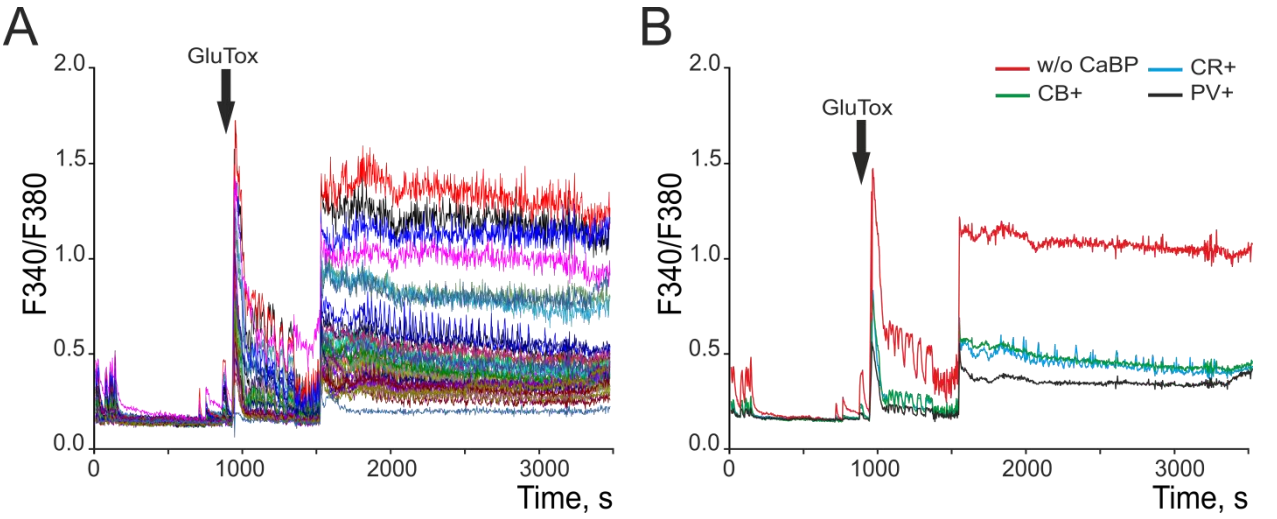

Fig. 2. Ca2+ signals of hippocampal neurons upon application of an excitotoxic concentration of glutamate, 100 μM in a magnesium-free medium with the addition of 10 μM glycine (GluTox). A – Ca2+ signals of all neurons in the field of view of the microscope upon application of GluTox. B – Average Ca2+ signals of GABAergic neurons containing buffer concentrations of calcium binding proteins – calbindin (CB+), calretinin (CR+) and parvalbumin (PV+). w/o CaBP – GABAergic neurons that do not contain any of the studied Ca2+-binding proteins

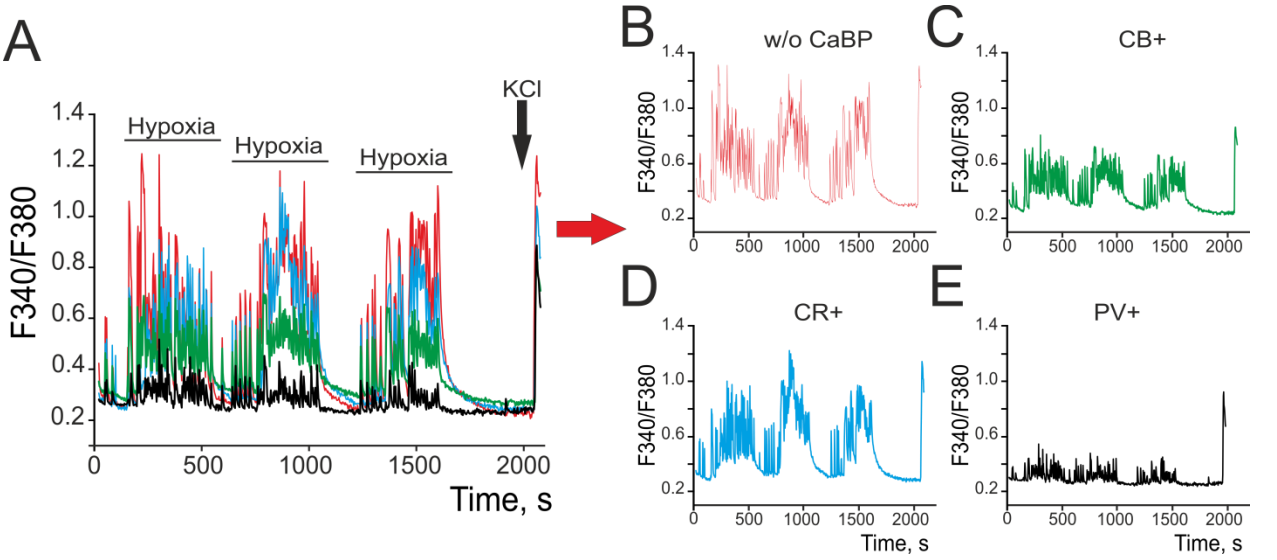

Fig. 3. Ca2+ signals of GABAergic neurons of the hippocampus to repeated episodes of hypoxia. A – Ca2+ signals of all GABAergic neurons in one experiment are presented. B, C, D, E – Ca2+ signals of GABAergic neurons without Ca2+-binding proteins (w/o CaBP, B), as well as those containing calbindin (CB+, C), calretinin (CR+, D) and parvalbumin (PV+, E) in buffer concentrations in the cytosol

the expression of parvalbumin (Fig. 3E) led to the generation of Ca2+ oscillations without increasing the basic level of [Ca2+]i. In addition, the presence of calbindin or parvalbumin in GABAergic neurons contributed to the generation of low-amplitude Ca2+ oscillations in response to hypoxia, and calretinin had virtually no effect on this parameter (Fig. 3D).

Thus, the expression of calcium-binding proteins in GABAergic neurons of the hippocampus is capable of determining the Ca2+ signals of cells under the excitotoxic effect of glu-

tamate and hypoxia. The presence of any of the studied CaBPs reduces the level of [Ca2+]i under the influence of glutamate, and is probably involved in the utilization of Ca2+ ions immediately after the generation of GluTox-induced Ca2+ signals. During hypoxia, calcium-binding proteins calbindin and parvalbumin determine the amplitude of Ca2+ impulses of GABAergic neurons, reducing it relative to GABAergic neurons without CaBPs or with calretinin.

Effects of expression of different types of calcium-binding proteins on mitochondrial potential, mitochondrial reactive oxygen species (ROS) production, and nitric oxide (NO) production in GABAergic neurons during glutamate excitotoxicity

Mitochondria are key organelles of neurons and provide energy for the functioning of all homeostatic systems of cells. Recording of the mitochondrial potential of neurons under GluTox conditions showed differences depending on the type of CaBP expressed (Fig. 4A). GABAergic neurons that do not contain CaBP are characterized by increased depolarization upon application of GluTox and a lack of cellular response to the application of the mitochondrial uncoupler FCCP at the end of the experiment (Fig. 4A - red curve). Expression of calbindin or calretinin resulted in less mitochondrial depolarization by GluTox, accounting for 29% and 40% of the depolarization of GABAergic neurons without CaBP (Fig. 4B). At the same time, the majority of GABAergic neurons containing parvalbumin are characterized by less mitochondrial depolarization during GluTox (Fig. 4A - black curve), when depolarization is less than 20% of the signal of GABAergic neurons without CaBP (Fig. 4B). Moreover, all GABAergic neurons containing any of the studied CaBPs retain mitochondrial potential until the end of the experiment and depolarize in response to the uncoupler FCCP.

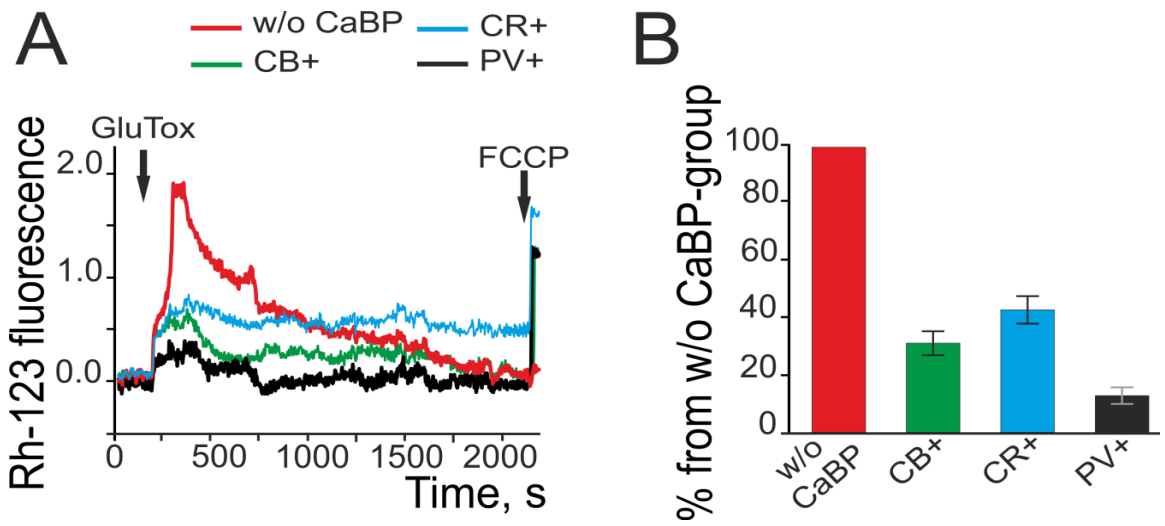

Fig. 4. Depolarization of mitochondria of GABAergic neurons under the influence of GluTox, depending on the type of calcium-binding protein expressed. A – Change in fluorescence of Rhodamine-123 (Rh-123) upon application of GluTox in GABAergic neurons without CaBPs (w/o CaBP) expressing calbindin (CB+), calretinin (CR+) or parvalbumin (PV+). FCCP is a depolarizing stimulus for mitochondria in the form of an application of 1 μM FCCP uncoupler. B – relative depolarization of mitochondria of GABAergic neurons depending on the type of expressed CaBP upon application of GluTox. Data are presented as percentages (%) relative to the depolarization of GABAergic neurons without CaBPs

Differences in the responses of mitochondria of GABAergic neurons depending on the type of expressed calcium-binding protein to GluTox application also suggest differences in the production of ROS by these neurons. Measurement of ROS production by mitochondria of

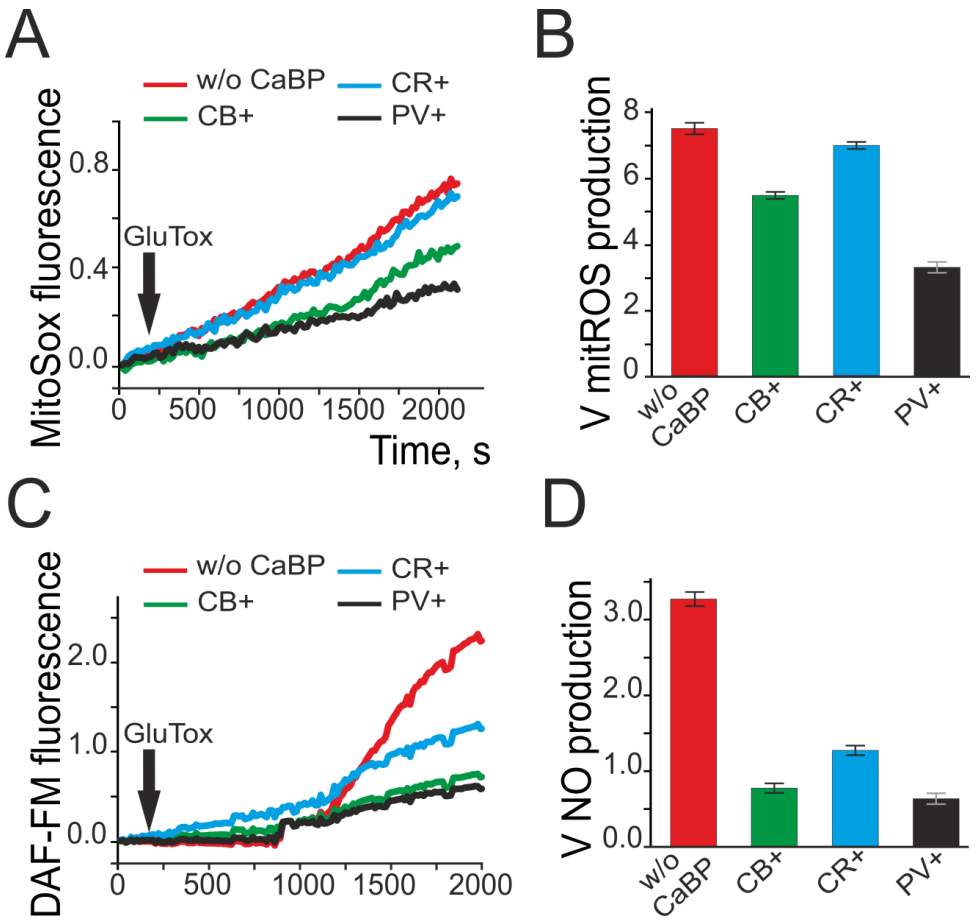

Fig. 5. ROS production by mitochondria and nitric oxide formation in GABAergic neurons depending on the type of calcium-binding protein expressed during GluTox. A, B – Averaged over 10 cells (for each curve) curves of ROS production by mitochondria (A) and the rate of ROS production (B) in GABAergic neurons depending on the type of calcium-binding protein. C, D – Curves of nitric oxide production (A) and the rate of nitric oxide production (B) in GABAergic neurons, averaged over 10 cells (for each curve), depending on the type of calcium-binding protein

GABAergic neurons without CaBPs and GABAergic neurons containing calretin (CR+) under GluTox showed similar rates of ROS production (Fig. 5A, B). ROS production by GABAergic neurons containing calbindin (CB+) and parvalbumin (PV+) was significantly lower when exposed to GluTox (Fig. 5C, D) compared to other types of GABAergic neurons.

Not only is nitric oxide an important signaling molecule, but excess NO production is a significant contributor to oxidative stress and neuronal damage. Measurements of NO production under GluTox showed that in GABAergic neurons without CaBPs, NO production occurs after a long lag period (Fig. 5C), but the rate of NO production under GluTox is greatest in this type of neuron (Fig. 5D). Expression of any of the CaBPs examined correlated with significantly lower NO production in GABAergic neurons (Fig. 5C, D). Moreover, the lowest rate of NO formation during GluTox was recorded in PV+ GABAergic neurons.

Discussion

Interneurons contain specific calcium-binding proteins (CaBPs) - parvalbumin (PV), calbindin (CB), and calretinin (CR), which may promote the survival of these neurons under pathological conditions (Fairless *et al.*, 2019; Schwaller, 2020). It has been shown that py-

ramidal neurons that do not contain PV and CB are damaged and die after ischemia, whereas interneurons that contain buffer concentrations of these proteins, on the contrary, survive (Freund *et al.*, 1990). Previously, we attempted to characterize GABAergic neurons by their Ca2+ signals depending on the presence of a certain type of Ca2+-binding protein. It has been established that the expression of parvalbumin in GABAergic neurons determines the shape of Ca2+ oscillations, creating a temporary delay in the generation of a Ca2+ pulse and desensitization of these neurons during high-frequency Ca2+ activity (Zinchenko *et al.*, 2016; Zinchenko *et al.*, 2016). In addition, we found that the lack of expression of CaBPs in GABAergic neurons is associated with the absence of a hypoxic preconditioning mechanism, and the presence of calcium-binding proteins promotes the survival of GABAergic neurons under hypoxic conditions. At the same time, the neuroprotective effectiveness was different for parvalbumin, calbindin and calretinin (Turovsky *et al.*, 2018). In this study, we were able to show that the expression of CaBPs significantly protects GABAergic neurons from GluTox by reducing [Ca2+]i levels. And under conditions of repeated episodes of hypoxia, calcium-binding proteins determine the amplitude of Ca2+ oscillations, which undoubtedly has an important signaling value. That is, calcium-binding proteins perform important signaling functions in GABAergic neurons under pathological conditions. For CaBPs, it has been well demonstrated that differences in the properties of the studied calcium-binding proteins differentially modulate the spatiotemporal aspects of intracellular signaling. CR and CB are fast CaBP capable of binding Ca2+ immediately after an increase in its concentration in the cytosol. They are 58% identical in structure and have six EF-hand type domains, of which five in CR and four in CB are functional and bind Ca2+ (Kuznicki *et al.*, 1996; Schwaller *et al.*, 1997). This is probably due to the very similar effects of calbindin and calretinin under GluTox and episodes of hypoxia in our experiments. PV has three domains, two of which are capable of binding both Ca2+ and Mg2+, and in the inactive state of the protein these domains bind only Mg2+. Ca2+ binding by parvalbumin has slower kinetics than CR and CB and is limited primarily by the magnesium dissociation constant (Choi *et al.*, 2008). However, parvalbumin, being a slow protein, is capable of binding a significantly larger number of Ca2+ molecules, which may determine the most pronounced effects of parvalbumin on the [Ca2+]i level under the influence of hypoxia and GluTox.

There is evidence that the bioenergetics of GABAergic neurons differs significantly from that of glutamatergic neurons. Fast inhibitory GABAergic interneurons are characterized by particular vulnerability of complexes I and IV of the mitochondrial respiratory chain (Kann *et al.*, 2011; Whittaker *et al.*, 2011), since in order to generate high-frequency action potentials that control oscillations of neural networks (gamma oscillations), neurons of this subtypes consume much more energy than other brain cells. It has also been shown that under ischemic conditions, HIF-1α expression occurs more consistently in interneurons than in pyramidal ones, which is associated with increased levels of intracellular glutathione in interneurons (Ramamoorthy & Shi, 2014). Thus, our data show that mitochondria of GABAergic neurons without calcium-binding proteins quickly and irreversibly depolarize under the influence of glutamate, while the presence of CaBPs correlates with less pronounced mitochondrial depolarization when neurons are able to maintain mitochondrial functioning under conditions of the excitotoxic action of glutamate and respond to a depolarizing stimulus mitochondrial uncoupler FCCP. This effect of CaBPs on mitochondrial functioning is also reflected in differences in ROS production. It is known that increased ROS production occurs with an increase in [Ca2+]i and is restored when the physiological concentration of [Ca2+]i returns. In glutamatergic neurons, ROS production increases when the extracellular Ca2+ concentration reaches 10 mM (Carriedo *et al.*, 1998). At the anatomical level, interneurons are characterized by increased mitochondrial density, high levels of cytochrome c oxidase (COX), and cytochrome C to maintain their

high energy capacity (Kageyama and Wong-Riley, 1982; Gulyas *et al.*, 2006). Preincubation of cells with an inhibitor of the electron transport chain of mitochondria - cyanide or rotenone - completely suppresses the production of ROS in response to stimulation of NMDA and AMPA/KA receptors in both GABAergic and glutamatergic neurons, which indicates the leading role of mitochondria in this process in both cell types (Carriedo *et al.*, 1998; Dugan *et al.*, 1995). ROS scavengers enhance the GABAergic component of neurotransmission during hypoxia and anoxia, as well as with the addition of exogenous H2O2, which is expressed in a shift in the outgoing current in response to stimulation (Hogg *et al.*, 2015), i.e., selective inhibition of ROS production in GABAergic neurons may be an effective strategy to enhance inhibition during excitotoxicity.

Thus, the expression of calcium-binding proteins in GABAergic neurons can determine their signaling at the level of regulation of [Ca2+]i and, as a result, overloading mitochondria with Ca2+ ions, maintaining the functioning of mitochondria under pathological conditions and limiting the formation of toxic concentrations of ROS.

Acknowledgements

The study was carried out with the financial support of the Russian Foundation for Basic Research within the framework of project No. 23-24-00328.

References

AILHAUD G. (2006): Adipose tissue as a secretory organ: from adipogenesis to the metabolic syndrome. *C R Biol*. **329** (8), 570–577.

AKHTER S., KUTUZOVA G.D., CHRISTAKOS S. & DELUCA H.F. (2007): Calbindin D9k is not required for 1,25-dihydroxyvitamin D3-mediated Ca2+ absorption in small intestine. *Arch Biochem Biophys.* **460** (2), 227–32.

AKOPOVA I., TATUR S., GRYGORCZYK M., LUCHOWSKI R., GRYCZYNSKI I., GRYCZYNSKI Z., BOREJDO J. & GRYGORCZYK R. (2012): Imaging exocytosis of ATP-containing vesicles with TIRF microscopy in lung epithelial A549 cells. *Purinergic Signal*. **8** (1), 59–70.

ATTILI S.M, MORADI K, WHEELER D.W. & ASCOLI G.A. (2022): Quantification of neuron types in the rodent hippocampal formation by data mining and numerical optimization. *Eur J Neurosci*. **55** (7), 1724–1741.

BAGUR R. & HAJNÓCZKY G. (2017): Intracellular Ca2+ Sensing: Its Role in Calcium Homeostasis and Signaling. *Mol Cell.* **66** (6), 780-788.

BELLIDO T., HUENING M., RAVAL-PANDYA M., MANOLAGAS S.C. & CHRISTAKOS S. (2000): Calbindin-D28k is expressed in osteoblastic cells and suppresses their apoptosis by inhibiting caspase-3 activity. *J. Biol. Chem.* **275** (34), 26328–26332.

BERRIDGE M.J., LIPP P. & BOOTMAN M.D. (2000): The versatility and universality of calcium signalin. *Nat. Rev. Mol. Cell Biol.* **1** (1), 11–21.

BOGUS-NOWAKOWSKA K., ROBAK A., KALINOWSKI D., KOZŁOWSKA A. & RÓWNIAK M. (2022): GABAergic and Glutamatergic Phenotypes of Neurons Expressing Calcium-Binding Proteins in the Preoptic Area of the Guinea Pig. *Int. J. Mol. Sci.* **23**, 7963.

CARRIEDO S.G., YIN H.Z., SENSI S.L. & WEISS J.H. (1998): Rapid Ca2+-entry through Ca2+-permeable AMPA/Kainate channels triggers marked intracellular Ca2+-rises and consequent oxygen radical production. *J. Neurosci*. **18**, 7727–7738.

CHOI W.-S., LEE E., LIM J. & OH Y.J. (2008): Calbindin-D28K prevents drug-induced dopaminergic neuronal death by inhibiting caspase and calpain activity. *Biochem. Biophys. Res. Commun*. **371** (1), 127–131.

COSTAS-FERREIRA C. & FARO LRF. (2021): Systematic Review of Calcium Channels and Intracellular Calcium Signaling: Relevance to Pesticide Neurotoxicity. *Int J Mol Sci*. **22** (24), 13376.

D'ORLANDO C., CELIO M.R. & SCHWALLER B. (2002): Calretinin and calbindin D-28k, but not parvalbumin protect against glutamate-induced delayed excitotoxicity in transfected N18-RE-105 neuroblastoma-retina hybrid cells. Brain *Res.* **945**, 181–190.

DUGAN L.L., SENSI S.L., CANZONIERO L.M.T., HANDRAN S.D., ROTHMAN S.M., *et al.* (1995): Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J. Neurosci.* **15**, 6377– 6388.

FAIRLESS R., WILLIAMS S.K. & DIEM R. (2019): Calcium-Binding Proteins as Determinants of Central Nervous System Neuronal Vulnerability to Disease. *Int J Mol Sci.* **20** (9), 2146.

FISH K.N, ROCCO B.R. & LEWIS D.A. (2018): Laminar Distribution of Subsets of GABAergic Axon Terminals in Human Prefrontal Cortex. *Front Neuroanat.* **16** (12), 9.

FREUND T.F., BUZSAKI G., LEON A., BAIMBRIDGE K.G. & SOMOGYI P. (1990): Relationship of neuronal vulnerability and calcium binding protein immunoreactivity in ischemia. *Exp Brain Res.* **83** (1), 55–66.

GALL D., ROUSSEL C., SUSA I., D'ANGELO E., ROSSI P., *et al.* (2003): Altered neuronal excitability in cerebellar granule cells of mice lacking calretinin. *J Neurosci*. **23** (28), 9320–7.

GULYAS A.I., BUZSAKI G., FREUND T.F. & HIRASE H. (2006): Populations of hippocampal inhibitory neurons express different levels of cytochrome c. *Eur. J. Neurosci.* **23**, 581–2594.

HEIZMANN C.W. & BRAUN K. (1992): Changes in Ca2+-binding proteins in human neurodegenerative disorders. *Trends Neurosci*. **15** (7), 259–264.

HEIZMANN C.W. (1993): Calcium signaling in the brain. Acta *Neurobiol Exp (Wars)*. **53**(1), 15–23.

HOGG D.W., PAMENTER M.E., DUKOFF D.J. & BUCK L.T. (2015): Decreases in mitochondrial reactive oxygen species initiate GABA(A) receptor-mediated electrical suppression in anoxia-tolerant turtle neurons. *J. Physiol*. **593** (10), 2311–2326.

KAGEYAMA G.H. & WONG-RILEY M.T. (1982): Histochemical localization of cytochrome oxidase in the hippocampus: correlation with specific neuronal types and afferent pathways. *Neuroscience* **7**, 2337–2361.

KANN O., HUCHZERMEYER C., KOVACS R., WIRTZ S. & SCHUELKE M. (2011): Gamma oscillations in the hippocampus require high complex I gene expression and strong functional performance of mitochondria. *Brain* **134**(2), 345–358.

KUZNICKI J., ISAACS K.R. & JACOBOWITZ D.M. (1996): The expression of calretinin in transfected PC12 cells provides no protection against Ca(2+)-overload or trophic factor deprivation. *Biochim. Biophys. Acta* **1313**, 194–200.

LUTZ W., FRANK E.M., CRAIG T.A., THOMPSON R., VENTERS R.A., *et al.* (2003): Calbindin D28K interacts with Ran-binding protein M: identification of interacting domains by NMR spectroscopy. *Biochem Biophys Res Commun*. **303** (4), 1186–92.

MCCORMICK D.A. (1992): Neurotransmitter actions in the thalamus and cerebral cortex and their role in neuromodulation of thalamocortical activity. *Progr Neurobiol*. **39**, 337–388.

MORGAN D.W., WELTON A.F., HEICK A.E. & CHRISTAKOS S. (1986): Specific in vitro activation of Ca,Mg-ATPase by vitamin D-dependent rat renal calcium binding protein (calbindin D28K). *Biochem Biophys Res Commun*. **138** (2), 547–53.

ORDUZ D., BISCHOP D.P., SCHWALLER B., SCHIFFMANN S.N. & GALL D. (2013): Parvalbumin tunes spike-timing and efferent short-term plasticity in striatal fast spiking interneurons. *J Physiol*. **591** (13), 3215–32.

PHILLIPS R.G., MEIER T.J., GIULI L.C., MCLAUGHLIN J.R., *et al.* (1999): Calbindin D28K gene transfer via herpes simplex virus amplicon vector decreases hippocampal damage in vivo following neurotoxic insults. *J. Neurochem*. **73** (3), 1200–1205.

RAMAMOORTHY P. & SHI H. (2014): Ischemia induces different levels of hypoxia inducible factor-1α protein expression in interneurons and pyramidal neurons. *Acta Neuropath. Commun*. **2**, 51.

REISNER P.D., CHRISTAKOS S. & VANAMAN T.C. (1992): In vitro enzyme activation with calbindin-D28k, the vitamin D-dependent 28 kDa calcium binding protein. *FEBS Lett.* **297** (1-2), 127–31.

SCHURMANS S., SCHIFFMANN S.N., GURDEN H., LEMAIRE M., LIPP H.P, *et al.* (1997): Impaired long-term potentiation induction in dentate gyrus of calretinin-deficient mice. *Proc Natl Acad Sci USA* **94** (19), 10415–20.

SCHWALLER B., DURUSSEL I., JERMANN D., HERRMANN B., COX J.A. (1997): Comparison of the Ca2+-binding properties of human recombinant calretinin-22k and calretinin. *J. Biol. Chem*. **272** (47), 29663–29671.

SCHWALLER B. (2009): The continuing disappearance of "pure" Ca2+ buffers. *Cell Mol Life Sci.* **66** (2), 275–300.

SCHWALLER B. (2010): Cytosolic Ca2+ buffers. *Cold Spring Harb. Perspect. Biol.* **2** (11), 004051.

SCHWALLER B. (2020): Cytosolic Ca2+ Buffers Are Inherently Ca2+ Signal Modulators. *Cold Spring Harb Perspect Biol.* **12** (1), 035543.

TUROVSKY E.A., ZINCHENKO V.P., GAIDIN S.G. & TUROVSKAYA M.V. (2018): Calcium-Binding Proteins Protect GABAergic Neurons of the Hippocampus from Hypoxia and Ischemia in vitro. *Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology* **12** (1), 74–84.

WHITTAKER R.G., TURNBULL D.M., WHITTINGTON M.A. & CUNNINGHAM M.O. (2011): Impaired mitochondrial function abolishes gamma oscillations in the hippocampus through an effect on fast-spiking interneurons. *Brain* **134** (7), 180.

ZINCHENKO V.P., TUROVSKY E.A., TUROVSKAYA M.V., BEREZHNOV A.V, SERGEEV A.I. & DYNNIK V.V. (2016): NAD causes dissociation of neural networks into subpopulations of neurons by inhibiting the network synchronous hyperactivity evoked by ammonium ions. *Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology* **10** (2), 118–125.

ZINCHENKOV.P., TUROVSKAYA M.V., TEPLOV I.YU., BEREZHNOV A.V. & TUROVSKY E.A. (2016): The role of parvalbumin-containing interneurons in the regulation of spontaneous synchronous activity of brain neurons in culture. *Biophysics, January* **61** (1), 85–93.