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# Epileptiform activity promotes decreasing of $Ca^{2+}$ conductivity of NMDARs, AMPARs, KARs, and voltage-gated calcium channels in $Mg^{2+}$-free model

Sergei G. Gaidin[a,*], Valery P. Zinchenko[a], Ilia Y. Teplov[a], Sultan T. Tuleukhanov[b], Artem M. Kosenkov[a,*]

[a] Institute of Cell Biophysics of the Russian Academy of Sciences, Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences", 142290, Pushchino, Russia
[b] Al-Farabi Kazakh National University, Almaty, Kazakhstan

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ABSTRACT

NMDA, AMPA, and kainate receptors are the principal excitatory receptors in the brain. These receptors have been considered as the main targets in the treatment of epilepsy in recent years. This work aimed to determine how the $Ca^{2+}$ conductivity of ionotropic glutamate receptors and voltage-gated $Ca^{2+}$ channels changes in an *in vitro* model of epilepsy. For induction of epileptiform activity, hippocampal neurons were exposed to $Mg^{2+}$-free medium. It has been shown that removal of $Mg^{2+}$ from the medium not only removes the block from the NMDA receptors but also stimulates the release of glutamate in a way that is independent of the NMDA receptors. Under these conditions, the structure of the bursts significantly differs from the spontaneous bursts arising in mature hippocampal cultures. We have demonstrated that the frequency and amplitude of $Mg^{2+}$-free medium-induced $Ca^{2+}$ oscillations decrease after the 60-min exposure. Besides, the $Ca^{2+}$ conductivity of ionotropic glutamate receptors and voltage-gated calcium channels significantly reduces. Thus, the decrease of $Ca^{2+}$ conductivity can be considered as one of the mechanisms of adaptation during epilepsy.

## 1. Introduction

Almost 50 million people around the world have epilepsy. This disease is characterized by recurring unprovoked seizures representing the abnormally synchronous activity of neurons. Dysregulation of the inhibitory GABAergic neurons, imbalance of extracellular ions concentration, and enhancement of the excitatory synaptic transmission can be considered as the causes of abnormal synchronization (Rogawski, 2013).

Currently, there are many models which allow to study the mechanisms of epileptogenesis, as well as to investigate the effects of various antiepileptic drugs. The synchronous epileptiform activity can be induced *in vitro* using various inhibitors, such as 4-aminopyridine ($K^+$-channels blocker), bicuculline or picrotoxin (GABA(A) receptor antagonists) (Avoli et al., 2002; Reddy and Kuruba, 2013; Salazar et al., 2003). Besides, the epileptiform activity can be induced by increasing extracellular $K^+$ concentration (Poolos and Kocsis, 1990) or reducing/removing $Mg^{2+}$ from the extracellular environment (Cho et al., 2017). In hippocampal cell cultures, the removal of $Mg^{2+}$ from the extracellular medium then induces neurons in the network to undergo synchronized oscillations of cytosolic $Ca^{2+}$ concentration (Wang and Gruenstein, 1997). Excessive excitation under these conditions can lead to glutamate-mediated excitotoxicity that is considered to be the leading cause of neuronal death during status epilepticus.

On the one hand, excessive glutamate release leads to intracellular $Ca^{2+}$ overload, oxidative stress, organelle swelling and rupture of intracellular membranes, activation of the proteases and necrosis (Dong et al., 2009; Vishnoi et al., 2016). On the other hand, increased activity triggers the adaptive processes of plasticity, which increase the stability of the system. We have previously shown that hyperexcitation leads to a rapid change in the activity of NMDA and AMPA receptors. It has been demonstrated in particular that the activity of NMDA receptors increases, while the activity of AMPA receptors decreases under ammonium-induced hyperexcitation (Kosenkov et al., 2018).

NMDARs, AMPARs, and kainate receptors (KARs) are the ionotropic glutamate receptors. Most AMPA and KA receptors are permeable to $Na^+$ but impermeable to $Ca^{2+}$. $Ca^{2+}$ permeability of AMPARs depends on the presence of GluA2 subunit. Only GluA2-lacking AMPARs are permeable to $Ca^{2+}$. Also, the receptor can be permeable to $Ca^{2+}$ if it contains an unedited GluA2 subunit (Wright and Vissel, 2012). KARs are $Ca^{2+}$-permeable only if they contain unedited GluK1 or GluK2 subunits (Perrais, 2010). In turn, NMDA receptors are permeable to

* Corresponding authors.
*E-mail addresses:* ser-gajdin@yandex.ru (S.G. Gaidin), kosenckov406@yandex.ru (A.M. Kosenkov).

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both $Na^+$ and $Ca^{2+}$. However, these receptors require prior depolarization for activation to remove the $Mg^{2+}$ block from the pore (VanDongen, 2009).

Taking into account that these receptors are the principal excitatory receptors in the brain, it is not surprising that much attention in recent years has been paid to studying these receptors as targets for the treatment of epilepsy (Schidlitzki et al., 2017). Nevertheless, there are only some works which are devoted to the changes of the properties of the receptors, especially subunit composition and ion permeability, in epilepsy (Amakhin et al., 2018; Egbenya et al., 2018). The present work aimed to investigate how the $Ca^{2+}$ conductivity of glutamate receptors and voltage-gated $Ca^{2+}$ channels changes during $Mg^{2+}$-free medium-induced epileptiform activity.

## 2. Materials and methods

All experimental protocols in this study were approved by the Bioethics Committee of the Institute of Cell Biophysics. Experiments were carried out according to Act708n (23 August 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 (22 September 2010) on the protection of animals used for scientific purposes. Pregnant female Sprague-Dawley rats were housed in the animal facility of Institute of Cell Biophysics at 25 ± 3 °C with a 12 h light/dark cycle and free access to food and water.

### 2.1. Cell culture preparation

Mixed neuroglial hippocampal cell cultures were prepared as described previously (Kosenkov et al., 2019; Turovskaya et al., 2019). Briefly, the animals were euthanized and then decapitated. The extracted hippocampus was put in a 1.5 ml tube with ice-cold $Mg^{2+}$- and $Ca^{2+}$-free Hank`s balanced salt solution (HBSS) and minced with clippers. The supernatant was carefully removed with a pipette, and 500 µl of 1% trypsin solution was added into the tube. Trypsinization of tissue fragments was performed at 37 °C and constant shaking (600 rpm, 10 min). To inactivate and remove trypsin, the minced tissue was washed two times with cold Neurobasal-A medium. Then, the tissue fragments were gently triturated with a pipette, and non-triturated debris was carefully removed with a small pipette tip. The cell suspension was centrifuged for 3 min at 2000 rpm, and the supernatant was removed. The pellet was resuspended in Neurobasal-A medium supplemented with B-27 (2 %) and glutamine (0.5 mM). The cells were seeded on polyethyleneimine-coated glass coverslips, which were placed in sterile Petri dishes, and covered with cell culture medium. Then, the dishes were placed in a $CO_2$-incubator. Cultures were grown at 37 °C and 95 % humidity. One-third of cell culture medium volume was replaced by fresh medium every 3–4 days. We used cell cultures after 12–13 days *in vitro* (DIV) in all experiments to avoid some possible age-dependent artifacts.

### 2.2. Fluorescence measurements

To detect $[Ca^{2+}]_i$ changes in neurons, the cells were stained with a fluorescent ratiometric $Ca^{2+}$-sensitive probe, Fura-2 AM. The probe was dissolved in HBSS solution composed of (mM): 156 NaCl, 3 KCl, 2 $MgSO_4$, 1.25 $KH_2PO_4$, 1.4 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.4. The cultures were incubated with 5 µM of Fura-2 for 40 min at 37 °C. Then, the cells were washed three times with HBSS. Glass coverslips with the cultures were then mounted into the microscopic chamber. An inverted motorized microscope Leica DMI 6000B equipped with a high-speed monochrome CCD camera Hamamatsu C9100 and the system for fast replacement of excitation filters Leica's Ultra-Fast Filter Wheels was used for the fluorescent measurements. Leica EL6000 illuminator with HBO 103 W/2 mercury lamp was used as an excitation source. FU2 filter set (Leica, Germany) with excitation filters BP340/30 and BP387/15, beam splitter FT410, and emission filter BP510/84 was used for excitation and registration of Fura-2 fluorescence. Applications of the reagents were made in a continuous flow of HBSS solution using a special perfusion system that allows quick replacement of the bathing solution. In the case of $Mg^{2+}$-free medium, $MgSO_4$ in HBSS was replaced by an osmotically equivalent concentration of $Na_2SO_4$. All imaging experiments were performed at temperature 28–30 °C.

### 2.3. Whole-cell patch-clamp recordings

Membrane potential from neurons was recorded at 28 °C with an Axopatch 200 B amplifier (Axon Instruments). Data were digitized by a low-noise Data Acquisition System (Axon DigiData 1440A digitizer) with pCLAMP 10 software from Axon Instruments (USA). The experiments were performed using a patch-pipette solution containing (mM): 5 KCl, 130 K-gluconate, 1 $MgCl_2 \times 6H_2O$, 0.25 EGTA, 4 HEPES, 2 $Na_2$-ATP, 0.3 Mg-ATP, 0.3 Na-GTP, 10 $Na_2$-phosphocreatine (305–310 mOsm, pH 7.2). The extracellular solution used for all recordings contained (mM): 156 NaCl, 3 KCl, 2 $MgSO_4$, 1.25 $KH_2PO_4$, 1.4 $CaCl_2$, 10 glucose and 10 HEPES, pH 7.4. In the case of $Mg^{2+}$-free medium, $MgSO_4$ was replaced by an osmotically equivalent concentration of $Na_2SO_4$.

### 2.4. Statistical and data analysis

The time series of images obtained in two different channels (excitation of Fura-2 at 340 and 380 nm) were processed with ImageJ. The changes in $[Ca^{2+}]_i$ are presented as the 340/380 ratio obtained from time-lapse images after background subtraction. ImageJ, Origin 2016, and MS Excel software was used for data analysis and graphs creation.

N – number of neurons which were analyzed in an experiment; n—number of the experiments. The results were analyzed for statistical significance using GraphPad PRISM version 8.0. The normality of data distribution was examined using Kolmogorov-Smirnov`s test. Differences were considered significant when $P < 0.05$. The differences between coefficient of variations were estimated by unpaired Kolmogorov-Smirnov`s *t*-test. The frequency and amplitude of $[Ca^{2+}]_i$ oscillations 5 and 55 min after $Mg^{2+}$-free medium application were compared using the paired Student's *t*-test because the data were normally distributed. In order to estimate the effect of $Mg^{2+}$-free-induced epileptiform activity on the activity ($Ca^{2+}$ conductivity) of NMDARs, AMPARs, KARs, and voltage-gated $Ca^{2+}$ channels, we used Two-way ANOVA, followed by Sidak's multiple comparison test.

### 2.5. Reagents

The following reagents were used in the study: (+)-MK 801 maleate, Diltiazem hydrochloride, N-methyl-D-aspartate (NMDA) (Sigma Aldrich, USA); D-AP5, NBQX disodium salt, UBP302, Domoic acid (Tocris Bioscience, UK); (S)-(-)-5-Fluorowillardiine (FW) (Santa Cruz Biotechnology, USA); Neurobasal-A medium, B-27 supplement, Trypsin (1 %) (Gibco, USA), Fura-2AM (Invitrogen, USA).

## 3. Results

### 3.1. $[Ca^{2+}]_i$ oscillations and burst-firing patterns of neurons in $Mg^{2+}$-free medium

Synchronous oscillations of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) appear in cultured neurons at 12–14 DIV. The elevation of cytosolic $Ca^{2+}$ concentration occurs for the opening of $Ca^{2+}$ channels during the bursts. The frequency of calcium oscillations and the basal level of $[Ca^{2+}]_i$ increase under $Mg^{2+}$-free conditions. In turn, the burst-firing under $Mg^{2+}$-free conditions markedly differ from control. The bursts do not have a well-defined structure in this case compared to

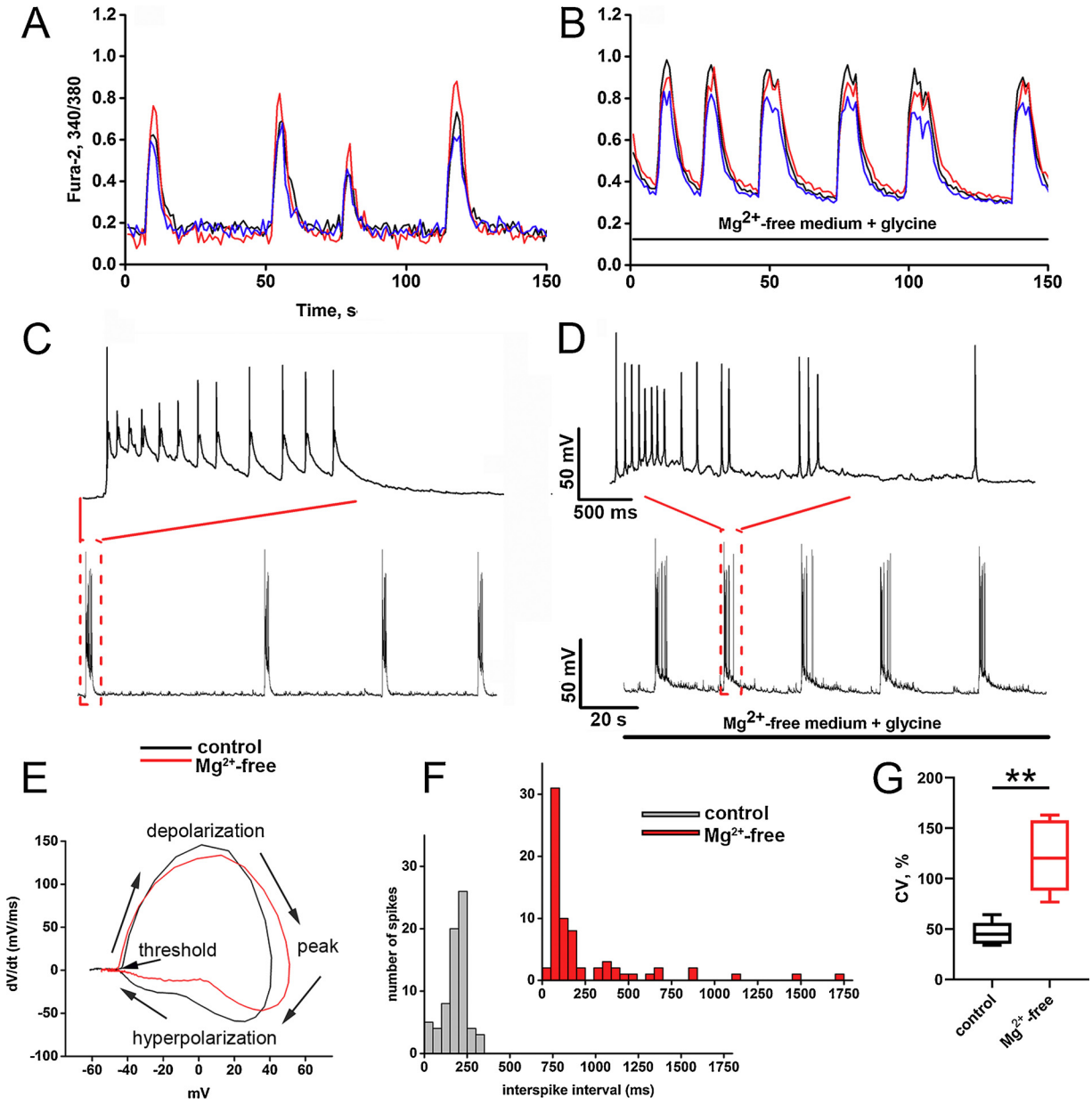


**Fig. 1. Comparison of $[Ca^{2+}]_i$ oscillations and bursts of neurons in control and $Mg^{2+}$-free medium.** $[Ca^{2+}]_i$ oscillations of neurons in control (A) and $Mg^{2+}$-free medium with the addition of glycine (20 μM) (B). Traces of representative neurons are shown in each figure. A total number of analyzed neurons in each experiment was 100 (N = 100). Each experiment was repeated three times (n = 3). Recordings of the membrane potential of neurons in control (C) and in $Mg^{2+}$-free medium with the addition of glycine (D), n = 5. (E) Phase portrait of time derivative of membrane potential (dV/dt) as a function of membrane potential (mV) during first spike of representative burst under normal conditions (black) and $Mg^{2+}$-free conditions (red). (F) Histogram reflecting the distribution of interspike intervals (ISIs) in control (black) and under $Mg^{2+}$-free conditions (red). ISIs were calculated for five representative bursts from three independent repeats. The normality of the distribution was examined by Kolmogorov-Smirnov test. (G) Box charts of ISIs coefficient of variation (CV). Unpaired Kolmogorov-Smirnov`s *t*-test, p** = 0.0079.

control (Fig. 1C). Separate action potentials (APs) arise as a result of a slow elevation of membrane potential (Fig. 1D). This peculiarity can be explained by the absence of the $Mg^{2+}$ block of NMDA receptors. Phase-portrait of membrane potential versus its time derivative for first AP in the burst demonstrates that depolarization and repolarization phases of APs under $Mg^{2+}$-free conditions differ from control (Fig. 1E). However, the threshold of AP generation under $Mg^{2+}$-free conditions is similar to the threshold in control. As shown in Fig. 1F, the distribution of interspike intervals (ISIs) under $Mg^{2+}$-free conditions is non-normal (p = 0.0001; Kolmogorov-Smirnov test) while ISIs in control are normally distributed (p = 0.2320). The variability of ISIs is also represented as a coefficient of variation (CV). CV for $Mg^{2+}$-free bursts is significantly higher compared to bursts in control (Fig. 1G) (Unpaired Kolmogorov-Smirnov`s *t*-test, p** = 0.0079; median [interquartile range]: 45 [35.28–56.08] for normal burst and 120.2 [88.16–157.9] for burst under $Mg^{2+}$-free conditions).

### 3.2. Mechanism of the $Ca^{2+}$ response to the application of $Mg^{2+}$-free medium

Removal of $Mg^{2+}$ from the medium induces $[Ca^{2+}]_i$ rise in all neurons (Fig. 2A). It is reasonable to assume that the $[Ca^{2+}]_i$ elevation is caused by the removal of the $Mg^{2+}$ block from NMDA receptors and the subsequent depolarization of the cell due to the receptor-mediated cation inflow. However, the $[Ca^{2+}]_i$ elevation is observed in the presence of the antagonists of NMDA receptors (Fig. 2B, C, D). Besides, the

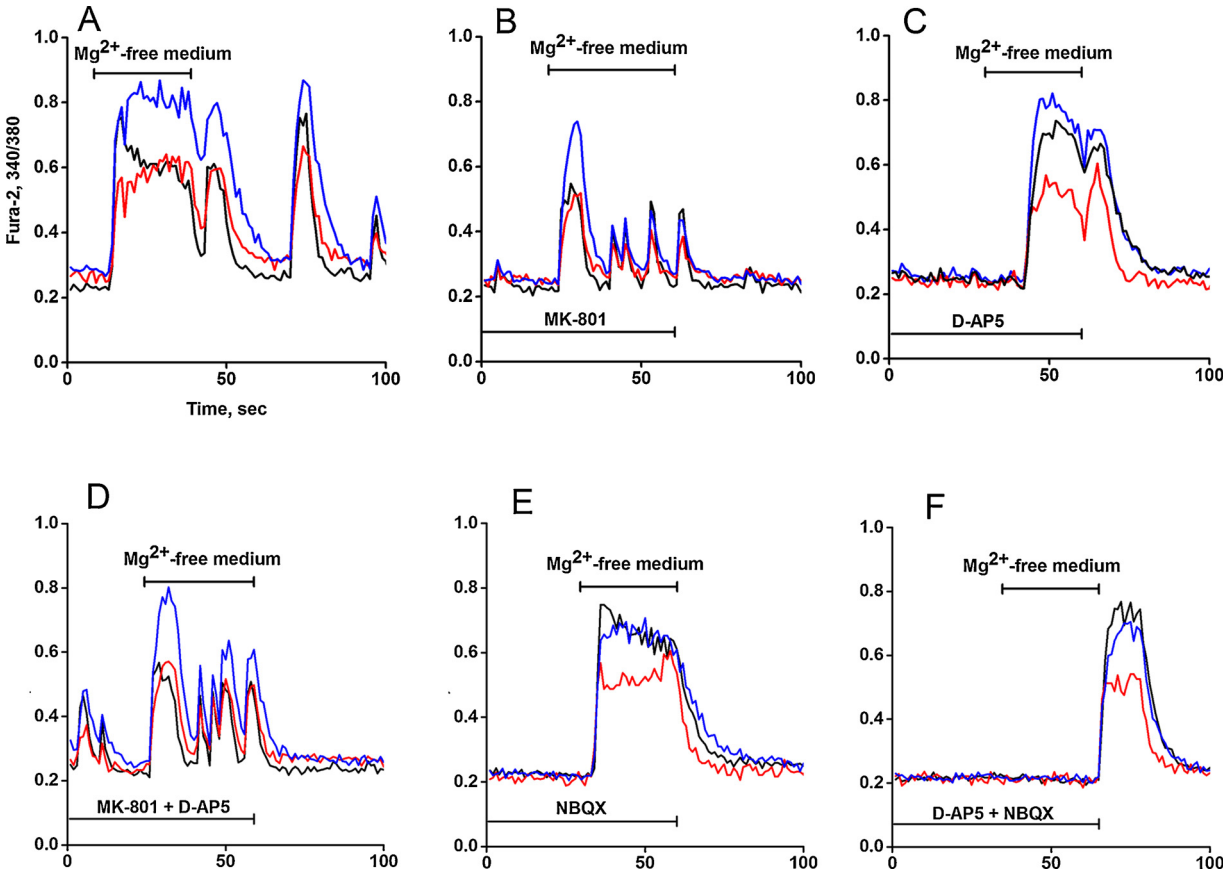


**Fig. 2. The effect of NMDA and AMPA receptors inhibitors on the $Ca^{2+}$ response to the application of $Mg^{2+}$-free medium.** $Ca^{2+}$ response of neurons to the application of the $Mg^{2+}$-free medium in the control (A), in the presence of a non-competitive antagonist of NMDA receptor MK-801 (20 μM) (B), a competitive antagonist of NMDA receptor D-AP5 (10 μM) (C), in the presence of both antagonists (D), in the presence of a competitive antagonist of AMPA receptor NBQX (10 μM) (E) and in the presence of both antagonists of NMDA and AMPA receptors (F). Traces of representative neurons are shown in each figure. A total number of analyzed neurons in each experiment was 100 (N = 100). Each experiment was repeated three times (n = 3).

calcium elevation is also observed in the presence of NBQX, AMPA receptor antagonist (Fig. 2E). Interestingly, the $[Ca^{2+}]_i$ rise is completely abolished only in the presence of both the NMDA and AMPA receptor antagonists (Fig. 2F).

It can be concluded from the above experiments that the $[Ca^{2+}]_i$ elevation in neurons occurs not only as a consequence of the removal of the $Mg^{2+}$ block since the $Ca^{2+}$ response remains even in the presence of MK-801, a non-competitive inhibitor of NMDA receptors. This inhibitor binds inside the ion channel, thus preventing the flow of ions through the channel, including $Ca^{2+}$ (Huettner and Bean, 1988). The $Ca^{2+}$ response is abolished only in the presence of both NMDAR and AMPAR inhibitors. Therefore, the $Mg^{2+}$-free medium-induced $Ca^{2+}$ response is mediated by AMPA as well as NMDA receptors. These findings may indicate that glutamate is released as a result of the removal of $Mg^{2+}$ from the medium. This suggestion is confirmed by the fact that D-AP5 and NBQX are competitive antagonists, so they block the glutamate binding site, but do not block the channel pore.

### *3.3. Changes in the $Ca^{2+}$-conductivity of ionotropic glutamate receptors and voltage-gated $Ca^{2+}$ channels in $Mg^{2+}$-free model of epileptiform activity*

We have compared the $Ca^{2+}$ conductivity of these receptors before and after exposure to $Mg^{2+}$-free medium. It should be noted that the frequency and amplitude of $[Ca^{2+}]_i$ oscillations induced by removal of $Mg^{2+}$ from the medium (Fig. 3) decrease during the first hour of exposure (paired Student's *t*-test; first vs. last: *p = 0.0368 for frequency; **p = 0.0014 for amplitude). This finding may indicate the adaptation of neurons to increased activity, which may occur, in particular, due to reducing the activity of ionotropic glutamate receptors. In order to examine this assumption, we have performed experiments in which we compared the amplitude of $Ca^{2+}$ influx through NMDARs, AMPARs, KARs, and voltage-gated $Ca^{2+}$ channels (VGCC) (Fig. 4). KCl application (35 mM) was used to depolarize the neurons and activate VGCC. The experiments were performed in the presence of the appropriate inhibitors to estimate the $Ca^{2+}$ influx through each subtype of glutamate receptors. Experiments have shown that $Ca^{2+}$ influx through ionotropic glutamate receptors markedly decreases as a result of epileptiform activity (Two-way ANOVA followed by Sidak's multiple comparison test; row factor F (3, 16) = 2.706, column factor F (1, 16) = 125.6; control vs. treatment (mean ± SD): 0.94 ± 0.13 vs. 0.45 ± 0.05 for NMDARs, p = 0.0001; 0.95 ± 0.05 vs. 0.6 ± 0.1 for AMPARs, p = 0.0002; 0.94 ± 0.05 vs. 0.6 ± 0.1 for KARs, p = 0.0003). In turn, the activity of VGCC is also reduced (0.95 ± 0.1 vs. 0.75 ± 0.05, p = 0.0248) but to a lesser extent compared to glutamate receptors (Fig. 4E). It is important to note that despite the fact that all NMDARs are $Ca^{2+}$-permeable under normal conditions, activation of these receptors in the presence of AMPARs, KARs, and VGCC L-type inhibitors does not lead to a significant increase in $[Ca^{2+}]_i$ in the soma of neurons.

As shown in Fig. 4F, only a small group of neurons responds to the application of AMPARs and KARs agonists (16 ± 3% and 12 ± 5% respectively) in the presence of the appropriate inhibitors. This data may indicate that non-responding neurons either do not express $Ca^{2+}$-permeable AMPARs / KARs or contain a relatively low quantity of receptors, which is insufficient for a marked increase in $[Ca^{2+}]_i$.

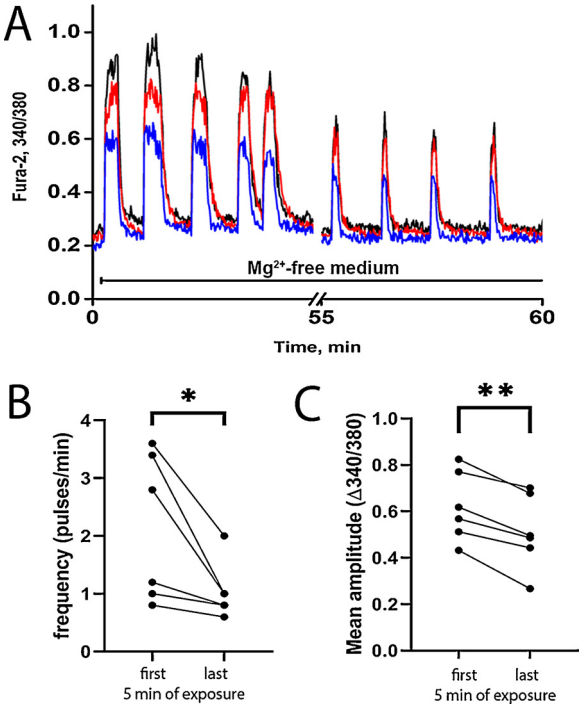


**Fig. 3. Changes in the frequency and amplitude of $Mg^{2+}$-free medium-induced $[Ca^{2+}]_i$ oscillations during the first hour of exposure.** (A) Traces of representative neurons during $Mg^{2+}$-free medium exposure. Comparison of the frequency (B) and amplitude (C) of $[Ca^{2+}]_i$ oscillations during the first and last 5 min of $Mg^{2+}$-free medium exposure. Glycine (20 μM) was contained in the medium. Each dot represents averaged values of the amplitude and frequency in an individual repeat before and after $Mg^{2+}$-free medium exposure. n = 6; N = 100 for each repeat. Statistical analyses were performed by paired *t*-test, *p = 0.0368, **p = 0.0014.

## 4. Discussion

The $Mg^{2+}$-free model has been used for more than thirty years to study the pathogenesis and therapy of epilepsy. It is believed that the removal of the $Mg^{2+}$ block of NMDA receptors is the leading cause of the epileptiform activity in this model. The pivotal role of NMDA receptors in the induction of epileptiform activity in this model is confirmed by the fact that antagonists of NMDA receptors suppress $[Ca^{2+}]_i$ oscillations (Pacico and Mingorance-Le Meur, 2014). In turn, spontaneous $[Ca^{2+}]_i$ oscillations in cultures are suppressed by AMPA receptor antagonists (Tanaka et al., 1996). The antagonists of NMDA receptors only reduce the intensity of $Ca^{2+}$ influx during the impulses in this case. However, $Mg^{2+}$ is involved in many cellular processes. $Mg^{2+}$ is known to play an essential role in energy metabolism, intracellular signaling, and synaptic transmission (Romani, 2011; Yamanaka et al., 2016). It was shown that $Mg^{2+}$ decreases the activation of voltage-gated channels at physiological concentrations via surface charge screening effects, thereby reducing neuronal excitability (Dribben et al., 2010). Besides, $Mg^{2+}$ blocks $Ca^{2+}$ influx and diminishes synaptic transmission at high concentrations (Slutsky et al., 2004). Given these facts, it is not surprising that the structure of bursts induced by $Mg^{2+}$-free medium differs from the structure of bursts in control. According to the record presented in Fig. 1, it can be assumed that the mechanisms of initiation and termination of bursts in $Mg^{2+}$-free model also differ from the mechanisms of generation of spontaneous bursts which occur in hippocampal neurons in cultures and slices (Liu et al., 2003; Tsukamoto-Yasui et al., 2007). In particular, this data may explain the lack of efficiency of some recognized anti-epileptic drugs in this model (Pacico and Mingorance-Le Meur, 2014).

Besides, we have shown that the removal of $Mg^{2+}$ from the medium leads to a release of glutamate, and this effect does not depend on the activity of NMDA receptors. Considering that the presence of $Mg^{2+}$ reduces the activity of voltage-gated channels (Dribben et al., 2010), it can be assumed that rapid removal of $Mg^{2+}$ from the medium leads to depolarization of neurons, followed by glutamate release.

In general, our findings suggest that the $Mg^{2+}$-free model is not well suited for testing anti-epileptic drugs, since the mechanisms of generation and maintenance of epileptiform activity may differ from the intact brain. However, the epileptiform activity resulting from the removal of $Mg^{2+}$ makes it possible to assess how the activity of various ionotropic channels changes. NMDA, AMPA, and KA receptors are the principal excitatory ionotropic receptors in the brain, and so the action of many antiepileptic drugs is based on the suppression of their activity. However, it has not been fully established yet how the activity of the receptors changes during epileptiform activity. In particular, despite the fact that the accumulation of intracellular $Ca^{2+}$ is one of the leading causes of neuron death in epilepsy (Delorenzo et al., 2005; Waldbaum and Patel, 2010) the mechanisms of changes of $Ca^{2+}$ conductivity have not been fully established yet. We have shown in this study that $Ca^{2+}$ influx mediated by NMDA, AMPA, and KA receptors reduces after exposure to $Mg^{2+}$-free medium in all neurons. In addition, the activity of VGCC also decreases. Besides, since NMDAR-mediated $Ca^{2+}$ influx makes a small contribution to the change in the $[Ca^{2+}]_i$, and the CP-AMPA/KA receptors are not expressed in all neurons, it can be assumed that the main influx of $Ca^{2+}$ during epileptiform activity is mediated by VGCC.

As is known, the processes of phosphorylation and dephosphorylation have a significant impact on the conductivity and trafficking of NMDA receptors. Phosphorylation of NMDA receptor by various protein kinases mainly leads to an increase in its activity. It was shown that phosphorylation of NMDA receptor by PKA increased the permeability of the receptor to $Ca^{2+}$ (Skeberdis et al., 2006). Besides, activation of PKC leads to upregulation of NMDA receptors (Lin et al., 2006; Xiong et al., 1998). In turn, the activation of the phosphatases leads to the opposite effect. It was shown that phospho-serine/threonine protein phosphatases 1 (PP1), 2A (PP2A), and 2B (PP2B or calcineurin) suppress the activity of NMDARs (Lieberman and Mody, 1994; Wang et al., 1994). Interestingly, calcineurin is activated by $Ca^{2+}$ entry through NMDA channels. Taking into account that $Ca^{2+}$ influx through NMDA receptor intensifies during epileptiform activity, it can be suggested that the reducing of the $Ca^{2+}$ inflow through NMDARs after exposure of $Mg^{2+}$-free medium can be realized via a feedback mechanism involving activation of calcineurin and other phosphatases.

Various reasons can cause a decrease in the amplitude of the $Ca^{2+}$ response to the application of AMPA receptor agonists after exposure to $Mg^{2+}$-free medium. It is known that the $Ca^{2+}$ permeability of AMPARs depends on the presence of GluA2 subunit. Receptors containing an unedited GluA2 subunit are not permeable to $Ca^{2+}$. It was shown using the pilocarpine model of epilepsy that epileptiform activity increases the expression of GluA2-containing AMPARs in rat cerebral cortex (Russo et al., 2013). Besides, it was found that the activation of extrasynaptic NR2B-containing NMDARs leads to a decrease in the activity of AMPA receptors (Kim et al., 2005; Sun and June Liu, 2007). As is known, the accumulation of extracellular glutamate during epileptiform activity promotes the activation of extrasynaptic NMDA receptors. It was demonstrated in another model of hyperexcitation that the presence of antagonists of NMDARs abolished the effect of a decrease in the activity of AMPARs (Kosenkov et al., 2018). Thus, it can be assumed that reducing of the amplitude of the $Ca^{2+}$ response may be associated with a decrease in the expression of $Ca^{2+}$-permeable AMPA receptors caused by activation of extrasynaptic NMDA receptors. In turn, not much is known about the regulation of the activity of KARs, especially about the regulation of $Ca^{2+}$ conductivity. However, it was demonstrated that GluK2 endocytosis mediated by Src phosphorylation could occur after prolonged stimulation (Zhu et al., 2014). Taking into account that the majority of GluK2-containing receptors are $Ca^{2+}$-

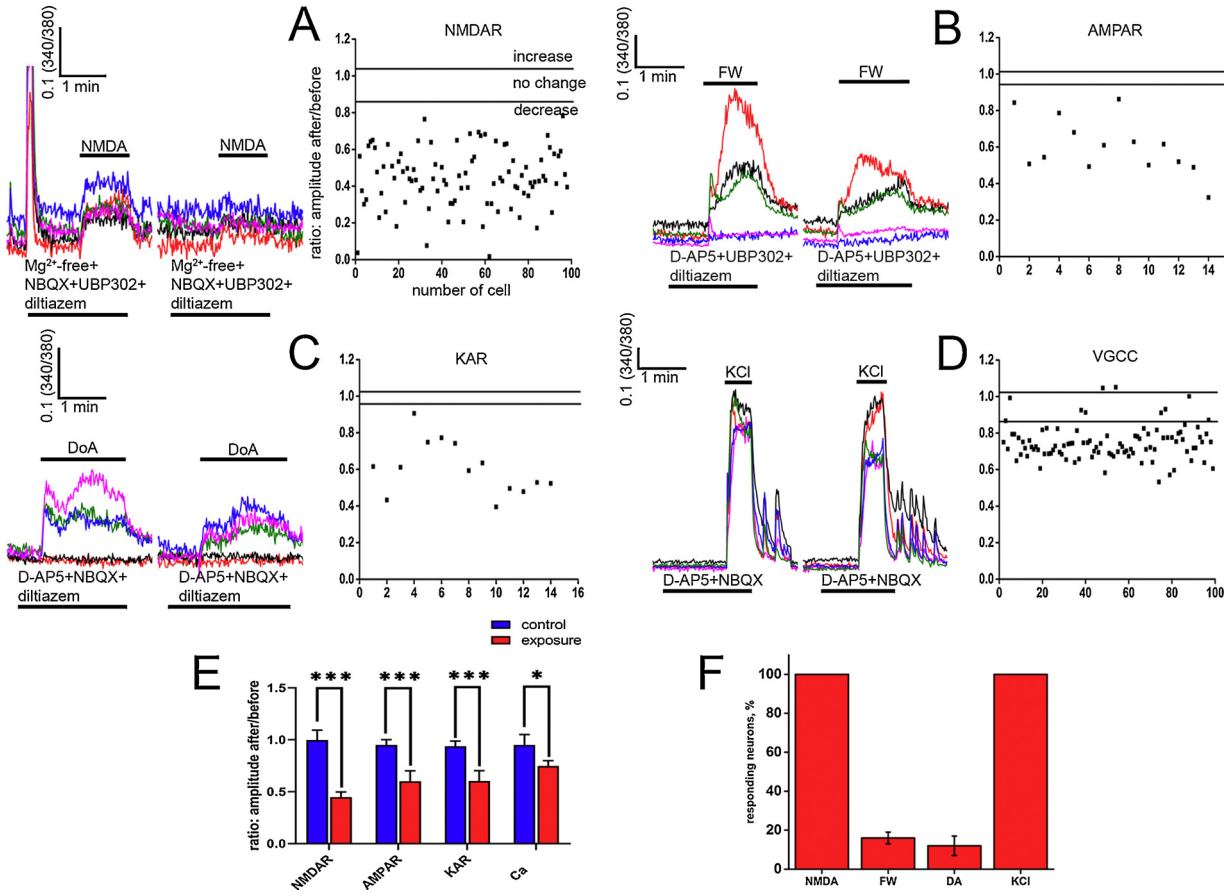


**Fig. 4. Changing of $Ca^{2+}$ conductivity of NMDARs, AMPARs, KARs, and VGCC during epileptiform activity.** A, B, C, D (left panel) – the response of neurons to the application of 10 μM NMDA, 500 nM FW (AMPAR agonist), 300 nM DA (KAR agonist) and 35 mM KCl before and after $Mg^{2+}$-free medium exposure. D-AP5 (10 μM) – an inhibitor of NMDARs, NBQX (2 μM) – an inhibitor of AMPARs, UBP302 (20 μM) – an inhibitor of KARs, diltiazem (300 μM) – an inhibitor of L-type VGCC. The exposure of $Mg^{2+}$-free medium lasted 1 h. Glycine (20 μM) was contained in the medium. Traces of representative neurons are shown in each figure. A total number of analyzed neurons in each experiment was 100 (N = 100). Each experiment was repeated three times (n = 3) A. B, C, D (right panel) - the ratio of $Ca^{2+}$ response amplitude to NMDA, FW, DA and KCl application after exposure of $Mg^{2+}$-free medium to the amplitude of the response before the exposure for separate neurons. The dots in plots represent the ratio of the amplitude after to the amplitude before for individual neurons. (E) Diagrams showing average changes in the amplitudes of the $Ca^{2+}$ responses of neurons to the application of agonists. Statistical analyses were performed by Two-way ANOVA, followed by Sidak's multiple comparison test. NMDAR ***p = 0.0001; AMPAR ***p = 0.0002; KAR ***p = 0.0003; VGCC *p = 0.0248. (F) Diagrams showing an average number of neurons responding to an agonist application.

permeable (Silva et al., 2001), this mechanism can be considered as a reason for the decrease in $Ca^{2+}$ influx through KARs during epileptiform activity.

Neuroglial culture is an excellent model for studying the functioning of neuronal networks in health and pathology. It has been demonstrated in this work that neurons can adapt to epileptiform activity by reducing activity and, in particular, the $Ca^{2+}$ conductivity of ionotropic glutamate receptors and VGCC, that leads to a decrease in the frequency and amplitude of $[Ca^{2+}]_i$ oscillations. Thus, activation of adaptive mechanisms prevents $Ca^{2+}$ overload in neurons under these conditions. This finding is significant because a prolonged increase in cytosolic $Ca^{2+}$ concentration can lead to the apoptosis and subsequent cell death. In addition, the fact that a decrease in $Ca^{2+}$ influx through NMDA and AMPA receptors is observed in all neurons indicates that this effect is systemic.

## Declaration of Competing Interest

None.

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