The Ionic Mechanisms Regulating Astrocytic Calcium Dynamic

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The aim of the study was to understand the effect of alterations in local ionic concentrations due to neuronal activity on calcium activity in astrocytes.

Materials and Methods. In this study we investigated astrocytic calcium dynamics using confocal microscopy on hippocampal slices from Wistar rats P15–18.

Results. Here we demonstrated that activation of metabotropic glutamate receptors on astrocytes led to an increase of the frequency and duration of calcium events. Elevations of the extracellular calcium concentration did not change the frequency and the duration. Elevations of the extracellular potassium concentration increased the frequency and reduced the duration of calcium events.

Conclusions. Neuronal activity causing alterations in local ionic concentrations might affect calcium activity in astrocytes, creating a feedback loop, controlling functioning of neuron-glia networks. These data indicate the complex nature of the effects, which modulate the interplay between neurons and astrocytes that cannot be considered only in the context of a receptor signaling.

Key words: hippocampus; astrocytes; calcium oscillations; metabotropic glutamate receptors; neuron-glia interaction.

Introduction. Astrocytes represent the most widely studied type of glial cells, due to their involvement in a number of crucial functions in the brain. Such as 1) trophic function [1, 2], by releasing trophic factors regulating proliferation of neurons, neurogenesis, synaptogenesis, axons grow; 2) homeostatic function, by maintaining the water balance via special channels — aquaporins, by removal glutamate from synaptic cleft, preventing excitotoxicity and desensitization of receptors and also by buffering potassium that accumulates extracellularly following action potentials in neurons [2, 3]; 3) metabolic function, by involvement in blood-brain barrier maintaining, providing energy support of neurons and removal of metabolites [2, 4, 5]; 4) signaling function, by generation of calcium signals [2, 6–8] and release of gliatransmitters, which can modulate synaptic transmission [3]. These calcium signals represent temporally restricted elevations of calcium in cytosol. They can be triggered mainly by activation of metabotropic receptors (mGluRs) on astrocytic membrane [9–12] and by mobilization from intracellular stores [9, 12–14]. Therefore, calcium plays a role of secondary messenger, that can trigger metabolic reactions, such as gene expression, release of neuroactive substances, gliatransmitters, including glutamate [1, 3, 6, 15], D-serine [16] and ATP [1, 11], which might affect synaptic transmission [17]. Calcium signals or oscillations occur when glutamate escape the synaptic cleft, diffuse and bind to mGluRs on astrocytic membrane. Activation of mGluRs I leads to triggering of the biochemical cascade inside of the astrocytes, involving phospholipase C (PLC) and generation of intracellular messengers such as inositol-1,4,5-phosphate (IP3), that activate the receptors to IP3 on endoplasmic reticulum (ER), leading to significant calcium efflux from ER [2, 7, 18–20]. Thus, neuronal activity is tightly connected with astrocytic activity via diffusion of neurotransmitters from synaptic cleft. Additionally, potassium efflux during repolarization phase of action potentials in neurons buffered by astrocytes might depolarize its membrane [3, 21–23], and might affect activation of astrocytic membrane receptors and IP3 receptors on ER, and therefore, calcium dynamic in astrocytes. However, the consequences of interactions between neurons and astrocytes remain not well understood. Specifically, it is not clear how the release of glutamate from neurons or potassium accumulation during action potentials affect calcium activity in astrocytes. Since calcium activity in astrocytes is very important for brain functioning, here we

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studied different factors of neuronal activity which might alter calcium dynamic in astrocytes.

The aim of the study was to investigate the effect of alterations in ionic concentration during neuronal activity on calcium activity in astrocytes.

Materials and Methods. Experiments were performed on 350 μm-thick hippocampal slices from Wistar rats (P15–18). All technical and housing conditions for animals were compliant with regulations from Ministry of Healthcare of Russia (No.267 from 19.06.03 "About rules on laboratory protocols in Russian Federation"). All experimental procedures were performed accordingly to the European Convention for Protection of Vertebrate Animals for Laboratory Practice (Strasbourg, 18.03.1986, approved 15.06.2006) with an agreement from Ethic Committee of Lobachevsky State University of Nizhni Novgorod. Animals were anesthetized with ether and then decapitated. The brain was removed and placed into the vibrato chamber MICROM HM650V (Thermo Scientific, USA) with an ice-cold solution, containing (in mM): 87 NaCl; 2.5 KCl; 8.48 $MgSO_4$; 1.24 NaH_2PO_4; 26.2 $NaHCO_3$; 0.5 $CaCl_2$; 11 D-glucose, at temperature 4°C, saturated with carbogen (95% O_2, 5% CO_2). Then slices were transferred into an incubation chamber for 1 h with the solution, containing (in mM): 119 NaCl; 2.5 KCl; 1.3 $MgSO_4$; 1 NaH_2PO_4; 26.2 $NaHCO_3$; 1 $CaCl_2$; 1.6 MgCl; 1 D-glucose, at temperature 34°C, saturated with carbogen. Then slices were stained with two fluorescent indicators: astrocyte-specific dye Sulforhodamine 101 (100 nM) and calcium indicator Oregon Green 488 BAPTA-1 AM (0.795 mM), diluted in dimethyl sulfoxide (DMSO) and pluronic acid (96:4) (Tocris Cookson, UK) (Figure 1 (a)). The dyes were diluted in the solution, containing (in mM): 119 NaCl; 2.5 KCl; 1.3 $MgSO_4$; 1 NaH_2PO_4; 26.2 $NaHCO_3$; 2.5 $CaCl_2$; 11 D-glucose, at temperature 34°C, for 40 min, saturated with carbogen. Then slice was transferred into the recording chamber with the same solution (perfusion speed 2–2.5 ml/min) saturated with carbogen of the microscope CarlZeiss LSM 510 (Germany). Sulforhodamine 101 was excited with the laser at 543 nm wavelength, and Oregon Green 488 BAPTA-1 AM with the laser at 488 nm. Emission was registered in diapasons 650–710 and 500–530 nm, accordingly. Cells stained with both dyes were identified as astrocytes and were used for the analysis (See Figure 1 (a)). Each recording lasted for 20–30 min at the speed 1–2 frames/s. Data were analyzed with custom-made software to obtain frequency and duration of calcium events in each recording. Statistical analysis was performed with Mann–Whitney and Wilcoxon's tests with significance when $p<0.05$. All results represented as average ± standard error of mean.

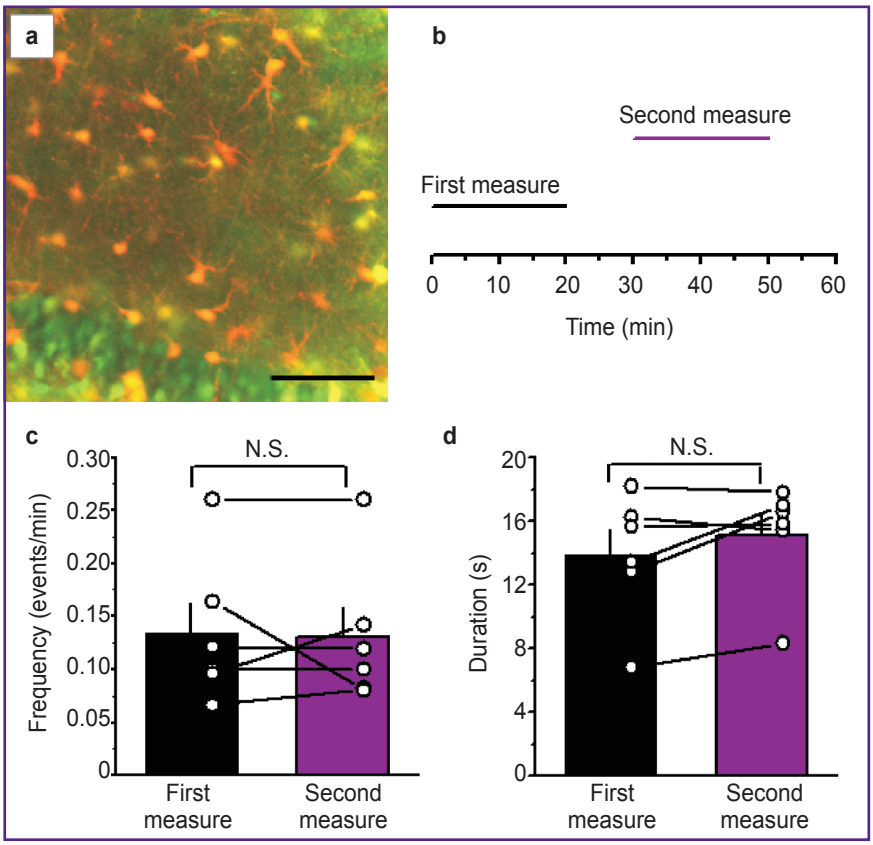

Figure 1. Calcium activity in astrocytes after laser excitation. (a) Astrocytes double stained with Sulforhodamine 101 and Oregon Oregon Green BAPTA1-AM, measurements were performed str. radiatum, scale 50 μm. (b) Two consequent separate measurements were obtained, 20 min duration each with 10 min interval between them. (c) The average frequency of calcium events did not change. (d) The duration of calcium events did not change. Wilcoxon test; N.S.: not significant

Results

Calcium dynamics in astrocytes. Optical imaging is a relatively non-invasive research technique, since does not require cell penetration unlike patch-clamp recordings. However, the energy transferred by the laser excitation over a long period of time might affect calcium activity in astrocytes. Therefore, we tested if the laser excitation energy might interfere with the results we obtain. We compare two sets of measurement of spontaneous calcium activity in astrocytes with 20 min length obtained consequentially. The second measurement started in 30 min after the first one (Figure 1 (b)). The average frequency of calcium activity in the first measurement was 0.135±0.023 events/min. When the average frequency of calcium activity in

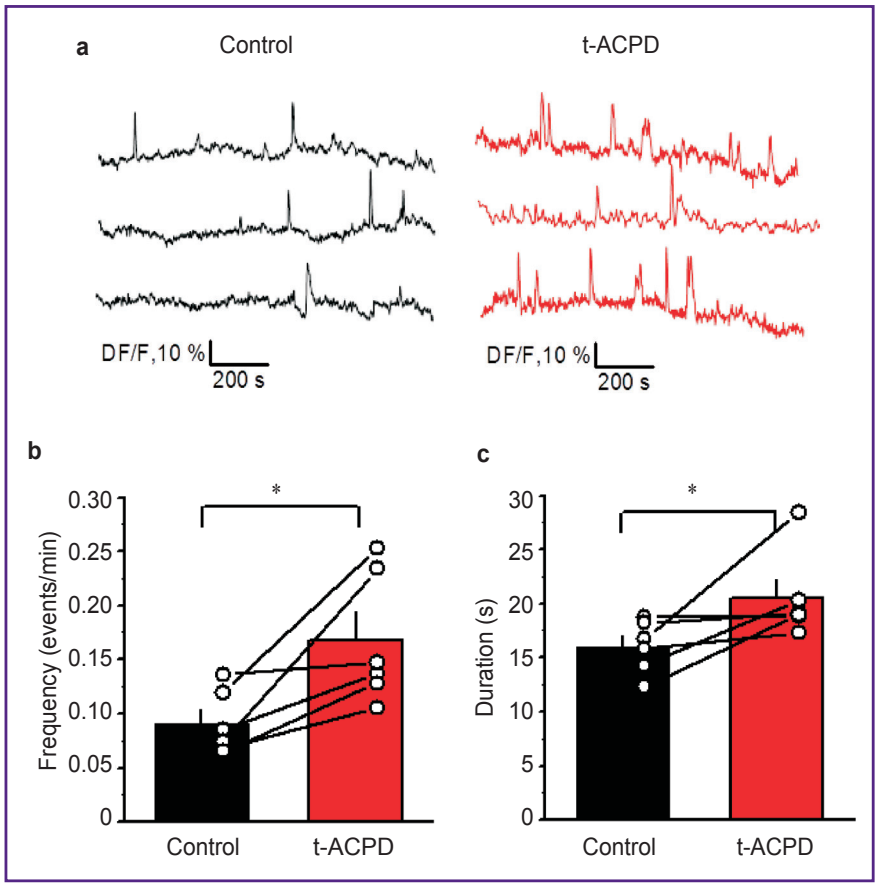

Figure 2. Calcium activity in astrocytes after activation of mGluRs by t-ACPD application. (a) The representative traces of calcium activity in control and after t-ACPD application. (b) The average frequency of calcium events increased after t-ACPD application. (c) The duration of calcium events increased after t-ACPD application. Wilcoxon test; * p<0.05

the second measurement was 0.131±0.028 events/min and was not statistically different from the first one (n=6; Wilcoxon test; p=0.999) (Figure 1 (c)). The duration of calcium events was also statistically not different (n=6; Wilcoxon test; p=0.313) (Figure 1 (d)), and was 13.88±1.62 s in the first, and 15.18±1.41 s in the second measurement. Thus we could conclude that our experimental conditions were stable over time and the laser excitation will not affect the measurements unequally during experiments.

Astrocytic calcium dynamics during activation of metabotropic glutamate receptors (mGluRs). Astrocytic processes mainly located in the proximity to synapses and have numerous glutamate transporter on the surface in order to perform glutamate uptake and prevent overexcitation of the synapse [15]. However, during high neuronal activity glutamate might partially escape the cleft [24]. The glutamate might diffuse to astrocytic mGluRs, activate them and cause changes in astrocytic calcium activity [12, 18–20, 25]. In order to test this possibility, we applied to the perfusion an agonist of mGluRI and mGluRII receptors (±)-1-Aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD), 10 μM (Figure 2 (a)). t-ACPD caused an increase in the frequency of calcium events on 184±3% of control compare to control level (0.09±0.01 events/min in control, 0.17±0.03 events/min after t-ACPD application; n=6; Wilcoxon test; p=0.03) (Figure 2 (b)), and the duration increased on 128±3% of control (n=6; Wilcoxon test; p=0.03) (Figure 2 (c)) from 15.99±1.08 s in control to 20.48±1.79 s after t-ACPD application. This effect on astrocytes could occur due to activation of mGluRI, triggering biochemical cascades, leading to an increase of IP3 production, activation of IP3R on ER, and as a consequence, prolonged duration of calcium events in astrocytes, similarly to the effect of glutamate escaping synaptic cleft.

Effect of elevation of extracellular concentration of calcium on frequency of astrocytic events. IP3R have two co-agonists: IP3 and calcium itself [14], the later triggers so called calcium-induced calcium release from ER. It had been shown, that depletion on internal stores, during high neuronal activity, as well as an increase of calcium concentration extracellularly, that occur in cell cultures, might cause alterations in astrocytic calcium dynamic [6, 14, 18, 26, 27]. This depletion might be related to an activation of astrocytic IP3R, calcium elevations, and release of ATP, that acts on purinergic receptors [2, 10, 11].

Therefore, we tested, how elevated extracellular calcium from 2.5 to 5 mM concentration might affect astrocytic calcium signaling (Figure 3 (a)). In case of 5 mM calcium we observed 280±24% of control higher frequency of events (0.12±0.21 in control, 0.34±0.05 with 5 mM of calcium; n=6; Mann–Whitney test; p=0.0022) (Figure 3 (b)), but the duration of calcium events did not change significantly (16.20±0.56 s in control, 16.14±1.12 s

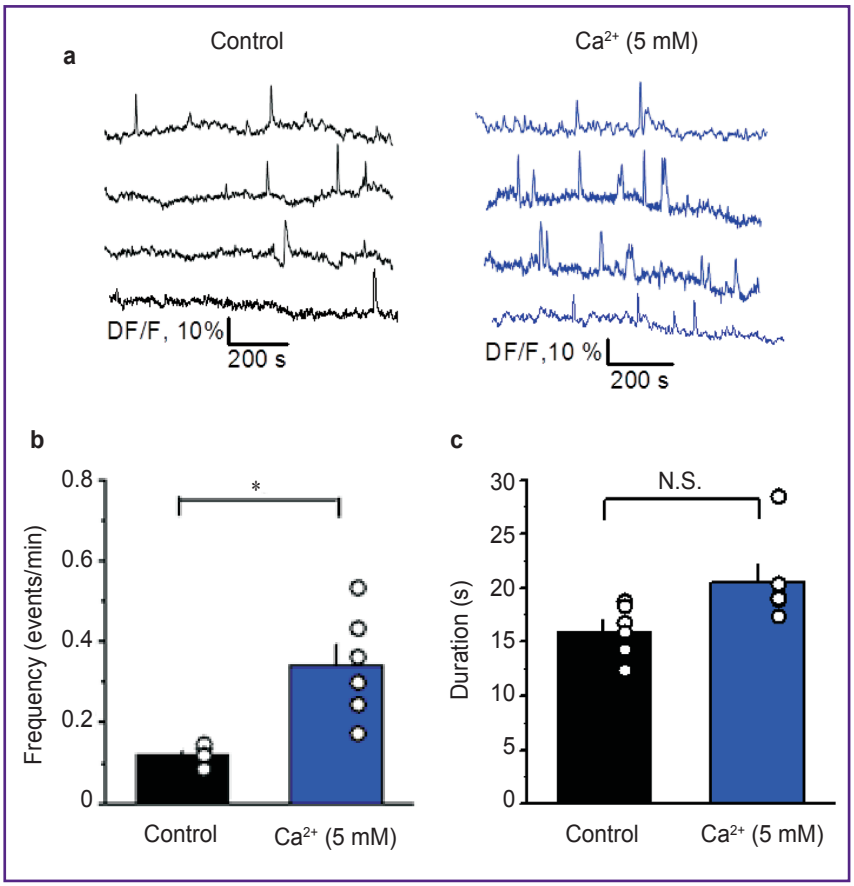

Figure 3. Calcium activity in astrocytes after elevation of extracellular calcium concentration. (a) The representative traces of calcium activity in control and after elevation of extracellular calcium concentration. (b) The average frequency of calcium events increased elevation of extracellular calcium concentration. (c) The duration of calcium events did not change after elevation of extracellular calcium concentration. Mann–Whitney test; * p<0.05; N.S.: not significant

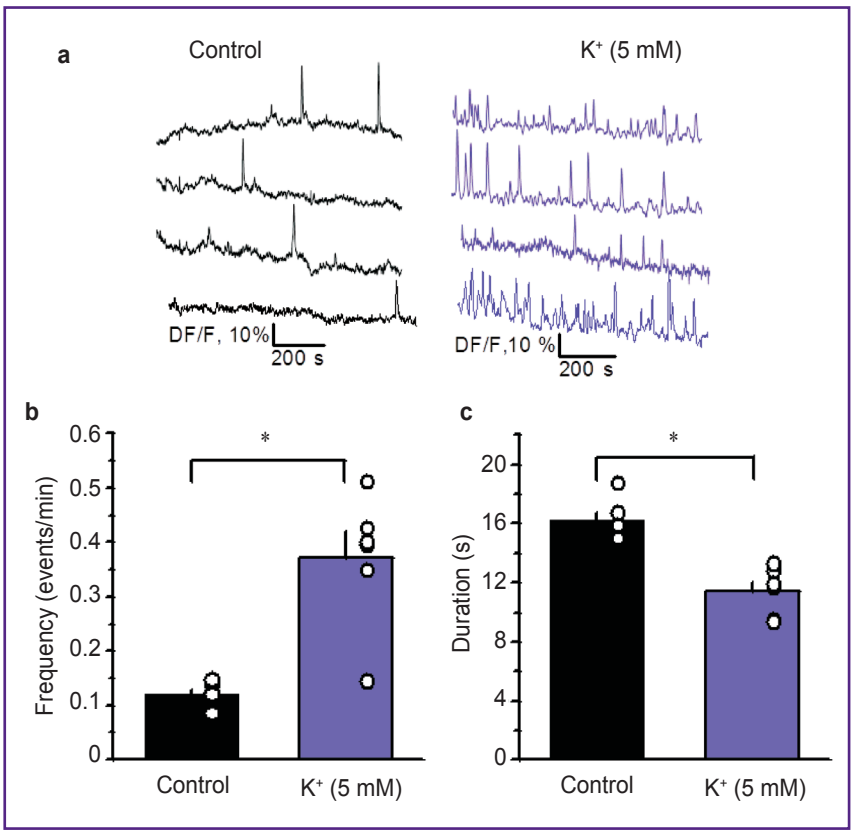

Figure 4. Calcium activity in astrocytes after elevation of extracellular potassium concentration. (a) The representative traces of calcium activity in control and after elevation of extracellular potassium concentration. (b) The average frequency of calcium events increased elevation of extracellular potassium concentration. (c) The duration of calcium events decreased after elevation of extracellular potassium concentration. Mann–Whitney test; * p<0.05

with 5 mM of calcium; n=6; Mann–Whitney test; p=0.9594) (Figure 3 (c)). Such an elevation of calcium extracellularly might affect the calcium influx via plasmatic membrane, causing an increase in calcium-induced calcium release from ER. The fact, that the duration of calcium events did not change, might support the idea that calcium itself, unlike IP3 does not cause an inactivation of IP3R [2].

Effect of elevation of extracellular concentration of potassium on frequency of astrocytic events. Astrocytic membrane potential is close to the reversal potential for potassium due to high permeability of astrocytes to these ions. When the neuronal network activity is high extracellular potassium concentration increases, that leads to depolarizing effect of potassium in astrocytes. That can cause an influx of potassium into the astrocytes [23], leading the depolarization of astrocytic membrane [28]. However, it is not clear if this depolarization might play a physiological role, and particularly in astrocytic calcium dynamic. To address this question, we compared calcium activity in astrocytes at 2.5 and 5 mM of extracellular potassium. This increase lead to 305±20% of control increase in frequency compare to normal potassium level (0.12±0.21 in control, 0.37±0.05 with 5 mM of potassium; n=6; Mann–Whitney test; p=0.0043) (Figure 4 (a), (b)). The duration of calcium events at 2.5 mM was 16.20±0.56 s and 11.38±0.67 s at 5 mM, showing a decrease 70±2% of control (n=6; Mann–Whitney test; p=0.0022) (Figure 4 (c)). Thus, increase of potassium as well as an escape of glutamate, both lead to an increase of calcium activity in astrocytes. However, they have different effect of duration of calcium events, which might be a compensatory mechanism during simultaneous action.

Discussion. In this work we studied the mechanisms of neuron-glia interaction. Traditionally, glutamate considered as a main mechanism of signal transmission. During high neuronal activity, the glutamate might escape the synaptic cleft and activate mGluRs on astrocytic membrane, triggering calcium activity in astrocytes [3, 4, 25]. However, the effect of changes in local ionic concentrations, occurring during neuronal activity, on neuron-glia interaction was not yet fully understood. Thus, in this study we investigated the effect of some of these changes on calcium activity in astrocytes. We demonstrated that elevated activation of mGluRs lead to an increase of frequency and duration of calcium events in astrocytes, which could be related to generation of IP3 and calcium efflux from ER. IP3 activates the receptors and prolongs relaxation time of calcium efflux, causing increase of events duration [2]. Elevation of calcium concentration in the extracellular space causes alterations in conductance of calcium channels in astrocytes, leading to larger calcium influx from extracellular space that acts on IP3R on ER and does not alter the duration of events. Thus, calcium itself does not affect the kinetics of activation/inactivation of IP3R. Accumulation of potassium in the extracellular space increases the frequency of calcium events in astrocytes, which can be related to the depolarizing effect of potassium entrance on astrocytic membrane, due to its reversal potential. Such accumulation of potassium can occur during repolarization phase of action potentials or during activation of neuronal NMDA and AMPA receptors [29]. That depolarization might affect activation of astrocytic voltage-dependent calcium channels, and calcium entered via these channels similarly might activate IP3R on ER and cause calcium-induced calcium release [8]. However, we observe the decrease of duration of calcium events, suggesting that high frequency might cause the depletion of intracellular calcium stores. These effects should be considered, since astrocytic calcium activity regulates the release of neuroactive substances and therefore neuronal activity in the brain [2, 8]. We showed the phenomenon of the influence of these factors of astrocytic calcium activity, however the mechanism are yet to be studied. Specifically, the results were obtained without blockade of neuronal activity and might be alternatively explain via secondary effect on astrocytes, while the main effect was produced on neuronal synaptic activity itself. However, we still consider that obtained results are the effect on astrocytes, because the application of the agonist of mGluRs increased the duration of events, when elevated calcium and potassium are not. Studying the potassium role as an agent of neuron-glia interaction might me crucial, because accumulation of potassium might occur not only in normal physiological [4], but also in pathophysiological conditions. For example, in conditions like stroke, migraine and epilepsy the potassium concentration elevates [2, 8], therefore understanding the potassium effects on astrocytic calcium activity can play an important role for developing new treatments of those conditions.

Conclusion. Neuronal activity causes local alterations in ionic concentrations, which can affect calcium signaling in astrocytes, creating an additional feedback for neuron-glia interaction in order to maintain normal neuronal functioning. These results point out a complex nature of neuron-glia interaction that cannot be considered exclusively in a frame of receptors interaction.

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Conflicts of Interest. The authors do not have any conflicts of interest.

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