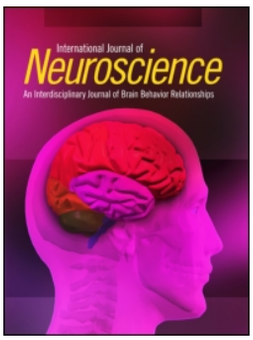

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Interleukin-10 restores glutamate receptor-mediated Ca^{2+}-signaling in brain circuits under loss of Sip1 transcription factor

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Abstract

The paper shows the connection between the mutation of the *Sip1* transcription factor and impaired Ca^{2+}-signaling, which reflects changes in neurotransmission in the cerebral cortex *in vitro*. Using fluorescence microscopy it was found that cortical neurons isolated from homozygous ($Sip1^{fl/fl}$) mice with the *Sip1* mutation demonstrate suppressed Ca^{2+} signals in a models of epileptiform activity *in vitro*. Wild-type cortical neurons are characterized by synchronous high-frequency and high-amplitude Ca^{2+} oscillations occurring in all neurons of the network in response to Mg^{2+}-free medium and bicuculline. But cortical $Sip1^{fl/fl}$ neurons only single Ca^{2+} pulses or attenuated Ca^{2+} oscillations are recorded and only in single neurons, while most of the cell network does not respond to these stimuli. This signal deficiency of $Sip1^{fl/fl}$ neurons correlates with a suppressed expression level of the genes encoding the subunits of NMDA, AMPA, and KA receptors; protein kinases PKA, JNK, CaMKII; and also the transcription factor Hif1α. These negative effects were partially abolished when $Sip1^{fl/fl}$ neurons are grown in media with anti-inflammatory cytokine IL-10. IL-10 increases the expression of the above-mentioned genes but not to the level of expression in wild-type. At the same time, the amplitudes of Ca^{2+} signals increase in response to the selective agonists of NMDA, AMPA and KA receptors, and the proportion of neurons responding with Ca^{2+} oscillations to a Mg^{2+}-free medium and bicuculline increases. Thus, IL-10 restores neurotransmission in neuronal networks with the *Sip1* mutation by regulating the expression of genes encoding signaling proteins.

Keywords: Sip1, neurons, epileptiform activity, glutamate receptors, protective genes, interleukin-10.

Introduction

Sip1 (Zeb2) is a transcription factor that is involved in the regulation of neurogenesis, and it mostly acts as a transcriptional repressor. Mutations of the *Sip1* gene in humans lead to the development of various pathologies, including Hirschsprung's disease and Mowat-Wilson syndrome [1, 2, 3]. The most common signs of Mowat-Wilson syndrome are pre- and postnatal microcephaly (identified in 97% of cases), epileptic seizures (82% of cases), Hirschsprung's disease (68%), congenital heart disease (47%) and agenesis of corpus callosum (29%). At the same time, mental retardation and craniofacial disorders are observed in 100% of cases of this syndrome [4].

One of the most common characteristics of Mowat-Wilson syndrome is epilepsy [4, 5]. The occurrence of epileptic seizures is often associated with an imbalance of excitation and inhibition in the cerebral cortex, hippocampus, striatum, and amygdala, as well as with impaired development of interneuron's. Van der Berghe and colleagues showed that *Sip1* is necessary for proper migration and maturation of GABAergic interneuron's [6].

Cortical neurons isolated from homozygous mice with the *Sip1* mutation have reduced sensitivity to AMPA and NMDA receptor activators *in vitro*, which may be considered as a sign of signaling insufficiency [7].

Mutations of the *Sip1* transcription factor in the cerebral cortex cause disturbances in the mechanisms of hypoxic preconditioning in neurons obtained from *Sip1* homozygous and heterozygous mice [8]. Short-term episodes of hypoxia, which may contribute to the formation of cell resistance to the damaging factors of hypoxia, on the contrary, lead to toxic effects in neurons with complete deletion of *Sip1* (homozygotes) [9]. One of the mechanisms responsible for hypoxic preconditioning, which is typical in wild-type and damaged by the Sip1 mutation, is the regulation of the ratio of subunits during the formation of the AMPA receptor tetramer and differences in the number and activity of GluA2 subunits. At the same time, there is a significant possibility to induce the phenomenon of hypoxic preconditioning with the *Sip1* mutation in neurons via the activation of an alternative signaling cascade with the participation of PI3K/Akt selective agonists or the anti-inflammatory cytokine IL-10. Long-term (up to 24 hours) exposure to IL-10 leads to the restoration of preconditioning in the cortical neurons, including increasing the expression of GluA2 subunits, which form AMPA receptors impermeable for Ca^{2+} ions [9].

Changes of $[Ca^{2+}]_i$ play a key role in the initiation and regulation of general and specialized cellular functions, such as proliferation, differentiation, secretion, contraction, as well as transmission of nerve impulses, immune response [10, 11]. Oscillatory Ca^{2+} activity in neuronal cultures correlates with the formation of new synapses and network development, i.e. maturation process [12]. It is known that during the first few days *in vitro* insignificant network activity is observed, but Ca^{2+} sporadic oscillations can be recorded in the process of cultivation. And at the age of 7–10 days *in vitro*, synchronous Ca^{2+} activity appears, which is observed in all neurons in the region of interest. In mature cultures both the density of synapses and the frequency of oscillatory activity increase [13]. Synchronization of neural networks recorded using electroencephalography represents the integral activity of neurons in one or several brain regions. It can ensure the normal functioning of the body, including cognitive functions and sleep, via the generation of both gamma rhythms and delta waves [14]. However, over-synchronization can also be a distinctive feature of epileptiform activity [15].

Thus, the close relationship of *Sip1* mutation with calcium signaling, excitatory receptor activity, and cell survival mechanisms can serve as an excellent model for studying the role of *Sip1* in synaptic transmission and developing methods for correcting *Sip1*-induced intracellular signaling disorders.

Materials and methods

Experimental protocols 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 on the protection of animals used for scientific purposes. Pregnant female mice 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.

Animals

We used *Sip1* mutants, obtained in Higashi laboratory [16]. Briefly, in these mice the seventh exon of *Sip1* is flanked by the loxP sites ($Sip1^{fl/fl}$) necessary for the Cre recombinase. Crossing $Sip1^{fl/fl}$ mice with NexCre mice (synthesize Cre recombinase only in post mitotic cells of neocortex), a conditioned mutant for the *Sip1* gene (knockout) is obtained. For genotyping of the mice we used

method and primers described early [9]. To obtain cortical cell cultures, we used 8 mice with the wild type genotype *Sip1*$^{wt/wt}$ and the homozygote genotype *Sip*$^{fl/fl}$.

Cell culture preparation

Mixed neuroglial cortical cell cultures were prepared as described previously [17, 18]. Briefly, the animals were euthanized and then decapitated. The extracted cortical tissue was washed in Mg^{2+}- and Ca^{2+}-free Versene medium and then minced with scissors. The tissue fragments were digested in 1% trypsin for 10 min at 37°C and washed twice with cold Neurobasal-A medium. The cell suspension was seeded on polyethyleneimine-coated glass coverslips and grown in Neurobasal-A medium supplemented with 2% B-27 and 0.5 mmol/L glutamine. Cell cultures at the age of 10 days in vitro were used for the experiments.

Fluorescence measurements

To detect $[Ca^{2+}]_i$ changes in neurons, the cells were loaded with a fluorescent ratiometric calcium-sensitive probe Fura-2 AM. To measure $[Ca^{2+}]_i$, we used Carl Zeiss Cell Observer on the basis of an inverted motorized microscope Axiovert 200M with a high-speed monochrome CCD-camera AxioCam HSm and a high-speed light filter replacing system, Ludl MAC5000. For Fura-2 excitation and registration, we used the 21HE filter set (Carl Zeiss, Germany) with excitation filters BP340/30 and BP387/15, beam splitter FT-409 and emission filter BP510/90, objective lens Plan-Neofluar 10x/0.3, excitation light source HBO 103 W/2. A series of images were obtained with an time-interval of 1 frame per 3 seconds. The recordings were performed in a custom-made imaging chamber in HEPES-buffered solution (HBSS), containing (in mM): 156 NaCl, 3 KCl, 1 $MgSO_4$, 1.25 KH_2PO_4, 2 $CaCl_2$, 10 glucose and 10 HEPES (pH 7.4) at ~30°C [19]. 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. For the identification of neurons a short-term (30 s) test additive of 35 mM KCl was provided. The KCl-induced depolarization promotes the opening of voltage-gated Ca^{2+} channels and induces rapid Ca^{2+}-responses exclusively in neurons. The conductivity and density of cation channels in astrocytes are insufficient to evoke a high-amplitude Ca^{2+}-response to KCl [20, 21, 22].

The obtained time series of two-channel images (at excitation light wavelengths of 340 and 380 nm) were processed in the ImageJ program with the plugin Time series analyzer. At the same time, is was measured the amplitude of single-cell calcium responses expressed as the ratio of Fura-2 fluorescence signals when excited at 340 and 380 nm, the amplitude and period of Ca^{2+} oscillations as well as the basic level of fluorescence which shows the cytosolic concentration of calcium ions. Results are presented as single cell signals or average cell signal in the 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. Analysis of variance was used to determine the significance of difference between groups of experiments (One-way ANOVA with post-hoc Tukey test).

Induction of epileptiform activity

Induction of epileptiform activity in the neuronal network we used two generally accepted models. In the Mg^{2+}-free model, Mg^{2+} ions were excluded from the HBSS medium [23] and added to the imaging chamber using a perfusion system. In the case of Mg^{2+} -free medium, $MgSO_4$ in HBSS was replaced by an osmotically equivalent concentration of Na_2SO_4. The second model for the induction of epileptiform activity was the application of GABA(A) receptor antagonist, bicuculline, 10 μM [24].

RNA extraction and Real-time polymerase chain reaction (RT-qPCR)

Total RNA isolation from the primary neuronal culture was performed with a Mag Jet RNA reagent kit in accordance with the manufacturer's instructions. RNA quality was assessed by electrophoresis in 2% agarose gel in TBE buffer in the presence of ethidium bromide (1 μg/ml). The RNA concentration was measured using a NanoDrop 1000c spectrophotometer (Wilmington, DE). The cDNA synthesis was performed using RevertAid H Minus First Strand according to the protocol specified by the manufacturer. The resulting single-stranded cDNA samples were used as a template for real-time PCR. qPCR was performed in 25 μl of a mixture containing: 5 μl of qPCRmix-HS SYBR), 1 μl (0.2 μmol) of each primer, 17 μl of water, 1 μl of cDNA [18]. The data were analyzed with DTlite software (DNA-technology, Moscow, Russia) and Origin 8.5 software (OriginLab Corporation, USA). It was conducted three independent qPCR experiments (three independent cell cultures of the cerebral cortex). In all experiments each cDNA sample was amplified in triplicate and average values were found. Primers specific for the studied genes were selected using the FAST PCR 5.4 programs and the NCBI Primer-BLAST system and synthesized by Evrogen (Moscow, Russia) (Supplementary 1, Table 1). The expression of the analyzed genes was normalized to the control Oaz1 gene encoding Ornithine decarboxylase enzyme (Oaz1). The results were calculated according to the standard method described by Livak [25].

Reagents

The following reagents were used in the study: bicuculline (Tocris, USA), Neurobasal-A medium, B-27 supplement, Trypsin (1%), glutamine (Gibco, USA), Fura-2AM (Invitrogen, USA), Mag Jet RNA reagent kit, RevertAid H Minus First Strand (Thermo Scientific, USA), qPCRmix-HS SYBR and primers (Evrogen, Russia).

Results

Ca^{2+} signals of cortical neurons in homozygous mice with a mutation of the Sip1 transcription factor in simulating epileptiform activity in vitro

The well-known classical methods of initiating epileptiform activity in vitro are the magnesium-free model, which is based on the exclusion of Mg^{2+} ions from the external environment [26, 27], and the bicuculline model - the blockade of the GABA(A)-receptors [28]. Replacing the medium with magnesium-free (Mg^{2+}-free) one causes the appearance of synchronous high-frequency Ca^{2+} oscillations (15 pulses per minute) with an increase in the basal level of $[Ca^{2+}]_i$ in neurons (Fig. 1A) in cell cultures obtained from wild-type mice. Inhibition of GABA(A) receptors of wild-type (WT) cortical neurons with the application of 10 μM bicuculline also leads to synchronous Ca^{2+} oscillations of a lower frequency (3 pulses per minute) without changing the basal level of $[Ca^{2+}]_i$ (Fig. 1B) in comparison with the magnesium-free model of epileptogenesis. In neurons obtained from the cerebral cortex of homozygous mice with the *Sip1* transcription factor mutation (*Sip1*$^{fl/fl}$) replacing the medium with a magnesium-free one initiates single Ca^{2+} oscillations consisting of 2–3 pulses also accompanied by an increase in the basal level of $[Ca^{2+}]_i$ (Fig. 1C). An increase in the incubation time of these cells in an Mg^{2+}-free medium for up to 25 minutes does not lead to the emergence of stable synchronous Ca^{2+} oscillations, but it shows a trend of a slow increase in the basal level of $[Ca^{2+}]_i$. At the same time, the addition of bicuculline to *Sip1*$^{fl/fl}$ deficient cortical neurons causes the appearance of nonsynchronous Ca^{2+} oscillations with an average frequency of 4 impulses per minute occurring at an increased basal level of $[Ca^{2+}]_i$ but quickly attenuated in most cells within 5–6 minutes (Fig. 1D). Also, it is worth noting that no more than 8% and 12% of the total number of *Sip1*$^{fl/fl}$ neurons in the network react to the application of the Mg^{2+}-free medium and bicuculline respectively, while in the case of wild type neurons the percentage of cells with synchronous Ca^{2+} oscillations is at the level 50–60%.

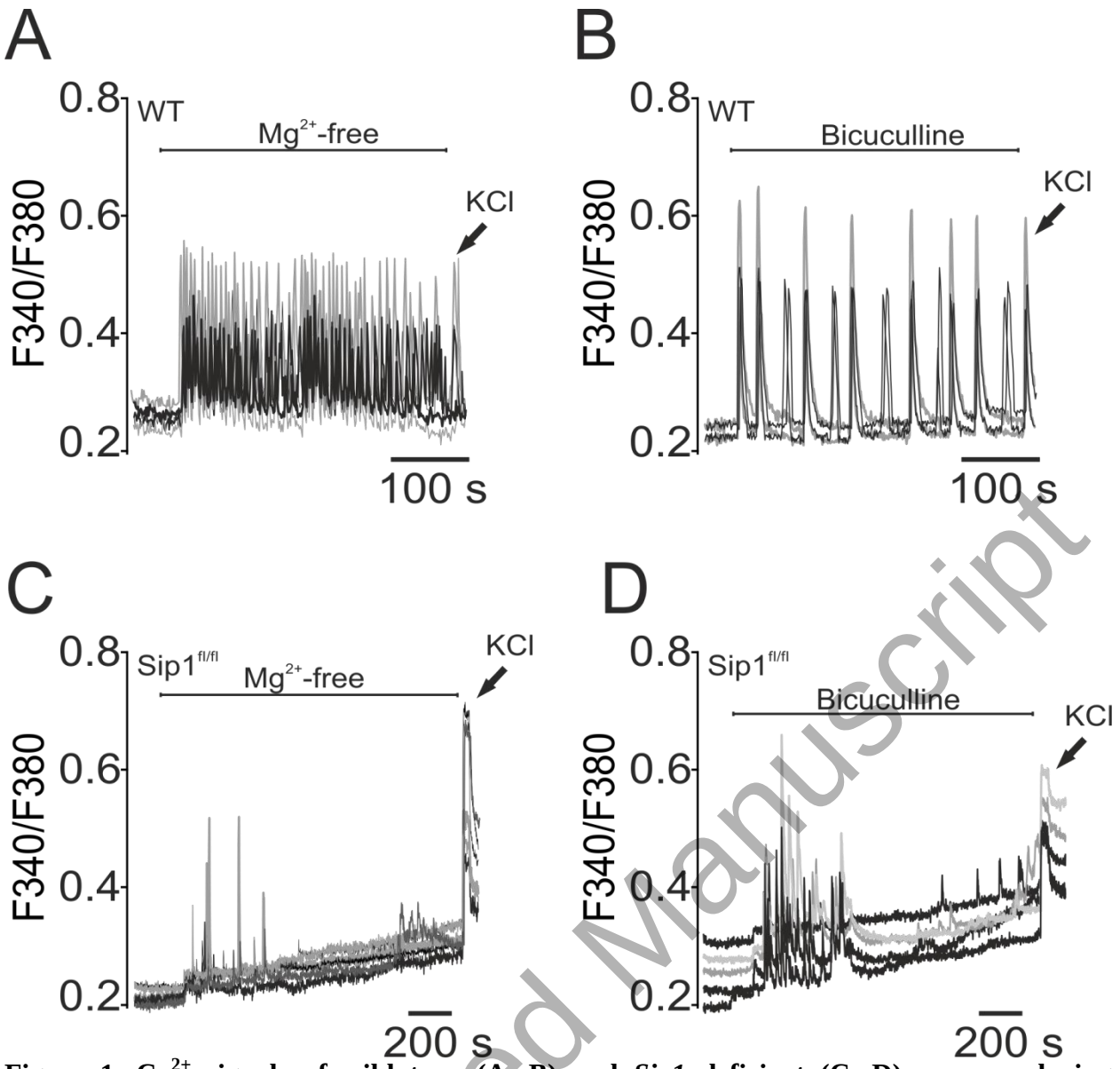

Figure 1. Ca^{2+} signals of wild type (A, B) and *Sip1* deficient (C, D) neurons during simulating of epileptiform activity.

A, C – Magnesium free model of epileptiform activity creating by wild type (A) and *Sip1* deficient (C) cortical neurons.

B, D – Initiation of epileptiform activity of wild type (B, ST) and *Sip1* deficient (D, $Sip1^{fl/fl}$) cortical neurons by inhibiting GABA(A) receptors with bicuculline (10 μM bicuculline).

The typical Ca^{2+}-signals of neurons are presented in figures. For $Sip1^{fl/fl}$ neurons it was performed short-term application of 35 mM KCl for identification of activity-dependent Ca^{2+} channels.

Thus, *Sip1* deficient cortical neurons showed decreased network activity compared to wild type cortical neurons in models of acute epileptiform activity. It manifested in the absence of stable synchronous Ca^{2+} oscillations as well as single Ca^{2+} pulses in most cells of neuronal networks . Such behavior of networks with the *Sip1* mutation may indicate a disruption of neurotransmission and Ca^{2+} signaling in the cortical neurons.

Activity of ionotropic glutamate receptors in the Sip1 deficient cortical neurons. A compensatory role of anti-inflammatory cytokine interleukin-10

We conducted experiments in which *Sip1* deficient cortical neurons were divided into 2 groups and were grown from day 1 under different conditions: the cells in the first group – under standard conditions, and the cells in the second group – in the presence of 1 nM interleukin-10 (IL-10). On the 10th day of cultivation a part of cells from each group was used to perform experiments with recording the $[Ca^{2+}]_i$ dynamics (Fig. 2 A – C) and the second part of the cells was used for total RNA extraction and qPCR analysis (Fig. 2 D – E). NMDA receptor agonist N-methyl-D-aspartate (NMDA) in a Mg^{2+}-free medium causes in the dose-dependent manner the generation of Ca^{2+} signals in $Sip1^{fl/fl}$ neurons grown under standard conditions (control, $Sip1^{fl/fl,}$ black curve) and in the presence of 1 nM interleukin-10 ($Sip1^{fl/fl}$ + IL-10, light curve) (Fig. 2a).: Interestingly, we observed unusual pattern of the dose-dependence in our experiments. Neurons grown under control conditions begin to respond to the application of NMDA at a concentration of 1 μM (Fig. 2A, black line, indicated by 4) and the average amplitude of Ca^{2+} signals increases insignificantly with increasing of the agonist concentration. However, it has been reported that the dependence of amplitude from the concentration is linear [28]. The group of neurons grown in media with IL-10 addition is characterized by higher amplitudes of Ca^{2+} signals on average and these neurons begin to respond by increasing $[Ca^{2+}]_i$ already with NMDA concentration of 0.5 μM (Fig. 2A, red line, indicated by 3).

The second excitatory ionotropic glutamate receptor is the AMPA receptor which activity in $Sip1^{fl/fl}$ neurons grown with the addition of IL-10 also significantly increases compared to $Sip1^{fl/fl}$ neurons grown under standard conditions. This effect can be judged by an increase in amplitude Ca^{2+} responses to the application of increasing concentrations of the selective agonist fluorowillardiine, added in the presence of 5 μM AMPA receptor desensitization inhibitor cyclothiazide (Fig. 3B, light curve, $Sip1^{fl/fl}$ + IL-10). In addition, Ca^{2+} signals in response to the agonist in neurons grown in the presence of IL-10 appear at fluorowillardiine concentrations of 0.01–0.03 μM (Fig. 2B, red line, indicated by numbers 2 and 3) while neurons grown in standard medium begin to respond with an increase in $[Ca^{2+}]_i$ only at 0.1 μM (Fig. 2B, black line, indicated by 4).

IL-10 act to the neuronal kainate receptors in a similar way (KAR, Fig. 2C) because the amplitudes of Ca^{2+} signals in response to domoic acid (KAR agonist) together with 30 μM of the AMPA receptor antagonist GYKI-52466 and the desensitization inhibitor KAR 200 μg/ml concanavalin A higher in the $Sip1^{fl/fl}$ + IL-10 group (Fig. 2C, light curve). At the same time, the amplitude differences in both groups are less pronounced for this receptor compared with the NMDA and AMPA receptors.

Chronic actions of active compounds recorded by changes in cell activity [29] are undoubtedly associated with changes in the level of gene expression. A comparative analysis of PCR data showed that the reduced Ca^{2+} signals of $Sip1^{fl/fl}$ neurons grown under standard conditions to activation of NMDA, AMPA and kainate receptors by selective agonists and coincide with the suppressed expression level of the genes encoding the subunits forming these receptors (Fig. 2D, $Sip1^{fl/fl}$) in comparison with wild-type cortical neurons (Fig. 2D, WT). The expression levels of Grin2a and Grin2b genes encoding the NR2A and NR2B subunits of the NMDA receptors, Gria1 and Gria2, encoding the GluA1 and GluA2 subunits of the AMPA receptors are reduced by more than 3–4.5 and 2.5–3 times respectively (Fig. 2D). As for kainate receptors, it was shown a 4-fold decrease in expression of Grik2 gene which encodes the kainate type subunit 2 KAR while the expression level of Grik1 (glutamate ionotropic receptor kainate type subunit 1) does not change during Sip1 mutation (Fig. 2D). At the same time, in cells grown in the presence of 1 nM IL-10 the expression of the Grin2a and Grin2b genes increases by 42.6% (Fig. 2D, $Sip1^{fl/fl}$ + IL-10 group). The expression level of the Gria1 gene does not change in the $Sip1^{fl/fl}$ + IL-10 group but the expression of Gria2 increases by 36% in comparison with cells of Sip1 homozygotes grown under standard conditions. As for the kainate receptor subunits in the $Sip1^{fl/fl}$ + IL-10 group a significant increase in their expression occurs due to the Grik2 gene whereas the Grik1 level does not change (Fig. 2D).

Protein kinase C (PKC) is one of the main kinases associated with the regulation of the activity of ionotropic glutamate receptors and is involved both in the processes of normal neuroplasticity and in the formation of pathologies [20, 30]. Expression of the genes encoding the PKC subunits is suppressed by mutation of the transcription factor *Sip1* at 4 or more times rather than in the wild type cortical cells (Fig. 2E). At the same time, interleukin-10 does not significantly affect the expression level of these three subunits (Fig. 2E, $Sip1^{fl/fl}$ + IL-10 group). IL-10 realizes its protective action via phosphorylation of phosphoinositide-3-kinase (PI3k) – the main kinase of IL-10R downstream signaling pathway. In Sip1 deficient neurons there is no significant change in the expression of the genes encoding the PI3K subunits (Pik3ca, Pik3cb, Pik3cg), i.e. this signaling cascade is not disturbed with a complete deletion of this transcription factor in cortical neurons (Fig. 2F, group $Sip1^{fl/fl}$). However, in cells that were grown in the presence of IL-10 the expression of the Pik3cb gene encoding the catalytic β-subunit of PI3K decreases and the expression level of the Pik3cg gene which encodes the catalytic γ-subunit increases by a factor of 7 (Fig. 2F, $Sip1^{fl/fl}$ + IL-10 group).

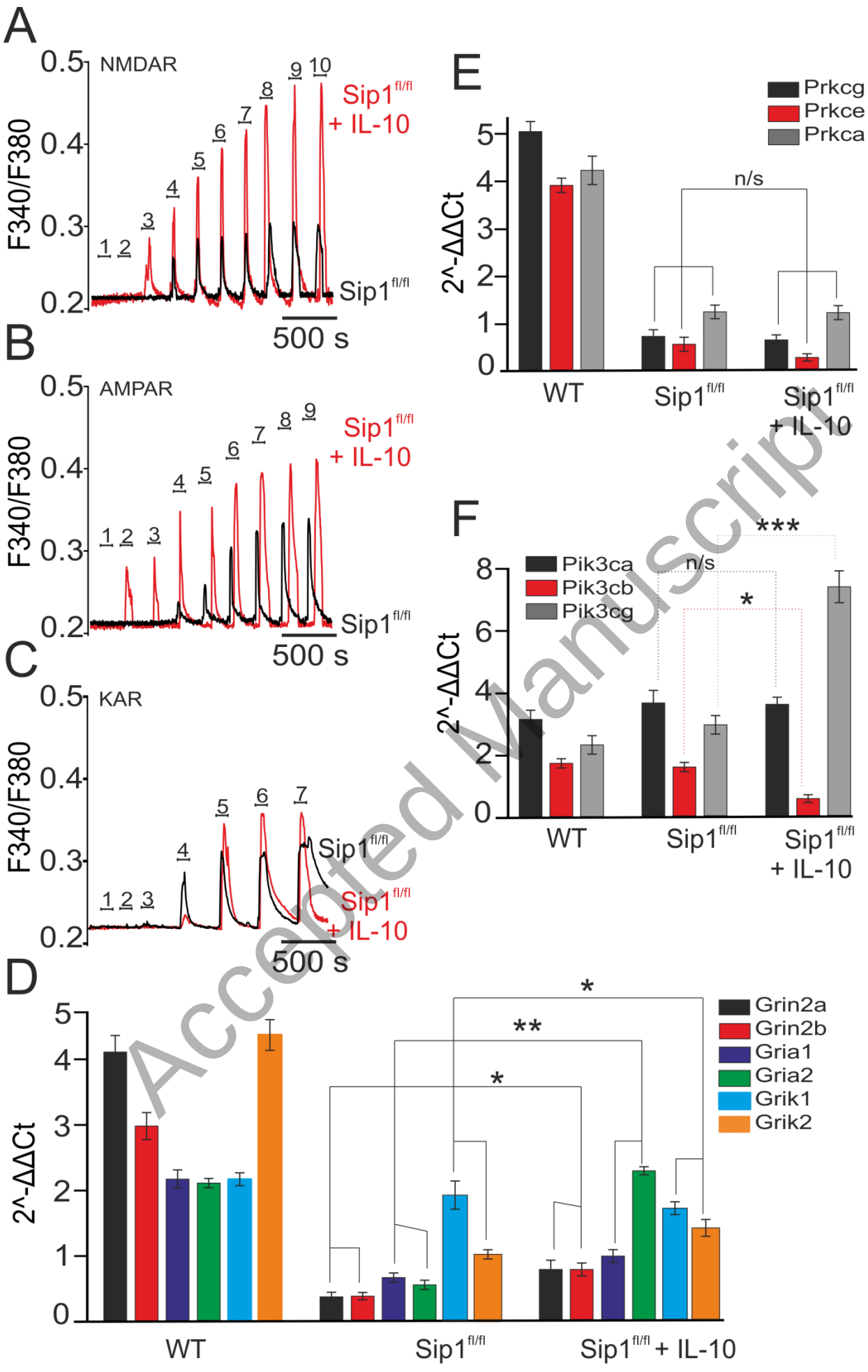

Figure 2. Interleukin-10 restores the activity of excitatory ionotropic glutamate receptors in cortical neurons of *Sip1*$^{fl/fl}$ mice via the regulation of the expression of genes encoding the receptors subunits and kinases.

A – Ca^{2+} responses of *Sip1*$^{fl/fl}$ cortical neurons grown under standard conditions (*Sip1*$^{fl/fl}$) and in the presence of 1 nM interleukin-10 (*Sip1*$^{fl/fl}$ + IL-10) to sequential addition of increasing concentrations of the NMDA-receptor agonist - NMDA in a Mg^{2+}-free medium. Legend: 1 – 0.1 μM, 2 – 0.3 μM, 3 – 0.5 μM, 4 – 1 μM, 5 – 3 μM, 6 – 5 μM, 7 – 10 μM, 8 – 30 μM, 9 – 50 μM, 10 – 100 μM.

B – Ca^{2+} responses of *Sip1*$^{fl/fl}$ cortical neurons grown under standard conditions (*Sip1*$^{fl/fl}$) and in the presence of 1 nM interleukin-10 (*Sip1*$^{fl/fl}$ + IL-10) to sequential addition of increasing concentrations of AMPA-receptor agonist – Fluorowillardiine together with 5 μM desensitization inhibitor of AMPA receptors – cyclothiazide. Legend: 1 – 0.01 μM, 2 – 0.03 μM, 3 – 0.05 μM, 4 – 0.1 μM, 5 – 0.5 μM, 6 – 1 μM, 7 – 3 μM, 8 – 5 μM, 9 – 10 μM.

C – Ca^{2+} responses of *Sip1*$^{fl/fl}$ cortical neurons grown under standard conditions (*Sip1*$^{fl/fl}$) and in the presence of 1 nM interleukin-10 (*Sip1*$^{fl/fl}$ + IL-10) to the sequential addition of increasing concentrations of the kainate-receptor (KAR) agonist – domoic acid together with 30 μM AMPA-receptor antagonist – GYKI-52466 and desensitization inhibitor KAR – 200 μg/ml concanavalin A. Legend: 1 – 0.01 μM, 2 – 0.05 μM, 3 – 0.1 μM, 4 – 0.3 μM, 5 – 0.5 μM, 6 – 1 μM, 7 – 5 μM

D – Expression of genes encoding NMDA-receptor subunits (Grin2a, Grin2b), AMPA-receptor (Gria1, Gria2) and kainate receptor (Grik1, Grik2) in the wild type cortical cells (WT), homozygotes with Sip1 mutation (*Sip1*$^{fl/fl}$) grown under standard conditions and homozygotes with the Sip1 mutation grown in the presence of 1 nM interleukin-10 (*Sip1*$^{fl/fl}$ + IL-10).

E – Expression of genes encoding protein kinase C, PKC (Prkca, Prkce, Prkcg) subunits in the wild type cortical cells (WT), homozygotes with Sip1 mutation (*Sip1*$^{fl/fl}$) grown under standard conditions and homozygotes with mutation Sip1 grown in the presence of 1 nM interleukin-10 (*Sip1*$^{fl/fl}$ + IL-10).

F – Expression of genes encoding phosphoinositide-3-kinase subunits, PI3K (Pik3ca, Pik3cb, Pik3cg) in the wild type cortical cells (WT), homozygotes with Sip1 mutation (*Sip1*$^{fl/fl}$) grown in standard conditions and homozygotes with the Sip1 mutation grown in the presence of 1 nM interleukin-10 (*Sip1*$^{fl/fl}$ + IL-10).

For panels A – C, Ca^{2+} responses averaged over several hundreds of neurons are presented. The level of gene expression presented on panels D – E corresponds to the calcium neuroimaging experiments presented on panels A – C since the one part of the cell culture was used for experiments using neuroimaging and the other part was used to total RNA isolation. The data were significant: *** – $p \leq 0.001$; ** – $p < 0.01$; * – $p < 0.05$; n/s – $p > 0.05$.

Therefore, mutation of the transcription factor *Sip1* in the cortical neurons leads to suppression of the activity of NMDA and AMPA receptors and decrease the expression of multiple genes encoding a subunits of these receptors as well as the genes encoding the PKC. At the same time, there are no changes of expression of one of the leading neuroprotective kinases – PI3K. The presence of IL-10 in the medium during first 10 days of cultivation of the *Sip1* deficient cortical cells leads to a significant increase of Ca^{2+} responses amplitudes of neurons when NMDA and AMPA receptors are activated by selective agonists. These effects are associated primarily with the regulation of interleukin-10 gene expression (upward) that encodes the subunits that form these receptors. Despite this, the level of expression of these genes in the presence of IL-10 does not match

the values for wild-type cells. The effect of IL-10 on the expression level of the subunits of protein kinase C is absent but there is an increase in the expression of the γ-subunit (catalytic) PI3K.

Interleukin-10 restores the oscillatory activity of Sip1$^{fl/fl}$ neurons.

In *Sip1*$^{fl/fl}$ neurons grown in the presence of interleukin-10 on day 10 of cultivation, spontaneous Ca^{2+} oscillations appear (Fig. 3A, black curve; Fig. 3B, light curve) which is at the same time a sign and a necessary condition for the development of the network. The application of Mg^{2+}-free medium to cells from the *Sip1*$^{fl/fl}$ + IL-10 group leads to the generation of Ca^{2+} oscillations with high amplitude and frequency (3 pulses per minute) which occur without an increase in the base value of $[Ca^{2+}]_i$ (Fig. 3A). At the same time, these oscillations spontaneously attenuate after an average of 7.3 minutes. They are not restored afterward which may be due to the activity of the desensitization mechanisms of receptors and negative feedbacks. Moreover, inhibition of GABA receptors with bicuculline leads to the generation of Ca^{2+} oscillations with high amplitude and frequency (4 pulses per minute on average) after a 5-minute lag-period (Fig. 3B). These oscillations do not cease during the experiment, and in some neurons, there is a trend toward a gradual increase in the basal $[Ca^{2+}]_i$.

The level of kinases responsible for the survival of brain cells and network development is reduced in the cortex of homozygous mice with the Sip1 mutation (Fig. 3C). The expression level of the gene encoding CaMKII is reduced by 3 or more times, JNK1 by 2.3 times, and gene expression of hypoxia-inducible factor-1 (Hif1) is reduced almost by 5 times (Fig. 3C). At the same time, the basal expression of the gene encoding caspase-3 (Casp3) is maintained at almost the same level in the cortical cells of wild type (WT) and homozygotes with the *Sip1* mutation (Fig. 3C, *Sip1*$^{fl/fl}$). The addition of IL-10 to the culture medium does not cause a significant change in the expression level of the genes Camk2a and JNK1, whereas the expression level of Hif1 increases by 2.2 times compared with the *Sip1*$^{fl/fl}$ group. The expression of Casp3, on the contrary, decreases by 23% (Fig. 3C, *Sip1*$^{fl/fl}$ + IL-10 group).

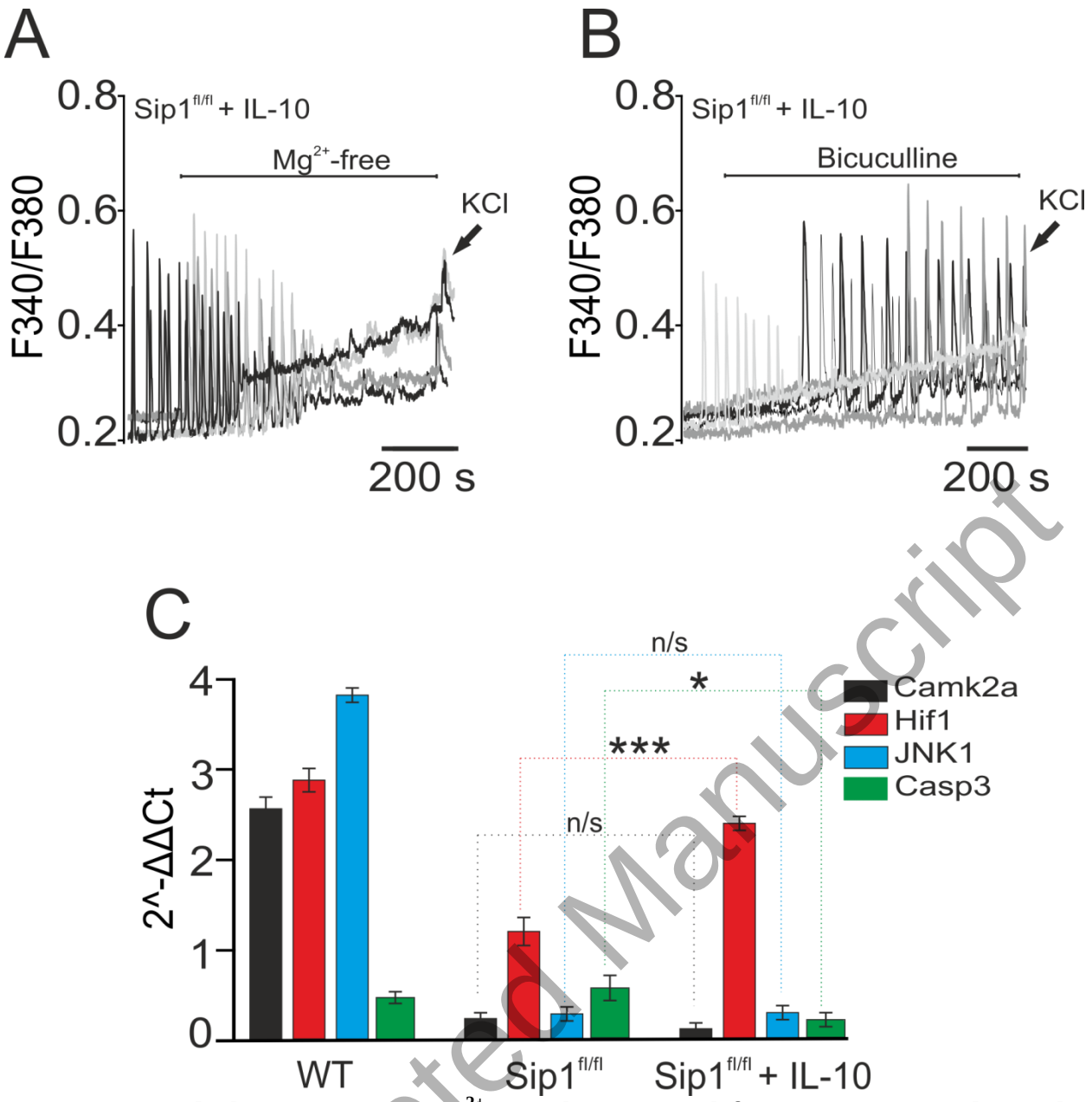

Figure 3. Interleukin-10 restores Ca^{2+} signals in *Sip1* deficient neurons. Role in the expression of genes encoding signal kinases.

A – Magnesium free medium (Mg^{2+} -free) causes Ca^{2+} oscillations in *Sip1*$^{fl/fl}$ cortical neurons grown in the presence of 1 nM interleukin-10.

B – Inhibition of GABA(A) receptors with 1 μM bicuculline causes the generation of Ca^{2+} oscillations in *Sip1*$^{fl/fl}$ cortical neurons grown in the presence of 1 nM interleukin-10.

C – The level of gene expression in the wild type cortical cells (WT), homozygotes with the *Sip1* mutation (*Sip1*$^{fl/fl}$) grown under standard conditions and homozygotes with the *Sip1* mutation grown in the presence of 1 nM interleukin-10 (*Sip1*$^{fl/fl}$ + IL-10). Legend: Camk2a is the catalytic subunit of Ca^{2+}/calmodulin-dependent protein kinase II (Camk2a); Hif1 is a hypoxia-inducible factor 1; JNK1 – c-Jun N-terminal kinase; Casp3 – caspase 3. The data were significant: *** – $p \leq 0.001$;* – $p < 0.05$; n/s – $p > 0.05$.

Ca^{2+} signals of single neurons are presented in panels A, B. It can be seen that spontaneous Ca^{2+} activity is observed in some neurons, showing the normal synaptic activity of the developed

network. The level of gene expression presented on panel C correlates with changes in the amplitudes of the Ca^{2+} signals presented on panels A, B, since the one part culture dishes from a passage was used in imaging experiments and the other part was used in RT-PCR.

Thus, IL-10-induced increase in the expression level of genes encoding excitatory glutamate receptor subunits which coincides with an increase in Ca^{2+} responses to activation by selective agonists (Fig. 2). This effect is also accompanied by the appearance of Ca^{2+} oscillations during inhibition of GABA receptors and removal of Mg^{2+}-block from NMDA-receptors which in the case of *Sip1* mutation can be assessed as a positive effect aimed at the neurotransmission restoring in the network. At the same time, the expression of genes encoding PI3K and Hif-1 increases after incubation with IL-10. In turn, the expression of the gene encoding pro-apoptotic caspase-3 decreases, which can help to maintain normal network functioning under pathological conditions.

Discussion

Cytosolic calcium oscillations are observed in cells of various brain regions – sympathetic, hypothalamic and suprachiasmatic nuclei, hippocampus and cerebral cortex [30–34]. Ca^{2+}-oscillations are also shown for other cell types [35, 36], but brain cells are characterized by a high degree of oscillations synchronization [37]. Ca^{2+} oscillations consisting of pulses of different frequencies and amplitudes in neuronal networks are necessary, on the one hand, for recording and processing information, differentiating neurons and the network, but on the other hand, they may be signs of epileptiform activity resulting in neuronal damage [38, 39, 40, 41, 42]. As early as 1987, it was discovered for the first time that in the culture of hippocampal neurons, the exclusion of Mg^{2+} ions from the extracellular medium causes repetitive synchronous calcium impulses. These Ca^{2+} oscillations were suppressed using NMDA receptor antagonist, which indicates the leading role of the NMDA receptor cation channels in synchronization of oscillations [43].

In our experiments with cell cultures of cerebral cortices (10 DIV) from the wild type mices we also observed synchronous oscillations with a high-frequency Ca^{2+} activity caused by the Mg^{2+}-free medium and bicuculline (Fig. 1A, B). This fact may indicate a high degree of neuronal differentiation and the development of the network. However, in the cells isolated from homozygous mice with the mutation of the *Sip1* transcription factor there is not observed such network behavior (Fig. 1C, D) which may indicate a disruption of the mechanisms of neuronal differentiation and maturation with the *Sip1* mutation. Previously using *in vitro* and *in vivo* models *Sip1* deletion was shown to damage the Wnt signaling pathway and disrupt these mechanisms [44]. In the hippocampus *Sip1* acts as a positive regulator of the non-canonical pathway of Wnt signaling. In particular, it blocks the expression of the Sfrp1 gene, which controls the activity of the Wnt signaling pathway. There are two known variants of non-canonical Wnt signaling – the first one via JNK kinase (c-Jun N-terminal kinase) and the second one via CaMKIIa (calcium-dependent calmodulin kinase II). Thus, the absence of *Sip1* increases the expression of Sfrp1 and inhibits the activity of JNK, which in both cases inhibits Wnt signaling [45]. In the calcium-dependent non-canonical Wnt signaling pathway, Wnt increases the level of extracellular $Ca^{2+,}$ which in turn leads to the activation of calcium-sensitive kinases, such as protein kinase C (PKC) and CaMKII [46, 47]. This correlates with our data showing that JNK, Camk2a, and PKC expression is significantly reduced in *Sip1* knockout neurons. According to the latest data, the disruption of Wnt signaling in neurons may cause epileptic seizures and the formation of epilepsy [48, 49], which correlates with the presence of epileptiform activity in *Sip1* mutant neurons owing to Wnt signaling inhibition.

There is a well-known relationship between Wnt signaling and regulation of excitatory neurotransmission. Wnt3a regulates the recirculation of synaptic vesicles in the hippocampus [50, 51] and may cause an increase in the frequency of mEPSC [52]. Inhibition of Wnt signaling disrupts long-term potentiation processes in hippocampal slices [53]. The addition of Wnt5a ligands enhances

the currents through the NMDA receptor channel and contributes to the formation of long-term potentiation [54, 55].

We have previously shown that Ca^{2+} responses of cortical neurons obtained from homozygous mice with the Sip1 mutation are characterized by reduced amplitude under the application of selective agonists NMDA, AMPA and to lesser extent kainate receptors [7]. Also, these neurons are characterized by the absence of such a neuroplastic phenomenon as a hypoxic preconditioning [8]. The recovery of this phenomenon occurred after 24-hours incubation of cell cultures with interleukin-10 due to changes in the functional expression of the subunits forming AMPA receptors [9]. Anti-inflammatory cytokine IL-10 protects brain cells from the damaging factors of hypoxia and ischemia [56]. However, it can also restore the damaged mechanisms of hypoxic preconditioning of homozygous mice with *Sip1* mutation in cortical neurons [9]. It is known that IL-10 regulates the expression of the GluA1 and GluA2 subunits of AMPA receptors *in vivo* [57]. Moreover, it was recently found that Wnt5a activates the expression and increases the level of IL-10 in dendritic cells [58]. The disruption of Wnt-signaling in *Sip1* deficient mice can lead to a decrease in the level of anti-inflammatory cytokines in the cerebral cortex.

Conclusion

Thus, our results show that neurons derived from *Sip1* deficient mice are characterized by impaired Ca^{2+} signaling in acute models of epileptiform activity *in vitro* due to a decrease in expression of subunits of NMDARs, AMPARs, and KARs. At the same time, the anti-inflammatory cytokine interleukin-10 is able to increase the expression level of the genes encoding the subunits of these receptors and partially restore the Ca^{2+} oscillations in response to the application of Mg^{2+}-free medium and bicuculline. Disruption of the transcription factor *Sip1* leads to disturbance of the neuronal Ca^{2+} signals in the cerebral cortex in response to the activation of the ionotropic glutamate receptors. Thus, we have shown the effect of *Sip1* deficiency on one of the central components of neurotransmission. A partial recovery of Ca^{2+} signaling by the anti-inflammatory cytokine shows the possibility of treating the symptoms of genetic disorders in the brain, albeit at the in vitro level.

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