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Mutation in the Sip1 transcription factor leads to a disturbance of the preconditioning of AMPA receptors by episodes of hypoxia in neurons of the cerebral cortex due to changes in their activity and subunit composition. The protective effects of interleukin-10

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ABSTRACT

The *Sip1* mutation plays the main role in pathogenesis of the Mowat-Wilson syndrome, which is characterized by the pronounced epileptic symptoms. Cortical neurons of homozygous mice with *Sip1* mutation are resistant to AMPA receptor activators. Disturbances of the excitatory signaling components are also observed on such a phenomenon of neuroplasticity as hypoxic preconditioning. In this work, the mechanisms of loss of the AMPA receptor's ability to precondition by episodes of short-term hypoxia were investigated on cortical neurons derived from the *Sip1* homozygous mice. The preconditioning effect was estimated by the level of suppression of the AMPA receptors activity with hypoxia episodes. Using fluorescence microscopy, we have shown that cortical neurons from the $Sip1^{fl/fl}$ mice are characterized by the absence of hypoxic preconditioning effect, whereas the amplitude of Ca^{2+}-responses to the application of the AMPA receptor agonist, 5-Fluorowillardiine, in neurons from the Sip1 mice brainstem is suppressed by brief episodes of hypoxia. The mechanism responsible for this process is hypoxia-induced desensitization of the AMPA receptors, which is absent in the cortex neurons possessing the Sip1 mutation. However, the appearance of preconditioning in these neurons can be induced by phosphoinositide-3-kinase activation with a selective activator or an anti-inflammatory cytokine interleukin-10.

1. Introduction

The transcription factor *Sip1* (Smad-interacting protein-1, Zinc finger homeobox (Zfhx1b), Zeb2) plays a leading role in pathogenesis of the Mowat-Wilson syndrome (MWS), a rare genetic autosomal dominant disease that affects one in every 50000–100000 newborns worldwide. This syndrome is characterized by the presence of mental retardation, short stature, specific facial features, as well as anomalies in the development of cardiovascular, renal and other systems. Deletion of *Sip1* disturbs the brain cells proliferation and differentiation that can lead to epilepsy and cognitive disorders [24] [35]; [1]. The *Sip1* mutation causes significant changes in the glutamatergic component of calcium signaling of the cortical neurons. In this case, sensitivity of homozygous mice to selective agonists of the NMDA- and AMPA-receptors was decreased, while sensitivity of heterozygous mice to these activators was increased [50]. It is known that AMPA receptors are more involved in the toxic effect of hypoxia, under certain conditions [36]. The change in the sensitivity of these receptors to glutamate may be critical in hyperexcitation occurring during hypoxia, ischemia, and some neurodegenerative diseases.

AMPA receptors (AMPARs) are ionotropic glutamate receptors which have a poor conductivity for Ca^{2+}, functioning to depolarize cells through an increase of pNa^{+}. Normally, AMPAR is almost impermeable to Ca^{2+} ions due to the presence of the GluA2 subunit. However, under certain conditions, for example, ischemia, there is an increase in expression of the GluA2-deficient (Ca^{2+}-permeable) AMPARs on postsynaptic membranes, causing increased Ca^{2+} permeability [18].

Even short-term cerebral ischemia can have a significant effect on the activity of AMPA receptors, reducing expression of the GluA2 subunit in the CA1 region of the hippocampus, and thus increasing the conductivity of AMPA receptors for Ca^{2+}, which can lead to the post-

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ischemic cell death [27]; [32]. However, there is evidence that a decrease of the GluA2 subunits expression is reversed, due to the formation of neuronal resistance to hypoxic conditions [17]. Changes in the expression or post-transcriptional splicing of the GluA2 subunits are also observed in the Alzheimer's disease and amyotrophic lateral sclerosis, when most neurons become very sensitive to the AMPA/kainate receptor-mediated injury [2,28].

The existing information on the effect of hypoxia, ischemia and excitotoxicity on functional expression of the AMPA receptors is largely contradictory. Taking into account close interconnection of genetic mutations, neurodegenerative diseases, hypoxic conditions, and glutamate component of the calcium signaling, a study of the endogenous neuronal protective mechanisms is of great interest.

2. Materials and methods

2.1. Animals

We used *Sip1* mutants, obtained in Higashi laboratory [12]. In mice of this line, the seventh exon of *Sip1* is flanked by the loxP sites ($Sip1^{fl/fl}$) necessary for the Cre recombinase. When crossing $Sip1^{fl/fl}$ mice with NexCre mice [9], which synthesize Cre recombinase only in post mitotic cells of their neocortex, a conditioned mutant for the *Sip1* gene (knockout) is obtained. The simultaneous presence of the *Sip1* gene with the loxP sites and Cre recombinase in post mitotic cells of the cerebral cortex leads to a partial deletion of *Sip1* and, as a consequence, translation of damaged protein product of *Sip1* with loss of its function. Mice were kept in SPF cages 40 × 25 × 15 cm under standard laboratory conditions: a 14 h light circuit, 22 °C. Animals had free access to food and water.

For genotyping, the tail cuts were dissolved in 0.3 ml of lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, 100 μg/ml proteinase K) at 55 °C for 2–10 h. The non-lysed tissue was removed by centrifuging the samples for 10 min at 10.000 rpm. The DNA was precipitated by adding an equal volume of isopropanol, then mixed and centrifuged (15 min, 13.000 rpm). The precipitated DNA was washed twice in 80% ethanol, air dried, and dissolved in 50 μl of sterile distilled water. All PCR reactions were carried out in a volume of 20 μl.

The following primers were used to determine the amplified product. Sip1-floxed allele and wild-type allele: 5′-TGGACAGGAACTTGC ATATGCT-3'; 5′-GTGGACTCTACATTCTAGATGC-3 '. Amplification program was as follows: 95 °C, 10 s; 59 °C, 20 s; 72 °C, 40 s; 35 cycles. The wild-type allele product is ~450 bp, Sip1-floxed ~600 bp. Primers for amplification of the NexCre allele and the wild type allele: 5′-CCG CATAACCAGTGAAACAG-3'; 5′-GAGTCCTGGAATCAGTCTTTTCT-3'; 5′-AGAATGTGGAGTAGGGTGAC-3'. Amplification program: 95 °C, 20 s; 54 °C, 30 s; 72 °C, 60 s; 35 cycles. The product of the wild-type allele is ~750 bp, the NexCre allele is ~500 bp.

Fourteen mice with the $Sip1^{fl/fl}$ genotype were used to obtain cell cultures.

2.2. Cell culture preparation

The mouse cerebellar cortex or brainstem was excised with clippers, put in a test-tube, incubated for 2 min and the supernatant was removed with a pipette. The cells were then covered with 2 ml trypsin (0.1% in Ca^{2+}- and Mg^{2+}-free Versene solution) and incubated for 15 min at 37 °C under constant shaking at 600 rpm. Trypsin was then inactivated by equal volume of cold embryo serum, and the preparation was centrifuged at 300 g for 5 min. The supernatant was discarded and cells were washed twice with Neurobasal A medium before being resuspended in Neurobasal medium containing glutamine (0.5 mM), B-27 (2%) and gentamicin (20 μg/ml). 200 μl of the suspension was put in a glass ring (internal diameter of 6 mm) resting on a round 25 mm coverslip (VWR International) which has been coated with poly-L-lysine (one hippocampus for five glasses). The glass ring was removed after a 5 h incubation period in a CO2-incubator (37 °C) and culture medium (2/3 of the volume) was replaced every 3 days. The density of plated cells was 15.000 cells/sq·cm and the age of the neuronal cell culture were 8–10 days in vitro (DIV).

2.3. Ca^{2+} measurements

The measurements of $[Ca^{2+}]_i$ were performed by fluorescence microscopy using Fura-2/AM (Molecular probes, USA), a ratiometric fluorescence calcium indicator. Neurons were loaded with the probe dissolved in Hanks balanced salt solution (HBSS) composed of (mM): 156 NaCl, 3 KCl, 2MgSO4, 1.25 KH2PO4, 2CaCl2, 10 glucose and 10 HEPES, pH 7.4, at a final concentration of 5 μM at 37 °C for 40 min with subsequent 15 min washout. Coverslip containing the cells loaded with Fura-2 was then mounted in the experimental chamber. To measure free cytosolic Ca^{2+} concentration, we used the Carl Zeiss Cell Observer and an inverted motorized microscope Axiovert 200 M with a high-speed monochrome CCD-camera AxioCam HSm with a high-speed light filter replacing system, Ludl MAC5000. Fura-2 excitation and registration was recorded, using a 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-Neo fluar 10 × /0.3, excitation light source HBO 103 W/2. Calcium responses were shown as a ratio of fluorescence intensities of Fura-2 excitation at 340 and 380 nm. To discriminate neuron and astrocytes, short-term application of 35 mM KCl in the end of experiments was used. We determined the amplitudes of Ca^{2+} responses to activators of AMPA receptors as (Δ) – Fmax-Fmin of Fura-2 fluorescence. ImageJ 2002 and Origin 8.5 software were used to analyze data, create graphs and perform statistical tests. All values are given as mean ± SEM or as responses of individual neurons. Data were statistically compared using one-way ANOVA, followed by post hoc Student-Newman-Keuls test or paired *t*-test and were considered significantly different at $p \leq 0.05$. All presented data were obtained from at least 3 coverslips and 2–3 different cell preparations.

2.4. The technique for short-term hypoxia episodes generation

Hypoxic conditions were created using a special vacuum system, where the dissolved oxygen was replaced from Hanks' medium by an inert gas, argon. Oxygen levels which correspond to moderate hypoxia were monitored using a Clark-type oxygen-sensitive electrode and foot up to 50–60 mm Hg. Each of the 3 short-term hypoxia episodes lasted 3 min and was created by supplying the hypoxic medium into the chamber, which contained the cortical or brainstem culture. The constant argon feed into the experimental chamber prevented the contact of the hypoxic medium with oxygen. After each short-term hypoxia experiment, neuronal cultures were washed 5 times with normal medium. These periods of oxygenation lasted 10 min, with subsequent brief activation of AMPA-receptors (30 s) with selective agonist, 5-Fluorowillardiine (FW). The effects of hypoxia on neurons were evaluated by the changes in the amplitude of cell calcium response to FW before and after 3 consecutive hypoxia episodes.

2.5. Immunocytochemical method for GluA2-subunits detection

Cells were fixed and subjected to an immunocytochemical assay using antibodies against the GluA2-subunits of the AMPA receptor. The cells were fixed with 4% paraformaldehyde in PBS for 30 min and then washed with PBS for 30 min. To block non-specific antibody binding sites, fixed cells were incubated in 10% bovine serum for 30 min at room temperature and then incubated in 1% bovine serum for 5 min at room temperature. The cells were then incubated with mouse Anti-GluR2 Antibody, clone 6C4, Alexa Fluor 488 Conjugate for 12 h at 4^0C (1:200 in 1% bovine serum). The fixed cells were subsequently washed with PBS for 15 min. The cells were visualized using a Leica TCS SP5 inverted confocal microscope equipped with an argon laser.

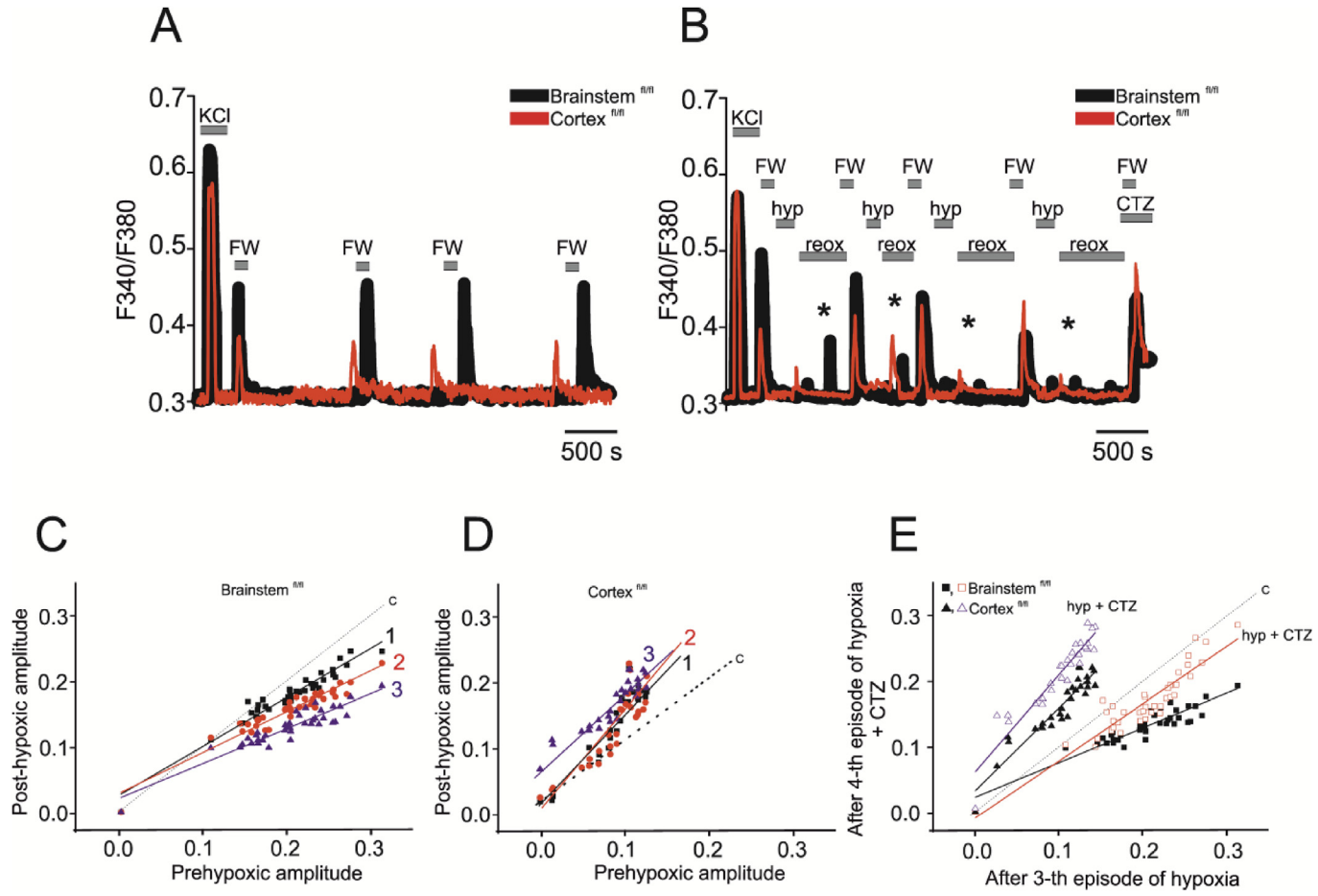

Fig. 1. Ca^{2+}-responses of the brainstem and cortical neurons derived from the *Sip1*$^{fl/fl}$ mice to the application of 0.1 μM FW in the control cells, after short-term episodes of hypoxia (hyp), and after reoxygenation (reox).

A – Average Ca^{2+}-responses of the brainstem (black curve, 96 cells) and the cortical (red curve, 112 cells) neurons to the FW application in the control (without episodes of short-term hypoxia);

B – Average Ca^{2+}-responses of the brainstem (black curve, 104 cells) and the cortical (red curve, 85 cells) neurons to the FW application, short-term hypoxia episodes (hyp) and reoxygenation (reox). After the fourth episode of hypoxia, cells were incubated with the desensitization inhibitor of AMPA-receptors, cyclothiazide (CTZ, 5 μM) prior to 0.1 μM FW application.

C – Dependence of the amplitude of Ca^{2+}-response to the FW application after short-term hypoxia episodes (y) on the amplitude of Ca^{2+}-response before hypoxia (x) in the brainstem neurons derived from the *Sip1*$^{fl/fl}$ mice.

D – Dependence of the amplitude of Ca^{2+} response to the FW application after short-term hypoxia episodes (y) on the amplitude of Ca^{2+} response before hypoxia (x) in the cortical neurons derived from the *Sip1*$^{fl/fl}$ mice.

E – Dependence of the amplitude of Ca^{2+}-response to the FW application after incubation with CTZ after four episodes of short-term hypoxia (hyp + CTZ, Y axis) on the amplitude of Ca^{2+}-response after three episodes of hypoxia (X axis), in the neurons obtained from brainstem (squares) and the brain cortex (triangles). The amplitudes of Ca^{2+}-responses and their approximations by a linear function after the third and the fourth episodes of hypoxia corresponding to panel B are presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. AMPA receptors of neurons derived from cerebral cortex of Sip1 homozygous mice are not preconditioned by short-term episodes of hypoxia

Previously, we showed that the mutation of the transcription factor *Sip1* reduced the amplitude of Ca^{2+}-responses to the AMPA receptor agonist, 5-Fluorowillardine, compared to neurons isolated from other brain regions and cells of the wild-type mice [50]. In this case, the short-term episodes of hypoxia increased the amplitude of Ca2+ responses to NMDA in the *Sip1*$^{fl/fl}$ neurons of the cerebral cortex, causing toxic effects and cell death [49]; [51]. To study the influence of the *Sip1* mutation on the effect of neuronal preconditioning, we registered changes of the cytosolic calcium concentration ($[Ca^{2+}]_i$) in response to applications of the selective AMPA receptor activator (5-Fluorowillardine, 0.1 μM), short-term episodes of hypoxia (hyp) and 10-minute reoxygenation periods (reox). In Fig. 1A, the Ca^{2+}-responses of cortical and brainstem neurons from homozygous mice with the *Sip1* mutation to the FW application without short-term hypoxia episodes are shown. Brainstem neurons from homozygous mice were used as a control because in this brain region the Cre-mediated conditional knockout did not affect the *Sip1* expression. It can be seen that the neurons of the brainstem (black curve) and the cortex (red curve) practically do not differ in the baseline of $[Ca^{2+}]_i$ and in the amplitude of the Ca^{2+}-response to a short-term application of 35 mM KCl. This data indicates that the mutation of the transcription factor *Sip1* does not influence the mechanism maintaining the constant concentration of Ca^{2+} ions in the cytosol and the activity of voltage-dependent Ca^{2+} channels, respectively. Ca^{2+}- signals to the sequential application of 0.1 μM FW, both in the brainstem and in the cortical neurons, were reproducible both in terms of amplitude and shape, but the Ca2+-signals amplitudes in the cerebral cortex neurons were lower by an average of 51 ± 6% (Fig. 1A, red curve), in comparison to brainstem.

A short-term hypoxia episodes consistently suppressed the Ca^{2+}-

signal amplitude to the AMPA receptors activation in the brainstem neurons by 15.7 ± 3, 31.6 ± 2 and 63.2 ± 8%, respectively (Fig. 1B, black curve), which is an attribute of hypoxic preconditioning of these ionotropic receptors. On the contrary, the amplitude of Ca^{2+}-responses to the FW application after each of three episodes of hypoxia in neurons of the cerebral cortex from the *Sip1*$^{fl/fl}$ mice (with induced mutation) was, on average, increased 17 ± 5, 18 ± 04, and 23 ± 8% fold (Fig. 1B, red curve). Hypoxia induced changes in the activity of ionotropic glutamate receptors are well approximated by a linear function and clearly demonstrate the appearance of hypoxic preconditioning phenomena, or toxic effects and hyperexcitability [54]. Analysis of the FW-induced Ca^{2+}-responses amplitude in the brainstem individual neurons after short-term hypoxic episodes from the Ca2+-response amplitude before hypoxia revealed the preconditioning effect. The effect expressed in the decreasing the line slope tangent that approximates these responses, after each hypoxic episode (Fig. 1C) – 0.74 ± 0.03, 0.62 ± 0.03 and 0.53 ± 0.03, respectively. At the same time, the cerebral cortex neurons with the *Sip1* mutation were characterized by a lack of preconditioning, and the slope of the linear approximation was always above 1 (corresponds to the slope of the straight line in the control experiments), namely, 1.64 ± 0.12 after the first episode of hypoxia, 1.46 ± 0.18, and 1.27 ± 0.32 after the second and third episodes (Fig. 1D), respectively.

1, 2, 3 – Linear function of Ca^{2+}-response to FW after each of the three hypoxia episodes. The equation for linear function: y = a + b * x. C – Linear approximation of the control data (without hypoxia). An asterisk (*) indicates a spontaneous increase in $[Ca^{2+}]_i$ in neurons during the reoxygenation period. For clarity, the figures are represented in the same scales of the X and Y axes.

The amplitude of the Ca^{2+-}responses to the AMPA receptors activation in the brainstem neurons of the *Sip1*$^{fl/fl}$ mice is consistently suppressed by the short-term hypoxia episodes, and the slope of the linear approximation of these amplitudes moves to the right in each individual neuron. At the same time it was shown the increase of the amplitude of the Ca^{2+-}responses to the AMPA receptors agonist for the cortical *Sip1*$^{fl/fl}$ neurons, and the slope of the linear approximation of these responses moves to the left. Thus, in the brainstem neurons from the *Sip1*$^{fl/fl}$ mice, the phenomenon of hypoxic preconditioning, which is detected in most cells after the first episode of hypoxia, is fully realized, whereas in neurons of the cortex this phenomenon is absent.

3.2. The role of desensitization and activity of Ca^{2+}-permeable AMPA receptors in the mechanism of hypoxic preconditioning

Cellular and molecular mechanisms involved in the hypoxic preconditioning are numerous and involve a wide range of signaling molecules. In this case, hypoxia, depending on the degree and time of exposure, can cause both short-term and long-term adaptive responses of neurons, which differ in the mechanism of formation. In this paper, we focused on the study of the mechanisms of rapid hypoxic preconditioning and the effect of the *Sip1* mutation on this process. It is known that at the early stages of brain development, no ischemic and glutamate-induced damage of neurons occurs [55]; [23]. Application of FW together with cyclothiazide (CTZ), desensitization inhibitor of the AMPA receptors, on the contrary, leads to their damage, which is suppressed by the AMPA/kainate receptor blockers. To investigate involvement of desensitization in the mechanism of hypoxic preconditioning of AMPA receptors in the brainstem neurons from the *Sip1*$^{fl/fl}$ mice and the absence of this phenomenon in their cortical neurons, activation of AMPA receptors was performed after incubation with 5 μM cyclothiazide. Fig. 2A illustrates that in the brainstem neurons (black curve), episodes of hypoxia do not reduce the amplitude of Ca^{2+}-responses to the FW application after incubation with CTZ, indicating the abolition of hypoxic preconditioning and its association with desensitization. In the neurons of cerebral cortex of the *Sip1*$^{fl/fl}$ mice (Fig. 2A, red curve), no hypoxic preconditioning was also registered after incubation with the desensitization inhibitor, but the retarded decrease of Ca^{2+}-response to the agonist washout indicated the increment of time necessary for activation of the pumping mechanisms $[Ca^{2+}]_i$.

Experiment in Fig. 1B (black curve) shows another evidence of the involvement of the AMPA receptors desensitization mechanism in hypoxic preconditioning. The powerful preconditioning effect in the *Sip1*$^{fl/fl}$ brainstem neurons after three episodes of hypoxia completely disappeared with CTZ after the fourth episode of hypoxia, and the amplitude of the Ca^{2+}-response increased by 16.2 ± 07%, compared with the amplitude of Ca^{2+}-response after the third episode of hypoxia in the absence of this inhibitor.

In Fig. 1E, linear dependencies approximating the amplitudes of neuronal Ca^{2+}-responses to FW after incubation with CTZ and four episodes of hypoxia are shown, which are presented in Fig. 1B. The Ca^{2+}-responses of brainstem neurons to the AMPA receptors activation after four episodes of hypoxia and incubation with CTZ are increases (Fig. 1B, black curve). The tangent of the straight line, approximating these Ca^{2+}-responses is 0.87 ± 0.06 (Fig. 1E, red squares), whereas in the same neurons after three episodes of hypoxia without CTZ this parameter was 0.53 ± 0.03 (Figure 1E, black squares), indicating the presence of preconditioning. For cortical neurons of the *Sip1*$^{fl/fl}$ mice, in which hypoxia did not cause a preconditioning effect (Fig. 1B, red curve), inhibition of desensitization led to the increase in average amplitude of the Ca^{2+}-signal by 17.5 ± 3%. The tangent of the straight line was also increased from 1.27 ± 0.09 (after three episodes of hypoxia without CTZ, Fig. 1E, black triangles) to 1.49 ± 0.1 (after four episodes of hypoxia + CTZ, Fig. 1E, blue triangles), which indicates formation of a toxic effect.

Thus, the desensitization of AMPA receptors is undoubtedly involved in the process of the hypoxic preconditioning in the brainstem neurons obtained from the *Sip1*$^{fl/fl}$ mice that are not affected by the mutation. At the same time, these effects of CTZ were not detected in the neurons of the cerebral cortex, which may be due to the effect of the *Sip1* mutation on the sensitizing mechanism. It should be noted that the mechanism of inhibition of the AMPA receptors desensitization by cyclothiazide is not fully understood, and is probably determined by its large effect on the activity of the GluA2-containing receptors.

The conductivity of the AMPA receptor for calcium is determined by the GluA2-subunit. A change in the subunit composition of AMPA receptors during ischemic stroke by suppressing expression of the GluA2-subunits results in calcium receptor permeability and toxic effects. On the contrary, preconditioning prevents expression of AMPA receptors lacking GluA2 [46]. Taking into account the fact that Ca^{2+}-responses of the cerebral cortex neurons obtained from homozygous mice with the *Sip1* mutation to the activation of AMPA receptor are characterized by lower amplitudes as compared with brainstem neurons from the same mice, it is interesting to investigate involvement of the GluA2-subunit activity in this process. In Fig. 2B it is shown that the amplitudes of Ca^{2+}-responses to repeated FW applications after incubation with CTZ remain unchanged both in the cortical and brainstem neurons. Incubation of cells with 1-Naphthyl acetyl spermine (NASPM, 30 μM), an antagonist of the calcium-conductive AMPA receptors, for 30 min did not affect the amplitude of the Ca^{2+}-signals. However, the effect of delayed Ca^{2+} reduction to the resting level after washout of the agonist in the *Sip1*$^{fl/fl}$ cortical neurons was increased (Fig. 2B, red curve). This period of Ca^{2+} reduction was 120 ± 11 s before incubation with NASPM, and 249 ± 31 s after 30-minute incubation.

However, there are some data indicating that the inhibitory effect of NASPM develops after long period of application, and effective concentrations may exceed 30–50 μM [25]; [33]. Thus, the cortical neurons of the *Sip1*$^{fl/fl}$ mutant were incubated with 100 μM NASPM for 24 h (Fig. 2C). It can be seen that 24 h incubation with a high concentration of the Ca^{2+}-permeable AMPA receptor antagonist also did not affect the amplitude of Ca^{2+}-responses of neurons to the application of even high FW concentration (5 μM), which contributes to the receptor

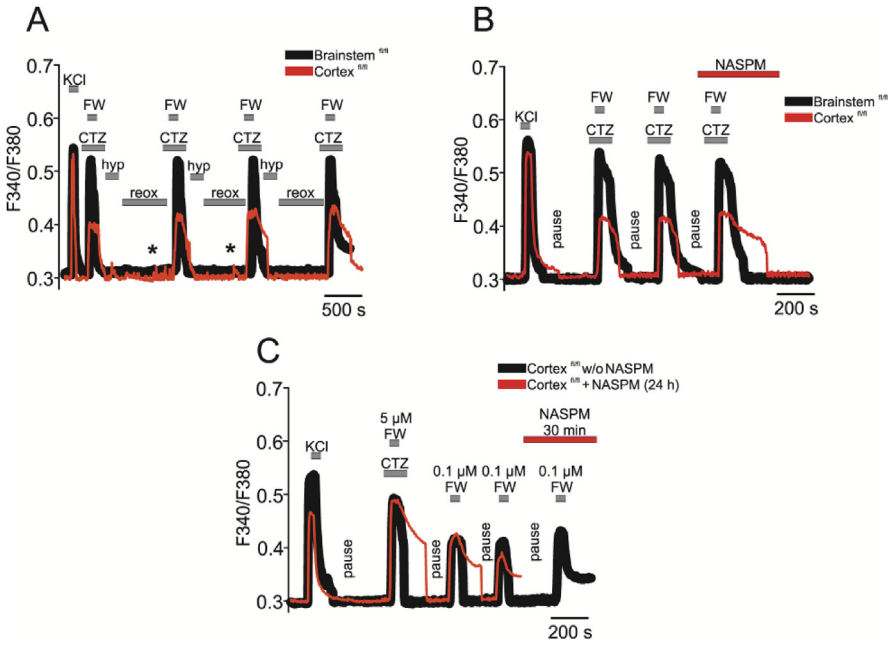

Fig. 2. The role of desensitization and activity of the Ca^{2+}-permeable AMPA receptors in generation of the Ca^{2+}-signals on 0.1 μM FW applications in the brainstem and cortical neurons derived from the *Sip1*$^{fl/fl}$ mutant mice.

A – Average Ca^{2+}-responses of the brainstem (black curve, 104 cells) and cortical neurons (red curve, 85 cells) to the FW application after incubation with desensitization inhibitor of AMPA receptors, CTZ (5 μM), before and after the short-term hypoxia episodes (hyp) and reoxygenation (reox);

B – Average Ca^{2+}-responses of the brainstem (black curve, 87 cells) and cortical neurons (red curve, 91 cells) to the FW application after incubation with desensitization inhibitor of AMPA receptors, CTZ (5 μM), without short-term hypoxia episodes (hyp), and after 30-minute incubation with Ca^{2+}-permeable AMPA receptor antagonist, 1-Naphthyl acetyl spermine (NASPM, 30 μM);

C – Average Ca^{2+}-responses of the cortical neurons to the FW application after 30 min (black curve, 25 cells) and 24 h (red curve, 53 cells) incubation with 100 μM NASPM. Pause – marked 10-minute periods when the recording of Ca^{2+}-dynamics was not performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Tangent of the slope of the straight line, approximating the amplitudes of Ca^{2+}-responses of neurons after 3 episodes of hypoxia. Influence of modulators of signal proteins.

	Control	CaMKII (inhibitor KN-93)	PI3K (inhibitor LY-294,002	PI3K (activator 740Y-P	IL-10 (anti-inflammatory cytokine
Brainstem *Sip1*$^{fl/fl}$	0.53 ± 0.03/R^2 0.86	0.62 ± 0.05/0.79	0.83 ± 0.04/0.87	0.44 ± 0.12/0.65	0.36 ± 0.08/0.71
Cortex *Sip1*$^{fl/fl}$	1.27 ± 0.08/R^2 0.88	1.22 ± 0.14/0.81	1.26 ± 0.08/0.77	**0.51** ± **0.04**/0.87	**0.35** ± **0.05**/0.67

desensitization in addition to the CTZ application. However, the delay time of the decrease of Ca^{2+} concentration to the resting level increased from 76 ± 8 s in the cortical neurons not pre-incubated with NASPM (Fig. 2C, black curve) to 177 ± 21 s after incubation with NASPM (Fig. 2C, red curve).

In this case, the effect of 100 μM NASPM was observed even after 30 min of incubation (Fig. 2C, black curve), also causing an increase in the delay time of $[Ca^{2+}]_i$ removal from 63 ± 11 s in the control (3rd application of FW after incubation with CTZ) to 186 ± 19 s after incubation with NASPM. Based on the data of the inhibitory analysis of the GluA2-lacking AMPARs activity in cortical and brainstem neurons derived from homozygous mice with the *Sip1* mutation, it is clear that their suppression in both cases does not affect the amplitude of Ca^{2+}-signals but determines the $[Ca^{2+}]_i$ recovery time after activation of AMPA receptors with selective agonist.

Taking into account the obtained data, it can be assumed that the phenomenon of rapid hypoxic preconditioning occurring in the brainstem neurons of the *Sip1*$^{fl/fl}$ mice, as well as the absence of this phenomenon in neurons of their cerebral cortex, is not determined by the activity of the GluA2-lacking AMPARs. However, inhibition of the Ca^{2+}-conducting AMPA channels increased the FW toxicity in the cortical neurons of the *Sip1*$^{fl/fl}$ mice. Therefore, the desensitization plays a key role in the mechanism of the hypoxic preconditioning in the wild type neurons, since the desensitization inhibitor CTZ completely abolished the preconditioning effect of hypoxic episodes. The Sip1 mutation is likely to disrupt the AMPA receptors desensitization mechanisms of the cortical neurons.

3.3. Interleukin-10 enhances the neuroprotective effect of hypoxic episodes in the brainstem neurons and activates the phenomenon of hypoxic preconditioning of AMPA receptors in the cortical neurons of the Sip1$^{fl/fl}$ *mutant mice*

It is known that the mechanism of hypoxic preconditioning is realized due to both Ca^{2+}-dependent and Ca^{2+}-independent activation of intracellular signaling proteins [3]. Moreover, it is well known that the *Sip1* mutation is characterized by a disturbance of such signaling pathways as Wnt, JNK and CaMKII [24] [58]; and prevents adaptive changes of the NMDA receptor activity [49]; [51]. Indeed, the inhibitor of CaMKII, KN-93, led to suppression of preconditioning of the AMPA receptors in the brainstem neurons obtained from the *Sip1*$^{fl/fl}$ mice, which was expressed in an increase of the slope of the straight line approximating Ca^{2+}-responses to FW from 0.53 ± 0.03 in the control experiment (after 3 episodes of hypoxia) to 0.62 ± 0.05 after 15 min of incubation with 1 μM KN-93 (Table 1). At the same time, this inhibitor did not influence the coefficient of slope of linear approximation of amplitudes of Ca^{2+}-responses to the FW application in the cerebral cortex neurons (Table 1) obtained from homozygous mice with the *Sip1* mutation that is not surprising due to the damage of this signal protein under mutation.

However, we hypothesized that the preconditioning phenomenon in the cortical neurons of the *Sip1*$^{fl/fl}$-mutant mice may be initiated through an alternative neuroprotective signaling cascade involving phosphoinositide-3-kinase (PI3K), which is probably not damaged with the Sip1 mutations. Inhibition of this enzyme with 10 μM LY-294,002 in brainstem neurons significantly increased the coefficient of slope of the linear approximation to 0.83 ± 0.12 and did not significantly affect the cortical neurons (1.26 ± 0.08) (Table 1). In this case, the activator of PI3K, 1 μM 740Y-P, initiated a decrease in the linear approximation coefficient in the cortical neurons from 1.27 ± 0.08 in the control

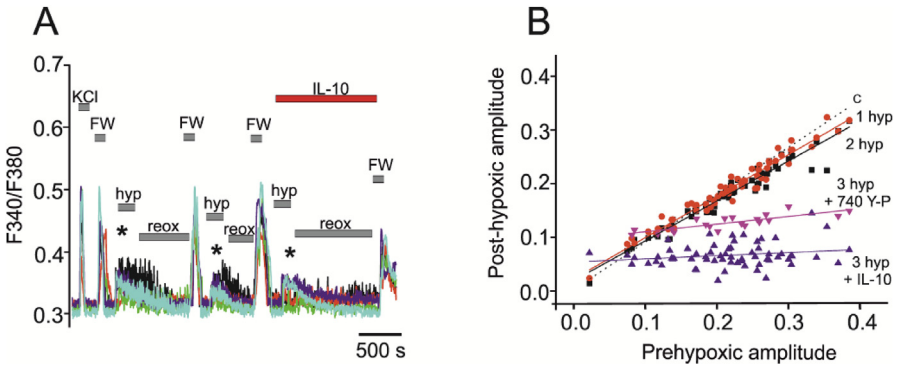

Fig. 3. Interleukin-10 initiates the hypoxic preconditioning of neurons obtained from the cerebral cortex of homozygous mice with a mutation of the transcription factor *Sip1*.

A – Incubation with IL-10 (1 nM) for 15 min activates the preconditioning of *Sip1*$^{fl/fl}$ cortical neurons, reducing the amplitude of Ca^{2+}-responses to the application of 0.1 μM FW after the third episode of hypoxia.

B – Influence of 15 min incubation of *Sip1*$^{fl/fl}$ cortical neurons with 1 μM activator of PI3K – 740Y-P (3hyp + 740Y-P) and 1 nM anti-inflammatory cytokine IL-10 on the amplitudes of Ca^{2+}-responses to the application of 0.1 μM FW before (X axis), and after (Y axis) the episodes of short-term hypoxia. 1 hyp and 2 hyp are Ca^{2+}-responses of neurons and their approximations by a linear function after the 1st and 2nd episodes of hypoxia. C – Linear approximation of the control data (without hypoxia).

* – increased Ca^{2+} in the cytosol of neurons during short-term episodes of hypoxia.

experiment (after 3 episodes of hypoxia) to 0.51 ± 0.04 after 15 min of incubation (Table 1, Fig. 3B, lilac triangles). In the brainstem neurons, 1 μM 740Y-P enhanced the preconditioning effect of short-term hypoxia episodes, and the slope of the straight line was 0.44 ± 0.12 (Table 1). The anti-inflammatory cytokine, interleukin-10, realizes many of its rapid effects through the activation of the signaling cascade involving PI3K. Previously, it was shown that IL-10 demonstrated high neuroprotective efficacy in the model of ischemia in vitro [48], and also restored the preconditioning effect in rat hippocampal GABAergic neurons after transient hypoxia [53]. In as little as 15 min of incubation with 1 nM IL-10, a hypoxic preconditioning phenomenon appears in the neurons of the cerebral cortex of the *Sip1*$^{fl/fl}$ mutant mice, being expressed in a decrease of the amplitude of Ca^{2+}-responses to the application of 0.1 μM FW (Fig. 3A), which did not occur before the incubation of these cells with IL-10. In addition, IL-10 is able to reduce the amplitude of the hypoxia-induced increase in Ca^{2+} in the cytosol (marked as *), apparently due to PKG-dependent inhibition of the IP3-receptor.

The slope coefficient of the straight line approximating Ca^{2+}-responses to FW before and after the first and the second episodes of hypoxia was 1.27 ± 0.08, but decreased to 0.35 ± 0.05 after 15 min incubation with IL-10 and the third episode of hypoxia (Fig. 3B, Table 1). Thus, IL-10 is the most effective neuroprotective compound, because its effect aimed at suppressing the amplitude of the Ca^{2+} signal with the activation of AMPA receptors is higher by 45% compared to the action of the selective activator of PI3K (740Y-P) (Fig. 3B). Increasing the incubation time of cortical neurons with 1 nM IL-10 up to 40 min or more leads to the appearance of a preconditioning effect after the first episode of hypoxia (data not shown).

Therefore, the intracellular signal proteins – CAMKII and PI3K kinases – are involved in the hypoxic preconditioning of the AMPA receptors. The inhibition of CAMKII and PI3K kinases leads to loss of the hypoxic preconditioning in the brainstem neurons of the *Sip1*$^{fl/fl}$ mutant mice, and conversely the activation of the CAMKII and PI3K kinases increases the preconditioning effect of hypoxic episodes. At the same time, only the PI3K activators were able to rescue the phenomenon of the hypoxic preconditioning in the cortical neurons of the *Sip1*$^{fl/fl}$ mutant mice, that can be explained by the disturbance of CAMKII kinase activity.

3.4. Interleukin-10 as a neuroprotector can mediate both rapid effects associated with the activity of AMPA-receptors and chronic effects that determine expression of the GluA2-subunits

The protective signal pathway involving PI3K, which is activated by hypoxia and ischemia, capable to enhance preconditioning in the glutamatergic neurons of hippocampus [52], is not affected by the mutation of the transcription factor *Sip1* and may be used as a perspective target to protect cortical neurons from damage during hypoxia. Indeed, even 15 min incubation with the selective activator of PI3K, 740Y-P, led to the appearance of preconditioning in cortical neurons obtained from the brain of homozygotes with the *Sip1* mutation (Fig. 2B), and in the brainstem neurons there was an increase in preconditioning (Table 1).

Incubation period of 24 h with IL-10 is most capable to suppressing the increase of Ca^{2+} in the cytosol of neurons and astrocytes in ischemia in vitro [49]; [51]. Indeed, 24 h incubation of cell cultures derived from the cerebral cortex of homozygote's with the *Sip1* mutation with 1 nM IL-10 led to hypoxic preconditioning after the first episode of hypoxia (Fig. 4B), whereas neurons from the same cell culture passage, but not incubated with IL-10, were characterized by an increase in the amplitude of Ca^{2+}-responses to FW after each episode of hypoxia (Fig. 4A). In addition, prolonged incubation with IL-10 resulted in a significant decrease in the sensitivity of cortical neurons to the episodes of short-term hypoxia, expressed in the almost total absence of Ca^{2+}- signals (marked *) in comparison with neurons not incubated with IL-10.

This effect of prolonged incubation with IL-10 leads to such an evident induction of preconditioning that after the third episode of hypoxia, the amplitudes of the Ca^{2+}-signals of the cortical neurons become comparable to those of the brainstem neurons obtained from the same *Sip*$^{fl/fl}$ mice (Fig. 4C). This is also confirmed by the slope coefficients of linear approximations of the Ca^{2+}-responses amplitudes of the cortical neurons after 24 h of incubation with IL-10. After three episodes of hypoxia, they were 0.59 ± 0.04 (Fig. 4D, red squares), and 1.4 ± 0.1 in the same neurons, but without IL-10 (Fig. 4D, black squares). The slope coefficient of the straight line approximating Ca^{2+}-responses to FW for the brainstem neurons was 0.61 ± 0.05 in the absence of IL-10 (not shown), decreasing to 0.32 ± 0.03 after 24 h incubation with 1 nM IL-10, which confirms the ability of this cytokine to enhance the preconditioning effects of episodes of hypoxia in normal neurons.

In this study, our main attention was focused on AMPA receptors and mechanisms, the absence or disturbance of which caused by a mutation of the transcription factor Sip1 prevents formation of hypoxic preconditioning as a normal way of adaptation to a low partial O_2 pressure. One of the reasons for the preconditioning effect after incubation with IL-10 can be the influence of IL-10 on expression of the GluA2-subunits. Indeed, after 24 h incubation of cortical neurons with IL-10, the number of neurons increased in which the expression of the GluA2-subunits of AMPA receptors is revealed (Fig. 5A, middle line). In addition, when considering single neurons in which the presence of the GluA2-subunits was detected, it was reliably established that the fluorescence intensity of AlexaFluor-488 was significantly higher after exposure to IL-10 compared to the brainstem and cortex neurons not exposed to this cytokine, indicating an increase in the number of these subunits on the membrane of neurons (Fig. 5B, C). It is possible that enhanced expression of the GluA2-subunits after treatment with IL-10 is one of the protective mechanisms, which activates preconditioning

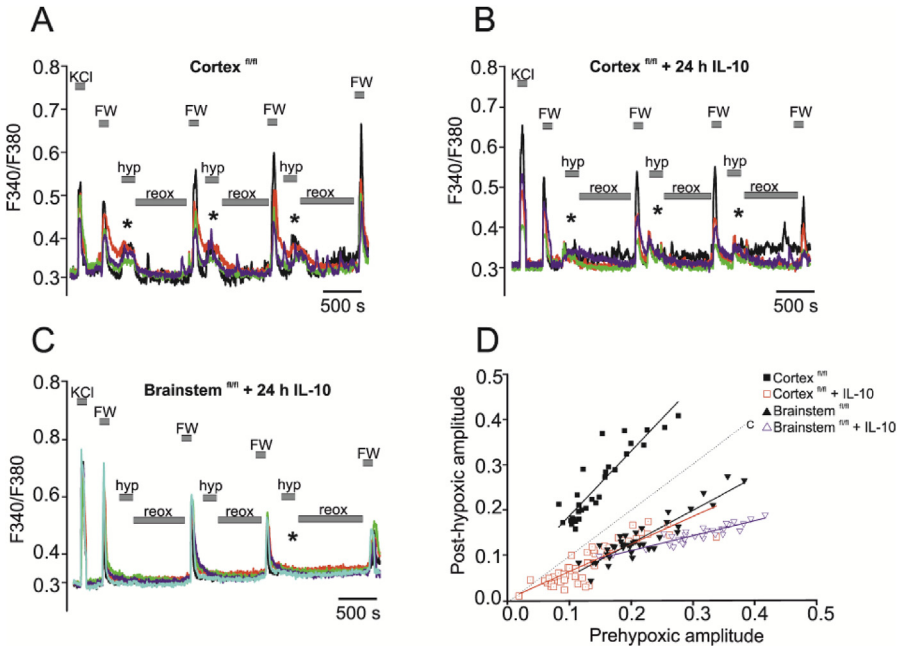

Fig. 4. Long-term (24 h) exposure to 1 nM IL-10 activates the phenomenon of hypoxic preconditioning in cortical neurons from the *Sip*$^{fl/fl}$ mice and enhances it in brainstem.

A – Ca^{2+}-signals of individual neurons of the cerebral cortex, obtained from a homozygous mouse with the *Sip1* mutation to the application of 0.1 μM FW, short-term episodes of hypoxia (hyp) and periods of reoxygenation (reox).

B – Ca^{2+}-signals of individual neurons of the cerebral cortex obtained from the same homozygous mouse with the Sip1 mutation (as in Fig. 5A) and grown with 1 nM IL-10 for 24 h to the application of 0.1 μM FW, short-term episodes of hypoxia (hyp) and periods of reoxygenation (reox).

C – Ca^{2+}-signals of individual brainstem neurons derived from a homozygous mouse with the *Sip1* mutation and grown with 1 nM IL-10 for 24 h to the application of 0.1 μM FW, short-term episodes of hypoxia (hyp) and periods of reoxygenation (reox).

D – Influence of a 24 h incubation of the cortex and brainstem neurons of the *Sip1*$^{fl/fl}$ mice with 1 nM of anti-inflammatory cytokine IL-10 on the amplitudes of Ca^{2+}-responses when applying 0.1 μM FW to (X axis) and after (Y axis) 3 episodes of short-term hypoxia.

* – increased Ca^{2+} in the cytosol of neurons during short-term episodes of hypoxia.

phenomenon.

Thus, it has been shown that the AMPA receptors of cortical neurons obtained from the homozygous mouse with the *Sip1* mutation are not preconditioned by the short-term episodes of hypoxia. The lack of preconditioning is associated with a disturbance of hypoxia-induced desensitization of the AMPA receptors, whereas inhibition of the Ca^{2+}-conducting AMPA receptors does not affect this process. Recovery of the preconditioning mechanism in the *Sip1*$^{fl/fl}$ cortical neurons is only possible when the PI3K-dependent signaling pathway is activated by the selective activator 740Y-P or the anti-inflammatory cytokine IL-10.

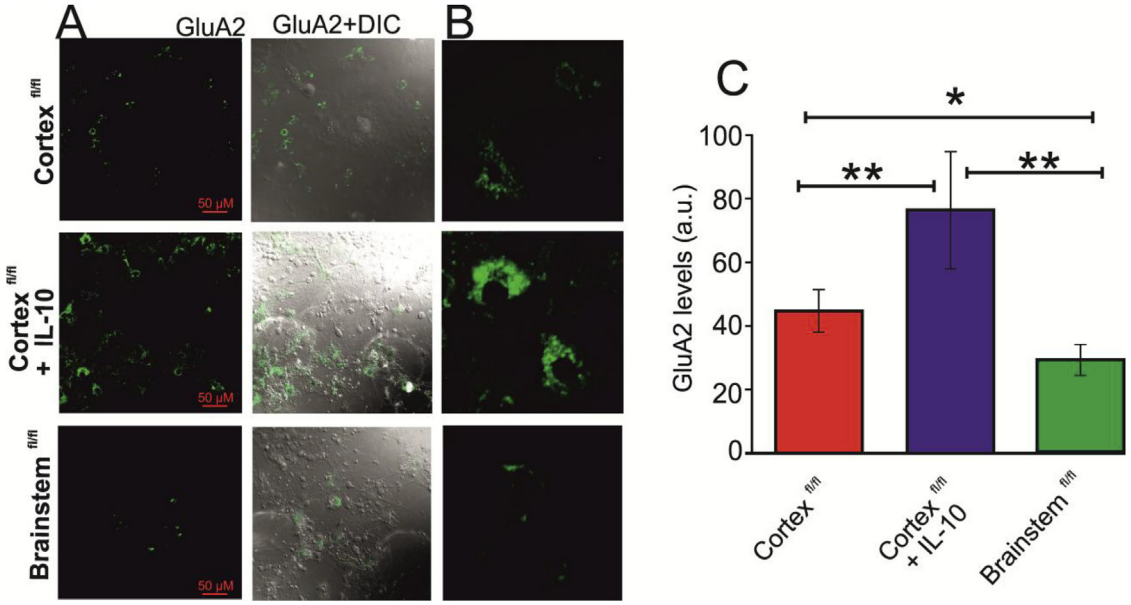

Fig. 5. Comparison of expression of GluA2-subunits of AMPA receptors in cortical and brainstem neurons from *Sip1*$^{fl/fl}$ mutant mice. IL-10-mediated increase of GluA2 surface levels in *Sip1*$^{fl/fl}$ cortical neurons.

A – Staining with specific antibodies against the GluA2-subunit of the AMPA receptors of the cortical (top line) and brainstem (bottom line) neurons derived from the homozygous mouse with the *Sip1* mutation. The midline of panel A shows the effect of 24-hour incubation of *Sip1*$^{fl/fl}$ cortical neurons with 1 nM IL-10. The GluA2 + DIC column shows the localization of the GluA2 subunit in neurons in the field of view of the microscope. Intensity levels of surface-expressed GluA2 were determined by confocal imaging and analyzed single neurons with fluorescence of Alexa Fluor 488 (green).

C – Influence of a 24 h incubation of cortical neurons from the *Sip1*$^{fl/fl}$ mutant mouse with 1 nM IL-10 on the expression level of the GluA2-subunit of the AMPA receptor. Qualitative data are presented on the expression of the GluA2-subunit in the form of an average GluA2 intensity values in summary bar charts represent the mean ± SEM from 300 cells for each column. *p = 0.006 versus cortical *Sip1*$^{fl/fl}$ neurons. **p ≤ 0.001 versus cortical or brainstem *Sip1*$^{fl/fl}$ neurons without IL-10. Statistical significance was assessed using one-way ANOVA, followed by post hoc Student-Newman-Keuls test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Hypoxia-induced desensitization of AMPA receptors as one of the mechanisms of rapid preconditioning

The damage and death of cells is directly related to the calcium-dependent processes triggered by glutamate [22]. Here, AMPA receptors can play the leading role, along with NMDA-receptors. Indeed, 24 h after short-term ischemia in vivo, the currents mediated by the activity of NMDA receptors in pyramidal neurons of the hippocampus are decreased while AMPAR currents are enhanced [47]. At the same time, under the conditions of a lack of oxygen in the brain cells, various mechanisms are activated that protect cells from irreversible disturbances in the functioning of innervated tissues and organs [42]. One of the main protective mechanisms is the phenomenon of hypoxic preconditioning, which normally develops in neurons within minutes and is expressed in a decrease in the amplitude of Ca^{2+}-signals for activation of AMPA- and NMDA receptors [54]. However, the *Sip1* mutation, leading to the development of the Mowat-Wilson syndrome, completely suppressed hypoxic preconditioning of the NMDA receptors of cortical neurons in vitro [49]; [51].

Given the fact that activation of AMPA receptors during hypoxia occurs together with NMDA receptors, the absence of preconditioning of AMPA receptors in neurons of homozygous mice with the *Sip1* mutation is due to the same impairments. The hypoxic preconditioning phenomenon involves a whole range of adaptation mechanisms triggered by various pathways of signal transduction and aimed at emergency desensitization of receptors. In neurons previously preconditioned with episodes of hypoxia, no pathological changes in the activity of AMPA receptors were detected. Rats survived after severe ischemia of the forebrain and CA1 area of the hippocampus [6]; [19].

Preconditioning of animals with moderate hypoxia prevents the abnormal reactivity of glutamatergic receptors in rat cortical slices through the inactivation of AMPA receptors and a change in functional coupling of group I mGlu receptors with calcium channels [39]. However, in our experiments with neurons obtained from the brain cortex of the $Sip1^{fl/fl}$ mutant mice, there was no decrease in the amplitude of Ca^{2+}-responses to the activation of AMPA receptors after episodes of hypoxia, but on the contrary, the signal increase was observed, probably due to the absence of a hypoxic inactivation mechanism. At the same time, the inhibitor of the AMPA receptors desensitization cyclothiazide (CTZ, Fig. 2) completely abolished the preconditioning effect in the brainstem neurons obtained from the same animals as the cortex neurons, which confirms the leading role of desensitization of AMPA receptors in the development of adaptation to hypoxia.

The mechanism of cyclothiazide action on AMPA receptors has not been established reliably, but there is an evidence that its inhibitory effect on the desensitization of AMPA receptors was the main reason for the toxic effects and death of retinal ganglion cells in vitro [29]. CTZ affects the desensitization of AMPA receptors, depending on the activity and the presence of various isoforms of the receptor subunits (in particular, GluA1) [30]; [44].

In our experiments, the CTZ application suppressed the desensitization of AMPA receptors and increased the amplitude of Ca^{2+}-responses by 25 ± 08% in cortical and brainstem (32 ± 06%) neurons from brain of the *Sip1* mutant mice in the absence of hypoxia (Fig. 1B, end of the experiment). It is known that receptor desensitization occurs due to the activity of protein kinases, phosphorylating hydroxyl groups of the serine, threonine and tyrosine residues of the carboxyl end group of the receptor [8]. Phosphorylation changes its three-dimensional conformation, and sensitivity can be restored by dephosphorylation of some from many cytosol phosphatases. In the case of the AMPA receptor, this protein kinase is calmodulin-dependent kinase II (CaMKII), whose activity and expression are significantly reduced in hypoxic conditions [11]; [37], explaining the negative effect of its inhibitor (KN-93, Table 1) on the preconditioning phenomenon in brainstem neurons. In this case, the *Sip1* mutation coincides with the disturbances of CaMKII in neurons of the cerebral cortex and, therefore, this signal protein as a regulator of desensitization of AMPA receptors cannot function, which explains the absence of the KN-93 effect (Table 1), as well as the desensitization inhibitor CTZ.

4.2. Relationship of preconditioning and functional expression of Ca^{2+}-permeable AMPA receptors

With AMPA receptor desensitization, the mechanism of hypoxic preconditioning is caused by the expression of subunits forming the AMPA-receptor [44]. In the processes of damage and death of neurons in hypoxic conditions, one of the key roles is played by Ca^{2+}-permeable AMPA receptors [45], which can determine the selective vulnerability of certain groups of neurons to the damaging effects of oxygen deficiency [31]. The permeability of the AMPA-receptor for Ca^{2+} ions is determined by the GluA2-subunit, in the presence of which the receptor channel is practically impermeable [14]. In normal conditions, almost all AMPARs are permeable only for Na^+ and K^+ [16]; [43].

In the regions of hippocampus, obtained from post-ischemic animals, the increased levels of cytosolic calcium in neurons from the CA1 region were detected, which is determined by Ca^{2+}-permeable AMPA receptors [26]; [59]. Hypoxia and ischemia cause a decrease in the expression of mRNA and GluA2-subunit protein of AMPA receptors in sensitive neurons, which contributes to their death [56]. Thus, Ca^{2+}-permeable AMPA receptors are the main mediators of hypoxic-ischemic encephalopathy and their antagonists (not antagonists of NMDA receptors) protect neurons from death in ischemia [20]; [15]. Immunocytochemical staining of $Sip1^{fl/fl}$ brainstem (Fig. 4 lower row) and cortical (Fig. 5 upper row) neurons of the brain with antibodies against the GluA2-subunit of AMPA-receptors showed that these subunits are less expressed in the brainstem neurons than in the cortex, which probably determines the initially large amplitude of Ca^{2+}-signals to the activation of AMPA receptors with an agonist. But this difference does not affect the preconditioning mechanism, as the desensitization of AMPA receptors (and especially after exposure to episodes of hypoxia) will be determined not by the initial amount of GluA2-subunits, but by a change in their activity, including the ratio of the GluA2-subunits with other subunits of this receptor in the formation of the tetrameric structure. In addition, in our case, the lack of rapid preconditioning in the cortical neurons with *Sip1* mutation is probably associated with disruption of phosphorylation and dephosphorylation of receptors because of changes in the subunit composition of AMPA receptors and in the appearance of more Ca^{2+}-permeable AMPA receptors, it takes more than an hour, whereas in neurons of the brainstem preconditioning develops after the first episode of hypoxia and reoxygenation (about 12–15 min).

AMPA-receptors also mediate the mechanism of p-CREB activation – through Ca^{2+}/calmodulin-dependent protein kinases (CaMKII and CaMKIV). In this case, the activation of CaMKII in neurons leads to the induction of apoptosis, whereas CaMKIV leads to the survival of neurons [4]; [38]. But in case of mutation of the transcription factor *Sip1*, expression N-terminal kinase of the c-Jun (JNK) is completely suppressed [24], as well as CaMKII [58] [24]; that also contributes significantly to the lack of adaptation of cortical neurons from $Sip1^{fl/fl}$ mice to hypoxia. The natural activator of PI3K and the anti-apoptotic signaling cascade is the anti-inflammatory cytokine – IL-10, previously shown to be highly effective in protecting the hippocampal neurons from OGD in vitro [49] [51]; [48]. IL-10 also activated hypoxic preconditioning of NMDA receptors in hippocampal GABAergic neurons [53]. In the cortical neurons, IL-10 activates the signaling cascade, which includes PI3-kinase and STAT-3, thereby decreasing brain damage in ischemic stroke [40]. Thus, the rapid effects of IL-10 aimed at enhancing the preconditioning of the AMPA receptors of the neuronal brainstem and the activation of this phenomenon in neurons of the cortex of the $Sip1^{fl/fl}$ mice are associated with the action of this cytokine

on the canonical signaling pathway involving PI3K/AKT which can inhibit calcium entry by phosphorylation of the regulatory subunits of AMPA receptors.

It has been shown that an elevated level of the GluA2 protein in the dorsal region of the hippocampus can represent an endogenous anti-epileptic mechanism that maintains the balance between excitation and inhibition after the status epilepticus [34] and reduces brain damage. During the status epilepticus, to which both the people with the Mowat-Wilson syndrome and the rodents with the *Sip1* mutation are inclined, the level of expression of the GluA2 subunit decrease only before the neurodegeneration process [10]. On the other hand, Western blotting has shown that hypoxia reduces the expression of the GluA1-subunit of AMPA receptors in hippocampal slices, and IL-10 is able to prevent this decrease with an increase of efficacy from 10 to 90 min after induction of hypoxia [60]. It is likely that this process also occurs in our experiments, and thus the second mechanism of the IL-10 protective action aimed at forming desensitization of AMPA receptors, is realized.

Thus, our study showed that the mutation of the transcription factor *Sip1* leads to the loss of the ability of neurons derived from the cerebral cortex of homozygous mice to adapt to the short-term episodes of hypoxia by disturbing the mechanisms of hypoxic preconditioning. The leading mechanism of rapid preconditioning, as a phenomenon of normal neuroplasticity in brainstem neurons (not damaged by a mutation), is the desensitization of AMPA receptors through the functioning of the MAPkinase signaling cascade and CaMKII. Disturbance of these signaling pathways in *Sip1* mutation is the reason for the absence of hypoxia-induced desensitization. Other mechanisms are also possible which are normal and are affected by *Sip1* mutation, including the ratio of subunits in the formation of the AMPA receptor tetramer, differences in the number and activity of GluA2-subunits, and many others. However, it is important to be able to induce hypoxic preconditioning in *Sip1* mutation by activating an alternative signaling cascade involving PI3K/AKT. Activation of this neuroprotective cascade with a selective agonist or anti-inflammatory cytokine-IL-10 is appearing by suppressing of AMPA receptor activity with short-term episodes of hypoxia after 15 min of incubation. Long-term (up to 24 h) exposure to IL-10 results to the recovery of preconditioning in cortical neurons, possibly including an increase in the expression of the GluA2-subunits of AMPA receptor's that interfere with the formation of the Ca^{2+}-permeable AMPA receptor's.

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