HYALURONIDASE-DEPENDENT CHANGES OF ADAR2 IN MICE HIPPOCAMPAL CELL CULTURES

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Abstract. The extracellular matrix plays an important role in brain function. Recent findings suggest that disruption of hyaluronan-based extracellular matrix can cause seizure-like activity (Vedunova et al., 2013). Epilepsy can be characterized by an excessive influx of Ca^{2+} ions through (Ca^{2+}) – permeable AMPA receptors, which may, in certain circumstances, contribute to seizures. Ca^{2+} – permeability of these receptors is dependent on RNA-editing of pre-mRNA transcript of GluA2 subunit at the Q/R site. Regulation of this process is carried out by a special nuclear enzyme, ADAR2 (Adenosine Deaminase Acting on RNA-2). Thus, the study of the principle of operation of this enzyme can contribute to understanding the mechanism of epileptogenesis.

Keywords: ADAR2, AMPA, RNA-editing, extracellular matrix.

List of Abbreviations

ADAR2 – Adenosine deaminase acting on RNA 2

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid –type of glutamate receptors

DAPI – 4′,6-diamidino-2-phenylindole, nuclear fluorescent stain

PBS – phosphate-buffered saline

ECM – extracellular matrix

HA – hyaluronic acid

CSPGs – chondroitin sulfate proteoglycans

WB – western blot

PCR-RT – real-time polymerase chain reaction

Introduction

Brain extracellular matrix (ECM) is a multicomponent complex. The members of the lectican family, chondroitin sulfate proteoglycans (CSPGs) such as neurocan, brevican, versican, and aggrecan with tenascins are anchored on hyaluronic acid (HA) backbone. Hyaluronic acid plays a pivotal role in synaptic plasticity during vascular cognitive impairment and dementia (Dityatev *et al.*, 2009). Extracellular remodeling is a common response to different types of injuries and diseases. Recent studies have shown that knockout mice lacking hyaluronic acid synthase demonstrate epileptic phenotype (Arranz *et al.*, 2014). Epileptic seizures are characterized by the over-synchronous firing of neurons and can be initiated and propagated by AMPA receptor stimulation. AMPA receptors are tetrameric receptor complexes in which GluA1-4 can be assembled in various combinations in which the GluA2 subunit plays the main role in the regulation of Ca^{2+} – permeability.

In the brain, essentially all AMPARs contain GluA2 edited by ADAR2 (Adenosine deaminase acting on RNA-2) at codon 607 within the Ca^{2+} pore (Farooq *et al.*, 2012). Conversion of this Adenine to Inosine (utilized as Guanine) causes Arg to be substituted for Gln and blocks Ca^{2+} entry. Thus, edited GluA2 is linked with normal low Ca^{2+} permeability, and suppression of GluA2 and its RNA editing can contribute to disease. Also, Marcucci's work (Marcucci et al., 2011) showed that mislocalized ADAR2 protein from the nucleus to cytoplasm study demonstrated reduced editing at the GluR2 Q/R.

Previously, we have found that enzymatic digestion of hyaluronic acid by exogenous enzyme Hyal leads to the delayed development of

epileptiform activity in cultured hippocampal neurons in vitro (Vedunova *et al.*, 2013).

In this study, we supposed that Q/R site RNA editing deficiency by the ADAR2 enzyme could be implicated in this phenomenon.

Methods

Cell cultures. All animals were treated in strict accordance with ethical animal research standards defined by the Russian law and approved by the Ethical Committee on Animal Health and Care of Privolzhsky Research Medical University of the Nizhny Novgorod.

C57BL6/j pregnant mice (E18) were used for the preparation of hippocampal cell cultures. Briefly, skin and skull bones were removed from embryos with tweezers, and the whole brain was placed on a dissecting glass plate. Before dissecting the hippocampus, all meninges were removed from the brain. All hippocampus tissues were collected in a 15 ml tube containing 0.25% trypsin-EDTA (Gibco, 25200056) for incubation 20 minutes at 37oC in a humidified incubator (Sanyo MCO – 18AIC). After incubation, the cell clump was triturated gently with a glass pasteur pipette and plated in six-well plates (TPP, 92206) in a medium containing (5% Fetal calf serum, (Paneco, FB-1001B/50м), 2% B-27 supplement (Gibco, 17504044), 0.5mM L-glutamine (Gibco, 25030081), 92.25% Neurobasal medium (Gibco, 21103049). On the next day, half of the medium was changed to medium containing (0.4% Fetal calf serum, 2% B-27, 1mM L-glutamine, 97.1% Neurobasal Medium) and then changed every other day up to DIV 17.

Hyaluronidase (Sigma-Aldrich, H3506) addition (75U/ml) was performed on the DIV17. A sampling of cell cultures for WB and PCR-RT assays were performed 2 and 9 days after the experiment. Sequence analysis and ICC were done after 2 days of hyaluronidase treatment.

The completeness of hyaluronan digestion was checked via immunocytochemistry staining against aggrecan.

PCR-RT. Total RNA from samples was extracted via RNA-extract kit (Evrogen) according to the manufacturer's instructions.

To investigate gene expression changes Real-time PCR method was used with CFX 96 (BioRad) instrument. TaqMan Gene Expression Assay (Hs00953730_m1, Thermo Fisher) was used. Gene expression of ADAR2 was normalized against such housekeeping gene Beta-Actin and measured by the ΔΔCt method.

GluA2 Q/R editing. To assess GluA2 editing, sequencing analysis was performed in agreement with the method described by Cantanelli (Cantanelli *et al.*, 2014). DNA fragments were amplified by PCR using primer pairs previously described (Cantanelli, 2014). Amplified products were confirmed by electrophoresis on 2% agarose gels with ethidium bromide staining. PCR products were purified with a Cleanup Standard purification kit (Erogen, Moscow, Russia) and sequenced on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using DNASTAR 7 software (Lasergen Inc., Houston, USA).

Immunocytochemistry. After treatment, hippocampal cells were washed twice with warm phosphate buffered saline (PBS) (Gibco) then fixed with PFA 4%/4% sucrose in PBS (pH = = 7.4) for 15-20 min and then washed three times in blocking buffer 1 (PBS,100 mM Glycine). Permeabilization and blocking were made with 0.2% Tween 20, 2% BSA in PBS for 20 min.

Samples were incubated with primary antibodies in blocking buffer 2 (5% normal goat serum, PBS) overnight at 4oC.

Primary antibodies against ADAR2 (Abcam, ab64830), Aggrecan (Abcam, ab216965) were used. After incubation, samples were washed with blocking buffer 3 (PBS, 0.1% BSA, 0.1% Tween 20) three times. Samples were then exposed to the corresponding secondary antibodies (PBS, 5% BSA, antibodies 1:400) solution for 45 min at room temperature with subsequent washing in the blocking buffer. Secondary antibodies conjugated with Alexa 488 (Thermo Fisher) were used. Coverslips were mounted with a fluoroshield mounting medium (Abcam, ab104139). Images were taken using an AxioScope A1 fluorescent microscope (Carl Zeiss, Germany).

Western Blotting. Analysis of changes in protein concentration on the 2nd and 9th days after adding hyaluronidase to the culture was carried out by Western blotting with preliminary separation into nuclear and cytoplasmic fractions to assess protein redistribution.

The division into nuclear and cytoplasmic fractions was carried out according to the Rockland method. Western blotting: electrophoresis was performed according to the Laemmli (Laemmli, 1970) method, under denaturing conditions in a gel with 12% acrylamide. Before introducing samples into the gel, the concentration was equalized to 100 mg/ml of total protein by the BCA method with NanoDrop1000, Thermo Fisher Scientific. The transfer of proteins to the PVDF membrane was performed in the Immobilon®-P system (Merck Millipore) using a tank blotting chamber (Bio-Rad) by the wet method according to the manufacturer's recommendations. Antibodies used for immunoblotting: mouse monoclonal a-beta-actin (C4) HRP, (Santa Cruz Biotechnology, sc-47778), GluA2 Polyclonal rabbit antibody (Synaptic System, 182 103), Anti-RED 1 antibody (Abcam, ab64830), secondary goat pAb to rabbit (HRP), Abcam, ab97080).

Results

Hyaluronidase treatment led to the complete elimination of perineuronal nets of the hyaluronan-based extracellular matrix that was confirmed by fluorescent staining against aggrecan (Fig. 1). According to obtained results, ADAR2 protein mislocalized upon hyaluronidase treatment (Fig. 2) – its localization changed from nuclei (Fig. 2A, 2B) to the cytoplasm (Fig. 2C).

Data of gene expression showed (Fig. 3) that upon hyaluronidase treatment, after 2 days ADAR2 gene expression is increased in comparison with the control group; however, after 9 days, there were no significant changes.

Sequencing of Q/R editing site revealed that in all the groups Q/R site was edited (Fig. 4): in normal conditions, the correctly-edited isoform of GluA2 transcript CAG during development is substituted by CGG (Arg), that provides Ca^{2+} impermeable channel. In case of a defect of transformation CAG codon (glutamine), stays unchanged which leads to uncontrollable Ca^{2+}- permeability. This process is inherent in a variety of neurodegenerative conditions, including amyotrophic lateral sclerosis, ischemia etc.

It was assumed that the application of hyaluronidase would cause the disruption of transformation and preservation of CAG, but the results of sequencing revealed a correctly edited form of the Q/R site.

Analysis of protein levels by Western Blotting showed that on the second and ninth days of cultivation, the level of $ADAR^{-2}$ in the cytoplasmic fraction did not differ significantly after hyaluronidase treatment. However, in the nuclear fraction, its concentration on the second day after the addition of hyaluronidase increases in comparison with the control (Fig. 5a), and on the ninth day, the ratio equates (Fig. 5b).

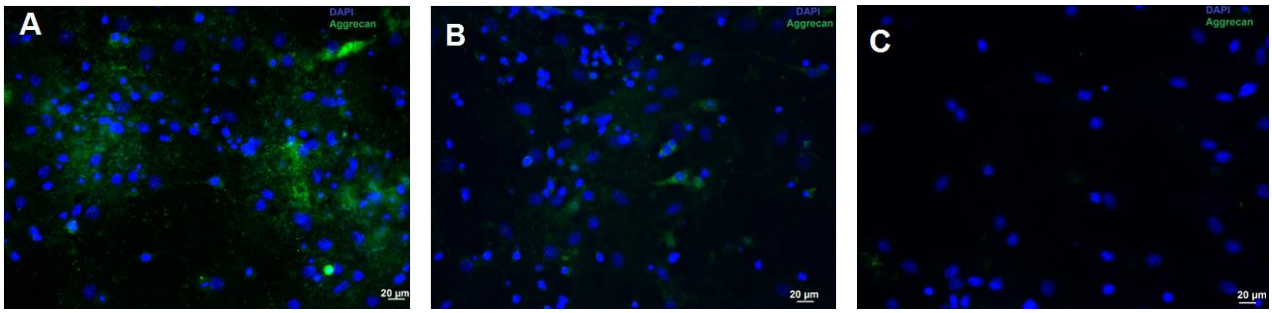

Fig. 1. A – perineuronal nets in intact neuronal culture after 2 days of experiment; B – perineuronal nets in control group (with addition of phosphate-buffered saline); C – elimination of perineuronal nets upon hyaluronidase treatment. Scale bar - 20 µm. Aggrecan - hyaluronic acid binding protein. DAPI – 4′,6-diamidino-2-phenylindole, nuclear fluorescent stain. Magnification 40x

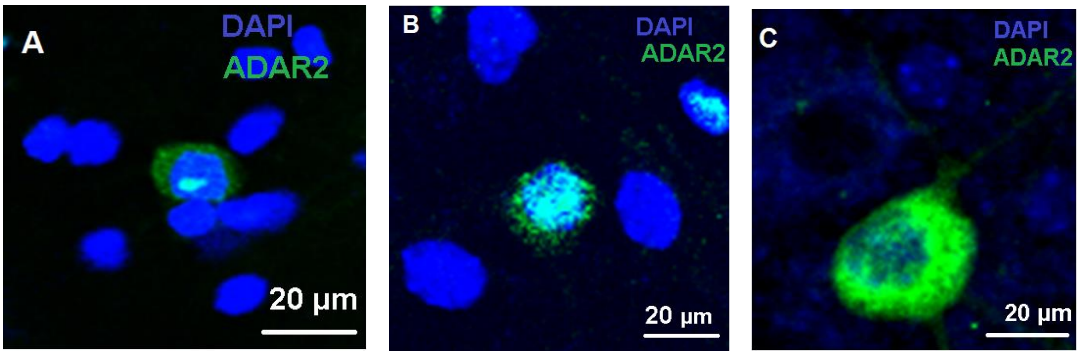

Fig. 2. A – intracellular localization of ADAR2 protein in intact neuronal culture after 2d of hyaluronidase treatment; B – intracellular localization of ADAR2 protein in control (with addition of phosphate-buffered saline) neuronal culture 2d of hyaluronidase treatment; C – intracellular localization of ADAR2 protein in experimental (with addition of hyaluronidase) neuronal culture 2d of hyaluronidase treatment. Scale bar – 20 μm, ADAR2 – adenosine deaminase acting on RNA2, DAPI – 4′,6-diamidino-2-phenylindole, nuclear fluorescent stain. Magnification 40x

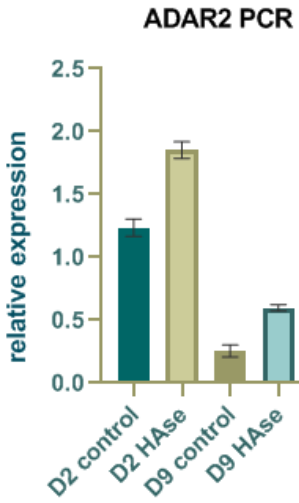

Fig. 3 Quantitative real-time PCR analysis of ADAR2 gene in neuronal cultures after 9 days of hyaluronidase treatment. Graph bars depict mean expression + SD levels of ADAR2 relative to the internal reference (Actin beta). Relative expression measured by ΔΔCt method

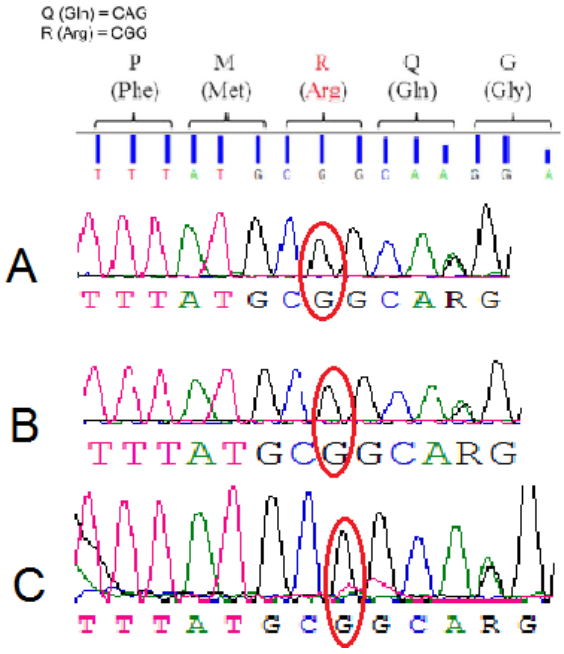

Fig. 4. Representative electropherogram of GluA2 Q/R editing-site sequence analysis. A – intact group after 2 days of experiment; B – control group after 2 days of experiment; C – HAse group after 2 days of experiment. Red circle shows editable nucleotides at Q/R editing-site

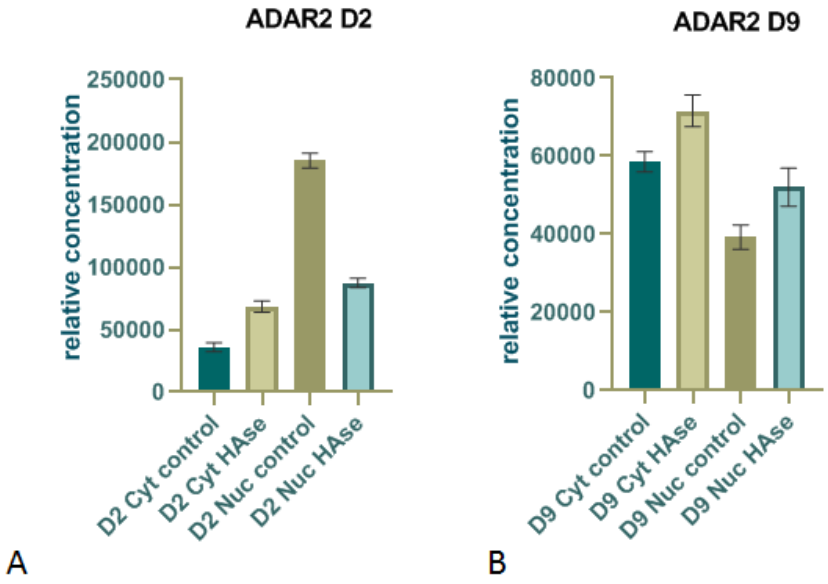

Fig. 5. Change of relative level of ADAR2 in cytoplasmic (Cyt) and nuclear (nuc) fractions of cells on the 2nd (D2) (Fig. A) and 9th (D9) (Fig. B) days after hyaluronidase (HAse) treatment

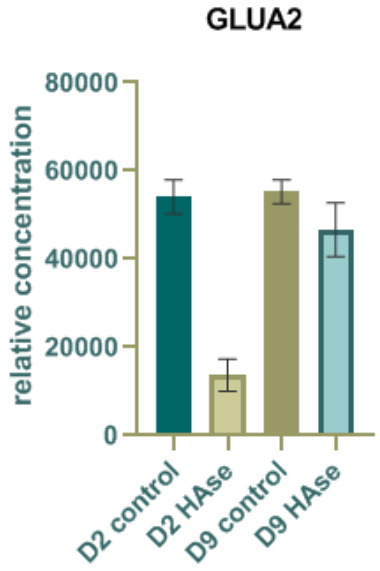

Fig. 6. Change of relative level of expression of GLUA2 in cell culture on the 2nd (D2) and 9th (D9) days after hyaluronidase (HAse) treatment

The level of GLUA-2 after the addition of hyaluronidase decreases in comparison with the control, and on the second day after the addition, this effect is more pronounced than on the ninth day (Fig. 6).

The redistribution of ADAR-2 between cell fractions, more pronounced on the second day after the addition of hyaluronidase than on the ninth day, is consistent with the PCR results showing an increase in expression by 1.5 times on the second day with a subsequent return to the initial value on the ninth day after cultivation.

Discussion

In response to enzymatic hyaluronan digestion, there is elevated calcium and electrical activity that have been found in works (Kochlamazashvili *et al.*, 2010; Vedunova *et al.*, 2013), which can subsequently activate Ca-dependent enzymes that can destroy nucleo-cytoplasmic transport because, in this study, we can see that

there is a problem of localization of the nuclear protein ADAR2. Such phenomena were described by Yamashita (Yamashita & Kwak, 2019) – where elevated Ca signaling through the unedited AMPA receptors cause activation of Ca-dependent protease calpain that disrupts nuclepore complex. In the work of Faustino (Faustino *et al.*, 2016), it was shown that calreticulin deficiency, i.e., calcium-binding deficiency, can cause abnormal nucleopore formation.

Even with the fact that there are no disturbances in RNA-editing processes upon hyaluronidase addition, understanding what exactly causes the problem of mislocalization of nuclear proteins should be elicited.

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