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Down-regulation of Homer1b/c expression protects cultured neurons after traumatic injury[☆]

Weidong Huang¹, Xiaobin Liu¹, Zhou Fei², Yuelin Zhang¹, Jun Yang¹

¹ Department of Neurosurgery, Shaanxi Provincial People's Hospital, Xi'an 710068, Shaanxi Province, China

² Department of Neurosurgery, Xijing Hospital, the Fourth Military Medical University of Chinese PLA, Xi'an 710032, Shaanxi Province, China

Abstract

Activation of metabotropic glutamate receptor 1a aggravates traumatic brain injury. The constitutively expressed protein Homer1b/c participates in delivering and anchoring metabotropic glutamate receptors in neurons. Here, we aimed to verify whether down-regulation of Homer1b/c by RNA interference could protect cultured rat cortical neurons from traumatic injury. We showed that 36 hours after transfection of Homer1b/c small interfering RNA, metabotropic glutamate receptor 1a was present only in the neuronal cytoplasm, but not in the dendrites. Calcium fluorescence intensity was also decreased significantly. Moreover, lactate dehydrogenase concentration was significantly decreased in Homer1b/c small interfering RNA-transfected cells compared with that in untransfected and control small interfering RNA-transfected cells 24 hours after traumatic neuronal injury. Our findings indicate that down-regulation of Homer1b/c could reduce metabotropic glutamate receptor 1a transfer from the cell body to the dendrite, relieve calcium overload, and protect neurons from traumatic injury.

Weidong Huang[☆], Ph.D., Associate professor, Department of Neurosurgery, Shaanxi Provincial People's Hospital, Xi'an 710068, Shaanxi Province, China

Corresponding author: Weidong Huang, Department of Neurosurgery, Shaanxi Provincial People's Hospital, Xi'an 710068, Shaanxi Province, China
huangwd73@sina.com

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Key Words

Homer; neuron; metabotropic glutamate receptor 1; RNA interference; calcium ion; lactate dehydrogenase; neuroprotection; neural regeneration

Research Highlights

Down-regulation of Homer1b/c expression could reduce metabotropic glutamate receptor 1a transfer from the neuronal cell body to the dendrite, relieve calcium overload, and decrease lactate dehydrogenase activity, thus protecting neurons against the secondary effects of traumatic injury.

Abbreviations

FIU, fluorescence intensity unit; IP₃R, 1,4,5-inositol triphosphate receptor; LDH, lactate dehydrogenase; mGluR, metabotropic glutamate receptor; siRNA, small interfering RNA

INTRODUCTION

Homer proteins belong to one of the post-synaptic density protein families and several splice variants of the *Homer* gene have been identified recently^[1-2]. This family comprises two major groups: the short-form protein (Homer1a) and the long-form proteins, including

Homer1b, Homer1c, Homer2, and Homer3^[3-5]. All the family members contain a highly conserved amino-terminal Ena/VASP homology 1 domain, which functions as a protein-protein interaction motif binding to the PPXXF consensus sequence that is present in the carboxy-terminus of the group I metabotropic glutamate

receptors (mGluRs)^[1, 5-7]. All long-form Homer proteins have a coiled-coil structure within their carboxy-terminal regions and can form homomeric and heteromeric oligomers^[3, 5, 8]; the short-form protein Homer1a can not multimerize because it lacks the coiled-coil structure^[1].

In our previous work, we showed that traumatic stimulation induced Homer1a expression, but had no effect on the expression of Homer1b/c^[9]. Dynamically expressed Homer1a and constitutively expressed Homer1b/c might modulate the distribution and function of group I mGluRs after traumatic neuronal injury. Homer1b/c may increase the cell surface expression of group I mGluRs and can link group I mGluRs to the 1,4,5-inositol triphosphate receptor (IP₃R) at the post-synaptic density, which is related to intracellular calcium release. Our previous studies also suggested that the group I mGluRs increased significantly and resulted in cellular excitotoxicity after diffuse brain injury^[10].

What role does Homer1b/c play in neuronal traumatic injury? Does decreased expression of Homer1b/c affect the accumulation of group I mGluRs at the post-synaptic density? Can neurons with low levels of Homer1b/c gene expression survive traumatic insult? Here, we used RNA interference to down-regulate Homer1b/c gene expression, and clarified the effect of Homer1b/c on mGluR1a distribution and neuronal survival after traumatic injury.

RESULTS

Homer1b/c small interfering RNA (siRNA) transfection inhibited Homer1b/c expression in cultured cortical neurons

siRNA-mediated down-regulation of Homer1b/c was detected by reverse transcription-PCR and western blot. The reverse transcription-PCR amplicons of Homer1b/c and β -actin were 672 bp and 249 bp long, respectively. The intensity of the 672-bp band was significantly lower in Homer1b/c siRNA-transfected cells than in untransfected cells ($P < 0.05$; Figure 1). There was no change in the intensity of the 249-bp β -actin band (Figure 1). These results proved that the expression of the Homer1b/c gene was significantly inhibited 36 hours after Homer1b/c siRNA transfection. Similar results were obtained by western blot: the intensity of the 47-kDa Homer1b/c band was decreased significantly in Homer1b/c siRNA-transfected cells ($P < 0.05$), but not in control cells; the expression of β -actin was unchanged (Figure 2).

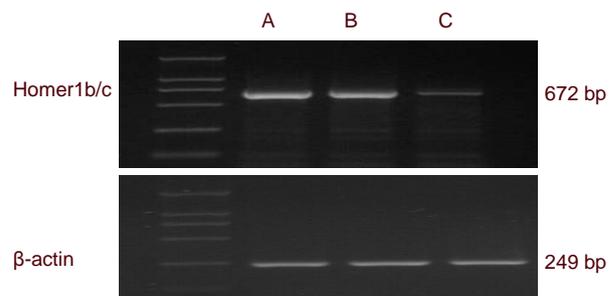


Figure 1 Reverse transcription-PCR analysis of Homer1b/c mRNA expression.

The intensity of the 672-bp band in the Homer1b/c siRNA-transfected group (C) was lower than that in the untransfected (A) and the control siRNA-transfected (B) groups. No change in the 249-bp β -actin band was observed among the groups. siRNA: Small interfering RNA.

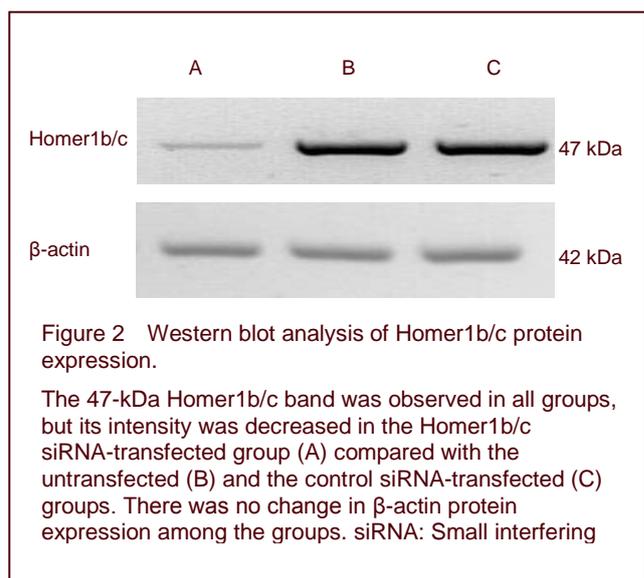


Figure 2 Western blot analysis of Homer1b/c protein expression.

The 47-kDa Homer1b/c band was observed in all groups, but its intensity was decreased in the Homer1b/c siRNA-transfected group (A) compared with the untransfected (B) and the control siRNA-transfected (C) groups. There was no change in β -actin protein expression among the groups. siRNA: Small interfering

Altered mGluR1a neuronal distribution after Homer1b/c siRNA transfection

In untransfected and control siRNA-transfected cells, mGluR1a was distributed in the dendrites and cytoplasm of neurons. In contrast, in Homer1b/c siRNA-transfected cells, mGluR1 was observed in the neuronal cytoplasm only (Figure 3). There were no differences among the groups in terms of neurofilament 200 staining (Figure 3).

Homer1b/c siRNA transfection reduced intraneuronal calcium concentration

The calcium fluorescence intensity in the untransfected and control siRNA-transfected groups was 51.8 ± 7.5 fluorescence intensity units (FIU) and 50.4 ± 6.0 FIU, respectively. In the Homer1b/c siRNA-transfected group, the fluorescence intensity was significantly lower at 23.4 ± 2.8 FIU ($P < 0.05$; Figure 4).

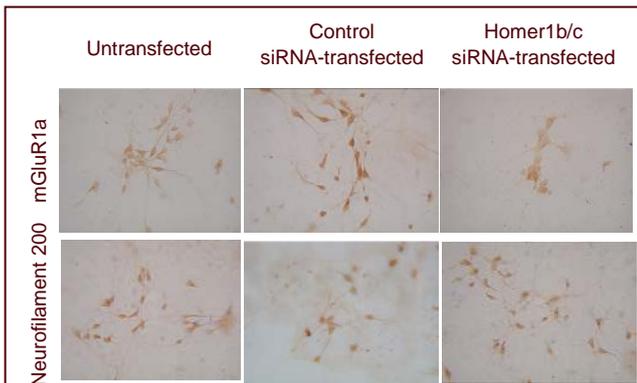


Figure 3 mGluR1a and neurofilament 200 immunohistochemical staining in neurons (inverted microscope, $\times 250$).

mGluR1a was distributed in the dendrites and cytoplasm of neurons in the untransfected and control siRNA-transfected cells, but it was observed in the neuronal cytoplasm only in the Homer1b/c siRNA-transfected group. There were no differences among the groups in terms of neurofilament 200 immunohistochemical staining.

siRNA: Small interfering RNA; mGluR: metabotropic glutamate receptor.

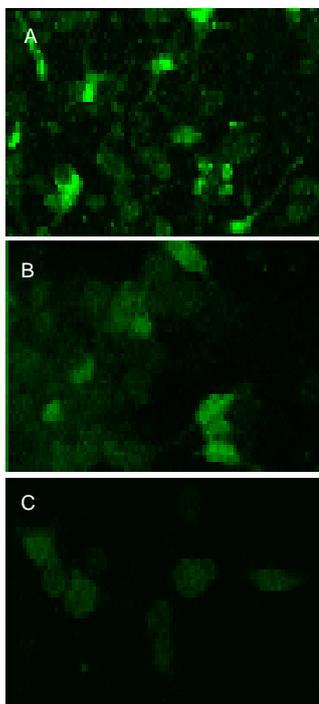


Figure 4 Fluo-3-AM staining in neurons (confocal microscope, $\times 400$).

The fluorescence intensity of calcium in the untransfected group (A) and the control siRNA-transfected group (B) was greater than that in the Homer1b/c siRNA-transfected group (C). siRNA: Small interfering RNA.

Homer1b/c siRNA transfection reduced lactate dehydrogenase (LDH) activity in neurons after traumatic injury

LDH activity

LDH concentration can be used as a marker of cell viability. Before mechanical injury, there was no significant difference in LDH concentration among the untransfected, control siRNA-transfected, and Homer1b/c siRNA-transfected groups ($P > 0.05$; Table 1). In contrast, 24 hours after traumatic neuronal injury, the LDH concentration was increased significantly in the untransfected and control siRNA-transfected groups compared with in the Homer1b/c siRNA-transfected group ($P < 0.05$; Table 1).

Table 1 Lactate dehydrogenase concentration (U/L) in untransfected, control siRNA-transfected, and Homer1b/c siRNA-transfected cells

Group	Before treatment	24 hours after treatment
Untransfected	78.4 \pm 19.5	158.4 \pm 23.0 ^a
Control siRNA-transfected	76.2 \pm 18.1	145.6 \pm 25.1 ^a
Homer1b/c siRNA-transfected	81.0 \pm 21.3	93.2 \pm 20.1

Data are expressed as mean \pm SEM ($n = 6$). By one-way analysis of variance, $F = 17.1$; $df = 2, 15$; ^a $P < 0.05$, vs. Homer1b/c siRNA-transfected group. siRNA: Small interfering RNA.

DISCUSSION

RNA interference is one of the most exciting discoveries of the past decade in functional genomics, and it is now an important research tool for gene silencing^[11-12]. Here, we used RNA interference to inhibit Homer1b/c expression in cultured cortical neurons, to examine the effects of reduced Homer1b/c on mGluR1 distribution, intraneuronal calcium concentration, and LDH activity. First, we demonstrated that down-regulation of Homer1b/c caused reduced mGluR1 in dendrites. Therefore, Homer1b/c may play an important role in the trafficking of mGluR1a from the neuronal cytoplasm to the dendrites, and act as an anchoring protein. Hayashi *et al*^[13] proposed that the HomerShank complex serves as a structural framework and as an assembly platform for other post-synaptic density proteins. In neurons lacking Homer1b/c, mGluR1a could not be delivered to the dendrites and efficiently anchored at the post-synaptic density^[14]. It has been shown that, when cells were transfected with both Homer1c and mGluR1a, more mGluR1a and Homer1c were associated with the cell surface compared with in cells transfected with mGluR1a alone^[15]. Cell surface immunostaining of mGluR1a showed that Homer1c could induce clustering of this receptor on the plasma membrane of HEK-293 cells^[16]. Our results are consistent with these previous

findings.

Second, we found a reduced calcium concentration in neurons after Homer1b/c siRNA transfection. This indicates that Homer1b/c functions in intracellular calcium release. Homer1b/c binds to mGluRs and IP₃Rs at the synapse^[17-18]. Decreased Homer1b/c expression results in reduced formation of these complexes, and low efficiency of second messenger IP₃ transduction^[19]; the intracellular calcium pool release would decrease accordingly. Homer1b/c participates in intracellular calcium release and is an important regulatory molecule in the mGluR1a-IP₃R-Ca²⁺ signal transduction pathway^[20]. Furthermore, a recent report has shown that Homer proteins have a novel high-affinity ligand that modulates ryanodine receptor Ca²⁺ release channels through an EVH1 domain that binds to proline-rich regions in its target proteins. Homer anchors proteins embedded in the surface membrane to the Ca²⁺ release channels in the endoplasmic reticulum and thereby anchors membrane or cytosolic proteins to the cytoskeleton^[21]. Therefore, the decreased calcium concentration in neurons after Homer1b/c down-regulation may partly result from ryanodine receptor Ca²⁺ release channel dysfunction.

The *in vitro* traumatic neuronal injury model has been shown to be highly reproducible and to induce consistent degrees of injury^[9]. In our results, neuroprotective effects were observed by decreasing Homer1b/c expression in neurons, which are threatened by secondary insults after traumatic injury. There are several possible mechanisms for this. First, insufficient Homer1b/c synthesis may cause retention of mGluR1a in the cytoplasm, which would lead to low mGluR1a expression on the neuronal surface. Second, without Homer1b/c, mGluR1a and IP₃R cannot form a complex at the synapse, thus impairing signal transduction. Third, down-regulation of Homer1b/c protects cortical neurons against excitotoxic injury by antiapoptotic activity. These neuroprotective effects are partly dependent on attenuating intracellular calcium-dependent intracellular reactive oxygen species production and improving endoplasmic reticulum and mitochondrial function^[22-23]. In conclusion, our results suggest that Homer1b/c down-regulation could protect neurons from excitatory toxic damage after primary traumatic injury.

MATERIALS AND METHODS

Design

A controlled observational study regarding cell biological research.

Time and setting

The experiments were conducted at the Department of Neurosurgery, Shaanxi Provincial People's Hospital, China, from January 2010 to December 2010.

Materials

Embryonic day 14 rats were provided by the Experimental Animal Center of the Fourth Military Medical University of the Chinese PLA, China (No. SCXK (Jun) 2002-006). The experimental procedures were carried out in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals* issued by the Ministry of Science and Technology of China^[24].

Methods

Isolation and culture of cortical neurons

Cortical neuronal cultures were derived from rat embryonic cortices. Morphology and immunocytochemistry (immunoperoxidase staining of neurofilament 200) were used to illustrate the cell type being cultured, as described previously^[9]. All experiments were performed on cultures at 7–10 days *in vitro*.

Homer1b/c siRNA transfection of primary cultured neurons

Homer1b/c siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) transfection into primary cultured neurons was performed according to the manufacturer's protocol^[22]. One day before the transfection, the media was aspirated and the cells were washed with sterile PBS. Antibiotic-free Dulbecco's modified Eagle's medium (Santa Cruz Biotechnology) supplemented with 20% fetal bovine serum was then added to the cultured neurons. Solutions A and B were prepared as follows (these volumes are suitable for a 24-well plate with a cell growth area of 2 cm², and should be adjusted as appropriate). Solution A: 3.6 μL of 10 μM Homer1b/c siRNA was added to 60 μL siRNA Transfection Medium (Santa Cruz Biotechnology). Solution A was mixed gently and kept at room temperature for 5 minutes. Solution B: 3.6 μL of siRNA Transfection Reagent (Santa Cruz Biotechnology) was added to 14.5 μL of siRNA Transfection Medium. Solution B was mixed gently and kept at room temperature for 5 minutes. After the required 5-minute incubation, Solutions A and B were combined to form the siRNA-siRNA Transfection Reagent Complex, which was mixed gently and incubated at room temperature for 20 minutes. The complex was added drop-wise to the culture dish wells with gentle rocking. The transfected neurons were incubated at 37°C for 30 hours. Cells in the untransfected control group were treated identically except no transfection reagents were added. The control siRNA-transfected cells were also treated identically

except that a control siRNA (si-Con; Santa Cruz Biotechnology), which has no homology to any known sequences in mouse, rat, or human RNA, was used.

Reverse transcription-PCR detection of Homer1b/c mRNA expression

Thirty hours after transfection, total RNA was extracted from the cultured neurons using Trizol (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocols. The forward and reverse primers were as follows. Homer1b/c: sense primer 5'-TTC AGC CAT CAG CAA ACA CT-3', antisense primer 5'-CCA ATT TTC AAA ACA CCC TCC-3'; β -actin: sense primer 5'-AAC CCT AAG GCC AAC CGT GAA AAG-3', antisense primer 5'-TCA TGA GGT AGT CTG TCA GGT-3'. The amplification conditions were: 5 minutes at 94°C; 35 cycles of 45 seconds at 94°C, 1 minute at 56°C, 1 minute at 72°C; followed by 10 minutes at 72°C. The experiments were repeated five times and the band intensities were quantified using an Image Analysis System (MiVnt, Shanghai, China).

Western blot detection of Homer1b/c protein expression

Proteins were extracted from neurons 30 hours after Homer1b/c siRNA transfection, electrophoresed, and transferred to a nitrocellulose membrane by electroblotting. The nitrocellulose membrane was incubated with primary antibody (polyclonal goat anti-Homer1b/c antibody, 1:50; Santa Cruz Biotechnology; or monoclonal goat anti-rat β -actin antibody, 1:50; Sigma, St. Louis, MO, USA) at 4°C for 12 hours, then was incubated with horseradish peroxidase-conjugated rabbit anti goat IgG (1:50; Santa Cruz Biotechnology) at room temperature for 2 hours. Signal was detected using Chemiluminescence Luminol Reagent (Boster, Wuhan, China). The band intensities were quantified using an Image Analysis System (MiVnt).

Immunocytochemical staining of mGluR1a

The expression of mGluR1a was analyzed by immunocytochemistry using a goat anti-rat mGluR1a monoclonal antibody (1:200; Sigma) 36 hours after Homer1b/c siRNA transfection. Neurofilament 200 was detected as a control, using a goat anti-rat neurofilament 200 monoclonal antibody (1:200; Sigma).

Intraneuronal calcium concentration

Calcium concentration was measured with a laser scanning confocal microscope 36 hours after *Homer1b/c* siRNA transfection^[22]. Briefly, 10 μ M Fluo-3-AM (Sigma)

was incubated with neurons for 30 minutes and then the plate was washed for three minutes with D-Hanks solution. Five to eight neurons were randomly selected to test the calcium fluorescence intensity by laser scanning confocal microscopy (Bio-Rad, Hercules, CA, USA).

Establishment of traumatic injury model in cultured neurons

Neuronal injury was induced according to the method of Faden *et al*^[25]. In brief, traumatic injury was induced in neuronal cultures using a punch device that consists of 28 joined stainless steel blades; these produce parallel cuts, 1.2 mm long, which are uniformly distributed throughout the cell layer. These cuts cause immediate death to the neurons under the blade, and are followed by secondary insults at a distance from the cuts. The experiments were performed 24 hours after traumatic injury.

Evaluation of cell viability

Cell viability was evaluated by LDH concentration in the neuronal culture medium, before and 24 hours after injury. LDH concentration can accurately reflect neuronal cell death in this model^[25]. The LDH levels were evaluated using an automatic biochemical analysis system (Hitachi, Tokyo, Japan). In brief, the sample was incubated with 2.0 mL reagent A (100 mM Tris- hydroxymethylamino-methane buffered solution, 100 mM potassium chloride, 52 mM L-lithium lactate) at 37°C for 5 minutes, then 0.6 mL NAD⁺ was added, and the mixture was kept at 37°C for further 1 minute.

Statistical analysis

Data were expressed as mean \pm SEM, and were analyzed by one-way analysis of variance using SPSS 9.0 statistical software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

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Author contributions: Weidong Huang was responsible for the experimental design and wrote the article. Xiaobin Liu contributed to the experimental research. Zhou Fei participated in the experimental design and direction. Yuelin Zhang performed the statistical analysis. Jun Yang conducted the experimental research.

Conflicts of interest: None declared.

Ethical approval: The experimental procedures followed the guidelines for animal care and experiments of the Shaanxi Provincial People's Hospital, China.

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