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Original Paper

Down-Regulation of Homer1b/c Protects Against Chemically Induced Seizures Through Inhibition of mTOR Signaling

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Key Words
Epilepsy • Homer • mGluRs • mTOR

Abstract
Background: Homer is a family of post synaptic density proteins functionally and physically attached to target proteins at proline-rich sequences. Reducing Homer1b/c expression has been shown in previous studies to be protective against excitotoxic insults, implicating Homer1b/c in the physiological regulation of aberrant neuronal excitability. ***Methods:*** To test the efficacy of a Homer1b/c reducing therapy for disorders with a detrimental hyperexcitability profile in mice, we used small interfere RNA (siRNA) to decrease endogenous Homer1b/c expression in mouse hippocampus. The baseline motor and cognitive behavior was measured by sensorimotor tests, Morris water maze and elevated plus maze tasks. The anti-epileptic effects of Homer1b/c knockdown were determined in two chemically induced seizure models induced by Picrotoxin (PTX) or pentylenetetrazole (PTZ) administration. ***Results:*** The results of sensorimotor tests, Morris water maze and elevated plus maze tasks showed that Homer1b/c reduction had no effect on baseline motor or cognitive behavior. In two chemically induced seizure models, mice with reduced Homerb/c protein had less severe seizures than control mice. Total Homer1b/c protein levels and seizure severity were highly correlated, such that those mice with the most severe seizures also had the highest levels of Homer1b/c. In addition, the phosphorylation of mammalian target of rapamycin (mTOR) and its target protein S6 was significantly inhibited in Homer1b/c down-regulated mice. Homer1b/c knockdown-induced inhibition of mTOR pathway was partially ablated by the metabotropic glutamate receptor 5 (mGluR5) agonist CHPG. ***Conclusion:*** Our results demonstrate that endogenous Homer1b/c is integral for regulating neuronal hyperexcitability in adult animals and suggest that reduction of Homer1b/c could protect against chemically induced seizures through inhibition mTOR pathway.

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Introduction

Epilepsy is one of the most common serious neurological disorders that affect approximately 65 million people worldwide [1]. People with epilepsy are at an increased risk of death, which is between 1.6 and 4.1 fold greater than that of the general population. Most current treatments are symptomatic therapies that suppress seizures without preventing the initial development or progression of epilepsy [2]. Although over twenty anti-seizure medications exist and are effective in some cases, more than one-third of patients with epilepsy are intractable to all medications [3].

The Homer family proteins are divided into the short form, Homer1a, and long forms that include Homer1b/c, Homer2 and Homer3 [4]. These recently discovered postsynaptic scaffolding proteins function as molecular adaptors that play a central role in Ca^{2+} signaling. They share a conserved N-terminal Ena/VASP homology 1 (EVH1) domain which acts as a protein-protein interaction motif to bind a proline-rich consensus sequence (PPXXFR) in various other scaffolding and signal transduction molecules, including metabotropic glutamate receptors (mGluRs) and 1,4,5-trisphosphate receptors (IP_3Rs), and assembles these signaling proteins into complexes [5]. Within Homer1 proteins, Homer1b/c can self-multimerize through a C-terminal coiled-coil (CC) domain and function as scaffolds of multi-protein complexes and mediators of group I mGluRs signaling. In contrast, Homer1a, which lacks the dimerization domain, can competitively bind to Homer1b/c-targeted proteins and behave as a dominant negative [6]. Substantial preclinical investigations have implicated that each of these Homer 1 variants are associated with the etiology of many neurological diseases, such as chronic pain, mental retardation syndromes, Alzheimer's disease (AD), Parkinson's disease (PD), schizophrenia, drug-induced addiction, and traumatic brain injury (TBI) [7, 8]. More recently, downregulation of Homer1b/c was shown to inhibit glutamate induced excitotoxicity and protect against traumatic neuronal injury in cortical neurons [9, 10]. However, no study to date has investigated the role of Homer1b/c in epileptic seizures.

In the present study, we tested directly the effects of reducing Homer1b/c expression on baseline behavior and chemically induced seizure severity. We also investigated the potential underlying molecular mechanism with focus on mGluR5 activity and the mammalian target of rapamycin (mTOR) pathway.

Materials and Methods

Cell culture

PC12 cells were purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibicon) plus 10% foetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% antibiotics (penicillin/streptomycin, Sigma) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was changed every 2-3 days.

RNA interference transfection

Double-stranded small interfering RNA (siRNA) transfection was used to down- regulate Homer1b/c expression *in vitro*. All siRNAs were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). PC12 cells were transfected with Si-H1b/c (Homer1b/c siRNA) using Lipofectamine RNAiMAX transfection Immunofluorescence reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). A control siRNA (Si-con), which has no homology to any known sequence, was used in parallel experiments. For *in vivo* knockdown, 50 μg Si-H1b/c or Si-con was infused into hippocampus by using a stereotaxic injection (Fig. 1). The mRNA and protein expression levels were measured 72 h after transfection.

Real-time RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen). After the equalization of the RNA quantity in each group, the mRNA levels were quantitated using a Bio-Rad iQ5 Gradient Real-Time PCR system (Bio-Rad Laboratories), and GAPDH was used as an endogenous control. Primers for all Real-Time PCR experiments were listed as follow: Homer1a: forward: 5'-GTGTCCACAGAAGCCAGAGAGGG-3', reverse:

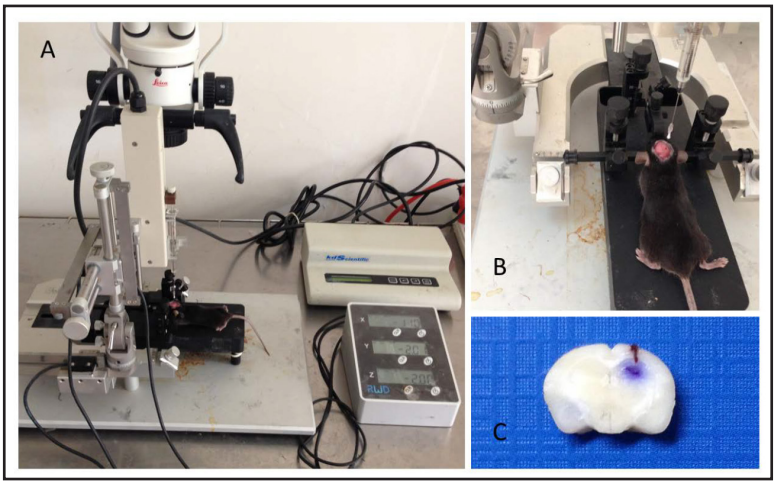

Fig. 1. *In vivo* transfection of Si-con or Si-H1b/c. A, The stereotaxic apparatus used for *in vivo* transfection. B, Injection into right hippocampus in mouse. C, Coronal sections of mouse brain showing the injection site.

5'-CTTGT -AGAGGACCCAGCTTCAGT-3'; Homer1b/c: forward: 5'-TCCGTCTAGCAGCCAAGC-3', reverse: 5'-TCTGTTGACGGTATTTCCTGTT-3'; GAPDH: forward: 5'-AAGGTGAAGGTCGGA -GTCAA-3', reverse: 5'-AATGAAGGGGTCATTGATGG-3'. Samples were tested in triplicates and data from five independent experiments were used for analysis.

Sensorimotor battery and 1 h locomotor activity

All mice were evaluated on a battery of sensorimotor tests designed to assess balance, strength, coordination and initiation of movement as described previously [11]. Locomotor activity was evaluated in all mice over a 1 h period using computerized photobeam instrumentation. General activity variables and indices of emotionality, including time spent, distance traveled, and entries made in a 33 × 11 cm central zone, were analyzed.

Morris water maze

Spatial learning and memory were evaluated in the Morris water maze as described previously [12]. The protocol included cued, place, and probe trials and all trials were performed in a 120-cm-diameter pool filled with opaque water. Escape path length, latency, and swimming speeds were calculated for the cued and place trials. Spatial bias for the target quadrant was analyzed by comparing the time spent in it versus the times spent in each of the other quadrants.

Elevated plus maze

As described previously [11], the elevated plus maze (EPM) apparatus is a four-arm maze shaped like a plus sign. One set of the opposing arms have walls (closed arms) and the other set is not enclosed (open arms). The number of entries made, time spent, and distance traveled in each set of arms were quantified. These three variables were also analyzed after normalizing the values to reflect percentages calculated out of the totals measured in both sets of arms.

Picrotoxin and pentylenetetrazole seizures

In vivo picrotoxin (PTX, Invitrogen) reverse microdialysis experiments from awake and freely moving mice were developed with modifications to the previously described method [11]. Pentylenetetrazole (PTZ, Invitrogen) was dissolved in sterile PBS at a concentration of 5 mg/ml. A dose of 80 mg/kg was delivered intraperitoneally for the experiments shown in Fig. 3C-3E. Each mouse was videotaped for 15 min to score seizure severity.

EEG recording and seizure severity scores

A previously published protocol was used to record EEG activity [11]. EEG spike frequency was assessed for the last 60 min and normalized to basal EEG of each mouse. The seizure severity score used was as follows: 0 = normal behavior; 1 = immobility; 2 = spasm, tremble, or twitch; 3 = tail extension; 4 = forelimb clonus; 5 = generalized clonic activity; 6 = jumping or running seizures; 7 = full tonic extension; and 8 = death.

Western blot analysis

Equivalent amounts of protein (40 μg per lane) were loaded and separated by 10% SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk solution in tris-buffered saline with 0.1% Triton X-100 (TBST, Sigma) for 1 h, and then incubated overnight at 4°C with the primary Homer1a antibody (1:200, Santa Cruz), Homer1b/c (1:800, Santa Cruz), p-mTOR (1:500, Cell Signaling), mTOR (1:1000, Cell Signaling), p-S6 (1:500, Cell Signaling), S6 (1:1000, Cell signaling), mGluR5 (1:800, Abcom) or β-actin (1:600, Cell Signaling) antibody dilutions in TBST. After that the membranes were washed and incubated with secondary antibody for 1 h at room temperature. Immunoreactivity was detected with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). An analysis software named Image J (Scion Corporation) was used to quantify the optical density of each band.

Statistical analysis

Statistical analysis was performed using SPSS 16.0, a statistical software package. Statistical evaluation of the data was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Down-regulation of Homer1b/c expression in vivo

To determine the functional effects of reducing Homer1b/c mRNA and protein *in vivo*, Homer1b/c specific targeted siRNA (Si-H1b/c) and a control siRNA (Si-con), which should not knock down any known proteins, were used in the following experiments. After confirming its ability to reduce Homer1b/c expression in murine PC12 cells (Fig. 2A), we infused 50 μg of Si-H1b/c into the hippocampus of adult mice using saline and Si-con as controls (Fig. 1). Three days after infusion, the hippocampus surrounding the injection site was analyzed for

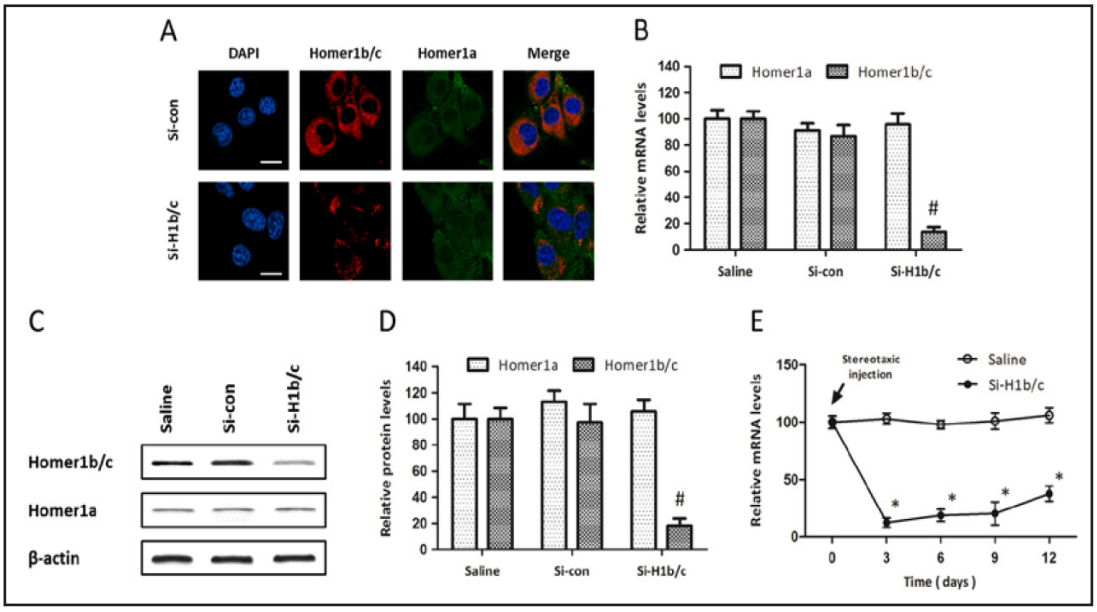

Fig. 2. Down-regulation of Homer1b/c expression *in vivo*. PC12 cells were transfected with Homer1b/c targeted siRNA (Si-H1b/c) or control siRNA (Si-con) for 72 h, and the expression of Homer1b/c (red) and Homer1a (green) were detected by fluorescence staining (A, n=3 times). Bar scale: 20 μM. Mice were infused with saline, Si-con, or Si-H1b/c in the hippocampus (n=8 mice), and the expression of Homer1a and Homer1b/c mRNA were measured by RT-PCR (B, n=3 times). The expression of Homer1a and Homer1b/c proteins were detected by western blot (C) and calculated (D) (n=3 times). Total Homer1b/c mRNA levels were analyzed up to 12 days after infusion (E, n=8 mice). The data are presented as the mean ± SEM. $^{\#}p < 0.05$ vs. Si-con. $^{*}p < 0.05$ vs. Saline.

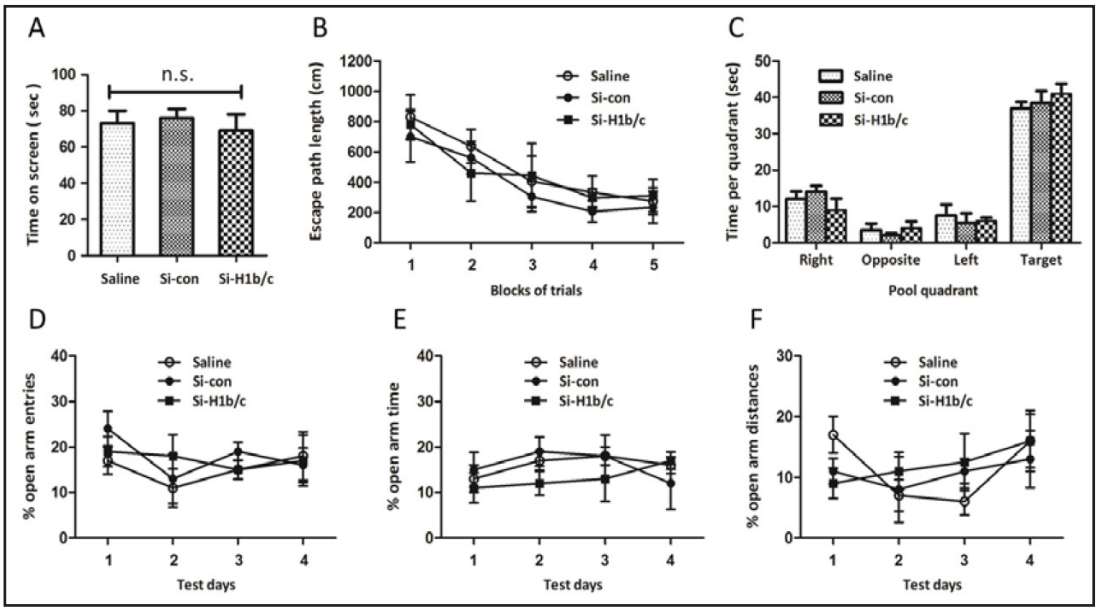

Fig. 3. Knockdown of Homer1b/c does not alter baseline behavior. Mice were infused with saline, Si-con, or Si-H1b/c in the hippocampus for 72 h. All three treatment groups performed similarly on the inverted screen task from the sensorimotor battery (A, n=8 mice). No significant differences were observed among the three treatment groups with regard to performance on the place trials in the Morris water maze (B) or spatial bias for the target quadrant during the probe trial (C) (n=6 mice). The three treatment groups also performed similarly on the EPM in terms of open arm entry percentage (D), percentage time spent in open arms (E), and open arm distance percentage (F) (n=6 mice). The data are presented as the mean ± SEM. n.s., not statistically significant.

Homer1b/c expression using RT-PCR (Fig. 2B) and western blot (Fig. 2C and 2D). Si-H1b/c infused in the hippocampus provided >70% reduction of Homer1b/c mRNA and protein, but had no effects on Homer1a expression. In addition, the results showed that the Homer1b/c mRNA levels remained >70% decreased at 9 days and >50% decreased at 12 days after Si-H1b/c infusion, indicating a long-term target knockdown of Homer1b/c *in vivo.*

Knockdown of Homer1b/c does not alter baseline behavior

Before assessing whether Homer1b/c knockdown can provide protection against seizures, we first analyzed the mice for any gross motor or cognitive abnormalities. Mice with decreased Homer1b/c levels performed similarly to bothsaline and Si-con groups in the inverted screen test (Fig. 3A). The Si-H1b/c infused mice did not exhibit any significant performance deficits on the place (Fig. 3B) or probe (Fig. 3C) trials in the water maze, suggesting that their spatial learning and memory were intact. Analysis of the EPM data also showed that the Si-H1b/c infused mice did not differ in levels of anxiety-related behaviors compared with the saline and Si-con groups, as evidenced by the results of percentage of open arm entries (Fig. 3D), percentage of open arm time (Fig. 3E) and percentage of open arm distance (Fig. 3F).

Reducing Homer1b/c expression protects against chemically induced seizure

The PTX induced seizure model was used to determine whether Homer1b/c reduction is protective against focal seizure, and the results showed that the Si-H1b/c infused mice showed a reduction in normalized spike frequency compared with the saline and Si-con groups (Fig. 4A). The total Homer1b/c protein levels in the left hippocampus of mice were highly correlated with normalized spike frequency (Fig. 4B), indicating a direct correction between lower Homer1b/c protein levels and reduced neuronal hyperexcitability. In addition, we also tested the effects of Homer1b/c reduction in a widely used seizure paradigm:

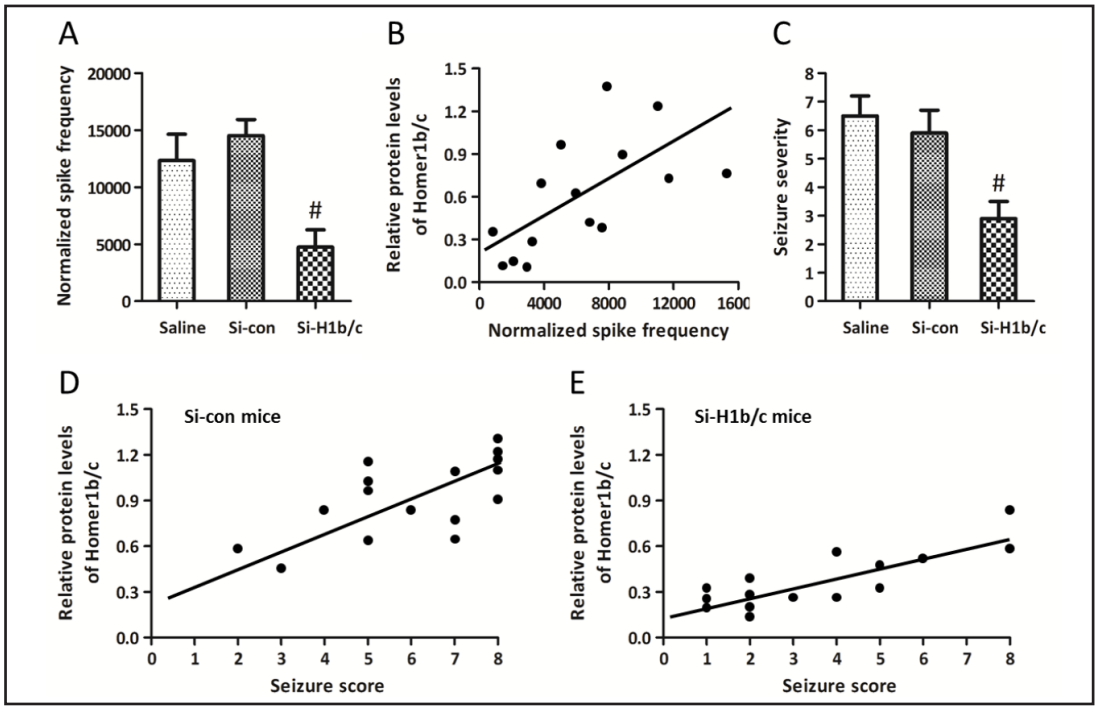

Fig. 4. Reducing Homer1b/c expression protects against chemically induced seizure. PTX were infused into the hippocampus of mice treated with saline, Si-con, or Si-H1b/c, and the spike frequency during the last hour of PTX infusion was calculated (A, n=8 mice). Total Homer1b/c protein levels in the hippocampus were plotted against normalized spike frequency (B, n=8 mice). PTZ (55 mg/kg) was given by intra-peritoneal injection to saline-, Si-con-, and Si-H1b/c-treated mice and final seizure stage was scored blinded (C, n=8 mice). Final seizure stage for each treated mouse plotted against the total Homer1b/c protein levels (3D and 3E) (n=8 mice). The data are presented as the mean ± SEM. $^{\#}p < 0.05$ vs. Si-con.

intraperitoneal PTZ injections. The results showed that those mice treated with Si-H1b/c had less severe seizures than both the saline and Si-con groups (Fig. 4C). The seizure severity and Homer1b/c protein levels correlated well in tested mice (Fig. 4D and 4E), providing evidence in a second inducible seizure model that a reduction in Homer1b/c is protective against seizures.

Knockdown of Homer1b/c inhibits the activation of mTOR signaling

The western blot analysis was used to measure the activation of mTOR pathway after Homer1b/c knockdown (Fig. 5A), and the results showed that phosphorylation of mTOR and S6 was significantly inhibited in Si-H1b/c treated mice (Fig. 5B). We also used CHPG, the selective agonist of mGluR5, to investigate the potential involvement of mGluR5 in Homer1b/c induced regulation of mTOR pathway (Fig. 5C). As a glutamate-site agonist, CHPG treatment did not alter the expression of mGluR5 protein (Fig. 5D). Potentiating the activity of mGluR5 by CHPG partially ablated the inhibition of mTOR pathway activation induced by Homer1b/c knockdown (Fig. 5E and 5F), indicating that Homer1b/c reduction-induced regulation of mTOR pathway might be associated with the attenuation of mGluR5 activity.

Discussion

Using siRNA infusion technology directed against endogenous murine Homer1b/c, the total Homer1b/c mRNA and protein levels were decreased throughout the hippocampus of adult mice, without affecting Homer1a expression. After Homer1b/c was reduced in the

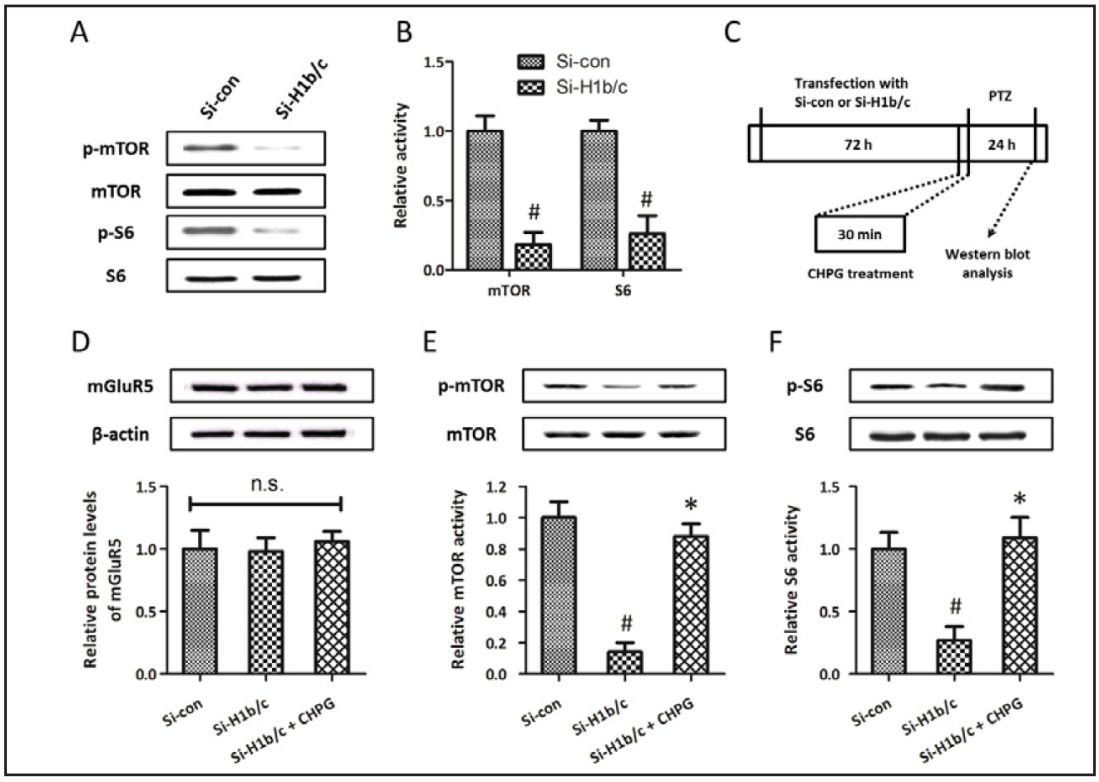

Fig. 5. Knockdown of Homer1b/c inhibits the activation of mTOR signaling. Mice were infused with saline, Si-con, or Si-H1b/c in the hippocampus for 72 h (n=8 mice), and the expression of p-mTOR, mTOR, p-S6 and S6 were detected by western blot (A) and calculated (B) (n=3 times). The mGluR5 agonist CHPG was given by intra-peritoneal injection together with PTZ to saline-, Si-con-, and Si-H1b/c-treated mice (C, n=8 mice). The expression of mGluR5 (D) and phosphorylation of mTOR (E) and S6 (F) were detected by western blot (n=3 times). The data are presented as the mean ± SEM. n.s., not statistically significant. $^{\#}p < 0.05$ vs. Si-con. $^{*}p < 0.05$ vs. Si-H1b/c.

adult mouse, no significant deviations from baseline were observed in a battery of motor and learning/memory behavior tasks, indicating that short-term Homer1b/c downregulation is well tolerated *in vivo*. In the setting of chemically induced seizures, Homer1b/c reduction protected against seizure severity, and a significant correlation between total Homer1b/c protein levels in the brain and seizure severity both in Si-H1b/c treated mice and in Si-con treated mice was observed. These data strengthen the link between total Homer1b/c expression levels and neuronal hyperexcitability regulation *in vivo* and demonstrate that the Homer1b/c reduction effect on neuronal hyperexcitability is likely a Homer1b/c mediated event and not a developmental phenomenon.

The involvement of Homer proteins in a number of neuropathological conditions, such as chronic inflammatory pain, drug addiction, major depression and sleep loss, has been investigated in recent years [7, 13, 14]. In this study, we provide the first direct evidence that Homer1b/c, a constitutively expressed long form of Homer proteins, plays an important role in chemically induced seizure models. Reducing Homer1b/c expression has been shown in previous studies to be protective against excitotoxic insults [10], implicating Homer1b/c in the physiological regulation of aberrant neuronal excitability. In addition, antagonizing Homer1b/c function through Homer1a overexpression was shown to attenuate neuronal injury in both *in vitro* and *in vivo* TBI models [15]. These reports, in conjunction with previous data using Homer1b/c targeted siRNA to protect neurons form traumatic injury [9] and our own *in vivo* Homer1b/c knockdown data in two different seizure models, support

the application of a Homer1b/c lowering therapy to regulate hyperexcitability. PTX and PTZ kindling are the most widely-accepted animal models used to study seizure mechanisms and to discover novel treatments for seizures [16, 17]. Given that the compounds that provide protection against PTZ seizures *in vivo* have generally been successful in subsequent human clinical trials [18], our findings of Homer1b/c knockdown using two different GABA antagonists will also apply broadly to epilepsy *in vivo* models and to human epilepsy.

The mTOR is a serine/threonine kinase that normally regulates a number of cellular functions, such as cell growth, proliferation, metabolism, protein synthesis, neuronal development and synaptic plasticity, many of which could affect neuronal excitability and epileptogenesis [19]. Dysregulation of mTOR pathway has been demonstrated in a variety of genetic and acquired epilepsies, and mTOR inhibitors was shown to prevent the development of seizures and associated cellular and molecular abnormalities that promote epileptogenesis in these models, such as glial proliferation, neuronal hypertrophy, and deficient glutamate transporters [20, 21]. In the present study, protection of Homer1b/c knockdown against seizures in two chemically induced models was accompanied by decreased phosphorylation of mTOR and its downstream target protein S6. This result confirms the involvement of mTOR pathway, however, it remains to be elucidated whether, in these animal models, mTOR is inactivated as a consequence of seizures or is involved in the epileptogenic process and/or seizure generation. Among numerous upstream signaling pathways that may regulate mTOR, the mGluRs pathway represents a logical candidate for mediating the effects of seizures, because seizures result in massive glutamate release and glutamate can stimulate several glutamate receptors [22]. Of the mGluRs family, group I mGluRs (type 1 and type 5) have gained the most attention and mGluR5 may participate in the events underlying long-term depression (LTD), a form of synaptic plasticity that likely contributes to learning and memory [23]. Moreover, activation of mGluR5 was shown to regulate intracellular Ca^{2+} influx and endoplasmic reticulum (ER) Ca^{2+} release through a Homer1b/c-dependent mechanism [10, 24, 25]. Our data showed that Homer1b/c knockdown-induced inhibition of mTOR pathway was partially ablated by mGluR5 agonist CHPG, with no effects on mGluR5 expression, indicating that the protection observed in our study might be associated with the inhibition of mGluR5-mTOR pathway, which might be demonstrate on mGluR5 knockout mice in further studies.

Although we clearly demonstrated the protective effects of Homer1b/c reduction against chemically induced seizures, these findings have some limitations. First, the reduction of Homer1b/c was induced by infusion of Si-H1b/c into the hippocampus, and this strategy had minor effects on the expression of Homer1b/c in other brain regions, such as striatum corpora and cerebral ganglion, which are also involved in the initial development or progression of epilepsy [26]. Some experiments using Homer1b/c knock-out mice might be helpful to confirm our findings. Second, Homer1a mRNA levels were shown to be upregulated in the acute period of the pilocarpine epilepsy model [27]. Although our data showed that the expression of Homer1a mRNA and protein was not altered by Si-H1b/c infusion, the induction of Homer1a mRNA after seizure might interfere activity of Homer1b/c and downstream signaling cascades in our models. Further studies should be done to determine the exact role of each of these Homer 1 variants under epileptic seizure conditions.

Conclusions

In conclusion, we have confirmed and extended the knowledge that knockdown of Homer1b/c, by inhibiting the mGluR5-mediated activation of mTOR pathway, has anti-antiepileptogenic (anti-seizure) properties. We have demonstrated for the first time that reducing Homer1b/c expression protect against chemically induced seizure without effects on baseline behavior. Therefore, the Homer1b/c reduction strategy outlined here may have potential to be translated to the clinical setting for patients with epilepsy.

Disclosure Statement

The authors report no conflicts of interest.

Acknowledgements

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