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Down-regulation of Homer1b/c attenuates group I metabotropic glutamate receptors dependent Ca^{2+} signaling through regulating endoplasmic reticulum Ca^{2+} release in PC12 cells

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ABSTRACT

The molecular basis for group I metabotropic glutamate receptors (mGluR1 and 5) coupling to membrane ion channels and intracellular calcium pools is not fully understood. Homer is a family of post synaptic density proteins functionally and physically attached to target proteins at proline-rich sequences. In the present study, we demonstrate that Homer1b/c is constitutively expressed in PC12 cells, whereas Homer1a, the immediate early gene product, can be up-regulated by brain derived neurotrophic factor (BDNF) and glutamate. Knockdown of Homer1b/c using specific target small interfering RNA (siRNA) did not interfere the expression of mGluR1, mGluR5 and their downstream effectors, including inositol-1,4,5-trisphosphate receptors (IP_3R), phospholipase C (PLC) and G_q proteins. By analyzing Ca^{2+} imaging in PC12 cells, we demonstrated that Homer1b/c is an essential regulator of the Ca^{2+} release from the endoplasmic reticulum (ER) induced by the activation of group I mGluRs, IP_3R and ryanodine receptors (RyR). Furthermore, the group I mGluRs activation-dependent refilling of the Ca^{2+} stores in both resting and depolarizing conditions were strongly attenuated in the absence of Homer1b/c. Together, our results demonstrate that in PC12 cells Homer1b/c is a regulator of group I mGluRs related Ca^{2+} homeostasis that is essential for the maintenance of normal Ca^{2+} levels in the ER.

1. Introduction

Glutamate is now recognized as the main excitatory neurotransmitter in the mammalian central nervous system (CNS). The glutamate released from synapses regulates cellular and synaptic activity and plasticity related to neuronal death and survival via two classes of surface expressed glutamate receptors: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) [1]. iGluRs mediate fast excitatory postsynaptic potentials through ligand-gated cationic-selective channels, whereas mGluRs can mediate slow excitatory and inhibitory synaptic potentials and are thus important for learning and memory, cognition, pain perception, emotional behaviors and motor activity [2]. Activation of group I mGluRs, including mGluR1 and mGluR5, evokes a complex synaptic response consisting of increased activity of $G\alpha_{q/11}$ proteins and phospholipase C (PLC), diacylglycerol (DAG)-mediated generation of inositol-1,4,5-trisphosphate (IP_3) and activation of protein kinases C (PKC), and followed Ca^{2+} release from endoplasmic reticulum (ER) stores [3,4]. However, it is largely unclear how group I mGluRs are linked to their secondary effector systems.

The Homer family of proteins is predominantly localized at the postsynaptic density (PSD) in mammalian neurons and acts as adaptor proteins for the polymeric network structure in the PSD [5]. These proteins share a highly conserved Ena/Vasp homology 1 (EVH1) N-terminal domain, which has been shown to bind a specific proline-rich sequence on target proteins that include group I mGluRs, IP_3 receptors (IP_3R), ryanodine receptors (RyR), transient receptor potential canonical (TRPC) ion channels and other PSD scaffolding proteins, such as Shank [6,7]. As a result of alternative splicing Homer protein has several variants, which are classified primarily into two groups: the long form proteins (Homer1b/c, Homer2 and Homer3) and the short form proteins (Homer1a and Ania3) [8]. With 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

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dimerization domain, can competitively bind to Homer1b/c-targeted proteins and behave as a dominant negative [8,9]. Previous studies have demonstrated that Homer1a and Homer1b/c play important roles in the trafficking, location, stability and function of group I mGluRs [7,10]. However, no study to date has demonstrated the functional consequences of Homer1b/c knockdown, without affecting Homer1a expression, on group I mGluRs dependent Ca^{2+} signaling in PC12 cells.

In the present study, acute modulation of Ca^{2+} transient by group I mGluRs was examined in PC12 cells in the presence or absence of Homer1b/c to determine its effect on mGluRs signaling. We also determined the involvement of IP_3R, RyR and depolarization-evoked Ca^{2+} entry in Homer1b/c-induced regulation of group I mGluRs dependent Ca^{2+} signaling.

2. Materials and methods

2.1. Cell culture and reagents

Rat 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. The group I mGluRs agonist 3,5-Dihydroxyphenylglycine (DHPG) and brain derived neurotrophic factor (BDNF) were obtained from Invitrogen. Primary antibodies to Homer1a, Homer1b/c, mGluR1, mGluR5, PLC, G_q, IP_3R and β-actin were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA).

2.2. RNA interference (RNAi)

Small interfering RNA (siRNA) transfection was used to down-regulate Homer1b/c expression, and all the siRNAs were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The Homer1b/c targeted siRNA (Si-Homer1b/c) and control siRNA (Si-Control) were dissolved separately in Optimem I (Invitrogen, CA, USA). After 10 min of equilibration at room temperature, each RNA solution was combined with the respective volume of the Lipofectamine 2000 solution, mixed gently and allowed to form siRNA liposomes for 20 min. The PC12 cells were transfected with the transfection mixture in antibiotic-free cell culture medium, and the mRNA and protein expression levels were measured 72 h after transfection.

2.3. Immunocytochemistry

Immunocytochemistry was used to detect the expression of Homer1a and Homer1b/c in PC12 cells. Briefly, after being fixed with 4% paraformaldehyde for 15 min at room temperature, PC12 cells were permeabilized with 0.2% Triton X-100, and incubated with primary antibodies (anti-Homer1a, 1:10 and anti-Homer1b/c, 1:50) overnight at 4 °C. The cells were then incubated with the Alexa-594-conjugated donkey-anti-goat secondary antibody (red, Invitrogen, 1:500) or the Alexa-488-conjugated goat-anti-mouse secondary antibody (green, Invitrogen, 1:500) for 2 h at 37 °C. 4,6-Diamidino-2-phenylindole (DAPI, 10 μg/ml) was used to stain nucleus. Images were captured with an Olympus FV10i Confocal Microscope (Tokyo, Japan).

2.4. Real-Time RT-PCR

Total RNA was isolated from PC12 cells using Trizol reagent (Invitrogen). After the equalization of the RNA quantity in each group, the mRNA levels of group I mGluRs and related molecules 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′-GTGTCCACAGAAGCC AGAGAGGG-3′, reverse: 5′-CTTGTAGAGGACCCAGCTTCAGT-3′; Homer1b/c: forward: 5′-TCCGTCTAGCAGCCAAGC-3′, reverse: 5′-T CTGTTGACGGTATT-TCCTGTT-3′; mGluR1: forward: 5′-TTCGACATC CCACAAATCGCC-3′, reverse: 5′-ATAG-GTCCAGTTGTACCGCTT-3′; mGluR5: forward: 5′-TCACAGATTTTCCGTTGGAGC-3′, reverse: 5′-TTAGGGTTTCCCCAGAGGACT-3′; G_q: forward: 5′-GGTCGGGCTAC TCTGA-CGA-3′, reverse: 5′-ACTTGTATGGGATCTTGAGCGT-3′; PLC: forward: 5′-CGCGGGAG-TAAGTTCATCAAA-3′, reverse: 5′-CCTCCAT GTTGGGTCCTGTC-3′; IP_3R: forward: 5′-CGTTTTGAGTTTGAAGGC GTTT-3′, reverse: 5′-CATCTTGCGCCAATTCCCG-3′; GAPDH: forward: 5′-AAGGTGAAGGTCGGAGTCAA-3′, reverse: 5′-AATGAAGGG GTCATTGATGG-3′. Samples were tested in triplicates and data from five independent experiments were used for analysis.

2.5. Western blot analysis

Equivalent amounts of protein were loaded and separated by 10% SDS–PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk solution in tris-buffered saline with 0.1% Triton X-100 (TBST) for 1 h, and then incubated overnight at 4 °C with the primary Homer1a antibody (1:200), Homer1b/c antibody (1:800), mGluR1 antibody (1:1200), mGluR5 antibody (1:1000), PLC antibody (1:800), G_q antibody (1:800), IP_3R antibody (1:800) or β-actin (1:600) antibody dilutions in TBST. Next, the membranes were washed and incubated with a secondary antibody for 1 h at room temperature. Immunoreactivity was detected with the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). The analysis software Image J (Scion Corporation) was used to quantify the optical density of each band.

2.6. Electrophysiology and Ca^{2+} imaging

Whole-cell recordings were performed following standard procedures using an EPC9 patch clamp amplifier as described previously [11]. Imaging of transient Ca^{2+} changes in PC12 cells was started 25–30 min after establishing the whole-cell configuration. A multipoint confocal microscope using dual spinning disc technology, attached to an upright microscope and equipped with a 403 objective was used to acquire fluorescence images in parallel to the patch clamp recordings.

2.7. IP_3 uncaging

For photolytic uncaging experiments, the internal saline was supplemented with NPE-IP3 (400 mM; Invitrogen). Uncaging of IP_3 was produced by directing the output of a diode laser (Coherent Cube; 375 nm, 15 mW at the laser head) onto the surface of the cultures with the use of a tapered lensed optical fiber [11].

2.8. 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 or unpaired t test (two groups). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of Homer1b/c in PC12 cells

Expression of Homer1a and Homer1b/c in PC12 cells was examined to test their feasibility in studying the biological function of Homer1 variants in mGluRs-related Ca^{2+} signaling. Immunostaining results showed that Homer1b/c immunoreactivity is localized in the cytoplasma, but not in the nucleus, which was counterstained with DAPI (Fig. 1A). Homer1a immunoreactivity was also observed in the cytoplasma, but its expression level was much lower than Homer1b/c. Because Homer1a can be dynamically regulated by synaptic activity in neuronal cells, we investigated the effects of glutamate and BDNF on the expression of Homer1a and Homer1b/c in PC12 cells at both mRNA and protein levels. The expression of Homer1a mRNA was significantly increased by glutamate and BDNF, but the expression of Homer1b/c mRNA was not altered (Fig. 1B). As shown in Fig. 1C and D, a similar result on protein expression levels was also observed.

3.2. Down-regulation of Homer1b/c does not alter the expression of group I mGluRs associated proteins

To elucidate the functional role of Homer1b/c, PC12 cells were transfected with specific targeted siRNA to knockdown the expression of endogenous Homer1b/c. The results of RT-PCR and Western blot showed that the expression of Homer1b/c was significantly decreased at both mRNA (Fig. 2A) and protein (Fig. 2B and C) levels by Si-Homer1b/c transfection, whereas the expression of Homer1a mRNA and protein were not altered. Because Homer1a and Homer1b/c can competitively bind to group I mGluRs, we also detected the expression of mGluR1 and mGluR5 after Homer1b/c knockdown (Fig. 2D–F). The mRNA and protein levels of these two receptors were not changed by Homer1b/c knockdown. In addition, we also verified that the specific deletion of Homer1b/c did not interfere significantly with the expression of PLC, G_q and IP_3R, three downstream effectors of group I mGluRs (Fig. 2G–I).

3.3. Down-regulation of Homer1b/c attenuates Ca^{2+} release from ER

To investigate the role of Homer1b/c in group I mGluRs-induced Ca^{2+} release from the ER stores, PC12 cells were treated with group I mGluRs agonist DHPG after transfection with Si-Homer1b/c or Si-control (Fig. 3A). The results showed that application of DHPG produced a local Ca^{2+} transient in Si-control transfected cells, but not in Si-Homer1b/c transfected cells (Fig. 3B). As shown in Fig. 3C, we also tested the responsiveness of ER Ca^{2+} stores to IP_3 by photolysis of NPE-caged IP_3 using optical fibers to deliver UV pulses. This stimulation produced large Ca^{2+} transients in Si-control transfected cells but only small transients in Si-Homer1b/c transfected cells (Fig. 3D). In line with this result, application of the RyR agonist caffeine evoked large Ca^{2+} transients in Si-control transfected cells (Fig. 3E), whereas the same application protocol was nearly ineffective in Si-Homer1b/c transfected cells (Fig. 3F).

3.4. Homer1b/c is required for refilling of ER Ca^{2+} stores

To determine the role of Homer1b/c in refilling ER Ca^{2+} stores in PC12 cells, we first measured the spontaneous refilling at resting potential by monitoring DHPG-induced Ca^{2+} release in Si-Homer1b/c and Si-control transfected cells. In Si-control transfected cells, the DHPG-induced Ca^{2+} release were abolished by

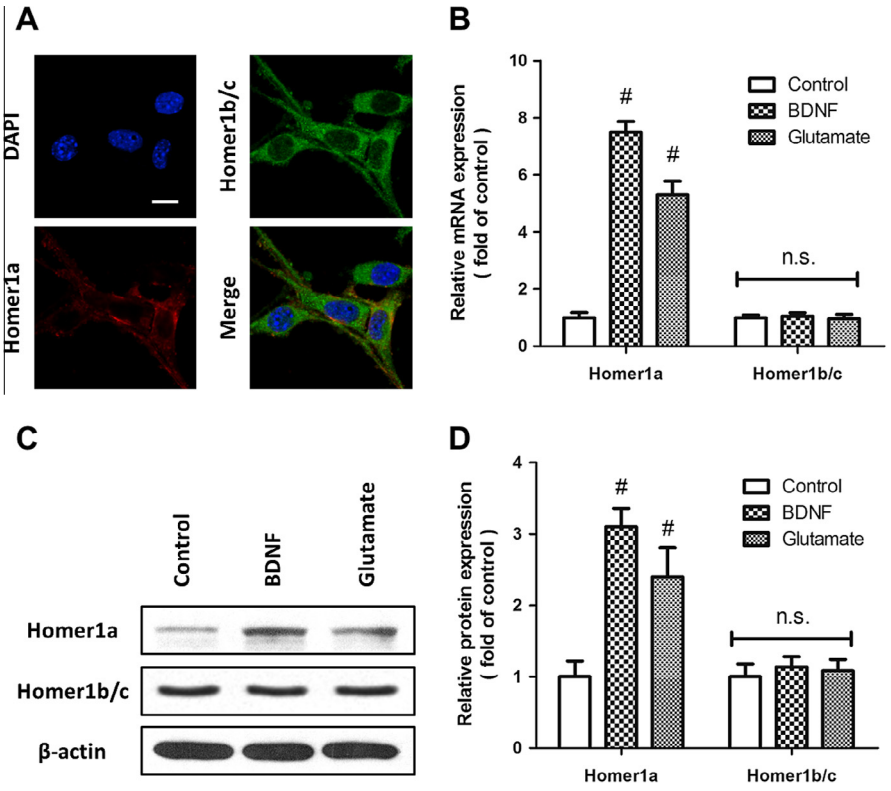

Fig. 1. Expression of Homer1b/c in PC12 cells. The expression of Homer1a and Homer1b/c in PC12 cells was detected by immunofluorescence staining, and the nuclei were stained by DAPI (A). PC12 cells were treated with BDNF or glutamate, and the expression of Homer1a and Homer1b/c was measured by Real-Time RT-PCR (B) and Western blot (C and D). Scale bars: 20 μm. Data are either representative of three similar experiments or are shown as mean ± SD of five experiments. $^{\#}p < 0.05$ vs. Control. n.s., not statistically significant.

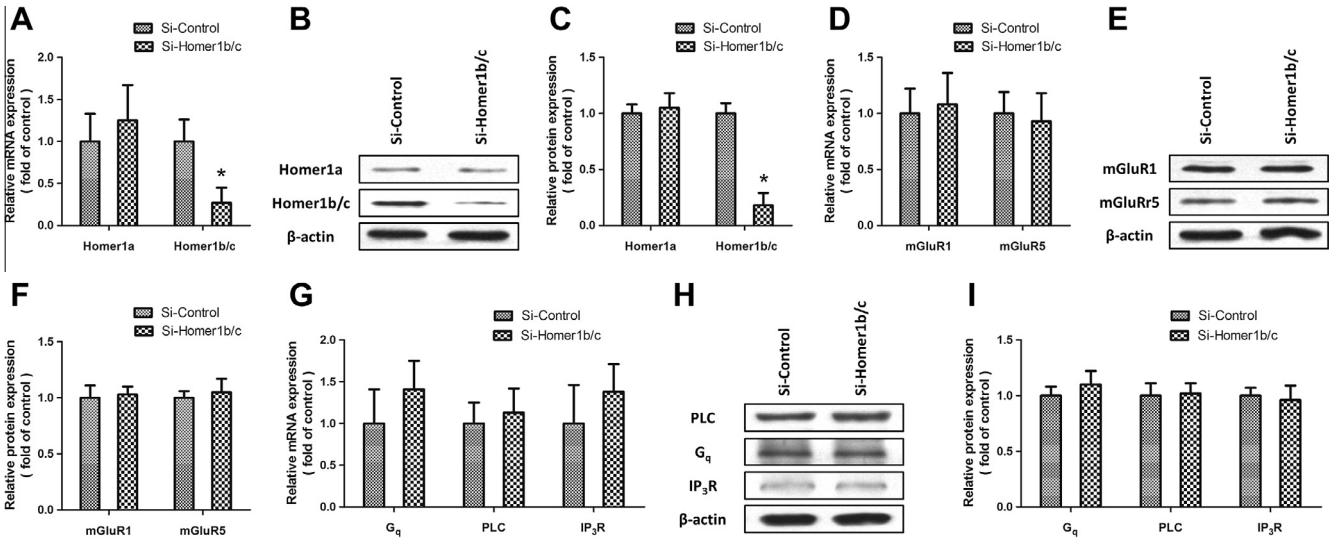

Fig. 2. Down-regulation of Homer1b/c does not alter the expression of group I mGluRs associated proteins. PC12 cells were transfected with Homer1b/c targeted siRNA (Si-Homer1b/c) or control siRNA (Si-Control) for 72 h. The expression of Homer1a, Homer1b/c, mGluR1, mGluR5, PLC, G_q and IP_3R were measured by Real-Time RT-PCR (A, D and G) and Western blot (B, C, E, F, H and I), respectively. Data are shown as mean ± SD of five experiments. $^*p < 0.05$ vs. Si-Control.

switching to Ca^{2+}-free extracellular medium, but recovered after returning back to Ca^{2+}-containing culture medium (Fig. 4A). In contrast, DHPG failed to evoke Ca^{2+} transients before, during and after the perfusion with Ca^{2+}-free culture medium (Fig. 4B). Next, we performed these experiments by adding depolarizing pulses. The results showed that DHPG application induced an augmented Ca^{2+} transient after depolarizing pulses in control cells (Fig. 4C), indicating an overloading of ER Ca^{2+} stores after depolarization. In Si-Homer1b/c transfected cells, DHPG application failed to induce Ca^{2+} release before depolarization, and evoked a decreased Ca^{2+} transient after depolarization compared to Si-control transfected cells (Fig. 4D).

4. Discussion

The results obtained in this study demonstrate that Homer1b/c is constitutively expressed in PC12 cells whereas Homer1a can be dynamically regulated by glutamate and BDNF. We show that Homer1b/c is required for groups I mGluRs-dependent ER Ca^{2+} release and for refilling the Ca^{2+} stores in resting and depolarizing cells. However, down-regulation of Homer1b/c did not interfere significantly with the expression of group I mGluRs and associated downstream effectors, including PLC, IP_3R and G_q. Importantly, both IP_3R-dependent and RyR-mediated release of Ca^{2+} from ER stores are affected by Homer1b/c knockdown.

Within Homer proteins, the well-studied Homer1 protein isoforms are shown to be associated with various neurological diseases, such as neuropathic pain, fragile x syndrome, drug addiction and stress-related cognitive deficits [12,13]. In previous studies, the alteration of Homer1 proteins is shown to be stimulus-dependent. Various synaptic activities associated with neural plasticity, traumatic injury, seizure, visual stimuli and cocaine administration dynamically increased the expression of Homer1a [5,14]. BDNF, VEGF and agonists of N-methyl-D-aspartate receptors (NMDAR) have been shown to up-regulate Homer1a expression via the ERK cascades in cultured neurons [15,16]. In contrast, Homer1b/c is constitutively expressed and keeps stable in mRNA and protein levels in response to neural activity [17]. Recently, the expression of Homer1a and Homer1b/c was also observed in PC12 cells [18], providing a new tool for studying Homer1-related signaling pathway. In the present study, we found that the expression of Homer1a, not Homer1ab/c, was significantly increased by glutamate and BDNF treatment, which is consistent with administration of apoptosis inducer tumor necrosis factor-α (TNF-α) with cycloheximide (CHX) in the same cell lines [19]. Up-regulation of Homer1a is believed to compete with Homer1b/c, disassemble the formation of multi-protein complexes, and thus behave as a dominant negative for Homer1b/c related downstream signaling pathways [20]. Moreover, overexpression of Homer1a was shown to exert protective effects after neuronal injury through regulating Homer1b/c-mGluR interaction and followed Ca^{2+} signaling [21]. Therefore, the undifferentiated PC12 cells were used in the present study to avoid the interference of Homer1a induction. We observed that Homer1a expression was at extreme low level in undifferentiated PC12 cells, and was not altered by Homer1b/c siNRA transfection, indicating that the results obtained in the present study was due to the knockdown of endogenous Homer1b/c alone.

To determine the involvement of Homer1b/c in group I mGluRs related signaling pathways, we also detected the expression of mGluR1/5 and downstream effectors, including PLC, G_q and IP_3R. Group I mGluRs is shown to be dynamically up-regulated and mediate excitotoxicity in brain ischemia, traumatic brain injury, neuropathic pain and several other neurological disorders [22]. A previous study showed that Homer1c acted as an anchoring protein that modulate the cell surface expression of mGluR1a, indicating the important role of Homer in the delivery of mGluR1 [23]. Here, we found that down-regulation of Homer1b/c did not alter the expression of all these molecules at both mRNA and protein levels. It is inconsistent with a recent finding that knockdown of Homer1b/c decreased the expression of mGluR1a after traumatic injury in cultured cortical neurons [10]. Actually, the modulation of the expression and function of group I mGluRs by other Homer family proteins, such as Homer2, Homer3, especially Homer1a, was fully investigated in previous studies [24–26]. The decreased expression of total mGluR1a protein observed by Fei et al. might be due to the function of Homer1a, which was demonstrated to be highly up-regulated by traumatic neuronal injury [24]. In the present study, we observed the effects of Homer1b/c knockdown on the expression of mGluR1 and mGluR5 in the absence of Homer1a induction. In addition, the function of group I mGluRs is largely dependent on the multi-protein complexes formed with other adaptor proteins and the cell surface expression of these receptors, but not the total protein expression [27,28]. Therefore,

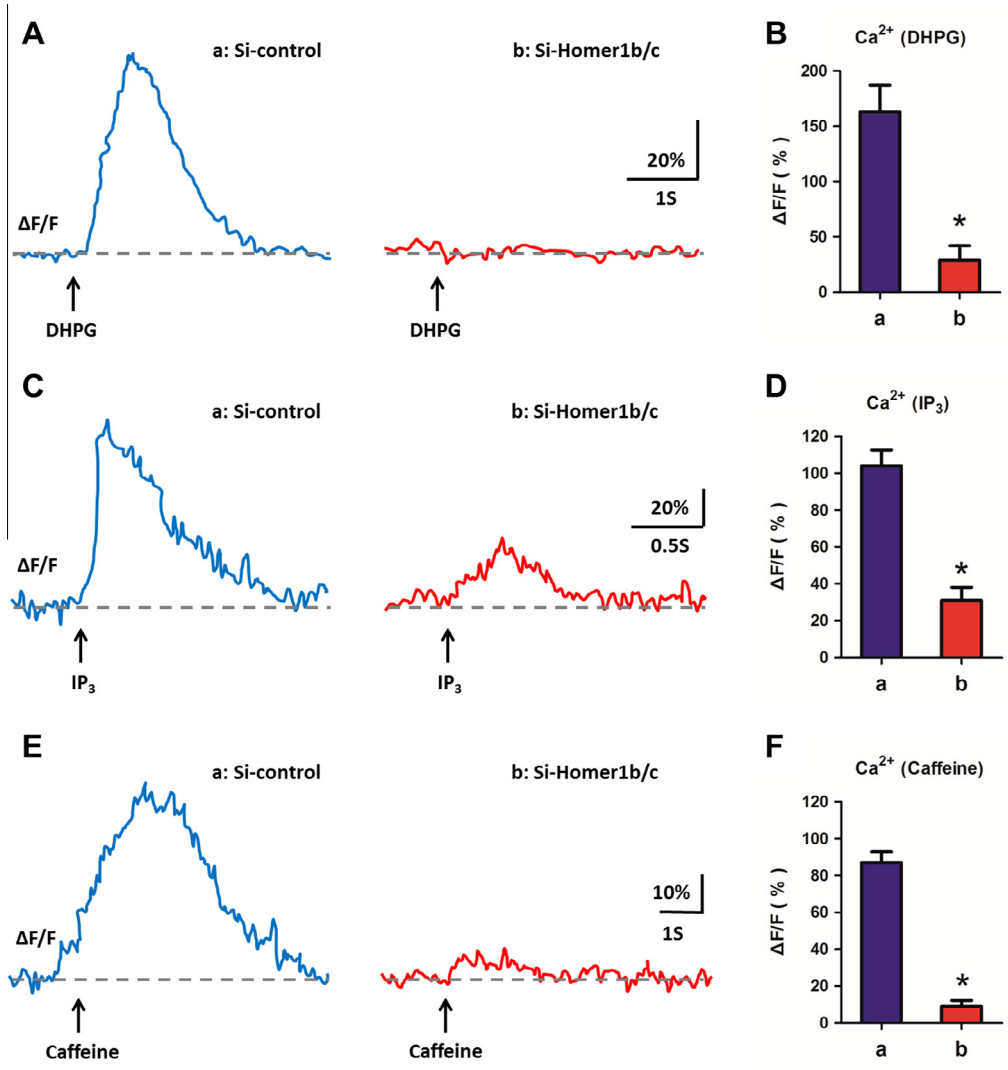

Fig. 3. Down-regulation of Homer1b/c attenuates Ca^{2+} release from ER. PC12 cells were transfected with Homer1b/c targeted siRNA (Si-Homer1b/c) or control siRNA (Si-Control) for 72 h. Relative changes in Ca^{2+} responses (ΔF/F) to the application of DHPG (200 μM, A), to the photolysis of caged IP_3 (C), or to the application of caffeine (80 mM, E) in Si-Control transfected cells (blue, left) and Si-Homer1b/c transfected cells (red, right) were detected, respectively. Summary of the Ca^{2+} responses experiments was shown in B, D and F. Data are shown as mean ± SD of five experiments. $^{*}p < 0.05$ vs. Si-Control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

we speculate that Homer1b/c might regulate group I mGluRs function through disturbing related multi-protein complexes formation or via altering their cell surface expression, which needs to be elucidated in future studies.

Intracellular Ca^{2+} mobilization in glutamatergic signaling is derived mainly from two sources, mGluRs-mediated Ca^{2+} release from the ER stores and Ca^{2+} influx from the extracellular spaces through iGluRs, especially via N-methyl-D-aspartate (NMDA) receptors [29,30]. Recent studies have shown that group I mGluRs are also involved in the regulation of Ca^{2+} influx after glutamate stimulation in neuronal cells. Activation of mGluR1 could generate a slow excitatory postsynaptic potential (sEPSP) through $G\alpha_q$-dependent regulation of TRPC3 channels [31,32]. The mGluR1 -dependent synaptic transmission and cerebellar motor behavior required the replenishment of Ca^{2+} stores that is mediated by stromal interaction molecule 1 (STIM1)-dependent opening of $Ca_v2.1$ and Orai channels [11]. In addition, previous studies have shown that Homer is required for gating of TRPC1 and L-type calcium channels [7,29,33]. In the present study, we found that knockdown of Homer1b/c attenuate group I mGluRs activation-mediated ER Ca^{2+} release, and also affect Ca^{2+} influx that refills intracellular Ca^{2+} store. It is well-known that empty ER Ca^{2+} stores can be refilled at both resting and depolarizing conditions through different mechanisms [11,34]. It takes place through a voltage-independent process at resting potential over a few minutes, whereas rapid activation of voltage-gated channels is required at depolarizing conditions [35]. Here, we found that ER Ca^{2+} refilling at resting potential and the depolarization-induced supercharging of Ca^{2+} stores were both abolished after Homer1b/c knockdown. These results indicated that Homer1b/c might act on IP_3R and/or RyR to mediate group I mGluRs activation induced ER Ca^{2+} release in PC12 cells, which is consistent with a previous study on dopamine neurons [29]. Importantly, after Homer1b/c is down-regulated, its roles in the opening of TRPC channels at rest would be decreased, and the activity of voltage-gated Ca^{2+} channels in the cellular membrane, such as $Ca_v2.1$ or L-type calcium channels, after depolarization would be reduced.

In conclusion, our results identify Homer1b/c as one of the important intracellular links between group I mGluRs and downstream Ca^{2+} signaling in undifferentiated PC12 cells. We

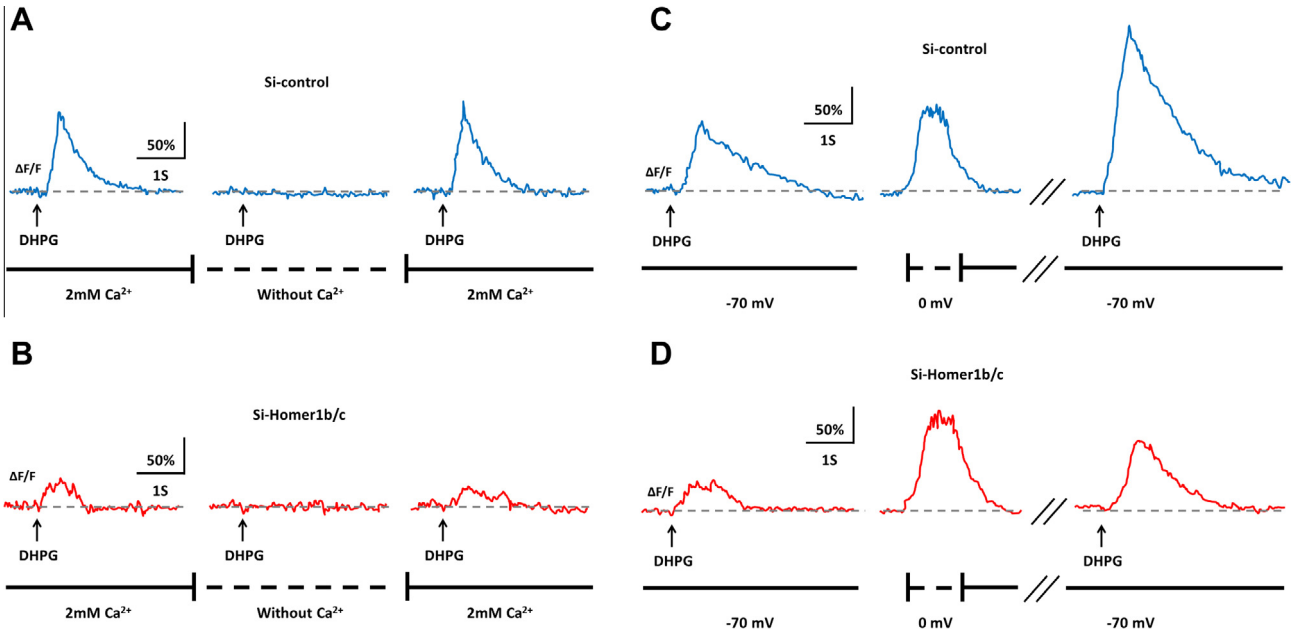

Fig. 4. Homer1b/c is required for refilling of ER Ca^{2+} stores. PC12 cells were transfected with Homer1b/c targeted siRNA (Si-Homer1b/c) or control siRNA (Si-Control) for 72 h. Ca^{2+} responses (ΔF/F) to the application of DHPG (200 μM) in medium containing 2 mM Ca^{2+} or Ca^{2+}-free medium were measured in Si-Control (A) or Si-Homer1b/c (B) transfected PC12 cells. Ca^{2+} transients in response to the application of DHPG (200 μM) before and after a depolarizing pulse (1 s, from −70 mV to 0 mV) were determined in Si-Control (C) or Si-Homer1b/c (D) transfected PC12 cells. Data are shown as mean ± SD of five experiments.

demonstrate that Homer1b/c is essential for IP_3R and RyR-mediated Ca^{2+} release form the ER and for refilling the Ca^{2+} stores in resting and depolarizing conditions. Because of the wide distribution of group I mGluRs-dependent signaling throughout the mammalian brain, Homer1b/c might be a key regulator of Ca^{2+} homeostasis in many other types of neuronal cells.

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