Neuroscience 150 (2007) 841–852

Homer1a REGULATES THE ACTIVITY-INDUCED REMODELING OF SYNAPTIC STRUCTURES IN CULTURED HIPPOCAMPAL NEURONS

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Abstract—Activity-dependent re-organizations of central synapses are thought to play important roles in learning and memory. Although the precise mechanisms of how neuronal activities modify synaptic connections remain to be elucidated, the activity-induced neuronal proteins such as Homer1a may contribute to the onset of synaptic remodeling. To further understand the physiological roles of Homer1a, we first examined prolonged effects of neuronal stimulation capable of inducing Homer1a on the distribution of a postsynaptic protein Homer1c by live imaging and immunostaining. We found that glutamate stimulation induced a biphasic change in the distribution of Homer1c, in which the postsynaptic clusters of Homer1c defused initially after 30 min to 1 h, and then reassembled more than the original level after 4–8 h. When other synaptic proteins (postsynaptic density-95 (PSD95), Filamentous actin (F-actin), glutamate receptors, synaptotagmin, synaptophysin and synapsin) were analyzed by immunocytochemical methods, the distribution of these proteins also showed a similar biphasic pattern, suggesting that glutamate stimulation induces a global alteration in synaptic structures. To further dissect the functions of Homer1a in the activity-induced synaptic remodeling, the short hairpin RNA (shRNA) vectors that specifically block the expression of endogenous Homer1a were constructed. When the shRNA of Homer1a was introduced to the cells, the activity-induced changes were almost completely suppressed. The expression of surface glutamate receptor 2 was also inhibited, suggesting that Homer1a may modulate the efficacy of synaptic transmission.

Furthermore, we found that Homer1a contributes to the presynaptic remodeling in a retrograde manner. Our data indicate that Homer1a regulates the activity-induced biphasic changes of post- and pre-synaptic sites.

Key words: Homer, F-actin, PSD95, AMPA receptor, synaptotagmin, shRNA.

Activity-dependent re-organization of central synapses is thought to play important roles in learning and memory (Mulle et al., 1998; Angelo et al., 2006). It is characterized by coordinated regulation of pre- and post-synaptic sites. Most of excitatory synapses are located on dendritic spines and neuronal activity induces a variety of changes in spine morphology and the distribution of postsynaptic proteins in spines (Matus, 1999; Fukazawa et al., 2003; Carlisle and Kennedy, 2005; Tada and Sheng, 2006; Alvarez and Sabatini, 2007; Schubert and Dotti, 2007).

The induction of Homer1a expression is one of these activity-dependent changes in neurons. Homer1a is expressed at a low level in the resting state and is induced by neuronal activities such as long-term potentiation (LTP) (Brakeman et al., 1997; Kato et al., 1997), seizure (Brakeman et al., 1997; Kato et al., 1997), and stimulations like inflammatory pain (Tappe et al., 2006), depolarization (Sato et al., 2001) and dopamine (Yamada et al., 2007; Zhang et al., 2007). Overexpression of exogenous Homer1a has been shown to inhibit spine morphogenesis and reduce the densities of postsynaptic proteins in spines (Sala et al., 2003). We have also reported that induction of endogenous Homer1a expression or overexpression of exogenous Homer1a causes a reduction of punctuate distribution of postsynaptic proteins Homer1c, a splice variant of Homer1 which is one of the scaffold proteins at postsynaptic density (PSD) (Kato et al., 1998; Xiao et al., 1998), and filamentous actin (F-actin) in dendrites (Inoue et al., 2004). Thus, Homer1a is expected to play an important role in the mechanism of activity-dependent synaptic re-organization, but there is little direct evidence to show the roles of endogenous Homer1a in synaptic re-organization.

Dispersion of Homer1c clusters was also caused by glutamate stimulation (Okabe et al., 2001; Shiraishi et al., 2003). Although the short-term (minutes) effects of the stimulation are well documented, the information about the consequences over hours after stimulation is limited.

In this study, we examined the roles of Homer1a in the glutamate-induced re-organizations of synapses, by live imaging or immunostaining of synaptic proteins for extended periods in cultured rat hippocampal neurons. The processes of synaptic re-organization were analyzed by monitoring the number and morphological properties of dendritic spines, labeled with postsynaptic proteins such as PSD95, Homer1c or F-actin, and presynaptic nerve terminals, marked by a presynaptic marker synaptotagmin. The expression of Homer1a protein was specifically suppressed by Homer1a short hairpin RNA (shRNA). We found that the induction of Homer1a is required not only for the dispersion but also for the subsequent re-clustering of postsynaptic proteins. Furthermore, we also found that inhibition of postsynaptic changes influences the presynaptic remodeling in a retrograde manner. Our data indicate that Homer1a plays a critical role in the activity-induced re-organization of both pre- and post-synaptic structures.

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Abbreviations: F-actin, filamentous actin; GluR, glutamate receptor; LTP, long-term potentiation; PBS, phosphate-buffered saline; PSD95, postsynaptic density-95 protein; shRNA, short hairpin RNA.

0306-4522/07$32.00+0.00
doi:10.1016/j.neuroscience.2007.09.081

EXPERIMENTAL PROCEDURES

Cell cultures

The primary cultures of hippocampal neurons were prepared as described previously (Kato et al., 2003). Briefly, hippocampi were isolated from Wistar rats at 18 days of gestation (E18), treated with papain (100 mg/ml, Worthington, Lakewood, NJ, USA) for 10 min at 37 °C. Dissociated neurons were plated at a density of 20,000∼30,000 cells/cm^2 onto coverglasses (Matsunami, Osaka, Japan) precoated with 1 mg/ml poly-L-lysine (Sigma), cultured in Neurobasal-A medium (Invitrogen) supplemented with 2% B-27 (Invitrogen) and 0.5 mM glutamine, in a humidified atmosphere of 5% CO_2 at 37 °C for 14–19 days. One half of the medium was exchanged every other week. All experiments conformed to the guidelines by Kyushu University Animal Welfare Committee on the ethical use of animals. Every effort was made to minimize the number of animals used and their suffering.

Constructions of plasmid DNAs

The cDNAs encoding Homer1c and actin were cloned in frame at *Eco*RI and *Bam*HI sites in pVenus-C1 (a gift from Dr. Miyawaki). Venus is a variant of yellow fluorescent protein (YFP) and has a merit of improved brightness (Nagai et al., 2002). Purified plasmid DNAs (1 μg/μl) were microinjected into the nuclei of pyramidal neurons at 14∼19 days *in vitro*.

Cell stimulation

Hippocampal neurons were stimulated with glutamate (100 μM 1 min). Coverglasses on which cells were cultured were dipped into the media containing the stimulants (or no stimulants for the controls) for fixed periods of time. These conditions of the stimulations caused no detectable damages to the cells, which appeared to remain healthy for at least 8 h after the stimulations.

Immunocytochemistry

Transfections of HEK293T cells were performed with 1 μg of Venus-Homer1a DNA by lipofection using Lipofectamine2000 (Life Technologies) according to the manufacturer's instructions. After 36 h of expression, these cells were used for live imaging, or were fixed for immunocytochemistry. Cultured neurons and HEK293T cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and incubated with 8% BSA in PBS for 30 min. The antibodies used were rabbit anti-Homer1c (1:400) (Kato et al., 2001), mouse anti-PSD95 (1:400, Affinity Bio Laboratory), mouse anti-tubulin (1:200, Chemicon), rabbit anti-Homer1a (1:100, Santa Cruz Biotechnology), rabbit anti-Synapsin (1:200, Zymed), mouse anti-synaptotagmin (1:200, Chemicon) and mouse anti-synaptophysin (1:200, Chemicon) antibodies. F-actin was stained with Alexa 633-phalloidin (1:1000, Molecular Probes).

Staining of glutamate receptor (GluR) 2 on synaptic surfaces

Cultured neurons were first incubated with 0.5% BSA in the conditioned medium for 15 min at 37 °C, and then reacted with a rabbit antibody against the N-terminal extracellular domain of GluR2 (1:60) in 0.1% BSA in the conditioned medium at 27 °C for 20 min. After being washed with 0.1% BSA in the conditioned medium, the cells were incubated with Cy3-conjugated anti-rabbit IgG antibody (1:60) at 27 °C for 20 min, washed with conditioned medium, and fixed with 4% paraformaldehyde in PBS for 0.5 h (Du et al., 2004).

Immunoblots of transiently expressed proteins in HEK293T cells

HEK293T cells were transfected with Venus-Homer1a by lipofection using Lipofectamine2000 (Life Technologies) according to the manufacturer's instructions. After 36 h, proteins were extracted in 2× sodium dodecylsulfate sample buffer. Equal amounts of cell extracts were analyzed by Western blotting using anti-Homer1a or anti-actin antibody. For measuring the expression levels of Homer1a and actin, PVDF membranes were fixed for 45 min with 4% PFA in PBS at 4 °C and rinsed three times for 20 min with PBS before blocking (Ishibashi et al., 2005).

Construction of Homer1a shRNA

To select the target sequences for the rat Homer1a-specific shRNA, we utilized the web-based tool of BLOCK-IT™ RNAi Designer (Invitrogen). The target sequences were chosen within the 3′-untranslated region specific to rat Homer1a and they met the following criteria: a GC content is between 40 and 55%, well-conserved regions among species, and sequences do not match with any other rat genes. The sequencing data for the rat Homer1a mRNA were obtained from GenBank (accession number: AB003726). We designed four Homer1a shRNA as follows: 5′-GCTGCAGAACAAGGAAATT-3′ (#1), 5′-GGTTTCCACCTCCACTTAA-3′ (#2), 5′-GCACTGAATACTGTGACAT-3′ (#3) 5′-GCAGGTTATAACCAGTCTT-3′ (#4). We found that the #1 shRNA is most effective and used for the experiments in this study. Plasmids were constructed using the pAVU6+27 vector with U6 RNA polymerase III promoter (a gift from Dr. David R. Engelke) (Paul et al., 2002). A scrambled sequence of Homer1a shRNA #1 (5′-GCTAAGAGAACAAGCGATT-3′) was used as a negative control.

Injection shRNA vectors

Homer1a-shRNA (1 μg/μl in PBS) together with other purified plasmid DNAs was injected into neurons 36 h before the glutamate stimulation. Successful injections were confirmed by immunocytochemical detection with a rabbit anti-Homer1a antibody (1:100, Santa Cruz). As controls, we used the same concentration of vector alone and scrambled Homer1a-shRNA.

Image analysis

Specimens were examined using LSM510 laser scanning confocal microscope (Zeiss). Confocal images were obtained using 40× objectives at 1024×1024 pixel resolution. The laser intensity and the gain were adjusted to avoid saturation of the maximum pixel intensity and photobleaching. Neurons of pyramidal shape were morphologically identified. Segments of 75 μm (from 5 μm to 80 μm distal from the soma) of major dendrites were examined. Once optimized, individual samples were imaged at a same optical setting. Images were projected in z-series consisting of three to five slices at 1–1.5 μm intervals, covering the entire z-plane of cultured neurons. Double-labeled neurons were chosen randomly from two to five coverslips. Each experiment was repeated at least three times. The numbers of neurons used for analyses are indicated in figure legends. Morphometric measurements were carried out with Image Browser (Zeiss). Immunoreactive clusters of synaptic proteins on dendritic spines and shafts were defined as spots of 0.5–2 μm in diameter over 50% of the maximum pixel intensity of examined areas unless otherwise specified. We defined dendritic spines as protrusions with an enlarged tip less than 4 μm in length and narrower than 1 μm in spine neck on dendrites expressing Venus.