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LOW ACCUMULATION OF DREBRIN AT GLUTAMATERGIC POSTSYNAPTIC SITES ON GABAergic NEURONS

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Abstract—Glutamatergic synapses form onto both glutamatergic and GABAergic neurons. These two types of glutamatergic synapses differ in their electrical responses to high-frequency stimulation and postsynaptic density protein composition. However, it is not known whether they differ in the actin cytoskeleton composition. In the present study, we used hippocampal neuronal cultures prepared from glutamate decarboxylase 67 (GAD67)-GFP knock-in mice and analyzed the differences in the actin cytoskeleton at glutamatergic synapses contacting GABAergic and glutamatergic neurons. Drebrin-binding actin filaments enriched in dendritic spines are known to play a pivotal role in spine formation. Immunocytochemical analyses demonstrated that drebrin accumulated at glutamatergic synapses on GABAergic neurons as well as at those on glutamatergic neurons. However, the density of drebrin clusters along dendrites in GABAergic neurons was significantly lower than those of glutamatergic neurons. Furthermore, the level of drebrin accumulating at glutamatergic synapses was lower on GABAergic neurons than on glutamatergic neurons. In neurons overexpressing drebrin, drebrin cluster density and accumulation levels in GABAergic and glutamatergic neurons were similar, suggesting that the low drebrin levels in the glutamatergic postsynaptic sites on GABAergic neurons may be because GABAergic neurons express low levels of drebrin. On the other hand, pharmacological analysis demonstrated that the postsynaptic localization of drebrin depended on actin cytoskeleton organization in both GABAergic and glutamatergic neurons. Together these results indicated that, although GABAergic and glutamatergic neurons share common regulatory systems affecting drebrin localization, the density of drebrin-positive glutamatergic synapses formed on GABAergic neurons is lower than those on glutamatergic neurons. This is probably due to the low expression of drebrin in GABAergic neurons. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: GABAergic neurons, drebrin, glutamatergic synapse, glutamate receptor, dendritic spine, actin cytoskeleton.

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Abbreviations: CytoD, cytochalasin D; DIV, day(s) *in vitro*; GABA, GABAergic neurons; GAD67, glutamate decarboxylase 67; Glu, glutamatergic neurons; HA-DA, HA-tagged drebrin A; LatA, latrunculin A; LTP, long-term potentiation; PBS, phosphate-buffered saline; PSD, postsynaptic density; vGluT1, vesicular glutamate transporter 1.

Although glutamatergic synapses form not only onto glutamatergic neurons but also onto GABAergic neurons, these two types of glutamatergic synapses show morphological and functional differences. On glutamatergic neurons, most glutamatergic synapses form at dendritic spines, which are small protrusions from the parent dendritic shaft. By contrast, on GABAergic neurons, most glutamatergic synapses form at dendritic shafts; only a small population of glutamatergic synapses also forms at dendritic spines (Acsady et al., 1998; Kawaguchi et al., 2006).

Functionally, while glutamatergic synapses of mossy fibers on glutamatergic CA3 pyramidal neurons show facilitation of excitatory postsynaptic currents (EPSCs) in response to high-frequency stimulation (Regehr et al., 1994), glutamatergic synapses of mossy fibers on GABAergic stratum-lucidum neurons show marked depression (Maccaferri et al., 1998). Structurally, the postsynaptic density (PSD) of glutamatergic synapses on GABAergic neurons contains high levels of citron-N (Zhang et al., 1999; Zhang and Benson, 2006), suggesting that glutamatergic synapses on GABAergic neurons may be distinct from those on glutamatergic neurons. The precise protein composition of glutamatergic postsynaptic sites on GABAergic neurons, however, remains to be fully elucidated.

Drebrin is a side-binding protein of F-actin and is highly expressed in the brain (for a review, see Sekino et al., 2007). Drebrin is involved in the dendritic spine formation of glutamatergic neurons during development and facilitates the accumulation of other postsynaptic proteins, such as PSD95 and F-actin, into dendritic spines (Takahashi et al., 2003; Aoki et al., 2005). Electron microscopic immunocytochemistry (EM-ICC) demonstrated that drebrin highly concentrates at glutamatergic postsynaptic sites but not at GABAergic postsynaptic sites (Aoki et al., 2005). On the other hand, immunohistochemical analysis revealed that hippocampal neurons in the hilus are immunoreactive for drebrin A *in vivo* (Aoki et al., 2005). Because these neurons in the hilus are most likely GABAergic, this suggests that GABAergic neurons express drebrin, as do glutamatergic neurons. However, whether drebrin is also concentrated at the glutamatergic synapses on GABAergic neurons has not been directly examined.

In the present study, we analyzed the localization of drebrin and other synaptic molecules at glutamatergic synapses made onto GABAergic neurons. These analyses were done on hippocampal neuronal cultures prepared from glutamate decarboxylase 67 (GAD67)-GFP knock-in

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mice. Our results identified differences in drebrin accumulation levels at glutamatergic postsynaptic sites on GABAergic and glutamatergic neurons.

EXPERIMENTAL PROCEDURES

Animals

All of the animal experiments in this study were performed according to the guidelines of the Animal Care and Experimentation Committee (Gunma University, Showa Campus, Maebashi, Japan). Every effort was made to minimize animal suffering and to reduce the number of animals used. The generation of GAD67-GFP (Δneo) mice has been described by Tamamaki et al. (2003), and these heterozygous mice, used in the present study, were termed GAD67-GFP knock-in mice. These mice specifically express GFP in GABAergic neurons, facilitating their identification. The mice were maintained with a genetic background of C57BL/6 at our animal facility. Embryos and tissues were obtained from timed matings. Noon of the day on which the vaginal plug was detected was designated as embryonic day 0.

Hippocampal cell cultures

Experiments were performed on Banker-style low-density hippocampal cultured neurons that had been cultured between three to four weeks *in vitro* (Takahashi et al., 2003). Hippocampi were dissected from embryonic day 16 fetal GAD67-GFP knock-in mice and digested in trypsin-containing medium. Cells were then dispersed by trituration (passing through a P-1000 pipette), plated at an approximate density of 5,000 cells/cm^2 on coverslips coated with poly-L-lysine, and then incubated in Minimum Essential Medium (MEM; Invitrogen, San Diego, CA, USA) supplemented with fetal bovine serum for 3 h. The coverslips were then transferred into a dish containing supporting glial cells, which were attached to the bottom of the culture dish, and maintained in serum-free MEM containing B27 supplement (Invitrogen). Cytosine β-D-arabinofuranoside (10 μM; Sigma, St. Louis, MO, USA) was added to the cultures at 4 days *in vitro* (DIV) to inhibit glial proliferation. Glial cells were prepared from the cerebral cortex of newborn Wistar rats (Charles River Laboratories, Yokohama, Japan). For the pharmacological experiments, latrunculin A (LatA) was obtained from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Cytochalasin D (CytoD) was obtained from Sigma.

cDNA microinjection

pCMV-HA vector (Clontech, Palo Alto, CA, USA) was used to construct the HA-drebrin A fusion vector. Drebrin A cDNA inserts were generated by PCR using rat drebrin A cDNA (Shirao et al., 1992) as a template. The drebrin A cDNA insert was amplified using a 5′ primer (AAAAGTCGACCATGGCCGGCGTCAGCTTCAGC) that contains a cDNA sequence encoding the N terminus of drebrin A and an additional *Sal*I site in frame with the HA sequence at its 5′ end and a 3′ primer (AAAGGTACCCTAATCACCACCCTCGAAGCC) that contains an antisense sequence encoding the C terminus of drebrin and an additional *Kpn*I site at its 3′ end. The products were digested with *Sal*I and *Kpn*I and cloned into a *Sal*I/*Kpn*I-digested pCMV-HA vector. The pCMV-HA vector was used as a control. The pmCherry-C1 vector (Clontech) was used for visualization of neuronal morphology.

For transfection of these vectors into neurons, we used a microinjection method. Glass micropipettes (Femtotips; Eppendorf, Hamburg, Germany) were filled with Tris–EDTA buffer (pH 8.0) that contained cDNA (0.5 μg/μl). We injected cDNA solution containing either mCherry and HA or HA-tagged drebrin A into nuclei of cultured hippocampal neurons at 21 DIV using a micromanipulator (Micromanipulator 5171; Eppendorf). Twenty-four hours after the injection, the neurons were fixed and analyzed immunocytochemically.

Immunocytochemistry

For immunocytochemical analyses, neurons were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Fixed neurons were permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. The neurons were then incubated overnight at 4 °C with either a monoclonal antibody against drebrin (clone M2F6; Shirao and Obata, 1986), or a monoclonal antibody against PSD95 (clone 7E3-1B8; Affinity BioReagents, Golden, CO, USA). After washing with PBS, the cells were incubated for 1 h at room temperature with rhodamine-conjugated goat antibody against mouse IgG (1:100; Chemicon, Temecula, CA, USA) as a secondary antibody. In some cases, the cells were further incubated with either rabbit anti-synapsin-I polyclonal antibody (1:1000; Chemicon) or anti-vesicular glutamate transporter 1 (vGluT1) polyclonal antibody (1:1000; Synaptic Systems, Goettingen, Germany), as the primary antibody, followed by Cy5-conjugated goat antibody against rabbit IgG (1:100; Chemicon), as the secondary antibody.

For double labeling of drebrin and F-actin, the cells were immunostained with anti-drebrin monoclonal antibody and Cy5-conjugated goat antibody against mouse IgG, and with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR, USA). For double labeling of synapsin I and F-actin, the cells were immunostained with anti-synapsin I polyclonal antibody and Cy5-conjugated goat antibody against rabbit IgG, and with rhodamine-conjugated phalloidin. For double labeling of drebrin and HA-DA, the cells were immunostained with anti-drebrin mouse monoclonal antibody and Cy5-conjugated goat antibody against mouse IgG, and anti-HA rat monoclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by biotin-conjugated goat antibody against rat IgG (1:100; Vector Laboratories, Burlingame, CA, USA) and AMCA-conjugated streptavidin (1:100; Vector Laboratories). After washing with PBS, the cells were mounted onto glass slides with Perma Fluor mounting medium (Thermo Shandon, Pittsburgh, PA, USA).

All fluorescence images of cells were obtained with a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with a Cool Snap FX cooled CCD camera (Photometrics, Tucson, AZ, USA), and operated with Meta Morph software (Universal Imaging, West Chester, PA, USA). A 63×, 1.4 numerical aperture objective lens (Zeiss) was used. A filter set (86000 Sedat Quad; Chroma, Brattleboro, VT, USA) was mounted into the excitation and emission filter wheels (Ludl Electronic Products, Hawthorne, NY, USA) of the microscope. All of the data were collected at 1300×1030 resolution at 12 bits/pixel. A single pixel in the image corresponded to a 106 nm square in the specimen plane. The images used for the comparisons carried out in this study were collected under identical conditions. Captured fluorescent images were analyzed using Meta Morph software. GFP signals were obtained through a filter for fluorescein isothiocyanate. Images for presentation were prepared using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

Quantification

Each protein cluster was defined as a round staining region having a peak fluorescent level that was twofold greater than the average fluorescent level of the dendrites, as described previously (Takahashi et al., 2003). For the measurements of immunofluorescence intensity, data are normalized to the average value of each molecule in control glutamatergic neurons from all dishes examined, which was defined as 100 relative units. In pharmacological ex-