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HNK-1 (HUMAN NATURAL KILLER-1) GLYCO-EPITOPE IS ESSENTIAL FOR NORMAL SPINE MORPHOGENESIS IN DEVELOPING HIPPOCAMPAL NEURONS

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Abstract—The human natural killer-1 (HNK-1) glyco-epitope possesses a unique structural feature, a sulfated glucuronic acid attached to lactosamine on the non-reducing termini of glycans. The expression of HNK-1 is temporally and spatially regulated by glucuronyltransferase (GlcAT-P) in the brain. Our previous report showed that mice lacking GlcAT-P almost completely lost HNK-1 expression in the brain and exhibited reduced long-term potentiation (LTP) at hippocampal CA1 synapses. GlcAT-P-deficient mice also showed impaired hippocampus-dependent spatial learning. Although HNK-1 plays an essential role in synaptic plasticity and memory formation, it remains unclear how HNK-1 regulates these functions. In this study, we showed that loss of the HNK-1 epitope resulted in an increase of filopodium-like immature spines and a decrease of mushroom-like mature spines in both the early postnatal mouse hippocampus and cultured hippocampal neurons. However, HNK-1 had no influence on spine density or filopodium formation. Immunofluorescence staining revealed that loss of HNK-1 altered the distribution of postsynaptic proteins such as α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA)-type glutamate receptor subunit GluR2 and PSD-95 from spine heads onto dendritic shafts without affecting synapse formation, resulting in an increase of shaft synapses in cultured GlcAT-P-deficient neurons. GluR2, a major HNK-1 carrier glycoprotein in postsynaptic density, has the ability to promote spine morphogenesis. Overexpression of GluR2 promoted spine growth in both wild-type and GlcAT-P-deficient neurons, but the increase in GlcAT-P-deficient neurons was lower than that in wild-type neurons. This is the first evidence that HNK-1 is a key factor for normal dendritic spine maturation and is involved in the distribution of postsynaptic proteins.

Key words: filopodium, glucuronyltransferase (GlcAT-P), GluR2, shaft synapse, spine maturation.

Glycosylation is one of the major post-translational protein modifications, especially for cell surface and secreted proteins, which play important roles in a variety of cellular functions (Ohtsubo and Marth, 2006). Several of the carbohydrates that are characteristically expressed on glycoproteins in the nervous system regulate synaptic plasticity (Kleene and Schachner, 2004). Among them, human natural killer-1 (HNK-1) is a well-characterized glyco-epitope found in the nervous system (Morita et al., 2008). HNK-1 is highly expressed in the brain during the early postnatal period, when the neural circuit is being actively formed. This epitope is carried by a series of cell adhesion molecules belonging to the immunoglobulin family cell adhesion molecules (NCAM, L1, P0, etc.) and extracellular matrix proteins (tenascin-R, phosphacan, etc.) (Saghatelyan et al., 2000; Liedtke et al., 2001). The HNK-1 glyco-epitope possesses a unique structure comprising a sulfated trisaccharide (HSO_3-3GlcAβ1-3Galβ1-4GlcNAc-) and is sequentially biosynthesized by one of two glucuronyltransferases (GlcAT-P or GlcAT-S) (Terayama et al., 1997; Seiki et al., 1999) and a sulfotransferase (HNK-1ST) (Bakker et al., 1997). To investigate the biological function of the HNK-1 epitope *in vivo*, we generated mice deficient in GlcAT-P, which is a major enzyme accounting for the majority of glucuronyltransferase activity in the nervous system (Yamamoto et al., 2002). The GlcAT-P-deficient mice showed an almost complete loss of HNK-1 expression in their brains and exhibited reduced long-term potentiation (LTP) at Schaffer collateral-CA1 synapses along with defects in spatial memory formation. Similarly, HNK-1ST-deficient mice also showed a reduction in LTP and abnormalities in spatial learning tasks (Senn et al., 2002). Recently, mice deficient in β4-galactosyltransferase-2 (β4GalT2), which is involved in Galβ1-4GlcNAc synthesis, were shown to exhibit markedly decreased HNK-1 expression as well as learning/memory impairment (Yoshihara et al., 2009). Although these lines of evidences strongly suggest that the HNK-1 epitope is essential for long-lasting changes in synaptic transmission, little is known about how HNK-1 modulates neural function.

Dendritic spines are highly specialized actin-rich small protrusions on neuronal dendrites and act as receptive sites for excitatory synaptic transmission. The size and number of spines are associated with synaptic plasticity including LTP and long-term depression (LTD), and it is considered that spine morphology is crucial for learning and memory (Segal, 2005). There are several different views on the origin of dendritic spines. One model is that spines initially form as dynamic filopodium-like protrusions and that these structures are then converted directly to

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionate; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DIV, days in vitro; Gal, galactose; GalT, galactosyltransferase; GlcA, glucuronic acid; GlcAT, glucuronyltransferase; GlcNAc, *N*-acetylglucosamine; GluR, glutamate receptor; HNK-1, human natural killer-1; HNK-1ST, HNK-1 sulfotransferase; LTP, long-term potentiation; PBS, phosphate-buffered saline; PSD, postsynaptic density; SDS, sodium dodecyl sulfate.

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mature spines, coincident with the process of postsynaptic specialization (Dailey and Smith, 1996; Ziv and Smith, 1996; Hering and Sheng, 2001). Another model is that dendritic spines arise from synapses located on dendritic shafts (shaft synapse) (Boyer et al., 1998; Fiala et al., 1998). AMPA type glutamate receptors mediate most of the fast excitatory synaptic transmission in the mammalian brain and control synaptic strength (Derkach et al., 2007; Isaac et al., 2007). The abundance of postsynaptic AMPA receptors is closely related to the size of dendritic spine heads and synapses, and their distributions regulate long-lasting synaptic changes. AMPA receptors are heterotetrameric complexes composed of various combinations of four subunits (GluR1–4). All subunits have an amino-terminal extracellular domain where four to six potential *N*-glycosylation sites are located (Pasternack et al., 2003). Among them, the extracellular domain of GluR2 has the ability to increase spine size and density in hippocampal neurons (Passafaro et al., 2003). The extracellular domain of GluR2 has four potential *N*-glycosylation sites, and the HNK-1 glyco-epitope is selectively expressed on the GluR2 subunit (Morita et al., in press).

In the present study, we showed that GlcAT-P-deficient mice had a large number of filopodium-like immature spines on their pyramidal neurons during the early postnatal period. However, filopodium sprouting and spine density were not affected by loss of the HNK-1 epitope. These observations indicate that HNK-1 plays an important role in the spine maturation process during conversion from filopodia to mature spines in a developmental stage-specific manner. Moreover, loss of HNK-1 also caused clustering of GluR2 and postsynaptic components on dendritic shafts instead of spine heads, indicating that abundant shaft synapses were formed. Our findings suggest that the HNK-1 glyco-epitope modulates the function of GluR2, resulting in normal spine morphogenesis in hippocampal neurons.

EXPERIMENTAL PROCEDURES

DNA constructs

The following mammalian expression plasmids were used: Venus (a GFP variant) attached to a membrane-targeted palmitoylation signal of GAP-43 (pCAGGS-GAP-Venus) was kindly provided by Drs. Y. Yoshihara and A. Miyawaki (RIKEN Brain Science Institute, Wako, Saitama, Japan) (Matsuno et al., 2006). GAP-Venus was released from pCAGGS-GAP-Venus with *Eco*RI and cloned into multi-cloning site A of the pIRES vector (Clontech, Mountain View, CA, USA). Then GlcAT-P was released from pEF-BOS-GlcAT-P (Terayama et al., 1997) with *Not*I and inserted into multi-cloning site B to yield pIRES-GAP-Venus-GlcAT-P. The 3.2 kb *Xho*I-*Xba*I GluR2 fragment derived from pKC24 (pBluescript) −GluR2, which was kindly provided by Dr. M. Mishina (Tokyo University, Tokyo, Japan) (Sakimura et al., 1990), was cloned into pcDNA3.1/myc-HisB (Invitrogen, Carlsbad, CA, USA) to yield the plasmid pcDNA3.1-GluR2.

DiI labeling

Mouse brains ($n=3$ for each genotype) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and sectioned into 100 μm slices using a vibratome (Leica Microsystems, Wetzlar, Germany). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) crystals (Molecular Probes, Eugene, OR, USA), a lipophilic tracer, were placed near the cell bodies of hippocampal CA1 pyramidal neurons to retrogradely label their dendrities. After 2 days, DiI diffusion on the pyramidal cell membrane was traced using confocal Z-series images taken at 0.75 μm depth intervals with the FLUOVIEW imaging system (OLYMPUS, Tokyo, Japan).

Cell cultures and transfection

Primary hippocampal cultures were prepared from postnatal day 0 mouse brains. Hippocampi were trypsinized for 10 min at 37 °C. Dissociated neurons were plated at 1.0×10^5 cells per well (BD BioCoat Poly-D-Lysine Cellware 4-Well CultureSlide (BD Biosciences, San Jose, CA, USA) coated with 2 μg/ml laminin) in Neurobassal medium (Gibco BRL, Grand Island, NY, USA) supplemented with 2% B27 (Gibco BRL) and 500 μM L-glutamine. Every 7 days, the cultures were fed by replacing half the medium with feeding medium. Hippocampal neurons were transfected using the MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA, USA).

Immunostaining and antibodies

Cultured neurons were fixed in ice-cold methanol at −20 °C and then washed with PBS. Neurons were incubated with 3% bovine serum albumin in PBS containing primary antibodies for 1 h at room temperature. HNK-1 monoclonal antibody (a hybridoma cell line was purchased from the American Type Culture Collection, Manassas, VA, USA), anti-GFP (Nacalai Tesque, Kyoto, Japan), anti-PSD-95 (Upstate, Lake Placid, NY, USA), and anti-synaptophysin (FabGennix Inc., Frisco, TX, USA), anti-MAP-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-GluR2 N-term antibodies (Zymed, San Francisco, CA, USA) were used. Alexa 448 and 546 secondary antibodies (Molecular Probes) were used for visualization. To label cytoskeletal F-actin, the neurons were fixed for 5 min in acetone at −20 °C and then stained with Alexa Fluor 546 Phalloidine (Molecular Probes).

Immunoprecipitation and immunoblot analysis

Cultured hippocampal neurons were lysed with 1 vol of 10 mM Tris–HCl (pH 7.4) buffer containing 1% sodium dodecyl sulfate and boiled for 5 min, and then five volumes of immunoprecipitation buffer (20 mM Tris–HCl (pH 7.4), 2% Triton X-100, and 0.3 M NaCl) and four volumes of H_2O were added to the lysate. To immunoprecipitate the GluR2 subunit, 4 μg/ml anti-GluR2/3 antibody (Upstate) and 20 μl of Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Piscataway, NJ, USA) were added into the lysate and rotated for 2 h at 4 °C. Immunoblot analysis was performed using standard protocols.

Image analysis and quantification

For the DiI labeling experiments, neurons were chosen randomly from three independent littermate pairs. For the analysis of GFP-transfected neurons, neurons were chosen from three to five independent experiments. Morphological analysis was carried out using MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA, USA). The length and width of the protrusions along dendrites were measured. Spines and filopodia were classified as follows: spines were defined as 0.5–1.5 μm length protrusions with mushroom-like heads, and filopodia were defined as protrusions longer than 1.5 μm without spine heads. In the case of GluR2 overexpression experiments, spines were defined as 0.5–2.0 μm length protrusions with mushroom-like heads, and filopodia were defined as protrusions longer than 2.0 μm without spine heads because of the unusual large spine heads. The densities of protrusions, spines and filopodia are indicated as their number per 10 μm of dendrite.