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OPPOSING FUNCTIONS OF CHONDROITIN SULFATE AND HEPARAN SULFATE DURING EARLY NEURONAL POLARIZATION

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Abstract—Axon-dendrite polarity of neurons is essential for information processing in the nervous system. Here we studied the functions of chondroitin sulfate (CS) and heparan sulfate (HS) in neuronal polarization using cultured dissociated hippocampal neurons. Immunohistochemical analyses of early cultured neurons indicated the distribution of these glycosaminoglycans to be quite different. While CS epitopes were accumulated in the focal contacts present in axons and cell bodies, those of HS were detected ubiquitously on the cell surface including on dendrites and axons. Treatment with chondroitinase (CHase) ABC, which degrades CS, and knockdown of a CS sulfotransferase, N-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (4,6-ST), which is involved in the biosynthesis of oversulfated structures, induced the formation of multiple axons in hippocampal neurons. Time-lapse recordings revealed the multiple axons of CHase ABC-treated neurons to be highly unstable, extending and retracting, repeatedly. CHase ABC-treatments suggested that CS is involved in the formation of phosphorylated focal adhesion kinase-positive focal contacts. Thus, CS may enhance integrin signaling in the nascent axons, supporting axon specification. On the other hand, when neurons were treated with heparitinases that specifically degrade HS, neurons with a single axon increased. The axons of HSase-treated neurons extended steadily and showed almost no retraction. These results suggest that CS stabilizes and HS destabilizes the growth of axons in an opposing manner, contributing to early neuronal polarization.

Key words: glycosaminoglycan, extracellular matrix, focal contact, hippocampal neuron, proteoglycan.

Neurons are highly polarized cells that typically have a single axon and multiple short dendrites, which are fundamental to the directional flow of electrical information in the nervous system. The process of neuronal polarization has been studied intensively using dissociated hippocampal neurons (Dotti et al., 1988; Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009). Shortly after plating, neurons extend several filopodia and form small protrusion veils (stage 1). After several hours, the neurons form several morphologically indistinguishable minor processes (stage 2). Then, one of these minor processes extends rapidly and becomes an axon, establishing the neuronal polarity (stage 3). A few days later, the other neurites grow and become dendrites (stage 4). After about 7 days, the neurons form synapses and establish neuronal networks (stage 5) (Dotti et al., 1988). Recent studies have identified numerous intracellular signaling molecules involved in this polarization process including phosphoinositide 3-kinase, the Rho family small GTPases, glycogen synthase kinase-3β and CRMP2 (Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009). These signaling molecules are considered to be asymmetrically activated by extracellular molecules such as brain-derived neurotrophic factor, netrin, and Wnt, leading to the axon differentiation (Prasad and Clark, 2006; Adler et al., 2006; Mai et al., 2009). However, little is known about the mechanism by which extracellular cues asymmetrically activate intracellular signaling pathways. Recently, Da Silva et al. (2005) indicated that plasma membrane ganglioside sialidase determines the axonal fate of hippocampal neurons, suggesting that cell surface glycoconjugates play critical roles in neuronal polarization. In this study, we investigated the possibility that glycosaminoglycans contribute to the asymmetrical activation of signaling during polarization of hippocampal neurons.

Glycosaminoglycans, especially chondroitin sulfate (CS) and heparan sulfate (HS), are major components of the cell surface of neurons and bind to many growth factors and extracellular matrix molecules including netrin and Wnt (Bandtlow and Zimmermann, 2000; Shipp and Hsieh-Wilson, 2007). Many studies have indicated that exogenously added CS and HS preparations modified the neuritogenesis of various neurons *in vitro* (Lafont et al., 1992; Fernaud-Espinosa et al., 1994; Bao et al., 2004; Mikami et al., 2009). Furthermore, when the ventricular surfaces of retinas were exposed to exogenous free CS preparations, the cell bodies and nerve fiber layers of retinal ganglion cells were repolarized to the opposite side of the neuroepithelium (Brittis and Silver, 1994). These observations suggested that glycosaminoglycans are involved in neuronal polarization, however, the mechanisms are unknown.

Previously, we revealed that neurons express various sulfotransferases required for the biosynthesis of glycosaminoglycans (Yabe et al., 2005; Ishii and Maeda, 2008a). It has been proposed that these sulfotransferases are responsible for the construction of micro-structural do-

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Abbreviations: BSA, bovine serum albumin; β-D-xyloside, 4-methylumbelliferyl β-D-xyloside; CHase, chondroitinase; CS, chondroitin sulfate; D structure, (GlcA(2-*O*-sulfate)β1-3GalNAc(6-*O*-sulfate); E16, embryonic day 16; E structure, GlcAβ1-3GalNAc(4,6-*O*-disulfate); FAK, focal adhesion kinase; GalNAc, N-acetylgalactosamine; GFP, green fluorescent protein; GlcA, glucuronic acid; HAase, hyaluronidase; HS, heparan sulfate; HSase, heparitinase; MCSP, melanoma chondroitin sulfate proteoglycan; NA, numerical aperture; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PTPζ, receptor-type protein tyrosine phosphatase ζ; UST, uronyl 2-*O*-sulfotransferase; 4, 6-ST, *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase.

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mains in glycosaminoglycans that selectively bind to various proteins and modify their physiological activities (Shipp and Hsieh-Wilson, 2007; Maeda, 2010). Highly sulfated regions, which are composed of oversulfated structures, often constitute the functional micro-structural domains in glycosaminoglycans. Glycosaminoglycans are present in the form of proteoglycans, in which they are covalently attached to the core proteins that determine the location of these polysaccharides in the cells (Bandtlow and Zimmermann, 2000; Maeda et al., 2010). Therefore, there is a possibility that neurons express glycosaminoglycans in restricted regions on their surface and asymmetrically activate signaling pathways for neuronal polarization. In fact, by using *in utero* electroporation, we revealed that knockdown of two CS sulfotransferases, *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (4,6-ST) and uronyl 2-*O*-sulfotransferase (UST), which are responsible for the biosynthesis of oversulfated structures in CS, suppressed neuronal migration in the cerebral cortex (Ishii and Maeda, 2008b). The knocked down cells showed a multipolar morphology, and it seemed that they cannot migrate radially along radial glial fibers probably because of an inability to adopt a bipolar shape, suggesting that neuronal CS plays critical roles in the polarization of cortical neurons *in vivo*.

In this study, we further examined the roles of endogenous glycosaminoglycans in neuronal polarization using dissociated hippocampal neurons. We found that CS and HS play opposite functions in neuronal polarization, with CS stabilizing and HS destabilizing the axons, probably through regulation of extracellular matrix molecules. Such opposing activities of these glycosaminoglycans may play important roles in neural development, plasticity and axon regeneration.

EXPERIMENTAL PROCEDURES

Animals

Pregnant ICR mice were purchased from Japan SLC (Shizuoka, Japan). All of the animal experiments were performed in accordance with the guidelines for animal research of the Japan Neuroscience Society and approved by the Animal Use and Care Committee of the Tokyo Metropolitan Institute for Neuroscience.

Cell culture

The mice were anesthetized with ether, and the embryos were dissected out. The hippocampi of embryonic day 16 (E16) mice were dissected and treated with a 0.025% trypsin-EDTA solution (Invitrogen, Carlsbad, CA, USA). Cells were dissociated by trituration with Pasteur pipettes and resuspended in Eagle's minimum essential medium containing 20% fetal bovine serum. The cells were plated on glass bottom culture dishes (Matsunami Glass, Osaka, Japan) coated with 0.1 mg/ml of poly-L-lysine (70–150 kDa; Sigma, St. Louis, MO, USA) at a density of ~2000 cells/cm^2. In some cases, poly-L-lysine-coated coverslips were further coated with 5 μg/ml of laminin (Sigma). After cells had attached to the glass coverslips, the medium was changed to a conditioned medium of astrocytes, and the cells were cultured under 5% CO_2 at 37 °C. Astrocytes were prepared from the cerebral cortices of E16 mice (Nishimura et al., 2007), and the conditioned medium was prepared by culturing the cells in serum-free Neurobasal medium (Invitrogen) containing B-27 supplement (Invitrogen) for 2–3 days. For time-lapse imaging, hippocampal neurons (~5000 cells/cm^2) were cultured in the presence of feeder zones of astrocytes located on the peripheral polystyrene part of the glass bottom culture dishes.

Protease-free chondroitinase (CHase) ABC, heparitinase (HSase) I, HSase II, hyaluronidase (HAase) SD (Seikagaku, Tokyo, Japan), and 4-methylumbelliferyl β-D-xyloside (Sigma) were added when the culture medium was changed to the conditioned medium of astrocytes.

Knockdown experiments

Immediately after dissociation, hippocampal neurons were transfected with shRNA constructs using the Amaxa Nucleofector device (Wako, Osaka, Japan) according to the manufacturer's instructions. In brief, 2~2.5$\times$10^6 cells were resuspended in 100 μl of Nucleofector solution containing 5 μg of plasmid, and the electroporation was performed using the program O-05. The cells were immediately resuspended in Eagle's minimum essential medium containing 20% fetal bovine serum, and cultured on glass bottom culture dishes as described above.

In utero electroporation was performed as described previously (Ishii and Maeda, 2008b). Briefly, timed pregnant ICR mice (E14) were anesthetized with sodium pentobarbital (Dainippon Pharma, Osaka, Japan) at 40 mg/kg of body weight, and the uterine horns were exposed. A plasmid DNA solution (4 μg/μl) was injected (~2 μl) into the left side of the lateral ventricle. The heads of embryos in the uterus were placed between the forceps-type electrode (Nepa Gene, Chiba, Japan), and four electrical pulses (35 V, 50 ms in duration at intervals of 950 ms) were delivered using an electroporator (CUY21; Nepa Gene). The uterine horns were returned into the abdominal cavity, and the embryos were allowed to develop until E18.

The construction of shRNA plasmids and details of their specificities and knockdown effects were described previously (Ishii and Maeda, 2008b). The target sequences were 5′-GGACTACGGTTTCTGCATG-3′ for UST, and 5′-AGACAAGAGTTGCATATGT-3′ for 4,6-ST.

Immunocytochemistry

For the cell surface localization of glycosaminoglycans, live neurons were incubated in the medium containing MO-225 or HepSS1 (Seikagaku) monoclonal antibodies (1:20 each) at 37 °C for 15 min. Neurons were washed three times with a solution containing 0.2% bovine serum albumin (BSA), 3% sucrose and phosphate-buffered saline (PBS) that had been prewarmed at 37 °C. They were then fixed with 4% paraformaldehyde (PFA)/3% sucrose/PBS for 15 min and washed three times with 0.02% BSA/PBS. Neurons were blocked with 1% BSA/4% normal goat serum/PBS for 15 min, and incubated in a solution containing Alexa Fluor 594 goat anti-mouse IgM (Molecular Probes, Eugene, OR, USA) (1:200) for 1 h. After being washed with 0.02% BSA/PBS, neurons were fixed again with 4% PFA/3% sucrose/PBS for 10 min. Then, they were permeabilized with 0.01% Triton X-100/1% BSA/PBS for 10 min, and incubated with a Tuj1 monoclonal antibody (Covance, Emeryville, CA, USA) (1:500) overnight at 4 °C. After being washed with 0.02% BSA/PBS, neurons were incubated with Alexa Fluor 488 goat anti-mouse IgG (1:200) for 1 h. After another wash with 0.02% BSA/PBS, neurons were observed under a confocal laser scanning microscope (FV-1000; Olympus, Tokyo, Japan) using a UplanSApo 60$\times$numerical aperture(NA) 1.42 oil lens. Results were then processed using Adobe Photoshop CS4 software (Adobe Systems, San Jose, CA, USA) with minimal adjustments of brightness, contrast and color balance applied to whole images.

In the case of triple labeling, neurons were first incubated with MO-225, then fixed and blocked as above. They were incubated in