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Research Paper

Requirement of keratan sulfate proteoglycan phosphacan with a specific sulfation pattern for critical period plasticity in the visual cortex

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ABSTRACT

Proteoglycans play important roles in regulating the development and functions of the brain. They consist of a core protein and glycosaminoglycans, which are long sugar chains of repeating disaccharide units with sulfation. A recent study demonstrated that the sulfation pattern of chondroitin sulfate on proteoglycans contributes to regulation of the critical period of experience-dependent plasticity in the mouse visual cortex. In the present study, we investigated the role of keratan sulfate (KS), another glycosaminoglycan, in critical period plasticity in the mouse visual cortex. Immunohistochemical analyses demonstrated the presence of KS containing disaccharide units of *N*-acetylglucosamine (GlcNAc)-6-sulfate and nonsulfated galactose during the critical period, although KS containing disaccharide units of GlcNAc-6-sulfate and galactose-6-sulfate was already known to disappear before that period. The KS chains were distributed diffusely in the extracellular space and densely around the soma of a large population of excitatory and inhibitory neurons. Electron microscopic analysis revealed that the KS was localized within the perisynaptic spaces and dendrites but not in presynaptic sites. KS was mainly located on phosphacan. In mice deficient in GlcNAc-6-*O*-sulfotransferase 1, which is one of the enzymes necessary for the synthesis of KS chains, the expression of KS was one half that in wild-type mice. In the knockout mice, monocular deprivation during the critical period resulted in a depression of deprived-eye responses but failed to produce potentiation of nondeprived-eye responses. In addition, T-type Ca^{2+} channel-dependent long-term potentiation (LTP), which occurs only during the critical period, was not observed. These results suggest that regulation by KS-phosphacan with a specific sulfation pattern is necessary for the generation of LTP and hence the potentiation of nondeprived-eye responses after monocular deprivation.

1. Introduction

Experience-dependent development of cortical functions has been investigated intensively in the visual cortex. Visual cortical neurons respond selectively to specific features of visual stimuli (Hubel, 1982), and this response selectivity is refined and maintained by visual experience during a postnatal developmental period called the critical period (Wiesel, 1982). Activity-dependent long-term synaptic modification has been considered to underlie the refinement of visual responsiveness (Bear et al., 1987; Katz and Shatz, 1996; Singer, 1995; Zhang and Poo, 2001). To test this hypothesis, ocular dominance plasticity in the visual cortex has been used. Visual deprivation of one eye during the critical period produces weakening and strengthening of visual responsiveness to the deprived and nondeprived eyes, respectively, in visual cortical neurons (Frenkel and Bear, 2004; Huang et al., 1999). Further, the depression of deprived-eye responses has been suggested to be mediated by N-methyl-D-aspartate (NMDA) receptor-mediated long-term depression (Heynen et al., 2003; Liu et al., 2008; Yoon et al., 2009). On the other hand, we suggested that T-type Ca^{2+} channel-dependent long-term potentiation (LTP) mediates the potentiation of nondeprived-eye responses (Yoshimura et al., 2008).

Extracellular matrix molecules regulate the development and function of the central nervous system, including experience-dependent development of the visual cortex (Wang and Fawcett, 2012). Proteoglycans, major components of the extracellular matrix, consist of a core protein and glycosaminoglycans, which are long sugar chains of repeating disaccharide units with sulfation. There are four types of sulfated glycosaminoglycans. They are chondroitin sulfate (CS), dermatan sulfate, heparan sulfate and keratan sulfate (KS). The functional role of CS

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has been intensely investigated, because CS is the major sulfated glycosaminoglycan in the brain. Digestion of CS in the adult visual cortex with chondroitinase ABC reactivates ocular dominance plasticity (Pizzorusso et al., 2002), suggesting that CS is involved in the regulation of developmental plasticity in the visual cortex. Moreover, it has been demonstrated that developmental changes in sulfation patterns of the CS determine the timing of the closure of the critical period (Miyata et al., 2012).

KS consists of repeating disaccharide units of *N*-acetylglucosamine (GlcNAc) and galactose, and exists in extracellular spaces as KS proteoglycan (Funderburgh, 2002). While KS plays an inhibitory role in axonal sprouting/regeneration after injury of the spinal cord like CS (Imagama et al., 2011; Ito et al., 2010), it is unknown whether KS is also involved in the regulation of developmental plasticity in the brain. Sulfation of GlcNAc at the C-6 position is necessary for the elongation of KS sugar chains (Kitayama et al., 2007). This GlcNAc-6-sulfation of KS is mediated by GlcNAc-6-*O*-sulfotransferase (GlcNAc6ST) (Uchimura, 2015). Among the members of the GlcNAc6ST enzyme family (Uchimura and Rosen, 2006), GlcNAc6ST1, GlcNAc6ST3 and GlcNAc6ST5 have been reported to mediate this sulfation in vivo (Akama et al., 2000; Hayashida et al., 2006; Hoshino et al., 2014; Zhang et al., 2006). In the present study, we examined whether KS is involved in the regulation of critical period plasticity in the visual cortex, by using GlcNAc6ST1-deficient mice we generated previously (Uchimura et al., 2004, 2005). The results demonstrated that KS with a specific sulfation pattern is required for T-type Ca^{2+} channel-dependent LTP and one component of ocular dominance plasticity.

2. Material and methods

Experiments were approved by the Animal Research Committee of Nagoya University, and performed in accordance with the guidelines of Nagoya University.

2.1. Animals

C57BL/6J mice were purchased from SLC Inc. (Hamamatsu, Japan). GlcNAc6ST1-knockout (KO) mice (Uchimura et al., 2004) and vesicular γ-aminobutylic acid (GABA) transporter (VGAT)–Venus transgenic mice were maintained on a C57BL/6J genetic background (Wang et al., 2009). All mice were maintained in the animal facilities of the Nagoya University Graduate School of Medicine and Research Institute of Environmental Medicine.

2.2. Antibodies

The following materials were obtained commercially from the indicated sources. The BCD4 anti-KS antibody and the 6B4 mouse monoclonal anti-phosphacan antibody were from Seikagaku Corporation (Tokyo, Japan); mouse anti-actin antibody (clone AC-40) was from Sigma (St. Louis, MO); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 was from Caltag (Burlingame, CA); HRP-conjugated goat anti-rabbit IgG (H + L) was from Cell Signaling Technology (Danvers, MA); rabbit anti-glutamate aspartate transporter (GLAST), rabbit anti-synaptophysin and guinea pig anti-VGAT polyclonal antibodies were from Frontier Institute Co., Ltd. (Hokkaido, Japan); rabbit anti-microtubule-associated protein 2 (MAP2), guinea pig anti-vesicular glutamate transporter 1 (VGlut1), and rabbit anti-parvalbumin (PV) polyclonal antibodies were from Millipore Inc. (Temecula, CA); Alexa488-conjugated goat anti-rabbit IgG (H + L), Cy™3-conjugated goat anti-mouse IgG1, HRP-conjugated goat anti-mouse IgG (H + L), Cy™3-conjugated goat anti-mouse IgG1, Alexa594-conjugated goat anti-rabbit IgG (H + L) and Cy™2-conjugated streptavidin were from Jackson ImmunoResearch Laboratories (West Grove, PA) and NeuroTrace™Fluorescent Nissl Stains, Alexa 488-conjugated goat anti-guinea pig IgG and Alexa Fluor 405 goat anti-rabbit IgG were from Life Technologies (Carlsbad, CA).

2.3. Western blotting

The brain was removed from the skull, after mice were anesthetized with 50 mg/kg of pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan) and then transcardially perfused with PBS. The dissected visual cortices were frozen immediately in liquid nitrogen. Fractionation of brain samples was performed as described previously (Hosono-Fukao et al., 2012). Snap-frozen mouse primary visual cortices (~25 mg) from critical period mice were homogenized in ice-cold Tris-buffered saline (TBS) containing cOmplete protease inhibitor cocktail (Roche). The material was ultracentrifuged at 100,000 ×g for 20 min at 4 °C. The supernatant was used as the "TBS-soluble fraction". The pellet was re-suspended in TBS containing 1% SDS and cOmplete protease inhibitor. The suspension was centrifuged at 12,000 rpm for 30 min at room temperature (RT). The supernatant was used as the "SDS-soluble fraction". Protein concentrations were measured by using a Proteostain Protein Quantification kit (Dojindo, Tokyo, Japan). Five to twenty micrograms of proteins in the TBS-soluble fraction was subjected to SDS-PAGE on 6%, 10% or 15% gels. To determine the effects of glycosaminoglycan-degrading enzymes, 1% Triton-soluble whole-tissue lysates were prepared and used as the "Triton-soluble fractions". The samples were treated with enzymes at 37 °C overnight before undergoing electrophoresis. The enzymes used were 50 mU chondroitinase ABC (Seikagaku) and 20 mU keratanase (Seikagaku). Electrophoresed samples were blotted onto polyvinylidene fluoride membranes (Millipore). Membranes were blocked with TBS containing 0.05% Tween-20 (TBS-T) and 3% skim milk for 1 h and then incubated with the primary antibody: R10G anti-KS antibody (1:500 dilution) or BCD4 anti-KS antibody (1:1000 dilution) in 5% skim milk/TBS-T overnight at 4 °C, or anti-actin antibody (1:10,000 dilution) for 1 h at RT. Membranes were washed with TBS-T and incubated for 30 min at RT with HRP-conjugated secondary antibodies. Bound antibodies were detected with NOVEX ECL chemiluminescent reagent (Invitrogen). Signals were quantified by using ImageJ (National Institutes of Health, Bethesda, MD).

2.4. Immunoprecipitations

Triton X-100 was added to 200 μg of proteins in TBS soluble fractions to a final concentration of 1% (w/v). The suspension was heated for 10 min at 95 °C. The samples were digested with chondroitinase ABC. The digested materials were mixed with the rabbit polyclonal anti-Ptprz-S antibody for 6 h at 4 °C. The immune complex was precipitated with 40 μl of a 50% (v/v) suspension of protein A Sepharose for 16 h at 4 °C. After centrifugation, the pellets were washed with PBS containing 1% Triton X-100. The immunocomplexes bound to the protein A beads were isolated by centrifugation and then subjected to immunoblotting.

2.5. Immunohistochemistry

Dissected brains were fixed for 24 h in 4% paraformaldehyde and then sunk in 30% sucrose in PBS. Thirty micrometer coronal sections were cut with a freezing microtome and then stored in a cryoprotective medium. Brain slice sections were permeabilized and blocked with PBS containing 0.3% Triton X-100 and 3% BSA for 1 h at RT. Sections were incubated with R10G (1:100 dilution) or BCD4 (1:100) anti-KS antibody, and anti-PV (1:500), anti-GLAST (1:1000), anti-VGlut1 (1:1000), anti-VGAT (1:1000), anti-MAP2 (1:200) or Streptavidin-Cy2 (1:50) in PBS containing 0.3% BSA overnight at 4 °C. Sections were then incubated with Cy3-conjugated goat anti-mouse IgG1 (1:250 dilution), Alexa 488-conjugated goat anti-rabbit IgG (1:250 dilution), or Alexa 488-conjugated goat anti-guinea pig IgG (1:200) with or without NeuroTrace (1:200) in PBS containing 0.3% BSA. Sections were mounted on glass slides (SF17293; Matsunami, Osaka, Japan) with FluorSave Reagent (Merck). Digital images were captured by