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ACTIVITY-DEPENDENT REMODELING OF CHONDROITIN SULFATE PROTEOGLYCANS EXTRACELLULAR MATRIX IN THE HYPOTHALAMO–NEUROHYPOPHYSIAL SYSTEM

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Abstract—The hypothalamo–neurohypophysial system (HNS) consisting of arginine vasopressin (AVP) and oxytocin (OXT) magnocellular neurons shows the structural plasticity including the rearrangement of synapses, dendrites, and neurovascular contacts during chronic physiological stimulation. In this study, we examined the remodeling of chondroitin sulfate proteoglycans (CSPGs), main extracellular matrix (ECM), in the HNS after salt loading known as a chronic stimulation to cause the structural plasticity. In the supraoptic nucleus (SON), confocal microscopic observation revealed that the immunoreactivity of 6B4 proteoglycans (PG) was observed mainly at AVP-positive magnocellular neurons but that of neurocan was seen chiefly at OXT-positive magnocellular neurons. The immunoreactivity of phosphacan and aggrecan was seen at both AVP- and OXT-positive magnocellular neurons. Electron microscopic observation further showed that the immunoreactivity of phosphacan and neurocan was observed at astrocytic processes to surround somata, dendrites, and terminals, but not synaptic junctions. In the neurohypophysis (NH), the immunoreactivity of phosphacan, 6B4 PGs, and neurocan was observed at AVP-positive magnocellular terminals, but the reactivity of *Wisteria floribunda agglutinin* lectin was seen at OXT-positive ones. The immunoreactivity of versican was found at microvessel and that of aggrecan was not detected in the NH. Quantitative morphometrical analysis showed that the chronic physiological stimulation by 7-day salt loading decreased the level of 6B4 PGs in the SON and the level of phosphacan, 6B4 PGs, and neurocan in the NH. These results suggest that the extracellular microenvironment of CSPGs is different between AVP and OXT magnocellular neurons and activity-dependent remodeling of CSPGs could be involved in the structural plasticity of the HNS.

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Abbreviations: AVP, arginine vasopressin; ChABC, chondroitinase ABC; CNS, central nervous system; CSPGs, chondroitin sulfate proteoglycans; CS-4-PG, chondroitin-4-sulfate-containing PG; CS-6-PG, chondroitin-6-sulfate-containing PG; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GAGs, glycosaminoglycans; G3PDH, glyceraldehydes-3-phosphate dehydrogenase; HNS, hypothalamo–neurohypophysial system; MMPs, matrix metalloproteases; NGS, normal goat serum; NH, neurohypophysis; OXT, oxytocin; PB, phosphate buffer; PBS, phosphate-buffered saline; PBST, PBS containing 0.3% Triton X-100; PFA, paraformaldehyde; PGs, proteoglycans; PNNs, perineuronal nets; RPTPζ, receptor-type protein-tyrosine phosphataseζ; RT-PCR, reverse transcription-polymerase chain reaction; SON, supraoptic nucleus; WFA, wisteria floribunda agglutinin.

Key words: plasticity, extracellular matrix, critical period, synaptogenesis, rearrangement.

Chondroitin sulfate proteoglycans (CSPGs) which are glycoproteins carrying sulfated glycosaminoglycans (GAGs) are known to be main components of extracellular matrix (ECM) in the CNS. Until now, more than 30 primary structures of proteoglycans (PGs) have been elucidated (for review, see Bandtlow and Zimmermann, 2000). Phosphacan and neurocan are major ECM components in the central nervous system (CNS) (for reviews, see Margolis et al., 1996; Margolis and Margolis, 1997; Oohira et al., 2000). Phosphacan is a secreted isoform of receptor-type protein-tyrosine phosphataseζ (RPTPζ) and lacks the intracellular tyrosine phosphatase domains (for review, see Margolis et al., 1996). Phosphacan has inhibitory effects on the neurite outgrowth in cultured retinal ganglion (Inatani et al., 2001) and dorsal root ganglion neurons (Sango et al., 2003), but it promotes the neurite outgrowth in cultured cortical neurons (Maeda and Noda, 1996) during neuronal development. The expression and proteolytic cleavage of neurocan is developmentally regulated (Rauch et al., 1991). The N-terminal fragment with 130 kDa core protein is named as neurocan-130 and C-terminal half fragment with 150 kDa core protein is called as neurocan-C (Rauch et al., 1991; Matsui et al., 1994). Full length form and two proteolytic isoforms of neurocan are expressed in juvenile CNS, whereas neurocan-130 and neurocan-C are mainly expressed in adult one. Neurocan is well known to inhibit the neurite outgrowth of cerebellar, retinal ganglion, and dorsal root ganglion neurons during neuronal development (Friedlander et al., 1994; Inatani et al., 2001; Sango et al., 2003). Aggrecan is a main component of perineuronal nets (PNNs), the ECM of many CNS neurons, surrounding neuronal cell bodies and dendrites in mesh-like structures (Matthews et al., 2002). Aggrecan is shown to inhibit the neurite outgrowth through Rho/ROCK signaling pathway in dorsal root ganglion neurons (Chan et al., 2008).

A lot of studies have proposed that CSPGs are possible molecules to stabilize neuronal connections and attenuate the structural plasticity or rearrangements in adult CNS (Rhodes and Fawcett, 2004; Pizzorusso, 2009). Following studies have supported this hypothesis: Firstly, PNNs composed of CSPGs and hyaluronan are firstly detected at the onset of the period during which the pattern of neuronal activity determines the mature synaptic circuitry and are increased in number and intensity during

0306-4522/10 $ - see front matter
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postnatal CNS development (Köppe et al., 1997; Brückner et al., 2000). In the visual cortex, neurocan and aggrecan appear around the end of the critical period and dark-rearing from birth prolongs the duration of the critical period concomitant with the attenuation of the expression of CSPGs (Lander et al., 1997). Moreover, the number of Cat-315-positive PNNs is reduced in the barrel cortex by the sensory deprivation of whiskers during postnatal CNS development. Secondly, the chondroitinase ABC (ChABC) injection into the visual cortex of adult animals removes CS GAGs from PNNs and thereby restores the ocular dominance plasticity as seen in juvenile ones (Pizzorusso et al., 2002, 2006). This result indicates that the removal of GAGs on CSPGs causes neurons to grow more freely into extracellular space and thereby regulates the neural circuit by forming new synapses (Fox and Caterson, 2002). The ChABC injection is also shown to promote the axonal regeneration or extension in various experimental models in adult CNS. The ChABC injection induces profuse outgrowth of terminal branches from Purkinje infraganglionic plexus in adult cerebellum (Corvetti and Rossi, 2005). Although glial scar containing CSPGs are inhibitory to axon growth and thereby regenerating axons stop at sites of CNS injury, the removal of CSPG GAGs with ChABC attenuates the inhibitory activity and promotes both ascending sensory projections and descending corticospinal tract axons (Bradbury et al., 2002; Kwok et al., 2008; Massey et al., 2006). It is further shown that rats receiving the combination of ChABC and specific rehabilitation show the improved manual dexterity (García-Alías et al., 2008, 2009). The ChABC injection into the amygdala specifically renders subsequently acquired fear memories susceptible to erasure (Gogolla et al., 2009).

The question now arises why certain populations of CNS neurons in adult are able to cause the structural plasticity, which is accompanied with the rearrangement of neuronal connections even after the establishment of neuronal circuits or over the critical period. Several proteases offer the key to an understanding of this issue; for instance, matrix metalloproteases (MMPs), which are known to cleave and degrade CSPGs, cause the neuronal plasticity in the CNS (Kaczmarek et al., 2002; Vaillant et al., 1999; Ethell and Ethell, 2007). Tissue-type plasminogen activator/plasminogen system is also shown to be concerned with the long-term potentiation, kindling, seizures, and motor learning by degrading CSPGs (Kaczmarek et al., 2002; Mataga et al., 2002, 2004; Oray et al., 2004; Yepes and Lawrence, 2004). Thus, numerous evidences have been suggested that extracellular proteases are necessary for the accomplishment of structural plasticity in adult CNS, but almost no evidence to demonstrate the activity-dependent remodeling of CSPGs has been documented in adult CNS.

The hypothalamo–neurohypophysial system (HNS) is a good model to elucidate how the remodeling of CSPGs is concerned with the structural plasticity in adult CNS (for review, see Miyata and Hatton, 2002). The HNS is a well-characterized peptidergic neuronal system consisting of arginine vasopressin (AVP) and oxytocin (OXT), which locate in the supraoptic nucleus (SON) and paraventricular nucleus and release them into blood capillary in the neurohypophysis (NH) (for review, see Cunningham and Sawchenko, 1991). The chronic physiological stimulation by salt loading and lactation causes the structural plasticity such as the apposition of neuronal membrane and multiple synapse formation in the SON (Modney and Hatton, 1989; Miyata et al., 1994; Hawrylak et al., 1998; Piet et al., 2004) and neurovascular contacts in the NH (Matsunaga et al., 2002; Miyata et al., 2001). This structural plasticity is observed uniformly in almost all magnocellular neurons of stimulated animals and hence quantitative structural and biochemical analyses are possible.

In our previous study, we have demonstrated that the expression of 6B4 PGs in the SON is downregulated with salt loading (Miyata et al., 2004), however, recent studies have reported that 6B4 epitope is same to Cat-315 and anti-6B4 PGs/Cat-315 antibody recognizes an O-mannose linked epitope of HNK-1 family on aggrecan and phosphacan/RPTPζ (Dino et al., 2006; Saitoh et al., 2008). Thus, our previous study is restrictive and does not explain the whole events of activity-dependent CSPG remodeling occurring in the HNS. Therefore, we extended the investigation of this issue to more understand the remodeling of CSPGs required for structural plasticity in the HNS; firstly, the subcellular and ultrastructural localizations of CS GAGs and CSPG core proteins such as phosphacan, 6B4 PGs, neurocan, and aggrecan were investigated by using specific antibodies and Wisteria floribunda agglutinin (WFA) lectin. Secondly, activity-dependent degradation of CSPGs was examined by the quantitative morphometrical immunohistochemistry, western blot, and reverse transcription-polymerase chain reaction (RT-PCR).

EXPERIMENTAL PROCEDURES

Animals

Wistar male rats (8–12 week old) were used for the present experiments. Rats were housed under standard conditions with a 12:12 h dark and light cycle. They were divided into two groups: unstimulated control male rats that were free access to food and water *ad libitum*; salt loading, chronically stimulated males whose drinking water had been replaced with 2% NaCl instead of normal tap water according to others (Theodosis et al., 1999). The fixation and sampling of brain tissues were performed on 10:00 to 12:00. All experimental protocols were performed in accordance with the guidelines for animal research of the Neuroscience Society of Japan to minimize the number of animals used and their suffering and approved by the committee of Kyoto Institute of Technology.

Antibodies and reagents

The following primary antibodies were used for investigating the localization of CSPGs: monoclonal anti-chondroitin-4-sulfate-containing PG (CS-4-PG; mouse IgG, Millipore-Chemicon, Temecula, CA, USA); monoclonal anti-chondroitin-6-sulfate-containing PG (CS-6-PG; mouse IgG, Millipore-Chemicon); monoclonal anti-6B4 PGs (mouse IgM, Maeda et al., 1995); monoclonal anti-phosphacan (clone 3F8, mouse IgG, Developmental Studies Hybridoma Bank, Iowa, IA, USA, Rauch et al., 1991); monoclonal anti-neurocan (clone 1G2, mouse IgG, Oohira et al., 1994); polyclonal anti-neurocan (PAb291, rabbit IgG, Matsui et al., 1994); polyclonal anti-versican (rabbit IgG, Millipore-Chemicon); polyclonal anti-ag-