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CONSTRUCTION OF PERINEURONAL NET-LIKE STRUCTURE BY CORTICAL NEURONS IN CULTURE

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Abstract—Perineuronal nets consisting of chondroitin sulfate proteoglycans and hyaluronic acid are associated with distinct neuronal populations in mammalian brain. Whether neurons or glia cells produce these surface-associated chondroitin sulfate proteoglycan perineuronal nets has remained in question. In the present study, we observed perineuronal net-like structure by rat cortical neurons in dissociated culture using *Wisteria floribunda* aggulutinin, hyaluronic acid binding protein, and the antibodies recognizing chondroitin sulfate proteoglycans. The double labeling experiments showed that perineuronal net-like structure labeled with *Wisteria floribunda* aggulutinin was observed often at parvalbumin-positive neurons in dissociated cortical culture without glia. Perineuronal net-like structure was not seen at the early stage of culture, but they became visible concomitantly with neuronal maturation after longer culture. High magnification observation further demonstrated that *Wisteria floribunda* aggulutinin labeling on cortical neurons was seen as numerous puncta along surface of somata and proximal dendrites, but not axons and synapses. Perineuronal net-like structure on cultured neurons was also visualized using chondroitin sulfate proteoglycan-specific antibodies and hyaluronic acid binding protein. Double labeling study demonstrated that perineuronal net-like structure in cultured cortical neurons was composed of chondroitin sulfate proteoglycans such as neurocan and phosphacan. The hyaluronidase treatment of live neurons abolished cellular labeling of hyaluronic acid binding protein and concomitantly diminished that of *Wisteria floribunda* aggulutinin.

These results indicate that cultured cortical neurons are able to construct perineuronal net-like structure without glial cells. © 2005 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: CS, chondroitin sulfate; CSPGs, chondroitin sulfate proteoglycans; CS-4-PG, chondroitin-4-sulfate-containing proteoglycan; CS-6-PG, chondroitin-6-sulfate-containing proteoglycan; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFAP, glial fibril acidic protein; HABP, hyaluronic acid binding protein; HRP, horseradish peroxidase; MAP2, microtubule-associated protein 2; NGS, normal goat serum; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.3% Triton X-100; PFA, paraformaldehyde; PGs, proteoglycans; RPTPβ, receptor protein tyrosine phosphatase β; TBST, 50 mM Tris-buffered saline containing 0.5% Tween-20; WFA, *Wisteria floribunda* aggulutinin.

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Proteoglycans (PGs) are glycoproteins that carry covalently bound unbranched up to 200 glycosaminoglycans chains through a serine residue and characteristic carbohydrate linkage regions (for review, see Bandtlow and Zimmermann, 2000). Depending on the disaccharides structures, glycosaminoglycans can be grouped into chondroitin sulfate (CS), dermatan sulfate, heparin/heparan sulfate, and keratan sulfate. Perineuronal nets are composed of several chondroitin sulfate proteoglycans (CSPGs) and hyaluronic acid, and known to surround neuronal somata and proximal dendrites to form lattice-like structures in distinct populations of neurons in the brain (for review, see Celio et al., 1998; Yamaguchi, 2000; Murakami and Ohtsuka, 2003; Rhodes and Fawcett, 2004). The neocortex contains high numbers of perineuronal nets in motor and primary sensory areas, whereas they are less numerous in the association and limbic cortices (Brückner et al., 1994; Hausen et al., 1996).

Perineuronal nets have been visualized using a variety of methods including the binding of lectins such as *Wisteria floribunda* agglutinin (WFA) and *Vicia villosa* aggulutinin that have high affinity to *N*-acetylgalactosamine (Nakagawa et al., 1986; Härtig et al., 1992; Brückner et al., 1993), the binding of hyaluronic acid binding protein (HABP) to hyaluronic acid (Brückner et al., 1998, 2000), and immunological staining for CSPGs (Brückner et al., 1998, 2000, 2003). During postnatal development, perineuronal nets first appear relatively late in postnatal age (Köppe et al., 1997; Brückner et al., 2000). Versican and aggrecan, components of perineuronal nets, are first detected at the onset of the period during which the pattern of neuronal activity determines the mature synaptic circuitry and neuronal phenotype in the visual cortex, and dark-rearing from birth prolongs the duration of the critical period and attenuates the expression of several CSPGs (Lander et al., 1997). Perineuronal nets are well preserved and maintained for 3–10 weeks in slice culture, indicating the differentiation of perineuronal nets is part of the developmental program maintained in brain tissue *in vitro* (Brückner and Grosche, 2001). It is also shown that the formation of perineuronal nets in brain slice culture is promoted via calcium signaling (Brückner and Grosche, 2001).

The functions of perineuronal nets are not beyond speculation which includes several aspects such as the involvement in synaptic stabilization (Hockfield et al.,

1990) and neuroprotection (Brückner et al., 1999). Recently, it is demonstrated that chondroitinase ABC digestion restores ocular dominance plasticity in the adult visual cortex, indicating that CSPG perineuronal nets are crucial factors in regulating experience-dependent plasticity (Pizzorusso et al., 2002). This result, moreover, implies that the degradation of CSPGs around perineuronal nets causes neurons to interact freely in the absence of CSPG extracellular matrix and neuronal processes such as axons and dendrites to grow more freely into extracellular space even in adult brains (Fox and Caterson, 2002). It is also shown that phosphacan/receptor-type protein–tyrosine phosphatase β is downregulated in the hypothalamic supraoptic nucleus with activity-dependent manner and possibly correlated with the structural plasticity (Miyata et al., 2004).

There has been a longstanding debate over the cellular origin of perineuronal nets, namely neurons or glia (Blümcke et al., 1995; Derouiche et al., 1996; for review, see Celio and Blümcke, 1994; Celio et al., 1998). The main reasons for glial origin are that perineuronal nets are associated with not only neuronal membrane surface but also fine astrocytic processes (Brückner et al., 1993) and glial cells but not neurons assemble hyaluronan-based pericellular matrices *in vitro* (Maleski and Hockfield, 1997). On the other hand, it is shown that aggrecan, brevican, and phosphacan are produced by cultured neurons (Lander et al., 1998; Seidenbecher et al., 2002; Hayashi et al., 2005). In the present study, therefore, we explored the possibility that neurons themselves are able to construct perineuronal nets structure in dissociated neuronal culture without glial cells. We detected perineuronal nets *in vitro* by the cytochemistry using WFA and HABP and the immunocytochemistry using anti-CSPG antibodies.

EXPERIMENTAL PROCEDURES

Cell culture

Neuronal cultures were prepared from the cerebral cortex of Wistar rats (E18) according to our previous method (Hayashi et al., 2005). All experimental protocols were performed in accordance with the guideline for animal research of the Neuroscience Society of Japan to minimize the number of animals used and their suffering. Briefly, cerebral cortices were removed and placed into phosphate-buffered saline (PBS) at 4 °C. They were then treated with 12 units/ml papain (Sigma-Aldrich Japan, Tokyo, Japan) and 0.01% DNase I (Sigma-Aldrich Japan) for 20 min at 37 °C. Tissues were suspended, triturated with Dulbecco's modified Eagle's medium (DMEM) containing 5% horse serum and 0.35% glucose, and centrifuged at 150×*g* for 3 min. The dissociated cells were plated on polyethyleneimine-coated glass coverslips at a density of 100,000 cells/cm^2 and grown in Neurobasal medium (Invitrogen, San Diego, CA, USA) containing B27 supplement (Invitrogen). To eliminate glial cells, 2.5 μM AraC (Sigma-Aldrich Japan) was added to the Neurobasal medium for 12 h on 2 days after plating. Immunocytochemical analyses were performed from neuronal culture at the day *in vitro* (DIV) indicated.

To obtain astrocytes, primary mixed glial cell cultures were generated from the cerebral cortex of neonatal rats according to our previous method (Hayashi et al., 2005). The glial cell culture was maintained on plastic dish in DMEM with 10% fetal bovine serum (FBS) for 7 days and the O2A progenitor and microglia were separated from type-1 astrocytes by shaking the cultures at 250 rpm for 18 h at 37 °C. After shaking, type-1 astrocytes were passaged with 0.05% trypsin in PBS, resuspended, and maintained in DMEM containing 10% FBS. The cultures were allowed to attach overnight then shaken to remove loosely adherent cells, and maintained to confluence. After the third passage in this manner, type-1 astrocytes were seeded on a plastic culture dish and maintained to confluence.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM was purchased from Pierce (Rockford, IL, USA). FITC-conjugated anti-rabbit and anti-mouse IgG and horseradish peroxidase (HRP) -conjugated anti-rabbit and anti-mouse IgG were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). The following primary antibodies were used: anti-chondroitin-4-sulfate-containing proteoglycan (CS-4-PG; mouse IgG, ICN Biomedicals, Costa Mesa, CA, USA), anti-chondroitin-6-sulfate-containing proteoglycan (CS-6-PG; mouse IgG, ICN Biomedicals), anti-β-COP (rabbit IgG, Affinity BioReagents, Golden, CO, USA), anti-glial fibril acidic protein (GFAP; mouse IgG, Sigma-Aldrich Japan), anti-microtubule-associated protein 2 (MAP2; mouse IgG, Chemicon), anti-neurocan PAb291 (rabbit IgG, Matsui et al., 1994), anti-neuroglycan C C1 (mouse IgG, Watanabe et al., 1995), anti-O4 (mouse IgM, R&D Systems Inc., Minneapolis, MN, USA), anti-OX-42 (mouse IgG, Serotec Ltd., Oxford, UK), anti-parvalbumin (mouse IgG, Sigma-Aldrich Japan), anti-phosphacan 6B4 (mouse IgM, Seikagaku Corporation, Tokyo, Japan), anti-synapsin (rabbit IgG, Molecular Probes, Eugene, OR, USA), and anti-tau antibodies (rabbit IgG, Chemicon).

Immunocytochemistry

For double labeling of WFA with several subcellular marker proteins or CSPGs, neuronal cultures were washed with PBS and then fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.5) for 20 min. Fixed cells were rinsed with PBS and treated with 25 mM glycine in PBS for 20 min. The cells were further immersed in methanol at −20 °C for 2 min, washed with phosphate-buffered saline containing 0.3% Triton X-100 (PBST) for 15 min, and incubated with 5% normal goat serum (NGS) in PBST for 30 min. The cells were then incubated with anti-β-COP (dilution 1:500), anti-GFAP (dilution 1:100), anti-MAP2 (dilution 1:200), anti-neuroglycan C (dilution 1:16), anti-parvalbumin (dilution 1:100), anti-phosphacan antibody (dilution 1:50), anti-synapsin (dilution 1:500), or anti-tau (dilution 1:200) antibody in PBST containing 5% NGS for 48 h at 4 °C. After several washes with PBST, they were incubated with FITC-conjugated anti-mouse IgG, anti-mouse IgM, or anti-rabbit IgG antibody (10 μg/ml) in PBST at 37 °C for 2 h. The cells were then incubated with biotinylated WFA (Sigma-Aldrich Japan, 10 μg/ml) or HABP (Sigma-Aldrich Japan, 5 μg/ml) in PBST for 48 h at 4 °C. They were then washed with PBST at 4 °C and incubated with Texas Red streptavidin (Vector Laboratories., Burlingame, CA, USA, 20 μg/ml) in PBST at 4 °C for 2 h.

For double labeling of WFA and FITC-conjugated phalloidin, cultured neurons were fixed with 4% PFA in phosphate buffer (pH 7.5) for 20 min, washed with PBS, treated with 25 mM glycine in PBS for 20 min, and washed with PBS. They were incubated with FITC-conjugated phalloidin (Sigma-Aldrich Japan, 2 μg/ml) in PBS for 1 h. After several washes with PBS, the cells were further incubated with biotinylated WFA (Sigma-Aldrich Japan, 10 μg/ml) in PBS for 48 h at 4 °C followed by Texas Red streptavidin (20 μg/ml) in PBS for 2 h at 4 °C.

For immunostaining of anti-CS-4-PG and anti-CS-6-PG antibodies, neurons were treated with chondroitinase ABC before the immunofluorescence procedure, since these two antibodies recognize CS-4-sulfated and CS-6-sulfated stub of core proteins

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