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Chondroitin sulfate demarcates astrocytic territories in the mammalian cerebral cortex

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ABSTRACT

Adjacent astrocytes in the grey matter of the mammalian cerebral cortex are organized in a tile-like manner and separated from one another, forming discrete domains named "non-overlapping territories". We have previously reported that an anti-chondroitin sulfate (CS) antibody, CS-56, marks a subpopulation of cortical astrocytes which we named the dandelion clock-like structure (DACS) based on its morphological characteristics. In the present study, we found that another anti-CS antibody (anti-CS-C) was also able to detect the DACS and the morphological analysis revealed that a single DACS enwrapped five to six neuronal somata on average, which indicated that DACS coincided with a single astrocyte territory. Double labeling of CS-C and glial fibrillary acidic protein (GFAP) showed a slight overlap between the two territories in the adult cerebral cortex of mice. The neuron number enwrapped by a single DACS was unchanged between 3- and 7-week-old mice, while more extensive processes of DACSs were found in 7-week-old mice compared with those in 3-week-old ones. Moreover, the measurement of a single DACS area was significantly increased by 45% between 3- and 7-week-old mice. In addition, DACSs were found in human, monkey, and domestic pig brains, but not mallard ones, indicating that DACS was conserved in mammalian species. Taken together, CS demarcates territories of a certain population of cortical astrocytes and the cerebral cortex is composed of CS-rich astrocytes and -poor astrocytes in a mosaic fashion.

Recent advances in techniques of dye injection into single cells and of green fluorescent protein expression in transgenic mice have enabled us to visualize the whole shapes of astrocytes. Although glial fibrillary acidic protein (GFAP) has been widely used to detect astrocytes, it cannot delineate the complete morphology of individual astrocytes. As GFAP labels less than 15% of their total volume [2], astrocytes are actually much larger, and have much more highly ramified processes, than is apparent in GFAP-labeled cell images. The outline of the ramified processes is cuboidal or round rather than star-shaped [12,14]. The processes of adjacent astrocytes are hardly overlapped each other, and each astrocyte has its own territory, called the non-overlapping territory [2,3]. This structural property has given rise to the idea that synapses within a territory might be under the control of a single astrocyte, because astrocytes can coordinate the activity of a local population of neighboring neurons by synchronously releasing glutamate into the synapses located within their territorial domain [5]. In this model, the astrocytic territory can group a certain number of neurons and synchronize their firing.

Chondroitin sulfate (CS) is a sulfated glycosaminoglycan composed of a repeated disaccharide unit (*N*-acetylgalactosamine and glucuronic acid). CS is covalently bound to a core protein via the serine residues and exists on cell surfaces as a chondroitin sulfate proteoglycan (CSPG). CSPGs are known to be inhibitory substances

Abbreviations: CS, chondroitin sulfate; DACS, dandelion-clock-like structure; GFAP, glial acidic fibrillary acidic protein; CSPG, chondroitin sulfate proteoglycan; d4S, delta-4S; d6S, delta-6S; d0S, delta-0S; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PBST, Triton X-100 in PBS; NGS, normal goat serum; DAPI, 4′,6-diamino-2-phenyl-indole dihydro-chloride; CS-4S, chondroitin-4-sulfate; CS-6S, chondroitin-6-sulfate; CS-2,6, SCS-2,6-sulfate.

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for neuronal regeneration and their upregulation in the injured central nervous system is thought to be a major reason for the failure of axonal regeneration in mammals [1,7]. In a previous study we revealed that the monoclonal anti-CS antibody CS-56 delineates a subpopulation of cortical astrocytes, and we named them dandelion clock-like structures (DACSs) based on their morphology [8]. DACSs are evenly distributed as patches throughout the cerebral cortex in the adult mouse brain. In this study, we found that another anti-CS antibody could detect DACSs and that these structures are conserved across mammalian species, including human. Further, we analyzed the colocalization of CS-C and GFAP immunoreactivities in the adult cerebral cortex of mice. Furthermore, structural relationships between DACSs and neurons and morphological changes of DACSs in postnatal development were also investigated.

Male C56BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and used as 7–10-week-old adults. The mice were housed in plastic breeding cages under standard laboratory conditions (23 °C, 55% humidity in a room with a light–dark cycle of 12 h) and had access to tap water and food ad libitum. Brains of domestic pigs (*Sus domesticus*) and mallards (*Anas platyrhynch*os) were obtained from the Department of Biotechnological Science, Kinki University.Brains of 11-year-old monkeys (*Macaca fuscata*) were sampled at the Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University. A brain sample of a woman who died in a traffic accident was obtained from the Department of Neurobiology and Anatomy, Nagoya City University. All animal protocols for these experiments were approved by the Animal Care Committee of Nara Medical University in accordance with the policies established in the NIH Guide for the care and use of laboratory animals.

The following primary antibodies were used: anti-CS-A (clone 2H6), -CS-C (clone MC21C), -delta-4S (clone 2-B-6, d4S), -delta-6S (clone 3-B-3, d6S) and -delta-0S (clone 1-B-5, d0S) from Seikagaku Biobusiness Corporation (Tokyo, Japan), CS-56 (Sigma–Aldrich, St. Louis, MO), -NeuN (Chemicon, Temecula, CA), -GFAP (Dakocytomation, Glostrup, Denmark), and -PSD-95 (Zymed Laboratory, Carlsbad, CA). The following secondary antibodies were purchased from Molecular Probes (Eugene, OR): Alexa 488-labeled anti-mouse IgM or IgG and Alexa 546-labeled anti-mouse IgG, -rabbit IgG, or -guinea pig IgG. Biotinylated anti-mouse IgM was purchased from Sigma–Aldrich.

The method used was as described previously [8,9]. Briefly, mice were deeply anesthetized with pentobarbital, and perfused transcardially with phosphate-buffered saline (PBS) and then 4% paraformaldehyde (PFA). Brains were dissected out and postfixed with the same fixative at 4 °C for 6 h. For other animal spices, brains were immersion-fixed with 4% PFA overnight. Fifty-micrometer sections were cut with a liner slicer (Pro. 7, DSK, Kyoto, Japan). For immunostaining, free-floating sections were washed with PBS, immersed with 25 mM glycine in PBS for 20 min, and permeabilized with 0.3% Triton X-100 in PBS (PBST). For staining with d4S, d6S, and d0S antibodies, sections were treated with chondroitinase ABC (Seikagaku, 0.1 U/ml) at 37 °C for 3 h. After blocking with 5%

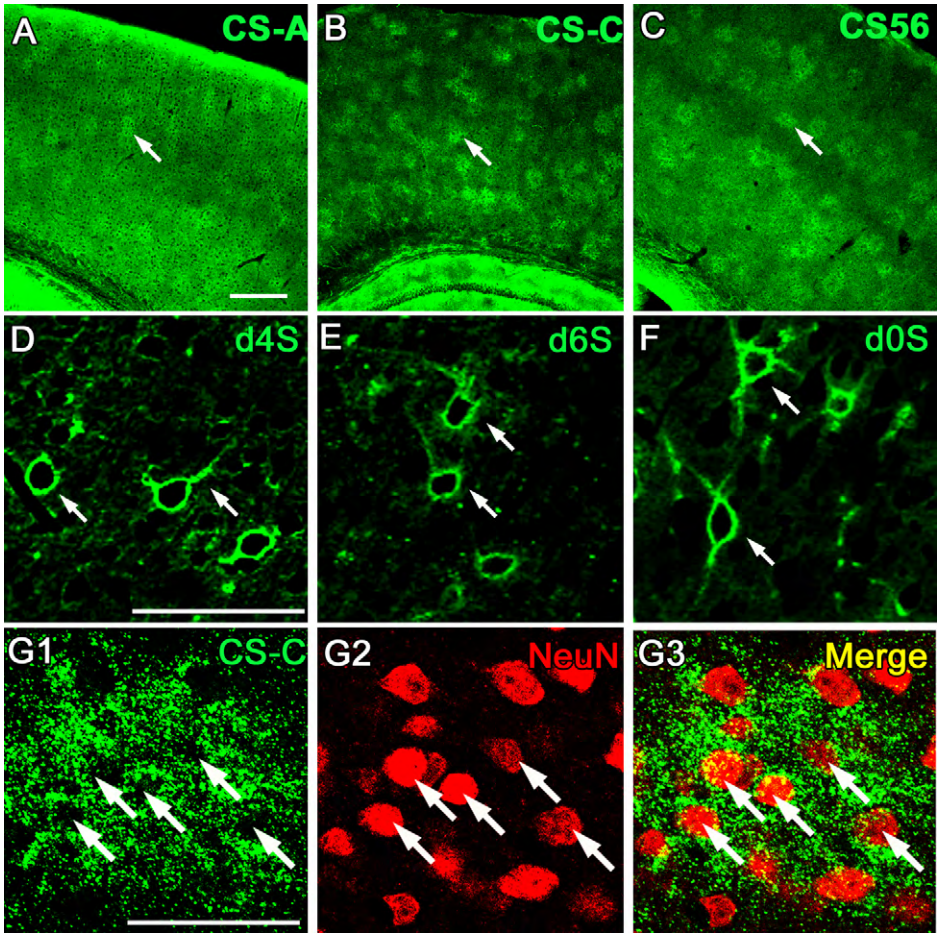

Fig. 1. Anti-CS-C antibody reveals DACS patterns and multiple enwrapped neurons in the adult cerebral cortex of mice. (A–F) Immunohistochemical images for anti-CS-A (A), -CS-C (B), CS-56 (C), -d4S (D), -d6S (E), and -d0S (F) antibodies. Arrows in A–C indicate a representative DACS. Arrows in D–F are typical staining patterns of perineuronal nets. (G) Double labeling with anti-CS-C (G1, green) and NeuN (G2, red) antibodies. A CS-C-labeled DACS enwraps $NeuN^+$ neurons (arrows). Scale bars = 200 μm (A–C) and 50 μm (D–G). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)