Psychiatry Research 270 (2018) 940–946

Contents lists available at ScienceDirect

Psychiatry Research

journal homepage: www.elsevier.com/locate/psychres

Pathological alterations of chondroitin sulfate moiety in postmortem hippocampus of patients with schizophrenia

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ARTICLE INFO

Keywords:
Postmortem brain
Hippocampus
Perineuronal net
Haloperidol

ABSTRACT

Perineuronal nets comprise chondroitin sulfate moieties and their core proteins, and their neuropathological alterations have been implicated in schizophrenia. To explore the molecular mechanism of the perineuronal net impairments in schizophrenia, we measured the immunoreactivity of chondroitin sulfate moieties, major components of perineuronal nets, in three brain regions (postmortem dorsolateral prefrontal cortex, caudate nucleus, and hippocampus) of schizophrenia patients and control subjects. Immunoblotting for chondroitin 4-sulfate and chondroitin 6-sulfate moieties revealed a significant increase in intensity of a 180 kD band of chondroitin 4-sulfate immunoreactivity in the hippocampus of patients, although we detected no significant alteration in their immunoreactivities with any other molecular sizes or in other brain regions. The levels of immunoreactivity were not correlated with postmortem interval, age, or storage time. We failed to find such an increase in a similar molecular range of the chondroitin 4-sulfate immunoreactivity in the hippocampus of the rats chronically treated with haloperidol. These results suggest that the level alteration of the chondroitin 4-sulfate moiety might contribute to the perineuronal net abnormality found in patients with schizophrenia.

1. Introduction

Schizophrenia is a mental disorder, whose biological etiology remains to be clarified, defined and characterized by the following symptoms: positive symptoms such as hallucination, delusions, and disorganized thoughts/speech, negative symptoms such as declining motivation, social withdrawal, and blunted affect, and cognitive dysfunctions such as impaired attention, executive dysfunction, and deficits in working memory (Owen et al., 2016). It is considered that the complex interactions between genetic vulnerability and environmental risk factors may contribute to the onset or pathology of this disease (Lewis and Lieberman, 2000; Ross et al., 2006). The evidence obtained from epidemiological, genetic, postmortem, neuroimaging, and animal studies on schizophrenia suggest that schizophrenia not only is a neurodevelopmental disorder but also shows several characteristics of a neurodegenerative disorder (Catts et al., 2013; Kasai et al., 2003; Kochunov and Elliot Hong, 2014; Lieberman et al., 2001; Rapoport et al., 2013; Rund, 2009). The abnormal neurodevelopmental processes of neurogenesis, neuronal migration, or synaptic plasticity of GABAergic cells have drawn the most attention to the neuropathology and pathophysiology of schizophrenia (Catts et al., 2013). Chondroitin sulfate (CS) is a glycosaminoglycan composed of a repetitive structure of disaccharide units, namely, glucuronic acid and *N*-acetylgalactosamine (GalNAc) (Avram et al., 2014; Galtrey and Fawcett,

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https://doi.org/10.1016/j.psychres.2018.10.062
Received 31 March 2018; Received in revised form 27 August 2018; Accepted 23 October 2018
Available online 30 October 2018
0165-1781/

2007; Kusche-Gullberg and Kjellén, 2003; Soleman et al., 2013; Sugahara et al., 2013). CS chains are covalently bonded to the core protein chondroitin sulfate proteoglycan (CSPG). CS chains can be mainly classified into 4-sulfated and 6-sulfated chondroitin (C4S and C6S, respectively) depending on the difference in the sulfated sites of GalNAc (Avram et al., 2014; Galtrey and Fawcett, 2007; Kusche-Gullberg and Kjellén, 2003; Soleman et al., 2013; Sugahara et al., 2013). It has been demonstrated that the expression levels of C4S and C6S inversely vary along with brain development (Miyata et al., 2012). The regulation of C4S and C6S expression has been implicated in the regulation of neuronal development and synaptic plasticity (Miyata et al., 2012). A lectin, Wisteria floribunda agglutinin (WFA), reacts with the CSPG linking to GalNAc and has been employed to detect the reticulated extracellular structure that is referred to as the perineuronal net (PNN) (Celio et al., 1998; Kurokawa et al., 1976). PNN is known to play important roles in neurodevelopmental processes such as synaptic refinement or myelination and neuronal protection from oxidative stress (Berretta et al., 2014; Bitanihirwe et al., 2016; Bitanihirwe and Woo, 2014; Cabungcal et al., 2013; Pantazopoulos and Berretta, 2016). Postmortem studies on schizophrenia have revealed the decrease in the WFA-positive PNN structure in patients' brain and suggested its pathophysiological contribution to this disorder (Enwright et al., 2016; Mauney et al., 2013; Pantazopoulos et al., 2015, 2010; Steullet et al., 2017).

The WFA-positive PNNs develop around the neurons, especially parvalbumin-positive interneurons (PV neurons) (Celio et al., 1998; Ueno et al., 2017). Alterations in the structure or expression of PNNs lead to the dysfunctions of PV neurons (Cabungcal et al., 2013; Shah and Lodge, 2013; Yoshioka et al., 2017). The levels of WFA-positive PNNs are decreased in the amygdala, prefrontal cortex, entorhinal cortex, and thalamic reticular nucleus of patients with schizophrenia (Enwright et al., 2016; Mauney et al., 2013; Pantazopoulos et al., 2010; 2015; Steullet et al., 2017). However, it has not yet been elucidated whether sulfation patterns are involved in the reported PNN reduction in schizophrenia. In the present study, we hypothesized that the quantitative abnormality of C4S and/or C6S is associated with schizophrenia neuropathology. To test this hypothesis, we measured and compared the immunoreactivities of C4S and C6S in the postmortem brains of patients with schizophrenia and control subjects using western blotting.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Ethics Committee of Niigata University School of Medicine. The use of postmortem brain tissues was authorized by the Ethics Committees of Matsuzawa Hospital, Fukushima Medical University, and Niigata University School of Medicine. The families of the control and patients with schizophrenia provided written informed consent to allow the use of brain tissues for pathological investigations. Human tissues were collected and stored according to the principles of the Declaration of Helsinki, and tissue use was in compliance with the Human Tissue Act 2004. The present animal experiments were approved by the Animal Experiment Committee of Niigata University and carried out in accordance with the Animal Use and Care Committee Guidelines of Niigata University, the ARRIVE guidelines, the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

2.2. Human tissue sampling

Postmortem brain tissue was collected from patients with chronic schizophrenia and controls with no history of neuropsychiatric disorders: 36 patients with chronic schizophrenia (21 men, 15 women; age 66.3 ± 8.50 years, mean ± SD) and 26 controls (18 men, 8 women; age 64.8 ± 18.0 years) (Supplemental Table S1). Almost all schizophrenics had taken antipsychotic drugs for prolonged periods. Our postmortem brain samples of the schizophrenia group were collected and frozen in 1982–2013 and some patients with schizophrenia had been diagnosed by previous criteria. Thus, we confirmed that each patient met the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) criteria for schizophrenia, reviewing their medical records. In each case, the left or right cerebral hemisphere was fixed in formalin for diagnostic examination, and the other hemisphere was frozen at −80 °C. All pathological analyses were performed at the authors' institutes. Tissue samples were taken from postmortem brains whose distinct regions were examined and did not exhibit neurodegenerative abnormalities with conventional pathological staining with the hematoxylin/eosin or Kluver-Barrera reagent (Shimizu et al., 2013). We defined post-mortem interval (PMI) as the elapsed time between a patient has clinically judged to be dead and the extracted brain tissue has been frozen (Table 1) and carefully evaluated the influences of PMI on our results (see below). The dorsolateral prefrontal cortex (approximately Brodmann area 46), hippocampus (including all the CA regions and dentate gyrus), and caudate nucleus were identified in frozen coronal slices according to a published human brain atlas (Haines, 2014).

Table 1
Profiles of patients and normal control subjects for the protein samples.

Area	Conditions	Control	Schizophrenia
Dorsolateral Prefrontal Cortex	*n* (M/F)	19 (11/8)	21 (13/8)
	age (y)	64.0 ± 12.7	63.9 ± 11.4
	PMI (h)	3.1 ± 1.1	4.6 ± 2.3*
	storage time (m)	196.9 ± 65.5	242.7 ± 114.5
Caudate Nucleus	*n* (M/F)	15 (10/5)	18 (10/8)
	age (y)	65.1 ± 12.3	64.2 ± 10.7
	PMI (h)	4.9 ± 5.0	4.7 ± 2.3
	storage time (m)	154.3 ± 51.8	193.7 ± 99.2
Hippocampus	*n* (M/F)	9 (7/2)	18 (9/9)
	age (y)	67.0 ± 10.0	68.1 ± 8.5
	PMI (h)	3.1 ± 1.2	10.4 ± 6.4**
	storage time (m)	194.7 ± 40.4	119.8 ± 54.5**

Data indicate the number (*n*) of subjects (M, male; F, female) and the mean ± SD of age (y; year), postmortem interval (PMI) (h; hpur) and storage time (m; month).
Significantly different from controls (*$p < 0.05$, **$p < 0.01$ by Mann–Whitney *U* test).

2.3. Neuroleptic treatment of animals

Male Wistar rats (7 weeks old, initial weight 200–220 g) were housed under a 12-h light–dark cycle with free access to food and water. The rats were treated with either haloperidol (target dose, 1–2 mg/kg/day) mixed with food pellets (1.217 g haloperidol/40 kg food) or pure food pellets for 12 months. These animals were anesthetized with pentobarbital (30 mg/kg) and brains were rapidly harvested after decapitation; each brain region was then dissected on ice.

2.4. Western blot analysis

2.4.1. Tissue lysate preparation

Frozen brain tissues were weighed and then sonicated in 10 volumes of Triton-X-100-containing buffer (20 mM Tris–HCl at pH 7.4, 10 mM EDTA, 1% Triton-X-100, 1:100 protease inhibitor cocktail [Complete, EDTA-free, Roche], 2 mM NaF, and 2 mM Na_3VO_4). The homogenate was centrifuged in a microcentrifuge at 6800 × *g* for 10 min, and the supernatant was collected and stored at −80 °C.

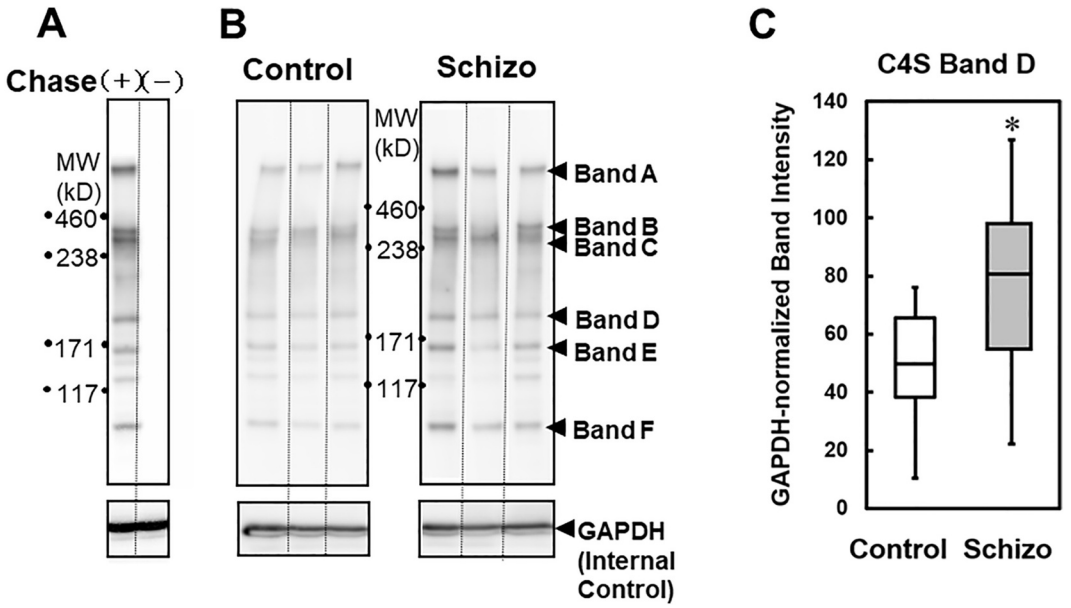

Fig. 1. (A) Human hippocampal tissue was homogenized and pretreated with (+) or without (−) chondroitinase ABC (Chase) to expose the epitope of C4S. The tissue homogenate was subjected to western blotting with the anti-C4S antibody. The bottom part of the blot was subjected to western blotting with the anti-GAPDH antibody as an internal loading control. (B) Hippocampal tissues of control subjects (Control) and patients with schizophrenia (Schizo) were homogenized, pretreated with chondroitinase ABC, and similarly subjected to western blotting with the anti-C4S antibody. Typical blots for the molecular range of > 100 kD are shown. The major bands for C4S immunoreactivity are referred to as Bands A–F in the order of higher molecular sizes. (C) The signal intensity of the 180 kD C4S immunoreactivity (Band D) was measured in all samples and normalized with that of GAPDH immunoreactivity in the same sample. The normalized values are shown as box-and-whisker plots for control and schizophrenia groups: the bottom and top of the box are the first and third quartiles, and the band inside the box is the second quartile (the median). *$p < 0.05$ by Mann–Whitney test.

2.4.2. Protein determination

The protein concentrations in the brain tissue extracts were determined using a Micro BCA kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

2.4.3. Chondroitinase ABC digestion

Brain tissue extract was treated with chondroitinase ABC (Chase) (Sigma-Aldrich, St. Louis, MO, USA) prior to western blotting to generate the epitope recognized by the anti-C4S/C6S antibody; the extract (25 µl) was mixed with 25 µl of sodium acetate buffer (50 mM sodium acetate, 1 M Tris pH 8.0, 10 mM EDTA) containing 25 mU of Chase and incubated for 2.0 h at 37 °C.

2.4.4. Electrophoresis and transfer

All samples were reduced with 10% mercaptoethanol-containing 2% sodium dodecyl sulfate (SDS), denatured for 10 min at 90 °C, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Tissue extracts were loaded (equal amounts of protein) onto pre-cast, 0.9 mm, 2–15% gradient SDS-PAGE gels (Multi Gel II Mini 2/15; CosmoBio Co., Ltd., Japan). Separated proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schull, Dassel, Germany).

2.4.5. Immunoreaction and quantitation

The membrane was incubated with blocking buffer (PVDF Blocking Reagent for Can Get Signal®; Toyobo Co., Ltd., Osaka, Japan) for anti-C4S or anti-C6S antibodies or with 10% bovine serum albumin for the other antibodies for 2 h. The membrane was then probed with anti-C4S (2B6) antibody (1:100; CosmoBio Co., Ltd., Japan), anti-C6S (3B3) antibody (1:100; CosmoBio Co., Ltd.), and anti- GAPDH antibody (1:200; Santa Cruz Biotechnology). After extensive washing, their immunoreactivity on the membrane was detected with anti-rabbit/mouse/rat immunoglobulin conjugated to horseradish peroxidase (Dako, Glostrup, Denmark), followed by chemiluminescence reaction with ImmunoStar LD (Wako Chemicals Inc., Japan) for C4S and C6S or with Western Lightning Plus-ECL (PerkinElmer Japan, Yokohama, Japan) for the other molecules. Chemiluminescence signals for C4S and C6S immunoreactivities were measured using a CCD camera system (G:BOX; Syngene, Co., Ltd., USA) and normalized by those for an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in the same lanes (Dittmer and Dittmer, 2006; Wu et al., 2012; Yu et al., 2011).

2.5. Statistical analysis

A nonparametric analysis involving Mann–Whitney test was used to compare immunoreactivity, age, PMI, and storage time between the schizophrenia and control groups. Fisher's exact probability test was used to compare the numbers of males and females in the two groups. Statistical correlations between the strength of the immunoreactivity and age, PMI, or storage time were assessed using Spearman's coefficient and Pearson's correlation test.

3. Results

We obtained postmortem samples of three brain regions (dorsolateral prefrontal cortex, caudate nucleus, and hippocampus), which are implicated in the neuropathology of schizophrenia. (Benes et al., 2007; Duan et al., 2015; Enwright et al., 2016; Harrison and Eastwood, 2001; Heckers and Konradi, 2015; Knable et al., 2004; Konradi et al., 2011; Lewis and Lieberman, 2000; Mauney et al., 2013; Roberts et al., 2009; Ross et al., 2006; Shah and Lodge, 2013; Tamminga et al., 2010). Tissue samples for each brain region were randomly taken from 18 to 21 chronic patients with schizophrenia and from 9 to 19 controls with no history of neuropsychiatric disorders; neither group exhibited apparent neurodegenerative features (Table 1) (see Supplemental Table S1 for details).

We matched the mean ages of schizophrenia patients and control subjects; however, it was unavoidable with the given postmortem samples that several other indices differed between groups. In the dorsolateral prefrontal cortex and hippocampus, PMI was larger in

patients than that in controls, and the storage time of hippocampal tissues was significantly longer in controls than that in patients. The influences of these indices were estimated by statistical correlation analyses (see below). All other indices were similar between the two groups.

To detect the C4S or C6S portions of CS chains, we employed monoclonal antibodies whose specificities were previously verified (Caterson, 2012). To expose 4-sulfated or 6-sulfated unsaturated disaccharide neoepitopes at the terminal of CS chains, tissue lysates were pretreated with chondroitinase ABC. In the absence of this pretreatment, there was no apparent immunoreactivity that was recognized by these antibodies on western blots, presumably reconfirming their authenticity (Fig. 1A). Similarly, tissue lysates from the dorsolateral prefrontal cortex, caudate nucleus, and hippocampus were treated with chondroitinase ABC and subjected to western blotting for C4S and C6S.

In the hippocampus, western blotting with the anti-C4S antibody identified six C4S-immunoreactive bands in the higher-molecular-weight range (> 100 kD) (Fig. 1B). The signal intensity of the 180 kD immunoreactivity (Band D), which was normalized with that of an internal control, GAPDH, significantly increased in the patient group compared with that in the control group ($U = 127$, $p = 0.017$) (Fig. 1C). All other C4S-immunoreactive bands (Bands A–C, E, and F) failed to exhibit a difference in their signal levels. There was no significant difference in the signal intensity of the internal control protein GAPDH between the groups ($U = 42$, $p = 0.055$).

As there were differences in the PMI and storage time of these hippocampal samples, we tested the correlation of PMI and storage time with the signal intensity of the 180 kD C4S immunoreactivity (Band D) to evaluate the effects of these sampling conditions (Fig. 2). Neither parametric nor nonparametric correlation analyses provided any significant correlations between Band D levels and these indices. In addition, there was no significant correlation between PMI and Band D levels in the dorsolateral prefrontal cortex or in the caudate nucleus ($rs = 0.048$, $p = 0.77$ for dorsolateral frontal cortex; $rs = 0.043$, $p = 0.82$ for caudate nucleus). These statistical results indicate that the signal intensity of the 180 kD C4S immunoreactivity (Band D) was not influenced by PMI. In other words, the PMI difference between schizophrenia and control groups presumably had no significant impact on the observed Band D levels. The signal intensity of Band D was not correlated with either age or sex (data not shown).

Western blotting with the anti-C6S antibody (3B3) revealed five C6S-immunoreactive bands in the hippocampal samples (Fig. 3). Compared with those in the control group, none of the C6S-immunoreactive bands in the patient group significantly increased or decreased in intensity.

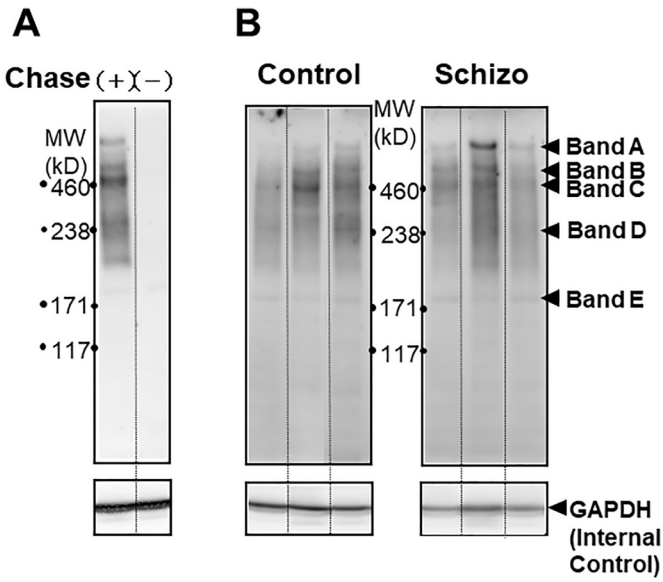

Fig. 3. (A) Human hippocampal tissue was homogenized and pretreated with (+) or without (−) chondroitinase ABC (Chase) to expose the epitope of C6S. The tissue homogenate was subjected to western blotting with the anti-C6S antibody. The bottom part of the blot was subjected to western blotting with the anti-GAPDH antibody as an internal loading control. (B) Hippocampal tissues of control subjects (Control) and patients with schizophrenia (Schizo) were homogenized, pretreated with chondroitinase ABC, and subjected to western blotting with anti-C6S antibody. Typical blots for the molecular range of > 100 kD are shown. The major bands for C6S immunoreactivity are referred to as Bands A–E in the order of higher molecular sizes.

In the dorsolateral prefrontal cortex and caudate nucleus, the signal intensities of C4S and C6S immunoreactivities were similarly determined. There were five C4S-immunoreactive and two C6S-immunoreactive bands on western blots that corresponded to molecular weights similar to those found in the hippocampal tissue (data not shown). These C4S-immunoreactive and C6S-immunoreactive bands,

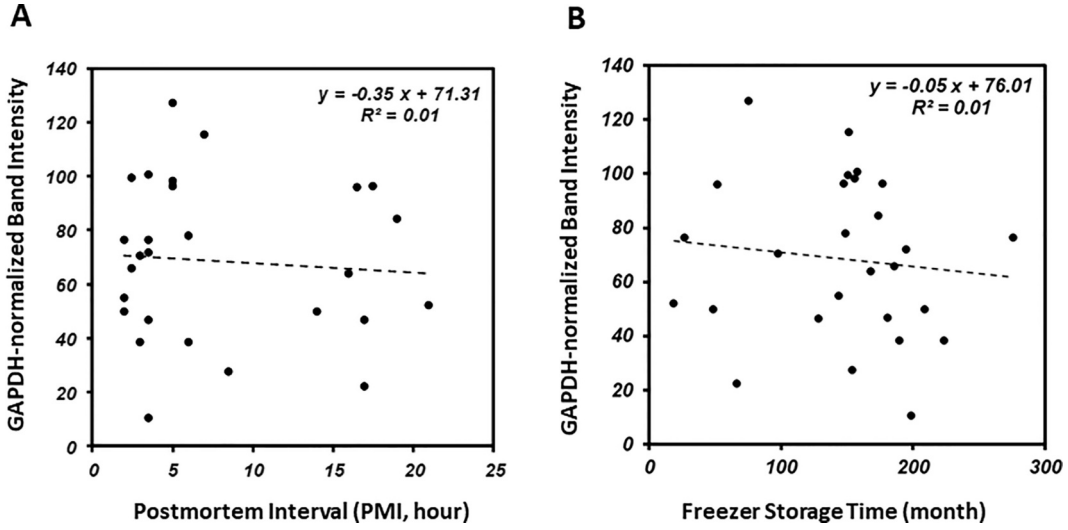

Fig. 2. With the given group differences of postmortem interval (PMI) and storage time in the hippocampal samples, we performed correlation analyses between PMI and the 180 kD C4S immunoreactivity (Band D) or between storage time and the Band D level. The immunoreactivity of Band D that was normalized by the GAPDH level was plotted with its PMI (hour) in (A) and with freezer storage time (month) in (B). No significant correlations were detected either by Spearman's correlation test or by Pearson's correlation test.

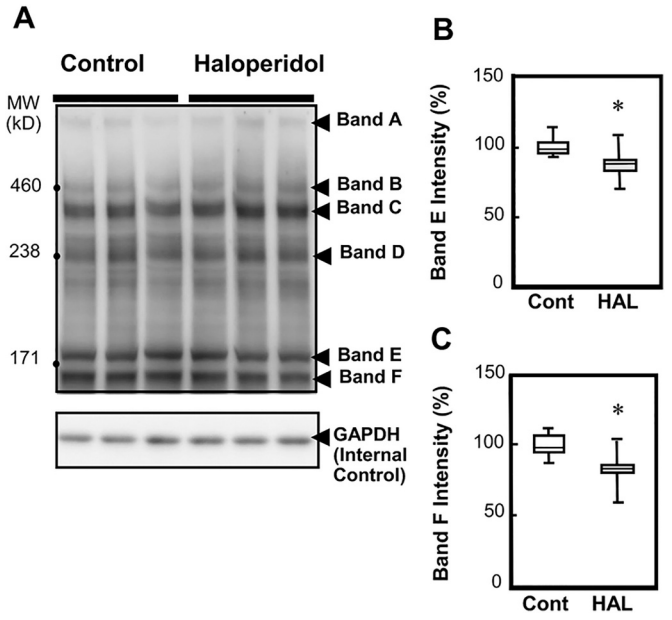

Fig. 4. (A) Adult male rats were treated with either haloperidol (target dose, 1–2 mg/kg/day) mixed with food pellets (1.217 g/40 kg food) or pure food pellets for 12 months ($n = 5$ rats for each group). Hippocampal tissues of control and haloperidol-treated rats were homogenized, pretreated with chondroitinase ABC, and subjected to western blotting with the anti-C4S antibody. Typical lanes are shown. The major bands for C4S immunoreactivity are referred to as Bands A–F in the order of higher molecular sizes. (B) The signal intensity of the band just above the 171 kD marker (i.e., Band E) was measured and normalized with that of GAPDH immunoreactivity. The normalized Band E levels are shown as box-and-whisker plots and compared between the haloperidol-untreated (Cont) and haloperidol-treated groups (HAL). (C) The signal intensity of the band just below the 171 kD marker (i.e., B and F) was measured and normalized with that of GAPDH immunoreactivity. The normalized Band F levels are shown as box-and-whisker plots and were compared between haloperidol-untreated (Cont) and haloperidol-treated groups (HAL). *$p < 0.05$ by Mann–Whitney test.

including Band D for the 180 kD C4S immunoreactivity, showed no significant quantitative differences between patients and control subjects in either the dorsolateral prefrontal cortex or the caudate nucleus ($U = 146–198$, $p = 0.153–0.979$ for the dorsolateral prefrontal cortex; $U = 104–152$, $p = 0.274–0.762$ for the caudate nucleus) (Supplemental Fig. S1).

The abovementioned disease-associated increase in C4S levels found in the hippocampus might have been influenced by neuroleptics given to the patients. To estimate such neuroleptic effects on the above 180 kD C4S immunoreactivity, haloperidol, a typical antipsychotic, was mixed with baits and chronically administered to adult rats for 12 months (see details in Materials and Methods). Western blotting with the anti-C4S antibody identified six C4S-immunoreactive bands (Fig. 4). Chronic treatment with haloperidol failed to elevate the signal intensity of any C4S-immunoreactive bands but rather decreased that of C4S-immunoreactive bands (i.e., Bands E and F) having molecular sizes of 160–180 kD in rat hippocampus.

4. Discussion

In the present study, we found that the 180 kD C4S immunoreactivity (Band D) was significantly increased in the hippocampus of patients with schizophrenia. This increase was not correlated with PMI, storage time, or age. Chronic treatment of adult rats with the antipsychotic haloperidol failed to mimic the change in C4S immunoreactivity. These observations suggest that the increase of C4S immunoreactivity in patients' hippocampus is associated with the neuropathology of schizophrenia. With the given limitation of postmortem samples, however, the regional specificity of the C4S change to the hippocampus needs to be verified in future studies. It also remains to be determined whether the observed change of C4S immunoreactivity reflects a quantitative alteration of the C4S sugar moiety alone or involves that of the CSPG core proteins carrying this C4S moiety.

The recent neuropathologic studies on chondroitin sulfate indicate that the C4S increase in PNN occurs in models of Alzheimer's disease and animal ageing to result in marked inhibitory actions on axon regrowth or synapse formation (Foscarin et al., 2017). That study suggests that the C4S increase leads to a decrease in neural plasticity and adversely affect learning and memory. In addition, transgenic mice overexpressing the CSPG phosphacan, which presumably results in the increase in CS levels, display abnormal excitatory neurotransmission in the hippocampus (Buxbaum et al., 2008; Takahashi et al., 2011). Accordingly, we assume that the alteration of C4S in the hippocampus associates with the cognitive deficits or the reported histopathology of patients with schizophrenia (Carstens et al., 2016; Brugger and Howes., 2017; Avery et al., 2018).

The hippocampus is a brain region that has been repeatedly discussed in terms of having an association with the neuropathology of schizophrenia (Benes et al., 2007; Harrison and Eastwood, 2001; Heckers and Konradi, 2015; Knable et al., 2004; Konradi et al., 2011; Shah and Lodge, 2013; Tamminga et al., 2010). It has been frequently reported that the neurochemical or electrophysiological functions of hippocampal interneurons are impaired in patients with schizophrenia (Benes et al., 2007; Harrison and Eastwood, 2001; Heckers and Konradi, 2015; Knable et al., 2004; Konradi et al., 2011; Tamminga et al., 2010). A rodent model study demonstrated that PNN depletion with chondroitinase ABC digestion induces the over-activation of dopamine neurons leading to schizophrenia-like behavioral deficits (Shah and Lodge, 2013). Moreover, the cytokines (epidermal growth factor and transforming growth factor-β) that are implicated in the neuropathology of schizophrenia are known to regulate the expression of CSPGs (Frydecka et al., 2013; Futamura et al., 2002; Smith and Strunz, 2005; Umeda-Yano et al., 2013). These previous studies indicate that schizophrenia appears to involve the deficits in the quantity or quality of CS chains in the brain.

In the present study, however, the molecular nature of the 180 kD C4S immunoreactivity (Band D) was unrevealed. Accordingly, it is difficult to illustrate the reason why the immunoreactivity for Band D alone showed the increase associated with schizophrenia. According to the reports that various environmental factors such as cytokines affect the length or chain number per its core protein as well as the expression of individual CSPG core proteins (Smith and Strunz, 2005; Kitagawa, 2014), we assume that the Band D change might reflect any region-specific neuropathologic condition involving certain inflammatory cytokines. We hope that future mechanistic insights into the Band D change will hint at the hippocampal neuropathology of schizophrenia.

Several technical limitations of using postmortem samples must be considered (Harrison, 1999). As we put the highest priority on matching the age of human subjects between groups, there were unavoidable differences in PMI and storage time. However, the following statistical and experimental evidence indicates that the quantitative alteration of C4S levels appears not to reflect the result of prolonged PMI or storage time of the postmortem samples used. Correlation analyses revealed that PMI and storage time did not affect C4S immunoreactivity in the hippocampus in either schizophrenic patients or control subjects. Medication influences on chronic schizophrenia were also taken into account in the present study because neurochemical changes in the schizophrenia studies often represent the influences of chronic antipsychotic medication rather than the core neuropathology of schizophrenia (Sokolov, 1998). The patients with schizophrenia used in the present study took various types of antipsychotics such as haloperidol (9 patients), levomepromazine (6 patients), risperidone (5

patients) etc. (see details in Supplemental Table S1). There should be fundamental differences in the antipsychotic effects between rats and human as well as between typical and non-typical antipsychotics (Gottschling et al., 2016). Under the given limitation of the animal studies, we examined the rats treated with a typical neuroleptic, haloperidol, for a year and determined its effects on C4S or C6S levels. At least, the chronic treatment of rats with haloperidol failed to mimic the above C4S change found in patients. With this respect we cannot fully rule out the possibility that the antipsychotic drugs other than haloperidol are involved in the present phenomenon.

Finally, we discuss the methodological limitation of western blotting. In the present western blotting, we employed monoclonal antibodies whose specificities for CS chains have been previously confirmed (Caterson, 2012). The emergence of the immunoreactivity for the anti-C4S or anti-C6S antibodies fully depended on the antigen exposure step with chondroitinase ABC digestion. In this respect, it is unlikely that these monoclonal antibodies might cross-react with sugar moieties other than CS.

Taken together, the results in the present study suggest the possibility that the alteration of C4S immunoreactivity in the hippocampus is associated with the pathogenesis of schizophrenia. However, there was no direct evidence that the alteration of C4S in the hippocampus would induce functional deficits of PV neurons or psychosis. Further studies are required to clarify the role of the hippocampal C4S abnormality in the pathophysiology of schizophrenia.

Author contributions

Toshiyuki Someya, Michihiro Igarashi, Nobuyuki Takei, and Hiroyuki Nawa designed the study; Takayuki Yukawa, Yuichiro Watanabe, Mami Saito, and Hiroyuki Nawa wrote the manuscript; Takayuki Yukawa, Yuriko Iwakura, Yuichiro Watanabe, and Kazuhiko Toyooka performed the experiments; Takayuki Yukawa and Yuriko Iwakura analyzed the data; Kazuhiro Niizato, Kenichi Oshima, Yasuto Kunii, Hirooki Yabe, Junya Matsumoto, Akira Wada, Mizuki Hino, Shuji Iritani, Shin-ichi Niwa, Ryoko Takeuchi, Hitoshi Takahashi, and Akiyoshi Kakita collected and examined postmortem tissues. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by Grants-in-Aid for Young Scientists (B) [No. 16K19753]; Scientific Research (C) [No. 18K06460]; Scientific Research on Innovative Areas [No. 18H05429]; from the Ministry of Education, Science, Sports and Culture of Japan; the Strategic Research Program for Brain Sciences from Japan Agency for Medical Research and Development (AMED), and a grant for Promotion of Niigata University Research Projects.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2018.10.062.

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Supplemental Table S1. Autopsy and clinical data

	Sex	Age at Death (years)	Cause of Death	Duration of Illness (year)	Drugs (mg/day)
Schizophrenia					
S-1	M	54	lung cancer	27	HPD 2.25 mg, pipamperone 100 mg
S-2	M	55	multiple organ failure	29	PAL 12 mg, LP 50 mg, BLS 24 mg
S-3	M	55	bleeding	37	HPD 24 mg
S-4	M	56	pancreatic cancer	28	Off-drug
S-5	F	57	acute heart failure	40	RIS 12 mg, QTP 600 mg
S-6	M	58	stomach cancer	30	HPD 22 mg, zotepine 150 mg, LP 100 mg
S-7	F	65	colon cancer, ileus	50	RIS 4 mg, LP 20 mg
S-8	M	66	pneumonia	36	Off-drug
S-9	F	68	chronic renal failure	40	HPD 3 mg, CP 150 mg, LP 25 mg
S-10	M	67	pneumonia	47	propericiazine 30mg
S-11	M	68	gastrointestinal bleeding	41	HPD 2 mg, mosapramine 60 mg
S-12	M	70	brainstem hemorrhage, pneumonia	39	RIS 8 mg, CP 75 mg, thiapride 150 mg
S-13	M	72	pneumonia	42	LP 25 mg, RIS 5mg
S-14	F	72	pneumonia	52	thioridazine 200 mg, HPD 10 mg
S-15	M	74	suffocation	52	RIS 4 mg
S-16	M	74	pneumonia, heart failure	52	LP 60 mg, thioridazine 60 mg
S-17	M	75	pneumonia	47	bromperidol 3 mg, thioridazine 75 mg
S-18	F	76	pneumonia	33	N.I.
S-19	F	79	leukocytoclastic vasculitis	60	HPD 3 mg
S-20	M	77	acute myocardial infarction	32	HPD 9 mg, bromperidol 9 mg
S-21	F	53	cardiac failure	N.I.	bromperidol 6 mg, nemonapride 10 mg, HPD 0.75 mg
S-22	M	78	gastrointestinal bleeding	N.I.	N.I.

S-23	F	70	DIC	N.I.	N.I.
S-24	F	71	N.I.	N.I.	N.I.
S-25	F	66	ascending colon cancer	25	N.I.
S-26	M	72	N.I.	N.I.	N.I.
S-27	F	82	congestive heart failure	N.I.	N.I.
S-28	F	83	respiratory failure	40	N.I.
S-29	F	87	suffocation	49	N.I.
S-30	M	60	hypoglycemia	N.I.	N.I.
S-31	M	43	pneumonia	N.I.	N.I.
S-32	F	48	N.I.	N.I.	N.I.
S-33	M	51	N.I.	N.I.	N.I.
S-34	F	59	pneumonia	35	N.I.
S-35	M	61	colon cancer	N.I.	N.I.
S-36	M	64	pneumonia	47	N.I.

Control

C-1	M	41	Ducchene muscular dystrophy
C-2	F	44	Gorham's disease
C-3	F	48	myasthenia gravis
C-4	F	49	Crow-Fukase syndrome
C-5	F	49	myopathy
C-6	M	51	pontine hemorrhage
C-7	M	59	myeloma
C-8	M	60	small infarcts
C-9	M	64	renal failure
C-10	M	64	renal failure
C-11	F	64	polymiositis
C-12	M	65	old contusion
C-13	M	68	cerebral infarction
C-14	M	70	multiple fresh infarcts

C-15	M	71	Crow-Fukase syndrome
C-16	M	71	multiple small infarcts
C-17	M	72	myasthenia gravis
C-18	F	73	myositis
C-19	M	75	pathymeningitis
C-20	M	76	gastrointestinal bleeding
C-21	F	79	Foix-Alajournine
C-22	M	82	multiple infarcts, gastric cancer
C-23	F	86	SCA6
C-24	M	55	myotonic dystrophy
C-25	M	71	cerebral infarction
C-26	M	77	myelodysplastic syndrome

M, male; F, female. HPD, haloperidol; RIS, risperidone; PAL, paliperidone; LP,levomepromazine; CP,chlorpromazine ; QTP, quetiapine; BLS,blonanserin;
Off-drug, no neuroleptic treatment for at least 3 months before death. PMI, postmortem interval (hour). NI, no information (transfer from other hospitals).
PFC, prefrontal cortex; HIP, hippocampus; CAU, caudate nucleus. Asterisk indicates available subjects in each region.
DIC, Disseminated intravascular coagulation; SCA, Spinocerebellar ataxia

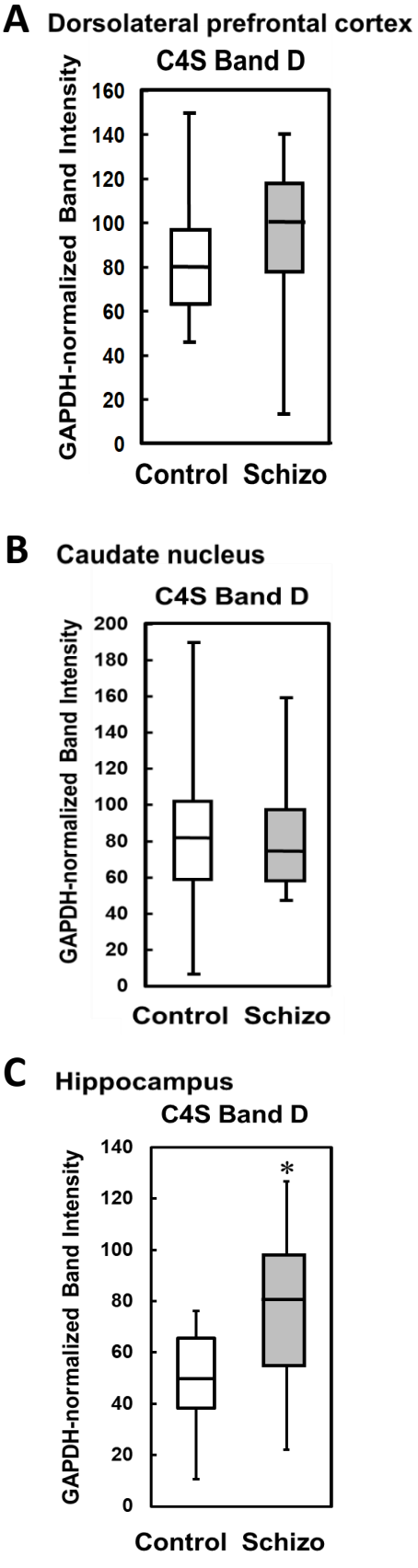

Figure S1; Relative comparison of Band D levels in the three brain regions of schizophrenia and control subjects.

We performed western blotting of the dorsolateral prefrontal (n=19 for control and n=21 for schizophrenia), caudate nucleus (n=15 for control and n=18 for schizophrenia), and hippocampus (n=9 for control and n=18 for schizophrenia) with the anti-C4S antibody, using 2 independent membranes. The inter-membrane variation of blotting efficacy was adjusted by the intensity of an internal control samples loaded to both membranes and the inter-lane variation of probing efficacy was normalized with the level of an internal control GAPDH in each lane. The intensity of Band D immunoreactivity was subjected to these normalizations and plotted. GAPDH-normalized Band intensity on the vertical axis is expressed with an arbitrary unit.