Hypersialylation is a common feature of neurofibrillary tangles and granulovacuolar degenerations in Alzheimer’s disease and tauopathy brains

Shun Nagamine,1 Tsuneo Yamazaki,2 Kouki Makioka,1 Yukio Fujita,1 Masaki Ikeda,1 Masamitsu Takatama,3 Koichi Okamoto,3 Hideaki Yokoo4 and Yoshio Ikeda1

1Department of Neurology, Gunma University Graduate School of Medicine, 2Department of Rehabilitation, Gunma University Graduate School of Health Sciences, 3Geriatrics Research Institute and Hospital, and 4Department of Human Pathology, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

Glycosylation is one of the major post-translational modifications of proteins. The status of sialylation of the neuropathological hallmarks of various neurodegenerative disorders was investigated in this study. Here, we report the novel findings that two phosphorylated tau (p-tau)-containing structures associated with Alzheimer’s disease (AD), that is, neurofibrillary tangles (NFTs) and granulovacuolar degenerations (GVDs), were hypersialylated. The NFTs, GVDs and dystrophic neurites of senile plaques (SPs) in AD hippocampi were clearly visualized by immunohistochemistry using an anti-sialic acid (SA) antibody. In contrast, the amyloid core of SPs was not sialylated at all. Interestingly, other p-tau-containing structures, that is, globose-type NFTs in progressive supranuclear palsy and Pick bodies and ballooned neurons in frontotemporal lobar degeneration with Pick bodies, were also hypersialylated. Unlike the p-tau-containing structures observed in tauopathies, the hallmarks of other neurodegenerative disorders, such as Lewy bodies in Parkinson’s disease, glial cytoplasmic inclusions in multiple system atrophy, Bunina bodies, skein-like inclusions and round inclusions in amytrophic lateral sclerosis, intranuclear inclusion disease and physiological bodies or granules (lipofuscin granules, corpora amylacea and melanin granules), were not immunolabeled by the anti-SA antibody. Because this antibody specifically identified NFTs and GVDs, immunostaining for sialylation represents a useful tool to screen these structures in a diagnostic setting. These results clearly indicate that the pathological hallmarks of various tauopathies are commonly hypersialylated, and that sialylation plays an important role in the process of p-tau accumulation in AD and other tauopathies.

Key words: Alzheimer’s disease, granulovacuolar degeneration, hypersialylation, neurofibrillary tangle, tauopathy.

INTRODUCTION

Tau is a major component of neurofibrillary tangles (NFT), which are one of the neuropathological hallmarks of Alzheimer’s disease (AD) brains.1 Hyperphosphorylation of tau promotes its self-assembly into paired helical filaments (PHFs).2 In AD brains, tau is aberrantly glycosylated with various oligosaccharides, including N-acetylneuraminic acid (NeuAc), which is a major member of the sialic acid (SA) family.3–5 In contrast, deglycosylation by glycosidases depresses the phosphorylation of tau.6 These findings suggest that aberrant glycosylation in AD brains facilitates the hyperphosphorylation of tau, resulting in the formation of NFTs.5 In support of this hypothesis, some biological changes of SAs and sialyltransferases have been reported in AD patients.7–10

With regard to the relationship between sialylation and amyloid β (Aβ) pathology of AD, it has been reported that the β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE-1) can cleave not only APP but the sialyltransferase ST6Gal-I, thus downregulating its transferase activity.11 In addition, a genome-wide association study of AD identified that the common variant of CD33, also known as Siglec-3 and a member of the sialic acid-binding immunoglobulin-like lectins, is a genetic risk factor for AD.12,13 The uptake and clearance of Aβ due to microglia...
are found to be inhibited by a transmembrane microglial protein CD33, so that CD33 could be a potential target to develop an anti-AD therapy.

Granulovacuolar degenerations (GVDs) are not always pathogenic, but are regarded as an additional pathological hallmark of AD.\(^{14}\) As GVDs contain phosphorylated tau (p-tau) and several tau kinases, such as casein kinase 1\(\delta\), GVDs are considered to be the site of tau phosphorylation.\(^{15}\) Considering that aberrant glycosylation facilitates the hyperphosphorylation of tau, it can be hypothesized that the p-tau present in GVDs is also hypersialylated.\(^{4,5}\) However, an association between GVDs and sialylation has not been reported so far. The present study reports the novel findings that both NFTs and GVDs were hypersialylated in AD brains. In addition, hypersialylation of tau was also confirmed in other pathological structures from tauopathies, such as progressive supranuclear palsy (PSP) and frontotemporal lobar degeneration with Pick bodies (PickD), although other well-known characteristic pathological structures of Parkinson’s disease (PD), multiple system atrophy (MSA), amyotrophic lateral sclerosis (ALS) and neuronal intranuclear inclusion disease (NIID) were not hypersialylated. These findings suggest the existence of a common mechanism in which p-tau is hypersialylated not only in AD, but also in other tauopathies, leading to its aggregation and subsequent neurodegeneration.

**MATERIALS AND METHODS**

**Subjects**

A total of 28 autopsied brain and spinal cord tissues (from 15 males and 13 females) and one biopsied skin tissue were collected from the Gunma University Hospital and the Geriatrics Research Institute and Hospital. These samples included six cases of AD, three cases of PD, five cases of MSA, six cases of ALS, one case of PSP, one case of PickD and one case of NIID, as well as six control elderly cases who did not have any neurodegenerative diseases. These autopsy and biopsy materials and their demographic findings are described in Table 1. All autopsies and the biopsy were performed in accordance with established procedures, and the specimens were processed after obtaining written informed consent from the patient or the patient’s family.

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M, male; F, female; AD, Alzheimer’s disease; PD, Parkinson’s disease; MSA, multiple system atrophy; ALS, amyotrophic lateral sclerosis; PSP, progressive supranuclear palsy; PickD, frontotemporal lobar degeneration with Pick bodies; NIID, neuronal intranuclear inclusion disease; Control, non-neurological control; n.a., not available; AMI, acute myocardial infarction; DOA, dead on arrival; OMI, old myocardial infarction; ARDS, adult respiratory distress syndrome; *, age at biopsy.
Patients with AD (average age at death, 75.8 years; two males and four females) were diagnosed as having clinically probable AD according to the clinical diagnostic criteria published by the Consortium to Establish a Registry for AD, and fulfilled the quantitative neuropathological criteria for diagnosing AD according to the National Institute on Aging – Alzheimer’s Association guidelines for the neuropathologic assessment of AD; that is, AD Neuropathologic Change scores of A3, B3 and C3.16–19

Patients with PD (average age at death, 79.0 years; two males and one female) were clinically diagnosed using the UK PD Society Brain Bank clinical diagnostic criteria, and the diagnosis was confirmed using histopathologic criteria.20,21

Patients with MSA (average age at death, 76.0 years; two males and three females) were clinically diagnosed using the criteria for definite MSA of the second consensus statement on the diagnosis of MSA.22 Patients with ALS (average age at death, 62.2 years; five males and one female) clinically fulfilled the diagnostic criteria for clinically definite ALS, clinically probable ALS, or clinically probable laboratory-supported ALS according to the revised El Escorial criteria, and were proven by autopsy examination.23 One male patient with PSP (71 years at death) showed parkinsonism, and fulfilled the neuropathologic criteria for PSP of the National Institute of Neurological Disorders and Stroke (NINDS).24 One male patient with PickD (73 years at death) was clinically diagnosed as having dementia and personality change, and fulfilled the neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration.25

One female patient with NIID (aged 69 years at the time of skin biopsy) who showed diffuse leukoencephalopathy on cerebral MRI with characteristic high intensities of subcortical U-fibers on diffusion-weighted imaging was pathologically diagnosed via confirmation of the skin biopsy findings of ubiquitin/p62-positive intranuclear inclusions in sweat gland cells and adipocytes.26,27

**Immunohistochemistry**

Five-micrometer-thick sections from formalin-fixed, paraffin-embedded tissues were deparaffinized and immunostained with primary antibodies using the streptavidin-biotin-peroxidase complex (ABC) method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). The sections were immersed in 0.3% hydrogen peroxide (Douchin Laboratories, Kumamoto, Japan) in methanol for 30 min, to block endogenous peroxidase activity, and autoclaved for 10 min in 10 mmol/L sodium citrate buffer (pH 6.0), for antigen retrieval. Moreover, for staining with the anti-Aβ antibody, the tissues were treated with 70% formic acid for 3 min. All sections were blocked in a solution supplied by the manufacturer for 30 min at room temperature, incubated with the primary antibodies overnight at 4°C, and then incubated with secondary antibodies raised against the animals that were used to produce the primary antibodies. Immunoreactivity was visualized using 0.5 mg/mL of 3,3′-diaminobenzidine tetrachloride (DAB) containing 0.03% hydrogen peroxide.

To examine the colocalization between SA and α-synuclein in MSA and PD brains, double immunohistochemistry was performed.26 The anti-SA antibody was visualized via the ABC method using DAB as a chromagen, and the anti-α-synuclein antibody was visualized via the ABC method using the VIP Substrate Kit (Vector Laboratories, Burlingame, CA, USA).

Because Bunina bodies, which are a neuropathological hallmark of ALS, can be easily recognized by HE staining, this method was initially used to identify the location of the spinal anterior horn cells containing Bunina bodies. After the HE sections were photographed, cover slips were removed from the slides in xylene, and the specimens were decolorized in alcohol, then restained using the respective immunolabeling.

To confirm specificity of the anti-SA antibody, the antibody was absorbed by sialyllactose sodium salt (S0885; Tokyo Chemical Industry, Tokyo, Japan) that contains an epitope of NeuAc2-3 bond recognized by the SA antibody. An aliquot of the SA antibody (0.4 μL) was preincubated at 4°C for 24 h with 38 mg of the sialyllactose sodium salt in a total volume of 200 μL solution containing 1% bovine serum albumin. Another aliquot of the anti-SA antibody was also preincubated without sialyllactose sodium salt in the same way, and served as a control. The preincubated SA antibodies were used for immunohistochemistry as described above.

All sections were counterstained with hematoxylin. The stained slides were evaluated under an Olympus DP72 microscope using the DP2-BSQ software (Olympus, Tokyo, Japan).

**Double immunofluorescence**

For immunofluorescence (IF), deparaffinization, antigen retrieval, blocking and labeling with the primary antibodies were performed as described above. Subsequently, the slides were incubated with a mixture of secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes-Invitrogen, Eugene, OR, USA) and Alexa Fluor 568-conjugated goat anti-mouse IgG (Molecular Probes-Invitrogen)) for 2 h. To avoid autofluorescence signals from the specimens, sections were immersed in Sudan Black (Wako Pure Chemical Industries, Osaka, Japan) for 5 min, then rinsed in 70% ethanol. The stained slides were mounted with Vectashield (Vector Laboratories) and evaluated under an FV1000 confocal microscope (Olympus).

**Primary antibodies**

The primary antibodies used in this study were as follows: rabbit polyclonal anti-α-synuclein antibody raised against amino
acids (aa) 111-131 (1:500, AB5038P; Millipore, Billerica, MA, USA); rabbit polyclonal anti-α-synuclein antibody raised against aa 116-131 (1:2000, prepared in-house);\(^{26,29}\) rabbit polyclonal anti-β antibody raised against the C terminal of β 1-42 (1:200, AB5078P; Millipore); mouse monoclonal anti-α-β antibody (clone 4G8) raised against aa 17-24 (1:5000, SIG-39220; Signet, Dedham, MA, USA); mouse monoclonal anti-PHF-tau (clone AT8) raised against purified human PHF-tau phosphorylated at Ser202 and Thr205 (AT8 antibody) (1:1000, MN1020; Thermo Fisher Scientific, Waltham, MA, USA); rabbit polyclonal anti-p62 antibody raised against aa 116-131 (1:2000, prepared in-house);\(^{28,29}\) rabbit polyclonal anti-cathepsin D antibody against aa 131-161 (1:500, AP1823a; ABGENT, San Diego, CA, USA); rabbit polyclonal anti-α2-macroglobulin antibody (clone HYB4) raised against IV3NeuAcLec4Cer, which recognizes a structure of α2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1’ Cer (1:500 for ABC the method and 1:100 for IF, 011-25171; Wako Pure Chemical Industries);\(^{31}\) rabbit polyclonal anti-lysosome-associated membrane protein-1 (LAMP1) antibody raised against aa 131-161 (1:500, AP1823a; ABGENT, San Diego, CA, USA); rabbit polyclonal anti-cathepsin D antibody (1:10, PU205-UP; BioGenex, San Ramon, CA, USA); rabbit polyclonal anti-cystatin-C antibody (1:2500, A0451; DAKO, Glostrup, Denmark); rabbit polyclonal anti-p62 antibody raised against aa 120-440 (1:1000, PM045; MBL, Aichi, Japan); and rabbit polyclonal anti-ubiquitin antibody (1:2000, Z0458, DAKO).\(^{32}\)

**RESULTS**

**NFTs, GVDs and DNs in both AD and control brains were hypersialylated**

Staining with the anti-SA antibody, which recognizes sugar chains containing a NeuAcα2-3 bond, led to the clear visualization of granular or filamentous structures in both AD and control brains. In the hippocampus of AD brains, flame-shaped materials (Fig. 1A, 1B) and granule-containing vacuoles in the neuronal cytoplasm (Fig. 1C), as well as clustered swollen structures located outside of neurons (Fig. 1D) were stained with this antibody. The staining pattern was observed as NFTs, GVDs and dystrophic neurites (DNs) of senile plaques (SPs), respectively.\(^{33}\) These structures labeled by this antibody were also found in the frontal (Fig. 1E-G) and occipital (Fig. 1H-J) cortices, the periaqueductal gray matter of the midbrain (PAG) (Fig. 1K,L), pontine nuclei (Fig. 1M, N), and the medulla (Fig. 1O,P). Immunohistochemistry showed that positive labeling by the anti-SA antibody was most prominent in the cornu ammonis 1 (CA1) area of the hippocampus in AD brains. The AT8 and p-tau Ser214 antibodies raised against p-tau detected NFTs and DNs, but not GVDs, in AD brains (data not shown). With regard to specificity of the anti-SA antibody, preincubation without sialyllactose sodium salt did not reduce the reactivity of the antibody (Fig. 1Q,R). However, the anti-SA antibody preincubated with sialyllactose sodium salt totally lost the reactivity for GVDs, NFTs and DNs (Fig. 1S,T), indicating this antibody specifically recognized the NeuAcα2-3 bond.

To examine whether these hypersialylated structures were actually the same as GVDs, NFTs or DNs, double IF analysis was performed. The amyloid core of SPs, which contains β, was not immunostained by the anti-SA antibody (Fig. 2A-F). In contrast, the DNs surrounding the SP core seemed to be labeled by this antibody (Fig. 2A-F). The p-tau-positive DNs (Fig. 2G-I) and NFTs (Fig. 2J-L) were both stained by the anti-SA antibody. The GVDs, which were negative for p-tau (Fig. 2G-L, circle), but positive for pPERK (Fig. 2M-O, circle) or pTDP-43 (Fig. 2P-R, circle), were also stained by an anti-SA antibody.\(^{30,34}\) To examine whether the granular structures that were positive for the anti-SA antibody were lysosomes, a double IF analysis using lysosome markers (LAMP1 and cathepsin D) was also performed (Fig. 2S-X). These lysosome markers were partially colocalized with the anti-SA antibody, but not to the extent observed for NFTs or GVDs (Fig. 2S-X).\(^{35,36}\)

**Characteristic pathological hallmarks of tauopathies, but not those of other neurodegenerative disorders, were also hypersialylated**

The status of sialylation of the characteristic neuropathological hallmarks of neurodegenerative disorders other than AD was also investigated. Lewy bodies (LBs), which are a...
Fig. 2 Double immunofluorescence analyses using the anti-sialic acid (SA) antibody in the hippocampus of Alzheimer’s disease (AD) brains. The Aβ-positive amyloid core of senile plaques (SPs, rectangle in A, and D) was not stained by the anti-SA antibody (rectangles in B, C, and E, F) in the hippocampus of AD brains. Conversely, the dystrophic neurites (DNs) of SPs were not stained by Aβ (A,D), but were stained by SA (B,C,E,F). The boxed areas in A, B and C are enlarged in D, E and F, respectively. The p-tau-positive DNs (G) and neurofibrillary tangles (NFTs, arrowheads in J) were also labeled by the anti-SA antibody (H,I,K,L, respectively). Granulovacuolar degenerations (GVDs, circles in G-L) were negative for p-tau, but positive for SA. The phosphorylated protein kinase-like ER kinase (pPERK)-positive and phosphorylated trans-activation response DNA-binding protein 43 (pTDP-43)-positive GVDs were colocalized with SA (circles in M-O and P-R, respectively). In contrast, SA-positive NFTs (arrowheads in N and Q) were not positive for pPERK or pTDP-43 (M,O,P,R, respectively). Two lysosome markers, the lysosomal-associated membrane protein-1 (LAMP1) and cathepsin D, were partially colocalized with SA (S-U and V-X, respectively), but not to the extent observed for NFTs or GVDs. Scale bars: A-C, and G-I, 50 μm; D-F, J-O, V-X, 25 μm; S-U, 10 μm.
Sialic acid (SA) immunoreactivity in the pathological hallmarks of various neurodegenerative disorders. A and B are serial sections of the substantia nigra of the same Parkinson’s disease (PD) brain. A and B are enlarged in C and D, respectively. The anti-α-synuclein antibody led to the visualization of Lewy bodies (LBs, A,C); however, the same LBs were not labeled by the anti-SA antibody (B,D). However, the smaller punctate structures were labeled by the anti-SA antibody (B,D). Double immunohistochemistry showed that the punctate structures stained by the anti-SA antibody (brown) appeared around LBs were also stained by the anti-α-synuclein antibody (purple) (E and enlarged in F). Some of the brown punctate structures detected in the vacuoles were similar to the appearance of granulovacular degenerations (GVDs, E,F). Double immunohistochemistry of the pontine nuclei of multiple system atrophy (MSA) brains using anti-SA (brown) and anti-α-synuclein (purple) antibodies showed that glial cytoplasmic inclusions (GCIs, purple) were not positive for the anti-SA antibody (G). However, the smaller punctate structures were labeled by both the anti-SA (brown) and anti-α-synuclein (purple) antibodies (G,H). HE staining showed the presence of Bunina bodies in the anterior horn cells (arrows in I) in the lumbar spinal cord of amyotrophic lateral sclerosis (ALS). The specimen shown in I was decolorized and re-stained with the anti-SA antibody, and no positive structures were confirmed in the same anterior horn cell (J). Serial sections of the lumbar cord of a patient with ALS showed that the skein-like inclusions (K) and round inclusions (M) were labeled by the anti-phosphorylated trans-activation response DNA-binding protein 43 antibody (pTDP-43), but not by the anti-SA antibody (K,L and M,N, respectively). Asterisks * and ** in K and L, and *** in M and N indicate the reference vessels that were used to confirm the position of the respective anterior horn cells. The anti-SA antibody labeled the globose-type neurofibrillary tangles (NFTs) in the substantia nigra (SN) neurons of a progressive supranuclear palsy (PSP) brain (O), Pick bodies in neurons from the cornu ammonis 1 (CA1) area (P and enlarged in Q), Pick bodies from the dentate gyrus (R and enlarged in S), and ballooned neurons or Pick cells from the temporal cortex and putamen, oculomotor nuclei, and PAG were also labeled by the anti-SA antibody (Fig. 3O,S,T). Moreover, staining with this antibody led to the clear visualization of Pick bodies in the neurons of other broad areas, including the temporal cortex, thalamus, putamen, and pallidum (data not shown). In the temporal cortex and putamen, the anti-SA antibody also stained the somata of larger cells, which were reminiscent of ballooned neurons (also known as swollen cells or Pick cells) (Fig. 3T). These ballooned neurons were clearly distinguishable from Pick bodies based on their size and appearance, that is, the ballooned neurons were larger and showed perinuclear staining, and Pick bodies were smaller and round- or oval-shaped (Fig. 3Q,S,T).

The p62/ubiquitin-positive neuronal intranuclear inclusions observed in the sweat gland cells and adipocytes of the skin biopsy tissue from a patient with NiID were not clearly labeled by the anti-SA antibody (Fig. 3U-W). Physiological or nonpathological structures were also analyzed in the control brains. The lipofuscin granules observed in neurons and the corpora amylacea in astrocytes of the CA1 area, and the melanin granules observed in SN neurons were not labeled by the anti-SA antibody (Fig. 3X-Z).

Double IF analyses revealed that the LBs detected in patients with PD were partially colocalized with α-synuclein and SA at the outer edge of LBs; however, the cores of LBs were negative for SA (Fig. 4A-C). The GCIs observed in MSA subjects that were stained with α-synuclein, were not obviously positive for SA (Fig. 3A-D). However, some punctate materials were labeled by the anti-SA antibody in the same neurons of the substantia nigra (SN) that contained LB and melanin granules (Fig. 3C,D). These findings were reproducibly confirmed in other SN neurons of three PD brains. Double immunohistochemistry showed that the small punctuate structures were stained with the anti-SA antibody (brown), and that LBs were independently stained for α-synuclein (purple) in the same cell (Fig. 3E,F). Moreover, some of the small granular structures were localized within the vacuole, which were similar to the appearance of GVDs (Fig. 3E,F).

In MSA brains, typical glial cytoplasmic inclusions (GCIs), which are a pathological hallmark of MSA and contain α-synuclein, were not obviously labeled by the anti-SA antibody (brown) (Fig. 3G). However, smaller punctate structures were positively labeled by both the anti-SA (brown) and anti-α-synuclein (purple) antibodies (Fig. 3G,H). Some of the smaller punctate structures were localized by the GCIs (Fig. 3G), whereas others were independent of GCIs (Fig. 3H).

The presence of Bunina bodies, which are a pathological hallmark of ALS, was confirmed by HE staining in the lumbar anterior horn cells of patients with ALS (Fig. 3I). The HE-stained slide shown in Figure 3I was decolorized and subsequently re-stained with the anti-SA antibody. Bunina bodies in the same slide shown in Figure 3I were not labeled by the anti-SA antibody (Fig. 3J). Moreover, other hallmarks of ALS, that is, skein-like inclusions (Fig. 3K) and round inclusions (Fig. 3M) that were positive for pTDP-43 were totally negative for the anti-SA antibody (Fig. 3L,N).

Globose-type NFTs, which are a hallmark of PSP, were detected by the anti-SA antibody in the SN neurons of a patients with PD were partially colocalized with α-synuclein and SA at the outer edge of LBs; however, the cores of LBs were negative for SA (Fig. 4A-C). The GCIs observed in MSA subjects that were stained with α-synuclein, were not obviously positive for SA (Fig. 3A-D). However, some punctate materials were labeled by the anti-SA antibody in the same neurons of the substantia nigra (SN) that contained LB and melanin granules (Fig. 3C,D). These findings were reproducibly confirmed in other SN neurons of three PD brains. Double immunohistochemistry showed that the small punctuate structures were stained with the anti-SA antibody (brown), and that LBs were independently stained for α-synuclein (purple) in the same cell (Fig. 3E,F). Moreover, some of the small granular structures were localized within the vacuole, which were similar to the appearance of GVDs (Fig. 3E,F).

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Globose-type NFTs, which are a hallmark of PSP, were detected by the anti-SA antibody in the SN neurons of a PSP midbrain (Fig. 3O). NFTs in the neurons of the putamen, olfactory nuclei, and PAG were also labeled by the anti-SA antibody in the same PSP subject (data not shown).

Pick bodies, which are a hallmark of PickD, were positive for SA in the neurons of the CA1 area of the hippocampus (Fig. 3P,Q) and of the dentate gyrus (Fig. 3R,S). The smaller granular materials observed within vacuoles, which were similar to the GVDs, were also labeled by the anti-SA antibody (Fig. 3Q). Moreover, staining with this antibody led to the clear visualization of Pick bodies in the neurons of other broad areas, including the temporal cortex, thalamus, putamen, and pallidum (data not shown). In the temporal cortex and putamen, the anti-SA antibody also stained the somata of larger cells, which were reminiscent of ballooned neurons (also known as swollen cells or Pick cells) (Fig. 3T). These ballooned neurons were clearly distinguishable from Pick bodies based on their size and appearance, that is, the ballooned neurons were larger and showed perinuclear staining, and Pick bodies were smaller and round- or oval-shaped (Fig. 3Q,S,T).

The p62/ubiquitin-positive neuronal intranuclear inclusions observed in the sweat gland cells and adipocytes of the skin biopsy tissue from a patient with NiID were not clearly labeled by the anti-SA antibody (Fig. 3U-W). Physiological or nonpathological structures were also analyzed in the control brains. The lipofuscin granules observed in neurons and the corpora amylacea in astrocytes of the CA1 area, and the melanin granules observed in SN neurons were not labeled by the anti-SA antibody (Fig. 3X-Z).

Double IF analyses revealed that the LBs detected in patients with PD were partially colocalized with α-synuclein and SA at the outer edge of LBs; however, the cores of LBs were negative for SA (Fig. 4A-C). The GCIs observed in MSA subjects that were stained with α-synuclein, were not obviously positive for SA (Fig. 3A-D). However, some punctate materials were labeled by the anti-SA antibody in the same neurons of the substantia nigra (SN) that contained LB and melanin granules (Fig. 3C,D). These findings were reproducibly confirmed in other SN neurons of three PD brains. Double immunohistochemistry showed that the small punctuate structures were stained with the anti-SA antibody (brown), and that LBs were independently stained for α-synuclein (purple) in the same cell (Fig. 3E,F). Moreover, some of the small granular structures were localized within the vacuole, which were similar to the appearance of GVDs (Fig. 3E,F).

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Globose-type NFTs, which are a hallmark of PSP, were detected by the anti-SA antibody in the SN neurons of a
Double immunofluorescence analyses of the pathological hallmarks of various neurodegenerative disorders using the anti-sialic acid (SA) antibody. In the substantia nigra of a Parkinson’s disease (PD) brain, Lewy body cores that were positive for α-synuclein were not immunostained by the anti-SA antibody. However, α-synuclein-positive smaller punctate structures located at the outer edge of the core were colocalized with SA (A-C). In the pontine nuclei of a multiple system atrophy (MSA) brain, glial cytoplasmic inclusions (GCIs) that were positive for α-synuclein were not clearly colocalized with SA; however, smaller punctate structures located nearby or independently of GCIs were colocalized with SA (D-I, arrowheads). In the lumbar anterior horn cells of an amyotrophic lateral sclerosis (ALS) spinal cord, skein-like inclusions and round inclusions that were positive for the phosphorylated transactivation response DNA-protein 43 (pTDP-43) (J and M, respectively), and Bunina bodies positive for cystatin-C (P) were not colocalized with SA (K and L, N and O, Q and R, respectively). The globose-type neurofibrillary tangles accumulated in the substantia nigra neurons of a progressive supranuclear palsy (PSP) brain were colocalized with p-tau and SA (S-U). Pick bodies in neurons from the cornu ammonis 1 area of a frontotemporal lobar degeneration with Pick bodies (PickD) brain were colocalized with p-tau and SA (V-X). Scale bars: 10 μm.

Fig. 4

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not obviously colocalized with SA (Fig. 4D-I). The smaller punctate structures that were independent of typical GCIs were colocalized with α-synuclein and SA; moreover, some of these structures were located in the proximity of GCIs (Fig. 4D-I, arrowheads). Skein-like inclusions (Fig. 4J-L) and round inclusions (Fig. 4M-O), which were both positive for pTDP-43 and Bunina bodies (Fig. 4P-R), were not obviously colocalized with SA in patients with ALS. The globose-type NFTs, which were positive for p-tau in a PSP brain, were colocalized with SA (Fig. 4S-U). Pick bodies, which were positive for p-tau in a PickD brain, were also colocalized with SA (Fig. 4V-X). The association between the above-mentioned characteristic neuropathological hallmarks of various neurodegenerative disorders and the status of sialylation is summarized in Table 2.

**DISCUSSION**

The present study clearly showed that prominent hypersialylation of NFTs, GVDs and DNs of SPs is a common pathological feature in AD and other tauopathies. Hypersialylation of these structures was confirmed in broad areas, including the CA1 area of the hippocampus, the frontal, temporal and occipital lobes, the PAG of the midbrain, the pons and the medulla of AD brains (Fig. 1). Double IF analyses revealed that, in AD and other tauopathies, p-tau was colocalized with SA (Figs 2G-L and 4S-X). In contrast, the amyloid cores of SPs, which are another important hallmark of AD pathology, did not show colocalization of Aβ and SA (Fig. 2A-F). These results indicate that p-tau, but not Aβ was hypersialylated in AD brains. As physiological or nonpathological structures, such as lipofuscin granules, corpora amylacea and melanin granules, and the characteristic pathological hallmarks of other neurodegenerative disorders, such as PD, MSA, ALS and NIID, were not sialylated at a level detected in AD brains (Figs 3 and 4), hypersialylation of NFTs, GVDs and DNs seems to be a highly specific finding of AD pathogenesis. Interestingly, the disease-specific pathological hallmarks of other tauopathies were also hypersialylated. The globose-type NFTs in a PSP brain and the Pick bodies and ballooned neurons in a PickD brain were also clearly hypersialylated at a level comparable to AD brains (Figs 3O-T and 4S-X). As these pathological hallmarks contained p-tau, p-tau present in the tauopathies may also be hypersialylated. These findings indicate that hypersialylation of p-tau is involved in the common pathological processes of AD and other tauopathies. Previous studies have shown that nonphosphorylated tau or p-tau in AD brains, but not normal human tau, are attached by various sugars, such as galactose, glucose, mannose, N-acetylglucosamine (GlcNAc) and NeuAc, via N-linked glycosylation. In addition, it has been reported that aberrant glycosylation facilitates hyperphosphorylation of tau, resulting in the formation of NFTs. Therefore, our results might support the association between hypersialylation and promotion of tau phosphorylation, causing subsequent accumulation of NFTs in AD and other tauopathies.

The LB cores detected in PD brains were not stained with the anti-SA antibody (Figs. 3A-F and 4A-C). Previous reports have shown that a subset of LB cores was stained with an anti-tau antibody in PD and dementia with LBs. According to these findings, it is speculated that tau is not hypersialylated in LB cores, and that the mechanism of tau accumulation in LBs is different from that involved in AD and other tauopathies. α-synuclein may be crucial as a nidus or a seed for tau accumulation at the outer edge of LBs. The LB cores and GCIs were also confirmed adjacent to the LBs or GCIs (Figs 3A-H and 4A-I). In PD brains, similar structures were...
previously reported to contain ER stress-responsive proteins or unfolded protein response (UPR) proteins, such as pPERK and peIF2α, which are involved in the aggregation and accumulation of α-synuclein.43 Similarly, in MSA brains, small structures were found to contain UPR proteins in oligodendroglia, which were activated at an early stage of MSA neurodegeneration.44 Moreover, the GVDs were also reported to contain UPR proteins, including pPERK, peIF2α, and pIRE1α.45 The smaller hypersialylated structures detected in PD and MSA brains (Figs 3A-H and 4A-I) suggest that the process of sialylation is closely associated with an activation of the UPR response, because glycosylation pathways can influence ER stress.45,46

Lectin is a collective term for the protein family that can bind to sugar chains. The *Maackia amurensis* (MAA) lectin was reported to detect PHF-tau on Western blot, and stains NFTs in the AD brain.4,47 However, it has not been reported that the GVDs could be detected by lectins.47 These findings suggest that the anti-SA antibody is able to detect the status of sialylation more sensitively than lectin proteins. Actually the anti-SA antibody was reported to recognize more diverse glycoproteins containing SA residues than the MAA lectin.31

In summary, we report the novel findings that the NFTs, GVDs and DNs in AD brains and the characteristic structures in other tauopathies, but not other pathological hallmarks in PD, MSA, ALS and NIID, were hypersialylated. These findings suggest that hypersialylation of tau is commonly associated with the pathogenesis of tauopathies. The UPR-associated smaller structures detected in PD and MSA brains were also hypersialylated, indicating a crucial role of sialylation for the activation of the UPR response. Although previously reported anti-tau antibodies can also detect NFTs, immunoreactivity for GVDs was not always observed by these antibodies.15,48 Because the anti-SA antibody specifically identified NFTs, GVDs and DNs, immunostaining for sialylation may represent a useful tool to screen these structures in a diagnostic setting. Clarification and a better understanding of the mechanism that leads to the hypersialylation and accumulation of tau will help to develop effective therapies for AD and other tauopathies.

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REFERENCES


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CONFLICT OF INTEREST

Nothing to report.

Hypersialylation of NFT and GVD


