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3-O-Sulfation of Heparan Sulfate Enhances Tau Interaction and Cellular Uptake

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Prion-like transcellular spreading of tau in AD is mediated by tau binding to cell surface heparan sulfate (HS) and the glycan determinants of this interaction are beginning to be decoded. In a microarray of structurally-defined HS oligosaccharides, an additional 3-O-sulfo group (3-O-S) resulted in significantly increased tau binding. SPR assays showed that an HS dodecasaccharides (12-mer) with one additional 3-O-S inhibited tau-HS interaction with a ~5-fold lower IC_{50} than an HS 12-mer without 3-O-S. In *Hs3st1*^{-/-} (3-O-sulfotransferase-1 knockout) cells, reduced 3-O-S levels of HS diminished cell surface binding and internalization of tau. An NMR titration mapped the 3-O-S recognition site in tau to PRR2 and R2 domains. Treatment of cells with the 3-O-S HS 12-mer reduced both tau-HS interaction and tau cellular uptake. This study established the important role of 3-O-S in tau-HS interaction, suggesting that 3-O-S represents a pharmacophore for glycan-based drug discovery targeting the spread of tau pathology.

Alzheimer's disease (AD) pathology is characterized by amyloid plaque and neurofibrillary tangles (NFTs). NFTs are composed of microtubule-associated protein tau (MAPT), whose normal functions include bundling and stabilizing microtubules (MTs) in neurons. Continued failure of anti-amyloid compounds in clinical trials has shifted the focus of AD research towards tau. In AD, tau becomes hyperphosphorylated, dissociates from microtubule and aggregates to form NFTs. In contrast with amyloid plaques, NFTs pathology correlates well with cognitive decline in AD¹. Recently, mounting evidence from cell culture^{2,3}, animal models⁴⁻⁶ and human pathology⁷ has established that tau NFTs spread through neural networks in an orderly and 'prion-like' manner, mediated by transcellular movement of tau⁸⁻¹⁰ (Fig. 1A). Because NFTs directly correlate to cognitive deficits, inhibiting the prion-like spread of tau is likely a viable strategy to slow down cognitive decline and the progression of AD in patients. Thus, there is a pressing need to understand the molecular mechanisms of NFT spread.

Figure 1. Cellular uptake of tau is mediated by HSPGs on cell surface. (A) Prion-like spread of tau pathology (represented by blue color) in AD brain. (B) Uptake of tau mediated by the binding to heparan sulfate proteoglycans (HSPGs). Microtubules are represented by a tube composed of α - and β - tubulins (yellow and purple). (C) Primary structure and sulfation pattern of heparan sulfate.

A key step in tau transcellular movement is tau binding to heparan sulfate proteoglycans (HSPGs) ^{11–14} on cell surface (Fig. 1B), followed by the endocytosis of tau. HSPGs are HS glycosaminoglycan (GAG) chains covalently linked to a protein core. HS is a linear, polyanionic GAG composed of disaccharide repeats of uronic acid (glucuronic acid or iduronic) and glucosamine with sulfation on the 3-OH, 6-OH and -NH of the glucosamine residue, and the 2-OH of the uronic acid residue (Fig.1C). Specific sulfation patterns are required for the recognition of HS by its protein binding partners ^{15,16}. In a previous study, we have identified the 6-O-sulfo groups as an important determinant in tau-glycan interaction ¹⁷, which was later confirmed by others with a variety of approaches, including the knockout of 6-O-sulfotransferases, HS biosynthetic enzymes responsible for carrying out cellular 6-O-S sulfation of HS ^{13,14}. The 2-O-sulfo group is not critical for tau-HS interaction, while controversial data exist regarding the role of *N*-sulfo groups ^{5,8,17}. The role of 3-O-S, a rare sulfation site in HS, has not been studied. Only six proteins are known to rely on the 3-O-S for binding specificity ^{18–20}. In the human, 3-O-sulfation of HS is catalyzed by seven isoforms of 3-O-sulfotransferase (*HS3ST*): *HS3ST1*, 2, 3A, 3B, 4, 5 and 6. *HS3ST1*, 2, and 5 are only expressed in the brain based on a tissue-based map of human proteome ²¹, and a recent study showed

that *Hs3st2* and *Hs3st4* were overexpressed in AD hippocampus²², suggesting the involvement of 3-O-sulfation in AD pathology. However, how 3-O-sulfation in HS contributes to AD is unknown.

Here, utilizing structurally defined HS oligosaccharide microarray, surface plasmon resonance (SPR), nuclear magnetic resonance spectroscopy (NMR), and cellular binding and uptake assays, we report for the first time that 3-O-S is a crucial determinant in tau-HS interaction and tau uptake. Our results provide molecular details of the link between 3-O-sulfation of HS and AD, pointing towards novel strategies for tau-targeted AD therapy.

Results

3-O-S enhances tau binding to HS in glycan array analysis. Previous

interaction studies of tau/glycan have relied on heparin as a substitute for HS but important structural and functional differences exist between heparin and HS. In this studies, tau/glycan interaction has been examined using HS. Structurally defined HS oligosaccharides were synthesized by a chemoenzymatic method as previously described^{19,23} and were then immobilized on a microarray chip, creating the low molecular weight HS (LMHS) array. Full-length tau binding (or lack thereof) to the HS array was visualized by fluorescently-labeled tau remaining on the chip after incubation and washing. As shown in Fig. 2, high fluorescence intensity was observed for a HS heptasaccharide (7-mer) on spot 4 (oligo-4), and three HS dodecasaccharides (12-mers) in spot 18, 19 and 20 (oligo-18, -19 and -20). Remarkably, oligo-4 which only differs from oligo-5 by a single additional 3-O-sulfo group, exhibits ~ 10-fold higher fluorescence intensity than oligo-5, indicating that the presence of 3-O-S increases the binding of tau protein. The significance of 3-O-S is also underscored from the binding of tau to longer oligosaccharides as demonstrated by the microarray analysis. HS 12-mer oligo-18 and oligo-19, containing two and one 3-O-S, respectively, displayed higher binding to tau compared to oligo-20, a HS 12-mer lack of 3-O-S. Oligo-21, which is not sulfated, exhibited negligible fluorescence.

Figure 2. Low molecular weight heparan sulfate (LMHS) array shows the crucial role of 3-O-sulfo group (3-O-S) in tau binding. Fluorescence intensity on each spot of array was shown in a bar graph, with the monosaccharide composition/sulfation pattern drawn for the HS oligosaccharides with high fluorescence intensity (tau binding). Complete results of the LMHS array can be found in Fig. S1 and S2.

3-O-S promotes inhibition of tau-HS interaction by oligosaccharides as demonstrated by SPR analysis. Binding kinetics and affinity between HS and tau have not been measured before. Here, HS from three different sources, porcine brain, porcine spine and porcine intestine, were prepared, biotinylated, and immobilized on a SA sensor chip for binding studies using full-length tau. Brain, spinal and intestinal HS exhibited similar binding pattern to tau, with a binding affinity (K_D) of 0.02 μ M (Fig 3A and Fig. S3), showing similar behavior in tau interaction of HS from these three different sources. The more accessible porcine intestinal HS was then used to further characterize the role of 3-O-sulfation in tau-HS binding, which likely resembles endogenous HS from brain tissues. Three synthesized HS 12-mers, oligo-19, oligo-20

122 and oligo-21 (the oligosaccharides in spots 19, 20, and 21 of the LMHS array, for
123 chemical structure see Fig. S1B) were tested by a solution/surface competition SPR
124 assay (Fig. 3B) to examine their ability to inhibit tau-HS interaction. Full-length tau
125 protein was individually pre-mixed with each of three HS 12-mer and then flowed over a
126 chip with surface-immobilized HS. The tau protein binding to 12-mer in solution
127 diminishes its interaction with the HS immobilized on the chip surface (Fig. 3B). With
128 increasing 12-mer solution concentrations, less and less binding to the surface was
129 detected. An IC_{50} of 0.9 μ M and 4.9 μ M for the inhibition of tau-HS interaction were
130 obtained for oligo-19 and oligo-20, respectively (Fig. 3C and 3D). Observed lower IC_{50}
131 value for oligo-19 is consistent with the stronger binding of tau to oligo-19 in HS
132 microarray analysis. This $\sim$ 5-fold lower IC_{50} indicates oligo-19 is much more effective in
133 the inhibition of the tau-HS interaction. In contrast, oligo-21 showed very little inhibition
134 of tau-HS interaction, with an IC_{50} higher than 700 μ M (Fig. 3E), also consistent with the
135 negligible fluorescence signal for oligo-21 in LMHS array. The significantly lower IC_{50} of
136 oligo-19 compared with that of oligo-20 and the lack of inhibition by oligo-21
137 demonstrates that sulfation is required for the ability of HS 12-mer to inhibit the tau-HS
138 binding, and that 3-O-S greatly enhances this inhibition.

Figure 3. HS 12-mer oligo-19 and oligo-20 inhibit full-length tau-HS binding with an IC_{50} of 0.9 μM and 4.9 μM , respectively. (A) Binding affinity of full-length tau-HS interaction was measured to be 0.02 μM by SPR binding kinetic assay for the first time. The association and dissociation curve of different tau concentrations were fitted (black line) by a 1:1 Langmuir kinetics model in Bio-evaluation. HS from three sources (porcine brain, porcine spine and porcine intestine) were tested (Fig. S3) and only porcine intestinal HS binding is shown here. **(B)** Scheme for Competition SPR. **(C)** Oligo-19 inhibits tau-HS binding with an IC_{50} of 0.9 μM . **(D)** Oligo-20 inhibits tau-HS binding with

an IC_{50} of 4.9 μ M. **(E)** Oligo-21 does not inhibit tau-HS binding, with an IC_{50} higher than 700 μ M.

***Hs3st* knockout reduces tau cell surface binding and cellular uptake.**

Based on the microarray and SPR data, we hypothesized that 3-O-S in HSPGs may play an important role in tau binding to the cell surface and its subsequent internalization. To test this hypothesis, we next carried out tau cell surface binding and cellular uptake assays using a pair of wild type (WT) and *Hs3st1* knockout (*Hs3st1*^{-/-}) mouse lung endothelial cell (MLEC) lines. The selection of *Hs3st1* was based on the expression profiles of HS 3-O-sulfo transferases in primary mouse cerebral cortex neurons determined by RNA-seq, with the highest expression level observed for *Hs3st1* among all *Hs3sts* (Fig. S4). The *Hs3st1*^{-/-} MLEC line was derived from the WT parent line using CRISPR-Cas9 gene-editing and expressed normal levels of NS, 6-O-S and 2-O-S (Fig. S5A), but reduced level of 3-O-S (confirmed by significantly reduced cell surface binding to antithrombin III requiring a 3-O-S for binding, Fig. S5B)²⁴. Biotinylated-tau was generated and incubated with cells, followed by washing and detection of surface-bound tau with streptavidin-HRP. Tau bound strongly to the surface of WT MLECs, while the binding was greatly diminished on *Hs3st1*^{-/-} MLECs surface, showing that 3-O-S strongly enhances HS binding of tau on the cell surface (Fig. 4A). We next incubated both WT and *Hs3st1*^{-/-} cells with Alexa488 labeled full-length tau (tau-Alexa) for 12 hrs., followed by detection with both flow cytometry (Fig. 4B) and confocal imaging (Fig. 4C) to further investigate the effects of 3-O-S deletion on the cellular uptake of tau. Large amounts of tau were internalized into the WT

MLECs, but internalization was greatly reduced in the *Hs3st1*^{-/-} MLECs, indicating that 3-O-S indeed enhances HSPG-mediated tau internalization. Here, we demonstrate another role for cell surface 3-O-S in tau pathology, in which it specifically recognizes extracellular tau and mediates efficient cellular uptake.

Figure 4. Deletion of *Hs3st1* diminishes tau cell surface binding and internalization. (A) The *Hs3st1*^{-/-} cells showed less (46.3% reduction) tau cell surface binding, compared with WT. After fixing and incubating with biotinylated full-length tau (500 ng/ml, 100 μ l/well) for 90 min at RT, the cell surface bound tau was measured after incubating with Streptavidin-HRP and color development. (B) The *Hs3st1*^{-/-} cells showed significantly less internalization of tau-Alexa (500 ng/ml) assessed by flow cytometry. (C)

The *Hs3st1*^{-/-} cells showed significantly less internalization of tau-Alexa by confocal images. The cells in 12-well plate were incubating with tau-Alexa (2 µg/ml, 500 µl/well) at 37°C for 3 h. The data shown are representative of 2-4 independent experiments.

Oligosaccharides with 3-O-S blocks tau cell surface binding and internalization. Interfering with tau-HS interaction using heparin (HP, a highly sulfated analog of HS) or its mimetics can block tau transcellular spreading in cell culture and animal models ¹¹. Designing glycan-based compound to disrupt the tau-HS interface represents a novel strategy to develop effective therapeutics for tauopathy in AD. We asked whether 3-O-sulfated oligosaccharides could be more effective at blocking tau cell surface binding and internalization than counterparts without 3-O-S. As expected, HP potently inhibits tau cell surface binding and internalization (Fig. 5). Oligo-19 and oligo-20, but not oligo-21, inhibit tau cell surface binding and internalization with similar pattern as to HP. Compared with oligo-20, oligo-19 exhibits significantly greater inhibition of the cell surface binding and internalization of tau, underscoring the crucial role of 3-O-sulfation for effectively blocking tau-HS interaction on cell surface and tau internalization. The addition of 3-O-S modification may lead to more potent HS-based therapeutics for tauopathy.

Figure 5. 3-O-S modification enhances the inhibitory potency of HS oligo on tau-cell interaction and tau cellular uptake. (A) HP, oligo-19 and oligo-20 inhibit tau cell surface binding by 46.3%, 28.0% and 13.0%, respectively. After fixing and incubating with biotinylated tau (500 ng/ml, 100 µl/well) without or with HP (50 ng), HS oligos (25 ng) for 90 mins at RT, the cell surface bound tau was measured after incubating with Streptavidin-HRP and color development. Oligo19 has a stronger inhibitory potency than oligo-20. Oligo-21 has no inhibition. **(B)** HP, oligo-19 and oligo-20 inhibit tau-Alexa (500 ng/ml) internalization assessed by flow cytometry. **(C)** HP, oligo-19 and oligo-20 inhibit tau internalization assessed by confocal images. The cells were incubated with tau-Alexa (2 µg/ml, 500 µl/well) without or with HP (10 µg/ml), HS oligo (2.5 µg/ml) at 37 °C for 3 h. Oligo-19 has a stronger inhibitory potency than oligo-20. Oligo-21 has no inhibition. The data shown are representative of 2-4 independent experiments.

214

215 **3-O-S is recognized by tau PRR2 and R2 regions in NMR titration.** We

216 next determined which regions of tau are responsible for the recognition of 3-O-S in HS.

217 The primary sequence of the longest tau isoform (441 residues) features the N-terminal

218 projection region (N1 and N2), the proline rich region (PRR1 and PRR2), and the

219 microtubule binding region (MTBR) and the C-terminal region (Fig. 6C). The MTBR

220 includes four internal repeat motifs (R1-R4), which mediates tau interactions with MTs

221 ^{25,26} and other proteins ²⁷, as well as tau aggregation³. We use full-length tau to map

222 the binding sites of 3-O-S. Shorter HS oligosaccharides, i.e. oligo-4 (HS 7-mer with 3-O-

223 S) and oligo-5 (HS 7-mer without 3-O-S), were used in the experiment, because tau

224 preferably binds to oligo-4 from the microarray analysis. Oligo-4 and oligo-5 were

225 individually added to ¹⁵N labeled tau and the refocused two-dimensional (2D) ¹H-¹⁵N

226 heteronuclear single quantum coherence (HSQC) NMR spectra of tau were recorded

227 before (blue peaks in Fig. 6A) and after the addition of the HS oligosaccharides (green

228 and red peaks in Fig. 6). Significant chemical shift perturbations (CSPs) in tau were

229 observed upon addition of both oligo-4 (resonance in red) and oligo-5 (resonance in

230 green) titration (Fig. 6). As expected, oligo-4 caused much larger CSPs than oligo-5,

231 due to the stronger binding conferred by the 3-O-S modification. Several isolated peaks

232 with large CSP are highlighted in Fig. 6B. The CSP differences (Δ CSP) between CSP

233 due to oligo-4 and CSP due to oligo-5 were plotted against the residue number (Fig. 6C)

234 to map the binding site of 3-O-S in tau (Fig. 6C). Significant Δ CSPs were located at the

235 PRR2 and R2 domains, in which residues V226, L243, and Q276 exhibit the largest

236 Δ CSPs, indicating a specific interaction between 3-O-S and the PRR2 and R2 of tau.

The hexapeptide $^{275}\text{VQIINK}^{280}$ in R2, which contributes to tau aggregation and MTs association, was previously identified as the main site of contact with HP^{17,33}. PRR regions of tau are not only important for MTs binding²⁸, but also hot spots for tau phosphorylation^{29,30} and protein interactions^{31,32}. The recognition of 3-O-S in HS by both PRR2 and R2 suggests HS interaction may modulate both tau aggregation and phosphorylation.

Figure 6. Chemical shift perturbation difference (ΔCSP) reveals specific interactions between 3-O-S and PRR2 and R2 domain of full-length tau. (A) Overlay of ^1H - ^{15}N HSQC spectra of full-length tau before (blue) and after 1:0.6 molar ratio addition of HS 7-mer oligo-5 (green) and HS 7-mer oligo-4 (red). **(B)** Zoomed-in NMR spectra of residues with biggest CSPs. **(C)** CSP differences (ΔCSP) reveals specific interaction between 3-O-S and tau PRR2 and R2 domain. Construct of tau is shown above the figure, PRR = proline-rich region, MRBR = microtubule binding region.

Discussion

Growing evidence has established that tau NFTs pathology propagates in a “prion-like” manner^{34,35}. While the mechanisms underlying the intercellular spread of tau are not completely understood, a required step in this process is that tau binding to HSPGs on the recipient cell surface³⁶. HS interactions with proteins are mainly driven by electrostatic forces, between positively charged side chains on proteins and negatively charged sulfo groups on HS³⁷. Although charge-based association is relatively non-specific, many HS-binding proteins require specific sulfation patterns in the glycan, e.g., heparin/antithrombin III (ATIII) interaction requiring a pentasaccharride sequence with a 3-O-sulfo group in its central residue. In contrast to the less stringent requirements for sulfation pattern reported for α -synuclein and A β binding to HS³⁸, tau requires more specific sulfate moieties^{13,17,38}. In previous work, we were the first to report that 6-O-S, but not 2-O-S, is required for tau binding, using structurally heterogeneous polysaccharides¹⁷.

Here, we demonstrate that the 3-O-sulfation strongly enhances the tau-HS interaction and cellular uptake of tau, using LMHS microarray, SPR, cellular binding and uptake assays, and NMR. Structurally defined HS 7-mer and 12-mer with additional 3-O-S exhibited significantly stronger binding to tau in LMHS array (Fig. 2). This was then confirmed in SPR competition assays showing that an HS 12-mer with one additional 3-O-S (oligo-19) inhibits tau-HS interaction with ~5-fold lower IC_{50} value than the same HS 12-mer without 3-O-S (oligo-20) (Fig. 3). The reduced cell surface binding and internalization of tau in *Hs3st1*^{-/-} cells indicates that 3-O-sulfation significantly enhances the cellular uptake of tau (Fig. 4). These data conclusively demonstrate that 3-O-S

modification plays a crucial role in tau-HS interaction and tau cellular uptake. Our data provide a mechanistic rationale for the recent observation that the expression of *Hs3st2* and *Hs3st4* is elevated in AD brain and that HS containing GAGs isolated from AD brain exhibit enhanced tau binding²².

To date, tau is only the seventh protein shown to specifically recognize 3-O-S in HS³⁹. Heparin/ATIII interaction has been the prime example of the specific interaction mediated by 3-O-S. Interestingly, 3-O-S also facilitates cellular entry of Herpes simplex virus (HSV-1), which has been linked to AD⁴⁰⁻⁴². 3-O-S enhances HS interaction with viral envelop glycoprotein D (gD)^{43,44}. Thus, both Herpes virus and tau entry into a cell are enhanced by the 3-O-S functional group, raising the possibility of mechanistic cross-talks between the spread of tau pathology and Herpes infection in the AD brain.

By establishing the critical role of the rare 3-O-S HS modification in tau-HS interaction, we provide one of the most important insights for developing HS-based therapies against the spread of tauopathy: to efficiently inhibit cellular uptake of tau, a 3-O-sulfo group is required. In this work, efficient inhibition of tau-HS interaction has been achieved with a HS 12-mer containing 3-O-S (oligo-19) with an IC_{50} of 0.9 μ M, in a SPR competition assay (Fig. 3C). Significant inhibition of cellular binding and uptake of tau was also observed (Fig. 5) by the same oligo-19. Based on these data, we propose that the 3-O-S and tau interface represents a novel target for AD disease-modifying therapy to block tau trans-cellular propagation in AD. As 3-O-sulfotransferases are overexpressed in AD brain²², inhibiting the expression or activity of 3-O-sulfotransferases may represent another avenue for inhibiting the propagation of NFT pathology.

NMR mapping shows 3-O-S (Fig. 6C) preferably bind to the PRR2 and R2 domain of full-length tau, which are the crucial regions for aggregation⁴⁵, MTs association^{28,46}, and interaction with heparin^{17,47} and other proteins^{31,32}. 6-O-S also binds to the R2 domain as previously studied. Taken together, we suspect there may be a synergistic effect between 3-O-S and 6-O-S that enhances the binding of HS to tau. Similarly, in ATIII-heparin interaction both 3-O-S and 6-O-S modification are critical for inducing the conformational change in ATIII⁴² needed for anticoagulant activity of heparin. Unlike ATIII, tau is an IDP without a fixed 3D structure, rendering it a more challenging system for conventional structural characterization. More work is needed to delineate the specific HS motifs (the combination of chain length, monosaccharide composition and precise sulfation pattern) required for tight binding to tau in human brain and in Alzheimer's disease, and to understand the structural basis of the specific interactions between 3-O-S and tau residues at atomic resolution.

In summary, our results demonstrate the key role of 3-O-S in the tau-HS interaction and cellular uptake of tau, uncovering a unique structural requirement of HS recognition by tau. This work represents a major step forward in our understanding of the mechanism of tau-HS interaction, with important implications for 3-O-S as a pharmacophore targeting the spread of tau pathology in the development of effective AD therapy.

Fig. S1. HS oligosaccharides immobilized on microarray chip (A) and chemical structure of oligo-19, 20 and 21 (B).

Fig. S2. Fluorescence image (A) and intensity (B) of each spot on LMHS array.

Fig. S3. Porcine Brain, spinal and intestinal HS exhibited similar binding pattern to full-length tau, with the binding affinity (K_D) of 0.02 μ M. The association and dissociation curve of different tau concentrations were fitted (black line) by a 1:1 Langmuir kinetics model in Bio-evaluation.

Fig. S4. Gene target selection in HS synthesis pathway for generating neuro-specific HS deficient mice with specific sulfation pattern. (A). HS structure and biosynthetic/remodeling genes. In mammals, the 3S level is determined by *Hs3st*. In MLECs *Hs3st1* is the most abundantly expressed *Hs3st*. **(B).** The expression profiles of *Hs6st*, *Sulfs* and *Hs3sts* in primary mouse cerebral cortex neurons determined by RNA-seq.

341 **Fig. S5. The *Hs3st1*^{-/-} MLEC line expressed normal levels of NS, 6-O-S and 2-O-S**
342 **(A), but reduced cell surface binding to antithrombin III (B).**

343

344

Methods

Materials. The overexpression and purification of full-length tau protein were performed as previously described^{48,49}. Chemoenzymatic synthesis of low molecular weight heparan sulfate (LMHS) was completed according to methods published previously^{50 51}. Heparan sulfate extracted from porcine intestine is a commercial product obtained as a side stream in the manufacture of porcine intestinal heparin⁵². Heparan sulfates from porcine brain and spine were purified and characterized as previously described⁵³.

The wildtype and *Hs3st1*^{-/-} MLEC lines were developed in our lab recently using Crispr-Cas9 or conditional Cre-LoxP gene editing technologies²⁴. The *Hs3st1* deletion selectively reduces 3-O-S and correspondingly, can be applied to specifically determine the requirement of 3-O-S in interaction with a protein ligand, respectively, in a cellular setting.

The binding preference of tau to HS using microarray assay. Full-length tau protein was labeled with fluorescence dye Alexa Fluor 488 5-SDP Ester (Life Technologies) according to the supplier's instructions. The degree of labeling (DOL) was 1-2 moles/mole of protein. A series of structurally defined HS oligosaccharides are immobilized on a microarray chip using a robotic printer as previously described⁵⁴. The fluorescently labeled tau protein is incubated with the slide for 1 h at room temperature and then washed. The wash process was repeated twice before analyzing the slide using the excitation wavelength of 488 nm on a GenePix 4300 scanner (Molecular Dynamics). Resolution was set at 10 μ m. The array images were analyzed by GenePix Pro 7.2.29.002 software. Spots were automatically found and spot deviations were

manually fit to correct. Mean median fluorescence intensities of arrays were obtained by Array Quality Control of software.

Characterization of tau-HS interaction by SPR assays.

Preparation of the HS biochip. Biotinylated HS was prepared by reacting sulfo-N-hydroxysuccinimide long-chain biotin (Thermo Scientific, Waltham, MA) with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain according to a published procedure⁵⁵. The biotinylated HS was immobilized to a SA chip based on the manufacturer's protocol. In brief, a 20 μ L solution of the HS-biotin conjugate (0.1 mg/mL) in HBS-EP running buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20 (pH 7.4)) was injected over flow cell 2 (FC2) of the SA chip at a flow rate of 10 μ L/min. Successful immobilization of HS was confirmed by the observation of an ~250 resonance unit increase in the sensor chip. The control flow cell (FC1) was prepared by a 1 min injection with saturated biotin.

Binding affinity of HS-tau interaction. Lyophilized full-length tau protein was resuspended in HBS-EP buffer. Different concentrations of the protein (0.1 μ M, 0.25 μ M, 0.5 μ M, 1.0 μ M, and 2.0 μ M) were injected at a flow rate of 30 μ L/min for 3 min. At the end of the sample injection, HBS-EP buffer was flowed over the sensor surface to facilitate dissociation. After a 3 min dissociation time, the sensor surface was regenerated by injection with 30 mL of 2 M NaCl. The response was monitored as a function of time (sensorgram) at 25 °C.

Competition assay of 12-mer. Competition SPR experiments were performed to study how the presence of 3-O-S impacts the inhibition of 12-mer on tau-HS interaction. Tau protein was premixed with three different 12-mer, separately, and flowed over the HS

chip at a flow rate of 30 μ L/min. After each injection, dissociation and regeneration were performed as described above. For each set of competition experiments on SPR, a control experiment (with only tau protein and no 12-mer) was performed to confirm that the surface was completely regenerated and that the results obtained between runs were comparable. A series of concentrations of 12-mer was tested and IC_{50} was obtained by fitting the data using the '[Agonist] vs. normalized response' equation in GraphPad Prism 8 software, $Y=100 \cdot X^H / (IC_{50}^H + X^H)$, where Y is the normalized binding of tau to HS biochip, X is the concentration of 12-mer, and H is the Hill slope describing the steepness of the curve.

Cell surface tau binding assay. ELISA was performed to determine cell surface tau binding. In brief, 3×10^4 MLECs, including wildtype and *Hs3st1*^{-/-} cells, were seeded at 200 μ L/well in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin in a 96 well plate. After culturing overnight, the cells were washed with DPBS (3 times x 5 min) and then fixed with 4% PFA (15 min, RT), washed with DPBS (3 times x 5 min) and blocked with DPBS containing 1% BSA (90 min, RT), the cells were incubated with 100 μ L DPBS containing BSA (50 ng), biotinylated Tau (50 ng/ml), or biotinylated Tau (50 ng) mixed with heparin (50 ng), oligo-19 (25 ng), oligo-20 (25 ng) or oligo-21 (25 ng) for 90 min at RT. Following, the cells were washed with DPBS (3 times x 5 min), incubated with Streptavidin-HRP (1:2000 dilution in DPBS containing 1% BSA, 30 min, RT), and then cell surface bound tau (represented by HRP activity) was measured using an Ultra TMB-ELISA kit (34028, Thermo Scientific) according to the manufacturer's protocol.

Tau internalization assay. MLECs (5×10^5), including wildtype and *Hs3st1*^{-/-} cells, were seeded at 600 µl /well DMEM containing 10%FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a 12 well plate. After culturing overnight, the cells were washed with DPBS twice and then incubated with 500 µl/well DMEM containing BSA (2 µg/ml), Tau-Alexa (2 µg/ml) or Tau-Alexa (2 µg/ml) mixed with heparin (10 µg/ml), oligo-19 (2.5 µg/ml), oligo-20 (2.5 µg/ml) or oligo-21 (2.5 µg/ml) at 37 °C for 3 h. Followingly, the cells were processed for image or flowcytometry analyses. For image analysis, the cells were covered with mounting medium DAPI and examined for internalized tau under confocal microscope for flowcytometry analysis, the cells were trypsinized and resuspended in DPBS containing 2 mM EDTA, 1% BSA and PI which stain dead cells, and then measured for internalized tau-Alexa with flow cytometer.

3-O-S Binding site mapping by NMR. NMR experiments with oligo-4 (HS 7-mer with 3-O-S) and oligo-5 (HS 7-mer without 3-O-S) were performed on full-length tau to map the binding site of 3-O-S on tau. ¹H-¹⁵N HSQC spectra were recorded on an 150 µM full-length tau sample before and after the adding of a 1:0.6 ratio of oligo-4 and oligo-5, separately. Normalized chemical shift perturbation (CSP) of tau for amide ¹H and ¹⁵N chemical shifts upon HS 7-mer addition were calculated using the equation $CSP = \sqrt{100 \times \Delta H^2 + \Delta N^2}$, where ΔH and ΔN are the differences between the chemical shifts of the free and bound forms of tau, respectively. As the only difference between oligo-4 and oligo-5 is an additional 3-O-S, a CSP difference (ΔCSP) calculated by CSP (due to oligo-4) minus CSP (due to oligo-5) was plotted against the residue number to map the binding site of 3-O-S in tau.

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Author contribution

C.W., L.W. and J.Z. conceived and designed the research and wrote the manuscript. J.Z. carried out SPR and NMR analysis. Y.Z. and X.S. generated the cell line data. Y.X. purified full-length tau protein and did fluorescence labeling. G.S. carried out LMHS array assay. X.L. assisted with data presentation. Z.W. synthesized HS 7-mer with 3-O-S. Y.M. and J.L. designed the microarray analysis and synthesized the HS oligosaccharides. J.G. and T.F.R. assisted with NMR analysis. G.L. offered some of the full-length tau protein. F.Z., D.E., G.L., and R.J.L assisted with data analysis and manuscript preparation.

Competing Financial Interests Statement

No competing financial interests.

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