

GLYCOBIOLOGY

Interplay between protein glycosylation pathways in Alzheimer's disease

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Deviations from the normal nucleoplasmic protein O-GlcNAcylation, as well as from normal protein sialylation and N-glycosylation in the secretory pathway, have been reported in Alzheimer's disease (AD). However, the interplay between the cytoplasmic protein O-GlcNAcylation and the secretory N-/O-glycosylation in AD has not been described. We present a comprehensive analysis of the N-, O-, and O-GlcNAc-glycomes in AD-affected brain regions as well as in AD patient serum. We detected marked differences in levels of glycan involved in both protein O-GlcNAcylation and N-/O-glycosylation between patients and healthy individuals and revealed brain region-specific glycosylation-related pathology in patients. These alterations are not general for other neurodegenerative conditions, such as frontotemporal dementia and corticobasal degeneration. The alterations in the AD glycome in the serum could potentially lead to novel glyco-based biomarkers for AD progression. Strikingly, negative interrelationship was found between the pathways of protein O-GlcNAcylation and N-/O-glycosylation, suggesting a novel intracellular cross-talk.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder and is the primary cause of dementia (1, 2). However, no disease-modifying treatment nor reliable biomarkers for early detection are available for AD or for mild cognitive impairment (MCI), which has come to be recognized as an intermediate state of clinical impairment before advanced AD (3, 4). The neuropathological hallmarks of AD are amyloid deposits (plaques) of the amyloid- β precursor protein (APP)-derived A β peptide accompanied by neurofibrillary tangles of the tau protein (5). In addition, a few studies suggest that the brain of AD patients displays an altered profile of protein O-GlcNAcylation, protein sialylation, and protein N-glycosylation (6–9). However, the interplay between the cytoplasmic protein O-GlcNAcylation and the secretory N-/O-glycosylation in AD has not been described.

Protein glycosylation, the enzyme-directed site-specific process that attaches glycans to proteins, is a ubiquitous posttranslational modification that regulates the folding and function of most proteins. The glycan modification serves to stabilize the target proteins, contributes to their high solubility, and plays a major role in almost all cellular processes, including cell signaling, immune recognition, and cell-cell interaction. Most proteins synthesized in the rough endoplasmic reticulum (ER) (that is, membrane or secreted) undergo glycosylation, which involves linkage of distinct saccharides. In this secretory pathway, two main types of glycosylation are known, according to the nature of the linkage between the "core" region of the oligosaccharide and the modified residue in the protein (10): N- and O-glycosylation. N-glycosylation occurs on asparagine residues and takes place in the rough ER and Golgi apparatus, whereas O-glycosylation occurs on serine or threonine residues and takes place mainly in the Golgi apparatus (10). N-glycosylation involves a covalent linkage of a variety of branched sugars, whereas O-glycosylation involves initial attachment of several monosaccharides, including N-acetylgalactosamine (GalNAc), mannose, fucose, and galactose. Additional monosaccharides are added to

the protein side chain one at a time. In many organisms, both N- and O-linked glycans are capped at the terminal position with sialic acids, which are also a form of monosaccharide units (11, 12). Another type of O-glycosylation is the cytoplasmic O-GlcNAcylation, which involves an attachment of N-acetylglucosamine on either serine or threonine residues and is, interestingly, competitive with phosphorylation of the same amino acids (13, 14). Subsequently, it was found that an extracellular pathway of O-GlcNAcylation also exists (15). Because of its broad cellular implications, deviations from normal protein glycosylation have been implicated in various diseases, including AD, diabetes, and cancer (9, 16–21).

Few reports have documented alterations of glycosylation pattern of specific AD-related proteins in the brains of AD patients versus healthy controls. These include tau (22), APP (23), acetylcholinesterase (24), and transferrin (25). A few publications also described vast, global glycosylation-related alterations in the brain of AD patients (6–9, 26–28). Specifically, global O-GlcNAcylation, N-glycosylation, and sialylation of proteins were reported to be altered in brain and cerebrospinal fluid (CSF) of AD patients, respectively (6–9). Several observations regarding the O-GlcNAcylation pathway suggest a causal role for altered O-GlcNAcylation in AD etiology. For example, reducing the extent of cellular O-linked N-acetylglucosamine (O-GlcNAc) removal from tau in tauopathy model mice increased the level of the O-GlcNAc on tau, hindered formation of tau aggregates, and decreased neuronal cell loss (29).

With the aim of examining the interplay between the various protein glycosylation pathways in the AD brain, we present here a comprehensive simultaneous analysis of the AD-related N-, O-, and O-GlcNAc-glycomes in various brain regions affected in AD etiology as well as in the serum of AD and MCI patients. Our results reveal massive alterations of global protein glycosylation in the various pathways in AD patients. In addition, our results suggest that there is brain region-specific glycosylation-related pathology in AD patients, both on cytoplasmic and membrane glycoproteins, and that this is not a general feature common to all neurodegenerative conditions. It appeared that the intracellular alterations in protein glycosylation in AD patients are mirrored in their serum samples. Strikingly, the global glycome analysis enabled us to observe a negative interrelationship between the pathways

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of protein O-GlcNAcylation and N-/O-glycosylation. In addition, we found that serum samples of AD patients have a unique glyco-fingerprint, which could be the basis for developing a novel class of biomarkers for AD progression.

RESULTS

Altered global protein O-GlcNAcylation in the brains of AD patients is region-specific

Global protein O-GlcNAcylation was reported to be decreased in the frontal lobe and cerebellum, and increased in the inferior parietal lobule, of postmortem brains of AD patients compared to those of healthy controls (9, 22, 30, 31). No study of protein O-GlcNAcylation levels in other brain regions nor of the cell membrane or secretome (that is, CSF and serum) has been published to date.

To further examine the alterations in global protein O-GlcNAcylation levels, we examined brain tissues from four regions known to be implicated in AD pathology, namely, frontal cortex, hippocampus, temporal cortex, and parietal cortex of postmortem brain samples of full-blown AD patients and healthy age-matched controls (see Table 2 and fig. S1A). From each region of the brains, we extracted total proteins from both the soluble cellular fraction (supernatant, that is, cytoplasmic) and the membrane (pellet) fraction. To validate the identity of these two purified fractions, we characterized them using a set of antibodies against bona fide cytoplasmic and membrane proteins (fig. S2).

We next evaluated the level of global protein O-GlcNAcylation in the two fractions by enzyme-linked immunosorbent assay (ELISA) using an O- β -GlcNAc-specific monoclonal antibody CTD 110.6. In agreement with previous studies (9, 22, 30), we found that global protein O-GlcNAcylation in the cytoplasmic fraction is markedly decreased (by ca. 47%) in the frontal lobe of full-blown AD patients compared with healthy controls (Fig. 1A). In contrast, significant increase in global protein O-GlcNAcylation level (ca. 60% more) was observed in the cytoplasmic fraction from the hippocampus of AD patients compared to that of healthy controls (Fig. 1A). No comparable significant differences between AD patients and healthy controls were observed in the cytoplasmic fraction from the two other brain regions examined (parietal lobe and temporal lobe).

The membrane fraction of all brain regions examined displayed significantly higher levels of O-GlcNAcylation in AD patients compared to those in healthy controls (Fig. 1B). The largest increase was observed in the hippocampus, with 28% higher protein O-GlcNAcylation levels in the brain of AD patients compared to those in healthy controls.

To assess levels of extracellular protein O-GlcNAcylation, we examined the serum samples of full-blown AD patients, MCI patients, and healthy controls (see Table 3 and fig. S1B). Similar to the results obtained for the membrane fraction, the serum samples of AD patients exhibited slightly, although not statistically significant, higher levels of O-GlcNAcylation compared with healthy controls (ca. 13%; Fig. 1C). MCI patients exhibited statistically significant increase in protein O-GlcNAcylation levels in their serum (by ca. 23%; Fig. 1C), similar to the pattern observed in AD patients. Collectively, the abovementioned results suggest that there is a brain region-specific O-GlcNAcylation-related pathology in AD patients, which is evident in their serum.

A brain region-specific global N-/O-glycosylation-related pathology in AD patients is mirrored in their serum samples

Two recent reports involving a systematic N-glycan profiling of CSF samples from AD patients, MCI patients, and healthy counterparts,

have described a decrease in the overall sialylation levels and an increase in species bearing bisecting GlcNAc in AD (28, 32). No comparable analysis of the O-glycome of AD patients has been described so far. We were prompted to use yet another global approach for glycan analysis, which is not specific for O-GlcNAc but will enable us to measure total glycan load on proteins. To that end, we used quantitative periodic acid-Schiff (PAS) for general staining of glycans (both N- and O-linked) in protein extracts. First, we calibrated it using the glycoproteins mucin (33) and casein (34) as well as with milk, which is enriched in glycoproteins (fig. S3) (35). PAS staining in microtiter plates positively detected, in a dose-dependent manner, the presence of glycans on both mucin and casein as well as in milk (fig. S3A). The negative control bovine serum albumin (BSA) protein, which lacks glycans, was hardly reactive with PAS staining. It is important to note that the PAS staining was

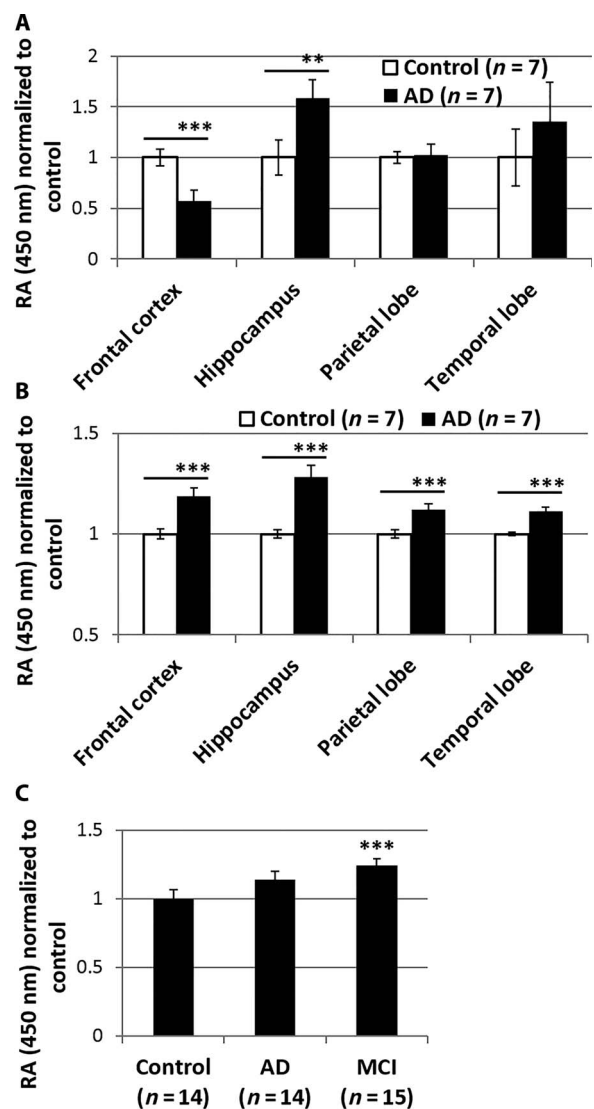


Fig. 1. Alterations of protein O-GlcNAcylation in AD. Protein O-GlcNAcylation levels were measured by ELISA using an O- β -GlcNAc-specific monoclonal antibody (CTD 110.6). Results of soluble cytoplasmic fraction (A) ($n = 7$ per group), membrane fraction (B) ($n = 7$ per group), and serum (C) ($n = 14$ to 15 per group) are presented as ratios, normalized to healthy controls. P values were calculated by Student's t test. ** $P < 0.05$ and *** $P < 0.001$.

found to be reactive in a glycan-dependent manner: Whereas galactose and mannose were highly reactive, glucose was not reactive at all (fig. S3B). We also verified that total protein lysates from either *Drosophila* or murine tissues, which contain a mixture of glycoproteins, are reactive with PAS staining (fig. S3C).

Remarkably, global glycan analysis using PAS staining indicated that full-blown AD patients display significantly higher (2.29-fold) glycan load than the control healthy group in the soluble fraction of the frontal cortex (Fig. 2A). No comparable significant differences were observed in other brain regions examined (Fig. 2A). PAS staining of the membrane fraction, which is enriched with both N- and O-linked glycoproteins, was significantly lower in the parietal and temporal lobes of AD patients relative to the staining of healthy control samples, by 26 and 32%, respectively (Fig. 2B). PAS staining of total proteins from serum samples (that is, secreted

proteins) was significantly weaker in AD and MCI patients (ca. 44 and 49%, respectively), compared to that in healthy controls (Fig. 2C), in accordance with the PAS staining of the membrane fractions (Fig. 2B). This lower PAS reactivity in the serum of AD patients cannot be attributed to altered levels of free glucose in the serum samples, because this sugar, which is highly abundant in the serum, is not reactive in the PAS assay (fig. S3B). These observations therefore suggest that there are lower levels of glycans on glycoproteins in the serum of AD patients as compared to healthy counterparts. It appears that there is a negative interrelationship between O-GlcNAcylation levels and global glycosylation levels (for example, AD patients have higher levels of O-GlcNAcylation and lower levels of total protein glycosylation in their serum compared to healthy counterparts). Collectively, our results imply that there is a brain region-specific global glycosylation-related pathology in AD patients, in addition to specific alterations in protein O-GlcNAcylation levels, which is also observed in their serum samples.

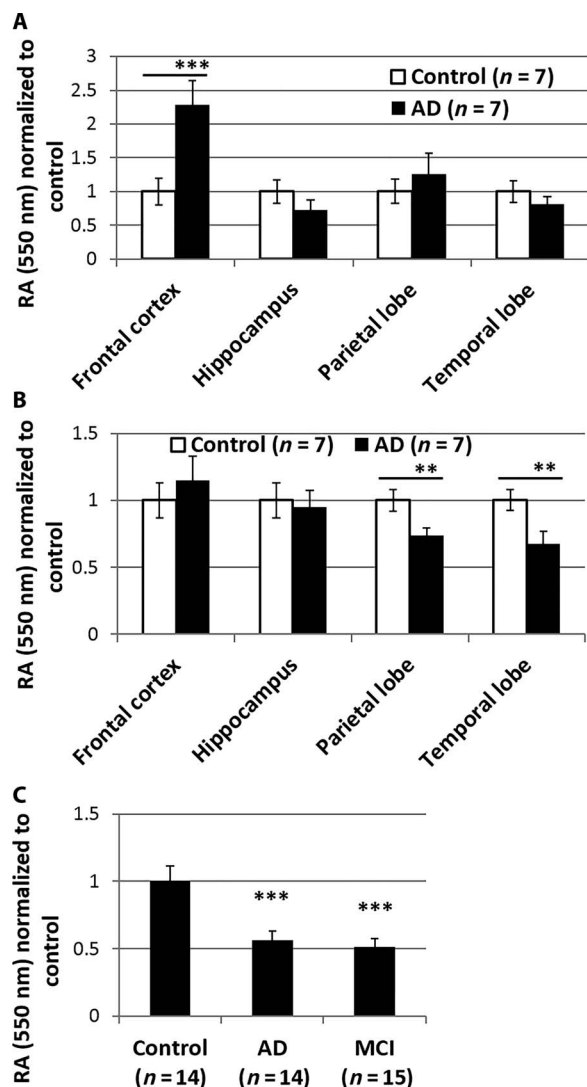


Fig. 2. Global glycosylation alterations in AD. Glycan levels were measured by quantitative PAS staining, which detects both N- and O-glycosylation. Results of the soluble cytoplasmic fraction (A) ($n = 7$ per group), membrane fraction (B) ($n = 7$ per group), and serum (C) ($n = 14$ to 15 per group) are presented as ratios, normalized to healthy controls. P values were calculated by Student's t test. ** $P < 0.05$ and *** $P < 0.001$.

The altered profile of protein glycosylation in AD is not a general feature common to all neurodegenerative conditions

Given the observed robust decrease in protein O-GlcNAcylation (by ca. 47%) and elevation (2.3-fold) in PAS staining of the soluble fraction of frontal cortex tissue of AD patients compared to that in healthy controls (Figs. 1A and 2A, respectively), we wondered whether these alterations are specific for the AD brain or occur in other neurodegenerative conditions as well. To test that, we obtained postmortem frontal cortex tissue samples from patients of various tauopathies, namely, progressive supranuclear palsy (PSP), frontotemporal dementia (FTLD) with parkinsonism (FTDP-tau Pick's disease and FTDP-tau *MAPT*) and corticobasal degeneration (CBD), as well as of additional age-matched controls (Table 4). We extracted their protein soluble fraction and subjected it to ELISA using the CTD 110.6 antibody and to qualitative PAS staining. Only FTDP-tau Pick's patients demonstrated significant altered protein O-GlcNAcylation levels—a decrease by ca. 27% (fig. S4A), similar to the decrease observed in AD patients (Fig. 1A). In addition, whereas no change in PAS signal was observed in PSP patients, a twofold increase was observed in both cases of FTLD (FTDP-tau Pick's and FTDP-tau) and a milder increase in CBD patients (fig. S4B). We therefore conclude that the elevation in glycan load on glycoproteins (as indicated by the PAS staining), accompanied by the decrease in O-GlcNAcylation levels (monitored by ELISA with an anti-O-GlcNAc antibody), of the soluble fraction of frontal cortex tissue of AD patients is not a general feature common to all neurodegenerative conditions, and each disease should be examined separately to better understand its glyco-related etiology.

AD patients have a unique glyco-fingerprint in their serum

If these glycan-specific alterations are also present in the serum of AD patients, they could provide a highly valuable source of novel biomarkers for AD pathology. To initiate a feasibility study along these lines, we used a high-throughput platform for characterizing the glycome fingerprint in a sensitive and high-resolution level using lectin chip microarray (LecChip). Using a LecChip, which contains 45 different lectins, we examined serum protein samples from AD and MCI patients as well as from healthy controls and found that they display unique lectin fingerprints [Fig. 3 and fig. S5 (for the list of glycans)]. Several lectins exhibited an increased signal in the serum of AD or MCI patients versus healthy counterparts, whereas others demonstrated an opposite trend. Specifically, nine lectins (20% of the lectins present on the LecChip) exhibited

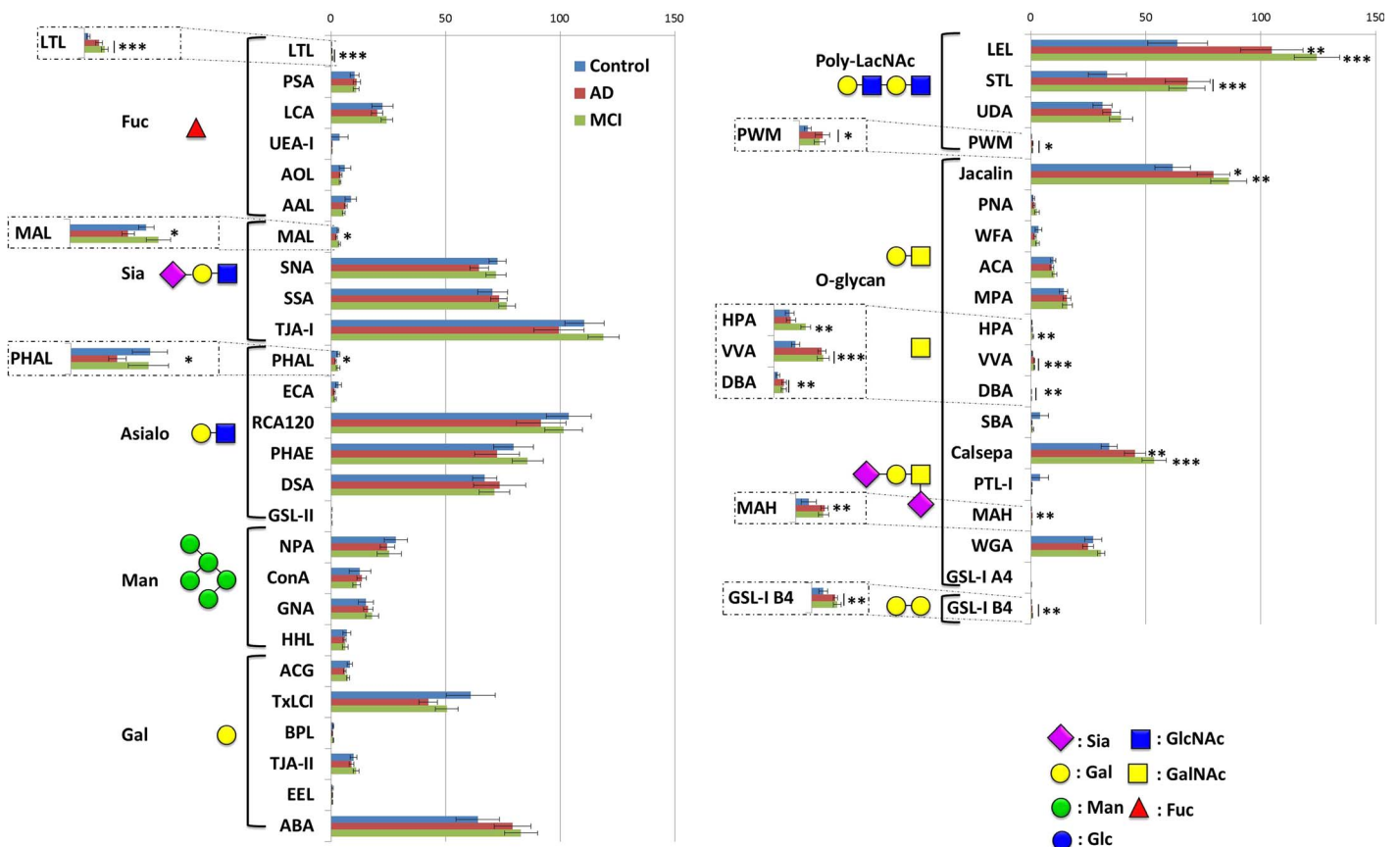


Fig. 3. Lectin array assay of serum samples from AD (red), MCI (green) patients, and healthy controls (blue) (n = 10). P values were calculated by analysis of variance (ANOVA) for normal distribution or by Kruskal-Wallis test for non-normal distribution. *P < 0.1, **P < 0.05, and ***P < 0.001.

Table 1. Interplay between levels of global protein O-GlcNAcylation and N-/O-glycosylation in brain and serum samples of AD patients. cx, cortex; ↓, down-regulation; ↑, up-regulation; –, no significant change; Sup, supernatant (this fraction is enriched with cytoplasmic proteins); Pellet (this fraction is enriched with membrane proteins).

Region	Frontal cx		Hippocampus		Parietal cx		Temporal cx		Serum	
	Sup	Pellet	Sup	Pellet	Sup	Pellet	Sup	Pellet	AD	MCI
O-GlcNAc	↓	↑	↑	↑	–	↑	–	↑	–	↑
N-/O-glycosylation	↑	–	–	–	–	↓	–	↓	↓	↓

a significantly higher signal for AD and MCI compared with healthy controls. These are as follows: fucose (lectin such as LTL), acetyl-lactosamine (lectins such as LEL, STL, and PWN), and GalNAc (lectins such as Jacalin, HPA, VVA, DBA, Calsepa, MAH, and GSL-I B4). The VVA lectin exhibited the most statistically significant altered signal (P < 0.003). In contrast, two lectins exhibited a significantly lower signal in AD serum compared to healthy controls: MAL and PHAL, which bind to sialic acid and asialo motif, respectively.

DISCUSSION

Using complementing glyco-detection approaches, we found that brain samples of AD patients have unique alterations in their protein glycome, in both sugar level and composition. It appeared that the intra-

cellular alterations in protein glycosylation in AD patients are reflected in their serum samples and possibly also in the serum of MCI patients. Moreover, our results imply that there is a brain region-specific glycosylation-related pathology in AD patients, which is not a general feature common to all neurodegenerative conditions. Our analysis of tissue samples of patients from various tauopathies indicated that the changes in protein glycosylation levels observed in AD patients were best correlated with changes in FTDP-tau Pick's patients, that is, both had decreased protein O-GlcNAcylation levels and an increased PAS signal in the soluble fraction of their frontal cortex. We note that glycosylation alterations identified by PAS staining in the soluble membrane fractions of AD patients could be partially attributed to alterations in glycosylation of molecules other than glycoproteins, such as glycolipids. A more comprehensive investigation toward the exact role of this

aberrant protein glycosylation is required for a detailed understanding of AD etiology and its progression and possibly of other tauopathies.

Previous studies reported that protein O-GlcNAcylation is altered in the AD brain. It was based on analyses of three brain regions only (frontal cortex, inferior parietal lobule, and cerebellum of AD patients) (9, 22, 30). Here, we observed a marked increase in protein O-GlcNAcylation levels in the hippocampus of AD patients (Fig. 1A). We observed brain-region specificity also when analyzing PAS staining from various brain regions of AD patients (Fig. 2, A and B). Being a complex disorder, involving lesions in various brain regions, we maintain that many aspects of AD pathology should be studied separately in a range of brain regions to gain more comprehensive insights into its etiology. This would likely be true for other complex brain disorders, such as tauopathies. The region-specific glycosylation profiles might be due to a variety of reasons, such as region-specific alterations in an expression profile of glycosylation-related genes or of glucose transporters (which will affect the intracellular availability of the sugar precursors for protein glycosylation) and tissue-specific alterations in glucose efflux. It would be interesting to further investigate the differences found between glycosylation levels on cytoplasmic and membrane proteins to better elucidate the underlying mechanisms. It appears that changes in glycosylation levels of membrane proteins, but not of cytoplasmic proteins, were better reflected in glycosylation levels of serum glycoproteins of AD patients. Specifically, it appears that changes in glycosylation levels of membrane proteins in the parietal and temporal lobes were best correlated with changes observed in glycosylation levels of serum glycoproteins of AD patients. It will be also intriguing to examine whether peripheral organs exhibit altered protein glycosylation in AD patients,

which might also contribute to the altered protein glycosylation pattern found in the serum of these patients.

The negative interrelationship that we observed between the levels of glycans belonging to the pathways of protein O-GlcNAcylation and N-/O-glycosylation is of particular interest (summarized in Table 1). For instance, AD patients have lower levels of protein O-GlcNAcylation and higher levels of global protein glycosylation in the cytoplasmic fraction of their frontal cortex compared to healthy counterparts. It appeared that this interplay exists both in the soluble cytoplasmic fractions as well as in the membrane fractions and serum samples of AD and MCI patients (supernatant and pellet, respectively, in Table 1), although the decrease in protein O-GlcNAcylation levels in the serum of AD patients was not statistically significant ($P = 0.114$). It would be intriguing to examine whether a similar negative interrelationship between the protein glycosylation pathways exists in other neurodegenerative diseases.

The mechanism generating this negative interrelationship is unknown. Ngoh *et al.* (36) reported that ER stress up-regulates O-GlcNAc signaling, which in turn results in cardioprotection. Therefore, they suggested that the up-regulation of O-GlcNAcylation might be a protective cellular response aimed at reducing ER stress-induced cell death. Along these lines, we propose that following ER stress and ER-associated protein degradation (ERAD), reported to occur in AD patients (37), the unfolded protein response machinery up-regulates the O-GlcNAcylation pathway. In this context, the decrease in overall N-/O-glycosylation levels seen in the membrane fractions from parietal and temporal lobes (Table 1), as well as in the serum, of AD patients might be a direct result of the ER stress that occurs in their brain.

Table 2. Human brain tissue of AD and control cases used in this study. PMD, postmortem delay; N/A, not applicable.

Case number	Gender	PMD (hours)	Age at death (years)	Braak stage	Brain bank identifier number
Con 1	F	22	80	II	BBN_20040
Con 2	M	53	84	N/A	BBN_18816
Con 3	M	45	90	N/A	BBN_14408
Con 4	M	26	65	I	BBN_10992
Con 5	M	25	67	I	BBN_10208
Con 6	M	23	63	N/A	BBN_10209
Con 7	F	9	92	II	BBN_10250
Mean ± SD		29 ± 14.9	77.28 ± 12.2		
AD 1	F	42	78	VI	BBN_19593
AD 2	F	20	74	VI	BBN_18818
AD 3	M	33	88	V	BBN_16191
AD 4	M	27	76	VI	BBN_15597
AD 5	M	22	67	VI	BBN_16202
AD 6	F	15	70	V–VI	BBN_15201
AD 7	M	24	78	V	BBN_11075
Mean ± SD		26.14 ± 9.0	75.86 ± 6.7		

Table 3. Human serum samples of AD, MCI, and control cases used in this study.

Case number	Gender	PMD (hours)	Age at death (years)	Braak stage	Amyloid*	NBB identifier number
Con 1	F	3	77	1	B	2009-022
Con 2	F	6.5	78	1	A	2000-032
Con 3	M	7	78	1	A	2000-049
Con 4	F	4.5	81	1	O	2011-028
Con 5	F	4.5	78	2	A	2012-059
Con 6	F	5.5	79	2	B	1999-052
Con 7	M	6	79	2	A	2012-070
Con 8	M	8	81	2	O	2007-082
Con 9	M	13.5	82	2	A	2000-030
Con 10	M	10	82	4	B	2003-084
Con 11	F	4.75	78	1	A	2001-028
Con 12	M	5.75	83	1	B	2011-017
Con 13	F	7.25	76	2	O	2011-072
Con 14	M	6.5	79	2	A	2012-104
Mean ± SD		6.85 ± 3.1	79.2 ± 1.8			
AD 1	M	8	74	5	C	2011-115
AD 2	M	5	79	5	C	2001-092
AD 3	F	4	79	5	C	2002-085
AD 4	M	4.5	85	5	C	2001-044
AD 5	M	5	85	5	C	2001-063
AD 6	F	5.5	73	6	C	2011-053
AD 7	F	5	78	6	C	1998-142
AD 8	M	10	78	6	C	2000-001
AD 9	F	5	81	6	C	2007-059
AD 10	F	5.5	82	6	C	2010-054
AD 11	F	5.5	76	6	C	1999-140
AD 12	M	4.5	80	6	C	2000-066
AD 13	M	4.5	77	5	C	2007-078
AD 14	M	6.25	86	5	C	2010-016
Mean ± SD		6.9 ± 3.3	79.6 ± 4.2			
MCI 1	M	10	72	1	B	2001-075
MCI 2	M	5	79	1	B	2006-033
MCI 3	F	4	82	1	B	2001-131
MCI 4	F	7	82	1	A	2008-104
MCI 5	F	6	76	2	O	2005-058
MCI 6	M	4.5	79	2	A	2008-048

continued on next page

Case number	Gender	PMD (hours)	Age at death (years)	Braak stage	Amyloid*	NBB identifier number
MCI 7	M	6	70	3	B	2013-030
MCI 8	F	4	76	3	B	2008-015
MCI 9	M	5	82	3	B	2008-016
MCI 10	F	5	83	3	B	2007-039
MCI 11	F	5.5	83	3	B	2007-061
MCI 12	F	5.5	85	3	C	1999-074
MCI 13	F	4.5	85	2	C	2001-061
MCI 14	F	4.5	89	1	C	2006-053
MCI 15	M	6.5	79	3	A	2009-064
Mean ± SD		5.5 ± 1	80.1 ± 4.6			

*Amyloid stage (59): stage A, amyloid deposits mainly found in the basal portions of the frontal, temporal, and occipital lobes; stage B, all isocortical association areas affected, whereas the hippocampal formation is only mildly involved, and the primary sensory, motor, and visual cortices are devoid of amyloid; and stage C, deposition of amyloid in these primary isocortical areas and, in some cases, the appearance of amyloid deposits in the molecular layer of the cerebellum and subcortical nuclei, such as striatum, thalamus, hypothalamus, subthalamic nucleus, and red nucleus.

Another mechanism that could account for the negative interrelationship between *O*-GlcNAcylation and *N*-/*O*-glycosylation pathways could be the GlcNAc moiety itself, which is common to both pathways. The brain of AD patients exhibits lower uptake of glucose into the cells due to insulin resistance and decreased levels of various glucose transporters (38, 39). Therefore, the intracellular availability of GlcNAc, which is derived from glucose through the hexosamine biosynthesis pathway, is limited in AD patients (22). The limited availability of GlcNAc, which is shared as a substrate by different glycosylation pathways, may result in their competition for this resource.

LecChip provided us with a simultaneous identification of a wide range of glycans as analytes in the serum of AD patients, requiring minimal sample consumption, as a glyco-biomarker screening platform. Broad unbiased approaches for glyco-based biomarker discovery, such as lectin arrays and high-throughput mass spectrometry, increasingly replace the traditional methods, which focused on glycosylation pattern of specific proteins (40–42). For instance, a high-throughput glycomic approach has been previously suggested for diagnosis of various types of cancer (43–46). Along these lines, the methods used in the present study enabled us to reveal new general insights, which would not have been discovered by looking only at specific proteins (for example, the negative interrelationship between protein *O*-GlcNAcylation and total protein *N*-/*O*-glycosylation).

Biologically available biomarkers from body fluids are especially sought after because they are easily accessible and can provide information regarding the underlying biochemical processes that occur in the brain. Currently, only three such biomarkers are included in clinical trials: CSF levels of Aβ42, total tau, and phosphorylated tau (47). It is likely that only a combined analysis of several biomarkers will define a patient-specific signature for AD diagnosis (48, 49). We believe that the alterations at the glycome levels of AD and MCI patients could potentially constitute a fingerprint biomarker for AD progression and lead to early diagnosis of AD using a simple blood test if further validated. For example, the lectin array analysis indicated that there is an increased reactivity of the VVA lectin, which binds specifically to the Tn antigen, in the serum samples of AD patients. This antigen is usually absent from the cell surfaces of normal cells and

was reported to be found on cancer cells (50). It is currently used as a serum biomarker in patients with carcinomas (breast, colon, and ovary) (51). Several mechanisms have been proposed to explain the increase in Tn antigen levels on cell surface in cancer patients, including dysregulation of glycosyltransferases involved in the initial steps of the *O*-glycosylation pathway and dysregulation of apomucin expression (52). These might also account for the observed increased levels of the Tn antigen in the serum of AD patients.

Because protein glycosylation is a ubiquitous posttranslational modification that regulates the folding and function of most proteins (53–58), deviations from normal protein glycosylation are expected to have severe implications. A causal relation between alterations in protein glycosylation and AD pathology is yet to be demonstrated. If this causal relation exists, alterations in protein glycosylation could lead to the development of novel treatment for AD, in addition to offering a new avenue for serum-based biomarkers of disease progression.

MATERIALS AND METHODS

Human brain tissue

Frozen autopsied frontal cerebral cortices, temporal cerebral cortices, parietal cerebral cortices, and hippocampal tissues from seven AD and seven controls were provided by the King's College London (KCL) Brain Bank (London, UK). The age, gender, postmortem delay, and Braak stages of these samples are listed in Table 2.

Serum samples of 10 AD patients, MCI patients, and controls were provided by the Netherlands Brain Bank (NBB). The age, gender, postmortem delay, Braak stages, and amyloid score of these samples are listed in Table 3.

The number of samples per group ($n = 14$ to 15 in the case of serum samples and $n = 7$ in the case of brain lysates) was calculated according to Mead's resource equation.

Frozen autopsied frontal cerebral cortices from 10 CBD cases, 5 PSP cases, 8 FTLD-tau Picks cases, 9 FTLD-tau MAPT exon 10 +16 mutation cases, and 8 controls were provided by the Manchester Brain Bank (Manchester, UK). The age, gender, and postmortem delay of these samples are listed in Table 4.

Table 4. Human frontal cortex samples of tauopathy and control cases used in this study.

Case number	Gender	PMD (hours)	Age at death (years)	MRC identifier number
Con 1	F	48	77	BBN_3378
Con 2	M	144	78	BBN_5761
Con 3	F	41	81	BBN_3447
Con 4	F	39	87	BBN_3454
Con 5	F	92.5	53	BBN_6067
Con 6	M	92	84	BBN_6071
Con 7	M	98	85	BBN_20005
Con 8	M	69.5	84	BBN_20006
Mean ± SD			78.6 ± 10.91	
CBD 1	M	N/A	75	BBN_3326
CBD 2	F	20	77	BBN_3335
CBD 3	M	N/A	65	BBN_3346
CBD 4	M	48	73	BBN_3381
CBD 5	M	40.5	70	BBN_3427
CBD 6	M	68.5	80	BBN_3431
CBD 7	M	53	90	BBN_3450
CBD 8	M	28	79	BBN_3474
CBD 9	M	119	64	BBN_6077
CBD 10	F	81	71	BBN_6082
Mean ± SD			74.4 ± 7.69	
PSP 1	F	N/A	N/A	BBN_3065
PSP 2	F	N/A	60	BBN_3083
PSP 3	M	25	76	BBN_3462
PSP 4	M	50.5	80	BBN_10262
PSP 5	F	43.5	80	BBN_21011
Mean ± SD			74 ± 9.52	
FTLD-tau Picks 1	F	N/A	60	BBN_3041
FTLD-tau Picks 2	M	N/A	56	BBN_3053
FTLD-tau Picks 3	F	N/A	84	BBN_3100
FTLD-tau Picks 4	F	N/A	58	BBN_3182
FTLD-tau Picks 5	M	N/A	77	BBN_3222
FTLD-tau Picks 6	M	N/A	69	BBN_3288
FTLD-tau Picks 7	M	102	75	BBN_3433
FTLD-tau Picks 8	M	125	69	BBN_6069

continued on next column

Case number	Gender	PMD (hours)	Age at death (years)	MRC identifier number
Mean ± SD			68.5 ± 9.96	
FTLD-tau MAPT 1	F	N/A	58	BBN_5696
FTLD-tau MAPT 2	M	N/A	55	BBN_5699
FTLD-tau MAPT 3	M	N/A	70	BBN_5710
FTLD-tau MAPT 4	F	N/A	65	BBN_5717
FTLD-tau MAPT 5	M	N/A	53	BBN_5733
FTLD-tau MAPT 6	F	36	60	BBN_5744
FTLD-tau MAPT 7	F	96	63	BBN_5760
FTLD-tau MAPT 8	F	48	58	BBN_5763
FTLD-tau MAPT 9	F	53	63	BBN_6081
Mean ± SD			60.5 ± 5.27	

The brain tissue was homogenized using Bullet Blender (Next Advance Inc.) after several washes with phosphate-buffered saline (PBS) in ice-cold homogenization buffer, consisting of 50 mM tris-HCl (pH 7.4), 8.5% sucrose, 2.0 mM EDTA, 100 mM GlcNAc, and cOmplete Protease Inhibitor Cocktail (Roche Molecular Biochemicals GmbH). The homogenates were centrifuged at 14,000 rpm for 20 min at 4°C, and the resulting supernatants were used for further analyses. The pellet fraction was later used for extraction of membrane proteins.

For preparation of protein lysate from the membrane fraction, the pellet was washed twice by resuspension in Tris-EDTA (TE) buffer supplemented with cOmplete Protease Inhibitor Cocktail and centrifuged at 40,000g for 10 min at 4°C. The pellet was then resuspended in TE buffer supplemented with 2% Triton X-100 for 30 min on ice for extraction of membrane proteins. The homogenates were centrifuged at 100,000g for 30 min at 4°C, and the resulting supernatants were used for further analyses of the membrane fraction. The use of the human brain tissue and serum samples was in accordance with all relevant Codes of Practice and Standards of the Human Tissue Authority and approved by our institutional review board.

SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis

Before electrophoresis, protein samples were brought to identical protein concentrations, and Laemmli protein sample buffer was added. SDS-polyacrylamide gel electrophoresis was performed in a GeBARunner apparatus (GeBA). The proteins were separated in 4 to 20% (w/v) polyacrylamide GeBaGels under reducing conditions and were transferred to a polyvinylidene difluoride membrane using a dry blot technique (iBlot, Life Technologies). The membranes were subjected to Western blot analysis.

Western blot analysis

The presence of O-GlcNAc on proteins was detected by Western blotting using a pan-GlcNAc mouse monoclonal antibody (CTD 110.6, Covance). For characterization of cytoplasmic and membrane fractions, we used a mouse monoclonal anti- β -actin antibody (ab8224, Abcam), a rabbit polyclonal anti-calnexin antibody (sc-11397, Santa Cruz Biotechnology), and a mouse monoclonal anti-Na(+)/K(+) adenosine triphosphatase α -1 subunit antibody (a6F, Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Briefly, the membrane was blocked for 1 hour using 3% BSA or 5% milk diluted in tris-buffered saline with Tween 20 (TBST) (0.1% Tween) while shaking. Antibodies, diluted 1:1000 in 3% BSA or 5% milk in TBST, were added to the membrane overnight, followed by several washes with TBST. Next, the membrane was incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature while shaking. Blots were developed after thorough TBST washes using an enhanced chemiluminescence system (EZ-ECL, Biological Industries) according to the manufacturer's manual.

Quantitative PAS staining

Before the experiment, protein samples were adjusted to identical protein concentrations [measurement was performed using bicinchoninic acid (BCA) reagent, PIR-2325, Pierce]. One hundred twenty microliters of 0.06% periodic acid solution (375810, Sigma-Aldrich) in freshly made 7% acetic acid was added to 25 μ l of protein lysate solution or serum (0.5 to 2 mg/ml) in each well of a 96-well plate. The microtiter plate was covered with a plastic seal and incubated at 37°C for 1.5 hours without shaking. The plate was allowed to cool to room temperature before 100 μ l of Schiff's reagent was added to each well (395-2, Sigma-Aldrich). The microtiter plate was covered again with a plastic seal and was shaken for 5 min. Color was allowed to develop at room temperature for an additional 30 min before absorbance was recorded at 550 nm. Experiments were performed in triplicate.

ELISA for measuring O-GlcNAcylation levels

Before the experiment, protein samples were adjusted to identical protein concentrations (measurement was performed by BCA reagent, PIR-2325, Pierce). ELISA 96-well plates were coated with 100 μ l of protein lysate solution or serum (50 μ g/ml). Plates were sealed and incubated overnight at 4°C. Then, plates were washed once with PBS and blocked with 300 μ l of 3% BSA in PBS overnight at 4°C. The next day, the plates were washed three times with PBS with Tween 20 (PBST) (0.05% Tween 20 in PBS) and 200 μ l of CTD 110.6 antibody were added (1:200 dilution in blocking solution) for overnight incubation at 4°C. Next, 200 μ l of secondary HRP-conjugated goat anti-mouse immunoglobulin M (IGM) antibody (SC-2064, Santa Cruz Biotechnology) were added at room temperature for 1 hour. Thereafter, plates were washed four times with PBST and developed with trimethylboron (TMB) as a substrate (100 μ l per well) (T0440, Sigma-Aldrich). The reaction was stopped by addition of 1.25 M H₂SO₄ (50 μ l per well), and absorbance was recorded at 450 nm. Experiments were performed in triplicate.

Labeling of proteins with Cy3

For analysis in the lectin chip microarray (LecChip), serum samples were first brought to identical protein concentrations (measurement was performed by BCA reagent, PIR-2325, Pierce) in PBS to obtain a 0.05-mg/ml solution. Cy3 NHS ester reagent (PA13101, GE Healthcare Life Sciences), dissolved in dimethyl sulfoxide, was added (final concentration, 0.05 mg/ml) for 1 hour at room temperature. Excess reactive reagent was blocked by inactivation with free lysine (final concentration, 0.05 mM; L5501, Sigma-Aldrich).

Lectin chip microarray

LecChip (GlycoTechnica Ltd.) was washed three times with Probing Solution (provided by the manufacturer), and Cy3-labeled serum samples (1 μ g/ml) ($n = 10$ per group) were added to the wells in Probing Solution (100 μ l per well). Samples were incubated overnight at 18°C, and the LecChip was washed with PBS and double-distilled water for 30 min each. The LecChips were scanned with a GenePix Professional 4200A Microarray scanner (Molecular Devices). Results were analyzed with Phoretix Array Professional software (BioGene).

Statistics

Student's t test, ANOVA, and the Kruskal-Wallis test were used to evaluate statistical significance of the observed differences. t test was used for statistical analysis of differences in PAS staining and O-GlcNAcylation levels between AD patients and healthy subjects in the various brain regions, whereas for analysis of differences in the serum, we used ANOVA. LecChip analysis was performed by examining the parameters' distribution using the Kolmogorov-Smirnov test. In the case of a normal distribution, ANOVA was used, whereas the Kruskal-Wallis test (nonparametric) was used for all other cases. Adjustment for multiple comparisons was performed using Bonferroni.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/3/9/e1601576/DC1>

fig. S1. Age distribution box plot of donors of sample of brains and serum.

fig. S2. Verification of the identity of the cytoplasmic and membrane fractions of two AD patients and two healthy counterparts.

fig. S3. Calibration of quantitative PAS staining.

fig. S4. Alterations of glycosylation in the soluble cytoplasmic fraction of the frontal cortex of tauopathy patients.

fig. S5. List and structure of glycans depicted in the LecChip.

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