Published in final edited form as:

J Pathol. 2011 September ; 225(1): 54–62. doi:10.1002/path.2912.

Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes

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Abstract

Brain glucose metabolism is impaired in Alzheimer's disease (AD), the most common form of dementia. Type 2 diabetes mellitus (T2DM) is reported to increase the risk for dementia, including AD, but the underlying mechanism is not understood. Here, we investigated the brain insulin–PI3K–AKT signalling pathway in the autopsied frontal cortices from nine AD, 10 T2DM, eight T2DM–AD and seven control cases. We found decreases in the levels and activities of several components of the insulin–PI3K–AKT signalling pathway in AD and T2DM cases. The deficiency of insulin–PI3K–AKT signalling was more severe in individuals with both T2DM and AD (T2DM–AD). This decrease in insulin–PI3K–AKT signalling could lead to activation of glycogen synthase kinase-3 β , the major tau kinase. The levels and the activation of the insulin–PI3K–AKT signalling components correlated negatively with the level of tau phosphorylation and positively with protein *O*-GlcNAcylation, suggesting that impaired insulin–PI3K–AKT signalling might contribute to neurodegeneration in AD through down-regulation of *O*-GlcNAcylation and the consequent promotion of abnormal tau hyperphosphorylation and neurodegeneration. The decrease in brain insulin–PI3K–AKT signalling also correlated with the activation of calpain I in the brain, suggesting that the decrease might be caused by calpain over-activation. Our findings provide novel insight into the molecular mechanism by which type 2 diabetes mellitus increases the risk for developing cognitive impairment and dementia in Alzheimer's disease.

Keywords

Alzheimer's disease; diabetes; insulin; PI3K; AKT; GSK-3 β ; calpain; β -arrestin-2

Introduction

Alzheimer's disease (AD) is the most common cause of dementia and results from age-associated, progressive, chronic neurodegeneration. Abnormal hyperphosphorylation and

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No conflicts of interest were declared.

Author contributions

YL designed and carried out experiments and wrote the draft of the manuscript; FL, IGI and KI were involved in analyses and interpretation of data and manuscript writing; CXG conceived the study, helped in experiment design, analysed and interpreted data and finalized the manuscript.

aggregation of tau protein, which forms neurofibrillary tangles (NFTs) in the AD brain, have been demonstrated to play a crucial role in Alzheimer's neurodegeneration [1,2]. The aetiology of sporadic AD is unknown, but ageing is the most important risk factor. Epidemiological studies have demonstrated that type 2 diabetes mellitus (T2DM), an age-associated chronic metabolic syndrome characterized by peripheral insulin resistance, is a risk factor for developing cognitive impairment and dementia, including AD [3,4]. However, several pathological studies failed to demonstrate an increase in Alzheimer's amyloid plaques and NFTs in the brains of AD patients with T2DM as compared to AD patients without T2DM [5]. Little is known about the mechanistic link between T2DM and AD.

We have previously shown that tau protein is modified by *O*-GlcNAcylation, a modification of nucleocytoplasmic proteins by β -*N*-acetyl-glucosamine (GlcNAc), and that this modification regulates phosphorylation of tau inversely [6]. Decreased brain glucose metabolism, which occurs in the AD brain [7,8], leads to down-regulation of tau *O*-GlcNAcylation and, consequently, hyperphosphorylation of tau [9–13]. Recent studies suggest that abnormal hyperphosphorylation of tau, instead of NFTs *per se*, promotes or leads to neurodegeneration in AD [2]. Thus, we hypothesize that decreased brain glucose metabolism contributes to neurodegeneration by facilitating abnormal hyperphosphorylation of tau via down-regulation of tau *O*-GlcNAcylation in AD [12].

Decreased glucose metabolism is known to precede the emergence of brain pathology and cognitive impairment in AD, but its initial causes are not well understood. In the periphery, glucose metabolism is regulated mainly by insulin signalling. It was previously thought that insulin did not play any significant regulatory role in the brain. However, recent studies have demonstrated that insulin not only regulates glucose and lipid metabolism in the brain, but also regulates neural development and neuronal activities and plays an important role in learning and memory [14,15]. Both insulin and insulin receptor (IR) are found in the brain, and IR is highly expressed in brain neurons [16,17]. Injection of streptozotocin into the lateral ventricle of rodent brain leads to brain insulin resistance [13,18,19] as well as decreased *O*-GlcNAcylation and increased phosphorylation of tau [13], suggesting that brain insulin resistance could result in decreased brain glucose metabolism, decreased tau *O*-GlcNAcylation and neurofibrillary degeneration.

Insulin signalling is initiated by the binding of insulin to its receptor, located in the cytoplasmic membrane (Figure 1A). This binding leads to rapid autophosphorylation and activation of the tyrosine kinase activity of IR, which recruits and phosphorylates different substrates, such as insulin receptor substrate-1 (IRS-1). Tyrosine-phosphorylated IRS-1 then displays binding sites for various downstream signalling partners, of which PI3K is the major one. Activation of PI3K by phosphorylation at the tyrosine residues of its regulatory subunit p85 leads to activation of the downstream kinase, 3-phosphoinositide-dependent protein kinase-1 (PDK1), by phosphorylating PDK1 at Ser241. Activated PDK1 then activates AKT by phosphorylating it at Thr308. Full activation of AKT also requires its phosphorylation at Ser473. A major target of AKT is GSK-3. The activity of GSK-3 is inhibited when it is phosphorylated at Ser21 of GSK-3 α or Ser9 of GSK-3 β by AKT, resulting in glycogen synthesis. GSK-3 β is also a major tau kinase [20–22]. Therefore,

down-regulation of insulin signalling could ultimately lead to both decreased glucose metabolism and increased tau phosphorylation through GSK-3 activation.

To learn whether decreased brain glucose metabolism is attributed by insulin resistance in AD brain, and whether insulin resistance also occurs in the brains of individuals with T2DM, we investigated the brain insulin-PI3K-AKT signalling pathway in AD and in T2DM. We found that the level and the activity of the brain insulin-PI3K-AKT signalling pathway were decreased in both AD and T2DM. The decrease of brain insulin signalling correlated to the hyperphosphorylation of tau and to the decrease in its *O*-GlcNAcylation. The decrease in the brain insulin-PI3K-AKT signalling pathway in AD and T2DM might result from increased degradation of the signalling components due to over-activated calpain I.

Materials and methods

Human brain tissue

Autopsied human brain tissue (frontal cortex; Table 1) was obtained from the Sun Health Research Institute Donation Program (Sun City, AZ, USA). All brain samples were confirmed pathologically and stored at -70°C until use. The use of the tissue was in accordance with the National Institutes of Health guidelines and was approved by our institutional review board.

Antibodies

The primary antibodies used in this study included monoclonal anti-IR β , polyclonal anti-phospho-PI3K (p85 α , Y580) and polyclonal anti- β -arrestin-2 from Abcam (Cambridge, MA, USA); polyclonal anti-IRS-1, polyclonal anti-PI3K (p85), polyclonal anti-PDK1, polyclonal anti-phospho-PDK1 (Ser241), polyclonal anti-AKT, polyclonal anti-phospho-AKT(Ser473) and polyclonal anti-phospho-GSK-3 β (Ser9) from Cell Signaling Technology (Danvers, MA, USA); polyclonal anti-calpain I and monoclonal anti-*O*-GlcNAc (clone RL2) from Affinity Bioreagents (Golden, CO, USA); polyclonal phosphorylation-dependent and site-specific tau antibodies from BioSource International (Camarillo, CA, USA); and polyclonal anti-GSK-3 β from our laboratory [23]. The secondary antibodies peroxidase-conjugated goat anti-rabbit, goat anti-mouse or rabbit anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Western blots

The frontal cortices were homogenized at 4°C in cold buffer containing 50 mM Tris-HCl, pH 7.4, 2.0 mM EDTA, 10 mM β -mercaptoethanol and 8.5% sucrose. The homogenates were centrifuged at $15000 \times g$ for 10 min, and the resulting supernatants (extracts) were assayed for protein concentrations by a modified Lowry method [24]. Western blots of the extracts were carried out using 10% or 7.5% SDS-PAGE and the blots were developed by using an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA). Densitometrical quantification of protein bands in blots was accomplished by using the TINA program (Rayrest IsotopenmeBgerate GmbH, Strau-Benhardt, Germany).

Correlation and statistical analysis

Comparison of means among groups was analysed by one-way ANOVA, using Statistica 6.0 (StatSoft, Tulsa, OK, USA). Pearson correlation analyses were carried out using the same software.

Results

Insulin signalling is impaired in the brains of individuals with AD and T2DM

To investigate whether the brain insulin signalling pathway is altered in AD and T2DM brains, we determined the level and activation status of each component of the insulin signalling pathway in the brain by quantitative western blots. The activation status was estimated by determining the level of phosphorylation, which determines the enzymatic activity, with the phosphorylation-dependent antibodies. We found that, in comparison to the age-matched control brains, the levels and the activation of most insulin signalling pathway components were decreased in both AD and T2DM brains (Figure 1B, C). In the majority of cases, the decrease was greater in T2DM brain than in AD brain, and the decrease was the greatest in the brains of individuals who had both AD and T2DM (T2DM-AD). These results indicate a generally more severe impairment of the brain insulin signalling pathway in T2DM than in AD.

AKT phosphorylated at Thr308, the major site phosphorylated by PDK1, was not detectable in human brain extracts with any anti-phospho-AKT(Thr308) antibodies we tested. Thus, the phosphorylated AKT shown in this study was that phosphorylated at Ser473, which is the major site phosphorylated by mTORC2 complex. Full Akt activation is achieved by the phosphorylation at both Thr308 and Ser473.

Impairment of insulin signalling appears to contribute to hyperphosphorylation of tau in the human brain

We recently demonstrated the hyperphosphorylation of tau in T2DM as well as AD brain [11]. To learn whether the decreased insulin signalling in AD and T2DM brain contributes to tau hyperphosphorylation, we carried out correlation analyses between the levels of site-specific tau phosphorylation and the levels as well as the activation (represented by the level of activated/phosphorylated kinases, except for GSK-3 β , the phosphorylated form of which represents the inactive GSK-3 β) of the insulin signalling pathway components. We observed a negative correlation between tau phosphorylation and the levels as well as the activation of the insulin signalling components (Table 2), although the negative correlation reached statistical significance only in some of these pairs.

Correlation analyses between the density of NFTs and the levels as well as the activation of the insulin signalling pathway components also yielded similar negative correlations, and some of them reached statistical significance (Table 2). These results support a role of decreased brain insulin signalling in abnormal hyperphosphorylation of tau and neurofibrillary degeneration in AD brain and T2DM brain.

Impairment of insulin signalling correlates to protein O-GlcNAcylation in the brain

Insulin signalling regulates glucose metabolism, which in turn regulates protein O-GlcNAcylation. Previously, we reported that decreased brain glucose metabolism contributes to neurodegeneration through decreased O-GlcNAcylation and, consequently, to hyperphosphorylation of tau [6,9,10,12]. A decreased level of O-GlcNAcylation is also seen in AD and T2DM brains [11]. To learn whether the decreased insulin signalling in AD and T2DM brains contributes to tau hyperphosphorylation also via down-regulation of O-GlcNAcylation in human brains, we carried out correlation analyses between the level of O-GlcNAcylation and the levels as well as the activation of the insulin signalling pathway components. We observed a significant positive correlation between O-GlcNAcylation and PDK1 as well as AKT (Table 3). Positive correlations were also apparent for other insulin signalling pathway components, but they did not reach statistical significance. Considering the large individual variations in human brain samples and the relative small sample size of this study, our observation suggests that the impaired insulin signalling might contribute to hyperphosphorylation of tau through down-regulation of O-GlcNAcylation in the human brain.

Impairment of insulin signalling may result from calpain over-activation in AD and T2DM brain

The overall similar sizes of the decreases in the levels of the insulin signalling pathway components and their activation/phosphorylation (Figure 1C) suggest that the decreases might result from increased degradation/turnover, rather than selectively decreased biosynthesis or decreased activation of the signalling in AD and T2DM brains. Previously we demonstrated that calpain I, a calcium-activated cysteine protease enriched in the brain, is over-activated in AD brain as the result of disturbance of calcium homeostasis [25], and this over-activation leads to proteolysis of calcineurin [25] and cAMP-dependent protein kinase [26]. To test whether the decreases in the insulin signalling pathway components could have resulted from calpain I over-activation in AD and T2DM brains, we determined the calpain I activation and performed correlation analyses between calpain activation and the levels of the insulin signalling pathway components.

Calpain is activated by autoproteolysis of its inhibitory domain from the inactive full-length (80 kDa) into the active, truncated form (76 and 78 kDa) [27,28]. We determined the activation of calpain I by quantification of the active/truncated form over the inactive/full-length form by western blots. We found that calpain I was over-activated in T2DM brain, although the over-activation was not as severe as in AD brain (Figure 2A, 2B). In the brains of individuals with both AD and T2DM, calpain I over-activation was most severe, with the full-length, inactive form of the enzyme barely detectable.

Correlation analyses indicated that the levels of the insulin signalling pathway components were negatively correlated with calpain activation, and the majority of the correlations reached statistical significance (Figure 2C). These results suggest that the decreases in the levels of the insulin signalling pathway components might result from increased degradation/turnover by over-activated calpain I in AD and T2DM brains.

β -arrestin-2 level is decreased in T2DM brain but not in AD brain

During the course of this study, Luan *et al* [29] reported that β -arrestin-2 plays an important role in regulating insulin signalling and is decreased in liver and muscle tissue of mouse models of T2DM. Therefore, we investigated whether β -arrestin-2 is also altered in AD and T2DM brains. We found that the level of β -arrestin-2 was indeed decreased significantly in T2DM brains but not in AD brains (Figure 3). The mean level of β -arrestin-2 was actually found to be higher in AD brains than in control brains, but the increase did not reach statistical significance. These results suggest that insulin resistance in AD brain and in T2DM brain might partially result from different mechanisms.

Discussion

Recent studies have indicated that insulin signalling regulates glucose metabolism in the brain, plays important roles in neural development and neuronal activities and affects learning and memory [14]. Neurons themselves express insulin [30,31], but the majority of the brain insulin originates from the periphery through the blood–brain barrier via a saturable transport mechanism [32]. A role for insulin dysfunction in AD has been postulated [33–37]. In the present study, we determined the level and activation of insulin signalling in AD brain with a short post mortem delay (1.5 to 4 h) and found dramatic decreases in almost all the signalling pathway components. These observations indicate that the insulin signalling pathway is indeed down-regulated in AD brain. In agreement with the present study, a previous study, although it employed autopsied brain tissue with a much longer post mortem delay (up to 14 h), reported a significant decrease in the level of IR, phospho-AKT and phospho-GSK-3 in AD brain [38].

Overwhelming studies have demonstrated that T2DM increases the risk for cognitive impairment and dementia, but whether T2DM also increases the risk for AD is still under debate. An analysis of nine high-quality studies demonstrated that individuals with probable T2DM have nearly a two-fold higher risk of AD than individuals without diabetes [39]. However, several pathological studies found mainly increased vascular changes, but not increased amyloid plaques and NFTs, in the brains of AD patients with T2DM as compared to AD patients without T2DM [5]. As it is more likely that the oligomerization of amyloid- β peptides and abnormal hyperphosphorylated tau, rather than the amyloid plaques and NFTs *per se*, underlie the pathogenesis of AD, our present study support the correlation between T2DM and AD and provide a possible mechanism by which T2DM increases the risk for AD via brain insulin resistance and impaired downstream signalling.

In a recent study, we found that the injection of streptozotocin into the lateral ventricle of rat brains leads to brain insulin resistance and, consequently, decreased glucose transporters and tau O-GlcNAcylation, and increased tau phosphorylation [13]. Taken together with these observations, the present study suggests a novel mechanism by which brain insulin resistance could promote neurodegeneration through decreased brain glucose metabolism, decreased tau O-GlcNAcylation and hyperphosphorylation of tau. Abnormal hyperphosphorylation of tau has been demonstrated to play a crucial role in neurodegeneration [2].

T2DM is well known to be caused by peripheral insulin resistance. However, it was not known whether insulin resistance also occurs in the brain in T2DM. In the present study, we found, for the first time, decreased levels of the insulin signalling pathway components in T2DM, suggesting that brain insulin resistance in T2DM is similar to that in AD. These findings suggest that T2DM may increase the risk for dementia and AD through brain insulin resistance that induces abnormal hyperphosphorylation of tau. Consistent with this hypothesis, decreased phosphorylation of tau have been observed in the brains of individuals with T2DM [11]. Reduced insulin signalling and hyperphosphorylation of tau have also been observed in the brains of a type 1 diabetes mouse model [40].

The role of decreased brain insulin signalling in hyperphosphorylation of tau is further supported by our correlation analyses, indicating a positive correlation between some insulin signalling pathway components and the level of *O*-GlcNAcylation as well as negative correlations between some insulin signalling pathway components and the level of tau phosphorylation at individual abnormal phosphorylation sites. Although not all of the correlations reached statistical significance, the correlations between all of these pairs, shown in Tables 2 and 3, strongly suggest that the correlations will most likely hold if a much larger cohort of samples is included. It is well known that large individual variations are present in human brain samples, which most likely contributed to the lack of statistical significance in some of the correlations. Several components of the insulin signalling pathway also crosstalk to other cell-signalling pathways. Thus, it is natural that, among the insulin signalling components, some correlations were stronger than the others. More significant correlations between PDK1/AKT and *O*-GlcNAcylation/phosphorylation of tau at many sites suggest a more direct relationship than the other components of the insulin signalling pathway. This is probably because PDK1 and AKT are directly modified by *O*-GlcNAcylation [41,42]. The fact that AKT also directly phosphorylates tau at several sites [43,44] may also contribute to the significant correlation between it and tau phosphorylation.

Because insulin signalling negatively regulates GSK-3 activity by phosphorylation at Ser21 of GSK-3 α or Ser9 of GSK-3 β , decreased insulin signalling would ultimately lead to over-activation of GSK-3 activity, which is consistent with our observation of decreased phospho-GSK-3 β (Ser9) in T2DM and T2DM–AD brains. However, we also observed a decrease in the total level of GSK-3 β in these groups. The decrease in GSK-3 β might compromise the activation of GSK-3 β .

The overall similar extents of the decreases in the levels of total and phosphorylated insulin signalling pathway components suggest increased degradation, instead of decreased activation, of the pathway. This notion is supported by our observations of calpain I over-activation in AD and T2DM and its negative correlation to the insulin signalling pathway components. Previous studies have shown that some of the insulin signalling pathway components can be degraded by calpain [45–49]. Besides increased degradation of the insulin signalling components, down-regulation of insulin signalling might also result from other mechanisms in AD. A recent study has shown that A β -derived diffusible ligands (ADDLs) cause major down-regulation of insulin receptor and that this down-regulation can be prevented by insulin [50]. Intracellular A β may also inhibit insulin signalling in neurons

by interfering with the association between PDK1 and AKT to preclude AKT activation [51].

β -Arrestin-2 is a newly identified regulator of insulin signalling [29]. Unlike the other components of the insulin signalling pathway, the level of β -arrestin-2 was found to be decreased in T2DM brain but not in AD brain. These results suggest that insulin resistance in T2DM brain might also be caused by additional mechanisms.

The role of impaired brain insulin signalling in the pathogenesis of AD is also supported by several recent studies showing improvements in cognition and memory by treatment with insulin or insulin sensitizers in AD patients [52–54] and in rodent models of AD and diabetes [55,56]. It is interesting to note that rosiglitazone, an anti-diabetic drug that increases insulin sensitivity, improves attention and memory in a subgroup of AD patients who do not carry the apoE4 allele [57]. A functional interaction between ApoE isoforms and efficacy of insulin action on cognition has been previously demonstrated [58].

Unfortunately, the cohort included in the present study was too small to study the effects of apoE isoforms on the brain insulin–PI3K–AKT signalling pathway. The autopsied brain tissues of the T2DM group included in this study were collected as controls for neurological disorders such as AD by the brain bank. Therefore, the clinical information about T2DM is incomplete. Only four and two of the 10 cases had a record of anti-diabetic treatments and duration of T2DM, respectively. None of the T2DM cases had cognitive impairment. With the limited clinical information, we did not find obvious correlation between the levels of brain insulin signalling components and the anti-diabetic treatments or the duration of T2DM.

In summary, we have observed that both the total and the phosphorylated components of the insulin signalling pathway were decreased in AD and T2DM brains. These decreases were most severe in the brains of individuals with both AD and T2DM. The levels of the insulin signalling pathway components correlated negatively to tau phosphorylation and positively to *O*-GlcNAcylation, suggesting that decreased insulin signalling may contribute to AD through down-regulation of *O*-GlcNAcylation and promotion of hyperphosphorylation of tau. The levels of the insulin signalling pathway components also correlated to calpain activation, suggesting a role of calpain activation in the impairment of brain insulin signalling in AD and T2DM. Finally, β -arrestin-2 was found to be decreased in T2DM brain but not in AD brain.

Acknowledgments

We thank Ms Janet Murphy for secretarial assistance and Ms Maureen Marlow for editorial assistance. We are also grateful to the Sun Health Research Institute Brain Donation Program of Sun City, AZ, USA, for the provision of post-mortem human brain tissue. The Brain Donation Program is partially supported by the National Institute on Ageing (Grant No. P30 AG19610, Arizona Alzheimer's Disease Core Center). This study was supported in part by the New York State Office for People with Developmental Disabilities and the US National Institutes of Health (Grant Nos R01 AG027429, R03 TW008123, R01 AG031969 and R01 AG019158) and the US Alzheimer's Association (Grant No. IIRG-10-170405).

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Figure 1.

The insulin signalling pathway and western blot analysis of the insulin signalling pathway components in human brains. (A) Diagram showing the insulin signalling pathway. Arrows, activation; $\perp$, inhibition; red font, components determined in this study. (B) Western blots of IR β , IRS-1, PI3K, PDK1, AKT and GSK-3 β of mid-frontal cortices from seven control, nine AD, 10 T2DM and eight T2DM-AD cases. Actin blots were included as loading controls. (C) Densitometrical quantifications (mean $\pm$ SE) of the blots shown in (B) after normalization by the actin blot. * $p < 0.05$ versus controls.

Figure 2.

Correlation between calpain I activation and the levels of the insulin signalling pathway components in human brains. (A) Western blots of calpain I of the mid-frontal cortical extracts from six control, nine AD, 10 T2DM and eight T2DM-AD cases. Actin blot was included as a loading control. (B) Densitometrical quantifications (mean $\pm$ SE) of the blots shown in (A). * $p < 0.05$ versus controls. (C) Correlation analysis between calpain I activation and the levels of the insulin signalling pathway components. The levels of the insulin signalling pathway components in the brain were determined by quantitative western blots, as shown in Figure 1. Bold type indicates statistically significant correlation ($p < 0.05$).

Figure 3.

Western blot analysis of β -arrestin-2 in postmortem human brains. (A) Western blot of β -arrestin-2 of the mid-frontal cortical extracts from six control, eight AD, nine T2DM and eight T2DM-AD cases. Actin blot was included as a loading control. (B) Densitometrical quantifications (mean $\pm$ SE) of the blot shown in (A) after normalization by the actin blot. * p < 0.05 versus controls.

Table 1

Human brain tissue used in this study

Case no.	Age at death (years)	Gender	PMI ^a (h)	Braak stage ^b	Tangle score ^c
Con 1	83	F	3.3	II	0.75
Con 2	85	F	2.8	II	5.00
Con 3	82	F	2.0	II	4.25
Con 4	70	F	2.0	I	0.00
Con 5	82	F	2.3	II	3.50
Con 6	85	M	3.2	II	4.25
Con 7	80	M	3.3	II	2.75
Mean ± SD	81.0 ± 5.2		2.7 ± 0.6		2.9 ± 1.9
AD 1	83	F	3.0	VI	12.40
AD 2	79	F	1.5	VI	14.66
AD 3	73	F	2.0	V	15.00
AD 4	74	M	2.8	VI	14.66
AD 5	81	M	3.0	V	11.00
AD 6	76	M	2.3	VI	15.00
AD 7	72	M	2.5	VI	15.00
AD 8	76	M	4.0	V	15.00
AD 9	78	M	1.8	VI	15.00
Mean ± SD	76.9 ± 3.7		2.5 ± 0.8		14.2 ± 1.5
T2DM 1	88	F	2.5	III	5.50
T2DM 2	88	F	3.5	III	2.50
T2DM 3	90	F	2.7	III	2.50
T2DM 4	89	M	1.5	IV	7.00
T2DM 5	80	M	2.2	I	1.00
T2DM 6	87	F	2.0	III	4.50
T2DM 7	79	M	2.0	II	2.50
T2DM 8	87	M	2.5	IV	5.30
T2DM 9	86	M	2.0	III	5.00
T2DM 10	78	M	1.7	I	0.00

Case no.	Age at death (years)	Gender	PMI ^a (h)	Braak stage ^b	Tangle score ^c
Mean ± SD	85.2 ± 4.4		2.3 ± 0.6		3.6 ± 2.2
T2DM-AD 1	91	M	3.3	V	12.00
T2DM-AD 2	86	M	3.0	V	11.25
T2DM-AD 3	89	F	2.5	VI	15.00
T2DM-AD 4	87	F	3.0	V	15.00
T2DM-AD 5	83	M	3.3	V	12.00
T2DM-AD 6	77	M	2.3	VI	15.00
T2DM-AD 7	84	F	2.2	V	10.50
T2DM-AD 8	84	F	3.0	VI	15.00
Mean ± SD	85.1 ± 4.3		2.8 ± 0.4		13.2 ± 2.0

^a PMI, postmortem interval

^b Neurofibrillary pathology was staged according to Braak and Braak [59].

^c Tangle score was a density estimate and was designated as none, sparse, moderate or frequent (0, 1, 2 or 3 for statistics), as defined according to the CERAD AD criteria [60]. Five areas (frontal, temporal, parietal, hippocampal and entorhinal) were examined, and the scores were added up for a maximum of 15.

Table 2

Correlation analyses between the level/activation of the insulin signalling pathway and the levels of tau phosphorylation at individual phosphorylation sites or the density of NFTs

		Phosphorylation sites of tau							NFTs density	
		T181	S202	S214	S262	S404	S422	Total	Frontal	Total
IR β	<i>r</i>	-0.058	-0.202	-0.251	-0.169	-0.277	-0.305	-0.222	-0.185	-0.185
	<i>p</i>	0.127	0.380	0.272	0.463	0.224	0.178	0.333	0.423	0.423
IRS-1	<i>r</i>	-0.258	-0.279	-0.233	-0.282	-0.371	-0.354	-0.201	-0.396	-0.396
	<i>p</i>	0.259	0.221	0.308	0.215	0.097	0.115	0.382	0.076	0.076
PI3K(p85)	<i>r</i>	-0.328	-0.310	-0.128	-0.564	-0.416	-0.580	-0.166	-0.546	-0.546
	<i>p</i>	0.147	0.172	0.582	0.008	0.061	0.006	0.473	0.010	0.010
pPI3K(p85)	<i>r</i>	-0.257	-0.458	-0.167	-0.387	-0.389	-0.511	-0.349	-0.464	-0.464
	<i>p</i>	0.260	0.037	0.468	0.083	0.081	0.018	0.121	0.034	0.034
PDK1	<i>r</i>	-0.570	-0.275	-0.527	-0.629	-0.626	-0.648	-0.232	-0.540	-0.540
	<i>p</i>	0.007	0.228	0.014	0.002	0.002	0.002	0.313	0.011	0.011
pPDK1	<i>r</i>	-0.029	-0.092	-0.134	-0.311	-0.158	-0.478	-0.041	-0.407	-0.407
	<i>p</i>	0.901	0.690	0.562	0.171	0.494	0.028	0.860	0.068	0.068
AKT	<i>r</i>	-0.428	-0.324	-0.296	-0.489	-0.370	-0.538	-0.133	-0.440	-0.440
	<i>p</i>	0.053	0.152	0.193	0.025	0.099	0.012	0.566	0.046	0.046
pAKT	<i>r</i>	-0.075	-0.375	-0.114	-0.267	-0.153	-0.176	-0.140	-0.182	-0.182
	<i>p</i>	0.753	0.103	0.633	0.381	0.520	0.445	0.544	0.444	0.444
GSK-3 β	<i>r</i>	-0.234	-0.507	-0.156	-0.150	0.010	-0.191	-0.137	-0.076	-0.076
	<i>p</i>	0.307	0.019	0.499	0.516	0.966	0.408	0.553	0.744	0.744
pGSK-3 β (S9)	<i>r</i>	-0.029	-0.245	-0.015	-0.140	-0.157	-0.291	-0.131	-0.298	-0.298
	<i>p</i>	0.900	0.287	0.949	0.545	0.495	0.199	0.572	0.189	0.189

The level and activation (represented by the level of the activated/phosphorylated form) of each component of the insulin signalling pathway and the level of tau phosphorylation at individual phosphorylation sites in the frontal cortices were determined by quantitative western blots. The density of NFTs was scored by 0 (none), 1 (sparse), 2 (moderate) and 3 (frequent) in each of the five areas (frontal, temporal, parietal, hippocampal and entorhinal), as defined according to CERAD AD criteria [60]. The total tangle density was calculated by adding up the scores from all five areas. The *p* values that reach statistical significance (*p* < 0.05) are printed in bold.

Table 3

Correlation analyses between the level/activation of the insulin signalling pathway and the levels of *O*-GlcNAcylation

	<u><i>O</i>-GlcNAcylation</u>	
	<i>r</i>	<i>p</i>
IRβ	0.154	0.505
IRS-1	0.057	0.808
PI3K(p85)	0.081	0.727
pPI3K(p85)	0.362	0.107
PDK1	0.714	0.000
pPDK1	0.047	0.840
AKT	0.492	0.024
pAKT	0.049	0.833
GSK-3β	0.269	0.239
pGSK-3β(S9)	0.294	0.195

The level and activation of each component of the insulin signalling pathway and the level of *O*-GlcNAcylation in the frontal cortex were determined by quantitative western blots. The *r* and *p* values that reach statistical significance ($p < 0.05$) are printed in bold.