Generation and characterization of a rabbit monoclonal antibody site-specific for tau O-GlcNAcylated at serine 400

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ARTICLE INFO

ABSTRACT

Aggregation of tau into paired helical filaments is a pathological process leading to neurotoxicity in Alzheimer's disease and other tauopathies. Tau is posttranslationally modified by O-linked *N*-acetyl-glucosamine (O-GlcNAc), and increasing tau O-GlcNAcylation may protect against its aggregation. Research tools to study the relationship between tau aggregation and tau O-GlcNAcylation have not been widely available. Here we describe the generation of a rabbit monoclonal antibody specific for tau O-GlcNAcylated at Ser400 (O-tau(S400)). We show the utility of this antibody for in vitro and in vivo experiments to investigate the function of O-GlcNAc modifications of tau at Ser400.
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1. Introduction

Tau is a component of the neuronal cytoskeleton where it is primarily associated with axonal microtubules [1]. In Alzheimer's disease and other tauopathies tau is aberrantly aggregated in neuronal cell somata, which may be causative in the neurodegenerative process during the disease course [1]. Recent evidence suggests that tau aggregation may be attenuated by increasing protein O-GlcNAcylation in brain [2] and thus modifying this mechanism may be a potential therapeutic approach to Alzheimer's disease and other tauopathies.

O-GlcNAcylation is a dynamic process in which proteins become posttranslationally modified through the attachment of *N*-acetyl-D-glucosamine (GlcNAc) moieties to the hydroxyl group of serine and threonine residues [3]. The attachment of GlcNAc is catalyzed by the enzyme O-GlcNAc transferase (OGT), which utilizes uridine 5′-diphospho-*N*-acetylglucosamine produced in the hexosamine biosynthesis pathway from glucose as substrate. Reversely, O-GlcNAc moieties are enzymatically removed from proteins by the glycoside hydrolase O-GlcNAcase (OGA) [4]. O-GlcNAcylation often occurs at or near amino acid residues that can be phosphorylated and thus it has been implied that O-GlcNAcylation can directly regulate phosphorylation of proteins.

Tau is subject to O-GlcNAcylation at several serine and threonine residues including Thr123, Ser 208, Ser356, Ser400 and either Ser409, 412, or 413 [5–7,2,8]. Ser400 is located within a cluster of phosphorylation sites (Ser396, Ser400, Ser404, Ser409, Ser412, Ser413, Ser416, and Ser422) at the C-terminal end of tau, several of which have been implicated in tau pathology [9]. Ser400 appears to play a predominant role in regulating the aggregation propensity of tau as a S400A mutation in a recombinant O-GlcNAcylated tau peptide abolished the aggregation attenuating effect on tau that was observed for the wild type O-GlcNAcylated version of the same peptide in vitro [2]. It is thus important to understand the regulation of tau O-GlcNAcylation at this particular site, but to date studies have been greatly hampered by the unavailability of O-GlcNAc tau site-specific antibodies.

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Here we describe the generation and characterization of a rabbit monoclonal antibody, named Otau(S400), specific for tau O-GlcNAcylated at Ser400. We demonstrate the utility of this antibody for biochemical applications in cells and tau transgenic mouse models. This important research tool will readily allow the study of tau O-GlcNAcylation and its implication for tau pathology in vitro and in vivo and will greatly facilitate the discovery of agents modifying tau O-GlcNAcylation at Ser400.

2. Material and methods

2.1. Generation of Otau(S400) antibody

The Otau(S400) antibody was generated at Epitomics, Inc.. In brief, two peptides, O-GlcNAc tau (cVYKSPVV-(O-GlcNAc)S-GDTSPRH) and unglycosylated tau (cVYKSPVVSGDTSPRH) were synthesized to ∼90% purity and conjugated to four different carrier proteins, KLH, OVA, Blue-Carrier, and BSA. Four three months old New Zealand Whites rabbits were immunized with KLH- or OVA-conjugated O-GlcNAc tau peptide employing a protocol of five subcutaneous injections and two test bleeds per rabbit. Serum titers against the BSA-conjugated O-GlcNAc tau and unglycosylated tau peptides indicated that all rabbits demonstrated a good immune response against the antigen (OD >0.3 at 1:64000 dilution). One rabbit was chosen for a final iv boost with Blue Carrier-conjugated O-GlcNAc tau peptide, followed by splenocyte collection for hybridoma fusion.>30 positive hybridoma clones were identified by ELISA against the BSA-conjugated O-GlcNAc tau peptide and subsequently counter-screened against BSA-conjugated unglycosylated tau peptide. Three clones were selected for subcloning by limited dilution. Ten subclones of each clone were selected based on ELISA screening against BSA-conjugated O-GlcNAc and unglycosylated tau peptides. One subclone was expanded and adapted to production medium and subjected to endotoxin-free protein A purification.

2.2. Antigen binding affinity ELISA

ELISA plates were coated with 50 μl/well of 1 μg/ml of BSA conjugated O-GlcNAc tau or unglycosylated tau peptide in bicarbonate coating buffer overnight at 4 °C. Antigen coated plates were blocked with 100 μl/well of 1% BSA (9048–46-8, Amersco) in TBS for 1 h at 37 °C, followed by thorough washes in TBST. Starting from 10 μg/ml 50 μl of 10-fold serial dilutions of the Otau(S400) antibody prepared in TBS containing 1% BSA were added to the ELISA plate and incubated for 1 h at 37 °C. After three washes in TBST anti-rabbit IgG conjugated with alkaline phosphatase (111–055-003, Jackson ImmunoResearch) was added at a dilution of 1:1000 and incubated for 30 min at 37 °C, washed again three times in TBST and developed by adding 50 μl PNPP substrate (4264–83-9, Amersco) freshly prepared in PNPP DEA buffer (34064, Pierce) for 15 min at RT before stopping with 50 μl 1 N NaOH. Absorbance was read at OD 405 nm. $EC_{50}s$ were calculated using GraphPad Prism software.

2.3. Plasmid cloning

The mammalian expression vector for myc-DDK tagged tau variant 2 (TrueORFGold expression validated cDNA clone RC213312; accession No. NM_005910.3) was obtained from OriGene. To generate tau-CGFP expression plasmids tau cDNA was transferred from the myc-DDK-tau expression vector to the destination vector pCMV6-AC-mGFP (PS100048, OriGene) following OriGene's Rapid-Shuttling Kit protocol. The human OGT cDNA was cloned from the OGT/pENTR221 vector (IOH27987, Invitrogen) into the pDEST26

destination vector (11809–019, Invitrogen) employing Gateway cloning technology (Invitrogen).

2.4. Transfection, ThiametG treatment and lysis of cells

To generate stable tau-expressing cell lines HEK293 cells (CRL-1573, ATCC) were transfected with myc-DDK-tau or tau-CGFP cDNA via Lipofectamine 2000 (11668–019, Invitrogen) lipofection according to the manufacturer's protocol. Transfected cells were cultured in MEM (11095, Invitrogen) containing 10% FBS (10099, Invitrogen) and penicillin/streptomycin (15140, Invitrogen) for 16 h after which cells were transferred to 6-well plates and cultured in the presence of 200 μg/ml geneticin (10131–027, Invitrogen) to 80% confluency. Cells were subsequently plated at a density of 1 cell/well in a 96-well plate and cultured until confluent. Individual clones were isolated and consecutively transferred into 24- and 6-well plates for expansion. Tau expressing clones were confirmed by Western blotting with the tau-5 antibody. For OGT/tau co-expression experiments OGT/pDEST26 cDNA was transfected in HEK293 cells stably expressing myc-DDK-tagged tau via Lipofectamine 2000 according to the manufacturer's protocol. Mock transfected HEKtau cells were used for control. For pharmacological inhibition of OGA, HEKtau or HEK293 cells were treated with either 10 μM ThiametG (MD08856, Carbosynth, UK) in 0.1% DMSO or 0.1% DMSO alone and incubated for 16 h at 37 °C. Cells from transfection or ThiametG treatment experiments were lysed in 10 mM Tris–HCl (T1080, Teknova), pH 7.5, 1% SDS (24730, Invitrogen), EDTA-free protease inhibitors (04693159001, Roche) and sonicated for 10 s, followed by incubation on ice for 10 min. Lysates were centrifuged at 1000 g for 3 min and the ensuing supernatants analyzed using the BCA protein assay kit (23227, Pierce) according to the manufacturer's protocol. 20–30 μg of protein lysate were applied for Western blot analysis.

2.5. Purification of O-GlcNAc tau from cell lysate

HEKtau cells were transiently transfected with OGT/pDEST26 as described above. Mock transfected HEKtau cells were used for control. After 24 h of expression cells were lysed and tau protein was isolated employing the Sigma FLAG Immunoprecipitation kit (FLAGIPT1, Sigma) following the manufacturer's instructions. Immunoprecipitated tau protein was eluted from the FLAG beads with 100 mM glycine, pH 2.0 and immediately dialyzed into PBS. Two micrograms of eluate was subjected to Western blotting as described below.

2.6. In-Cell Western assay

HEKtau-CGFP cells were maintained in MEM (11095, Invitrogen), 10% FBS (10099, Invitrogen), 200 μg/ml geneticin (10131–027, Invitrogen). Cells were plated in 96-well plates at 50000 cells/100 μl per well and incubated overnight. The following day serial dilutions of ThiametG in media with a final DMSO concentration of 0.5% or vehicle were added to the cells and incubated for 16 h. Cells were washed in PBS and 3.7% formaldehyde in PBS was added for fixation followed by permeabilization in 0.1% Triton X-100 in PBS. After blocking for 1.5 h in LiCOR Odyssey blocking buffer (927–40000, LiCOR Biosciences Inc.) at 22 °C the cells were probed with the Otau(S400) antibody at a concentration of 1 μg/ml followed by detection with a goat anti-rabbit IRDye800CW antibody (926–32213, LiCOR Biosciences Inc.). The bound secondary antibody was quantified on a LiCOR Odyssey infrared imager (LiCOR Biosciences Inc.). For the pre-adsorption experiments the Otau(S400) antibody was incubated with either 5 μg/ml O-GlcNAc tau peptide (VYKSPVV-(O-GlcNAc)S-GDTSPRH) or 5 μg/ml unglycosylated tau peptide (VYKSPVVSGDTSPRH) or 100 mM N-acetyl-glucosamine (A8625, Sigma Aldrich) in LiCOR Odyssey blocking

buffer for 1 h at 22 °C prior to addition to the cells. EC_{50} values were calculated using GraphPad Prism software.

2.7. ThiametG treatment of Tg21221, JNPL3 mice and tau knockout mice

Tg21221 mice were generated as described elsewhere [10] and bred at the McLaughlin Research Institute (MRI). 3 months old females and male Tg21221 (n = 15/group) were used. Eighteen weeks old male JNPL3 mice were obtained from Taconic Farms (NY, USA) and 16 weeks old male tau knockout mice (B6.129-Mapt^{tm1Hnd}/J) were obtained from Jackson Laboratory (Bar Harbor ME, USA). All experiments were approved by the EMD Serono Research & Development Institute and MRI Institutional Animal Care and Use Committees (IACUC). ThiametG was dissolved in water and administered po at a concentration of 500 mg/kg/day for five consecutive days in JNPL3 mice and for 1 or 14 consecutive days in Tg21221 mice. ThiametG was administered at a single dose in tau knockout mice. Water was used for the vehicle treated groups.

2.8. Brain lysis and tau immunoprecipitation

Animals were euthanized 4 h after administration of the last dose of ThiametG. Hemi-forebrains were rapidly dissected and frozen on dry ice. Tissue samples were homogenized in Phosphosafe Buffer (71296–4, EMD Millipore), followed by centrifugation (15 000 g) to remove cellular debris. The ensuing supernatant was assayed by DC protein assay (500–0113, 500–0114, 500–0115, BioRad) to determine protein concentrations. Twenty micrograms of brain lysate were subjected to Western blotting as described below. To immunoprecipitate tau protein from JNPL3 brain lysate a Crosslink Immunoprecipitation kit (26147, Thermo-Scientific) was used according to the manufacturer's instruction. In brief, protein A/G resin was crosslinked to 20 µg of the tau-5 antibody (MS-247-P1, ThermoScientific). Two hundred and fifty micrograms of brain lysates as described above was incubated with the resin-coupled tau antibody overnight at 4 °C. Samples were eluted with 50 µl of low pH elution buffer and immediately neutralized with 5 µl 1 M Tris, pH 9.5.

2.9. Western blotting

Protein samples were subjected to 4–20% Tris–glycine SDS–PAGE (WT4121A, Invitrogen) for separation of cell lysate and to 4–15% Tris–HCl SDS–PAGE (345–0028, BioRad) for brain lysate, followed by semi-dry transfer to nitrocellulose membranes (IB301–01, Invitrogen). Membranes were blocked in LiCOR Odyssey blocking buffer (927–40000, LiCOR Biosciences Inc.), then incubated with the appropriate primary antibody for 2 h at RT or overnight at 4 °C, extensively washed in TBST, followed by labeling with IRDye680LT- or IRDye800CW-conjugated goat-anti-rabbit or -mouse secondary antibody (926–68021, 926–32212, LiCOR Biosciences Inc.) at a dilution of 1:10 000 for 1 h at RT. After extensive washing in TBST membranes were scanned on the LiCOR Odyssey infrared imager (LiCOR Biosciences Inc.). The following primary antibodies and dilutions were used in this study: RL2 (MA1072, ThermoScientific) at 1:200, tau-5 (MS-247-P1, ThermoScientific) at 1:5000, HT7 (MN1000, ThermoScientific) at 1:50 000, and Otau(S400) at 1:1000.

3. Results

3.1. Specificity of Otau(S400) against tau peptide O-GlcNAcylated at Ser400

In order to generate a rabbit monoclonal antibody against O-GlcNAc modified tau at Ser400 two peptides spanning the

sequence around Ser400 were synthesized, one of which carried an O-GlcNAc moiety at serine 400: O-GlcNAc tau peptide (cVYKSPVV-(O-GlcNAc)S-GDTSPRH) and unglycosylated tau peptide (cVYKSPVVSGDTSPRH). Rabbits were immunized with KLH-conjugated O-GlcNAc tau peptide by subcutaneous injections, followed by splenocyte collection and hybridoma fusion. Positive hybridoma clones identified by using a standard ELISA protocol against BSA-conjugated O-GlcNAc tau peptide were further subcloned. One ELISA positive hybridoma subclone was expanded and protein A purified. The purified antibody was designated Otau(S400). Antibody antigen binding activity testing by ELISA against BSA-conjugated O-GlcNAc tau and unglycosylated tau peptide showed that Otau(S400) has highly specific binding activity with a calculated EC_{50} of 785 nM towards the O-GlcNAc-tau peptide and was non-reactive to the unglycosylated peptide (Fig. 1). The lowest O-GlcNAc tau peptide specific signal could be detected at 1 ng/ml antibody concentration.

3.2. Specificity of Otau(S400) for O-GlcNAcylated tau in OGT transfected cells

To further demonstrate the specificity of the Otau(S400) antibody in a cellular context we overexpressed His-tagged human OGT in HEK293 cells stably expressing myc-DDK-tagged human 2N4R tau (HEKtau). Western blot analysis of cell lysate with an antibody (RL2) that recognizes O-GlcNAc modifications on a broad spectrum of proteins confirmed that OGT was active when heterologously expressed in HEKtau cells as indicated by the stronger intensity of bands in the OGT transfected as compared to mock transfected cells (Fig. 2A). A similar increase in signal intensity was observed in the parental HEK293 cell line in the presence of OGT (Fig. 2A). When probed with the Otau(S400) antibody a strong immunopositive band with an apparent molecular mass of ~60 kDa was detected in OGT transfected, but not in mock transfected HEKtau cells (Fig. 2A). This band overlapped with a band detected with an antibody recognizing total tau (tau-5), suggesting that Otau(S400) indeed recognized O-GlcNAcylated tau in cells. A band with similar intensity was detected with the tau-5 antibody in the mock transfected HEKtau cells confirming equal loading of protein lysates on the gel (Fig. 2A). Interestingly, the tau-5 antibody revealed a duplet of bands, of which only the band with lower molecular mass co-localized with the Otau(S400) band (see overlay, Fig. 2A). This suggests that only a subpopulation of tau is substrate for O-GlcNAcylation in HEKtau cells. As a further demonstration of the antibody specificity, no immunoreactive bands were observed with the Otau(S400) antibody in the absence or presence of OGT in HEK293 cells that do not express tau (Fig. 2A).
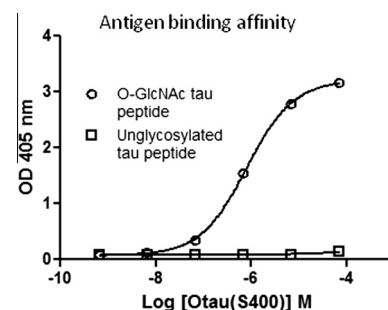

Fig. 1. ELISA with BSA-conjugated O-GlcNAc tau and unglycosylated tau peptide demonstrated highly specific binding activity of Otau(S400) for the O-GlcNAc-tau peptide. EC_{50} for the O-GlcNAc tau peptide was 785 nM and lowest limit of detection was at 1 ng/ml Otau(S400).
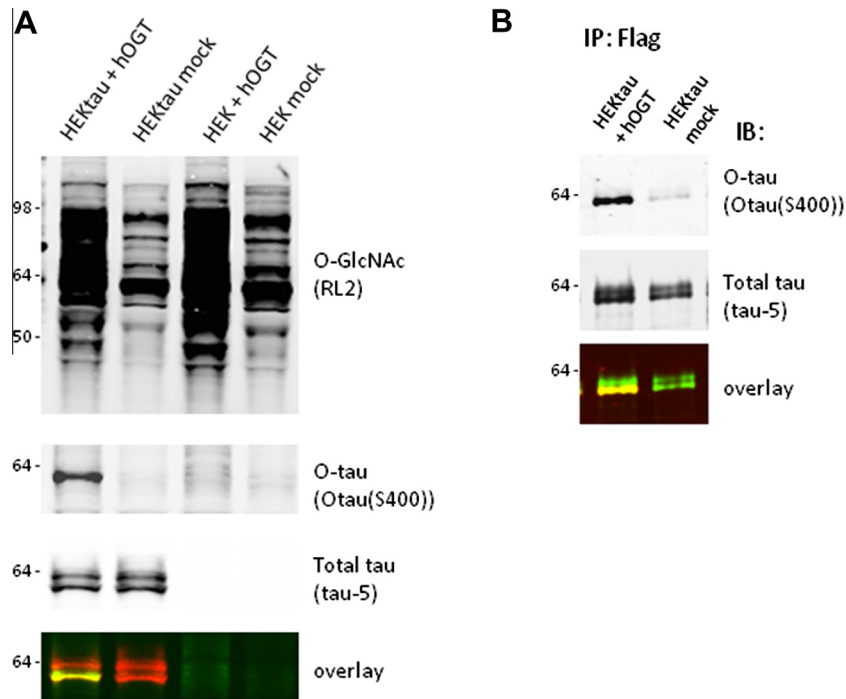
Fig. 2. (A) Western blot of lysates from HEKtau and HEK cells, OGT or mock transfected, and probed with antibodies against total O-GlcNAc levels (RL2), O-GlcNAc tau at Ser400 (Otau(S400)) and total tau (tau-5). (B) Western blot of tau immunoprecipitated from lysates of OGT or mock transfected HEKtau cells and probed with antibodies against O-GlcNAc tau at Ser400 (Otau(S400)) and total tau (tau-5).

To corroborate that the immunopositive band in the OGT transfected HEKtau cells represented O-GlcNAcylated tau we affinity-purified myc-DDK-tagged tau from OGT transfected HEKtau cells via a FLAG antibody that recognizes the DDK epitope and subjected it to Western blotting with the Otau(S400) antibody. A strong immunoreactive band was observed at ∼60 kDa in the tau preparation isolated from OGT transfected HEKtau cells, but only a very faint signal was seen in the tau preparation from mock transfected HEKtau cells (Fig. 1B). Tau-5 staining confirmed that the immunoprecipitated proteins were indeed tau (Fig. 1B). Similar to our previous observation, Otau(S400) positive tau only overlapped with the lower molecular mass tau (see overlay, Fig. 2B).

3.3. Specificity of Otau(S400) for O-GlcNAcylated tau in ThiametG treated cells

OGT overexpression in cells may result in unphysiologically high levels of protein O-GlcNAcylation. To demonstrate that Otau(S400) can detect tau O-GlcNAcylation under more physiological conditions cellular O-GlcNAcylation was increased by incubating HEKtau cells in the presence of the potent and selective OGA inhibitor ThiametG [11]. ThiametG treatment elevated global protein O-GlcNAcylation in HEKtau and HEK cells as determined with the RL2 antibody (Fig. 3A). Otau(S400) detected a band at ∼60 kDa only in the ThiametG treated, but not vehicle treated HEKtau cells, indicating that the antibody is sensitive enough to detect tau O-GlcNAcylation at substoichiometric levels in cells (Fig. 3A). Furthermore, this also demonstrates that tau is a substrate for endogenously expressed OGT in HEK cells. Bands with similar intensities were detected with the tau-5 antibody in both, Thiamet G and vehicle treated HEKtau cells, confirming equal loading of protein lysates on the gel (Fig. 3A). HEK293 cells that did not express tau did not show any significant immunoreactivity with Otau(S400) with or without ThiametG treatment (Fig. 3A).

In order to be able to rapidly identify small molecule OGA inhibitors that elevate tau O-GlcNAcylation at Ser400 a sensitive cellular assay is needed that allows for high throughput screening of compounds. To this end we employed an In-Cell Western assay platform in which HEKtau-CGFP cells were treated with increasing concentrations of Thiamet G (300 pM–30 μM; Fig. 3B) and O-GlcNAc tau was detected with the Otau(S400) antibody. Thiamet G increased tau O-GlcNAcylation in a concentration dependent manner with an EC_{50} of 10 nM. Importantly, adding 5 μg/ml O-GlcNAc tau peptide during the primary antibody incubation completely abolished the detection of O-GlcNAc tau, but no significant change in O-GlcNAc tau signal was seen in the presence of 5 μg/ml of the unglycosylated version of the peptide (EC_{50} = 14.3 nM). Notably, adding 100 mM free N-acetyl-glucosamine to the primary antibody did not interfere with the detection of O-GlcNAc tau (EC_{50} = 12.7 - nM). Taken together, this clearly indicates that the Otau(S400) antibody requires the pendant O-GlcNAc residue as well as the peptide backbone for binding (Fig. 3B).

3.4. Specificity of Otau(S400) for O-GlcNAcylated tau in ThiametG treated Tg21221 and JNPL3 mice

Accumulating evidence suggests that increasing O-GlcNAc levels in brain via the pharmacological inhibition of OGA may provide a therapy for the treatment of Alzheimer's disease and other tauopathies [2]. To establish that Otau(S400) recognizes O-GlcNAcylated tau in vivo we employed two different tau transgenic animal models. First, we treated Tg21221 mice, a transgenic mouse line that overexpresses human wild type tau (variant 0N4R), with 500 mg/kg ThiametG for 1 or 14 days [10]. To demonstrate that oral ThiametG administration is effective in increasing O-GlcNAc levels in brain we probed brain lysate from singly and repeatedly treated animals with the RL2 antibody. A single dose of ThiametG slightly increased global protein O-GlcNAcylation as compared to vehicle
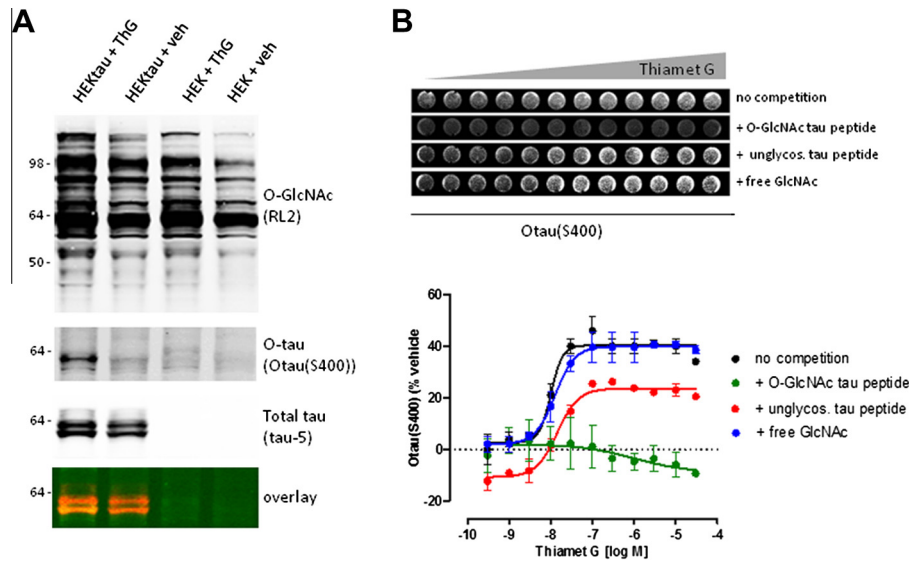
Fig. 3. (A) Western blot of lysates from HEKtau and HEK cells treated with 10 μM ThiametG and probed with antibodies against total O-GlcNAc levels (RL2), O-GlcNAc tau at Ser400 (Otau(S400)) and total tau (tau-5). (B) In-Cell Western assay of HEKtau-CGFP cells treated with ThiametG (300 pM–30 μM) and detected with Otau(S400) in the absence (no competition) or presence of O-GlcNAc tau peptide (5 μg/ml), unglycosylated tau peptide (5 μg/ml) or 100 mM free GlcNAc. The graph depicts a ThiametG concentration dependent increase of cellular O-GlcNAc tau (no competition, $EC_{50} = 10$ nM), with unglycosylated tau peptide ($EC_{50} = 14.3$ nM), with free GlcNAc ($EC_{50} = 12.7$ nM), but no signal in the presence of O-GlcNAc tau peptide.
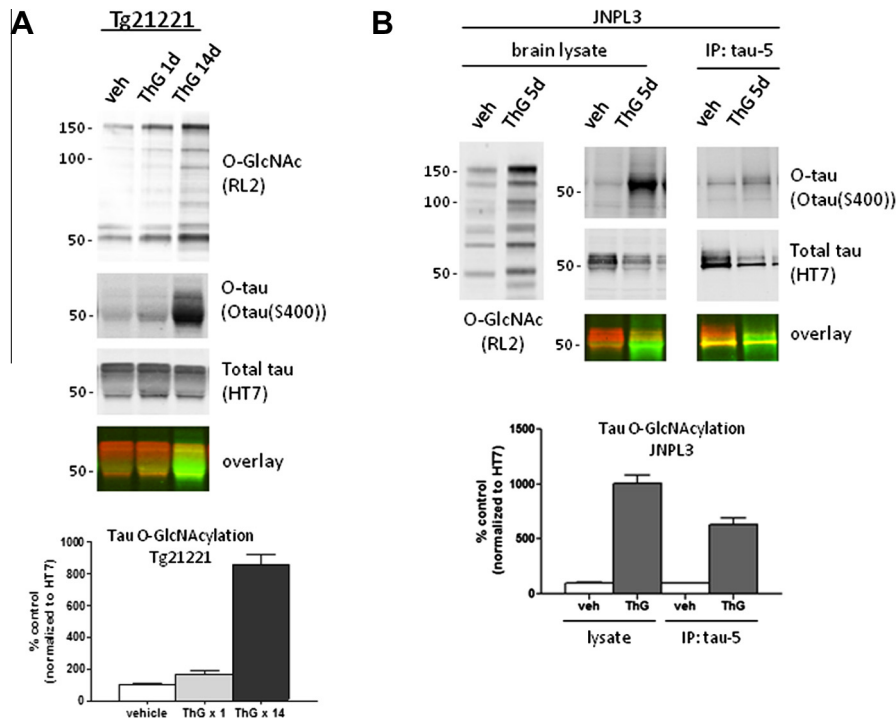

Fig. 4. (A) Western blot of lysates from Tg21221 mice treated with 500 mg/kg ThiametG or vehicle for 1 or 14 days and probed with antibodies against total O-GlcNAc levels (RL2), O-GlcNAc tau at Ser400 (Otau(S400)) and total tau (HT7). The graph depicts an increase in O-GlcNAc tau signal in response to 14 doses of ThiametG. (B) Western blot of lysates and immunoprecipitated tau from JNPL3 mice treated with 500 mg/kg ThiametG or vehicle for 5 days and probed with antibodies against total O-GlcNAc levels (RL2), O-GlcNAc tau at Ser400 (Otau(S400)) and total tau (HT7). The graph depicts an increase in O-GlcNAc tau signal after 5 daily doses of ThiametG.

treated animals, whereas repeated administration of ThiametG resulted in a robust increase in global protein O-GlcNAcylation (Fig. 4A). A single dose of ThiametG did not significantly increase tau O-GlcNAcylation at Ser400 as compared to vehicle treated animals (Fig. 4A). However, two weeks of daily administration of ThiametG elicited a significant ~ninefold increase in signal with the Otau(S400) antibody, indicating that subchronic OGA inhibition

strongly increases O-GlcNAc modifications on human wild type tau that can be detected with the Otau(S400) antibody (Fig. 4A). The overlay showed a partial overlap of O-GlcNAcylated tau with total tau, suggesting that only a subspecies of tau is subject to O-GlcNAcylation in this treatment paradigm.

Tg21221 mice do not exhibit the typical tau pathology that can be observed in JNPL3 mice, a widely used transgenic mouse model

for the study of Alzheimer's disease related tau pathology carrying the P301L mutant form of tau [12]. To demonstrate that Otau(S400) can recognize O-GlcNAc tau in this mouse model JNPL3 mice were treated with 500 mg/kg ThiametG or vehicle daily for five days. Subchronic treatment with ThiametG elicited a robust increase in global protein O-GlcNAcylation as detected with the RL2 antibody (Fig. 4B). Similar to Tg21221 mice, we observed a highly significant ~ninefold increase in O-GlcNAc tau signal as compared to vehicle treated animals. To confirm that the Otau(S400) immunopositive bands represented tau we immunoprecipitated tau from JNPL3 brain lysate with the tau-5 antibody and subjected it to Western blotting with the Otau(S400) antibody. Strong immunoreactive bands were observed around ~50 kDa in the tau preparation isolated from ThiametG treated mouse brain, but a much fainter signal was seen the tau preparation from vehicle treated animals (Fig. 4B). Staining with the HT7 antibody that recognizes human tau confirmed that the immunoprecipitated proteins were indeed tau (Fig. 4B).

Lastly, to demonstrate the low cross-reactivity of the Otau(S400) antibody with other O-GlcNAcylated proteins we probed brain lysates from tau knockout mice (tau KO) that were treated with a single dose of 500 mg/kg ThiametG po or vehicle with the Otau(S400) antibody (Fig. 5). A very faint signal could be seen at ~50, ~130 kDa and at the top of the gel only after scanning the Western blot at highest sensitivity, indicating that the Otau(S400) antibody shows only limited cross-reactivity with other O-GlcNAcylated proteins unrelated to tau. Tau-5 antibody staining revealed no signal, confirming that tau was absent in brains of tau KO mice (Fig. 5)

Taken together, this suggests that Otau(S400) is a valid tool to study changes in tau O-GlcNAcylation at Ser400 in mouse models of tauopathy.

4. Discussion

O-GlcNAcylation is a ubiquitous posttranslational modification that has been implicated in a multitude of vital cellular processes including transcriptional regulation, cell signaling and proteasomal degradation [13]. O-GlcNAc is also found on many cytoskeletal proteins. Although O-GlcNAcylation of proteins emerges as being as abundant as phosphorylation [14], the former has by far not as intensely studied than the latter. This is partly due to the relative paucity of research tools that allow for the specific analysis

Fig. 5. Western blot of lysates from tau KO mice treated with a single dose of 500 mg/kg ThiametG or vehicle and probed with antibodies against O-GlcNAc tau at Ser400 (Otau(S400)) and total tau (tau-5).

of this modification on proteins of interest. Recent years have brought forward the development of elegant biochemical methods to sensitively label and identify O-GlcNAcylated proteins [15,16]. However, these methods are based on the enrichment of a pool of O-GlcNAcylated proteins that require additional biochemical methods to subsequently determine the identity of the O-GlcNAc modified protein. The availability of epitope specific antibodies that unambiguously recognize O-GlcNAc moieties on a specific protein of interest is limited. Earlier attempts to generate O-GlcNAc-site specific antibodies for individual proteins have resulted in antibodies that recognize O-GlcNAc modifications on many proteins in a relatively peptide-independent manner [17,18]. More recently, a polyclonal rabbit antibody recognizing the neuronal protein tau when O-GlcNAcylated at Ser400, termed 3925, has been described [7]. While the authors were able to clearly demonstrate that 3925 recognizes recombinant O-GlcNAc tau in vitro, the antibody showed strong cross-reactivity with other proteins in rat brain lysates limiting its use for in vivo studies. Here we describe the generation of a new rabbit monoclonal antibody, named Otau(S400), that is specific for human tau O-GlcNAcylated at Ser400. Our antibody showed high binding affinity for a tau peptide O-GlcNAc modified at Ser400 by ELISA and only recognized a single band corresponding to tau in cells in which O-GlcNAcylation was increased either by overexpression of OGT or by pharmacological treatment with the OGA inhibitor ThiametG. Furthermore, in tau transgenic animal models expressing either wild type or mutant tau Otau(S400) showed strong immunoreactivity for O-GlcNAc tau in response to treatment with ThiametG, but not in vehicle treated animals. Binding of Otau(S400) to O-GlcNAcylated tau could be completely abolished with a tau peptide O-GlcNAcylated at Ser400, but not with the unmodified version of the same peptide or with free N-acetyl-glucosamine. This observation is in good agreement with a number of studies of O-GlcNAc epitope specific antibodies that show no or incomplete competition with free GlcNAc [19,20,7]. This strongly suggests that Otau(S400) specifically recognizes the O-GlcNAc moiety at Ser400 in the context of the tau peptide backbone and is consistent with the minimal cross-reactivity of Otau(S400) in brain lysate of tauKO mice. In summary, these data highlight the power of this new antibody for the investigation of tau O-GlcNAcylation at Ser400 in a cellular or tissue context.

Tau is a major constituent of neurofibrillary tangles, a characteristic pathological feature of Alzheimer's disease and other tauopathies [1]. Neurofibrillary tangle formation involves aggregation and hyperphosphorylation of tau in neurons, ultimately leading to neuronal cell death. Intense research efforts have been made to elucidate mechanisms of tau aggregation in order to identify molecular drug targets to interfere with tau aggregation. Recent data support a role for O-GlcNAcylation in stabilizing tau against aggregation [2]. While the exact mechanism by which O-GlcNAcylation may protect tau from aggregating remains to be determined, it is feasible that O-GlcNAc moieties impose a conformation on tau that makes it less prone to aggregation. Notably, O-GlcNAc modification at Ser400 appears to play an important role in inhibiting tau oligomerization in vitro [2] and an increase in tau O-GlcNAcylation at Ser400 correlated with a reduction in tau pathology and slowed neurodegeneration in a mouse model of tauopathy [2]. It is thus important to be able to monitor changes in tau O-GlcNAcylation at Ser400 in response to genetic or pharmacological manipulation to understand its role in tau function. With the Otau(S400) antibody we have established a tool that can readily detect changes in tau O-GlcNAcylation at this epitope in vitro and in vivo as demonstrated in two tau transgenic models, JNPL3 and Tg21221 mice. This will greatly facilitate the understanding of the role of O-GlcNAcylation in tau aggregation and the discovery of efficacious drugs aimed at increasing O-GlcNAcylation of tau.
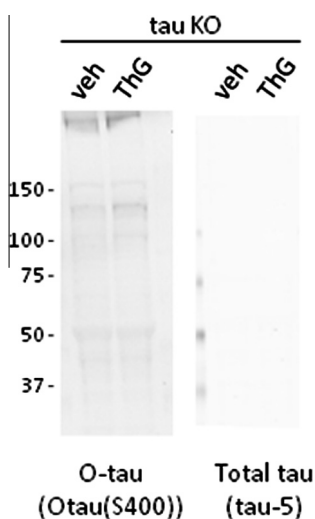
Acknowledgments

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