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(54) Title: O-GlcNAc TAU ANTIBODY AND USE THEREOF

(57) Abstract: The invention relates to an antibody that is capable to bind the O-GlcNAcylated tau isoform 2N4R at serine 400. Another object of the invention concerns the use of said antibody for screening molecules for OGA inhibitory activity, and a method for administering a therapeutic molecule to a patient in need of treating Alzheimer's disease. The invention also relates to a method for preparing a monoclonal rabbit antibody.

O-GlcNAc tau antibody and use thereof

The invention relates to an antibody that is capable to bind the O-GlcNAcylated tau isoform 2N4R at serine 400. Another object of the invention concerns the use of said antibody for screening molecules for OGA inhibitory activity, and a method for administering a therapeutic molecule to a patient in need of treating Alzheimer's disease. The invention also relates to a method for preparing a monoclonal rabbit antibody.

A wide range of cellular proteins, both nuclear and cytoplasmic, are post-translationally modified by the addition of the monosaccharide 2-acetamido-2-deoxy-β-D-glucopyranoside (β-N-acetyl glucosamine) which is attached via an O-glycosidic linkage. This modification is generally referred to as O-linked N-acetylglucosamine or O-GlcNAc. The enzyme responsible for post-translationally linking β-N-acetylglucosamine (GlcNAc) to specific serine and threonine residues of numerous nucleocytoplasmic proteins is O-GlcNAc transferase (OGT). A second enzyme, known as O-GlcNAcase (OGA), removes this post-translational modification to liberate proteins making the O-GlcNAc-modification a dynamic cycle occurring several times during the lifetime of a protein.

O-GlcNAc-modified proteins regulate a wide range of vital cellular functions including, for example, transcription, proteasomal degradation and cellular signaling. O-GlcNAc is also found on many structural proteins. For example, it has been found on a number of cytoskeletal proteins, including neurofilament proteins, synapsins, synapsin-specific clathrin assembly protein AP-3 and Ankyrin-G. O-GlcNAc modification has been found to be abundant in the brain. It has also been found on proteins clearly implicated in the etiology of several diseases including Alzheimer's disease (AD) and cancer.

For example, it is well established that AD and a number of related tauopathies including Downs' syndrome, Pick's disease, Niemann-Pick Type C disease and amyotrophic lateral sclerosis (ALS) are characterized, in part, by the development of neurofibrillary tangles (NFTs). These NFTs are aggregates of paired helical filaments (PHFs) and are composed of an abnormal form of the cytoskeletal protein "tau". Normally, tau stabilizes a key cellular network of microtubules that is essential for distributing proteins and nutrients within neurons. In AD patients, however, tau becomes hyperphosphorylated, disrupting its normal function, forming PHFs and ultimately aggregating to form NFTs. Six isoforms of tau are found in the human brain. In AD patients, all six isoforms of tau are found in NFTs, and all are markedly hyperphosphorylated. Tau in healthy brain tissue bears only 2 or 3 phosphate

groups, whereas those found in the brains of AD patients bear, on average, 8 phosphate groups. A clear parallel between NFT levels in the brains of AD patients and the severity of dementia strongly supports a key role for tau dysfunction in AD. The precise causes of this hyperphosphorylation of tau remain elusive. Accordingly, considerable effort has been dedicated toward: a) elucidating the molecular physiological basis of tau hyperphosphorylation; and b) identifying strategies that could limit tau hyperphosphorylation in the hope that these might halt, or even reverse, the progression of Alzheimer's disease. Several lines of evidence suggest that up-regulation of a number of kinases may be involved in hyperphosphorylation of tau, although very recently, an alternative basis for this hyperphosphorylation has been advanced.

In particular, it has recently emerged that phosphate levels of tau are regulated by the levels of O-GlcNAc on tau. The presence of O-GlcNAc on tau has stimulated studies that correlate O-GlcNAc levels with tau phosphorylation levels. The recent interest in this field stems from the observation that O-GlcNAc modification has been found to occur on many proteins at amino acid residues that are also known to be phosphorylated. Consistent with this observation, it has been found that increases in phosphorylation levels result in decreased O-GlcNAc levels and conversely, increased O-GlcNAc levels correlate with decreased phosphorylation levels. This reciprocal relationship between O-GlcNAc and phosphorylation has been termed the "Yin-Yang hypothesis" and has gained strong biochemical support by the recent discovery that the enzyme OGTforms a functional complex with phosphatases that act to remove phosphate groups from proteins. Like phosphorylation, O-GlcNAc is a dynamic modification that can be removed and reinstalled several times during the lifespan of a protein. Suggestively, the gene encoding O-GlcNAcase has been mapped to a chromosomal locus that is linked to AD. Hyperphosphorylated tau in human AD brains has markedly lower levels of O-GlcNAc than are found in healthy human brains. Very recently, it has been shown that O-GlcNAc levels of soluble tau protein from human brains affected with AD are markedly lower than those from healthy brain. Furthermore, PHF from diseased brain was suggested to lack completely any O-GlcNAc modification whatsoever. The molecular basis of this hypoglycosylation of tau is not known, although it may stem from increased activity of kinases and/or dysfunction of one of the enzymes involved in processing O-GlcNAc. Supporting this latter view, in both PC-12 neuronal cells and in brain tissue sections from mice, a nonselective N-acetylglucosaminidase inhibitor, which also potently inhibits lysosomal hexosaminidases, was used to increase tau O-GlcNAc levels, whereupon it was observed that phosphorylation levels decreased. The implication of these collective results is that by maintaining healthy O-GlcNAc levels in AD patients, such as by inhibiting the

action of O-GlcNAcase (OGA), one should be able to block hyperphosphorylation of tau and all of the associated effects of tau hyperphosphorylation, including the formation of NFTs and downstream effects. However, because the proper functioning of the lysosomal β-hexosaminidases is critical, any potential therapeutic intervention for the treatment of AD that blocks the action of O-GlcNAcase would have to avoid the concomitant inhibition of both lysosomal hexosaminidases A and B.

Consistent with the known properties of the hexosamine biosynthetic pathway, the enzymatic properties of O-GlcNAc transferase (OGT), and the reciprocal relationship between O-GlcNAc and phosphorylation, it has been shown that decreased glucose availability in brain leads to tau hyperphosphorylation. The gradual impairment of glucose transport and metabolism leads to decreased O-GlcNAc and hyperphosphorylation of tau (and other proteins). Accordingly, the inhibition of O-GlcNAcase should compensate for the age-related impairment of glucose metabolism within the brains of health individuals as well as patients suffering from AD or related neurodegenerative diseases.

These results suggest that a malfunction in the mechanisms regulating tau O-GlcNAc levels may be vitally important in the formation of NFTs and associated neurodegeneration. Good support for blocking tau hyperphosphorylation as a therapeutically useful intervention comes from studies showing that when transgenic mice harboring human tau are treated with kinase inhibitors, they do not develop typical motor defects and, in another case, show a decreased level of insoluble tau. These studies provide a clear link between lowering tau phosphorylation levels and alleviating AD-like behavioral symptoms in a murine model of this disease.

There is also a large body of evidence indicating that increased levels of O-GlcNAc protein modification provides protection against pathogenic effects of stress in cardiac tissue, including stress caused by ischemia, hemorrhage, hypervolemic shock, and calcium paradox. For example, activation of the hexosamine biosynthetic pathway (HBP) by administration of glucosamine has been demonstrated to exert a protective effect in animal models of ischemia/reperfusion, trauma hemorrhage, hypervolemic shock and calcium paradox. Moreover, strong evidence indicates that these cardioprotective effects are mediated by elevated levels of protein O-GlcNAc modification. There is also evidence that the O-GlcNAc modification plays a role in a variety of neurodegenerative diseases, including Parkinson's disease and Huntington's disease.

Humans have three genes encoding enzymes that cleave terminal β-N-acetyl- glucosamine residues from glycoconjugates. The first of these encodes the enzyme O-glycoprotein-2-acetamido-2-deoxy-β-D-glucopyranosidase (O-GlcNAcase or OGA). O-GlcNAcase is a member of family 84 of glycoside hydrolases. O-GlcNAcase acts to hydrolyze O-GlcNAc off of serine and threonine residues of post-translationally modified proteins. Consistent with the presence of O-GlcNAc on many intracellular proteins, the enzyme O-GlcNAcase appears to have a role in the etiology of several diseases including type II diabetes, AD and cancer. Although O-GlcNAcase was likely isolated earlier on, about 20 years elapsed before its biochemical role in acting to cleave O-GlcNAc from serine and threonine residues of proteins was understood. More recently O-GlcNAcase has been cloned, partially characterized, and suggested to have additional activity as a histone acetyltransferase.

However, a major challenge in developing inhibitors for blocking the function of mammalian glycosidases, including O-GlcNAcase, is the large number of functionally related enzymes present in tissues of higher eukaryotes. Accordingly, the use of non-selective inhibitors in studying the cellular and organismal physiological role of one particular enzyme is complicated because complex phenotypes arise from the concomitant inhibition of such functionally related enzymes. In the case of β-N-acetylglucosaminidases, existing compounds that act to block O-GlcNAcase function are nonspecific and act potently to inhibit the lysosomal β-hexosaminidases.

Another challenge is the site-specific detection of O-GlcNAC modifications. Tau is subject to O-GlcNAcylation at several serine and threonine residues including Thr123, Ser 208, Ser356, Ser400 and either Ser409, 412, or 413. 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. 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. 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.

Biochemical methods to sensitively label and identify O-GlcNAcylated proteins 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 (Snow et al., J Cell Biol 1987 104(5): 1143-1156; Teo et al., Nat Chem Biol 2010 6: 338-343). The deficiencies of polyclonal antibodies are also widely recognized. The pattern seen when such antibodies are used on mammalian brain material shows a high degree of nonspecific binding. A polyclonal rabbit antibody recognizing the neuronal protein tau when O-GlcNAcylated at Ser400, termed 3925, has been described (Yuzwa et al., Amino Acids 2011 40(3): 857-868) but the antibody showed strong cross-reactivity with other proteins in rat brain lysates limiting its use for in-vivo studies. Immunocytochemistry with such an antibody must be viewed as of dubious provenance. Further, it is widely known that Western blots are low-throughput and semi-quantitative in nature. Western blots are not amenable to compound screening campaigns.

There is a need for a specific antibody that is site-specific for O-GlcNAc tau. Therefore, the technical problem forming the basis of the present invention is to provide an antibody, which allows the reliable and unequivocal detection of O-GlcNAc modification. It is another problem to provide a cellular assay for detecting O-GlcNAc modifications of proteins.

The present invention solves the first problem by providing a monoclonal antibody comprising a heavy chain variable domain V_H having an amino acid sequence of SEQ ID NO: 1 and/or a light chain variable domain V_L having an amino acid sequence of SEQ ID NO: 2, or an antigen-binding variant, mutant, part of the amino acid sequence or at least 95% homologous sequence thereof.

It has been surprisingly demonstrated by the inventors that said antibody specifically recognizes O-GlcNAcylated tau at serine 400. As tau comes in several different isoforms, this serine has a different number in each isoform. Serine 400 typically refers to tau isoform 2N4R (NCBI Reference Sequence NP_005901). However, all the other isoforms are subsumed under the scope of protection and are applicable in the invention. Reference to serine 400 associated with the specified isoform 2N4R shall not be understood to limit the scope of protection since the protein members of the group represented by tau may be replaced by each other. The teaching of the present specification concerning a specific tau isoform, such as 2N4R, is considered as valid and applicable without restrictions to other members of the tau group if expedient therein. It is also not excluded that further tau isoforms will be characterized, which also exhibit a high homology to some or even all members of the tau group as well as a O-GlcNAc modification at serine. Therefore, the

teaching of the present invention is not restricted to the currently known tau isoforms, but shall cover each tau isoform of high homology to them and O-GlcNAc modification at serine. The isoforms of EEF1A can be easily assigned by the accession numbers, which are generally accepted and fixed in numerous data bases, such as NCBI, GenBank, SwissProt and the like.

The antibody of the invention is particularly mono-specific, which guarantees an exclusive and directed interaction with the O-GlcNAc modification site. In an aspect of the invention, the antibody binds the Ser-400 tau O-GlcNAc modification with an EC50 of less than 1 µM. In another aspect of the invention, the antibody binds the Ser-400 tau O-GlcNAc modification with a specificity which outnumbers the nonspecific binding by at least an order of magnitude, preferably two orders of magnitude. As a consequence, the superiority of the antibody according to the invention is that it appears to be cleaner. Further, the antibody of the invention that bind the O-GlcNAcylation of the substrate tau in a site-specific manner can be advantageously used in a cellular assay, which is more quantitative, reproducible and higher throughput compared with Western blots in the art. In an aspect of the invention, a signal specific to the Ser-400 tau O-GlcNAc modification is detected with the antibody in a concentration of 100 ng/ml or less, preferably 10 ng/ml or less, m ore preferably 1 ng/ml or less. The antibody and its use in a cellular assay according to the invention are powerful tools to measure changes of GlcNAcylation directly on the protein of interest.

The inventive antibody denotes a polypeptide encoded by an immunoglobulin gene, or fragments thereof. The antibody comprises at least one light chain and/or at least one heavy chain, preferably at least one light chain and at least one heavy chain, more preferably two light chains and two heavy chains, each of them as defined hereunder. That means, the light chain comprises at least a single CDR, particularly of rabbit origin, in the variable region of said light (V_L) chain and optionally at least a single FR in the variable region of said light (V_L) chain, preferably at least said CDR and at least said FR. The heavy chain comprises at least a single CDR, particularly of rabbit origin, in the variable region of said heavy (V_H) chain and/or at least a single FR in the variable region of said heavy (V_H) chain, preferably at least said CDR and at least said FR. Within the antigen-binding portion of an antibody, the CDRs directly interact with the epitope of the antigen while the FRs maintain the tertiary structure of the paratope. In both the light chain and the heavy chain of immunoglobulins, there are three to four framework regions (FR-1 through FR-4) separated respectively by three complementarity determining regions (CDR-1 through CDR-3). The

CDRs or hyper-variable regions, in particular the CDR-3 regions, more particularly the heavy chain CDR-3, are largely responsible for antibody affinity and specificity.

In an aspect of the invention, the light chain variable region (V_L) comprises two CDRs, more preferably three CDRs, most preferably together with the same number of FRs or even one FR more. In still another preferred embodiment of the invention, the heavy chain variable region (V_H) comprises two CDRs, more preferably three CDRs, most preferably together with the same number of FRs or even one FR more. In another aspect of the invention, the antibody of the invention comprises the light chain variable region (V_L) and the heavy chain variable region (V_H), each of the regions comprises two CDRs, most preferably three CDRs, highly preferably together with the same number of FRs or even one FR more.

In other words, the antibody of the invention shall comprise at least that minimum scaffold from a variable region of a single chain, which confers binding capacity to the Ser-400 tau O-GlcNAc modification. According to the invention, the antibody can also be present as a number of other well-characterized fragments of an immunoglobulin or even as an intact immunoglobulin provided that the aforementioned minimum scaffold is given. Fragments are preferably selected from the group comprising heavy chain (H), light chain (L), variable regions (V), single chain variable fragment (scFv), F_{ab} fragments consisting of a covalently bound antibody light chain and a portion of the antibody heavy chain (F_d), and the like.

The light chain of the antibody can additionally comprise a constant region of the light (C_L) chain. Similarly, the heavy chain of the antibody can additionally comprise a constant region of the heavy (C_H) chain, or a portion thereof, wherein the portion especially refers to the constant region within the F_d region. The F_d fragment is the major determinant of antibody specificity and retain epitope-binding ability in isolation. The antibody of the invention can also be completed by F_c fragment as effector of the complement cascade, which is not involved in antigen binding. Fragments, such as F_{ab} and F_c fragments, can be produced by cleavage using various peptidases. Furthermore, fragments can be engineered and recombinantly expressed, preferably scFv.

In the scope of the invention, the antibody is of monoclonal origin. The great advantages of monoclonal antibodies include an immortal source of reagents, stable antibody properties and precise specificity. Popular techniques for producing monoclonal antibodies, such as the hybridoma technology, are also well-known to the skilled artisan. In contrast, polyclonal antibodies are usually produced in mammal organisms when an immune response is

caused by antigens being strange to the organism and having a molecular weight that exceeds 3.000 g/mol.

Favorable host species for antibody production comprise rat, goat, rabbit and mouse, more preferably rabbit. The rabbit antibodies, more preferably rabbit monoclonal antibodies, exhibit higher affinity along with a wider range of epitope recognition than mouse monoclonals, while due to divergence in the immune systems, and extended CDRs, stronger responses to epitopes, preferably human epitopes, can be produced compared to murine responses. It shall be understood that chimeric antibodies can be genetically engineered, which CDRs, FRs and/or constant regions are derived from different mammalian sources provided that one or more CDRs have a rabbit source. Accordingly, chimeric antibodies can be obtained by replacing not only the CDR but the whole variable regions of the light and heavy chains of non-rabbit origin. The affinity of the antigen-binding sites can be alternatively influenced by selective exchange of some amino acids within the variable regions.

The basic principal for making monoclonal rabbit antibodies are described in WO 2011/020529, for example. Following the immunization of rabbits, the spleen is taken from those rabbits producing polyclonal serum. The isolated rabbit B cells of the immunized rabbits are fused with a rabbit plasmocytoma cell line to produce stable hybridomas. The hybridoma cells are tested for secretion of antibodies, which are specific for the immunogen, and they can be subsequently cloned. The original establishment of the rabbit hybridomas fusion partner cell line is described by Spieker-Polet et al., PNAS USA 1995, 92(20): 9348-9352. Further developments of the fusion partner cell line are disclosed in US 7,429,487 B2. Still further methods are published in the US Appl. Nos. 10/705,109; 10/266,387; 10/313,881; 10/350,841 and 11/476,277. The cDNA of inserts encoding the antibody is preferably cloned, sequenced and inserted in an expression vector to allow production of wholly defined antibodies. The skilled artisan knows suitable techniques for the recombinant production of antibodies, such as in the EBNA cell expression system according to Pham et al., Biotech Bioeng 2003, 84(3): 332-342.

It shall also be understood that variants, mutants, parts of said amino acid sequences or homologous sequences having the same function are included in the scope of definition as well as protection. The degree of alteration between the original sequence and its derivatives is inevitably limited by the requirement of antigen recognition within the structural context. A couple of methods are known to the skilled artisan to generate equivalent peptides and proteins, i.e. amino acid sequences that are analogous in function

to those of the inventive teaching by realizing the benefits of the invention to a large extent. Therefore, the invention also contains the alterations as listed herein. Variants of the amino acid sequences underlying the antibody of the invention can arise from modifications (e.g. alkylation, arylation or acetylation of at least a single amino acid), incorporation of enantiomers, addition of at least a single amino acid and/or fusion with another peptide or a protein. Possible mutations comprise deletion, insertion, substitution, translocation and/or inversion. Parts of the amino acid sequences and antibodies, respectively, relate to a restriction to those regions that are sufficient for the expression of a specific function. The parts of the antibody can be very small due to the characterization of the paratope, for instance, which also binds to an antigen as tau. In the meaning of the invention, it is to be clearly distinguished between parts of any size and homologous sequences; the homology of the latter is related to the entire sequence. Preferably, the homology between an original sequence and its derivatives having the same features amounts to at least 80%, more preferably at least 95%, most preferably at least 98%. Similarly, the homology is to be considered if the aforementioned part of any size is altered to a variant or mutant. The present teaching if solving the problem of the invention covers all peptide derivatives, which are developed on the basis of the present ingredients by such procedures.

Moreover, several techniques are described in prior art to generate non-homologous peptides with the same function. Herein, non-homologous peptides denote amino acid sequences having less homology compared to the preferred amounts of homology above. For example, it is possible to replace a single amino acid or multiple amino acids without adversely affecting the activity with respect to accomplishing the object of the present invention. For replacement of such amino acids, reference is made to appropriate standard textbooks of biochemistry and genetics. As well-known to those skilled in the art, some amino acids have analogous physicochemical properties and hence, these amino acids can be advantageously replaced by each other. These include the amino acid groups (a) glycine, alanine, valine, leucine and isoleucine, (b) serine and threonine, (c) asparagine and glutamine, (d) aspartic acid and glutamic acid, (e) lysine and arginine, and (f) phenylalanine, tyrosine and tryptophan. Amino acids within one and the same group (a) to (f) can be replaced among one another. Further alterations are possible in accordance with the teaching of Schneider et al., PNAS 1998, 95: 12179-12184; WO 1999/62933 or WO 2002/38592, describing one way of generating functionally analogous amino acid sequences. All amino acid sequences, sequence parts or structures comprising sequences, which are designed by using the cited methods and starting from any amino acid sequence of the invention, are considered as sequences in the meaning of the

invention, and they shall be included in the teaching according to the invention, provided they accomplish the object of the invention.

Object of the invention is also a polynucleotide encoding the antibody according to the invention, or a fragment thereof. The term “polynucleotide” refers to a natural or synthetic polymer of single or double-stranded DNA or RNA alternatively including synthetic, non-natural or modified nucleotides, which can be incorporated in DNA or RNA polymers. Each nucleotide consists of a sugar moiety, a phosphate moiety, and either a purine or pyrimidine residue. The nucleic acids can be optionally modified as phosphorothioate DNA, locked nucleic acid (LNA), peptide nucleic acid (PNA) or spiegelmer. The term "polynucleotide encoding" refers to that part of a gene which enciphers a protein, a polypeptide or a part thereof. The regulatory sequences and/or elements controlling the initiation or termination of transcription are excluded. The coding sequence and/or the regulatory element can normally be found in cells, in which case it is referred to as autologous one or endogenic one, or it cannot be located in cells, in which case it is referred to as heterologous one.

In a preferred embodiment of the present invention, the polynucleotide encoding the antibody of the invention comprises one or two nucleic acid sequences selected from the group of SEQ ID NOs: 3 and 4. The prior teaching of the present specification concerning the antibody and specific amino acid sequences thereof is considered as valid and applicable without restrictions to the polynucleotide and specific nucleic acid sequences if expedient.

Another object of the invention concerns a vector comprising the antibody-encoding polynucleotide according to the invention as described above. The term "vector" denotes a recombinant DNA construct which can be a plasmid, a virus, an autonomously replicating sequence, a phage, or a nucleotide sequence, which is linear or circular, consisting of single or double-stranded DNA or RNA, wherein a number of nucleotide sequences are linked or recombined to form a unique construction, and which is capable of introducing a promoter fragment and a DNA sequence of a selected gene product in sense or antisense orientation into a cell, together with suitable non-translated 3' sequences.

It is preferred that a plasmid comprises the antibody-encoding polynucleotide of the invention, particularly to clone and express recombinant genes of the inventive antibody or a fragment thereof. In the meaning of the invention, plasmids are genetic elements which are stable inherited without being part of the chromosome of their host cell. They may

comprise DNA or RNA, and they can be both linear and circular. Plasmids encode molecules ensuring their replication and stable inheritance during cell replication. The starting plasmids disclosed in the present specification are either commercially available, accessible to the public, or can be constructed from available plasmids by routine use of well-known, published methods. Many plasmids and other cloning and expression vectors, which can be used according to the invention, are well-known and easily available to the skilled artisan. Furthermore, a person skilled in the art can easily construct any number of other plasmids suitable for the use in this invention.

The vector shall be suitable for introduction into host cells. Accordingly, a host cell comprising the vector with the antibody-encoding polynucleotide is still another object of the invention. The present invention preferably relates to isolated prokaryotic or eukaryotic cells, but it shall also cover cell cultures, tissues, organs, and the like, and even organisms, which comprise the host cell of the invention, including an above-described vector. The term "host cell" denotes a cell that has been genetically modified by the transfer of a chimeric, heterologous or autologous nucleic acid sequence or derivatives thereof still including said sequence. These cells are also referred to as transgenic cells. Where an autologous nucleic acid sequence is transferred, the number of copies of this sequence in the host cell is higher than that of the naturally occurring sequences.

The invention also relates to a method for preparing a rabbit antibody comprising the steps of: (i) immunizing a rabbit with an immunogen comprising an amino acid sequence of SEQ ID NO: 5; (ii) obtaining a polyclonal antiserum comprising polyclonal antibodies from the rabbit; and (iii) preparing the monoclonal antibody. The prior teaching concerning antibody alterations is considered to be valid and applicable without restrictions to altered immunogens of step (i) if expedient. As obvious to the skilled artisan, the present invention shall not be construed to be limited to the full-length peptide. Physiological or artificial fragments and secondary modifications of the peptide, species-dependent alterations as well as allelic variants of the peptide are also encompassed by the present invention. In this regard, an "allelic variant" is understood to represent the gene product of one of two or more different forms of a gene or DNA sequence that can exist at a genetic single locus. Artificial fragments preferably encompass a peptide produced synthetically or by recombinant techniques, which at least comprises the epitopes of diagnostic interest.

The mammal immunization and serum extraction of steps (i) and (ii) follow well known techniques and good laboratory practice, such as described in the course of the specification and examples. Sera of step (ii) are subsequently tested for the presence of

polyclonals, and the detected antibodies are screened for antigen recognition. Suitable tests and screens are available to those skilled in the art. Further, the antibody preparation is continued to the species of mono-specific, identical antibodies, i.e. monoclonals of step (iii). Monoclonal antibodies are typically made by fusing myeloma cells with the spleen cells from the mammal that has been immunized according to step (iii). A selective HAT medium containing hypoxanthine, aminopterin and thymidine is particularly used in which only fused cells can grown. The so-called hybridomas are then diluted and clones are grown from single parent cells on microtiter wells. The antibodies secreted by the different clones are tested for their ability to bind to the O-GlcNAc tau peptide.

Although the most productive and stable clone can be grown in culture medium to a high volume, the monoclonal of choice is preferably expressed in a recombinant fashion. It requires cDNA cloning of the antibody encoding inserts, sequencing and inserting in expression vectors to allow production of wholly defined antibodies. Subsequently, the invention also relates to a method for manufacturing a recombinant monoclonal antibody or a fragment thereof comprising the steps of (1) introducing vector(s), which comprises nucleic acid sequence(s) of SEQ ID NOs: 3 and/or 4 into a host cell, (2) cultivating the host cell in a culture medium, thereby expressing the encoded antibody or fragment thereof, and (3) purifying the expressed antibody or fragment thereof. The vector can be introduced by any method of the art, such as transformation, transfection or transduction. It shall be understood that prokaryotic cells, including bacteria and archaea, are particularly transformed, such as Escherichia species or Bacillus species, whereas eukaryotic cells are particularly transfected, such as CHO, HeLa, and the like. The three domain systems can also be transducted by viral vehicles. The vector can comprise either one or more nucleic acid sequences encoding the monoclonal antibody or a fragment thereof. It shall be understood that several vectors are favorably different by bearing only a single sequence of said SEQ ID NOs above. It is preferred in step (1) to introduce two vectors, each of them bearing one sequence of said SEQ ID NOs above.

It is still another object to provide a method for detecting a Ser-400 tau O-GlcNAc modification, comprising the steps of: (a) permeabilizing cells with a surfactant; (b) blocking the cells with a serum protein; (c) incubating the cells with the antibody of the invention; (d) incubating the cells with a fluorescent dye-conjugated secondary antibody; and (e) detecting the Ser-400 tau O-GlcNAc modification by correlating an amount of a fluorescence signal, or change in fluorescence signal, with the presence of said modification.

Cell lysis can be performed in suitable, well-known lysis buffers, which may cause an osmotic shock and perforate the cell membrane. The stability of the cell structure can also be destroyed by mechanical forces, such as ball mill, French press, ultrasonic, etc., by enzymatic degradation of cell wall and cell membrane, respectively, and/or by the action of tensides. The tau protein can be further purified to remove disturbing substances or concentrated in the sample. Downstream-processing and/or concentrating are preferably performed by the method of precipitation, dialysis, gel filtration, gel elution, or chromatography, such as HPLC or ion exchange chromatography. It is recommended to combine several methods for better yields.

Before using antibodies to detect proteins by immunohistochemistry (IHC), all epitopes on the sample should be blocked to prevent the nonspecific binding of the antibodies. Otherwise, the antibodies or other detection reagents may bind to any epitopes on the sample, independent of specificity. In principle, any protein that does not have binding affinity for the target or probe components in the assay can be used for blocking. In practice, however, certain proteins perform better than others, because they more readily bind to the nonspecific sites (also called reactive sites) or stabilize the function of other system components. In fact, no single protein or mixture of proteins works best for all IHC experiments, and empirical testing is critical to obtain the best possible results for a given combination of specific antibodies and substrate system. The blocking step for IHC is performed after all other sample preparation is completed and just prior to incubating the sample with the primary antibody. The general protocol is to incubate the fixed, embedded, mounted, cleared and unmasked IHC sample with the appropriate blocking buffer for a time period from 30 minutes to overnight at either ambient temperature or 4ºC based on the optimized protocol specific to each antibody and target antigen. Sufficient washing after the blocking step is critical to remove excess protein that may prevent detection of the target antigen. Normal serum is a common blocking reagent, because the serum carries antibodies that bind to reactive sites and thus prevents the nonspecific binding of the secondary antibodies used in the assay. A critical factor, though, is to use serum from the species that the secondary antibody was generated in, as opposed to the species of the primary antibody. Serum from the primary antibody species would bind to reactive sites, but the secondary antibody would recognize those nonspecifically-bound antibodies along with the antibodies bound to the target antigen. Besides serum, concentrated protein buffers made with 0.1 to 5% bovine serum albumin (BSA), gelatin or nonfat dry milk is often used to coat all proteins in a sample. This approach essentially forces primary antibodies to out-compete the blocking protein for binding to cognate ligands while reducing nonspecific binding because the antibodies have no greater binding affinity for nonspecific epitopes

than do the buffer proteins. While these buffers can be easily made in the lab, for best results they must be made fresh prior to use, which increases the time and workload of the IHC staining. Ready-made blocking buffers are also available to block samples in preparation for antibody treatment. These buffers can contain highly-purified concentrations of single proteins or proprietary protein-free compounds. A benefit of using commercial blockers is that there are many available options that perform better than gelatin or casein and have improved a shelf life (Thermo Fisher Scientific Website).

In a preferred embodiment of the invention, the cells are blocked with goat serum and BSA in step (b), preferably 5-15% goat serum and 0.5-3% BSA.

In another preferred embodiment of the invention, the cells are incubated with the antibody in a concentration of 100 ng/ml or less, preferably 10 ng/ml or less, more preferably 1 ng/ml or less.

The antibody of the invention is incubated with the cells for detecting the Ser-400 tau O-GlcNAc modification. The cells are contacted with the antibody of the invention for a distinct period, which depends on the kind of cellular material, antibody and/or antigen. The incubation process also depends on various other parameters, e.g. the sensitivity of detection, which optimization follows routine procedures known to those skilled in the art. Adding chemical solutions and/or applying physical procedures can improve the accessibility of the target structures in the sample. Specific incubation products are formed as result of the incubation.

An assay suitable to detect and/or quantify the Ser-400 tau O-GlcNAc modification is preferably based on substances specifically interacting with the primary antibody of the invention. The term “specific substances” as used herein comprises molecules with high affinity to the antibody of the invention in order to ensure a reliable binding. The substances are preferably specific to parts of the antibody, e.g. constant regions, particularly rabbit constant regions, more particularly an F_c fragment, if any. There are a distinct number of specific antibodies against rabbit antibodies existing. Parts represent a restriction to those regions which are sufficient for the expression of a specific function, i.e. the provision of a structural determinant for recognition. In the context of the present invention, the term “recognition” - without being limited thereto - relates to any type of interaction between the specific substances and the target antibody, particularly covalent or non-covalent binding or association, such as a covalent bond, hydrophobic/ hydrophilic interactions, van der Waals forces, ion pairs, hydrogen bonds, ligand-receptor interactions, interactions between

epitope and antibody binding site, nucleotide base pairing, and the like. Such association may also encompass the presence of other molecules such as peptides, proteins or other nucleotide sequences.

The specific substances are composed of biological and/or chemical structures capable to interact with the target molecule in such a manner that makes a recognition, binding and interaction possible. The specific substances express a sufficient sensitivity and specificity in order to ensure a reliable detection. A specific substance has at least an affinity of 10^{-7} M for the antibody of the invention. The specific substance has preferably an affinity of 10^{-8} M or even more preferred of 10^{-9} M for its target molecule. As the skilled artisan will appreciate, the term specific is used to indicate that other biomolecules present in the sample do not significantly bind to the substance specific for antibody of the invention. Preferably, the level of binding to a biomolecule other than the target molecule results in a binding affinity of only 10% of the affinity of the target molecule, more preferably only 5% or less. Most preferably, the substances are mono-specific in order to guarantee an exclusive and directed interaction with the chosen primary antibody of the invention. A highly preferred specific substance will fulfill both the above minimum criteria for affinity as well as for specificity.

In particular, the specific substances are selected from the group of proteins, peptides, nucleic acids, carbohydrates, polymers and small molecules having a molecular weight between 50 and 1000 Da, preferably proteins and nucleic acids. The proteins or peptides are preferably selected from the group consisting of antibodies, cytokines, lipocalins, receptors, lectins, avidins, lipoproteins, glycoproteins, oligopeptides, peptide ligands and peptide hormones. More preferably, antibodies are used as specific substance. The nucleic acids are preferably single or double stranded DNA or RNA, primers, antisense oligonucleotides, ribozymes, DNA enzymes, aptamers and/or siRNA, or parts thereof. More preferred nucleic acid probes are aptamers, most preferably RNA aptamers since the 2'-hydroxyl group available in RNA promotes a couple of intra- and intermolecular contacts. Aptamers can be synthesized using standard phosphoramidite chemistry. In addition, RNA aptamers having more than approximately 30 nucleotides can be favorably synthesized in large amounts by in-vitro transcription. Selection, synthesis, and purification of aptamers are well-known to those skilled in the art.

The specific substances can be labeled; in doing so the labeling depends on the inherent features of specific substances and specific incubation products to be monitored, as well as the detection method to be applied, i.e. the required sensitivity, ease of conjugation,

stability requirements, and available instrumentation and disposal provisions. A labeling method is not particularly limited as long as a label is easily detected. A "labeled specific substance" is one that is bound, either covalently through a linker or a chemical bond, or non-covalently through ionic, van der Waals, electrostatic, hydrophobic interactions or hydrogen bonds, to a label such that the presence of the antibody of the invention may be detected by detecting the presence of the label.

Specific immunological binding of an antibody to a protein can be detected directly or indirectly. Hereunder, the antibody-to-protein pair shall be understood to include either the primary antibody of the invention or a secondary antibody directed to the primary antibody. Preferred examples of suitable detection methods according to the present invention are luminescence, particularly fluorescence, furthermore VIS coloring and/or radioactive emission.

Luminescence concerns the emission of light as a result of chemiluminescence, bioluminescence or photoluminescence. Chemiluminescence involves the emission of visible light as a result of a chemical reaction, whereas bioluminescence requires the activity of luciferase. The presently preferred photoluminescence, which is also known as fluorescence stimulation, is caused by the absorption of photons, preferably provided by radiation, which is released again as photon with a shift in wavelength of 30 to 50 nm and within a period of approximately 10^{-8} seconds. The instruments for fluorescence detection include, but are not limited to typical benchtop fluorometers, fluorescence multi-well plate readers, fiber optic fluorometers, fluorescence microscopes and microchips/microfluidics systems coupled with fluorescence detection.

VIS coloring denotes the visualization of any achromatic substance in order to be visible to the naked eye. Preferably, the intensity of coloring is measured by a photometer.

Radioactive radiation of isotopes is measured by scintillation. The process of liquid scintillation involves the detection of beta decay within a sample via capture of beta emissions in a system of organic solvents and solutes referred to as the scintillation cocktail. The beta decay electron emitted by radioactive isotopes such as ^{3}H, ^{14}C, ^{32}P, ^{33}P and ^{35}S in the sample excites the solvent molecule, which in turn transfers the energy to the solute. The energy emission of the solute (the light photon) is converted into an electrical signal by a photo-multiplier tube within a scintillation counter. The cocktail must also act as a solubilizing agent keeping a uniform suspension of the sample. Gamma ray photons often arise as a result of other decay processes (series decay) to rid the newly

formed nucleus of excess energy. They have no mass and produce little if any direct ionization by collision along their path. Gamma photons are absorbed for detection and quantization by one or more of three mechanisms: the Compton effect, the photoelectric effect and pair production. A favorable gamma decay isotope of the present invention is ^{125}I.

Direct labels include fluorescent or luminescent tags, metals, dyes, radionuclides, and the like, attached to the antibody. An antibody labeled with iodine-125 (^{125}I) can be used. A chemiluminescence assay using a chemiluminescent antibody specific for the protein is suitable for sensitive, non-radioactive detection of protein levels. An antibody labeled with fluorochrome is also suitable. Examples of fluorochromes include, without limitation, DAPI, fluorescein, Hoechst 33258, R-phycocyanin, B-phycoerythrin, R-phycoerythrin, rhodamine, Texas red, and lissamine. Indirect labels include various enzymes well known in the art, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), β-galactosidase, urease and the like. The horseradish-peroxidase detection system can be used, for example, with the chromogenic substrate tetramethylbenzidine (TMB), which yields a soluble product in the presence of hydrogen peroxide that is detectable at 450 nm. The alkaline phosphatase detection system can be used with the chromogenic substrate p-nitrophenyl phosphate, for example, which yields a soluble product readily detectable at 405 nm. Similarly, the ß-galactosidase detection system can be used with the chromogenic substrate o-nitrophenyl-ß-D-galactopyranoxide (ONPG), which yields a soluble product detectable at 410 nm. A urease detection system can be used with a substrate, such as urea-bromocresol purple.

In a preferred embodiment of the present invention, the antibodies are labeled with detectable moieties, which include, but are not limited to, radionuclides, fluorescent dyes, e.g. AlexaFluor488, fluorescein, fluorescein isothiocyanate (FITC), Oregon Green™, rhodamine, Texas red, tetrarhodimine isothiocynate (TRITC), Cy3, Cy5, IRDye680LT, IRDye800CW, etc., fluorescent markers, e.g. green fluorescent protein (GFP), phycoerythrin, etc., auto-quenched fluorescent compounds that are activated by tumor-associated proteases, enzymes, e.g. luciferase, HRP, AP, etc., nanoparticles, biotin, digoxigenin, and the like.

In another preferred embodiment of the present invention, the nucleic acids are labeled with digoxigenin, biotin, chemiluminescence substances, fluorescence dyes, magnetic beads, metallic beads, colloidal particles, electron-dense reagents, enzymes, or radioactive

isotopes. Preferred isotopes for labeling nucleic acids in the scope of the invention are ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S or ^{125}I, more preferred ^{32}P, ^{33}P or ^{125}I.

A variety of immunoassay techniques, including competitive and non-competitive immunoassays, can be used (e.g. WO 2010/051882). The term "immunoassay" encompasses techniques including, without limitation, flow cytometry, FACS, enzyme immunoassays (EIA), such as enzyme multiplied immunoassay technique (EMIT), enzyme-linked immunosorbent assay (ELISA), IgM antibody capture ELISA (MAC ELISA) and microparticle enzyme immunoassay (MEIA), furthermore capillary electrophoresis immunoassays (CEIA), radio-immunoassays (RIA), immunoradiometric assays (IRMA), fluorescence polarization immunoassays (FPIA) and chemiluminescence assays (CL). If desired, such immunoassays can be automated. Immunoassays can also be used in conjunction with laser induced fluorescence. Liposome immunoassays, such as flow-injection liposome immunoassays and liposome immunosensors, are also suitable for use in the present invention. In addition, nephelometry assays, in which the formation of protein/antibody complexes results in increased light scatter that is converted to a peak rate signal as a function of the marker concentration, are suitable for use in the methods of the present invention. In a preferred embodiment of the present invention, the incubation products are detected by ELISA, RIA, fluoro immunoassay (FIA) or soluble particle immune assay (SPIA).

A signal from the direct or indirect label can be analyzed, for example, using a spectrophotometer to detect color from a chromogenic substrate, using a radiation counter to detect radiation, such as a gamma counter for detection of ^{125}I, or using a fluorometer to detect fluorescence in the presence of light of a certain wavelength. For detection of enzyme-linked antibodies, a quantitative analysis can be made using a spectrophotometer, such as an EMAX Microplate Reader (Molecular Devices; Menlo Park, CA) in accordance with the manufacturer's instructions. If desired, the assays of the present invention can be automated or performed robotically, and the signal from multiple samples can be detected simultaneously. Optical images viewed and optionally recorded by a camera or other recording device (e.g. a photodiode and data storage device) are optionally further processed in any of the embodiments herein, e.g. by digitizing the image and storing and analyzing the image on a computer. A variety of commercially available peripheral equipment and software is available for digitizing, storing and analyzing a digitized video or digitized optical image. One conventional system carries light from the specimen field to a cooled charge-coupled device (CCD) camera, in common use in the art. A CCD camera includes an array of picture elements (pixels). The light from the specimen is imaged on the

CCD. Particular pixels corresponding to regions of the specimen are sampled to obtain light intensity readings for each position. Multiple pixels are processed in parallel to increase speed. The apparatus and methods of the invention are easily used for viewing any sample, e.g. by fluorescent or dark field microscopic techniques.

In another embodiment of the screening method, the detection of the OGA inhibitory activity can be additionally refined. For this purpose, the level of Ser-400 tau O-GlcNAc modification is determined by correlating an amount of signal, or change in signal, with the modification level in the system. In other words, the modification level of the O-GlcNAcylated tau form correlates with an amount of an emitted physical signal, or change in an emitted physical signal. The cellular system of the invention is incubated with various concentrations of an identified OGA inhibitor. The amount of emitted signal, or change in signal, observed in the presence of the inhibitor is indicative of the change in modification level experienced by the inhibitor. The change can be then related to the concentration of the inhibitor in the sample, i.e. the calibration curve enables the meter-reading of a matching concentration. Preferably, the calibration curve is based on the Lambert-Beer equation if using UV/VIS coloring or luminescence.

Further, the invention may be practiced as a kit comprising the antibody, polynucleotide, vector or host cell, each of them according to the present invention, in order to perform the inventive use of detecting the Ser-400 tau O-GlcNAc modification. The kit of the invention may include an article that comprises written instructions or directs the user to written instructions for how to practice the method of the invention. In an embodiment, the kit further comprises a reporter moiety or a reporter apparatus. The prior teaching of the present specification concerning the kit ingredients and the use thereof is considered as valid and applicable without restrictions to the kit if expedient.

Object of the present invention is also a method for screening molecules for OGA inhibitory activity, comprising performing the method steps (a) to (e) above, and the further steps of: (a-1) immobilizing cells on a solid support, incubating the cells with one or more molecules for at least 8h in 5-10% CO_2, and fixing the cells with a cross-linking agent, before step (a); and (f) comparing levels of the Ser-400 tau O-GlcNAc modification in the cells incubated with the molecules and control cells not incubated with the molecules, wherein an increased level in the cells incubated with the molecules indicates the OGA inhibitory activity of said molecules.

The term “inhibition” denotes any reduction in glycosidase activity, which is based on the action of specific new biologic entities (NBEs) or new chemical entities (NCEs) capable to interact with the target glycosidase in such a manner that makes recognition, binding and blocking possible. It shall be understood that the term “new” relates to the new functional property of OGA inhibitory activity, which is not necessarily associated with a new structure (e.g. screening of library with known compounds). The chemical entities are characterized by such an appreciable affinity to at least one glycoside hydrolase which ensures a reliable binding and preferably a complete blocking of glycosidase activity.

In a preferred embodiment of the present invention, the glycosidase comprises glycoside hydrolases, more preferably family 84 glycoside hydrolases, most preferably O-glycoprotein-2-acetamido-2-deoxy-β-D-glucopyranosidase (OGA), highly preferably a mammalian O-GlcNAcase. It is particularly preferred that the chemical entities selectively bind an O-GlcNAcase, e.g. thereby selectively inhibiting the cleavage of 2-acetamido-2-deoxy-β-D-glucopyranoside (O-GlcNAc) while they do not substantially inhibit a lysosomal β-hexosaminidase.

A cellular system is defined to be any subject provided that the subject comprises cells. The cell refers to any type of primary cells or genetically engineered cells, whether in the isolated status, in culture, as cell line, assembled in tissue, organs or intact laboratory mammals, provided that they are capable of expressing, or expressing, the glycosidase and tau. It shall also be understood that the cell expresses the glycosidase and tau as inherent pre-condition to put the methods of inhibition into practice. Although it is particularly preferred that the cells are capable of expressing or do express the glycosidase and tau, it shall not be excluded that glycosidase-deficient and/or tau-deficient cells can be used and the glycosidase and tau are artificially added to the cellular system. The assay of the invention can be even completely performed in-vitro such that the cell is waived but a glycosidase is contacted with at least one molecule according to the invention and/or physiologically acceptable salts thereof. Hence, an amount of isolated glycosidase and tau is provided in crude or purified form for this purpose. This teaching of the present specification is also valid and applicable without restrictions to the method for detecting a Ser-400 tau O-GlcNAc modification if appropriate.

The cell sample is stored, such as frozen, cultivated for a certain period or immediately subjected to the assay. Before incubating it with molecules to be screened, the cell sample could be divided into multiple portions. If doing so, at least two portions are provided; one is used for screening while the other one serves as control. Preferably, the number of

portions for screening exceeds the number of control portions. Usually, numerous portions are subjected to a high-throughput screening.

The cells can be immobilized onto a variety of solid supports, such as magnetic or chromatographic matrix particles, the surface of an assay plate (e.g. microtiter wells), pieces of a solid substrate material or membrane (e.g. plastic, nylon, paper) and the like. An assay strip can be prepared by coating the cells in an array on a solid support. This strip can then be dipped into the test sample and processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot. The analysis can be carried out in a variety of physical formats. For example, the use of microtiter plates or automation could be used to facilitate the processing of large numbers of test samples. Alternatively, single sample formats could be developed to facilitate diagnosis or prognosis in a timely fashion. Useful physical formats comprise surfaces having a plurality of discrete, addressable locations for the detection of a plurality of samples. Such formats include protein microarrays or protein chips.

The molecules are composed of biological and/or chemical structures capable to interact with a target molecule. Herein, any component of genomics or proteomics signaling shall be considered as “target molecule”, which is not limited to genes, or a regulator protein or a gene product thereof, or a component of a signal transduction pathway comprising a gene or gene products thereof. Consequently, the specific interaction of molecules may involve either the mere targeting or the induction of alterations in cell function, or it may even include both effects simultaneously.

The molecules to be screened in the method of the invention are not restricted anyway. In particular, the molecules are selected from the group of nucleic acids, peptides, carbohydrates, polymers, small molecules having a molecular weight between 50 and 1000 Da and proteins. These molecules are often available in libraries. It is preferred to incubate a single molecule within a distinct portion of the cell sample. However, it is also possible to investigate the cooperative effect of molecules by incubating at least two molecules within one portion. While a fraction of the cellular system is incubated with one or more molecules to be analyzed, a further portion of cells is incubated in the absence of the molecules and this additional non-treated fraction of the system is used as negative control. It is also possible that the system acts simultaneously as test and control system by determining the status before OGA inhibition and comparing it with the status thereafter.

The term “incubation” denotes the contacting of the molecules with the cells for a distinct period, which depends on the kind of molecules and/or target. The incubation process also depends on various other parameters, e.g. the cell type and the sensitivity of detection. The incubation procedure can be realized without a chemical conversion of inhibitors or may involve a metabolic conversion of pro-inhibitors. In a preferred embodiment of the invention, the cells are incubated with one or more NCEs in step (a-1). For example, WO 2013/028715 A1 discloses suitable NCEs in the meaning of the invention. In another preferred embodiment of the invention the cells are incubated for at least 12 hours in step (a-1), more preferably for at least 16 hours.

To ensure free access of the antibody to its antigen, the cells must be fixed and permeabilized. In general, fixation strengths and times are considerably shorter for cells than on the thicker, structurally complex tissue sections. For immunocytochemistry, sample preparation essentially entails fixing the target cells to the slide. Perfect fixation would immobilize the antigens, while retaining authentic cellular and subcellular architecture and permitting unhindered access of antibodies to all cells and subcellular compartments. Wide ranges of fixatives are commonly used, and the correct choice of method will depend on the nature of the antigen being examined and on the properties of the antibody used. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture. Cross-linking reagents (such as paraformaldehyde) form intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components, and require the addition of a permeabilization step, to allow access of the antibody to the specimen (IHC World Website).

The advantageous biological activity of the molecules can be demonstrated in the cell-culture based assay according to the invention. When testing molecules described herein, or in the prior art (e.g. WO 2013/028715) in the cellular assay, an increase in O-GlcNAcylation (due to the inhibition of OGA) is preferably measured. EC50 is the effective concentration of a molecule that produces 50% of the maximum possible response for that molecule. The molecules exhibit EC50 values in the range of 0.1 µM to 100 µM. It is preferred that the molecules have an activity, as expressed by an EC50 standard, of less than 100 µM, more preferably less than 10 µM, most preferably less than 1 µM, highly preferably less than 0.2 µM.

The identification of effective molecules in the meaning of the invention is indirectly performed by determining the Ser-400 tau O-GlcNAc pattern. The determination is performed at a specified moment and correlated to the signal strength at the beginning of the experiment and the control. For example, the control system is not incubated with the molecules (negative control), or the control system is incubated with a standard molecule having no OGA inhibitory activity (negative control). The control system can also be incubated with a standard molecule having OGA inhibitory activity (positive control). The activity is revealed by a change in glycosylation. Preferably, the modification levels in cells with inhibitor exposure are compared to the modification levels in cells that were not exposed to inhibitors. Pair-wise comparisons are made between each of the treatments. A pair-wise comparison involves that the modification data for tau under a given treatment condition are compared to the modification data for this protein under a second treatment condition. The comparison is performed using suitable statistical technique with the assistance of known and commercially available software programs.

The OGA inhibitory activity of molecules is diagnosed by comparing the level of Ser-400 tau O-GlcNAc modification in the sample with known levels of cells treated with inhibitors and/or not. It shall be understood that the known modifications are statistically proven, therefore representing a certain level or range, respectively. The direction and strength of said modification can also be figured out by the differential glycosylation analysis of the marker protein tau of the invention such that a distinct increase with a certain factor is recognized. Any measured modification, which differs from the modification level of non-stimulated cells, indicates an abnormality of the tested cell sample, whereas a molecule cannot be classified as inhibitor at a modification level which is comparable to the level of non-stimulated cells. It is preferred to measure concentrations, which are higher than that of non-stimulated cells, for detecting OGA inhibition. Using this method, the inventors demonstrated sensitivity to submicromolar or even nanomolar concentrations. The calibration plot reveals that the method can be applied in a dynamic range that spans over a couple of magnitude. Therefore, the method of the invention includes that a level of OGA activity is screened by comparing the level of Ser-400 tau O-GlcNAc modification in the test system with the level in the control system.

It is another preferred aspect of the invention that the inherent inhibitory activity is detected if the level of the Ser-400 tau O-GlcNAc modification is increased in the cellular system in comparison with a negative, positive or negative control system, or if the modification levels are substantially identical in the system and a positive control system. In detail, (A) a higher modification level in the test system in comparison with a negative control system, which is

not incubated with any molecule, indicates said activity, or (B) a substantially identical or higher modification level in the test system in comparison with a positive control system, which is incubated with a standard molecule having OGA inhibitory activity, indicates said activity, or (C) a higher modification level in a relative control system, which is incubated with molecules having a concentration other than that in the test system with the proviso that a lower concentration is assigned to the relative control system, indicates said activity. It shall be understood that it is not required to determine the level by two or all comparisons set forth above but the indication of OGA inhibitory activity resulting from any comparison of modification levels under (A), (B) or (C) inevitably implies said activity in any other comparative determination (whether performed or not).

In another more preferred aspect of the invention, the glycosylation level is increased by a factor of at least 1.5, more preferably at least 2, most preferably at least 5, highly preferably at least 9.

In another preferred embodiment of the invention, the signal is normalized against the cell density determined by Hoechst staining. Hoechst staining is done as a counter-stain to determine if cell density is changed as a result of molecule treatment (i.e., to determine if the molecule is cytotoxic, or stimulates proliferation, etc.). Cell density is used to normalize the signal from the O-tau staining.

As discussed herein, the glycosidase-signaling pathways are relevant for various diseases, preferably neurodegenerative diseases, diabetes, cancer and stress. Accordingly, the molecules are useful in the prophylaxis and/or treatment of diseases that are dependent on the said signaling pathways by interaction with one or more of them. The present invention therefore relates to molecules as inhibitors of the signaling pathways described herein, preferably of the OGA-mediated signaling.

In detail, another aspect of the invention relates to a method for administering a therapeutic molecule to a patient, comprising performing the method steps (a-1) and (a) to (f) with a series of molecules, and the further steps of: (g) identifying the molecule having the greatest inhibitory activity within the series, and (h) administering said molecule to the patient in need of treating Alzheimer's disease.

The susceptibility to treatment with the molecules can be particularly determined by tests, whether in the course of research or clinical application. Typically, a culture of the cell is combined with a molecule, optionally at various concentrations for a period of time, which is

sufficient to allow the active agents to modulate glycosidase activity, usually between about one hour and one week. In-vitro treatment can be carried out using cultivated cells from any sample or cell line.

The host or patient can belong to any mammalian species, for example a primate species, particularly humans; rodents, including mice, rats and hamsters; rabbits; horses, cows, dogs, cats, etc. Animal models are of interest for experimental investigations, providing a model for treatment of human disease.

The inhibition can be monitored by the techniques described in the course of the present specification. An in-vitro use is preferably applied to samples of humans suffering from neurodegenerative diseases, diabetes, cancer and stress. Testing of several specific molecules makes the selection of that active ingredient possible that is best suited for the treatment of the human subject. The in-vivo dose rate of the chosen molecule is advantageously pre-adjusted to the glycosidase susceptibility and/or severity of disease of the respective subject with regard to the in-vitro data. Therefore, the therapeutic efficacy is remarkably enhanced.

The invention relates to the administration of the molecule as a medicament. A "medicament" in the meaning of the invention is any agent in the field of medicine, which comprises one or more molecules or preparations thereof (e.g. a pharmaceutical composition or pharmaceutical formulation) and can be used in prophylaxis, therapy, follow-up or aftercare of patients who suffer from diseases, which are associated with OGA activity, in such a way that a pathogenic modification of their overall condition or of the condition of particular regions of the organism could establish at least temporarily.

Consequently, the invention also relates to the administration of a pharmaceutical composition comprising the active molecule in an effective amount with pharmaceutically tolerable adjuvants and/or excipients. The pharmaceutical composition of the invention is produced in a known way using common solid or liquid carriers, diluents and/or additives and usual adjuvants for pharmaceutical engineering and with an appropriate dosage. In the meaning of the invention, an "adjuvant" denotes every substance that enables, intensifies or modifies a specific response against the active ingredient of the invention if administered simultaneously, contemporarily or sequentially. Known adjuvants for injection solutions are, for example, aluminum compositions, such as aluminum hydroxide or aluminum phosphate, saponins, such as QS21, muramyldipeptide or muramyltripeptide, proteins, such as gamma-interferon or TNF, M59, squalen or polyols. Further, the amount of

excipient material that is combined with the active ingredient to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Suitable excipients include organic or inorganic substances that are suitable for the different routes of administration, such as enteral (e.g. oral), parenteral or topical application. Examples of suitable excipients are water, vegetable oils, benzyl alcohols, alkylene glycols, polyethylene glycols, glycerol triacetate, gelatin, carbohydrates, e.g. lactose or starch, magnesium stearate, talc and petroleum jelly.

Pharmaceutical formulations can be adapted for administration via any desired suitable method, for example by oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intra-muscular, intravenous or intradermal) methods. Pharmaceutical formulations adapted for preferred oral administration can be administered as separate units, such as, for example, capsules or tablets; powders or granules; solutions or suspensions in aqueous or non-aqueous liquids; edible foams or foam foods; or oil-in-water liquid emulsions or water-in-oil liquid emulsions. Such formulations can be prepared using processes known in the pharmaceutical art by, e.g., combining the active ingredient with the excipient(s) or adjuvant(s). It goes without saying that, in addition to the above particularly mentioned constituents, the formulations may also comprise other agents usual in the art with respect to the particular type of formulation; thus, for example, formulations which are suitable for oral administration may comprise flavors.

Accordingly, the invention also relates to the administration of a pharmaceutical composition comprising as active ingredient an effective amount of at least one molecule together with pharmaceutically tolerable adjuvants for oral administration, optionally in combination with at least another active pharmaceutical ingredient.

The terms “effective amount” or “effective dose” or “dose” are interchangeably used herein and denote an amount of the pharmaceutical molecule having a prophylactically or therapeutically relevant effect on a disease or pathological conditions, i.e. which causes in a tissue, system, animal or human a biological or medical response which is sought or desired, for example, by a researcher or physician. A “prophylactic effect” reduces the likelihood of developing a disease or even prevents the onset of a disease. In the meaning of the invention, prophylactic treatment is advisable if the subject possesses any preconditions for the aforementioned physiological or pathological conditions, such as a familial disposition, a genetic defect, or a previously passed disease. A “therapeutically relevant effect” relieves to some extent one or more symptoms of a disease or returns to

normality either partially or completely one or more physiological or biochemical parameters associated with or causative of the disease or pathological conditions. In addition, the expression “therapeutically effective amount” denotes an amount which, compared with a corresponding subject who has not received this amount, has the following consequence: improved treatment, healing, prevention or elimination of a disease, syndrome, condition, complaint, disorder or side-effects or also the reduction in the advance of a disease, complaint or disorder. The expression “therapeutically effective amount” also encompasses the amounts which are effective for increasing normal physiological function.

The respective dose or dosage range for administering the pharmaceutical composition according to the invention is sufficiently high in order to achieve the desired prophylactic or therapeutic effect of reducing symptoms of the aforementioned diseases. It will be understood that the specific dose level, frequency and period of administration to any particular human will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general state of health, gender, diet, time and route of administration, rate of excretion, drug combination and the severity of the particular disease to which the specific therapy is applied. Using well-known means and methods, the exact dose can be determined by one of skill in the art as a matter of routine experimentation.

Pharmaceutical formulations can be administered in the form of dosage units which comprise a predetermined amount of active ingredient per dosage unit. The concentration of the prophylactically or therapeutically active ingredient in the formulation may vary from about 0.1 to 100 wt %. Preferably, the molecules are administered in doses of approximately 0.5 to 1000 mg, more preferably between 1 and 700 mg, most preferably 5 and 100 mg per dose unit. Generally, such a dose range is appropriate for total daily incorporation. In other terms, the daily dose is preferably between approximately 0.02 and 100 mg/kg of body weight. The specific dose for each patient depends, however, on a wide variety of factors as already described in the present specification (e.g. depending on the condition treated, the method of administration and the age, weight and condition of the patient). Preferred dosage unit formulations are those which comprise a daily dose or part-dose, as indicated above, or a corresponding fraction thereof of an active ingredient. Furthermore, pharmaceutical formulations of this type can be prepared using a process which is generally known in the pharmaceutical art.

Although a therapeutically effective amount of a molecule has to be ultimately determined by the treating doctor or vet by considering a number of factors (e.g. the age and weight of the animal, the precise condition that requires treatment, severity of condition, the nature of the formulation and the method of administration), an effective amount of a molecule for the treatment of neurodegenerative diseases, for example Alzheimer's disease, is generally in the range from 0.1 to 100 mg/kg of body weight of the recipient (mammal) per day and particularly typically in the range from 1 to 10 mg/kg of body weight per day. Thus, the actual amount per day for an adult mammal weighing 70 kg is usually between 70 and 700 mg, where this amount can be administered as a single dose per day or usually in a series of part-doses (such as, for example, two, three, four, five or six) per day, so that the total daily dose is the same. An effective amount of a salt or solvate or of a physiologically functional derivative thereof can be determined as the fraction of the effective amount of the molecule per se. It can be assumed that similar doses are suitable for the treatment of other conditions mentioned above.

The pharmaceutical composition can be employed as medicament in human and veterinary medicine. According to the invention, the molecules are suited for the prophylactic or therapeutic treatment and/or monitoring of diseases that are caused, mediated and/or propagated by OGA activity. It is particularly preferred that the diseases are neurodegenerative diseases, diabetes, cancer and stress, more preferably neurodegenerative diseases, most preferably tauopathies, highly preferably Alzheimer's disease.

The neurodegenerative disease or condition is more preferably selected from the group of Alzheimer's disease, Amyotrophic lateral sclerosis (ALS), Amyotrophic lateral sclerosis with cognitive impairment (ALSci), Argyrophilic grain dementia, Bluit disease, Corticobasal degeneration (CBP), Dementia pugilistica, Diffuse neurofibrillary tangles with calcification, Down's syndrome, Familial British dementia, Familial Danish dementia, Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), Gerstmann-Straussler-Scheinker disease, Guadeloupean parkinsonism, Hallevorden-Spatz disease (neurodegeneration with brain iron accumulation type 1), Multiple system atrophy, Myotonic dystrophy, Niemann-Pick disease (type C), Pallido-ponto-nigral degeneration, Parkinsonism-dementia complex of Guam, Pick's disease (PiD), Postencephalitic parkinsonism (PEP), Prion diseases (including Creutzfeldt-Jakob Disease (GJD), Variant Creutzfeldt-Jakob Disease (vCJD), Fatal Familial Insomnia, Kuru, Progressive supercortical gliosis, Progressive supranuclear palsy (PSP), Richardson's syndrome, Subacute

sclerosing panencephalitis, Tangle-only dementia, Huntington's disease and Parkinson's disease. Most preferred is Alzheimer's disease.

All references cited herein are incorporated by reference in their entirety in the disclosure of the invention, particularly WO 2010/051882, WO 2011/020529 and WO 2013/028715.

It is to be understood that this invention is not limited to the particular molecules, pharmaceutical compositions, uses and methods described herein, as such matter can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention, which is only defined by the appended claims. As used herein, including the appended claims, singular forms of words such as "a," "an," and "the" include their corresponding plural referents unless the context clearly dictates otherwise. Thus, e.g., reference to "a molecule" includes a single or several different molecules, and reference to "a method" includes reference to equivalent steps and methods known to a person of ordinary skill in the art, and so forth. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art to which this invention belongs.

The techniques that are essential according to the invention are described in detail in the specification. Other techniques which are not described in detail correspond to known standard methods that are well known to a person skilled in the art, or the techniques are described in more detail in cited references, patent applications or standard literature. Other microorganisms, cell lines, plasmids, promoters, resistance markers, replication origins, and the like, which are not mentioned in the application, are commercially available. Provided that no other hints in the application are given, they are used as examples only, they are not considered to be essential according to the invention, but they can be replaced by other suitable tools and biological materials. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable examples are described below. The following examples are provided by way of illustration and not by way of limitation. Within the examples, standard reagents and buffers that are free from contaminating activities (whenever practical) are used. The examples are particularly to be construed such that they are not limited to the explicitly demonstrated combinations of features, but the exemplified features may be unrestrictedly combined again provided that the technical problem of the invention is solved. Similarly, the features of any claim can be combined with the features of one or more other claims.

FIGURE LEGENDS

Figure 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. EC50 for the O-GlcNAc tau peptide was 785 nM and lowest limit of detection was at 1 ng/ml Otau(S400).

Figure 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). (C) Western blot of lysates from HEKtau and HEK cells treated with 10 µM ThiametG and probed with antibodies against O-GlcNAc tau at Ser400 (Otau(S400)) and total tau (tau-5).

Figure 3: (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 O-GlcNAc tau at Ser400 (Otau(S400)), total tau (HT7) and GAPDH. 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 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.

Figure 4: Effects of acute or subchronic ThiametG on O-GlcNAcylation and phosphorylation in Tg4510 mice. A, Total O-GlcNAcylation levels were increased in mouse hemi forebrain 4h after a single administration of ThiametG or 4h after 14 daily repeated treatments with ThiametG. B, Immunoprecipitated tau (HT7 antibody) was strongly O-GlcNAcylated at S400 in animals treated for 14 days as compared to vehicle controls. C, Tau O-GlcNAcylation protein levels were slightly increased in mouse hemi forebrain 4h after a single treatment of ThiametG (%) and significantly increased 4h after the last of 14 daily treatments with ThiametG. D, Tau phosphorylation was decreased at epitopes S202/205, S262, and S396 4hrs after single administration of ThiametG, but returned to normal levels following 14 daily treatments with ThiametG. Tau phosphorylation at S356 was significantly reduced following a single and repeated (14day) administration of ThiametG (1 way ANOVA * $p < .05$). Western blot data (N=13-15/group) are expressed as mean±s.e.m. percentage of vehicle-treated controls. 1 way ANOVA; * $P < .05$ as compared to control.

Figure 5: Effects of chronic ThiametG treatment on tau O-GlcNAcylation and pathological tau in Tg4510 mice. A, Tau O-GlcNAcylation levels remain elevated in mouse hemi forebrain following 4 months administration of ThiametG. B, Hyperphosphorylated pathological tau (64 kD) is dramatically reduced at epitopes pS202/205, pS400, pS356, and pS262 following 4 months administration of ThiametG. C, Localized expression of O-GlcNAc tau (Otau(S400) antibody; top panel) and AT8 (middle panel), which recognizes hyperphosphorylated aggregated tau was detected in the CA1 region of the hippocampus in Tg4510 mice. Dual-immunostaining was performed to demonstrate no colocalization of O-GlcNAc tau with pathological tau (bottom panel, 63x image). D, Tau phosphorylation status of the 50-60 kD tau species was unchanged following 4 months repeated administration with ThiametG. Western blot data (N=13-15/group) are expressed as mean±s.e.m. percentage of vehicle-treated controls. 1 way ANOVA; * $P < .05$ as compared to control.

Figure 6: Effects of chronic ThiametG treatment on tau dystrophic neurons and tangles in the hippocampus. A, AT8 positive neurons are significantly reduced in the CA1 and CA3 region of the hippocampus following 4 months administration of ThiametG. B, Agyrophilic fibers (as measured via Bielschowsky stain) are significantly reduced in the CA1 region of the hippocampus, but not the CA3 region following 4 months administration of ThiametG. IHC and Bielschowsky quantification (N=13-15/group) are expressed as mean±s.e.m. percentage of vehicle-treated controls.

Figure 7: (A) Western blot of lysates from HEKtau-CGFP, -NGFP and HEKtau cells OGT- or mock transfected and probed with antibodies against O-GlcNAc tau at Ser400 (Otau(S400)) and total tau (tau-5). (B) GFP/terbium signal ratio in TR-FRET experiment with HEKtau-CGFP and HEKtau cells OGT- or mock transfected and incubated with 3 nM Tb-Otau(S400).

EXAMPLE 1: Generation and characterization of a rabbit monoclonal antibody site-specific for tau O-GlcNAcylated at serine 400

MATERIALS & METHODS

Generation of Otau(S400) antibody: Two peptides were synthesized to ~90% purity and conjugated to four different carrier proteins, KLH, OVA, Blue-Carrier, and BSA:

(i) O-GlcNAc tau peptide: cVYKSPVV-(O-GlcNAc)S-GDTSPRH (SEQ ID NO: 5)

(ii) Unglycosylated tau peptide: cVYKSPVVSGDTSPRH (SEQ ID NO: 6).

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 1:64,000 dilution). One rabbit was chosen for a final i.v. 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.

An appropriately sized column containing recombinant protein A (rPA) resin was equilibrated in 5 column volumes of equilibration buffer (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0) and subsequently loaded with culture supernatant from the hybridoma cells. After washing in 5 volumes wash buffer (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0) the loaded column was transferred to an AKTA FPLC station for antibody elution. Bound antibodies were eluted in elution buffer (50 mM sodium phosphate, 150 mM sodium chloride, pH2.5) and collected in fractionation tubes and immediately adjusted to pH 6.0 with neutralization solution (1 M Tris base). Tubes containing peak fractions of purified antibodies were pooled and Tween80 was added to a final concentration of 0.05%. The purified antibodies were filtered through a 0.22 µM filter and subjected to endotoxin and SDS-PAGE analysis to determine the purity of the sample.

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 OD405 nm. EC50s were calculated using GraphPad Prism software.

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. 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).

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 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 1000g 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.

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. 2 µg of eluate was subjected to Western blotting as described below.

ThiametG treatment of Tg21221 and JNPL3 mice: 18 weeks old male JNPL3 mice were obtained from Taconic Farms. Tg21221 mice were generated as described elsewhere (Hoover et al. Neuron 2010 68: 1067-1081) and bred at the McLaughlin Research Institute (MRI). 3 months old females and male Tg21221 (n=15/group) were used. 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 p.o. at a concentration of 500 mg/kg/day for 5 consecutive days in JNPL3 mice and for 1 or 14 consecutive days in Tg21221 mice. Water was used for the vehicle treated group.

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 (15,000g) to remove cellular debris. The ensuing supernatant was assayed by DC protein assay (500-0113, 500-0114, 500-0115, BioRad) to determine protein concentrations. 20 µg of brain lysate were subjected to Western blotting as described below. To immunoprecipitate tau protein from JNPL3 brain lysate a Crosslink Immunoprecipitation kit (26147, ThermoScientific) 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). 250 µg 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.

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 blocking buffer (927-40000, Licor), then incubated with the appropriate primary antibody for 2 h at room temperature 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) at a dilution of 1:10,000 for 1 h at room temperature. After extensive washing in TBST membranes were scanned on the Licor Odyssey infrared imager. 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, GAPDH at 1:1000 (ab9484, Abcam) and Otau(S400) at 1:1000.

RESULTS

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

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 EC50 of 785 nM towards the O-GlcNAc-tau peptide and was non-reactive to the unglycosylated peptide (Figure 1). The lowest O-GlcNAc tau peptide specific signal could be detected at 1 ng/ml antibody concentration.

(ii) 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 His-tagged human OGT was overexpressed 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 (Figure 2A). A similar increase in signal intensity was observed in the parental HEK293 cell line in the presence of OGT (Figure 2A). When probed with the Otau(S400) antibody a strong immunopositive band with an apparent molecular mass of ~60 kD was detected in OGT transfected, but not in mock transfected HEKtau cells (Figure 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 (Figure 2A). 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, Figure 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 (Figure 2A).

To corroborate that the immunopositive band in the OGT transfected HEKtau cells represented O-GlcNAcylated tau, myc-DDK-tagged tau was affinity-purified 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 kD 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 (Figure 1B). Tau-5 staining confirmed that the immunoprecipitated proteins were indeed tau (Figure 1B). Similar to the previous observation, Otau(S400) positive tau only overlapped with the lower molecular mass tau (see overlay, Figure 2B).

(iii) 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. ThiametG treatment elevated global protein O-GlcNAcylation in HEKtau cells as determined with the RL2 antibody (data not shown). Otau(S400) detected a band at ~60 kD 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 (Figure 2C). Furthermore, this also demonstrates that tau is a substrate for endogenously expressed OGT in HEK cells. HEK293 cells that did not express tau did not show any significant immunoreactivity with Otau(S400) with or without ThiametG treatment (Figure 2C).

(iv) 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. To establish that Otau(S400) recognizes O-GlcNAcylated tau in-vivo, two different tau transgenic animal models were employed. First, Tg21221 mice, a transgenic mouse line that overexpresses human wild type tau (variant 0N4R), were treated with 500 mg/kg ThiametG for 1 or 14 days. A single dose of ThiametG did not significantly increase tau O-GlcNAcylation at Ser400 as compared to vehicle treated animals (Figure 3A). However, two weeks of daily administration of ThiametG elicited a significant ~9fold 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 (Figure 3A). Tg21221 mice do not exhibit 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. 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. Similar to Tg21221 mice a highly significant ~9fold increase in O-GlcNAc tau signal was observed as compared to vehicle treated animals. To confirm that the Otau(S400) immunopositive bands represented tau, tau was immunoprecipitated from JNPL3 brain lysate with the tau-5 antibody and subjected to Western blotting with the Otau(S400) antibody. Strong immunoreactive bands were observed around ~50 kD in the tau preparation isolated from ThiametG treated mouse brain, but a much fainter signal was seen the tau preparation from vehicle treated animals (Figure 3B). Staining with the HT7 antibody that recognizes human tau confirmed that the immunoprecipitated proteins were indeed tau (Figure 3B). 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.

DISCUSSION

The generation of a new rabbit monoclonal antibody, named Otau(S400), is described, which is specific for human tau O-GlcNAcylated at Ser400. The 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 with O-GlcNAc tau in response to treatment with ThiametG, but not in control animals. 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. It is particularly 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 a tool has been established 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.

EXAMPLE 2: Antibody sequencing

In order to obtain the mRNA sequence of the variable region of the Otau(S400) antibody about one million hybridoma cells were subjected to total RNA isolation and RT-PCR. Regions of interest were amplified with specific primers targeting the IgH/K leader and CH1/CK regions of rabbit IgG and ensuing PCR products were re-amplified with nested primers followed by DNA sequencing. Uniform VH and VK gene sequences were detected from several individually cloned PCR amplicons.

EXAMPLE 3: Increased O-GlcNAcylation reduces pathological tau without affecting its normal phosphorylation in a mouse model of tauopathy

MATERIALS & METHODS

Animals: Tg(tauP301L)4510 mice were generated as previously described (Santacruz et al., 2005, Science 309: 476-481). Animals were bred and housed at the McLaughlin Research Institute (Great Falls, Montana). All experiments were approved by the MRI Institutional Animal Care and Use Committee (IACUC). The acute (1day treatment) and subchronic (14 day treatment) effects of ThiametG were evaluated in male and female 3 month old Tg4510 mice. The chronic (4 month) effects of ThiametG were evaluated in male and female Tg4510 mice beginning at 2 months of age. ThiametG was dissolved in water and administered po, at a concentration of 500 mg/kg/day.

O-GlcNAc tau specific antibody (Otau(S400)): To generate a rabbit monoclonal antibody specific for tau O-GlcNAcylated at serine 400 rabbits were immunized with a peptide (cVYKSPVV-(O-GlcNAc)S-GDTSPRH) corresponding to amino acids 393 to 407 on 2N4R human tau. Lymphocytes from rabbits with high titer antisera were isolated and hybridomas generated. IgG antibodies were purified from supernatant of positive hybridoma subclones. The specificity of the antibody was confirmed on Western blots with samples of recombinant O-GlcNAcylated tau and lysates from HEK293 cells coexpressing OGT and human 2N4Rtau (data not shown).

Tau immunoprecipitation: To immunoprecipitate tau protein from brain lysates a Crosslink Immunoprecipitation kit (Pierce 26147) was used. The A/G resin was crosslinked to 10 µg of the HT7 tau antibody (Thermo Scientific MN1000) or control mouse IgG (Santa Cruz Biotech sc-2025) via the manufacturer's protocol. 250 µg of brain lysates prepared as described below was incubated with the resin-coupled tau antibody overnight at 4ºC. Samples were eluted with 50 µl of low pH Elution buffer and immediately centrifuged into collection tubes containing 5 µl of 1M Tris, pH 9.5. Immunoprecipitated tau was subjected to Western blotting as described below.

Western Blot: To examine changes in O-GlcNAcylation and phosphorylation, animals were euthanized 4h after injection in the acute or subchronic studies and 24h after the last injection in the chronic study. Hemi-forebrains were rapidly dissected and frozen on dry ice. Tissue samples were homogenized in Phosphosafe Buffer (EMD Chemicals), followed by a low-speed centrifugation (15,000 g) to remove cellular debris. The resulting supernatant

(low-speed supernatant, Lss) was assayed to determine protein concentrations by Lowry method. O-GlcNAcylation and phosphorylation were determined in 20 µg protein samples subjected to 4-15% SDS-PAGE (Tris-HCl gels, Bio-Rad), followed by a transfer to nitrocellulose membranes (Invitrogen, IBlot system). Membranes were blocked in Licor blocking buffer at room temperature for 1h and incubated in primary antibody overnight at 4°C. Total protein O-GlcNAcylation was detected using the RL2 antibody (1:500, ThermoScientific), tau O-GlcNAcylation was detected using the Otau(S400) antibody (1:500) and tau phosphorylation was detected using AT8 (1:500, ThermoScientific), pS396, pS262, pS356 (1:500, Abcam) and pS400 (1:5000, GenScript). GAPDH (1:1000, Abcam) or total tau (1:50,000 ThermoScientific) antibodies served as internal loading controls. Membranes were incubated with species-specific fluorophore-conjugated secondary (1:10,000; Licor) antibodies for 1h at room temperature and detected using the Licor Odyssey.

Tau fractionation: To analyze 50-60 kD versus 64 kD tau, the Lss fraction containing both 50-60 kD and 64 kD tau species was centrifuged at high speed (110,000 g for 15 min). The supernatant (S1 fraction) containing the 50-60 kD tau proteins was removed and assayed to determine protein concentrations. To analyze changes in 50-60 kD and 64 kD tau, the Lss and S1 fractions were subjected to 10% SDS-PAGE (Tris-HCl gels, Bio-Rad) followed by transfer as described above. 64 kD tau appeared as one compact band with an apparent mass of ~64 kD in whole brain lysate (Lss fraction), but was absent in the supernatant (S1 fraction) after high speed centrifugation, which separates 50-60 kD from 64 kD tau (Figure 5B). 50-60 kD tau appears as several bands with an apparent mass ranging from ~50-60 kD.

Immunohistochemistry: To examine changes in tangle pathology, a hemibrain was dissected and immersion fixed in 10% neutral buffered formalin for 24-48h and subsequently embedded in paraffin blocks. 10 micron serial coronal sections were mounted onto Superfrost Plus slides, and stained using Bond Intense R kit. To detect dystrophic AT8 neurons, mounted slides were pretreated with antigen retrieval solutions for 10 min followed by washes with BOND wash buffer. Sections were subsequently quenched with hydrogen peroxide in Bond Intense R kit, blocked with M.O.M. blocking buffer (M.O.M Immunodetection Kits, Vector Laboratories), and then incubated with pS202/205 primary antibody (1:500; ThermoScientific). Sections were then incubated sequentially with biotinylated donkey anti-mouse secondary antibody (1:200; Jackson Immunoresearch), Streptavidin-HRP, and 3,3'-diaminobenzidine (both BOND Intense R kit). To detect agyrophilic tangles, sections were deparafinized, rehydrated in distilled water and treated

with formaldehyde (4%) overnight at 37°C. Sections were washed in tap water, incubated in a 20% silver nitrate solution for 15 min in the dark, washed, incubated with ammoniated silver solution for 10 min in the dark, washed in ammonia water, and treated with developer. Sections were subsequently washed in ammonia water, distilled water, thiosulfate sodium, dehydrated and mounted.

Immunofluorescence: To examine the colocalization between O-GlcNAcylated tau and AT8, sections were blocked with 5% Normal Goat Serum (Jackson Immunoresearch) for 1h, followed by a sequential incubation with Otau(S400); (1:100, 1h) and AT8 (1:500, 1h). After washing, sections were incubated with secondary FITC conjugated goat anti-rabbit and Texas Red conjugated goat anti-mouse (Invitrogen) antibodies in PBS for 1h. After washing, slides were coverslipped with Prolong Gold anti-fade reagent (Invitrogen).

Statistical Analysis: Protein O-GlcNAcylation and phosphorylation changes were analyzed by one-factor ANOVA, followed by Dunnets post hoc comparisons or by t-test for those studies with only two treatment groups. Immunohistochemistry was analyzed by t-test.

RESULTS

(i) Acute and subchronic OGA inhibition increases tau O-GlcNAcylation and transiently reduces tau phosphorylation

To investigate the effects of increased O-GlcNAcylation on tau phosphorylation, the Tg4510 mouse model was chosen because it closely mimics human tauopathy and represents an important model for the study of tau-related neurodegenerative diseases. Tg4510 mice received either a single or repeated injection of the OGA inhibitor ThiametG or vehicle. ThiametG is a potent inhibitor of OGA with an IC50 of ~5 nM. OGA catalyzes the removal of O-GlcNAc residues from proteins and thus inhibition of OGA results in a relative increase of O-GlcNAc modification on proteins. A significant increase in total protein O-GlcNAcylation in the CNS was observed following either a single injection of ThiametG ($F_{(2, 43)} = 20.98$; $p < .01$ as compared to vehicle-treated; Figure 4A) or 14 days of administration ($F_{(2, 43)} = 12.57$; $p < .01$). The increase in total protein O-GlcNAcylation following 14 days of ThiametG was significantly higher than that following a single injection ($p < .05$).

To specifically investigate the effects of ThiametG treatment on tau O-GlcNAcylation, a rabbit monoclonal antibody specific to O-GlcNAcylation of tau at serine 400 (Otau(S400)) was generated. S400 can be modified by O-GlcNAcylation (Yuzawa et al., 2010, Amino Acids 40: 857-868) and is located between S396 and S404, which are phosphorylation

sites known to be implicated in tau pathology. To confirm that Otau(S400) indeed recognized O-GlcNAcylated tau, tau was immunoprecipitated with a pan-specific tau antibody (HT7) from brains of Tg4510 mice that had been subchronically treated with ThiametG and probed with the Otau(S400) antibody. The Otau(S400) antibody strongly recognized immunoprecipitated tau in the ThiametG treated animals, but only to a much lesser extent in the vehicle-treated animals (Figure 4B). Interestingly, O-GlcNAcylated tau was detected at the lower molecular mass bands of tau, indicating that only a subset of tau was O-GlcNAcylated. Only a small increase in tau O-GlcNAcylation was detected following a single injection of ThiametG. However, repeated injection of ThiametG produced a 9-fold increase in tau O-GlcNAcylation ($F_{(2, 42)} = 22.04$; $p < .05$ as compared to vehicle-treated; Figure 4C). This confirms that tau is a substrate for O-GlcNAcylation and that OGA inhibition robustly increases O-GlcNAc on tau at serine 400 in a mouse model of tau pathology.

A single injection of ThiametG reduced tau phosphorylation at epitopes S202/205 ($F_{(2, 43)} = 43.49$; $p < .05$), S262 ($F_{(2, 43)} = 27.36$; $p < .05$), S356 $F_{(2, 43)} = 33.31$; $p < .05$ and S396 ($F_{(2, 43)} = 22.48$; $p < .05$; Figure 4D). Acute ThiametG treatment did not alter tau phosphorylation at S400, suggesting that O-GlcNAcylation does not regulate tau phosphorylation at this epitope. Interestingly, repeated treatment with ThiametG did not produce a greater reduction in tau phosphorylation at the investigated epitopes. In the case of S202/205, S262 and S396 phosphorylation returned towards basal levels following 14 days of ThiametG, whereas phosphorylation at S356 was still significantly reduced ($F_{(2, 43)} = 26.72$; $p < .05$), but showed a trend towards increased phosphorylation.

(ii) Chronic inhibition of OGA reduces tau pathology

To examine the chronic effects of ThiametG on tau pathology, Tg4510 animals received 4 months of treatment with ThiametG beginning at 2 months of age. Mice were intentionally selected at this age to start the treatment paradigm before any signs of pathological tau accumulation and neurodegeneration. Twenty-four hours after the last injection brain tissue was collected for tau protein analysis via western blot as well as histological analysis for tangles. The levels of total protein O-GlcNAcylation following 4 months of ThiametG were similar (185%) to that produced after the 14-day treatment (data not shown). Furthermore, tau O-GlcNAcylation remained elevated 9-fold following 4 months of dosing, comparable to the level of tau O-GlcNAcylation after 14 days of ThiametG treatment, indicating that tau O-GlcNAcylation reached a steady state already after 2 weeks of OGA inhibition ($T_{27} = 18.95$; $p < .0001$; Figure 5A). Notably, O-GlcNAcylation appeared on tau at the lower molecular mass bands and was absent from the 64 kD band representing pathological tau,

suggesting that only non-pathological tau is O-GlcNAcylated. To corroborate that pathological tau is not O-GlcNAcylated, dual-labeling immunofluorescence experiments were performed on brain slices of ThiametG treated Tg4510 mice with the Otau(S400) antibody (Figure 5C, top panel) and the AT8 antibody (Figure 5C, middle panel), which recognizes hyperphosphorylated aggregated tau. Individual neurons in the CA1 region of the hippocampus showed strong AT8-immunoreactivity in the soma and neurites (Figure 5B, middle panel), whereas O-GlcNAc-tau immunoreactivity was mainly localized to neuronal cell bodies (Figure 5C, top panel). No colocalization of O-GlcNAc-tau with pathological tau was observed (Figure 5C, bottom panel), which agrees with the biochemical analysis that pathological tau species are not O-GlcNAcylated in Tg4510 brains.

Hyperphosphorylated pathological tau was biochemically identified by differential centrifugation of brain homogenate from Tg4510 mice and detection with phospho-tau specific antibodies. Pathological tau appeared as one compact high molecular mass band at around 64 kD in whole brain homogenate (low speed spin fraction; Lss), but was absent in the supernatant after high speed centrifugation (S1 fraction), which separates normal from pathological tau (Figure 5B). The S1 fraction contained tau species with an apparent molecular mass ranging from ~50-60 kD. Chronic treatment with ThiametG significantly decreased 64 kD tau as detected with phosphorylation-specific antibodies directed at S202/205 (T_{27} = 2.984; $p < .01$), S400 (T_{27} = 2.769; $p < .01$), S356 (T_{27} = 2.132; $p < .05$) and S262 (T_{27} = 3.030; $p < .01$; Figure 5B) of tau, indicating that a sustained increase in tau O-GlcNAcylation prevents the accumulation of pathological tau.

There was no change in the phosphorylation state of the 50-60 kD tau species (Figure 5D) at various epitopes implicated in tau aggregation, namely S202/205, S356, and S262. This suggests that a sustained increase in O-GlcNAcylation does not regulate the phosphorylation of the 50-60 kD tau species and thus may prevent pathological tau accumulation independent of the phosphorylation level. This contrasts with the reduction in tau phosphorylation observed after a single injection of ThiametG and is inconsistent with the notion in the art that tau phosphorylation is directly regulated by O-GlcNAcylation through competitive or adjacent site occupancy.

To confirm the effect of OGA inhibition on tau aggregation, tau pathology was assessed histologically in brain slices of ThiametG treated Tg4510 mice. Consistent with the biochemical analysis, chronic treatment with ThiametG significantly reduced pS202/205 (AT8) positive dystrophic neurons in CA1 (T_{26} = 3.053, $p < .01$) and CA3 (T_{25} = 3.046, $p <$

.01) region of the hippocampus (Figure 6). Furthermore, to demonstrate that AT8 immunoreactive neurons indeed reflect tangle bearing neurons, Bielschowsky staining was performed on brain slices of ThiametG and vehicle treated animals. Consistent with AT8 immunohistochemistry, a significant reduction of tangle pathology in the CA1 region of the hippocampus was found ($T_{(25)} = 2.309$; $p < .05$; Figure 6B). However, no difference was observed in tangle burden in the CA3 region of the hippocampus. This may be due to differences in sensitivity for early tau aggregates between the methodologies.

Taken together, the results suggest that increasing O-GlcNAc levels on tau attenuates the formation of pathological tau species in the Tg4510 mouse model.

DISCUSSION

It was shown that chronic pharmacological treatment of the Tg4510 tau mouse model with a potent and selective inhibitor of OGA, ThiametG, results in a significant reduction in tau pathology as measured biochemically and pathologically. A highly significant reduction in pathological 64 kD tau was observed in brain homogenates of ThiametG treated animals. This tau species represents a distinct low speed soluble, but high speed sedimentable pool of aggregated tau, most likely consisting of tau dimers and oligomers. These early tau aggregates precede NFT formation and correlate better with neuronal dysfunction and degeneration than that of sarkosyl-insoluble tau or NFT in Tg4510 mouse brain. Similarly, in certain areas of Alzheimer's Disease (AD) brain neuronal loss and NFT pathology are topographically distinct with the number of degenerated neurons far greater than that of NFT bearing neurons, implying that NFT are unlikely to be the primary neurotoxic agent during disease progression. Moreover, abnormal tau structurally similar to the pathological 64 kD tau species in Tg4510 mice is found in human tauopathies, making these aggregated tau intermediates a potential target for therapeutic treatment. With this study, it was clearly demonstrated that the pathological 64 kD species of tau can be reduced through long term inhibition of OGA, making OGA an attractive molecular target for drug discovery. This observation is also in close agreement with the immunohistological findings that showed significantly fewer neurons immunoreactive with the AT8 antibody, a marker for pathological tau aggregates, in animals treated with ThiametG.

Importantly, markedly stronger O-GlcNAcylation of tau was found in response to chronic OGA inhibition, which may account for the more pronounced effect on pathological tau. The difference in tau O-GlcNAcylation may be explained by the use of the Tg4510 tau mouse model in this study, which transgenically expresses tau at a higher level than the JNPL3 mouse model. Additionally, the site-specific O-GlcNAc-tau antibody may have higher

affinity for tau O-GlcNAcylated at S400 as the 3925 antibody. Notably, in this study O-GlcNAc modification at S400 was only found on tau that migrated at lower molecular mass on polyacrylamide gels and was absent from AT8 immunopositive neurons, suggesting that only non-pathological tau was O-GlcNAcylated. This agrees with the notion that O-GlcNAcylation maintains tau in a state that renders it less prone to aggregation. Consistently, non-pathological tau immunopurified from brains of AD patients was found to be more O-GlcNAcylated than hyperphosphorylated pathological tau.

Chronic OGA inhibition decreased the abundance of pathological tau aggregates in Tg4510 mouse brain without affecting phosphorylation levels of non-pathological tau. This suggests that O-GlcNAcylation may not directly regulate the phosphorylation of tau, but attenuate tau aggregation through a phosphorylation-independent mechanism. Although it cannot be completely ruled out that other O-GlcNAc dependent mechanisms are responsible for the effect on tau aggregation, it is likely that O-GlcNAcylation of tau directly lessen its oligomerization propensity as has been demonstrated in-vitro with truncated forms of O-GlcNAc-modified tau. In this context it is important to note that O-GlcNAcylation at S400 appears to play a predominant role in inhibiting tau oligomerization, which is consistent with the highly significant 9-fold increase in tau O-GlcNAcylation at S400 and the concurrent reduction in tau aggregation in response to chronic OGA inhibition as observed in this study. This protective effect of O-GlcNAcylation on protein aggregation is not singular to tau, as O-GlcNAcylated versions of TAB1 and alpha-synuclein peptides were less prone to oligomerization as compared to their unmodified counterparts. As O-GlcNAcylation prevents different types of amyloidogenic proteins from aggregating, OGA inhibition may provide a therapeutic strategy to a multitude of diseases caused by aberrant protein aggregation beyond AD.

In summary, these data, for the first time, demonstrate that a chronic increase in tau O-GlcNAcylation protects against the formation of hyperphosphorylated tau aggregates, which are closely linked to neurotoxicity observed in AD and other tauopathies. This study strongly supports OGA as a molecular target for a disease-modifying therapy to attenuate the progression of tau pathology in AD and other tauopathies.

EXAMPLE 4: Cellular O-GlcNAcylation assay

B35 rat neuroblastoma cells (ATCC; CRL-2754) were plated in 96 well poly-D-lysine treated plates (BD Falcon; 354640) at a density of 10,000 cells per well in a total volume of 90 µl complete medium. The following day cells were treated with appropriate concentration of a solution of inhibitor for 16h at 37°C in 5% CO_2. Cells were fixed in 100 µl

4% paraformaldehyde for 15 min at room temperature, followed by three washes in PBS buffer. The cells were then permeabilized with 0.1% Triton X-100 for 60 min at room temperature. After three washes in PBS the cells were blocked with 10% goat serum containing 1% BSA in PBS buffer for two hours at room temperature. The cells were then incubated with a monoclonal rabbit antibody specific for tau O-GlcNAcylated at serine 400 (Epitomics) at a 1:1000 dilution overnight at 4°C. The primary antibody was washed off and the cells were incubated with a goat anti-rabbit AlexaFluor488-conjugated secondary antibody (Molecular Probes; A11034), and Hoechst 33342 nuclear dye at a concentration of 1 µg/ml were added. Cells were read on the Acumen Explorer eX3 plate reader. To calculate an EC_{50} the total peak intensity was plotted against the concentration of inhibitor to produce a sigmoidal dose response curve.

EXAMPLE 5: Otau(S400) antibody – LanthaScreen

MATERIALS & METHODS

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-NGFP and tau-CGFP expression plasmids tau cDNA was transferred from the myc-DDK-tau expression vector to the destination vectors pCMV4-AN-mGFP (PS100040, OriGene) and pCMV6-AC-mGFP (PS100048, OriGene) following OriGene's RapidShuttling 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).

TR-FRET assay: 0.85 mg of Otau(S400) antibody was custom-labeled with LanthaScreen amine reactive terbium chelate (Invitrogen) and dialyzed into HBS. The terbium/IgG labeling efficiency was determined as 5.5. HEKtau-CGFP or HEKtau cells were transfected with OGT/pDEST26 in 100 mm dishes with Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. After 16 h incubation cells were transferred to white flat bottom 384 well plates (3570, Corning) at 20,000 cells/well and incubated for 24 h. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.6, 5 mM EDTA, 150 mM NaCl, 1% NP40) containing 3 nM terbium labeled O-tau(S400) antibody for 4 h at room temperature in darkness. Plates were read on an Envision plate reader (Perkin Elmer) with the following settings: excitation filter 340/30 nm, detection filter 1: 520/25 nm, detection filter 2: 495/10 nm, mirror D400/D630 (Lance Dual).

RESULTS

(i) High throughput TR-FRET assay to detect Ser400 tau O-GlcNAcylation

In order to rapidly identify small molecule OGA inhibitors that are able to elevate tau O-GlcNAcylation at Ser400 a sensitive cellular assay is needed that allows for high throughput screening of compounds. To this end the LanthaScreen technology was employed, which is based on time resolved fluorescence resonance energy transfer (TR-FRET) between a fluorescence donor (terbium) and a fluorescence acceptor (GFP). When terbium and GFP labeled probes are brought into proximity, energy transfer takes place causing an increase in acceptor fluorescence and a decrease in donor fluorescence. These fluorescent signals can be read in a time-resolved manner to reduce assay interference and increase data quality. The TR-FRET value is determined as a ratio of the FRET specific signal to that of the signal specific to terbium. HEK293 cells stably expressing human 2N4R tau N- or C-terminally fused to GFP (HEKtau-NGFP or -CGFP) were generated as fluorescence acceptor, and terbium-conjugated Otau(S400) antibody (Tb-Otau(S400)) was obtained as fluorescence donor. FRET will only occur if Tb-Otau(S400) is bound to O-GlcNAcylated tau-GFP. To prove the principle of this assay, human OGT was expressed in HEKtau-NGFP and -CGFP cells. Similarly to OGT transfected HEKtau cells, strong immunoreactivity was observed with the Otau(S400) antibody in cell lysates from both HEKtau-NGFP and -CGFP cells when subjected to Western blotting (Figure 7A), indicating that GFP-tagged tau is also substrate for O-GlcNAcylation in HEK293 cells. To determine the optimal antibody concentration to detect O-GlcNAcylated tau-GFP, a range of Tb-Otau(S400) concentrations was tested on OGT transfected HEKtau-CGFP cells, and a concentration of 3 nM of Tb-Otau(S400) was identified to be optimal (data not shown). Using this optimized protocol a ~3 fold increase was detected in the GFP/Tb signal ratio between HEKtau-CGFP cells transfected with OGT as compared to mock transfected HEKtau-CGFP cells (Figure 7B). Importantly, in the absence of OGT the GFP/Tb signal ratio in HEKtau-CGFP cells was as low as in HEKtau cells (with or without OGT), indicating that background fluorescence from tau-CGFP was low.

EXAMPLE 6: Pharmaceutical preparations

(A) Injection vials: A solution of 100 g of an active ingredient according to the invention and 5 g of disodium hydrogen phosphate in 3 l of bi-distilled water was adjusted to pH 6.5 using 2 N hydrochloric acid, sterile filtered, transferred into injection vials, lyophilized and sealed under sterile conditions. Each injection vial contained 5 mg of active ingredient.

(B) Suppositories: A mixture of 20 g of an active ingredient according to the invention was melted with 100 g of soy lecithin and 1400 g of cocoa butter, poured into moulds and allowed to cool. Each suppository contained 20 mg of active ingredient.

(C) Solution: A solution was prepared from 1 g of an active ingredient according to the invention, 9.38 g of $NaH_2PO_4 \cdot 2\ H_2O$, 28.48 g of $Na_2HPO_4 \cdot 12\ H_2O$ and 0.1 g of benzalkonium chloride in 940 ml of bi-distilled water. The pH was adjusted to 6.8, and the solution was made up to 1 l and sterilized by irradiation. This solution could be used in the form of eye drops.

(D) Ointment: 500 mg of an active ingredient according to the invention were mixed with 99.5 g of Vaseline under aseptic conditions.

(E) Tablets: A mixture of 1 kg of an active ingredient according to the invention, 4 kg of lactose, 1.2 kg of potato starch, 0.2 kg of talc and 0.1 kg of magnesium stearate was pressed to give tablets in a conventional manner in such a way that each tablet contained 10 mg of active ingredient.

(F) Coated tablets: Tablets were pressed analogously to EXAMPLE E and subsequently coated in a conventional manner with a coating of sucrose, potato starch, talc, tragacanth and dye.

(G) Capsules: 2 kg of an active ingredient according to the invention were introduced into hard gelatin capsules in a conventional manner in such a way that each capsule contained 20 mg of the active ingredient.

(H) Ampoules: A solution of 1 kg of an active ingredient according to the invention in 60 l of bi-distilled water was sterile filtered, transferred into ampoules, lyophilized under sterile conditions and sealed under sterile conditions. Each ampoule contained 10 mg of active ingredient.

(I) Inhalation spray: 14 g of an active ingredient according to the invention were dissolved in 10 l of isotonic NaCl solution, and the solution was transferred into commercially available spray containers with a pump mechanism. The solution could be sprayed into the mouth or nose. One spray shot (about 0.1 ml) corresponded to a dose of about 0.14 mg.

CLAIMS

1. A monoclonal antibody comprising a heavy chain variable domain V_H having an amino acid sequence of SEQ ID NO: 1 and/or a light chain variable domain V_L having an amino acid sequence of SEQ ID NO: 2, or an antigen-binding variant, mutant, part of the amino acid sequence or at least 95% homologous sequence thereof.

2. The antibody according to claim 1, wherein the antibody is of rabbit origin.

3. The antibody according to claim 1, wherein the antibody binds specifically a Ser-400 tau O-GlcNAc modification.

4. The antibody according to claim 3, wherein the antibody binds mono-specifically and preferably having an EC50 of less than 1 µM.

5. The antibody according to claim 4, wherein the antibody binds the O-GlcNAc modification of tau isoform 2N4R at serine 400 with a specificity which outnumbers the nonspecific binding by at least an order of magnitude, preferably two orders of magnitude.

6. A polynucleotide encoding the antibody, or an antigen-binding fragment thereof, according to claim 1.

7. The polynucleotide according to claim 6, wherein the heavy chain variable domain V_H of the antibody is encoded by a nucleic acid sequence of SEQ ID NO: 3 and/or the light chain variable domain V_L of the antibody is encoded by a nucleic acid sequence of SEQ ID NO: 4.

8. A method for preparing the monoclonal antibody according to claim 1, comprising the steps of:
(i) immunizing a rabbit with an immunogen comprising an amino acid sequence of SEQ ID NO: 5;
(ii) obtaining a polyclonal antiserum comprising polyclonal antibodies from the rabbit; and
(iii) preparing the monoclonal antibody.

9. A method for detecting a Ser-400 tau O-GlcNAc modification, comprising the steps of:
(a) permeabilizing cells with a surfactant;
(b) blocking the cells with a serum protein;
(c) incubating the cells with the antibody according to claim 1;
(d) incubating the cells with a fluorescent dye-conjugated secondary antibody; and
(e) detecting the Ser-400 tau O-GlcNAc modification by correlating an amount of a fluorescence signal, or change in fluorescence signal, with the presence of said modification.

10. The method according to claim 9, wherein the cells are blocked with goat serum and BSA in step (b), preferably 5-15% goat serum and 0.5-3% BSA, and/or the cells are incubated with the antibody in a concentration of 10 ng/ml or less, preferably 1 ng/ml or less.

11. A method for screening molecules for OGA inhibitory activity, comprising performing the method according to claim 9, and the further steps of:
(a-1) immobilizing cells on a solid support, incubating the cells with one or more molecules for at least 8h in 5-10% CO_2, and fixing the cells with a cross-linking agent, wherein step (a-1) is performed before step (a); and
(f) comparing levels of the Ser-400 tau O-GlcNAc modification in the cells incubated with the molecules and control cells not incubated with the molecules, wherein an increased level in the cells incubated with the molecules indicates the OGA inhibitory activity of said molecules.

12. The method according to claim 11, wherein the cells are incubated with NCEs in step (a-1).

13. The method according to claim 11, wherein the cells are incubated for at least 12 hours in step (a-1), preferably for at least 16 hours.

14. The method according to claim 11, wherein the signal is normalized against cell density determined by Hoechst staining in step (e).

15. A method for administering a therapeutic molecule to a patient, comprising performing the method according to claim 11 with a series of molecules, and the further steps of:
(g) identifying the molecule having the best inhibitory activity, and
(h) administering said molecule to the patient in need of treating Alzheimer's disease.

FIGURE 1

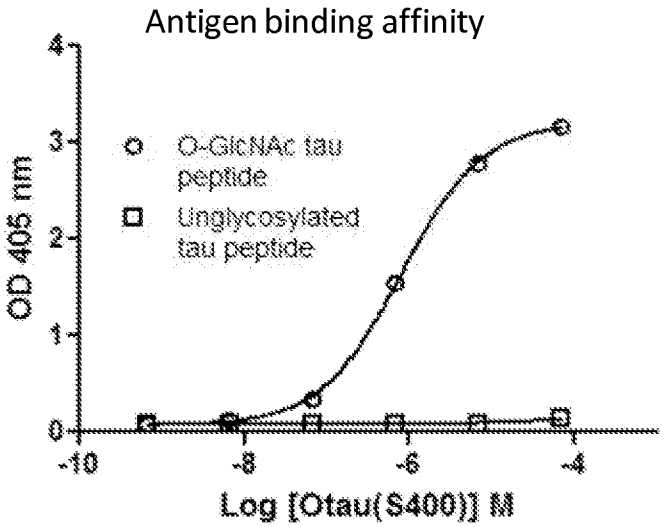
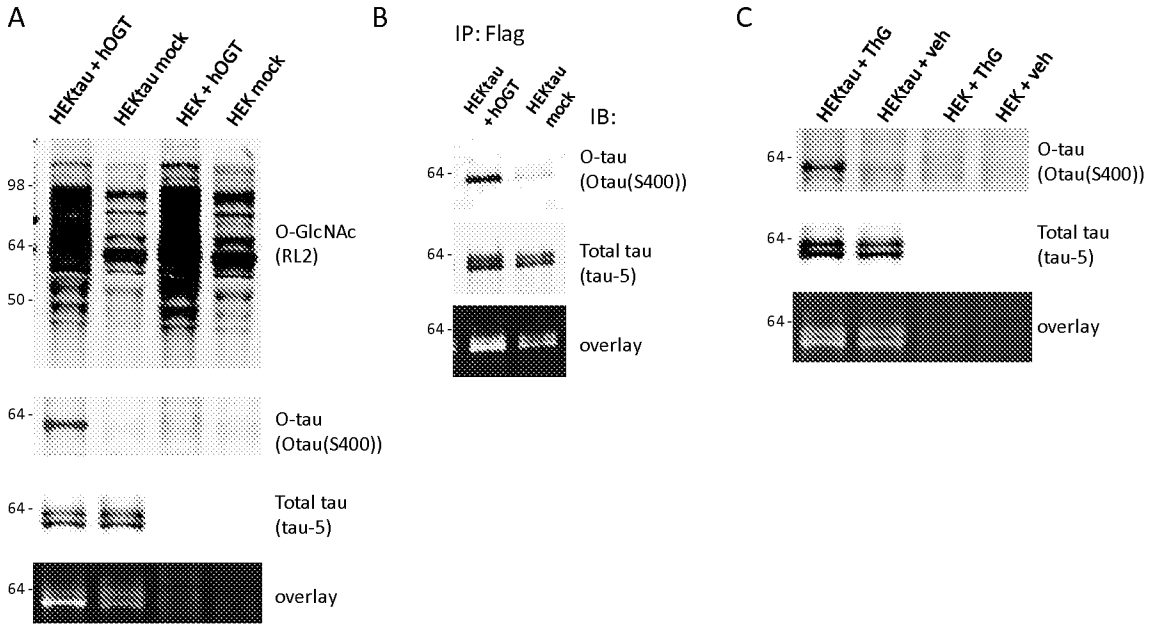

Antigen binding affinity
OD 405 nm
O-GlcNAc tau peptide
Unglycosylated tau peptide
Log [Otau(S400)] M
4
3
2
1
0
-10
-8
-6
-4

FIGURE 2


A
HEKtau + hOGT
HEKtau mock
HEK + hOGT
HEK mock
98
64
50
O-GlcNAc
(RL2)
64
O-tau
(Otau(S400))
64
Total tau
(tau-5)
64
overlay
B
IP: Flag
HEKtau
+hOGT
HEKtau
mock
IB:
64
O-tau
(Otau(S400))
64
Total tau
(tau-5)
64
overlay
C
HEKtau + ThG
HEKtau + veh
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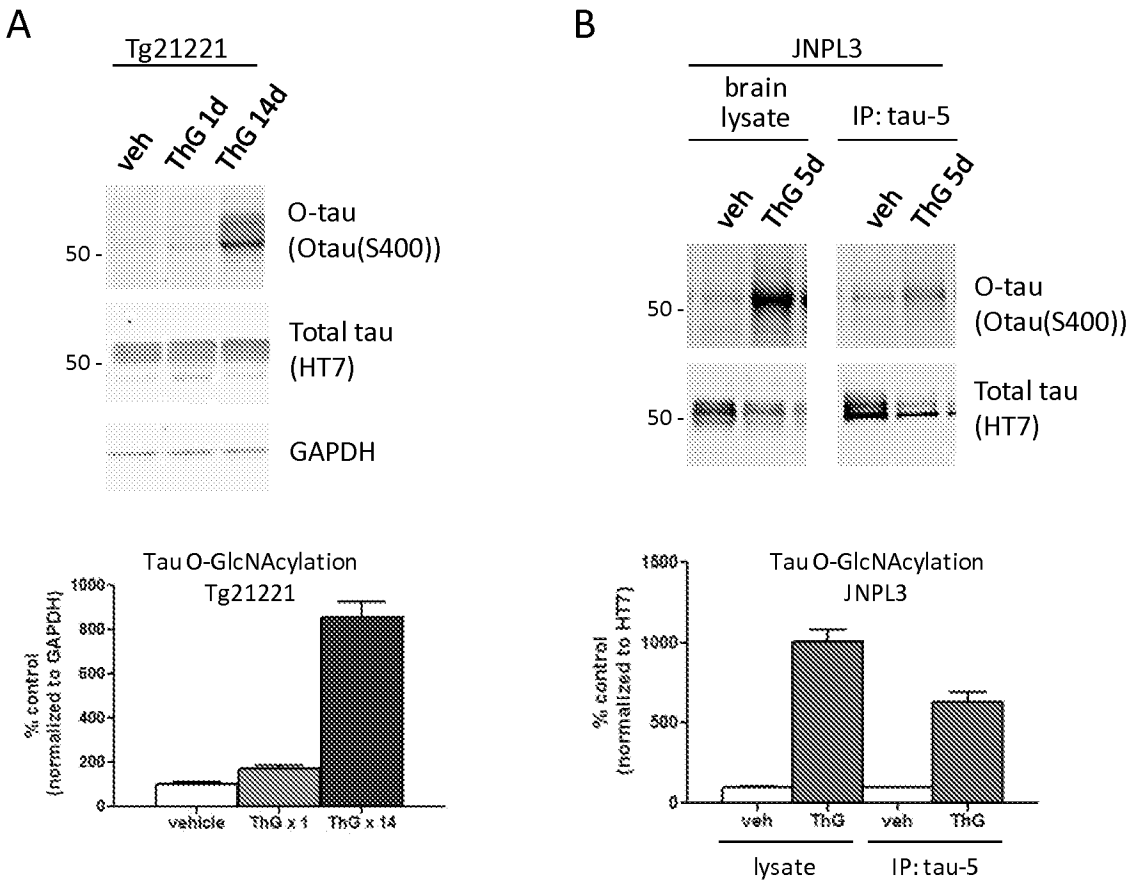
HEK + veh
64
O-tau
(Otau(S400))
64
Total tau
(tau-5)
64
overlay

FIGURE 3


A
Tg21221
veh
ThG 1d
ThG 14d
50 -
O-tau
(Otau(S400))
50 -
Total tau
(HT7)
GAPDH
Tau O-GlcNAcylation
Tg21221
% control
(normalized to GAPDH)
1000
800
600
400
200
0
vehicle
ThG x 1
ThG x 14
B
JNPL3
brain
lysate
IP: tau-5
veh
ThG 5d
veh
ThG 5d
50 -
O-tau
(Otau(S400))
50 -
Total tau
(HT7)
Tau O-GlcNAcylation
JNPL3
% control
(normalized to HT7)
1000
500
0
veh
ThG
veh
ThG
lysate
IP: tau-5

5

FIGURE 4

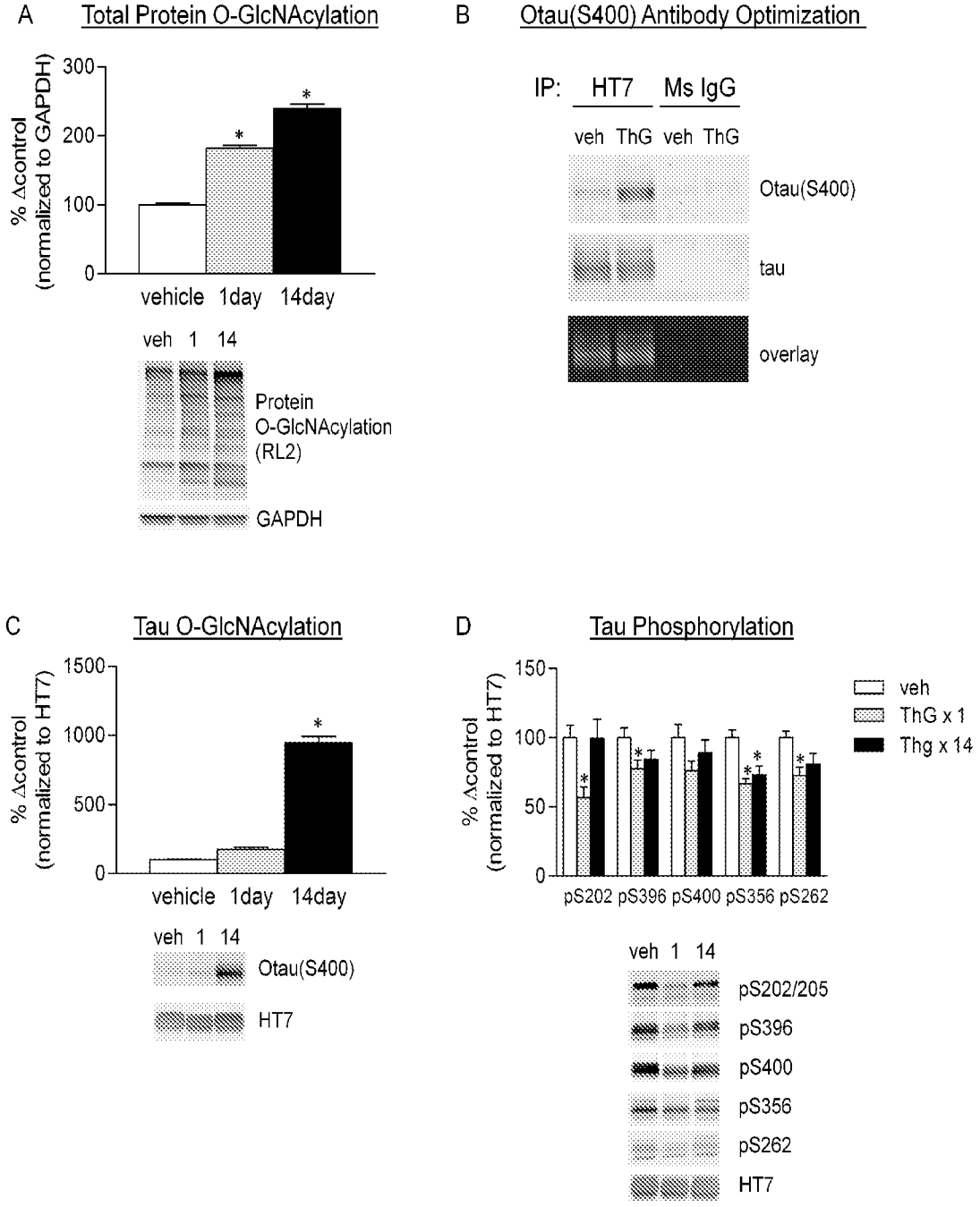

A
Total Protein O-GlcNAcylation
% Δcontrol (normalized to GAPDH)
300
200
100
0
*
*
vehicle 1day 14day
veh 1 14
Protein O-GlcNAcylation (RL2)
GAPDH
B
Otau(S400) Antibody Optimization
IP: HT7 Ms IgG
veh ThG veh ThG
Otau(S400)
tau
overlay
C
Tau O-GlcNAcylation
% Δcontrol (normalized to HT7)
1500
1000
500
0
*
vehicle 1day 14day
veh 1 14
Otau(S400)
HT7
D
Tau Phosphorylation
% Δcontrol (normalized to HT7)
150
100
50
0
veh
ThG x 1
Thg x 14
pS202 pS396 pS400 pS356 pS262
veh 1 14
pS202/205
pS396
pS400
pS356
pS262
HT7

FIGURE 5

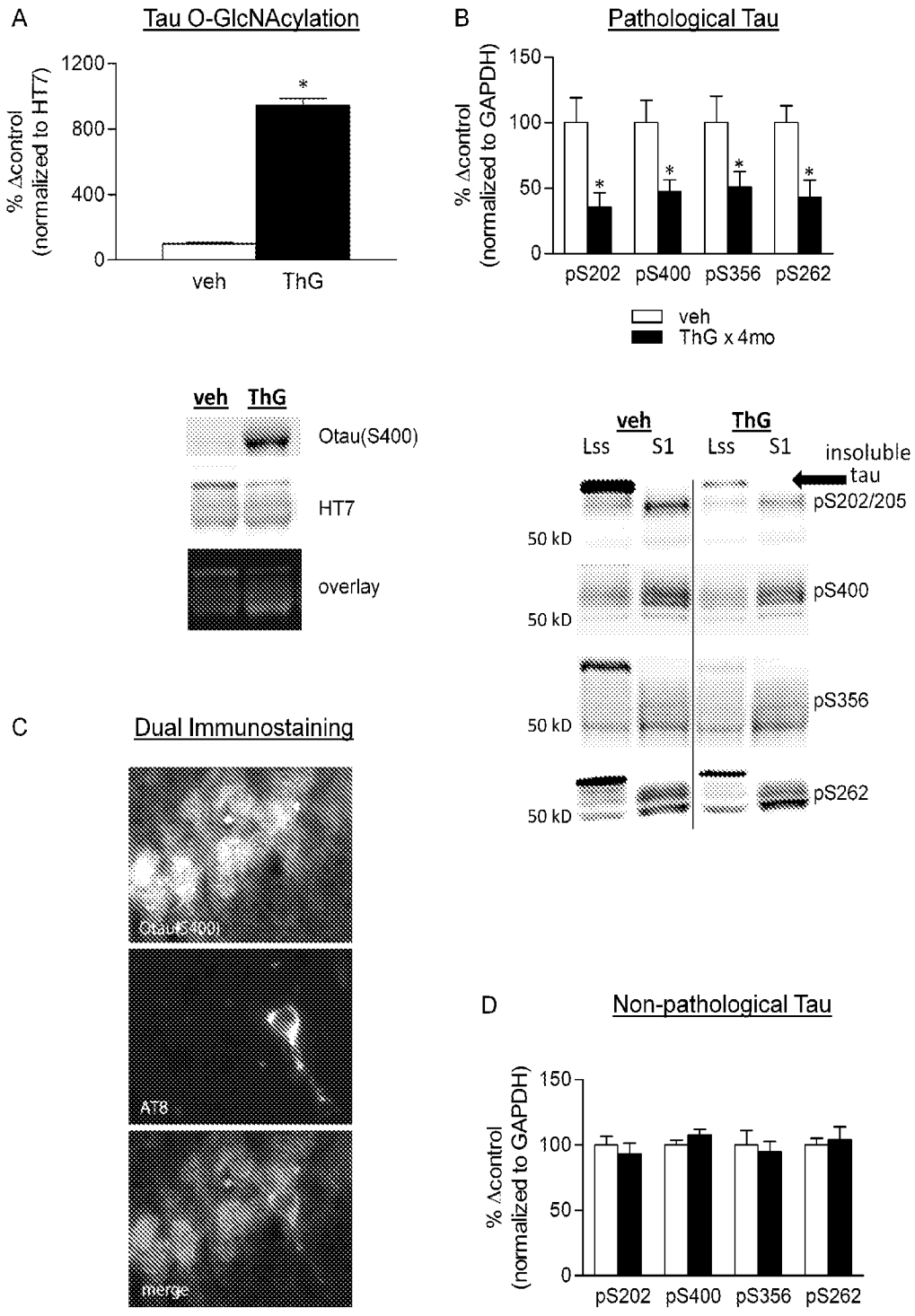
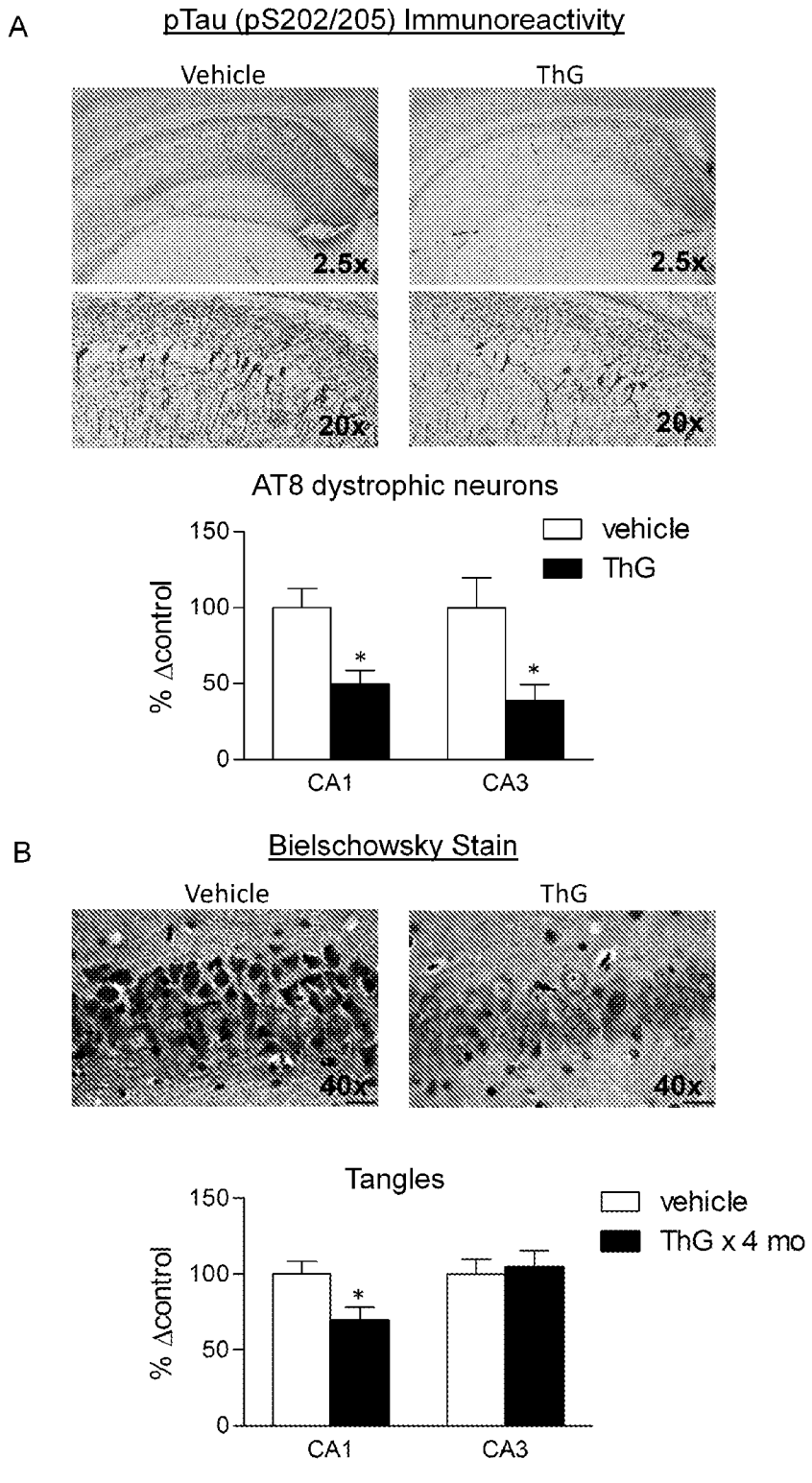
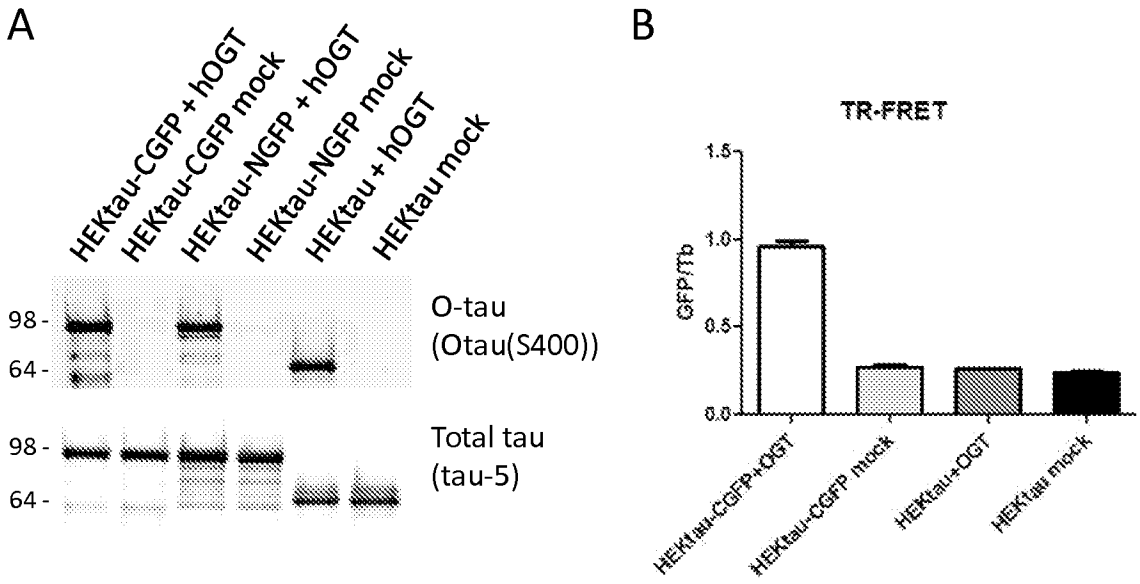

A
Tau O-GlcNAcylation
% Δcontrol (normalized to HT7)
1200
800
400
0
*
veh
ThG
B
Pathological Tau
% Δcontrol (normalized to GAPDH)
150
100
50
0
pS202
pS400
pS356
pS262
veh
ThG x 4mo
veh
ThG
Otau(S400)
HT7
overlay
veh
ThG
Lss
S1
Lss
S1
insoluble tau
pS202/205
50 kD
pS400
50 kD
pS356
50 kD
pS262
50 kD
C
Dual Immunostaining
Otau(S400)
AT8
merge
D
Non-pathological Tau
% Δcontrol (normalized to GAPDH)
150
100
50
0
pS202
pS400
pS356
pS262

FIGURE 6


A
pTau (pS202/205) Immunoreactivity
Vehicle
ThG
2.5x
2.5x
20x
20x
AT8 dystrophic neurons
% Δcontrol
150
100
50
0
vehicle
ThG
*
*
CA1
CA3
B
Bielschowsky Stain
Vehicle
ThG
40x
40x
Tangles
% Δcontrol
150
100
50
0
vehicle
ThG x 4 mo
*
CA1
CA3

5

FIGURE 7


A
HEKtau-CGFP + hOGT
HEKtau-CGFP mock
HEKtau-NGFP + hOGT
HEKtau-NGFP mock
HEKtau + hOGT
HEKtau mock
98 -
64 -
O-tau
(Otau(S400))
98 -
64 -
Total tau
(tau-5)
B
TR-FRET
GFP/Tb
1.5
1.0
0.5
0.0
HEKtau-CGFP+OGT
HEKtau-CGFP mock
HEKtau+OGT
HEKtau mock