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Supplementary Information for

Hydrogen sulfide is neuroprotective in Alzheimer's disease by sulfhydrating GSK3β and inhibiting Tau hyperphosphorylation.

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Supplementary Information Text

Materials and Methods

Immunoprecipitation assays and Western blot analysis

Cells were lysed in buffer (IP buffer), containing 50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10 % glycerol protease inhibitors (cOmplete™, EDTA-free Protease Inhibitor Cocktail from Sigma) and phosphatase inhibitors (PhosSTOP™ phosphatase inhibitor, Sigma) and lysates were cleared by centrifugation at 16,000 g for 10 min followed by recovery of the supernatant. Protein was quantified by Bradford assay and samples were normalized for protein content. Inputs were reserved and 500 μg of protein was incubated with GST beads overnight at 4 °C with rotation. Beads were washed four times in IP buffer, followed by elution into 1X LDS buffer (Stock 4X containing 40% glycerol, 4% lithium dodecyl sulfate (LDS), 0.8 M triethanolamine-Cl pH 7.6, 4% Ficoll®-400, 0.025% phenol red, 0.025% Coomassie G250, 2 mM EDTA disodium from Thermo Fisher Scientific, USA) with 1 mM DTT at 95 °C for 5 min. Samples and inputs were loaded on a mini NuPAGE 4-12% Bis-Tris gel (Thermo Fisher, Scientific USA) and electrophoresed in 1X NuPAGE MES (2-(N-morpholino) ethanesulfonic acid) SDS running buffer (Thermo Fisher Scientific, USA) and immunoblotted with the indicated antibodies. Antibodies used include anti-CSE, generated in-house (1) (1: 4000), anti-FLAG (1: 3000, Sigma), anti-GST-HRP (1:10,000, Sigma), anti-Tau (1: 1000, Santa Cruz Biotechnology), anti-GFP (1:1000, Cell Signaling Technology), anti-p396Tau (1:1000, Santa Cruz Biotechnology), anti-GSK3β (1: 1000, Santa Cruz Biotechnology), anti-β-actin HRP (1:10,000, Santa Cruz Biotechnology). For the *in vitro* immunoprecipitation assays, either anti-Tau antibody or normal mouse IgG control was incubated with Protein A/G agarose overnight at 4 °C with rotation. Antibody-agarose mixture was incubated with purified wild type Tau for 6 h at 4 °C with rotation, washed three times in IP buffer, and incubated with CSE overnight at 4 °C with rotation. Beads were washed four times in IP buffer followed by elution into LDS buffer with 1 mM DTT at 95 °C for 5 min. Samples and inputs were analyzed by western blotting as described above.

Use of the H_2S donor, NaGYY

In this study we have used a sodium salt of an established H_2S generating molecule, GYY4137, NaGYY (2, 3). The reasons for this were due to confounding chemicals present in commercial sources of GYY4137, sold through all major research chemical suppliers is a morpholine salt, present at a 1:1 ratio with the parent compound. However, morpholine itself is biologically active and highly toxic with a well characterized toxicity profile, and LD50 at doses commonly used for GYY4137 (e.g. 200-400 mg/kg i.p.; http://www.inchem.org/documents/ehc/ehc/ehc179.htm). As such its presence would confound interpretation of results generated. In addition, commercial GYY4137 contains an undisclosed amount of carcinogenic solvent (dichloromethane) present as

part of the crystal lattice structure (as $xCHCl_2$) and it is well known to be metabolized to carbon monoxide *in vivo*. This is particularly complicated since the CO and H_2S have similar pharmacological properties and it is possible that many of the reported effects of commercially sourced GYY4137 *in vivo* may be due to CO rather than H_2S. Moreover, with undisclosed amounts of dichloromethane (at least 0.5 molecules per molecule of GYY4137 (3), the molecular mass of commercial compound is not accurate e.g. molecular weight of commercial GYY4137 is 376.6 with additional unknown quantities of dichlromethane mwt-84.9 (so an additional 22% to the final mass. For these reasons, we have used the pharmaceutically more acceptable sodium salt which is devoid of these confounding chemicals (3, 4). The decomposition (hydrolysis) pathway and H_2S generation are identical and both salts are freely water soluble which offer considerable advantages over H_2S releasing molecules which require organic solvents such as DMSO or ethanol (themselves biologically active) such as dithiolethione and thiohyrdoxybenzamide derivatives (2, 3).

Animals and treatment

The 3xTg-AD mouse model was obtained from Jackson laboratories (Bar Harbor, Maine). Animals were housed on a 12-h light–dark schedule and received food and water ad libitum.6-mo old 3xTg-AD mice and their wild type controls were injected with either 100 mg/kg NaGYY or vehicle (saline) intraperitoneally for 12 wk and behavioral studies conducted at 9-mo.

Post-mortem brain samples

Post-mortem samples from normal and AD patients (Braak stage 6) were obtained from the Johns Hopkins Brain Center from J. Troncoso and O. Pletnikova.

Hydrogen sulfide production assays

HEK293 cells were transfected with indicated plasmids for the indicated time periods. For experiments with purified protein, purified recombinant CSE and Tau was used. Lysates or purified proteins were incubated for 6 h at 37 °C purged with nitrogen in 100 mM K+ PBS, .5% Triton X-100, 50 μM pyridoxal phosphate, 10 mM cysteine. Samples were injected with 125 μL 1% zinc acetate and 2.5 μL 10 N NaOH and incubated shaking at RT for 1 h. 500 μL of deionized water, 100 μL 20 mM N-N-dimethyl-p-phenylenediamine sulfate in 7.2M HCl, and 100 μL 30 mM $FeCl_3$ in 1.2M HCl were added to each sample and absorbance at 670 nm was subsequently measured. In addition, production of H_2S by CSE was measured in a spectrophotometric assay in which the reaction of H_2S with lead acetate to form lead sulfide was monitored at 390nm (5). Reaction buffer contained 1 mM of cysteine and 0.4 mM lead acetate in HEPES buffer (50 mM, pH 7.4) at 37 °C. hCSE (0.22 μM) or hCSE/hTau (0.22 μM/0.44 μM) were preincubated for 5 minutes at 37 °C (to allow the interaction and activation of the enzyme) before they were added into the buffer. H_2S

production was monitored for 30 minutes by measuring the absorbance spectrum every 4 seconds. The reaction between lead acetate and L-cysteine in HEPES buffer was used as a control.

Sulfhydration/persulfidation assays

Sulfhydration assays were conducted using the modified biotin switch assay, the dimedone switch assay and by mass spectrometry as described previously (6-8). Briefly, HEK293 cells were transfected with wild type Tau for 24 h and treated with 100 μM NaSH for 24 h, as indicated. Cells were lysed in HEN buffer (250 mM HEPES–NaOH, pH 7.7, 1 mM EDTA, 0.1 mM Neocuproine) with 1% Triton X100, protease, and phosphatase inhibitors and cleared by centrifugation at 16,000 g at 4 °C for 10 min. Protein was quantified by Bradford assay and samples were normalized for protein content. Sample free thiols were blocked in methyl methanethiosulfonate (MMTS) at 50 °C for 20 min with shaking. Protein in samples was precipitated with cold acetone and washed twice with at RT with 70% acetone. Protein pellets were resuspended in HENS (HEN buffer with 1% SDS) buffer and biotin-HPDP for biotinylation of sulfhydrated cysteines. Samples were incubated for 75 min at RT with rotation. Proteins were precipitated twice with cold acetone followed by resuspension in HENS buffer. Neutralization buffer (20 mM HEPES-NaOH, pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) was added, and inputs were reserved for loading on SDS-PAGE. Neutravidin agarose beads were added to the samples and incubated at 4 °C overnight with rotation. Beads were washed with neutralization buffer with high salt (Neutralization buffer with 600 mM NaCl) seven times followed by addition of elution buffer (20 mM HEPES-NaOH, pH 7.7, 100 mM NaCl, 1 mM EDTA, 1% β-mercaptoethanol) and incubation at 95 °C for 5 min. LDS buffer with DTT was added and samples were loaded on SDS-PAGE and immunoblotted with indicated antibodies. The dimedone switch assay was conducted essentially as described earlier (7).

Mass Spectrometry for Identification of Sulfhydrated Cysteines

Purified GST-GSK3β was incubated with 100 uM NaSH in HEN buffer (HEPES–NaOH, pH 7.7, 250 mM EDTA, 1 mM Neocuproine 0.1 mM) for 1 h at 37 °C followed by FASP digest on a 30 kDa filter by trypsin overnight at 37 °C. Filter was washed, acidified, and peptides were eluted with 60% ACN/.1% TFA and were run immediately on high-resolution mass spectrometry for analysis. MS-MS spectra was searched using PEAKSX against the database created from MASCOT. Fragment Mass Error Tolerance was 0.012 Da and Parent Mass Error Tolerance was 6 ppm. MASCOT was set to search NP_002084.2 (glycogen synthase kinase-3 beta isoform 1 [*Homo sapiens*]). Human recombinant Tau 441 was incubated with H_2O_2 and H_2S mixture to induce protein persulfidation and labeled with dimedone switch method as described earlier (39). Untreated samples served as a control. Proteins were subjected to trypsin digestion and LC/MS/MS analysis. Obtained spectra were analyzed with PEAKSX. The search settings were: precursor Δm tolerance = 10 ppm,

fragment ∆m tolerance = 0.2 Da, missed cleavages = 2, -10logP>50, modifications of lysine: NBF (163.0012), modifications of cysteine: NBF (163.0012), dimedone (138.0681),

Purified Recombinant proteins

WT Tau and Tau C291S/C322S were purified from BL21 bacterial cells transformed with pTrcHis-Tau or pTrcHis-Tau C291S/C322S, respectively. Site-directed mutagenesis was carried out to generate the Tau mutant plasmid. Capturem His-Tagged Purification Maxiprep Columns (Takara) were used to purify the recombinant Tau proteins and visualized by SDS gel electrophoresis on 4-12% Bis-Tris polyacrylamide gels, followed by Coomassie staining using SimplyBlue SafeStain (Thermo Fisher Scientific). GST-CSE was purified by affinity chromatography using glutathione-agarose (Sigma) as per the manufacturer's recommendations.

Tau phosphorylation assays

HEK293 cells were transfected with indicated plasmids for 24 h. Cells were treated with 100 μM NaSH for 24 h and then lysed in IP buffer with clearance of lysates by centrifugation at 16,000 g for 10 min. Protein was quantified by Bradford assay and samples were normalized for protein content. LDS buffer with DTT was added and samples incubated at 95 °C for 5 min. Samples were loaded on SDS-PAGE and immunoblotted with indicated antibodies. For the *in vitro* assays, the kinase activity assay provided with the SignalChem GSK3β purified protein. Either 10 μL of GSK3β (0.02 μg/uL) diluted in kinase dilution buffer III (KDBIII), containing 5 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 5 mM $MgCl_2$, 1 mM EGTA, 0.4 mM EDTA and 50 ng/μL BSA or 10 μL of KDBIII, 5 μL of 15 μM purified Tau, and 5 μL of dH_2O or 0.5 mM NaSH in dH_2O (stock solution). Subsequently 5 μL of 0.25 mM ATP was added to each reaction and incubated in the shaking incubator for 15 min at 30 °C. Next, LDS (+DTT) was added to stop the reactions and incubated at 95 °C heat block for 5 min followed by Western blot analysis.

Radioactive assay for determination of GSK3β activity: For the radioactive *in vitro* assays, the kinase activity assay using GSK3β purified protein (SignalChem) was utilized. Either 10 μL of GSK3β (0.02 μg/uL) diluted in kinase dilution buffer III (KDBIII), containing 5 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 5 mM $MgCl_2$, 1 mM EGTA, 0.4 mM EDTA and 50 ng/μL BSA or 10 μL of KDBIII, 5 μL of 1 mg/mL peptide substrate (YRRAAVPPSPSLSRHSSPHQpSEDEE, derived from glycogen synthase), and 5 μL of dH_2O or 0.5 mM NaSH in dH_2O (stock solution). Subsequently 5 μL of 0.25 mM [γ-^{32}P] ATP (1 mCi/100 μl) was added to each reaction and incubated in the shaking incubator for 15 min at 30 °C. Next, the reaction mixture was spotted onto individually precut strips of phosphocellulose P81 paper, which were air dried and washed with 1% phosphoric acid in double distilled water three times and subjected to scintillation counting to estimate the radioactivity incorporated into the peptide.

Mouse behavioral studies

Barnes maze test: 6-mo old WT and 3xTg-AD mice were treated with daily intraperitoneal injections of NaGYY (100 mg/kg) or saline (vehicle) as indicated for 12 wk until behavioral assays were performed as indicated at 9-mo. Mice were trained on the Barnes Maze platform twice a day for four days followed by testing on the fifth and twelfth days. Training and testing were carried out as follows: mice were placed in the middle of a raised platform with visual cues in the periphery and 20 holes along the edge, one of which contained an escape path for the mouse. For a maximum of three min, the number of nose pokes into correct and incorrect holes as well as the time until the mouse nose poked the correct hole and the time until the mouse entered the escape path were recorded. If a mouse entered the escape path prior to the three-min limit, the session was cut short.

Open field test. The mouse cohort used for the Barnes maze test was run on an open field chamber with IR beams recording their locomotor activity. Mice were allowed to freely roam the chamber for 45 min.

Antibody array assessment of GSK3β sulfhydration. Antibody-array like detection of GSK3β sulfhydration from human brain samples. The GSK3β antibody (sc-377213, Santa Cruz) was immobilized on a 96 well plate with NHS-activated surface as described previously (7). Considering that proteins are labelled with NBF (green) for total load and with Cy5 (red) for sulfhydration, measuring the ratio of the two signals yields the levels of GSK3b sulfhydration. As a negative control 488-labelled albumin (instead of antibody) was used to block the available surface and then incubated with control lysates.

Statistical Analysis. Results are presented as means ± SEM for at least three independent experiments. The sample sizes used were based on the magnitude of changes and consistency expected. Statistical significance was reported as appropriate. *P* values were calculated with Student's *t* test. In behavioral analyses, the experimenter conducting the test was blinded to the genotype or treatment of the animals under study. Statistical significance was calculated using one-way ANOVA/post-hoc Tukey test.

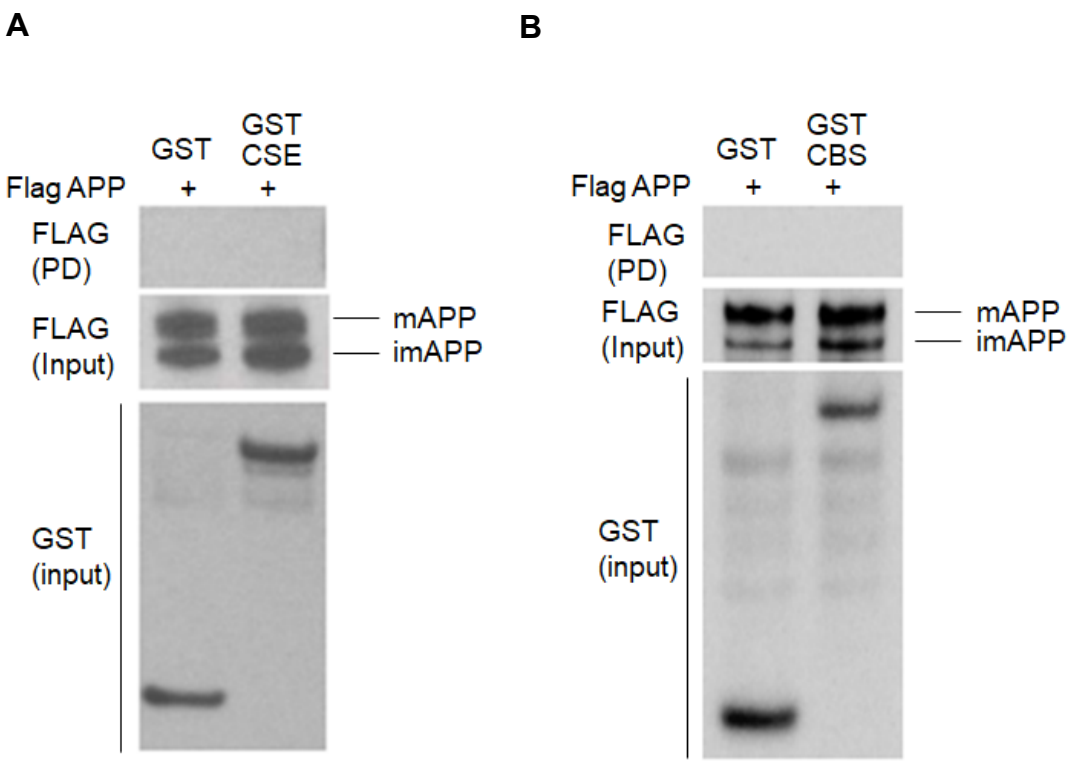

Fig. S1. Amyloid precursor protein (APP) does not interact with cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS). (*A*) CSE does not bind APP. HEK293 cells were transfected with FLAG APP and either GST alone or GST-CSE for 24 h and GST pulldown conducted using glutathione agarose. Western blot analysis was performed after the pull-down, APP exists in cells as two forms, the immature form (imAPP), which is *N*-glycosylated and *O*-glycosylated. CSE does not bind to APP as seen in the pull-down (PD) using anti-FLAG antibodies. (*B*) CBS does not bind APP. HEK293 cells were transfected and processed as described above and analyzed by Western blot analysis.

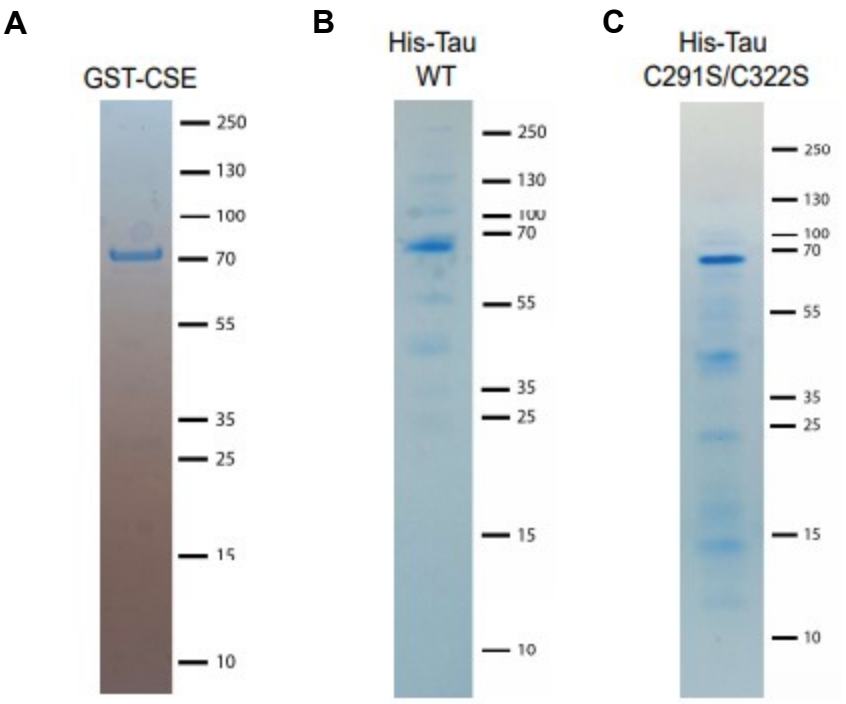

Fig. S2. Protein purification profiles of Tau, its mutants and cystathionine γ-lyase (CSE). (*A*) GST- tagged CSE was purified using glutathione agarose and analyzed by Coomassie staining. (*B,C*) His-tagged wild type Tau and the double cysteine mutant, Tau C291S/C322S was purified using CaptureEm purification systems (Takara) and purity confirmed by SDS gel electrophoresis and Coomassie staining as described earlier.

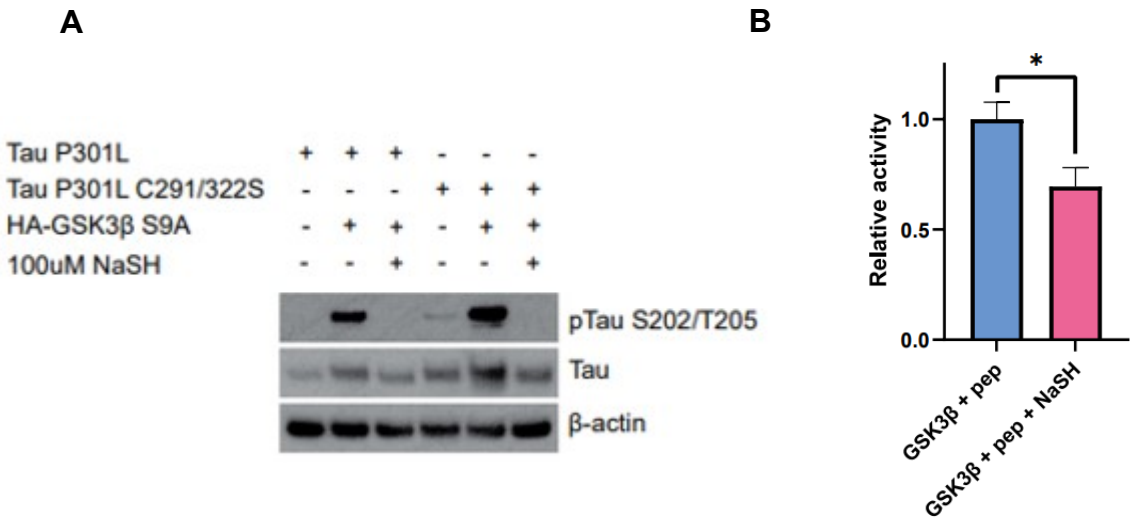

Fig. S3. Glycogen synthase kinase 3β (GSK3β) activity assays. (*A*) H_2S inhibits phosphorylation of Tau P301L by GSK3β. HEK293 cells were transfected with Tau P301L or Tau P301LCys291/Cys322 and GSK3β and treated with 100 μM NaSH for 24 h and analyzed for phosphorylation of Tau at Ser202 and Thr205 by western blotting. While GSK3β phosphorylated Tau, NaSH prevented this phosphorylation. (*B*) The H_2S donor, NaSH prevents phosphorylation of a peptide substrate of Tau by GSK3β (YRRAAVPPSPSLSRHSSPHQ**pS**EDEE, derived from glycogen synthase). Active GSK3β, peptide substrate (pep) and [γ-^{32}P] -ATP were incubated at 30 °C for 15 min, followed by termination of the reaction by spotting onto phosphocellulose P81 paper. The samples were air-dried and washed three times with 1% phosphoric acid, followed by quantitation of remaining radioactivity in a scintillation counter (n=6, SEM, **P* <0.05).

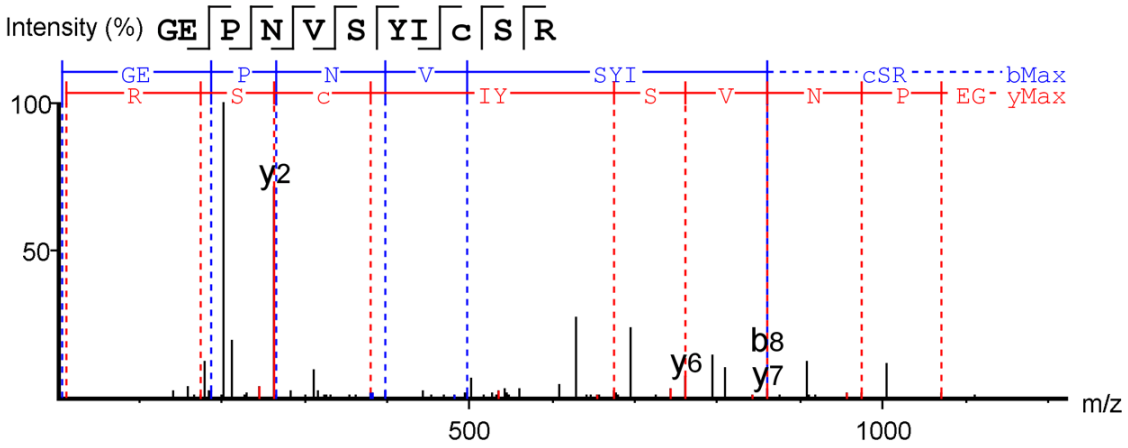

Fig. S4. LC-MS/MS spectra for identification of sulfhydration of GSK3β at Cysteine 218. Blue represents b-ions and red y-ions. Unmodified amino acids are labelled with capital letters. Sulfhydrated cysteine 218 (containing another S atom, m/z 32) is marked as c.

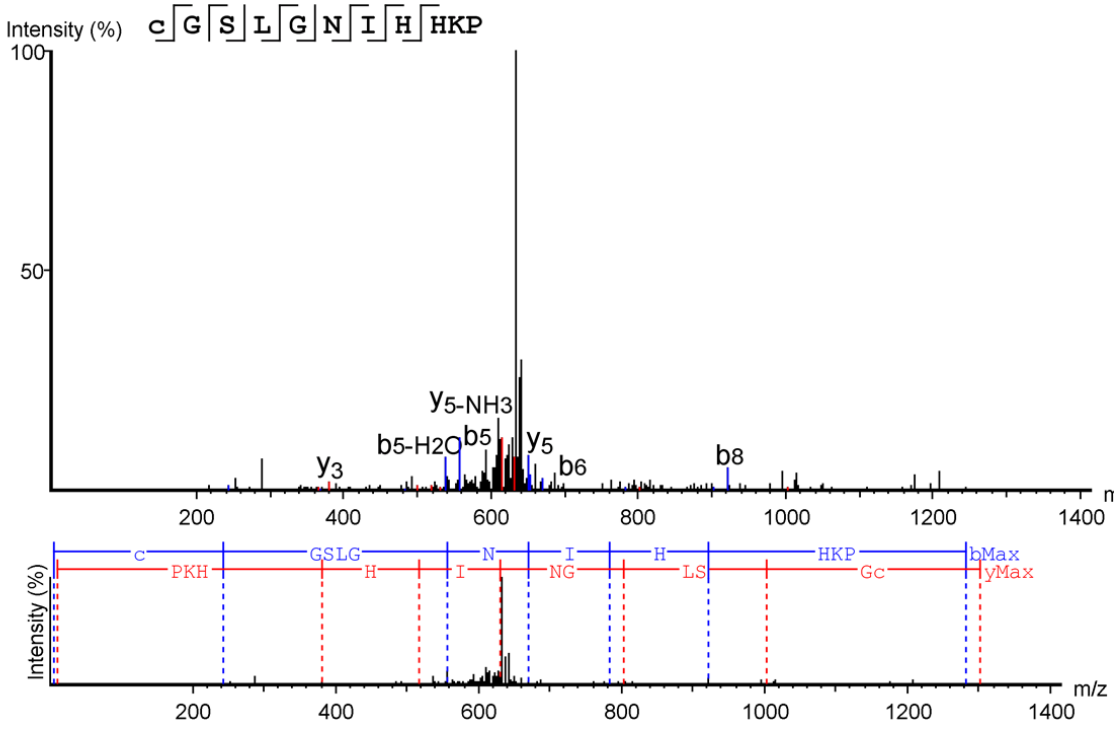

Fig. S5. MS/MS spectra of sulfhydrated C322 containing peptide of Tau labeled with dimedone, using dimedone switch method. Blue represents b-ions and red y-ions. Unmodified amino acids are labelled with capital letters. Dimedone labelled cysteine 322 (m/z 138) is marked as c.

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