Dysregulated protein phosphorylation in a mouse model of FTLD-tau

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Author’s contributions
IF, PAB, and ES designed the study; PAB prepared the colonies; JFI, KA, and ES performed the proteomic analysis; IF, PAB, JAR, KA, PC, ML, JFI, and ES analyzed and interpreted the data; IF, PAB, and ES wrote the manuscript. All authors critically revised the manuscript.

Abstract

The neocortex of P301S mice, used as a model of fronto-temporal lobar degeneration linked to tau mutation (FTLD-tau), and wild-type mice, both aged 9 months, were analyzed with conventional label-free phosphoproteomics and SWATH-MS (sequential window acquisition of all theoretical fragment ion spectra mass spectrometry) to assess the (phospho)proteomes. The total number of identified dysregulated phosphoproteins was 328 corresponding to 524 phosphorylation sites. The majority of dysregulated phosphoproteins, most of them hyper-phosphorylated, were proteins of the membranes, synapses, membrane trafficking, membrane vesicles linked to endo- and exocytosis, cytoplasmic vesicles, and cytoskeleton. Another group was composed of kinases. In contrast, proteins linked to DNA, RNA metabolism, RNA splicing, and protein synthesis were hypo-phosphorylated. Other pathways modulating energy metabolism, cell signaling, Golgi apparatus, carbohydrates, and lipids are also targets of dysregulated protein phosphorylation in P301S mice.
The present results, together with accompanying immunohistochemical and western blotting studies, show widespread abnormal phosphorylation of proteins, in addition to protein tau, in P301S mice. These observations point to dysregulated protein phosphorylation as a relevant contributory pathogenic component of tauopathies.

Key words: tau, (phospho)proteomics, tauopathy, membranes, cytoskeleton, kinases

Introduction

Phosphorylation is one of the most common and essential mechanisms of protein function that mostly derives from activation/inhibition of protein function and/or recruitment of interacting proteins with structurally conserved domains [1-6].

Alzheimer's disease (AD) generates dysregulated phosphorylation of a large number of proteins [7-10]. Differentially regulated phosphoproteins are components of cell membranes and membrane signaling, cytoskeleton, synapses including neurotransmitter receptors, serine-threonine kinases, proteins involved in energy metabolism, and RNA processing and splicing, among others [7, 8, 10]. Dysregulated protein phosphorylation has also been reported in transgenic mouse models of AD expressing abnormal β-amyloid deposition [11-16]. These observations point to the fact β-amyloid may promote abnormal protein phosphorylation in humans and mice. Yet, we cannot rule out the possibility that abnormal protein phosphorylation in AD is also associated with tau pathology.

Abnormal protein phosphorylation has been described in a few human tauopathies including aging-related tau astrogliopathy (ARTAG) a common old-age 4Rtauopathy involving astrocytes [17], and globular glial tauopathy (GGT) a rare 4Rtauopathy with characteristic phospho-tau inclusions in neurons, astrocytes, and oligodendrocytes [18].

However there is little information about differential protein phosphorylation in transgenic models of pure tauopathy [13]. For this reason, the present study is focused on altered protein phosphorylation in transgenic mice expressing human P301S tau (line PS19), a validated model of fronto-temporal lobar degeneration linked to P301S mutation in the tau gene (FTLD-tau). Our hypothesis is that in addition to abnormal tau phosphorylation, dysregulated protein phosphorylation involving key proteins is a common and relevant molecular pathology in tauopathies.

Materials and methods

Animals

The experiments were carried out in heterozygous transgenic mice expressing human P301S tau (line PS19: B6;C3-Tg(Prnp-MAPT*P301S)PS19Vle/J; The Jackson Laboratory), and wild-type (WT) littermates in a C57BL/6 background. The number of mice was nine per group with equal numbers of males and females per group. Transgenic mice were identified by genotyping genomic DNA isolated from tail clips using the polymerase chain reaction conditions indicated by Jackson Laboratory (Bar Harbor, ME). Animals were maintained under standard animal housing conditions in a 12-hour dark-light cycle with free access to food and water. All animal procedures were carried out following the guidelines of the European Communities Council Directive 2010/63/EU and with the approval of the local ethical committee (C.E.E.A: Comitè Ètic d'Experimentació Animal; University of Barcelona, Spain; ref. 426/18). Animals were killed at the age of nine months by cervical dislocation and their brains were then rapidly removed and processed for study. The left cerebral hemisphere was dissected on ice, immediately frozen, and stored at -80°C until use for biochemical studies. The right hemisphere, brainstem, and cerebellum were fixed in 4% paraformaldehyde, cut in coronal sections, and embedded in paraffin. De-waxed sections were stained with haematoxylin and eosin, or processed for immunohistochemistry.

Immunohistochemistry

De-waxed sections, 4 microns thick, were processed for immunohistochemistry. The sections were boiled in citrate buffer pH=6 (20 min). Endogenous peroxidases were blocked by incubation in 10% methanol-1% H_2O_2 solution (15 min), followed by 3% normal horse serum solution. The sections were incubated at 4ºC overnight with one of the primary antibodies listed in Table 1. After incubation with the primary antibody, the sections were incubated with EnVision + system peroxidase (Dako, DK) for 30 min at room temperature. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Control of immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody.

Double-labeling immunofluorescence and confocal microscopy

De-waxed sections, 4 microns thick, were stained with a saturated solution of Sudan black B (Merck) for 15 min to block autofluorescence of lipofuscin granules present in cell bodies, and then rinsed in 70% ethanol and washed in distilled water. The sections were boiled in citrate buffer to enhance antigenicity and blocked for 30 min at room temperature with 10% fetal bovine serum diluted in PBS. Then, the sections were incubated at 4ºC overnight with combinations of primary antibodies against different proteins. The characteristics of the antibodies, the dilutions, and the suppliers are listed in Table 1. After washing, the sections were incubated with Alexa488 or Alexa546 (1:400, Molecular Probes) fluorescence secondary antibodies against the corresponding host species. Nuclei were stained with DRAQ5™ (1:2,000, Biostatus). After washing, the sections were mounted in Immuno-Fluore mounting medium (ICN Biomedicals), sealed, and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope.

Gel electrophoresis and western blotting

Frozen samples of the posterior part of the left hemisphere from 3 WT and 4 P301S mice were homogenized in RIPA lysis buffer composed of 50mM Tris/HCl buffer, pH 7.4 containing 2mM EDTA, 0.2% Nonidet P-40, 1mM PMSF, protease, and phosphatase inhibitor cocktail (Roche Molecular Systems, USA). The homogenates were centrifuged for 20 min at 12,000 rpm. Protein concentration was determined with the BCA method (Thermo Scientific). Equal amounts of protein (12µg) for each sample

were loaded and separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membranes (Amersham, Freiburg, GE). Non-specific bindings were blocked by incubation in 3% albumin in PBS containing 0.2% Tween for 1 h at room temperature. After washing, membranes were incubated overnight at 4°C with antibodies against different forms of tau protein (Table 1). Protein loading was monitored using an antibody against β-actin. Membranes were incubated for 1 h with appropriate HRP-conjugated secondary antibodies (1:3,000, Dako); the immunoreaction was revealed with a chemiluminescence reagent (ECL, Amersham). Results were analyzed statistically with SPSS 19.0 (SPSS Inc., USA) software and GraphPad PRISM (GraphPad Software, Inc.) software. Data were presented as mean ± standard error of the mean (SEM). The unpaired student's t-test was used to compare groups. Significance level was set at * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$.

(Phospho)proteomic analysis

Brain samples of the anterior left hemisphere from WT and P301S mice (n = 8) were homogenized in a lysis buffer containing 7M urea, 2M thiourea, 50mM DTT supplemented with protease, and phosphatase inhibitors. The homogenates were spun down at 100,000 x g for 1 hour at 15°C. Protein quantitation was performed with the Bradford assay kit (Bio-Rad). The (phospho)proteomes and the corresponding proteomes were independently analyzed by conventional label-free phosphoproteomics [19] and SWATH-MS (Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry) [20], respectively.

Label-free phosphoproteomics: 600µg of protein was used to obtain the phosphorylated fractions. For protein digestion, reduction was performed by addition of DTT to a final concentration of 10mM and incubation at RT for 30 minutes. Subsequent alkylation with 30mM (final concentration) iodoacetamide was performed for 30 minutes in the dark at room temperature. An additional reduction step was performed with 30mM DTT (final concentration), allowing the reaction to stand at room temperature for 30 minutes. The mixture was diluted to 0.6M urea using MilliQ-water, and after addition of trypsin (Promega) (enzyme:protein, 1:50 w/w), the sample was incubated at 37ºC for 16h. Digestion was quenched by acidification (pH<6) with acetic acid. After protein enzymatic cleavage, peptide cleaning was performed using Pierce™ Peptide Desalting Spin Columns (ThermoFisher). To obtain the phosphorylated peptide fractions, the High-Select™ TiO_2 Phosphopeptide Enrichment Kit (Thermo Scientific) was used according to the manufacturer's instructions. Phosphopeptide mixtures were separated by reversed-phase chromatography using an Eksigent NanoLC ultra 2D pump fitted with an Acclaim™ PepMap™ 100 C18 column (0.075x250 mm, particle size 3µm; ThermoFisher). Samples were first loaded for concentration into an Acclaim™ PepMap™ 100 C18 trap column (0.1x20 mm, particle size 5µm; ThermoFisher). Mobile phases were 100% water 0.1% formic acid (FA) (buffer A), and 100% Acetonitrile 0.1% FA (buffer B). Column gradient was developed in a gradient from 2% B to 40% B in 120 min. Column was equilibrated in 95% B for 10 min and 2% B for 10 min. During the entire process, the precolumn was in line with the column and flow maintained all along the gradient at 300nl/min. Eluting peptides were analyzed using a 5600 Triple-TOF mass-spectrometer (Sciex). Information data acquisition was acquired upon a survey scan performed in a mass range from 350 m/z up to 1250 m/z in a scan time of 25ms. Top 15 peaks were selected for fragmentation. Minimum accumulation time for MS/MS was set to 200 ms giving a total cycle time of 3.3 s. Product ions were scanned in a mass range from 100 m/z up to 1500 m/z and excluded for further fragmentation during 15 s. The raw MS/MS spectra searches were processed using the MaxQuant software (v 1.6.7.0) and searched against the Uniprot proteome reference for *Mus Musculus* (Proteome ID: UP000000_10090, March 2021). The parameters used were as follows: initial maximum precursor (15 ppm) fragment mass deviations (20 ppm);

fixed modification (Carbamidomethyl (C)); variable modification (Oxidation (M); Acetyl (Protein N-terminal; Phospho (STY)); enzyme (trypsin) with a maximum of 2 missed cleavage; minimum peptide length (7 aminoacids); and false discovery rate (FDR) for PSM and protein identification (1%). Frequently observed laboratory contaminants were removed. Perseus software (version 1.6.14.0) was used for statistical analysis and data visualization.

SWATH-MS: For the SWATH-MS assay library, a pool of all samples included in the analysis (4 WT and WT PS19; 20ug/mice) was used. Twenty micrograms were diluted in Laemmli buffer and loaded into a 0.75 mm thick polyacrylamide gel with a 4% stacking gel cast over a 12.5% resolving gel. Total gel was stained with Coomassie Brilliant Blue, and 12 equal slides from each pooled sample were excised from the gel and transferred into 1.5mL Eppendorf LoBind tubes. Protein enzymatic cleavage was carried out with trypsin (Promega; 1:20, w/w) at 37°C for 16 h as previously described [21]. Purification and concentration of peptides were performed using C18 Zip Tip Solid Phase Extraction (Millipore). The peptides recovered from in-gel and in-solution digestion processing were reconstituted into a final concentration of 0.5µg/µL of 2% ACN, 0.5% FA, 97.5% MilliQ-water prior to mass spectrometric analysis. MS/MS datasets for spectral library generation were acquired on a TripleTOF 5600+ mass spectrometer (Sciex, Ontario, CA) interfaced to the Eksigent nanoLC ultra 2D pump system (Sciex) as previously described. MS/MS data acquisition was performed using AnalystTF 1.7 (Sciex), and spectra files were processed through ProteinPilot v5.0 search engine (Sciex) using the Paragon™ Algorithm (v.4.0.0.0) [22] for database search. To avoid using the same spectral evidence in more than one protein, the identified proteins were grouped based on MS/MS spectra with the Progroup™ Algorithm, regardless of the peptide sequence assigned. False discovery rate (FDR) was determined using a non-lineal fitting method [23] and displayed results were those reporting a 1% global FDR or better. Then, individual protein extracts from all sample sets (n=8) were subjected to in-solution digestion, peptide purification, and reconstitution prior to mass spectrometric analysis. Protein extracts (20µg) from each sample were reduced, adding DTT to a final concentration of 10mM and incubation at room temperature. Reduction was performed by the addition of DTT to a final concentration of 10mM and incubation at room temperature for 30 minutes. Subsequent alkylation by 30 mM iodoacetamide was performed for 30 minutes in the dark. An additional reduction step was performed with 30mM DTT, allowing the reaction to stand at room temperature for another 30 minutes. The mixture was then diluted to 0.6M urea using MilliQ-water, and after addition of trypsin (Promega) (enzyme:protein, 1:50 w/w), the sample was incubated at 37ºC for 16h. Digestion was quenched by acidification with acetic acid. The digestion mixture was dried in a SpeedVac. Purification and concentration of peptides was made using C18 Zip Tip Solid Phase Extraction (Millipore). The peptides recovered were reconstituted into a final concentration of 1µg/µL of 2% ACN, 0.5% FA, 97.5% MilliQ-water prior to mass spectrometric analysis. For SWATH-MS-based experiments, the TripleTOF 5600+ instrument was configured as described [24]. Using an isolation width of 16Da (15Da of optimal ion transmission efficiency and 1Da for the window overlap), a set of 37 overlapping windows was constructed covering the mass range 450–1000 Da. In this way, 1µL of each sample was loaded into an Acclaim™ PepMap™ 100 C18 trap column (0.1x20 mm, particle size 5 µm; ThermoFisher) and desalted with 100% water 0.1% formic acid at 2µL/min for 10 min. The peptides were loaded into an Acclaim™ PepMap™ 100 C18 column (0.075x250 mm, particle size 3µm; ThermoFisher) equilibrated in 2% acetonitrile 0.1% FA. Peptide elution was carried out with a linear gradient of 2 to 40% B in 120 min (mobile phases A:100% water 0.1% formic acid (FA) and B: 100% acetonitrile 0.1% FA) at a flow rate of 300nL/min. Eluted peptides were infused in the mass-spectrometer. The Triple TOF was operated in SWATH mode, in which a 0.050s TOF MS scan from 350 to 1250m/z was performed, followed by 0.080s

product ion scans from 230 to 1800 m/z on the 37 defined windows (3.05s/cycle). Collision energy was set to optimum energy for a 2+ ion at the center of each SWATH block with a 15 eV collision energy spread. The resulting ProteinPilot group file from library generation was loaded into PeakView® (v2.1, Sciex), and peaks from SWATH runs were extracted with a peptide confidence threshold of 99% confidence (Unused Score ≥ 1.3) and FDR lower than 1%. For this, the MS/MS spectra of the assigned peptides were extracted by ProteinPilot, and only the proteins that fulfilled the following criteria were validated: (1) peptide mass tolerance lower than 10ppm, (2) 99% confidence level in peptide identification, and (3) complete b/y ions series found in the MS/MS spectrum. Only proteins quantified with at least two unique peptides were considered. Then, quantitative data were analysed using the Perseus software (version 1.6.14.0) for statistical analysis and data visualization. MS data and search result files were deposited in the Proteome Xchange Consortium via the JPOST partner repository (https://repository.jpostdb.org, accessed on 1 March 2022) [25], with the identifier PXD031961 for ProteomeXchange and JPST001509 for jPOST (https://repository.jpostdb.org/preview/1738288420621e76ca4fcef; Access key 8489)

Bioinformatics: The identification of significantly dysregulated regulatory/metabolic pathways in proteomic datasets was performed using Metascape [26]. Network analysis was performed submitting the corresponding protein IDs to the STRING (Search Tool for the Retrieval of Interacting Genes) software (http://stringdb.org/) [27]. Proteins are represented with nodes and all the edges were supported by at least one reference from the literature or from canonical information stored in the STRING database. To minimize false positives as well as false negatives, only interactions tagged as “high confidence” (> 0.7) in STRING database were considered.

Results

P301S transgenic mice

Transgenic mice at the age of 9 months showed phosphorylated tau deposition, as revealed with the AT8 antibody, in the cytoplasm of neurons and proximal dendrites in the entorhinal and piriform cortex, CA1 region of the hippocampus, dentate gyrus, somatosensory cortex, and cingulate cortex; the amygdala, hypothalamus, striatum, thalamus, septal nuclei, nuclei of the basal forebrain, and granule cells of the cerebellum were also immunostained with AT8 antibodies. Neurons of the brainstem, particularly those in the pontine nuclei, tegmentum, and raphe nuclei also contained AT8-positive deposits. Neuropil threads in the grey matter, plexiform layers of the hippocampus, hilus of the dentate gyrus, and molecular layer of the cerebellum also contained AT8-positive deposits. Antibodies PHF1, directed against tau-P Ser396/404, and phospho-tau Ser422 showed a similar distribution and localization of phosphorylated tau. However, the antibody MC1, directed against conformational tau isoforms, stained lesser numbers of neurons in all regions; and the antibody tau C-3, directed against truncated tau at aspartic acid 421, stained only a few neurons in the CA1 region and dentate gyrus (Supplementary Figure 1). Tau deposits were homogeneous and diffuse and have the appearance of neurofibrillary pre-tangles (cytoplasmic tau immunoreactivity in neurons without apparent formation of fibrillary structures) and more rarely tangles. Astrocytes and oligodendrocytes were devoid of phosphorylated tau deposits. These findings were in agreement with our previous observations in P301S transgenic mice [28] and are summarized in Table 2. Immunohistochemistry with anti-4Rtau antibodies revealed a positive neuropil, but not increased 4Rtau deposits in any cell type. 3Rtau immunohistochemistry was negative. Neurons also contained deposits of tau nitrated at Tyr29 (tau-N Tyr29) in the same regions and localizations like those revealed with AT8 antibodies (Supplementary Figure 1; Table 2).

Neurons also contained phosphorylated microtubule associated protein, as recognized with the MAP2-P Thr1620-1623 antibody, with the same extension and localization as tau AT8 (Figure 1A-C). In contrast, only very rarely did neurons localized in the hippocampus and cerebral cortex exhibit phosphorylated light neurofilament (NFL-P Ser473) immunoreactivity (Figure 1D). A subpopulation of neurons was positive with anti-casein kinase δ antibodies (Figure 1E, F). Subpopulations of neurons also showed cytoplasmic granules positive with antibodies against active p38 (p38-P Thr180/tyr182), phosphorylated tyrosine-protein kinase SRC (SRC-P Tyr416) (only in the entorhinal cortex), and phosphorylated stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK-P Thr183/185) (Figure 1G-J). GSK-3β-P Ser9 immunoreactivity was negative. Markers of reticulum stress eIF2α-P Ser51 and IRE-P Ser274 were negative. Only a few neurons in the dentate gyrus and threads in the hilus showed ubiquitin deposits (Figure 1K). LC3-immunoreactive granules were not observed. Immunostaining with AKT-P Ser473, PKAα/β-P Tyr197, SRPK1, and SRPK2-P Ser497 antibodies was negative. SRPK1 immunoreactivity was localized in neuronal and glial cell nuclei; inclusions were negative (Table 2).

Double-labeling immunofluorescence and confocal microscopy to AT8 and active p38 showed co-localization of tau deposits and the active tau kinase in the cytoplasm). Similarly, neurons with AT8-immunoreactive deposits co-localized SAPK/JNK-P-positive granules in the cytoplasm (Supplementary Figure 2).

WT mice showed weak 4Rtau immunoreactivity in the neuropil. The AT8 antibody revealed only nuclear immunostaining without positivity in the cytoplasm or dendrites of neurons. Immunohistochemistry with the rest of the antibodies did not show abnormal deposits (Table 2).

GFAP-immunoreactive astrocytes were abundant in the CA1 region and dentate gyrus when compared with age-matched WT controls. Microglial cells, as revealed with the Iba1 antibody, showed a significant increase in the CA1 subfield of the hippocampus

and dentate gyrus in P301S mice. Glial responses were in line with our previous observations in these mice [40].

Western blotting of total brain homogenates in WT and P301S

Western blots of the anterior part of the left cerebral hemisphere were carried out in 3 WT and 4 P301S transgenic mice. The antibody Tau 5, which identifies total tau, showed strong bands of variable molecular weight in P301S when compared with WT mice. 4Rtau was present in WT mice, but it was strongly increased in P301S, showing two bands of 68kDa and 64kDa. In contrast, 3Rtau was absent in WT and P301S mice. Phosphorylated tau at Thr181 and MC1 tau were expressed in P301S, but they were almost absent in WT mice. Two bands of 68kDa and 64kDa were observed with the antibodies P-tau Thr181 and MC1 in P301S mice (Figure 2).

(Phospho)proteomics

The increment in the tau phosphorylation of four serines (positions 396, 404, 422 and 202) previously observed by immunohistochemistry, were confirmed by mass-spectrometry (for more details, see JPOST ID JPST001509 in https://repository.jpostdb.org). Four additional residues (S400, S416, S198, S199) were also phosphorylated in P301S transgenic mice.

Moreover, 524 phosphosites were also identified by our phosphoproteomic approach, with 215 being hypo-phosphorylated and 309 hyper-phosphorylated (Figure 3A and JPOST ID JPST001509 in https://repository.jpostdb.org). Three-hundred twenty-eight phosphoproteins were differentially regulated in P301S transgenic mice when compared with WT mice; 179 up-regulated, 134 down-regulated, and 16 both up-regulated and down-regulated in different phosphorylation sites. Fifty-five percent of the differentially regulated phosphoproteins were quantified at the level of the total protein, showing that the levels of phosphorylation were not dependent on the total protein levels. Regarding the remaining 45%, total levels of these proteins were not quantified, and we cannot assume that 100% of the differences were due exclusively to phosphorylation.

Enrichment analysis of obtained data revealed that the main differentially regulated phosphoproteins were categorized with the cellular component GO terms related to (i) synapse, including presynapse (GO:0098793) and postsynapse (GO:0098794), and related components (Figure 3B) such as endocytic vesicle (GO:0030139) and glutamatergic synapse (GO:0098978); (ii) structural components of synaptic and neuronal architecture and cytoskeleton (Figure 3B), such as axon (GO:0030424), cell body (GO:0044297), cell junction (GO:0005911), and neuron projection (GO:0044306); and also (iii) membrane and vesicle terms (Figure 3B), such as clathrin-coated pit (GO:0005905) or cell projection membrane (GO:0031253) (Figure 3B). Accordingly, the molecular function categories most significantly enriched correspond to tubulin/actin binding (GO:0015631; 0003779), GTPase regulatory activity (GO:0030695), serine kinase activity (GO:0106310), calmodulin kinase, and phospholipid-binding properties (GO: 0019900; 0005516; 0005543). This functional mapping pointed out a significant disruption in biological processes associated to neuron projection organization (GO:0031175; 0031344), modulation of trans-synaptic signaling (GO:0099537; 0050804) and protein phosphorylation (GO:0006468) between others (Figure 3B; for more details about all these functional annotations, see JPOST ID JPST001509 in https://repository.jpostdb.org).

Identification of differentially regulated phosphoproteins

The pinpoints of the differentially regulated proteins were assessed manually using available databases.

Two-hundred eighty nine differentially regulated phosphoproteins were classified according to their localization and function into six major groups: kinases; proteins linked to the cytoskeleton; proteins linked to the cellular membranes, membrane

metabolism, synapsis, vesicles, endocytosis, and exocytosis; proteins linked to the DNA, RNA, and protein synthesis; proteins linked to autophagy and ubiquitin-proteasome system (UPS); and phosphatases. Other differentially regulated phosphoproteins were involved in cell signaling, energy metabolism, and lipid and carbohydrate metabolism, among other functions.
The remaining 39 differentially regulated phosphoproteins were not considered in the study because their function in the nervous system was barely known.
Thirty-seven differentially regulated phosphoproteins were kinases: 23 were up-regulated, 12 down-regulated, and two had both up-regulated and down-regulated phosphorylation sites in P301S compared with WT mice (Supplementary table 1).
Thirty-four differentially regulated phosphoproteins were related to the cytoskeleton, 18 up-regulated, 12 down-regulated, and four with both up- and down-regulated phosphorylation sites in P301S compared with WT mice (Supplementary Table 2).
One-hundred and thirteen differentially regulated phosphoproteins were integral membrane proteins, linked to membrane metabolism including ion channels, synapsis, vesicles, endocytosis, and exocytosis. Eighty seven were up-regulated, 18 down-regulated, and eight with both up- and down-regulated phosphorylation sites in P301S compared with WT mice (Supplementary Table 3).
Forty dysregulated phosphoproteins were linked to DNA and RNA metabolism, RNA splicing, and protein synthesis; 37 phosphoproteins were down-regulated, and 3 up-regulated in P301S compared with WT mice (Supplementary Table 4).
Sixteen differentially regulated phosphoproteins were components of the cellular degrading systems, autophagy, and UPS. Five phosphoproteins were up-regulated and 11 down-regulated (Supplementary Table 5).
Nine dysregulated phosphoproteins were phosphatases, five were up-regulated, and four down-regulated in P301S when compared with WT mice (Supplementary Table 6).
Thirty-nine differentially regulated phosphoproteins (22 up-regulated, 17 down-regulated, and one with up- and down-regulated phosphorylation sites) were involved in cell signaling, energy metabolism, lipid metabolism, and carbohydrate metabolism, among other functions (Supplementary Table 7). The classification is considered instrumental as many proteins are involved in different pathways.

Phosphoprotein interactomes in P301S mice

Network analysis was performed submitting the corresponding protein IDs to the STRING. A schematic representation is shown in Figure 4. Abbreviations and quantitative values are detailed in https://repository.jpostdb.org (JPOST ID JPST001509). The image illustrates the complementary phosphoprotein network dysregulated in P301S tauopathy. There is a close interaction between proteins of the membranes and cytoskeleton, and proteins with kinase activity.

Discussion

Validation of the model

P301S transgenic mice develop hyper-phosphorylated tau deposits in neurons with the appearance of pre-tangles in the cerebral cortex, amygdala, hippocampus, striatum, and thalamus; and neuropil threads in the cerebral cortex, and plexiform layers of the hippocampus. Tau deposits are positive with phospho-specific anti-tau antibodies AT8 (Ser202/Thr205), PHF1 (Ser396/404), Tau-P Ser422, Tau 100 (Tau-P Thr212/Ser214), and antibodies against epitopes within amino acids 312-322 (conformational antibody MC1). Truncated tau at the aspartic acid 421 (Tau C-3) is also expressed in a subset of neurons at advanced stages of the tauopathy [28]. Affected neurons in P301S transgenic mice also contain nitrated tau (Tau-N Tyr29). Tau phosphorylation is accompanied by activation of p38, SAPK/JNK, and SRC kinases, as identified with the antibodies p38-P Thr180/182, SAPK/JNK-P Thr183/Thr185, and SRC-P Tyr416, respectively. Affected neurons also show rare granular casein kinase δ immunoreactivity. In addition to phospho-tau, neuronal deposits are strongly immunoreactive with the MAP2-P Thr1620/1623 antibody. Markers of endoplasmic reticulum stress and autophagy (eIF2α-P Ser51, IRE1-P Ser 274, LC3, and LAMP) are negative. Early synaptic pathology, changes in the synaptic proteome, altered neuroinflammatory gene regulation, oxidative stress, mitochondrial dysfunction, and brain lipid modifications are characteristic alterations in P301S transgenic mice [28-32]. Increased CaMKII phosphorylation, and S-nitrosylation of E3 ubiquitin-protein ligase RNF213, have also been identified in P301S mice [33].

Altered protein phosphorylation in P301S mice

Previous phosphoprotemics disclosed ten dysregulated phosphoproteins in P301S mice [13]. In the present study, 328 differentially regulated phosphoproteins were identified in P301S transgenic mice. Dysregulated phosphoproteins, either hyper-, hypo-, or hyper- and hypo-phosphorylated in different sites, included kinases, proteins linked to the cytoskeleton, proteins linked to the cellular membranes and membrane metabolism, synapsis, vesicles, endocytosis, and exocytosis, proteins linked to the DNA, RNA, and protein synthesis, proteins linked to autophagy and ubiquitin-proteasome system, and phosphatases. Other differentially regulated phosphoproteins were involved in cell signaling, energy metabolism, and lipid and carbohydrate metabolism. A detailed identification of abnormally regulated phosphoproteins and their function is found in Supplementary Tables 1-7.

Validation of dysregulated phosphoproteins is difficult because of the lack of suitable commercial antibodies. Yet, phospho-tau sites and MAP2 phosphorylation have been identified in tissue sections immunostained with phosphor-specific antibodies.

The number of dysregulated phosphoproteins is probably higher than those identified in the current phosphoproteomics study. P38-P and SAPK/JNK-P immunohistochemistry has revealed the presence of these active kinases co-localizing phosphor-tau deposits in P301S transgenic mice.

Commonalities of dysregulated protein phosphorylation in P301S mice and human tauopathies

Proteins with altered phosphorylation in P301S mice are similar to those identified in ARTAG and GGT [17, 18], but also particular disease-specific proteins.

In ARTAG, 109 proteins were hyper-phosphorylated and 31 hypo-phosphorylated compared with controls. Dysregulated phosphoproteins were components of the neuronal and astrocyte cytoskeleton, kinases, proteins linked to calcium/calmodulin signalling, cAMP signalling and DNA repair, nuclear and nucleolar regulators, proteins linked to tight junctions, proteins linked to proteolysis, and synaptic proteins [17].

In GGT, phosphoproteome profiling revealed 74 dysregulated proteins in the frontal cortex and 15 in the white matter. Cytoskeleton, axon guidance, exocytosis, synapses,

chaperone-mediated protein folding, and myelination were part of the significantly over-represented dysregulated biological processes in the frontal cortex. In the frontal cortex, alterations corresponded to oligodendrocytes (12%), astrocytes (10.5%), and neurons (5%). In the white matter, 18% of proteostatic alterations were specific of oligodendrocytes, and 3% of astrocytic and neurons [18].

Functional implications

Phosphorylation is one of the most common and essential mechanisms of protein function [1-6]; in consequence, abnormal protein phosphorylation results in altered protein function. In the present context, abnormal protein phosphorylation in P301S transgenic mice, and human tauopathiers [17, 18], implicates altered kinase activation, abnormal membrane signaling and cytoskeletal function, altered neurotransmission, membrane and cytoplasmic vesicle metabolism, DNA, RNA, and protein metabolism functions. Such altered functions in vital subcellular organelles, structures, and signaling pathways must be considered, besides direct effects of abnormal tau, pathogenic components in P301S transgenic tau mice, and human tauopathies.

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Figure legends

Figure 1: Representative images of P301S transgenic mice of the entorhinal cortex (EC), CA1 region of the hippocampus (CA1), and dentate gyrus (DG) stained with MAP2-P Thr1620/1623 (A-C), NFL-P Ser473 (D), casein kinase δ (E, F), p38-P Thr180/Tyr182 (G, H), phosphorylated tyrosine-protein kinase SRC: SRC-P Tyr 416, SAPK/JNK-P Thr183/Thr185 (J), and ubiquitin (K). MAP2-P immunoreactivity occurs in the cytoplasm and dendrites of neurons and in neuropil threads in all these regions. In contrast, NFL-P antibodies decorate a few neurons in the CA1 region. Casein kinase δ antibodies strongly stain the cytoplasm of a subpopulation of neurons distinguishing them from the rest of the neurons that show basal casein kinase δ immunostaining. p38-P, SRC-P, and SAPK/JNK-P antibodies show strong granular cytoplasmic immunoreactivity in some neurons (thick arrows in G, H, and J) and diffuse immunostaining in others. Finally, ubiquitin immunoreactivity is observed only in a few neurons of the dentate gyrus and threads and grains in the hilus. Paraffin sections with slight haematoxylin counterstaining, bar = 50µm, excepting J, bar =20 µm.

Figure 2: Gel electrophoresis and western blotting of total brain homogenates from wild type (WT) and P301S transgenic mice at the age of 9 months; blots are processed with Tau 5, 4Rtau, 3Rtau, phosphorylated tau at Thr181 (tau-Pthr181), and MC1 antibodies. Different blots are shown. β-actin is used as a control of protein loading; the representative image is obtained from the re-probed tau 5 western blot. A weak band of 4Rtau is present in WT, but 4Rtau is increased in P301S mice showing two bands of 68kDa and 64kDa. In contrast, 3Rtau is absent in both genotypes. Phosphorylated tau at Thr181 and MC1 tau are expressed in P301S but almost absent in WT mice. Two bands of 68kDa and 64kDa are observed with the antibodies P-tau Thr181 and MC1 P301S mice. Graphs representing mean ± SEM densitometry values of the bands: * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$.

Figure 3: Differentially expressed phosphosites in frontal cortex area in P301S mice. A) Principal Component Analysis (PCA) based on (phospho)feature abundance levels across runs, separating WT and P301S/PS19 mice according to abundance variation (upper panel). Heatmap representing the fold-change of identified phosphosites with associated p-values from the pair-wise quantitative comparisons. 524 phosphosites were identified using phosphoproteomic approaches, with 215 being hypo-phosphorylated and 309 hyper-phosphorylated. Significantly up-regulated phosphosites between pair-wise comparisons are labelled in red and significantly down-regulated phosphosites are labelled in green (lower panel). B) Gene Ontology mapping of differential phosphoproteins across subcellular compartments, molecular functions and biological processes.

Figure 4: Phosphoprotein interactomes in P301S mice showing a close interaction between proteins of the membranes and cytoskeleton, and proteins with kinase activity. Abbreviations and quantitative values are detailed in Repository

Supplementary Table 1: List of altered phosphoproteins in P301S in comparison with WT mice related to kinases and their functions. The number of dysregulated phosphosites and direction (hyper- or hypo-phosphorylation) are indicated for each protein.

Supplementary Table 2: List of altered phosphoproteins in P301S in comparison with WT mice related to the cytoskeleton in addition to tau protein. The number of dysregulated phosphosites and direction (hyper- or hypo-phosphorylation) are indicated for each protein.

Supplementary Table 3: List of altered phosphoproteins in P301S in comparison with WT mice related to membrane and membrane-linked proteins. The number of deregulated phosphosites and direction (hyper- or hypo-phosphorylation) are indicated for each protein.

Supplementary Table 4: List of altered phosphoproteins in P301S in comparison with WT mice related to DNA, RNA metabolism, and protein synthesis. The number of dysregulated phosphosites and direction (hyper- or hypophosphorylation) are indicated for each protein.

Supplementary Table 5: List of altered phosphoproteins in P301S in comparison with WT mice related to autophagy and the ubiquin-proteasome system (UPS). The number of dysregulated phosphosites and direction (hyper- or hypo-phosphorylation) are indicated for each protein.

Supplementary Table 6: List of altered phosphoproteins in P301S in comparison with WT mice related to phosphatases and their activity. The number of dysregulated phosphosites and direction (hyper- or hypo-phosphorylation) are indicated for each protein.

Supplementary Table 7: List of altered phosphoproteins in P301S in comparison with WT mice related to energy metabolism, cell signaling, Golgi apparatus, carbohydrates, and lipids. The number of dysregulated phosphosites and direction (hyper- or hypo-phosphorylation) are indicated for each protein.