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Changes in proteome solubility indicate widespread proteostatic disruption in mouse models of neurodegenerative disease

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Abstract

The deposition of pathologic misfolded proteins in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, frontotemporal dementia and amyotrophic lateral sclerosis is hypothesized to burden protein homeostatic (proteostatic) machinery, potentially leading to insufficient capacity to maintain the proteome. This hypothesis has been supported by previous work in our laboratory, as evidenced by the perturbation of cytosolic protein solubility in response to amyloid plaques in a mouse model of Alzheimer's amyloidosis. In the current study, we demonstrate changes in proteome solubility are a common pathology to mouse models of neurodegenerative disease. Pathological accumulations of misfolded tau, α -synuclein and mutant superoxide dismutase 1 in CNS tissues of transgenic mice was associated with changes in the solubility of hundreds of CNS proteins in each model. We observed that changes in proteome solubility were progressive and, using the rTg4510 model of inducible tau pathology, demonstrated that these changes were dependent upon sustained expression of the primary pathologic protein. In all of the models examined, changes in proteome solubility were robust, easily detected, and provided a sensitive indicator of proteostatic disruption. Interestingly, a subset of the proteins that display a shift towards insolubility were common between these different models, suggesting that a specific subset of the proteome is vulnerable to proteostatic disruption. Overall, our data suggest that neurodegenerative proteinopathies modeled in mice impose a burden on the proteostatic network that diminishes the ability of neural cells to prevent aberrant conformational changes that alter the solubility of hundreds of abundant cellular proteins.

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Author Contributions

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Keywords

Proteostasis; Protein Misfolding; Neurodegeneration; Proteinopathy; Proteomics

Introduction

Neurodegenerative disorders, including Alzheimer's disease (AD), Frontotemporal Dementia (FTD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are characterized pathologically by the accumulation of misfolded proteins (often referred to as "proteinopathies") [9, 21, 22, 41, 85, 86]. Considerable evidence supports the idea that the accumulation of misfolded proteins could be deleterious to the proteostasis network [5, 8, 15, 24, 44, 56, 62, 68, 73, 90, 95]. This network includes chaperones, the ubiquitin-proteasome system, and the autophagic system working in unison to maintain protein folding or degrade misfolded/aggregated protein species. Aggregated forms of misfolded tau are degraded in the autophagy-lysosomal system, while the accumulation of misfolded tau in CNS tissues of AD, and other tauopathies, is associated with lower 26S proteasome activity [39, 55, 69, 89]. Similar reductions in proteostatic activity have been reported in the presence of α -synuclein (α Syn) pathology [16, 26, 28, 104], and in tissues from mouse models that over-express ALS-associated variants of superoxide dismutase 1 (SOD1) [6, 10, 14, 50, 51, 79]. Accumulations of misfolded protein have also been associated with overburdened chaperone function, leading to impaired folding and maturation other proteins (reviewed in [5, 44]). This idea was originally elaborated in *C. elegans* where muscle cells expressing aggregating mutants of huntingtin, or SOD1, failed to correctly fold co-expressed temperature-sensitive metastable proteins [33, 34]. Accordingly, it has become increasingly clear that "mixed" proteinopathies are common in neurodegenerative disease [13, 42, 46, 87, 92]. For example, humans with tauopathy, as well as transgenic mice that model tauopathy, often have pathologic deposition of TDP-43 and α Syn [12, 42, 60, 63, 103]. Thus, there is evidence that disruptions in proteostatic function could degrade proteome integrity in neurodegenerative disease. To date, however, there is limited understanding of how changes in these proteostatic functions could impact overall proteome folding in the mammalian nervous system.

We have previously shown in a mouse model of Alzheimer's amyloidosis that, as A β deposition increases, the CNS proteome begins to lose solubility and a fraction of cytosolic proteins become aberrantly detectable in detergent-insoluble fractions [102]. Given that A β is largely deposited extracellularly, it was difficult to conclude that our observed outcomes could be explained by misfolded A β peptides directly over-burdening cytosolic protein chaperone functions. To assess whether the cytoplasmic, intracellular, accumulation of misfolded proteins, such as tau, might impose a burden on protein folding, we have examined proteome solubility in transgenic mouse models of mutant tau, mutant SOD1, and mutant α Syn proteinopathies. Similar to prior effort in amyloidosis models, we used bottom-up label-free proteomics to identify CNS proteins that lose solubility in detergent-containing buffers as pathology develops. The study design was to identify CNS proteins in control nontransgenic animals that are normally consistently detectable in aqueous fractions and of low abundance, or absent, from the detergent-insoluble fractions. We then assessed whether

these proteins aberrantly fractionate into insoluble fractions at a greater frequency in the CNS lysates of our neurodegenerative mouse models. To study the effects of tau pathology on proteome solubility, we utilized the rTg4510 mouse (human tau-P301L), an established model of conditional tauopathy [75, 77]. We also examined changes in proteome solubility in the JNPL3 model (human tau-P301L) of spinal tau pathology [58], the M83 model (human α Syn-A53T) of spinal α Syn pathology [31], and the G93A model (human SOD1-G93A) of spinal SOD1 pathology [37]. In all of these models, we observe >100 proteins that lose solubility in buffers containing SDS as pathology develops. Although some proteins were unique to each model, many were common to all four models, with some additional overlap with A β amyloidosis models. Overall, our data are consistent with the hypothesis that the accumulation of misfolded proteins associated with neurodegenerative disease imposes a burden on proteostatic function such that a subpopulation of CNS cytosolic proteins fail to maintain normal soluble conformations.

Materials and Methods

Transgenic animals

The JNPL3 mouse model of tauopathy (maintained on the Swiss Webster background from Taconic) expresses mutant human tau (P301L, 4R0N) under the mouse prion promoter and develops robust tau pathology in the spinal cord and brain stem with other regions being modestly affected [59]. All of the JNPL3 mice used in this study were female due to an earlier onset of tau pathology and motor dysfunction. The rTg4510 model of tau pathology (maintained on a hybrid 129S6/FVB background) consists of bigenic mice that contain both human tau (P301L, 4R0N) behind by a minimal promoter disrupted by tetracycline responsive elements, and the tet-transactivator (tTA) driven by a Ca^{2+} calmodulin kinase II promoter (forebrain-specific); promoting high levels of mutant tau within the hippocampus and neocortex [75]. All of the rTg4510 mice used in this study were female due to the potential for differences in the severity of tau pathology between males and females [4]. The rTg21221 mice express the wild-type human tau (4R0N) gene under the same promoter systems as that of rTg4510 mice [45]. To model α -synucleinopathy, we used the M83 mouse model (maintained on the hybrid B6/C3 background). This model overexpresses mutant (A53T) human α Syn under the mouse prion promoter [31], and develops α Syn pathology primarily within the spinal cord, brain stem midbrain, hypothalamus, thalamus and periaqueductal gray regions (other brain regions less affected) resulting in a severe motor phenotype and paralysis. This pathology and phenotype occurs between 8–16 months of age in homozygous M83 mice, but later than 21 months in hemizygous M83 mice [31]. Mice expressing the G93A mutant of human SOD1 have been previously characterized [37]. The mutant gene is expressed under the human SOD1 promoter within a 12 Kb fragment of genomic human DNA. The G93A mice used for the current study were maintained on the FVB background and develop paralysis at about 150 days of age.

Sequential protein detergent extraction from mouse forebrain/spinal cord and preparation of protein samples for proteomics analysis

The protocol used here is based on changes in protein solubility in buffers containing detergents and was developed in previous studies of proteome solubility in heat-stressed

cultured cells and the brains of APP^{swe}/PS1^{dE9} (Line 85 or L85) mice that model Alzheimer's amyloidosis [101, 102]. Altered protein solubility in buffers containing either nonionic (e.g. NP40) or ionic detergents (e.g. SDS) is a well-known criterion used to distinguish natively folded proteins from those that are misfolded and/or aggregated [32, 54, 64, 80, 91, 93]. The sequential extraction and sedimentation technique was used to reduce the complexity of samples that were subjected to LC-MS/MS. Mice were anesthetized with isoflurane, perfused with PBS, followed by the extraction of the forebrain or spinal cord. These tissues were then homogenized in 4 ml PBS on ice with 1% protease inhibitor cocktail in DMSO (Sigma Aldrich, St. Louis, MO) followed by sequential detergent extraction and ultracentrifugation. The sequence of detergents used was nonidet-P40 (NP40), followed by deoxycholate (DOC), and then sodium dodecyl sulfate (SDS) according to our previously utilized protocol [102]. We ultimately compared PBS-soluble (PBS-S) and SDS-insoluble fractions, the latter of which was resuspended in 300 μ l of 1x Laemmli buffer, in chromatography tandem mass spectrometry (LC-MS/MS). 30 μ l of each SDS-insoluble sample or 20 μ l of each PBS-soluble sample was loaded into a 4–20% Tris-HCl (Bio-Rad, Hercules, CA) gel and subjected to SDS-PAGE until proteins migrated about 1.5 cm into the gel. The gel was then stained with Coomassie blue and each lane was individually excised from the gel and cut into ~ 1 mm³ pieces. This was followed by trypsin (Sigma Aldrich, St. Louis, MO) digestion and peptide extraction from the gel by standard protocols (Online Resource 1) executed by personnel in the University of Florida Interdisciplinary Center for Biotechnology Research Proteomics and Mass Spectrometry Core (Gainesville, FL).

Calibration, relative quantification and bioinformatics of proteomics data

The number of MS/MS spectra identified for a particular protein is presumed to be largely proportional to its abundance in a sample. To determine whether a particular protein identified in SDS-insoluble fractions from CNS tissues of transgene positive mice was over-represented relative to nontransgenic controls, we used two statistical tools to analyze the total unweighted spectrum counts for each protein from the Scaffold data on protein identity (see Online Resource 1 Table S1 for a summary of the number of animals compared by the different statistical approaches). One of the statistical tools we used is the *G*-test (likelihood ratio test for independence) [70, 74], which can be programmed into Microsoft Excel. In analyzing data for rTg4510 and APP^{swe}/PS1^{dE9} mice across ages, we used littermate controls (Online Resource 1, Table S1). In studies of the JNPL3 tau, G93A SOD1, M83 α Syn mice, we grouped all of the data for nontransgenics together to produce a collective control group of *n*=6 (Online Resource 1, Table S1). For many proteins, the average number of spectral counts for a given protein was <1 in the nontransgenic littermate control samples. In such cases, the corresponding transgenic sample must have contained at least 5 spectra for the protein analyzed to achieve *G*-test significance (*p*< 0.05). In cases in which the average number of spectra for a given protein in controls was ≥ 1 , we identified proteins in the transgenic sample that presented with 3-fold more spectra and used an unpaired *t*-test, as well as the *G*-test (at least 5 spectra in the transgenic sample), to establish whether the spectral count numbers were significantly different. GraphPad Prism (Version 7.0h, La Jolla, CA) was used when *t*-tests were a statistical method of comparison. In order to identify proteins that were statistically consistent by genotype and age, we used SAINT (Significance Analysis of INteractome) analysis with a SAINT score threshold of 0.9 as

the cut off for confidence in over-representation in SDS-insoluble fractions [11]. The SAINT scoring algorithm factors protein molecular weight in its calculation of statistical probability of over-representation. To identify proteins with the highest possible confidence level as being over-represented in SDS-insoluble fractions from samples from transgene-positive rTg4510 mice, we used the SAINT scoring system and grouped all nontransgenic littermate controls for all ages together as collective control group (n=34). Bioinformatics analysis was conducted using the PANTHER (Protein Analysis Through Evolutionary Relationships) database (version 12.0, Los Angeles, CA) [65, 66]. SAS JMP pro (version 13) was used to perform cluster analysis and generate heat maps (Cary, NC).

Western blot validations of LC-MS/MS data

30 μ L of SDS-insoluble or 5 μ L of PBS-soluble protein fractions from each animal were loaded onto a Criterion 4–20% tris-glycine gel (Bio-Rad, Hercules, CA). After overnight transfer to nitrocellulose membrane, the proteins were identified by overnight incubation in primary antibody following standard procedures. Primary antibodies included ubiquitin carboxyl-terminal hydrolase isozyme 1 (UCHL1, 1:1000 Proteintech, Rosemont, IL), alpha-enolase (ENO1, 1:200, Santacruz Biotechnology, Dallas, TX), fructose biphosphate aldolase C (ALDOC, 1:1000, Encore Biotechnology, Gainesville, FL), phosphoglycerate mutase 1 (PGAM1, 1:5000, Sigma Aldrich, St. Louis, MO), malate dehydrogenase (cytoplasmic) (MDH1, 1:1000, Abcam, Cambridge, MA) and superoxide dismutase 1 (SOD1, 1:4000, generated in house).

N2a cell culture and K18-mediated human tau seeding *in vitro*

Neuro 2a (N2a) cells were purchased from the American Type Culture Collection (ATCC) and cultured in media containing an equal amount of Dulbecco's Modified Eagle Media (Thermo Fisher Scientific, Hampton, NH, USA) and Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific). Media was supplemented with 5% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA). Cell transfections were conducted using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. The vector expressing 4R/0N human tau P301L has been previously described [88]. Twenty minutes post-transfection, wild-type human K18 tau fibrils were added to the cell media to achieve a 1 μ M concentration. Cells were harvested 48 hours post-transfection followed by detergent extraction as previously described [100]. Briefly, cells were initially harvested in PBS with 1% protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) followed by sequential homogenization/extraction in different detergents (NP40 and DOC), yielding the DOC-insoluble protein fraction which was resuspended in 1x Laemmli buffer prepared from a 4x Laemmli buffer stock solution. These samples were analyzed by LC-MS/MS as described above and in Online Resource 1.

Results

Neurofibrillary tangle pathology perturbs the solubility of CNS cytosolic proteins

The rTg4510 model of tau pathology produces robust neurofibrillary tangle pathology that progressively worsens with age [75, 77]. Guided by previous studies of this model, we

produced cohorts of mice aged to 2.5, 4.5, 7.0 and 9.5 months before harvesting. Following methods described in Material and Methods (and Online Resource 1), we produced PBS-soluble and SDS-insoluble fractions from the homogenized forebrains of rTg4510 mice and nontransgenic littermates for proteomics analysis. The proteins we focused on were those that were readily detected in PBS-soluble fractions from the nontransgenic mice (having at least 5 peptide spectra detected in all nontransgenic PBS-soluble fractions analyzed per group). We viewed these proteins as molecules that should normally be highly soluble. We used two separate criteria for distinguishing when these proteins became over-represented in SDS-insoluble fractions of mutant mice relative to the nontransgenic controls. One criterion required at least a 3-fold difference in the number of peptide spectra in the SDS-insoluble fraction in transgenic samples relative to the average value for nontransgenic insoluble samples. There were many proteins where the average spectral counts for nontransgenic samples was less than 1 or zero. In these cases, only proteins in which there were at least five peptide spectra for that protein in the transgenic sample met statistical criteria (G -test; $p < 0.05$) for over-representation [43, 70, 84]. In each individual animal analyzed the number of proteins that met criteria for over-representation in SDS-insoluble fractions increased progressively by age group (Fig. 1a; Online Resource 2, Table S2). Notably, the brains of rTg4510 mice at all ages exhibited significantly higher levels of insoluble tau as compared to nontransgenic mice, with the levels of insoluble tau rising steadily across the 4 age groups (Fig. 1b). The rTg4510 mouse expresses mutant human tau-P301L conditionally, using the tet-off system in which the administration of doxycycline suppresses transgene expression. In a paradigm similar to the initial characterization of this model [75, 77], two groups of rTg4510 mice received doxycycline, beginning either at 4.5 or 5.5 months with harvest at 7.0 months of age, or beginning at 5.5 months with harvest at 9.5 months of age. When we conducted proteomics analyses on rTg4510 mice that received doxycycline, number of proteins meeting criteria for over-representation in SDS-insoluble fractions was reduced in parallel to reduced levels of insoluble tau (Fig. 1a-b, shaded area). Overall, these data demonstrate that changes in protein solubility worsen as the level of misfolded tau increases and that suppression of transgene expression attenuates the shifts in proteome solubility.

The proteins identified as losing solubility in the younger rTg4510 animals were also identified in older rTg4510 animals. Of the 148 unique proteins that were identified collectively as over-represented in the SDS-insoluble fractions for the 2.5 month old mice, 128 (86%) were identified in insoluble fractions from the forebrains of 7.0 and 9.5 month old animals; however, for many of these proteins there was considerable animal-to-animal variability in spectral count numbers (Online Resource 2, Table S2). Therefore, we used two statistical approaches to compare data between mice of different age groups to identify proteins that were consistently over-represented in the SDS-insoluble fractions. We initially used an approach in which we identified proteins that were over-represented in SDS-insoluble fractions in 7 of 10 7.0 month old rTg4510 mice and all three 9.5 month old rTg4510 mice. By this approach, we identified >200 proteins (including tau) that met statistical criteria for over-representation, relative to littermate controls (Online Resource 2, Table S3). Between the mice from the two age groups, there were 169 proteins that were common, with 255 unique proteins identified when both age groups were considered together. To further refine the data, we applied a second statistical analysis, called SAINT

scoring [11], that accounts for variability in spectral data between animals and for differences in protein molecular weight in calculating the probability of over-representation in a set of samples. The SAINT system was originally developed for analyzing protein:protein interaction data, and compares spectral count numbers between samples from two different conditions. In our study, the two conditions compared were spectral counts from controls versus transgene positive animals. In this approach, we analyze all of the data collectively, comparing the transgene positive rTg4510 mice to all nontransgenic mice of any age (see Online Resource 1, Table S1). Table 1 lists the 30 proteins other than tau with the largest fold-change in spectral counts as compiled by SAINT score analysis (see Online Resource 2, Table S3 for complete protein lists). Under this more stringent analysis, we identified 5 proteins that met a confidence level of 90% (0.9) probability to be over-represented in the SDS-insoluble fractions of forebrains from 2.5 month old rTg4510 mice (Fig. 1c; Online Resource 2, Table S4). All 5 of the proteins were also detected in insoluble fractions of 7.0 and 9.5 month old mice (Fig. 1c). Further, with these more stringent criteria, we observed a high level of overlap in protein identifications as over-represented in SDS-insoluble fractions among all age groups of rTg4510 mice (Fig. 1c). In the two oldest age groups (7.0 and 9.5 months), SAINT scoring identified 190 proteins that were common, with 277 unique proteins (aside from tau) identified when both age groups were considered together. Collectively, these data indicate that a nonrandom subpopulation of the murine CNS proteome is over-represented in SDS-insoluble fractions from the brains of rTg4510 mice with more severe tau pathology.

To further compare changes in protein solubility across all rTg4510 mice that were analyzed, we performed a cluster analysis of SDS-insoluble peptide spectra from all of the mice within our rTg4510 cohort. Based upon this analysis, which groups data sets by similarity, rTg4510 mice that received Dox from 4.5/5.5 months to 7.0 months clustered together with 2.5 and 4.5-month-old rTg4510 mice (Online Resource 3). Only two out of five 9.5-month-old mice that received Dox beginning at 5.5 months clustered with aged rTg4510 mice (7.0 and 9.5 months) that did not receive Dox. This analysis indicates that changes in proteome solubility were partially mitigated by suppressing tau expression in the 4.5–5.5 month age window. Importantly, the effect of Dox on mutant tau expression was not complete as the treated mice still express the human tau-P301L transgene at levels that are 2-fold over endogenous mouse tau [77]. Thus, in mice treated with Dox at 4.5 or 5.5 months of age, the tau pathology still worsens progressively when aged to 9.5 months [77]. Overall, the data strongly indicate that the number of CNS proteins that lose solubility in rTg4510 mice is highly correlated with the severity of tau pathology.

As indicated above, the techniques used here to analyze the rTg4510 mice are very similar to what we have used in the past to examine changes in proteome solubility in the brains of the APPswe/PS1dE9 model of Alzheimer's amyloidosis [102]. The technology in mass spectrometry has evolved rapidly in the last few years and we thus conducted a replication study of proteome solubility in the APPswe/PS1dE9 mice so that we could make better comparisons of data between the two models. Our prior study with older mass spectrometry instruments identified 28 proteins in addition to A β that were over-represented in SDS-insoluble fractions of 16 months old APPswe/PS1dE9 mice. Importantly, we were able to identify 21 of the same proteins, including A β , in the new data sets, which grew to a list of

179 proteins in 20 month old APP^{swe}/PS1^{dE9} mice (Online Resource 4 and 5). Between the APP^{swe}/PS1^{dE9} and rTg4510 models, there were 141 common proteins that could be identified as over-represented in SDS-insoluble fractions despite the very distinct locations of A β and tau pathology (Fig. 1d; Online Resource 2, Tables S4 and S5). Overall, however, the total number of proteins that show changes in solubility in the brains of rTg4510 mice was much higher than the APP^{swe}/PS1^{dE9} mice.

Validation of LC-MS/MS data in tauopathy

To validate the LC-MS/MS data, we conducted immunoblot confirmations for a subset of proteins identified. We obtained antibodies from commercial sources and confirmed detection of a band of the appropriate size for proteins of interest by immunoblots of PBS-soluble fractions. Importantly, the proteins that we tested spanned a wide range of relative abundance in our samples (estimated by spectral counts). We included in this validation rTg21221 mice expressing wild-type (WT) human tau, which do not exhibit NFT pathology [45]. No proteins consistently lost solubility in this model when analyzed at 9.5 months of age (see Online Resource 2; Table S2). We also included rTg4510 animals at 2.5, 4.5, 7.0, and 9.5 months of age, doxycycline-treated rTg4510 mice, and nontransgenic control mice. Because the composition and level of proteins in the insoluble fractions was expected to vary considerably by genotype and age, we normalized all fractions by volume. For each tissue sample, we initially generated a 10% weight by volume homogenate in PBS. The final volume of each fraction generated from a given sample was adjusted to be equivalent to the starting volume of the initial PBS homogenate (10% weight by volume). We then loaded an equivalent volume of sample in each lane, meaning that each lane should contain the same volumetric fraction of the original 10% homogenate. Focusing on SDS-insoluble fractions, we conducted immunoblots to ubiquitin carboxyl-terminal hydrolase isozyme 1 (UCHL1), alpha-enolase (ENO1), fructose biphosphate aldolase C (ALDOC), phosphoglycerate mutase 1 (PGAM1) and malate dehydrogenase (cytoplasmic; MDH1) (Fig. 2a). These proteins were easily detected in PBS-soluble fractions of nontransgenic control mice, indicative of their normally soluble state (Fig. 2a). In good alignment with the LC-MS/MS data, we observed an increase in the intensity of the band corresponding to these proteins in samples from 4.5 and 7.0 month old untreated rTg4510 mice. The intensity of the band for each protein was variably lower in Dox-treated mice. Overall, the intensity of the band for each protein tracked well with the LC/MS-MS data at 4.5 and 7.0 months of age (Online Resource 2, Table S6), but for unknown reasons the intensity of immunoreactivity for these proteins in 9.5 month old rTg410 mice was consistently lower than expected. By 9.5 months of age, we note significant animal-to-animal variability in the spectral count data and that the intensity of immunoreactivity for each protein in SDS-insoluble fractions does not entirely correlate to spectral count numbers. For example, the spectral count numbers for Eno1 are much higher than that of Uchl1, but the immunoblot data would suggest that the latter protein should be more abundant (Online Resource 2, Table S6). Overall, the immunoblot data provide a measure of validation of the mass spectrometry data.

Because the complexity of the PBS-soluble fractions that were analyzed by LC-MS/MS was much greater than that of the SDS-insoluble fractions, direct comparisons of protein abundance by spectral counting is difficult. Spectral count numbers for peptides from any

given protein can be affected by co-elution with other abundant peptides and the number of spectra for any given peptide can reach a saturation point from sample loading. From an analysis of serially diluted PBS-soluble fractions, we observed that each protein exhibits different saturation points. In the present study, the amount of sample that was loaded for LC-MS/MS of PBS-soluble fractions was biased towards detecting enough spectra from proteins of lesser abundance to provide confidence of protein identification. In this setting, the spectral counts for very abundant proteins were at saturation. Based on the intensity of immunostaining of proteins detected in SDS-insoluble fractions relative to PBS-soluble fractions in immunoblots, we surmised that a relatively small fraction of the total amount of any given protein shows shifts in solubility in the brains of the aged rTg4510 mice. To confirm this assumption, we compared the relative intensity of the immunoreactivity for MDH1 in the PBS-soluble fraction from nontransgenic brains to that of 7.0 month old rTg4510 mice. As expected, the levels of MDH1 in the PBS-soluble fraction of brain lysate from rTg4510 mice were not significantly different from that of brain lysates from age-matched nontransgenic littermates (Fig. 2b-c). Immunoblotting for SOD1 was used as a loading control because SOD1 is only detected in PBS-soluble fractions in both nontransgenic and transgenic mice (see Online Resource 2; Table S2). We conclude that at steady-state, a relatively small fraction of these proteins exhibits altered solubility. Whether these non-natively folded proteins are cleared or whether they may be in equilibrium with a natively folded state is unknown at present.

We next asked whether the proteins detected in SDS-insoluble fractions would potentially be visible as inclusions in tissue from mice with high levels of neurofibrillary tangle pathology and if changes in the distribution of these proteins would change by Dox treatment. As with validating LC-MS/MS data using immunoblot, obtaining reliable antibodies for histology was also a challenge, particularly with less-studied proteins. One of the proteins that had a large differential in spectral counts in SDS-insoluble fractions from nontransgenics relative to older rTg4510 mice was Hspa4 (Table 1). We were able to identify an antibody that appeared to be specific for Hspa4 and demonstrated increased immunostaining for this protein in older rTg4510 mice (Online Resource 6). The increased staining for Hspa4 did not appear to overlap with tau reactivity in NFT structures, suggestive that Hspa4 was not co-depositing with tau in vivo.

Differential effects of pathological tau versus amyloid- β on proteome solubility

As noted above, there was a somewhat surprising degree of overlap in the identity of proteins that lost solubility in the APPswe/PS1dE9 and rTg4510 models. We used cluster analysis to compare the data from these two models to get a more in-depth assessment of similarity. For comparison, we also included data from analysis of SDS-insoluble proteins in the brains of rTg21221 mice expressing WT human tau. All rTg21221 mice clustered into a distinct subgroup, which was expected given the lack of tau pathology and misfolded tau in this model (Fig. 3). The data from rTg21221 brain analysis clustered together with younger (2.5 months) rTg4510 samples and one 4.5-month-old rTg4510 sample. The three remaining 4.5-month-old rTg4510 mice were clustered into a separate group, exemplifying the variability in spectral count data from the SDS-insoluble brain fractions of this age group (Fig. 3). Interestingly, these three mice at this stage of tauopathy were more similar to aged

APPswe/PS1dE9 mice than to their older rTg4510 counterparts. As expected, all older rTg4510 mice (both 7- and 9.5-month groups) clustered together aside from one 7.0 month old animal, reflecting their similarities in the number and identity of proteins that aberrantly fractionate in the SDS-insoluble fraction (Fig. 3). APPswe/PS1dE9 animals clustered according to age, with older mice (20 months of age) exhibiting more severe proteome insolubility compared to younger mice (9, 12 and 16 months of age) and accurately reflects our previous findings in this model [102]. Older groups of both rTg4510 and APPswe/PS1dE9 mice were more similar to each other regarding their insoluble proteomes compared to their younger counterparts. Thus, it appears that as pathological burden increases with age (highest in older mice of these lines) a specific subpopulation of the proteome becomes over-represented in SDS-insoluble fractions from the brains of these mice.

Analysis of changes in proteome solubility in mouse models of spinal proteinopathy

To further extend our analysis of changes in proteome solubility that occur in neurodegenerative disorders, we analyzed the SDS-insoluble proteomes of mice that model SOD1-linked ALS, spinal tauopathy and spinal α -synucleinopathy (Online Resource 5), using the same criteria and statistical analyses as described above. The G93A SOD1 model develops an ALS-like paralytic phenotype accompanied by motor neuron degeneration [37] and the accumulation of aggregated mutant SOD1 that is insoluble in non-ionic detergent [48, 53, 93, 94]. Our proteomics analysis of G93A SOD1 mice revealed many cytosolic proteins that aberrantly fractionate as SDS-insoluble in spinal cords of paralyzed G93A mice. Specifically, 177 proteins were identified that exhibited a SAINT score of 0.9 based on spectra from 4 independent replicates of G93A SOD1 mice compared to 6 nontransgenic controls (see Online Resource 2, Table S4; proteins with greatest differential listed in Table S7). Notably, prior studies have demonstrated that mutant SOD1 aggregates in G93A spinal cord are solubilized by SDS [53]. Neither human nor mouse SOD1 peptides were detected in SDS-insoluble fractions at levels that met statistical significance criteria (see Online Resource 2, Table S4). Thus, in the G93A model, proteins that fractionate in SDS-insoluble fractions are clearly not doing so because of some association with an SDS-insoluble SOD1. By contrast, in rTg4510 mice, tau peptide spectra were easily detected at high frequency in SDS-insoluble fractions (see Online Resource 2, Table S4). In comparing the identities of proteins with abundant peptide spectral counts in the SDS-insoluble fractions of the G93A and rTg4510 mice (based on spectral counts), there were multiple proteins in common (Fig. 4a-b). In the data for A β , tau and SOD1 models, there were 91 shared proteins that were over-represented in the SDS-insoluble fractions G93A spinal cords and the brains of APPswe/PS1dE9 and rTg4510 models (Fig. 4c; Online Resource 2, Table S8).

To compare the effects of tau and α Syn pathology in the spinal cord on proteome solubility, we utilized the JNPL3 (human tau-P301L [59]) and M83 (human α Syn-A53T, [31]) mouse models. Both models develop abundant spinal tau or α Syn inclusions, respectively, as well as severe motor dysfunction. We used M83 mice in two separate paradigms. These included homozygous M83 mice that intrinsically develop α Syn pathology with age [31] and hemizygous M83 mice induced to develop CNS pathology via peripheral intramuscular (IM) injection of pre-formed α Syn fibrils [76] (see Online Resource 1, Supplemental Methods). In mice from all three models that exhibited severe spinal pathology and motor deficits, we

identified over 100 proteins that showed 3-fold or greater over-representation in the SDS-insoluble fraction (Fig. 5a). The harvest age for each model varied according to the age of paralytic impairment, ranging from 13 to 18 months in JNPL3 mice, 12 to 15 months in homozygous M83 mice, 5.4 to 5.8 months in the seeded M83 IM-injected mice (hereafter referred to as M83 seeded), and at 6 months in G93A SOD1 mice. The control mice for these studies, which also varied in age, were grouped as a single control population (n=6; see Online Resource 1, Table S1). Individual samples for the JNPL3 mice produced a wide variation in the number of proteins that met criteria (3-fold difference, with minimum of 5 spectral counts in the transgenic sample), ranging from 50 to 233 (134 average; Fig. 5a). SDS-insoluble fractions from individual spinal cords of aged homozygous M83 mice exhibited a range of 102 to 180 affected proteins (161 average; Fig. 5a). Similar results were achieved in heterozygous seeded M83 mice, ranging from 142 to 195 proteins identified (173 average, Fig. 5a). In comparison, in the G93A model, the range of proteins meeting criteria was 195 to 223 (210 average; Fig. 5a).

To identify proteins in the SDS-insoluble fractions that were common between the models, we used SAINT score analysis (using a score threshold of 0.9), which compared data across all models and controls in statistical analyses (individual lists per transgenic model can be found in Online Resource 2, Table S4). Among these different models, we identified 43 proteins that were commonly identified as over-represented in SDS-insoluble fractions (Online Resource 2, Table S9). To further define shared features of these models, we used cluster analysis based upon peptide spectra for detergent-insoluble proteins. The proteins identified and the number of peptide spectra for SDS-insoluble fractions from tissues from each model clustered largely by genotype (Online Resource 7). Additionally, compared to the data from rTg4510 mice, the different models of spinal proteinopathy grouped together, with G93A SOD1 mice being most similar to M83 seeded mice (accelerated pathology model). Meanwhile, JNPL3 mice displayed the greatest degree of variability, having the most similarity to aged homozygous M83 mice. Overall, the proteomic data from these models clustered first according to pathological localization and second according to genotype. In summary, our data demonstrate changes in the solubility of a subpopulation of CNS proteins and identify 43 proteins that are aberrantly detectable in SDS-insoluble fractions of spinal cord from mice with mutant SOD1, tau, or α Syn pathology.

Identification of proteins in SDS-insoluble fractions from N2a cell lysates prepared with tau aggregates

To obtain additional evidence that what was observed in our proteomics analysis of transgenic mouse tissues from tau mice was not due to the binding of cellular proteins to pathologic tau aggregates, we utilized an established paradigm of exposing mouse Neuro2a (N2a) cells to human tau aggregates [30, 36]. N2a cells were expected to express many of the same proteins found in CNS tissues. Cells were transfected with vectors to express human mutant P301L tau in conjunction with seeding the cells with pre-formed aggregates of a synthetic tau fragment (K18) [36] (see Online Resource 1, Supplemental Methods). In this paradigm, a subset of cells produce full-length human tau aggregates [36]. We hypothesized that, if proteins identified in SDS-insoluble fractions from the tau mice were simply binding to misfolded tau during the fractionation process, then such proteins should

exhibit similar behavior if they are present in the N2a cells. In all of the mouse studies above, the samples underwent sequential extraction starting with PBS, then in buffers containing NP40, DOC, and SDS (see Materials and Methods). In this cell culture model, we simplified the protocol to separate PBS soluble proteins from proteins insoluble in buffered DOC. We had previously determined that high quality LC-MS/MS data can be obtained from cultured cells with this simplified method [100, 101]. Proteins in which the spectra were at least 3-fold more abundant in DOC-insoluble fractions of cells seeded to induce tau aggregation compared to naïve cells in 2 out of 3 replicates were considered to meet criteria for over-representation. If a protein was absent from the DOC-insoluble protein fractions of naïve cells, then there must have been five peptide spectra present for the protein in the insoluble fractions from the tau seeded condition to meet criteria (G-test $p > 0.05$). As previously described, the data was filtered to focus on proteins that are normally readily detectable in PBS-soluble fractions of naïve cells but aberrantly fractionate into detergent-insoluble fractions in cells exposed to aggregated tau. Of the 20 proteins with the highest number of spectral counts in SDS-insoluble fractions from the brain of rTg4510 mice (see Fig. 4b), 7 met criteria for detection in insoluble fractions generated from the seeded N2a cells (having an average spectral count = 5) while 13 were detected only in PBS-soluble fractions (Fig. 6a-b). Importantly, only tau and one other protein (Fasn) met criteria for over-representation in the DOC/SDS-insoluble fractions of both N2a cells and rTg4510 mice (Fig. 6a). As expected, the number of spectral counts for tau in the insoluble fractions from the seeded N2a cells was much higher than any other protein (Fig. 6a). The vast majority of these tau spectral counts were derived from the recombinant tau seeds, which comprise the amino acids 244 to 372 of human tau. We identified 16 proteins that met criteria for over-representation in detergent-insoluble fractions in tau seeded N2a cells, 12 of which were unique to this paradigm and not present in either rTg4510 or JNPL3 mice (Fig. 6c-d). There were 3 proteins aside from Fasn (Acly, Glis and Tpi1) that were over-represented in the DOC-insoluble fraction of N2a cells that also met criterion for SDS-insoluble fractions from either rTg4510 or JNPL3 mice (Fig. 6d); however, the spectral counts for these three proteins were relatively low (Online Resource 5). Of the 453 proteins detected only in PBS-soluble fractions of tau seeded N2a cells, 102 of these proteins were among those that were over-represented in detergent-insoluble fractions of CNS lysates from the rTg4510 and JNPL3 models (Fig. 6d). Collectively, these data indicate that the proteins identified as over-represented in detergent-insoluble fractions of the tau models are not likely to be associating with misfolded tau aggregates during the fractionation process.

Bioinformatic analysis of proteins that aberrantly fractionate as SDS-insoluble reveals commonly affected protein classes

Our data to this point indicate that the brains of mice with distinct pathological features show common signatures of altered protein solubility. To determine whether particular classes of proteins might be selectively vulnerable, we conducted bioinformatics analyses on all proteins that met statistical criteria across the neurodegenerative proteinopathy models. For this gene ontology analysis, we honed in on protein classes that were significantly over-represented (statistical over-representation test, PANTHER database, see Materials and Methods) in the SDS-insoluble fraction relative to the mouse proteome. This revealed the common occurrence of transferases, oxidoreductases, dehydrogenases, kinases, ligases,

chaperonins, chaperones and lyases as aberrantly fractionating into insoluble fractions in the presence of neurodegenerative pathology (Fig. 7). Many protein classes were present in three or more proteinopathy models, including cytoskeletal proteins, actin family cytoskeletal proteins, non-motor actin binding proteins, aminoacyl-tRNA synthetases and hydrolases. Other affected protein classes were unique to the presence of a particular type and/or location of pathological inclusion, such as anion channels being specific to the M83 seeded model and cysteine proteases/proteases being specific to G93A SOD1 mice. Meanwhile, several protein classes were specifically affected in the presence of cortical tauopathy (Online Resource 8). Overall, the data indicate significant commonality in the identities and classes of proteins that aberrantly fractionate into SDS-insoluble fractions of CNS tissues from neurodegenerative mouse models.

Discussion

In the current study, we have determined the identity of CNS proteins that exhibit changes in detergent solubility in the presence of robust neurodegenerative pathology. Building on a prior study of mice that model Alzheimer's amyloidosis, the models examined here included mice that develop tau, α Syn, or SOD1 pathology. In mice that model cortical tau pathology, we demonstrate that the number of proteins that become aberrantly detectable in SDS-insoluble fractions increases as pathology worsens and the levels of insoluble tau increase. To control for possible associations between aggregated tau and cytosolic proteins, we used an N2a cell culture model of seeded tau aggregation. In these seeding experiments, only a fraction of the cells contain tau aggregates and, thus, this paradigm provides a control for potential interactions between tau aggregates and cytosolic proteins during cell lysis and fractionation. Our data suggest that the proteins that were over-represented in SDS-insoluble fractions of rTg4510/JNPL3 mice were not merely binding to and co-sedimenting with tau aggregates during the fractionation process. Cluster analysis of the detergent-insoluble peptide spectra across the different models demonstrated that mice with high levels of amyloidosis (aged APPswe/PS1dE9 model) clustered together with older rTg4510 mice. 141 of the 179 proteins identified as over-represented in insoluble fractions from the brains of APPswe/PS1dE9 mice by SAINT score were also found in insoluble fractions from the brains of rTg4510 mice (277 total proteins by SAINT score). Interestingly, 91 proteins identified in SDS-insoluble fractions of brains from APPswe/PS1dE9 or rTg410 mice were also found in insoluble fractions from spinal cords of G93A SOD1 models. Additionally, there were 43 proteins that were present in the insoluble fractions from spinal cords of G93A SOD1 mice that were also found in spinal fractions from α Syn and tau models. Importantly, the shifts in proteome solubility in the spinal cords of G93A mice (177 proteins total) occurred despite the fact that misfolded mutant SOD1 was not itself SDS-insoluble [53]. Collectively, these data provide strong arguments against the notion that the cytosolic proteins found in SDS-insoluble fractions of CNS tissues from these mouse models are simply binding to, or otherwise specifically co-aggregating with, a primary pathologic protein.

Proteomic approaches have been used extensively to investigate proteins that may associate with pathological features in neurodegenerative disorders (Online Resource 1, Table S13). One aspect of our study that must be emphasized is that the tissues we have subjected to our

detergent extraction protocol were all freshly isolated. When we first began to use our protocol to analyze mice that develop Alzheimer's amyloidosis, we noticed a remarkable difference in the LC-MS/MS data between freshly prepared and frozen tissue [102]. This aspect of our study must be taken into account when comparing our findings to previous work of human and mouse samples that had been frozen prior to tissue analysis (Online Resource 1, Table S13; one study used tissue embedded in paraffin [25]). We harvest and homogenize fresh tissue in order to avoid changes in solubility that could occur due to freeze/thaw. Nonetheless, despite methodological differences, a subset of the proteins we identified had also been identified in other studies as associated with pathological features or pathological proteins. Multiple studies have used a co-immunoprecipitation paradigm with either A β , or tau, on human AD and tauopathy tissues (Online Resource 1, Table S13). Of the collective 352 proteins identified in these studies, 109 overlapped with our data set (Fig. 8, left panel). One of these co-immunoprecipitation studies, identified over 100 proteins that bind to a synthetic β -structure peptide [71], but our data showed minimal overlap with this data set (only 7 proteins; Online Resource 1, Table S13). Altogether, of the 277 proteins (across all ages) we identified as over-represented in SDS-insoluble fractions of brain lysates from the rTg4510 mice, there were 115 that had been previously identified as binding Tau (Online Resource 2, Table S10). Laser capture microdissection another approach that has been used to enrich for proteins adjacent to pathological features in human tissues [25, 61, 96] (Online Resource 1, Table S13). Of the 296 proteins identified by these approaches, 94 overlap with our data set (Fig. 8, left panel). Notably, of the 36 proteins identified to be associated with Lewy bodies present in AD brains, 17 were also present in SDS-insoluble protein fractions of rTg4150 mice (Online Resource 1, Table S13) [99]. Collectively, these prior studies identified 143 proteins (in addition to tau itself) that were associated with pathologic features that we also detected as over-represented in SDS-insoluble fractions from rTg4510 mice (Online Resource 2, Table S11). Several studies have identified proteins in tissues from AD and tauopathy patients that were insoluble in detergents or otherwise exhibited altered solubility [1, 3, 35, 38, 81, 98] (Online Resource 1, Table S13), and our study identified many of the same proteins ($n = 60$; Fig. 8, right panel). One study of mice expressing tau-P30L transgenes, similar to our JNPL3 mice, identified proteins with shifts in solubility [17], and our study identified 10 of the 28 proteins previously characterized as altered (Online Resource 1, Table S13). Altogether, of the 277 proteins we identified as over-represented in SDS-insoluble fractions from the brains of 7.0–9.5 month old rTg4510 mice, 121 have not been identified in any previous proteomic study of neurodegenerative pathology (Online Resource 9).

Previous work in G93A SOD1 mice had looked for detergent-insoluble proteins that may specifically become sequestered into SOD1 aggregates [82]. An analysis of proteins extracted from frozen tissues that were insoluble in NP40 detergent identified mutant SOD1 as the only protein uniquely present in samples derived from the paralyzed G93A mice. Another study in G93A SOD1 mice identified 29 proteins enriched in triton-insoluble fractions of spinal cords from 26 week-old mice [7]; however, only 11 of these 29 proteins overlapped with our own data from this line of mice. Overall, the vast majority of proteins we identify in SDS-insoluble fractions from the spinal cords of G93A SOD1 mice have not been previously recognized as binding aggregated SOD1. Moreover, as noted above,

aggregated SOD1 is largely soluble in SDS and, thus, the insoluble proteins we detected in spinal cords of these mice were not bound to SOD1.

The process by which a subset of cellular proteins become insoluble in SDS in these models requires further study. None of the proteins identified here have been previously described to form inclusions in CNS tissues of the mouse models we examined. While it is difficult to completely exclude interactions between misfolded pathologic proteins (tau, SOD1, α Syn, etc.) and a subset of cytosolic proteins, we also envision a scenario in which proteins form SDS-resistant conglomerates as a result of extended existence in a non-natively folded state. These non-natively folded proteins could persist longer in neural cells in neurodegenerative disease either because there is insufficient chaperone function to fold them or insufficient proteasome/autophagic function to degrade them. In non-native states of folding, hydrophobic regions within these proteins could be exposed, causing them to associate into amorphous aggregates that rapidly coalesce into larger sedimentable aggregates when the tissues are initially homogenized in PBS. Notably, in prior studies of proteostatic stress in cell culture models, we have observed that acute thermal stress causes shifts in protein solubility, but the structures generated are not resistant to SDS [101]. We hypothesize that the proteins we identify as aberrantly insoluble in mouse models of tauopathy, α -synucleinopathy, and SOD1 pathology may exhibit this property due to extended existence in a non-natively folded state. There is well-established precedence for non-natively folded proteins to organize into large aggregates in the presence of detergents from studies of misfolded prion protein (PrP). Misfolded PrP^{Sc} that accumulates in the brains of hamsters infected with prion rapidly organizes into large aggregates when membranes isolated in aqueous solutions are exposed to detergents [64]. Notably, the misfolded PrP^{Sc} in the brains of infected hamsters often accumulates without organizing into recognizable intracellular inclusions [64]. We hypothesize that a similar type of reorganization of dispersed, misfolded, cellular proteins in the brains of our models could cause these proteins to lose solubility in SDS.

There have been multiple studies that have documented changes in proteasome function in tissues from mouse models of neurodegenerative disease as well as human tissues [10, 26, 28, 49–51, 55, 69, 79, 89, 104]. Diminished proteasome or autophagic function could cause non-natively folded proteins to persist longer than normal. Similarly, there is ample evidence that insufficient chaperone function could play a role in causing proteins to lose solubility. Reports have described associations between tau many different chaperones that are engaged in multiple functions, including the facilitation of microtubule binding and its proteolytic turnover [19, 23, 27, 47, 52, 67, 72, 78]. Tau also associates with other proteostatic factors, including the E3 ligases CHIP, TRAF6, and Axotrophin/MARCH7 [2, 29, 72, 83]. Both the ubiquitin/proteasome and autophagic systems contribute to tau degradation [2, 18, 20, 27, 39, 40, 57, 72, 97]. It has also recently been discovered that a deubiquinating enzyme, OTUB1, regulates the ubiquitination state of tau [95]. As previously mentioned, proteasomal impairment has been linked to tauopathy in a wide range of settings [18, 40, 55, 57, 69, 89]. Based upon the extensive degree of proteostatic elements and processes involved in tau homeostasis, it is reasonable to propose that when pathological tau accumulates to the degree that is seen in rTg4510 mice, the balance of cellular proteostasis may be compromised, leading to insufficient capacity to maintain other cellular proteins.

One of the aspects of the rTg4510 model that is particularly useful is that the expression of mutant tau is driven by promoter elements that can be regulated by treating the mice with analogs of tetracycline such as doxycycline [75]. Tau suppression was effective in improving proteome solubility when animals were harvested 2.5 or 1.5 months after initiating treatment at 4.5 or 5.5 months of age, respectively, but much less effective when the mice were aged longer (with doxycycline administered at 5.5 months of age and harvested at 9.5 months). Previous work in the rTg4510 model has characterized the effects that tau reduction has on NFT pathology, tau solubility, and cognitive performance [77]. Suppression of tau expression at 4.5 or 5.5 months of age has a limited effect on the evolution of tau pathology due to the baseline expression of the mutant tau gene (estimated to be 2-fold over endogenous in the presence of doxycycline) [77]. Our mass spectrometry data, however, show that the levels of SDS-insoluble tau are lower in mice treated with doxycycline. In prior studies of rTg4510 mice, doxycycline to suppression of mutant tau at 5.5 months of age led to statistically significant improvements (but not a full recovery) in cognitive performance when tested at either 7.0 or 9.5 months of age [77]. We observed that far fewer proteins were identified as over-represented in SDS-insoluble brain fractions from mice treated with doxycycline at 5.5 months and harvested at 7.0 months or 9.5 months. The alignment of these data suggests that changes in proteome solubility could contribute to cognitive dysfunction and neuronal death. Notably, significant losses in hippocampal neurons are first evident in rTg4510 mice at 5.5 months age, which is beyond the age that we first observe changes in proteome solubility (4.5 months; see Fig. 1a).

The present work, coupled with a prior effort using mice that model Alzheimer's amyloidosis, demonstrates that one of the common signatures of disease in mouse models of neurodegenerative proteinopathy is a loss in proteome integrity. In each of the models we examined, we identified >100 proteins that aberrantly fractionated into detergent-insoluble fractions. This study focused on proteins that were consistently detected in PBS-soluble fractions with minimal tendency to spontaneously lose solubility with age (see Online Resource 2, Table S2). A large body of literature has described changes in protein quality control networks in neurodegenerative disease including changes in the function of chaperones, the ubiquitin-proteasome system, and the autophagy-lysosomal pathway. How these changes impact cellular function has largely been uncharacterized. Here, we identify the cellular proteins and specific protein classes that are most impacted by the disruptive features of misfolded A β , tau, α Syn, and SOD1. Although each proteinopathy exhibits some unique signatures of altered cellular proteome solubility dysfunction, we identified a core of vulnerable proteins that were consistently detected in SDS-insoluble fractions at much higher frequency in the transgenic animal models analyzed here. For any given cellular protein, the portion that aberrantly fractionated as SDS-insoluble was relatively modest and this limited alteration in solubility seems unlikely to be sufficient to cause loss of function of any particular protein that we identified. However, it is possible that our method does not fully account for changes in proteome folding because some, or most, of the non-natively folded proteins in the CNS of these models might not coalesce into detergent-resistant aggregates. In conclusion, our study provides the first systematic comparison of proteome solubility across different models of neurodegenerative disease and reveals the identity of

proteins that consistently show aberrant fractionation into SDS-insoluble fractions and, thus, could be useful molecular biomarkers of proteostatic disruption.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Quantification of changes in protein detection in SDS-insoluble fractions in brains of rTg4510 mice.

(a) Numbers of proteins that meet criteria for over-representation in SDS-insoluble fractions in rTg4510 mice at different stages of tauopathy, both with (shaded area) and without tau suppression via doxycycline. Data are presented as mean $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$. Proteins were accepted if they achieved (i) at least a 3-fold increase in SDS-insoluble peptide spectra from nontransgenic to transgenic counterparts, (ii) when nontransgenic SDS-insoluble fractions yielded an average of <1 spectra for any given protein, the transgenic SDS-insoluble fraction contained at least 5 peptide spectra identified, and (iii) each individual protein in a given animal achieved G -test significance ($p < 0.05$). For doxycycline (DOX) treatment, the number shown within parentheses indicates the age (in months) at which the mice received doxycycline to suppress tau expression. Orange symbols were animals treated with DOX beginning at 4.5 months and the black symbols were animals that were treated with DOX beginning at 5.5 months of age, with all harvested at 7.0 months of age. n.s.; not significant.

(b) Numbers of SDS-insoluble tau spectra corresponding to each animal analyzed. (a-b) Each data point symbol represents an individual animal with each symbol corresponding to the same animal analyzed in panel (a). (c) Venn diagram of the affected proteins that reach SAINT score threshold (> 0.9) in each individual age group of rTg4510 mice (Online Resource 2, Table S4). (d) Venn diagram of the overlap between rTg4510 and APP^{swe}/

PS1dE9 mice based upon LC-MS/MS analysis of SDS-insoluble proteins. The protein list was compiled based upon the lists of proteins generated in Online Resource 2, Table S4.

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Fig. 2. Immunoblot validations of LC-MS/MS data in rTg4510 mice.

(a) SDS-insoluble fractions were analyzed for rTg4510, nontransgenic littermate, and rTg21221 (wild-type) tau-expressing control mice and probed with antibodies for MDH1, PGAM1, ALDOC, UCHL1 and ENO1. 30 μ L of the SDS-insoluble fractions (of the total ~300 μ L volume) from the forebrains of rTg4510 or nontransgenic mice were separated on a 4–20% tris-glycine gel. DOX treated animals received the drug at 4.5 months of age (7.0 month group) or 5.5 months of age (9.5 month group). The spectral count data from the proteins analyzed by immunoblotting are provided in Online Resource 2, Table S6. (b) MDH1 immunoblot of PBS-soluble fractions of 7-month-old rTg4510 mice and nontransgenic controls. 5 μ g of protein were loaded per well. Immunoblotting for SOD1 served as a loading control. All lanes in which samples were loaded are shown in each blot, with the blot cropped to include the relevant weight marker for each protein detected. (c) Quantification of MDH1 signal; data are presented as mean $\pm$ SD; n.s., not significant; A.U., arbitrary units.

Fig. 3. Two-way clustering of spectral count data from SDS-insoluble fractions from rTg4510, rTg21221 and APPswe/PS1dE9 mice.

Clustering is based upon detergent-insoluble peptide spectra for 222 total proteins identified as affected in either rTg4510, rTg21221 or APPswe/PS1dE9 Line 85 (L85) mice. Red is indicative of the highest number of peptide spectra for a given protein relative to nontransgenic control mice, while blue is indicative of absence of the peptide in SDS-insoluble fractions, or absence of a difference between transgenic and nontransgenic samples. Figure generated using JMP Pro Statistical Discovery from SAS (version 13.0, Cary, NC, USA).

Fig. 4. Comparison of spectral count data between rTg4510 and G93A-SOD1 mice.

(a-b) Average spectral counts of the 20 most affected proteins in either paralyzed G93A mice (a) or 7-month rTg4510 (b) mice relative to either mutant SOD1 or tau, demonstrating low levels of mutant SOD1 in the SDS-insoluble fractions of spinal cord from G93A SOD1 mice. The small black arrow marks the position for tau and SOD1 in each graph.

(c) Venn diagram of the overlap between rTg4510, APP^{swe}/PS1^{dE9} (L85), and G93A SOD1 mice based upon LC-MS/MS analysis of SDS-insoluble proteins. There were 91 proteins that were identified as enriched in SDS-insoluble fractions in G93A spinal cord by SAINT score analysis that were common between the three models.

Fig. 5. Neurodegenerative models of spinal proteinopathy characterized by paralysis also induce impairments in proteome solubility.

(a) Numbers of proteins that abnormally shift to an insoluble state in JNPL3 ($n = 7$), homozygous M83 ($n = 5$), hemizygous M83 seeded ($n = 4$) and G93A SOD1 ($n = 4$). Graph displays the number of proteins in each model that were identified as over-represented in SDS-insoluble fractions of the transgenic animal relative to nontransgenic control mice. JNPL3 (all female), homozygous M83 (3 female, 2 male), hemizygous M83 seeded (2 male, 2 female) and G93A SOD1 (2 male, 2 female) mice were analyzed at end-stage phenotype (one or more limbs exhibiting paresis). The figure was generated using GraphPad Prism (version 7.0h). Significance was assessed by unpaired, two tailed T-test *, $p < 0.01$; **, $p < 0.005$. Data are presented as mean $\pm$ SD.

(b) Venn diagram of the proteins that met criteria by SAINT score (0.9 threshold) as over-represented in SDS-insoluble fractions among all spinal models.

Fig. 6. Analysis of cellular protein co-sedimentation with tau aggregates in mouse N2a cell models.

(a) Average DOC-insoluble spectral counts in the seeded N2a cells for a subset of proteins that were identified as aberrantly fractionating to SDS-insoluble fractions from the brains of rTg4510 mice.

(b) Average PBS-soluble spectral counts for the same proteins displayed in panel (a), indicating the level of detectability of the proteins in PBS-soluble fractions.

(c) Spectral count data for the small number of proteins that meet criteria for over-representation in DOC-insoluble fractions from N2a cells seeded for tau aggregation. Proteins were accepted if they (i) achieved at least a 3-fold increase in DOC-insoluble spectra from untransfected to tau seeded cells for at least 2 out of 3 experimental replicates and (ii) during instances where untransfected cells yielded 0 peptides for any given proteins, the tau seeded condition yielded a significant G-test ($p < 0.05$).

(d) Venn Diagram comparing DOC-insoluble and PBS-soluble proteins from the N2a cells with the proteins identified as over-represented in SDS-insoluble fractions from either brain or spinal cord of rTg4510 and JNPL3 mice, respectively.

Fig. 7. Bioinformatic analysis of protein classes that are statistically over-represented in SDS-insoluble fractions of proteinopathy models analyzed via LC-MS/MS. Statistical over-representation tests ($p < 0.05$) with Bonferroni correction for multiple testing were conducted using the PANTHER (Protein Analysis Through Evolutionary Relationships) database (version 12.0). The graph was generated using Microsoft Excel (version 16.0). The protein list was compiled based upon the lists of proteins generated in Online Resource 2, Table S4.

Fig. 8. Overlapping protein identifications between SDS-insoluble fractions from Tg4510 mice and previous proteomic studies of disease-associated pathological features.

Venn diagram demonstrating the number of proteins identified from previous studies listed in Online Resource 1 (Table S13). These prior studies used various techniques including laser capture microdissections of amyloid plaques and tau tangles (LCM), affinity capture with antibodies to tau, A β -42, or synthetic β -structure proteins (IP), isolation of detergent-insoluble proteins from transgenic mouse and human disease tissue (Detergent-Insoluble), and density gradient (Other) methodologies.

Table 1
List of the proteins that showed the highest differential of over-representation in SDS-insoluble fractions from the forebrain of 7-month-old rTg4510 mice.

Spectral counts for each protein were averaged from data from 10 individual rTg4510 animals versus 34 nontransgenic controls. All spectral count comparisons in this table exhibited a SAINT score of 0.9 (n=10) for over-representation in SDS-insoluble fractions. nTg = nontransgenic, Tg = transgenic, PBS-S = PBS soluble.

Gene Symbol	Protein	Accession Number	MW kDa	Average Spectral Counts		
				SDS-insoluble		PBS-S
				Ctrl	rTg4510	nTg
Pgk1	Phosphoglycerate kinase 1	PGK1_MOUSE	45	4.47	58.2	96.8
Pkm	Pyruvate kinase	KPYM_MOUSE	58	3.74	49.8	164
Uba1	Ubiquitin-activating enzyme E1	B9EHN0_MOUSE	118	3.44	47.7	103.3
Fasn	Fatty acid synthase	FAS_MOUSE	272	1.79	37.6	40.5
Ldhb	L-lactate dehydrogenase B chain	LDHB_MOUSE	37	3.91	36.4	89
Gpi	Glucose-6-phosphate isomerase	G6PI_MOUSE	63	3.26	35.7	107
Crmp1	Dihydropyrimidinase-related protein 1	Q6PIJ1_MOUSE	62	4.41	33.7	72
Rap1gds1	RAP1 GTPase-GDP dissociation stimulator 1	Q3TU36_MOUSE	66	1.62	32.7	52.5
Cand1	Cullin-associated NEDD8-dissociated protein 1	CAND1_MOUSE	136	3.74	31	59.3
Pfkfb1	ATP-dependent 6-phosphofructokinase, liver type	PFKFB1_MOUSE	85	2.03	27.9	25
Gdi1	Rab GDP dissociation inhibitor alpha	GDIA_MOUSE	51	3.47	27.6	84.8
Synj1	Synaptojanin-1	D3Z656_MOUSE	176	3.62	26.6	54.3
Vcp	Transitional endoplasmic reticulum ATPase	TERA_MOUSE	89	0.74	26.6	56.3
Tkt	Transketolase	TKT_MOUSE	68	0.85	25.8	58
Mdh1	Malate dehydrogenase, cytoplasmic	MDHC_MOUSE	37	3.85	25.4	56
Fscn1	Fascin	FSCN1_MOUSE	55	2.38	24.9	30.8
Ldha	L-lactate dehydrogenase A chain	Q3UDU4_MOUSE	36	1.03	23.2	61.3
Prkca	Protein kinase C	Q4VA93_MOUSE	77	2.74	23.2	12.5
Eef2	Elongation factor 2	EF2_MOUSE	95	2.09	21.8	72.3
Gstp1	Glutathione S-transferase P 1	GSTP1_MOUSE	24	2.56	21.2	35.5
Glul	Glutamine synthetase	GLNA_MOUSE	42	3.65	21.1	50
Idh3b	Isocitrate dehydrogenase [NAD] subunit, mitochondrial	Q91VA7_MOUSE	42	2.94	21.1	24
Pgam1	Phosphoglycerate mutase 1	PGAM1_MOUSE	29	2.09	20.8	73.3
Vps35	Vacuolar protein sorting 35	Q3TRJ1_MOUSE	92	2.18	20.8	36
Qdpr	Dihydropteridine reductase	DHPR_MOUSE	26	1.00	20.7	16.8
Cs	Citrate synthase, mitochondrial	CISY_MOUSE	52	4.15	20.6	35.8
Ppia	Peptidyl-prolyl cis-trans isomerase A	PPIA_MOUSE	18	4.76	20.3	54.3
Gdi2	Rab GDP dissociation inhibitor beta	Q3UC72_MOUSE	57	3.09	19.7	53.75
Hspa4	Heat shock 70 kDa protein 4	Q571M2_MOUSE	94	0.56	19.6	52.5
Aldoc	Fructose-bisphosphate aldolase C	ALDOC_MOUSE	39	2.53	19.3	96