Cytosolic proteins lose solubility as amyloid deposits in a transgenic mouse model of Alzheimer-type amyloidosis

Guilian Xu1,2,3, Stanley M. Stevens, Jr 4,5, Brenda D. Moore1,2, Scott McClung4,∗ and David R. Borchelt1,2,3,*

1Department of Neuroscience, 2Center for Translational Research in Neurodegenerative Disease, 3SantaFe HealthCare Alzheimer’s Disease Research Center and 4Interdisciplinary Center of Biotechnology Research (ICBR), University of Florida, Gainesville, FL 32610, USA and 5Department of Cell Biology, Microbiology and Molecular Biology, University of South Florida, Tampa, FL 33620, USA

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The extracellular accumulation of β-amyloid peptide is a key trigger in the pathogenesis of Alzheimer’s disease (AD). In humans, amyloid deposition precedes the appearance of intracellular inclusion pathology formed by cytosolic proteins such as Tau, α-synuclein and TDP-43. These secondary pathologies have not been observed in mice that model Alzheimer-type amyloidosis by expressing mutant amyloid precursor protein, with or without mutant presenilin 1. The lack of secondary pathology in these models has made it difficult to establish how amyloid deposition initiates the cascade of events that leads to secondary intracellular pathology that characterizes human AD. In transgenic mice that model Alzheimer-type amyloidosis, we sought to determine whether there is evidence of altered cytosolic protein folding by assessing whether amyloid deposition causes normally soluble proteins to misfold. Using a method that involved detergent extraction and sedimentation coupled with proteomic approaches, we identified numerous cytosolic proteins that show specific losses in solubility as amyloid accumulates. The proteins identified included glycolytic enzymes and members of the 14-3-3 chaperone family. A substantial accumulation of lysine 48-linked polyubiquitin was also detected. Overall, the data demonstrate that the accumulation of amyloid by some manner causes the loss of solubility intracellular cytosolic proteins.

INTRODUCTION

The amyloid cascade hypothesis of Alzheimer’s disease (AD) predicts that the deposition of β-amyloid (Aβ) peptide is a critical early event in disease that precedes the appearance of intracellular neurofibrillary tangles (NFTs) (1). This hypothesis is grounded in the genetics of early-onset AD in which mutations in genes encoding the amyloid precursor protein (App) and presenilin 1 (Psen1), or Psen2, lead to alterations in the proteolytic processing of amyloid precursor protein (APP) to enhance the relative production of the Aβ 42 peptide (Aβ42) (reviewed in 2). Studies in mouse models have demonstrated that one of the primary consequences of expressing mutant APP, with or without mutant PS1, is an earlier appearance of amyloid pathology (3–6). In humans, amyloid pathology antedates the appearance of NFTs being the most common intracellular pathology (reviewed in 7). In AD with Lewy bodies, an accumulation of α-synuclein containing Lewy body inclusions is the major intracellular pathology that occurs along with amyloid pathology (reviewed in 8). Moreover, recent studies have demonstrated that 20–50% of AD cases show intracellular inclusion pathology containing TDP-43 (9). One hypothesis that could explain the pathogenic cascade of AD is that the accumulation of amyloid, in some manner, causes a disruption in protein
homeostasis (proteostasis), which in turn leaves neurons less capable of preventing the accumulation of misfolded intracellular proteins (reviewed in 10,11).

Proteostasis is viewed as being a delicate balance of the intracellular chaperone network and/or protein degradation systems (reviewed in 10). Recent studies in Caenorhabditis elegans models have led to the hypothesis that a disturbance in protein homeostasis caused by the accumulation of misfolded intracellular proteins (mutant huntingtin or human superoxide dismutase) (12,13) can produce broad effects on the folding of multiple ‘bystander’ proteins that are inherently metastable at physiologic temperatures (14). In the present study, we have asked whether the accumulation of amyloid in the brain impinges on the cytosolic protein homeostatic network, creating an environment in which proteins lose solubility.

RESULTS

To investigate whether amyloid deposition associated with AD may perturb the proteostasis network, we determined whether cytosolic brain proteins lose solubility in APPswe/PS1dE9 transgenic mice (5) with high amyloid burden (16 months of age). We used a method in which brains were first homogenized in phosphate buffered saline (PBS) followed by sequential extraction and centrifugation in buffers containing detergent of increasing strength [NP40, sodium deoxycholate (DOC) and SDS], following a protocol developed in cell models of heat shock (15) (see Supplementary Material, Fig. S1). It is well-established that the loss of solubility in detergent distinguishes misfolded proteins in multiple settings including prion protein, amyloid beta peptide, superoxide dismutase 1, α-synuclein and huntingtin (16–22). There are examples of misfolded proteins that are insoluble in non-ionic detergent but soluble in SDS, such as mutant superoxide dismutase 1 (23), and examples of misfolded proteins that remain insoluble in SDS, such as amyloid beta peptide and huntingtin (17,21). In our experimental application of detergent extraction and sedimentation, we took advantage of the propensity of misfolded proteins to expose hydrophobic surfaces, which induces a cascade of aberrant protein–protein interactions that leads to the formation of large, heterogeneous, insoluble protein aggregates (24,25). In the fractionation of the mouse brains, we produced the following samples: PBS-soluble and -insoluble, NP40-soluble and -insoluble, DOC-soluble and -insoluble and SDS-soluble and -insoluble. The primary goal of this scheme was to reduce the complexity of protein mixtures that were ultimately analyzed by SDS–PAGE, in-gel trypsin digestion and liquid chromatography coupled to mass spectrometry (LC-MS/MS). The strategy used here was very similar to a strategy we recently used to identify proteins in SH-SYSY and CCF-STTG1 cells that lose solubility upon heat shock (15).

Preliminary assessments of the various fractions by SDS–PAGE and LC-MS/MS indicated that the SDS-insoluble fractions were of a sufficiently low complexity to allow high-quality protein identification. Ultimately, we decided to compare the SDS-insoluble fraction of non-transgenic mice with that of the transgenic mice. We also analyzed PBS-soluble fractions from non-transgenic mice as a means to identify proteins that should normally be soluble.

To begin to identify proteins that lose solubility, two completely independent LC-MS/MS experiments were performed with pairs of 16-month-old transgenic mice with same-gender non-transgenic littermates as controls. Starting with SDS–PAGE gels of PBS-soluble fractions from brains of NTg mice and SDS-insoluble fractions from brains of NTg and APPswe/PS1dE9 mice (see Supplementary Material, Fig. S2 for an example of the gels used in proteomic analyses), we identified a total of 749 proteins (Supplementary Material, Table S1) from the combination of all the samples (see Materials and Methods for an explanation of criteria for protein identification). In comparing the data from the two independent analyses, we identified 28 proteins that appeared to lose solubility in mice with amyloid deposition (Table 1). The criteria we used to identify such proteins were the following: (i) there was a ≥5-fold difference in the number of peptide identifications (spectra) for the specific protein between SDS-insoluble fractions of APPswe/PS1dE9 and NTg mice in both experiments; (ii) when the SDS-insoluble fraction of NTg mice lacked spectra for a given protein, the number of spectra for that protein in the SDS-insoluble fraction of the APPswe/PS1dE9 mice was at least five in both experiments. Additionally, to further confirm which proteins may be over-represented in the SDS-insoluble fraction of the APPswe/PS1dE9 mice, we used a G-test (see Materials and Methods) to estimate significance (P < 0.05) (Table 1). In cases in which no peptides for a given protein were identified in the SDS-insoluble fraction of brains from NTg mice, statistical significance in the G-test was met only when we identified at least five peptides for that protein in the SDS-insoluble fraction of brains from APPswe/PS1dE9 mice. This level of stringency focused attention on the most robust signals, which we anticipated would be the most easily reproduced. Ultimately, the list of proteins generated included several very abundant proteins in which we identified well over 10 spectra per protein. Additionally, for most of the proteins identified in the SDS-insoluble fraction from the brains of APPswe/PS1dE9 mice, we found abundant spectra for those proteins in the PBS-soluble fraction of non-transgenic animals (Table 1; Supplementary Material, Table S1).

To validate the MS data from the mice, we turned to immunoblotting of fractions prepared in an identical manner from an independent set of mice (Fig. 1; Supplementary Material, Fig. S3). The first major hurdle we encountered was obtaining high-quality antibodies to the proteins identified by LC-MS/MS. Ultimately, we identified several antibodies that seemed to be highly specific and that recognized a band of the expected molecular weight on immunoblots. The antibodies used recognized proteins that were representative examples of proteins that spanned the range of spectral counts (low to high abundance) in the SDS-insoluble fractions. The validation set of APPswe/PS1dE9 mice included animals that were 20 (n = 3), 6 (n = 3) and 2 months old (n = 2), along with non-transgenic littermate controls (one from each age). Focusing on the SDS-insoluble fraction, we analyzed immunoblots with antibodies to γ-enolase (Eno2 or NSE), α-enolase (Enol1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 14-3-3 eta and phosphoglycerate mutase 1 (PGAM1) (Fig. 1A). Each of these
proteins was identified as over-represented in the SDS-insoluble fractions of 16-month-old APPswe/PS1dE9 mice by LC-MS/MS (Table 1) and each of these proteins was specifically detected only in the SDS-insoluble fractions of 20-month-old APPswe/PS1dE9 mice by immunoblot (Fig. 1A). No insoluble forms of these proteins were detected in younger APPswe/PS1dE9 mice or NTg mice of any age. In general, we were able to validate a sampling of our LC-MS/MS data by immunoblotting.

Other aspects of the data provided additional validation of the LC-MS/MS data set. As one would expect, we identified a signature peptide for Aβ in almost every segment of gel from the SDS-insoluble fractions of older APPswe/PS1dE9 brains (one peptide of Aβ was repeatedly identified by LC-MS/MS; see Supplementary Material, Fig. S4 for representative spectra). We completely validated the presence of Aβ in multiple gel segments by detecting Aβ immunoreactivity in immunoblots as a smear throughout the gel (Fig. 1B). Apart from Aβ, no other APP peptides were detected in SDS-insoluble fractions. APP was abundantly detected in PBS-soluble fractions of the APPswe/PS1dE9 mice with antibodies specific for the human Aβ domain of the transgene-derived protein (Fig. 1A). ApoE and clusterin were also detected that were only found in PBS-soluble fractions (Supplementary Material, Table S1), and we confirmed the presence of this protein in these fractions by immunoblot (Fig. 1A).

In all of the fractions we analyzed (including pilot studies of fractions not detailed here), a total of 280 proteins were detected that were only found in PBS-soluble fractions [never detected in any detergent insoluble fraction in any brain sample (see Supplementary Material, Table S2)]. Proteins such as tau, α-synuclein and superoxide dismutase 1 were examples of proteins that were detected only in the PBS-soluble fractions. Immunoblots of SDS-insoluble fractions of both NTg and APPswe/PS1dE9 mice (See Supplementary Material, Table S1), and we confirmed the presence of this protein in these fractions by immunoblot (Fig. 1A).

Table 1. Detergent-insoluble proteins identified in APPswe/PS1dE9 (line 85) mouse forebrains

<table>
<thead>
<tr>
<th>Gene product symbol</th>
<th>Protein</th>
<th>Accession number</th>
<th>MW (kDa)</th>
<th>pI</th>
<th>Experiment A</th>
<th>Experiment B</th>
<th>G-test</th>
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<tr>
<td>NSF</td>
<td>Vesicle-fusing ATPase</td>
<td>IP000656325</td>
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<td>6.8</td>
<td>0</td>
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<td>20</td>
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<td>APOE</td>
<td>Apolipoprotein E</td>
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<td>0</td>
<td>24</td>
<td>3</td>
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<td>GADPH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>IP000273646 (+1)</td>
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<td>8.6</td>
<td>3</td>
<td>23</td>
<td>55</td>
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<tr>
<td>DNMI</td>
<td>Isoform 3 of dynamin-1</td>
<td>IP000466504</td>
<td>96</td>
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<tr>
<td>HSPA8</td>
<td>Heat shock cognate 71 protein</td>
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<td>CKB</td>
<td>Creatine kinase B-type</td>
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<td>62</td>
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<td>ENO1</td>
<td>Alpha- enolase</td>
<td>IP000462072</td>
<td>47</td>
<td>6.6</td>
<td>0</td>
<td>12</td>
<td>64</td>
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<td>YWHAZ</td>
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<td>2</td>
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<td>34</td>
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<td>0</td>
<td>10</td>
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<td>YWHAQ</td>
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<td>4.9</td>
<td>0</td>
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<td>22</td>
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<td>ACTC1</td>
<td>Actin, alpha cardiac muscle 1</td>
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<td>0</td>
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<td>11</td>
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<tr>
<td>CLTC</td>
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<tr>
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<tr>
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<td>4.9</td>
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<td>9</td>
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<td>ACCO2</td>
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<td>27</td>
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<td>0</td>
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<td>DPYSL2</td>
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<tr>
<td>STXBP1</td>
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<td>IP000415402 (+1)</td>
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<td>EIF1A1</td>
<td>Elongation factor 1-alpha 1</td>
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<td>ALDOA</td>
<td>Fructose-bisphosphate aldolase A</td>
<td>IP000221404</td>
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<td>ARFI</td>
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<td>CLU+</td>
<td>Clusterin</td>
<td>IP000320420 (+1)</td>
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<td>5</td>
<td>0</td>
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<td>UBA52a</td>
<td>Ubiquitin</td>
<td>IP000621468 (+1)</td>
<td>8.5</td>
<td>6.8</td>
<td>0</td>
<td>2</td>
<td>3</td>
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</table>

*Western blot validated in this study.
By focusing on the SDS-insoluble fractions, we reduced the probability that the proteins found in the insoluble fractions might be non-specifically bound to amyloid. We expected that the ionic detergent would dissociate any weakly bound proteins. To further control for the possibility that any of the proteins found in the SDS-insoluble fraction from the older mice were non-specifically associated, we conducted the following control experiment. We took synthetic peptides of Ab40 and 42 and aggregated the peptides in vivo. We then mixed these aggregates, using an amount that would be predicted to be similar to the amyloid load of older APPswe/PS1dE9 mice, with brain homogenates of older non-transgenic mice at the initial stage of homogenization. The samples were then processed exactly as we had processed the APPswe/PS1dE9 mice to separate soluble and insoluble proteins. We then probed the SDS-insoluble fractions and PBS-soluble fractions with the same panel of antibodies used in Figure 1 (Fig. 3A). Tubulin, which was found in insoluble fractions by LC-MS/MS of non-transgenic mice described in Figure 1, was similarly detected in these fractionation experiments (Fig. 3A). Immunoblotting these same fractions with antibody to Ab demonstrated the sedimentation of Ab aggregates in the SDS-insoluble fractions derived from brain homogenates spiked with pre-formed aggregates (Fig. 3B).

In the LC-MS/MS experiments, we identified ubiquitin spectra in multiple gel segments. Using the same set of animals described above, we confirmed that mice with high amyloid burden accumulate high-molecular-weight SDS-insoluble polyubiquitin (Fig. 4A). Ubiquitin-conjugated proteins contain a diglycine remnant of ubiquitin covalently attached to a lysine residue that is resistant to trypsin proteolysis (29,30). The vast majority of peptides possessing the K-GlyGly ion were ubiquitin itself. We were unable to detect the K-GlyGly motif on any peptides from proteins listed in Table 1. Ubiquitinated ubiquitin peptides were found in multiple gel pieces, corresponding to various molecular weight ranges. The primary linkage of the polyubiquitin was Lys-48 to form polyubiquitin chains (Fig. 4B).

Collectively, this series of immunoblot studies provided a measure of validation of the LC-MS/MS analyses and identified several cytosolic proteins that lose solubility as amyloid accumulates. We interpret these data as an indication that the accumulation of amyloid is associated with a secondary accumulation of insoluble cytosolic enzymes. To determine whether the proteins that lost solubility were unique in some manner, we calculated the theoretical protein theoretical isoelectric point (pI) and hydrophobicity of proteins identified in insoluble fractions. The pI of the SDS-insoluble proteins from APP/PS1dE9 mice was slightly more acidic than that expected from the mouse proteome (Supplementary Material, Fig. S7A); however, the proteins in the insoluble fractions were no more hydrophobic than the mouse proteome (Supplementary Material, Fig. S7B). Thus, we identified no obvious distinguishing feature of the proteins that lost solubility.

An obvious question is whether the proteins that we identify as losing solubility are expressed in neurons or astrocytes. To address this issue, we used expression data for each of these proteins...
genes that are available on the Allen Brain Atlas (http://mouse.brain-map.org/). All of the genes listed in Table 1 have been analyzed for expression by in situ hybridization and as expected all were expressed at some level in the brain. The cellular pattern of the expression of these genes was largely neuronal, although about half the genes were also expressed at some level in white matter (Table 2). Only four of the genes showed strong expression in astrocytes in the hippocampus: APOE, CKB, EE1A1 and CLU (Table 2). Of these four, CKB and EE1A1 were also expressed in neurons. APOE and CLU were exclusively astrocytic. Thus, based on the expression patterns of these genes, it would appear that the secondary accumulation of insoluble proteins is likely to be occurring in neurons.

Bioinformatic analysis of the LC-MS/MS data suggested that certain types of proteins were vulnerable to ‘bystander’ misfolding. Using gene ontology analysis (PANTHER tools) to classify the 28 detergent-insoluble proteins from APPswe/PS1dE9 brains, the most abundant classes of proteins identified were chaperones (9 out of 28 proteins: 6 members of 14-3-3 family and HSP90AA1, HSPA8, HSP90AB1, \( P = 1.68 \times 10^{-9} \)) and enzymes involved in glycolysis (6 out of 28 proteins, including GAPDH, ENO1, PGAM1, ALDOA, PGK1 and ENO2, \( P = 1.35 \times 10^{-7} \) by biological process, \( P = 1.99 \times 10^{-10} \) by pathway) (Fig. 5 and Supplementary Material, Fig. S8A). The relationship between the 28 insoluble proteins in Table 1 and their cellular location was analyzed using Pathway Studio (Supplementary Material, Fig. S8B). Most of the insoluble proteins from the APPswe/PS1dE9 mouse brains were cytosolic proteins; only one protein was localized in the nucleus.
In the present study, we have used proteomic approaches to analyze brain tissues from mice that model Alzheimer-type amyloidosis, asking whether the deposition of amyloid leads to secondary ‘bystander’ misfolding of cytosolic proteins. We identified 25 neuronally expressed cytosolic proteins that appeared to specifically lose solubility in the brains of APPswe/PS1dE9 mice with high amyloid burden. Immunoblotting of a separate cohort of animals validated a subset of the LC-MS/MS data providing confirmation that at least six proteins show specific losses in solubility as amyloid accumulates. Additionally, the brains of these mice accumulate insoluble K-48-linked polyubiquitin, which is potentially an indication of an overwhelmed proteasome (15). APPswe/PS1dE9 mice with high amyloid burden displayed both of these phenotypes, whereas none of these biomarkers were present in young APPswe/PS1dE9 transgenic mice or older non-transgenic mice. These proteins were not found in insoluble fractions when pre-aggregated Aβ was spiked into brain homogenates and then fractionated to separate detergent-insoluble proteins. One explanation that is consistent with our findings is that the deposition of amyloid, by some manner, increases the burden of the protein homeostasis network, leading to diminished folding of bystander cytosolic proteins in a collateral damage model of action.

Importantly, the loss of solubility by normally cytosolic proteins in the older APPswe/PS1dE9 mice was not global in nature. There were still a large number of proteins that were found only in the PBS-soluble fractions, including abundant proteins such as tau, α-synuclein or SOD1. Thus, it appears that in these mice a select subset of cytosolic proteins is vulnerable to bystander misfolding. Although in humans tau and α-synuclein are prone to misfold when amyloid deposition occurs, it appears that the murine forms of these proteins are resistant to bystander misfolding. Whether the appearance of misfolded forms of these proteins in human disease is solely a consequence of bystander misfolding or some other more complex mechanism is clearly a topic for further study.

Previous studies have utilized proteomic approaches to identify proteins in human AD brain that show reduced solubility (31,32). We note a 30–40% overlap in the identities of proteins identified in these human AD studies with our study of the mouse model (Table 3). A major caveat in interpreting studies of human post-mortem tissues is how ante-mortem events may impact the brain and how the handling of tissues may introduce artifacts. In our study, the brains were quickly removed, chilled, homogenized and then fractionated. Additionally, human AD brains exhibit all the secondary intracellular pathology that is normally associated with the disease, making it impossible to define the role of amyloid.

Our studies in mice provide key evidence that amyloid deposition can impose a burden on protein homeostasis such that abundant constituents of the neuronal proteome lose solubility.

The accumulation of K-48-linked polyubiquitin in the symptomatic APPswe/PS1dE9 mice is also consistent with diminished proteostasis function. However, it is also clear that the inhibition of the proteasome can induce the accumulation of K-48-linked polyubiquitin (33). Hence, with the accumulation of K-48 polyubiquitin, we cannot distinguish proteostasis stress from proteasome dysfunction. Similar to our mouse model, an accumulation of polyubiquitin linked by K-48, as well as K-11- and K-63-linked, polyubiquitin has been detected in human AD brain (34). However, this study of human brain did not separate detergent-soluble and -insoluble fractions. Our study, suggests that K-48-linked polyubiquitin in the brains of the APPswe/PS1dE9 mice is, in some manner, associated with detergent-insoluble proteins.

There are three reports in the literature in which investigators have identified proteins associated with pathologic features of AD. In general, it is not straightforward to relate our findings to these published works because these studies start with frozen tissue and we avoided freezing tissues in our study to avoid inducing changes in protein solubility by freeze/thaw. However, we noted some similarities between our findings in mice and human studies. In a study in which amyloid plaques were isolated by laser capture microdissection, there were 22 proteins identified as associated with plaques (35); and 8 of the proteins we identified in detergent-insoluble fractions of APPswe/PS1dE9 mice were identical (Table 3). A much longer list of proteins has been reported to be associated with NFT pathology (36); and 21 of the insoluble proteins in our study overlap with those associated with tangles (Table 3). Finally, we also note significant

| Table 2. Expression pattern of proteins that lose solubility in older APPswe/PS1dE9 mice |
|---------------------------------|-----------------|-----------------|
| Neuronal ISH | Neuronal and white matter ISH | Astrocytic ISH |
| NSF, DNM1, ENO1, YWHAG, CLTC, YWHAG, YWHAB, STXBP1, PGAMA1, ALDOA, ARF1, ENO2 | GAPDH, HSPA8, CKB, HSPA90AB1, YWHAQ, ACTC1, HSPA90AA1, YWHAE, PGK1, ACO2, GDH1, DFPYL2, EEF1A1 | APOE, CKB, EEF1A1,CLU |

The gene product names are listed in order of appearance in Table 1.

**DISCUSSION**

In the present study, we have used proteomic approaches to analyze brain tissues from mice that model Alzheimer-type amyloidosis, asking whether the deposition of amyloid leads to secondary ‘bystander’ misfolding of cytosolic proteins. We identified 25 neuronally expressed cytosolic proteins that appeared to specifically lose solubility in the brains of APPswe/PS1dE9 mice with high amyloid burden. Immunoblotting of a separate cohort of animals validated a subset of the LC-MS/MS data providing confirmation that at least six proteins show specific losses in solubility as amyloid accumulates. Additionally, the brains of these mice accumulate insoluble K-48-linked polyubiquitin, which is potentially an indication of an overwhelmed proteasome (15). APPswe/PS1dE9 mice with high amyloid burden displayed both of these phenotypes, whereas none of these biomarkers were present in young APPswe/PS1dE9 transgenic mice or older non-transgenic mice. These proteins were not found in insoluble fractions when pre-aggregated Aβ was spiked into brain homogenates and then fractionated to separate detergent-insoluble proteins. One explanation that is consistent with our findings is that the deposition of amyloid, by some manner, increases the burden of the protein homeostasis network, leading to diminished folding of bystander cytosolic proteins in a collateral damage model of action.

Importantly, the loss of solubility by normally cytosolic proteins in the older APPswe/PS1dE9 mice was not global in nature. There were still a large number of proteins that were found only in the PBS-soluble fractions, including abundant proteins such as tau, α-synuclein or SOD1. Thus, it appears that in these mice a select subset of cytosolic proteins is vulnerable to bystander misfolding. Although in humans tau and α-synuclein are prone to misfold when amyloid deposition occurs, it appears that the murine forms of these proteins are resistant to bystander misfolding. Whether the appearance of misfolded forms of these proteins in human disease is solely a consequence of bystander misfolding or some other more complex mechanism is clearly a topic for further study.

Previous studies have utilized proteomic approaches to identify proteins in human AD brain that show reduced solubility (31,32). We note a 30–40% overlap in the identities of proteins identified in these human AD studies with our study of the mouse model (Table 3). A major caveat in interpreting studies of human post-mortem tissues is how ante-mortem events may impact the brain and how the handling of tissues may introduce artifacts. In our study, the brains were quickly removed, chilled, homogenized and then fractionated. Additionally, human AD brains exhibit all the secondary intracellular pathology that is normally associated with the disease, making it impossible to define the role of amyloid.

Our studies in mice provide key evidence that amyloid deposition can impose a burden on protein homeostasis such that abundant constituents of the neuronal proteome lose solubility.

The accumulation of K-48-linked polyubiquitin in the symptomatic APPswe/PS1dE9 mice is also consistent with diminished proteostasis function. However, it is also clear that the inhibition of the proteasome can induce the accumulation of K-48-linked polyubiquitin (33). Hence, with the accumulation of K-48 polyubiquitin, we cannot distinguish proteostasis stress from proteasome dysfunction. Similar to our mouse model, an accumulation of polyubiquitin linked by K-48, as well as K-11- and K-63-linked, polyubiquitin has been detected in human AD brain (34). However, this study of human brain did not separate detergent-soluble and -insoluble fractions. Our study, suggests that K-48-linked polyubiquitin in the brains of the APPswe/PS1dE9 mice is, in some manner, associated with detergent-insoluble proteins.

There are three reports in the literature in which investigators have identified proteins associated with pathologic features of AD. In general, it is not straightforward to relate our findings to these published works because these studies start with frozen tissue and we avoided freezing tissues in our study to avoid inducing changes in protein solubility by freeze/thaw. However, we noted some similarities between our findings in mice and human studies. In a study in which amyloid plaques were isolated by laser capture microdissection, there were 22 proteins identified as associated with plaques (35); and 8 of the proteins we identified in detergent-insoluble fractions of APPswe/PS1dE9 mice were identical (Table 3). A much longer list of proteins has been reported to be associated with NFT pathology (36); and 21 of the insoluble proteins in our study overlap with those associated with tangles (Table 3). Finally, we also note significant
Aging C. elegans

Beta-structure
AD Lewy bodies
Density gradient
ACO2, AP2A1, CLTC, DPYSL2, GAPDH, GPI, HSP90AB1, OTUB1, Aβ, APOE, DNMT1, YWHAH, YWHAZ
8 of 22

AD plaques
Laser capture microdissection
Abeta, ATP6V1B2, DNMT1, PFKP, UBA1, YWHAH, YWHAE, YWHAZ
8 of 22

AD tangles
Laser capture microdissection
ACTB, APOE, CFL1, CLTC, DNMT1, DPYSL2, EEF1A1, ENO1, GAPDH, GIP, HSPA6, MDH1, PGK1, PKM2, PPIA, PRDX1, STXB1, SYN2, UBA52
21 of 99

AD Lewy bodies
Density gradient
ACO2, AP2A1, CLTC, DPYSL2, GAPDH, GIP, HSP90AB1, OTUB1, UBA1, UBA52
10 of 38

Beta-structure binding
Affinity chromatography
CLTC
1 of 49

Aging C. elegans
Trition/DOC/SDS insoluble
ACO2, GAPDH, HSPA8, PPIA, RPL6, YWHAE, YWHAZ
7 of 123

*123 proteins were identified in an aging-accelerated, Fem-1 mutant, C. elegans model as showing a >5-fold increase in abundance in the insoluble fractions. A much longer list of proteins was identified as showing 1.5- to 5-fold changes in abundance in insoluble fractions.

overlapping proteins were identified in an aging-accelerated, Fem-1 mutant, C. elegans model as showing a >5-fold increase in abundance in the insoluble fractions. A much longer list of proteins was identified as showing 1.5- to 5-fold changes in abundance in insoluble fractions.

We note very little overlap between our list of insoluble proteins in older APPswe/PS1de9 mice and proteins identified as binding beta structures (38) or proteins identified as becoming detergent-insoluble in a model of accelerated aging in C. elegans (39) (Table 3). The lack of overlap with these data sets provides an additional measure of confidence, beyond our control experiments, that the proteins identified in older APPswe/PS1de9 mice as acquiring detergent insolubility are not inherently prone to adhere to aggregated beta structures, nor simply prone to lose solubility with aging.

Alzheimer’s disease is thought to involve a pathogenic cascade in which the deposition of amyloid antedates secondary intracellular inclusion pathology caused by the accumulation of misfolded tau, α-synuclein or TDP-43 (7–9). It has been suggested that the accumulation of misfolded cytosolic proteins may be a consequence of diminished function of the cellular protein quality control network, or proteostasis network (12,40). This network, which includes chaperones of the cytosol and endoplasmic reticulum, the autophagic system and the ubiquitin/proteasome system, functions in the folding, post-translational modification and degradation of proteins involved in multiple functional pathways (reviewed in 10,40). Loss of function in these systems could cause collateral damage by disrupting protein homeostasis in a manner that compromises the ability of other bystander proteins to correctly fold (12,13). Our study suggests that amyloid deposition causes the secondary misfolding of intracellular proteins. For reasons that remain unclear, the accumulation of amyloid in the brains of mice does not result in the secondary intracellular inclusion pathology that characterizes AD. However, our results demonstrate that amyloid accumulation, by a yet-to-be-defined mechanism, disrupts the folding of cytosolic proteins, providing a potential mechanistic explanation for how amyloid deposition could trigger a pathogenic cascade.

**MATERIALS AND METHODS**

**Transgenic mice**

Line 85 transgenic mice co-expressing mutant human PS1 (with exon 9 deletion) and mouse/human chimeric APP695 (humanized Aβ domain) harboring the Swedish (K594M/N595L) mutation used in this study have been described previously (5). All the mice were B6C3 hybrid background. Both transgenes were expressed under the control of the mouse prion protein promoter (MoPrP.Xho), which drives high protein expression in neurons and astrocytes of the central nervous system (26,41). Two pairs of 16-month-old mice and their same-gender non-transgenic littermates were used for LC-MS/MS. Mice of other ages, including 2, 6, 16 and 20 months, were used for immunoblot analysis to validate the MS data. Sixteen-month-old non-transgenic mice of the same strain were used for control experiments in which brains homogenized in PBS were spiked with pre-aggregated Aβ. Animals of both genders were used. All procedures involving animals were approved by the University of Florida Institutional Animal Care and Use Committee.

**Preparation of Aβ aggregates**

Hexafluoro-2-propanol-pre-treated Aβ1–40 and Aβ1–42 peptides (California Peptide) were dissolved at 2.2 mM in 50 mM NaOH by vortex for 30 s. Aggregation reactions were initiated by incubating monomeric Aβ peptides in 150 mM NaCl, 20 mM Tris–HCl (pH 8.0) at 37°C. Aggregation kinetic parameters were obtained by monitoring the reaction with thioflavin T. After 17 h incubation of Aβ1–42 and 31 h incubation of Aβ1–40, reactions were centrifuged (19,000 x g for 5 min) to separate supernatant and pellet fractions. Pellet fractions were resuspended in 150 mM NaCl, 20 mM Tris–HCl (pH 8.0) and collected for future experiments.
Sequential detergent extraction of brain proteins and proteomic analysis

The mice were euthanized by CO₂ inhalation and then the forebrains were quickly removed and homogenized in 5 ml of PBS, followed by sequential detergent extraction to fractionate the proteins according to their solubility in NP40, DOC and SDS (see Supplementary Material, Fig. S1 for detailed methods). The samples for LC-MS/MS were separated by 4–20% Tris–glycine SDS–PAGE, 45 μl of each fraction was loaded per lane. From the Coomassie blue-stained SDS–PAGE gel, each lane was separated out and then subsequently cut into five to nine pieces, from the lowest to highest molecular weight. Standard in-gel trypsin digestion was used prior to LC-MS/MS protein identification following a protocol used by the Protein Chemistry Core (ICBR, University of Florida, Gainesville, FL, USA). See Supplementary Material for additional detailed descriptions of methods used in protein identification and bioinformatic analysis.

LC-tandem mass spectrometry analysis

The digested peptides from all the samples in this study were analyzed with QSTAR® XL (Applied Biosystems, Foster City, CA, USA), a hybrid quadrupole time-of-flight mass spectrometer. A 120 min gradient was used for LC separation. Tandem mass spectra were extracted by Analyst QS (version 1.1). Charge-state deconvolution and deisotoping were not performed. Depending on sample source, all MS/MS samples were analyzed using Mascot (version 2.2.0) to search IPI MOUSE database (version 3.32, 49 427 entries) (42), assuming digestion enzyme trypsin. Both Mascot and X! Tandem (www.thegpm.org; version 2007.01.01.1) was used to search a subset of the IPI MOUSE database (version 3.80, 1002 entries) databases, also assuming trypsin. Both Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 0.30 Da. Iodoacetamide derivatives of cysteine were specified in Mascot and X! Tandem as a fixed modification. S-carbamoylmethylcysteine cyclization (N-terminus) of the N-terminus, de-amination of asparagine and glutamine, oxidation of methionine and ubiquitination signatures of lysine were specified in Mascot and X! Tandem as variable modifications.

Scaffold (version 2.01_02, Proteome Software, Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Later, data were converted and modified with a new version of Scaffold (version 3.00_07). Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm (43). Protein identifications were accepted if they could be established at >99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (44). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

SDS–PAGE and immunoblot

Proteins from different fractions (10 or 20 μl) were separated by 4–20% Tris–glycine SDS–PAGE for immunoblot, using antibodies against ubiquitin [5–25 (1:5000), Signet] according to standard protocols. Antibodies against 14-3-3 eta (1:5000, EnCor, Gainesville, FL, USA), GAPDH (1:10 000, EnCor), PGAM1 (1:500, Sigma), ENO2 (NSE, 1:500, Sigma), Clusterin (C-18: sc-6419, 1:200, Santa Cruz), ENO1 (H-300: sc-15343, 1:200, Santa Cruz), ubiquitin [1:1000, DAKO or MCA-Ubi-1(1:1000), EnCor], tubulin βIII (1:5000, Covance), Aβ [6E10 (1:1000), Signet] and tau (mTau 5157, 1:5000, a gift from Dr Naruhiko Sahara, University of Florida) were used.

Gene ontology and other bioinformatics analysis

pl and hydrophobicity

The pl and hydrophobicity of the whole mouse proteome (EBI database ipi.MOUSE.v3.72 from European Bioinformatics Institute) were calculated by Protein Digestion Simulator (version 2.0) developed by the Department of Energy (PNL, Richland, WA, USA). The Kyte–Doolittle scale, a widely used method for delineating the hydrophobic character of a protein, was used to calculate the hydrophobicity of the proteins.

Biological process, protein class and pathway

Gene ontology analysis of the specific insoluble proteins identified from APPswe/PS1dE9 (line 85) transgenic mouse brain was classified based on the PANTHER (Protein ANalysis Through Evolutionary Relationships) system (http://www.pantherdb.org) (45,46). The PANTHER Protein Class ontology was adapted from the PANTHER/X molecular function ontology, and includes commonly used classes of protein functions.

Protein relationship network

The Pathway Studio software (version 8.0, Ariadne Genomics, Rockville, MD, USA) was used to analyze the relationship between the identified insoluble proteins and the enriched pathways.

Data compilation and semi-quantification

The number of unweighted spectrum counts per protein was tabulated from the unfiltered Scaffold data for comparison. Unweighted spectrum counts from the different conditions were compared between samples; those proteins with a significant difference in the number of spectra were analyzed in more detail. To test whether the abundance of a particular protein was significantly higher in one sample than another, a spectral counting technique was used (47). The method for relative quantitation followed published protocols (48,49) in which the change in abundance was determined by the ratio of the total number of identified MS/MS spectra (normalized unweighted spectral count from Scaffold) for a particular protein in the APPswe/PS1dE9 and non-transgenic mice,
respectively. A statistical G-test (likelihood ratio test for independence) was then utilized to determine the statistical probability that the abundance of a particular protein in a particular fraction was higher or lower than expected (48,50). To increase statistical power for G-test analysis and overall spectral counting sensitivity of proteins identified with high confidence (99% protein confidence, 95% peptide confidence and containing two unique peptides), we included in the data sets peptides with lower Mascot or Sequest scores that represent true positive identifications at 50% probability to match. Differences in protein composition between fractions were considered highly significant if the G-test significance was \( P < 0.05 \).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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*Conflict of Interest statement.* None declared.

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**REFERENCES**


