Molecular and functional signatures in a novel Alzheimer’s disease mouse model assessed by quantitative proteomics

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Abstract

**Background:** Alzheimer’s disease (AD), the most common neurodegenerative disorder, is characterized by the deposition of extracellular amyloid plaques and intracellular neurofibrillary tangles. To understand the pathological mechanisms underlying AD, developing animal models that completely encompass the main features of AD pathologies is indispensable. Although mouse models that display pathological hallmarks of AD (amyloid plaques, neurofibrillary tangles, or both) have been developed and investigated, a systematic approach for understanding the molecular characteristics of AD mouse models is lacking.

**Methods:** To elucidate the mechanisms underlying the contribution of amyloid beta (Aβ) and tau in AD pathogenesis, we herein generated a novel animal model of AD, namely the AD-like pathology with amyloid and neurofibrillary tangles (ADLPAPT) mice. The ADLPAPT mice carry three human transgenes, including amyloid precursor protein, presenilin-1, and tau, with six mutations. To characterize the molecular and functional signatures of AD in ADLPAPT mice, we analyzed the hippocampal proteome and performed comparisons with individual-pathology transgenic mice (i.e., amyloid or neurofibrillary tangles) and wild-type mice using quantitative proteomics with 10-plex tandem mass tag.

**Results:** The ADLPAPT mice exhibited accelerated neurofibrillary tangle formation in addition to amyloid plaques, neuronal loss in the CA1 area, and memory deficit at an early age. In addition, our proteomic analysis identified nearly 10,000 protein groups, which enabled the identification of hundreds of differentially expressed proteins (DEPs) in ADLPAPT mice. Bioinformatics analysis of DEPs revealed that ADLPAPT mice experienced age-dependent active immune responses and synaptic dysfunctions.

**Conclusions:** Our study is the first to compare and describe the proteomic characteristics in amyloid and neurofibrillary tangle pathologies using isobaric label-based quantitative proteomics. Furthermore, we analyzed the hippocampal proteome of the newly developed ADLPAPT model mice to investigate how both Aβ and tau pathologies regulate the hippocampal proteome. Because the ADLPAPT mouse model recapitulates the main features of AD pathogenesis, the proteomic data derived from its hippocampus has significant utility as a novel resource for the research on the Aβ-tau axis and pathophysiological changes in vivo.

**Keywords:** Alzheimer’s disease, Animal disease model, Aβ, Tau, Quantitative proteomics, 10-plex tandem mass tag

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Background

Alzheimer’s disease (AD) is the most common neurodegenerative disorder and is characterized by extracellular amyloid plaque deposition, intracellular neurofibrillary tangle (NFT) development, and memory impairments [1, 2]. Although the disease has been extensively studied over the past several decades, the exact mechanisms and pathological causes of AD remain unclear. Genetically modified mouse models that recapitulate the major features of AD pathologies are invaluable in determining the underlying disease mechanisms and evaluating new therapeutic approaches. Few transgenic mouse models, such as the triple transgenic mice, 3xTg-AD model, have been introduced to develop the concomitant manifestation of both amyloid plaques and NFTs [3]. Because this model showed no neuronal cell death in the hippocampus, the development of a new mouse model that fully mimics AD pathologies is still needed.

One of the most widely used models in AD research is the 5xFAD (Tg6799) model, which contains five familial AD mutations within the APP and PSEN1 genes. The 5xFAD model is considered to be an effective AD model because of the rapid progression of the amyloid pathology [4]. The JNPL3 mouse model, which expresses P301L-mutant human tau, has been widely used to examine intraneuronal NFTs [5]. To recapitulate the main features of AD pathogenesis, we herein developed a new mouse model that carries six mutations within transgenes encoding human amyloid precursor protein (APP), presenilin-1 (PSEN1), and tau. The resulting transgenic mouse model, Alzheimer’s disease-like pathology with APP, PSEN1, and MAPT transgenes (ADLPAPT), exhibited Aβ accumulation, NFTs, early neuronal loss in the brain, and subsequent memory impairments. The pathological phenotypes of ADLPAPT mice, which feature both Aβ deposition and NFTs, were compared with those of ADLPAPP/PS1 (Aβ deposition, no NFTs) and ADLPTau (no Aβ deposition, NFTs) mice of the same genetic background. Understanding the interplay between Aβ accumulation and NFTs is imperative for elucidating the pathogenesis of AD. Thus, the ADLPAPT mouse model, which shows both robust amyloid and NFT pathologies, should be an excellent model for examining the Aβ-tau axis in vivo.

The hippocampus is known to play an important role in memory formation [6]. Thus, understanding the pathological status of the hippocampus under AD is crucial for studying the mechanisms of AD-related memory impairments. Although some mechanisms that contribute to AD pathogenesis have been uncovered by studies on individual genes or proteins, systematic analysis of the pathological changes of the hippocampus is lacking. Mass spectrometry (MS)-based proteomics is expected to be an appropriate tool for systematic analysis [7, 8]. Although MS-based proteomics have been limited because of the incomplete coverage of the proteome, recent technological advances allowed researchers to study comprehensively up to 10,000 proteins from a single cell line [9]. However, this level of coverage requires extensive pre-fractionation, large samples, and several months of instrument time [10]. In addition, the reliable MS-based quantitation under several perturbation states requires the use of biological and technical replicates, thus increasing the complexity of MS experiments. Importantly, these shortcomings can be overcome by an isobaric labeling strategy, such as the application of tandem mass tags (TMTs) [11]. The recent expansion of multiplexing capacity up to 10 samples per MS injection has markedly increased the scope of quantitative proteomics [12]. In TMT experiments, protein quantification is accomplished by comparing the intensities of reporter ions produced during MS/MS [11]. Since this approach enables sensitive and precise protein quantification, many research groups have successfully used TMT-based strategies [13–16].

With the aid of 10-plex TMT quantification strategy combined with high-resolution MS, we constructed a comprehensive proteome map of the newly developed mouse models. We have successfully discovered nearly 10,000 proteins and quantified 7000 proteins from the hippocampus of wild type, ADLPAPP/PS1, ADLPTau, and ADLPAPT mice. The protein abundances of ADLPAPT mice were compared with those of other single transgenic mice to discover differentially expressed proteins and characterize functional signatures of ADLPAPT mice via bioinformatics analysis. Furthermore, our network analysis could suggest the presence of interacting proteins that connect between amyloid and NFT pathologies. In conclusion, new ADLPAPT mice and their hippocampal proteome dataset may help to offer a novel insight of pathogenesis of AD in further studies targeting the concurrent molecular network of amyloid and NFT pathologies.

Methods

Experimental design

The aim of this study was to construct a mouse model of Alzheimer’s disease that carries mutant human genes and to introduce its molecular and functional characteristics. The protein expressions of newly constructed mouse models were assessed by quantitative proteomics combined with LC-MS/MS and TMT isobaric labeling. A total of 36 hippocampi samples were used in the proteomic experiments. (4 mouse types * 3 age-points * biological triplicates), which were randomly divided into four 10-plex TMT experimental sets. All samples were analyzed twice via MS. Three to twelve mice were sacrificed accordingly to the type of biochemical experiment, which includes western blotting and immunostaining. Behavioral tests of the AD model mice were performed by investigators in blind with respect to genotypes. No data were excluded.
Reagents and materials
Tandem mass tag (TMT) 10-plex isobaric reagents, bicinchoninic acid (BCA) assay kit - reducing agent compatible, tris (2-carboxyethyl) phosphine (TCEP), and LC/MS-grade solvents such as acetone, acetonitrile (ACN), and water were purchased from Thermo Fisher Scientific (Waltham, MA). Other reagents and materials were purchased from the following companies: Dithiothreitol (DTT) and urea from AMRESCO (Solon, OH), Sodium dodecyl sulfate (SDS), Trizma base from USB (Cleveland, OH) and sequencing-grade modified trypsin from Promega Corporation (Madison, WI), POROS20 R2 bead from Applied Biosystems (Foster City, CA). High-purity (>97%) mass spectrometry (MS) grade ovalbumin from Protea (Morgantown, WV), HLB Oasis column from Waters (Milford, MA). All other reagents, unless noted, were purchased from Sigma-Aldrich (St. Louis, MO).

Transgenic mice
5XFAD mice (Tg6799; Jackson Laboratory, Stock#006554) express both mutant human APP with the Swedish, Florida, and London mutations and mutant human PSEN1 with the M146 L and L286 V mutations under the murine Thy1 promoter. JNPL3 mice (TauP301L-JNPL3; Taconic, Stock#2508 homozygote) carry mutant human tau with the P301L mutation under the murine prion protein promoter. Due to the mixed genetic background of JNPL3 mice, ONPL3 mice were backcrossed with B6SJL (C57BL/6 X SJL) mice. The resulting JNPL3 mice on the B6SJL genetic background were crossed with 5XFAD mice to create a novel animal model, ADLP animal model. This carries the three human mutant genes and its corresponding mutations mentioned before. Only female mice were used for pathological characterization due to earlier signs of aggraved pathologies and memory deficit than male mice.

Immunohistochemistry (IHC)
Mice were anesthetized and perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The brain tissues were fixed with 4% PFA for 20 h at 4 °C, incubated in 30% sucrose (wt/vol) for 72 h and then frozen. The frozen brains were cut into 30 μm coronal sections using a Leica CM 1850 Cryostat. Brain slices were washed with PBS and then incubated in 70% formic acid in PBS for 20 min to perform antigen retrieval when amyloid plaques and NFTs were stained. Brain slices were permeabilized and blocked with blocking solution (0.3% Triton X-100, 5% horse serum, and 0.05% BSA solution) for 1 h at 20 °C prior to incubating it with primary antibodies overnight. Amyloid plaques were stained with the biotin-4G8 antibody (1:700, COVANCE), followed by the streptavidin-488-conjugated secondary antibody (Invitrogen). Hyperphosphorylated tau was examined by using the AT8 (1:300, Thermo Scientific) and AT180 (1:300, Thermo Scientific) antibody, which recognize the Ser202/Thr205 epitopes and Thr231 of human tau, followed by biotinylated anti-mouse IgG (Vector Laboratories) and streptavidin-594-conjugated secondary antibody (Invitrogen). To visualize astrocytes and microglia, anti-GFAP (1:1000, Invitrogen) for astrocytes and anti-Iba-1 (1:500, Wako) antibodies were used respectively. Hippocampal neurons of the CA1 layer were visualized by staining with anti-NeuN (1:1000, Millipore) antibody. Stained brain slices were incubated with goat anti-rat Alexa 488, donkey antirabbit Alexa 488, and donkey anti-mouse Alexa 647 antibody (1:500, Life Technologies) for 1 h at 20 °C. Images were obtained using LSM 700 (Carl Zeiss). At least six serial sections of each sample were imaged to consider the volume of cells in brain slices. When the number of neuronal cells in CA1 layer stained by anti-NeuN antibody were counted, one middle region of the hippocampus tissue was imaged to avoid an overlap of CA1 pyramidal neurons. All images were quantified using ImageJ software (NIH).

Sarkosyl-insoluble tau fractionation
One side of the hippocampus was homogenized in 8 volumes of Tris buffer solution (TBS) including phosphate inhibitors and protease by tissue grinder [TBS solution; 25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, phenylmethylsulfonyl fluoride (PMSF), protease 1, and 2]. The homogenates were centrifuged at 14,000 x g at 4 °C for 15 min. The supernatant was collected for further fractionation. The supernatant was incubated with N-Lauroylsarcosine sodium salt solution 20% solution (1% final concentration) at 37 °C rotator for 1 h and then ultracentrifuged at 150,000 x g at 25 °C for 1 h. The resulting sarkosyl-insoluble pellets were resuspended in TBS solution for washing pellets. The mixture was concentrated by ultracentrifuge again at 150,000 x g at 25 °C for 1 h. The resulting pellets containing tau aggregates were suspended with 5xSample buffer (Serva Blue G) and heated at 70 °C for 10 min to prevent further aggregation.

Behavioral test
For Y-maze test, after introduction to the middle of the maze, the mouse was allowed to freely explore new environments for 8 min. Spatial memory function was measured as the percent of spontaneous alteration [17]. The number of total arm entries and the sequence of the Y-maze arm into which mice entered were recorded in order to calculate the percentage of spontaneous alteration. The number of alternations was counted when the mouse entered into the three different maze arms consecutively. The percent of spontaneous alteration was calculated as the number of alterations divided by the total entry number multiplied by 100.
RT-PCR analysis

To verify the amount of mouse or human tau mRNA levels in ADLP\textsuperscript{Apt} and ADLP\textsuperscript{APT} mice, RT-PCR analysis was carried out with 10-month-old ADLP model mice. Total RNA was extracted from the hippocampus with the RNaseasy Mini kit (QIAGEN). All RNA samples were converted into cDNA using Maxime RT PreMix Kit (Intron BIOTECH). Quantitative RT-PCR was carried out in triplicates using KAPA SYBR FAST ABI Prism qPCR kit (KAPA Biosystems). For mouse Tau, the primers 5′-AGCCCTAAGACTCCCTCCA-3′ and 5′-TGCTGTAGGC GCCGCATT CGTTTGTGCAGTG-3′ were used. Human tau was amplified with GAPDH which was amplified with the primers 5′-GCCCTTGACTGTGCCGTTGAATTT-3′ and 5′-CCTTGCTCAGGTC AACTGGT-3′. The mRNA levels of mouse Tau and human Tau were normalized with GAPDH which was amplified with the primers 5′-GGCCCTTGTGCTGCGTTGAATTT-3′ and 5′-ACA GCGCATC TTCTTGTGCAGTG-3′. Once the reaction was completed, the RT-PCR products were evaluated/analyzed via gel-electrophoresis to measure/calculate the specificity of human tau primers.

Western blot analysis

Western blot analysis was used to confirm tau aggregates from sarkosyl-insoluble tau fractionation and the validation of proteomic analysis results. After isolated tau aggregates were heated at 70 °C for 10 min to prevent further tau protein aggregation, the same volume of sarkosyl-insoluble tau aggregate samples was loaded per lane of 4–12% Bis-Tris polyacrylamide precast gels (NuPAGE system, Invitrogen). Following electrophoresis, proteins were transferred to a PVDF membrane. Membranes were blocked with 5% skim milk solution and then incubated with primary antibodies against human tau (Tau13, Abcam, 1:1000) and total tau (endoogenous tau and human tau) (Tau5, Abcam, 1:1000). Primary antibodies against ABCA1 (Abcam, 1:500), Ptpcr (CD45, Abcam, 1:1000) and Hcls1 (HS1, CST, 1:1000) were used for the validation of proteomic analysis. For confirmation of kinase expression levels, CK18 (Abcam, 1:5000), RSK1 (CST, 1:2000) and GSK-3β (CST, 1:2000) were used as primary antibodies. Anti-mouse or rabbit IgG conjugated HRP was used to detect primary antibodies and West Save Gold (Ab frontier) was used for their visualization. Since sarkosyl-insoluble fractionation only extracts protein aggregates, certain proteins generally used for normalization such as GAPDH and β-actin were not detected in the sarkosyl-insoluble pellets. Thus, total antibody signals of tau aggregates or each signal for distinct sizes of tau aggregates were quantified for quantification.

Mass spectrometry-based proteomics

Mouse hippocampus tissues were resected and subjected to the previously described sample preparation methods with some modifications [18–20]. Detailed procedures including protein digestion, peptide labeling, fractionation, and MS analysis are described in Additional file 1: Supplementary Methods.

Quantification of protein abundance and statistics

The quantification and statistical processing methods described below are related to proteomic data. First of all, among the 9814 identified proteins, only 6964 proteins satisfying the following criteria were used for subsequent quantitative analysis; identification in all channels (7022 proteins), high protein confidence (6970 proteins, assessed by Proteome Discoverer), and possessing 1 or more unique peptides (6964 proteins). The protein abundance ratio of individual samples to pooled sample (named “normalized protein abundance”) was generated by dividing the reporter ion intensity of each channel by the intensity of the pooled sample channel in its corresponding experimental TMT set (Additional file 2: Figure S3B). There was no significant difference between the values of the pooled sample channels (Additional file 2: Figure S5E). Thus, the denominators were considered to be common and eliminated. The fold-change values used in the bioinformatics analysis were generated by dividing the normalized protein abundance of each transgenic mouse by the value of the age-matched wild type mouse. The distribution of ratiometric data was almost normal (Additional file 2: Figure SSG) but this was not thoroughly tested.

Statistical processes for the proteomic data were performed based on the normalized protein abundance using Perseus [21]. Initially, total identified proteins were filtered based on the 6964 proteins that quantified in all mouse samples. The statistical cut-off value for significance was set to \( p \)-value <0.05 for the Student’s \( t \)-test, while Benjamini-Hochberg FDR adjusted \( p \)-value cut-off [22] of 0.05 was applied for the ANOVA test. The normalized protein abundances were subjected to z-normalization followed by hierarchical clustering. The statistical tests for the other biochemical experiments were described in each figure legend.

Bioinformatics analysis

The Gene Ontology (GO) of the proteins was classified using DAVID bioinformatics tool (version 6.8) [23]. The GO classification was evaluated by Fisher’s exact test to obtain a set of \( P \)-values, which were then filtered at a cut-off value of 0.01. Canonical pathways, downstream biological functions, and upstream regulators were enriched using Ingenuity Pathway Analysis (IPA, QIAGEN) [24]. The analytical algorithms embedded in IPA uses input protein list (here differentially expressed proteins) to predict putative upstream regulators such as transcription factors and growth factors, as well as downstream effects on known biological pathways. IPA derives these protein
set-pathway (or regulator) relationships from their own large-scale causal network database, named Ingenuity Knowledge Base. Because the algorithm cannot determine with certainty which causalities in its database can explain our experimental results, the tool performs statistical tests (i.e., Fisher’s exact test) to assess the reliability of predicted upstream genes and pathways. Finally, IPA also assigns activation states (activated or inhibited) to putative regulators or pathways based on the quantitative values of protein members. The user will be given a confidence in the P-value obtained from the Fisher’s exact test and the magnitude of activation as a Z-score, respectively. In this study, the P-value cut-off criteria for the enrichment was 0.01 for Fig. 6a and b and the predictive activation Z-score cut-off was 1. For the Fig. 4b, the cut-off value was 0.05 and the predictive activation Z-score cut-off was 1. The initial pathway diagrams were obtained by IPA but were manually modified. Protein-protein interactions (PPIs) for the network analysis was interrogated from STRING database (http://www.string-db.org) [25]. The PPIs in network model were visualized using Cytoscape [26].

Results
Pathological symptoms of ADLP\textsuperscript{APT} mice
Four mouse models with the same genetic background were generated: wild-type, ADLP\textsuperscript{APP/PS1} (Aβ accumulation only), ADLP\textsuperscript{TAU} (NFTs only), and ADLP\textsuperscript{APT} (both Aβ and NFTs) mice. To confirm the amyloid plaque burden in the hippocampus of the ADLP mice, we stained coronal brain sections of 4-, 7-, and 10-month-old ADLP mice with the biotin-4G8 antibody that recognizes amino acids 17–24 of Aβ peptides. Both ADLP\textsuperscript{APP/PS1} and ADLP\textsuperscript{APT} mice started showing slight extracellular accumulation of Aβ at 4 months and exhibited large amyloid plaques at 7 months; no significance was observed between-model difference in their amyloid plaque burdens (Fig. 1a and c).

To investigate the NFT pathology in the hippocampus, we first stained phosphorylated tau using the AT8 antibody, which detects residue Ser202/Thr205 of phosphorylated tau in paired helical filaments (PHFs). Intense immunoreactivity of PHFs localized only at the soma region to detect the residue Thr231 of phosphorylated tau. The residue Thr231 was strongly stained at the soma and dendrites of CA1 neurons starting from 4 months of age. In 10-month-old mice, we observed phosphorylated tau localized only at the soma region as a form of NFTs, indicating that ADLP\textsuperscript{APT} mice developed severe NFT pathology compared with ADLP\textsuperscript{TAU} mice (Additional file 2: Figure S1A).

To validate the expression of tau in the hippocampus, mRNA levels of endogenous mouse tau were confirmed by RT-PCR analysis. The results indicated no significant differences among the ADLP mouse models. Furthermore, the human tau mRNA expression was not different between ADLP\textsuperscript{TAU} and ADLP\textsuperscript{APT} mice, indicating accelerated NFT pathology in ADLP\textsuperscript{APT} mice without quantitative difference in tau protein (Additional file 2: Figure S1B–1D).

Constructing a Hippocampal proteome database of the ADLP mouse models
To obtain insights into the molecular basis of AD pathogenesis, we performed quantitative proteomic analysis using three replicates of hippocampus resected from each mouse model at three time points (4, 7, and 10 months of age; Fig. 2a). Briefly, each hippocampus was homogenized and digested via filter-associated sample preparation [28],
Fig. 1 (See legend on next page.)
Gab2 factors, such as samples. Notably, we also detected low-abundance risk Tardbp TDP-43 ([37][29]), indicating that our proteome exhibited a profound correlation value of 0.991 (Additional file 2: Figure S5F). Thus, our extensive proteome profiling of the hippocampal CA1 layer of ADLP mice was stained with the AT8 antibody against phosphorylated tau (Ser202/Thr205). A significant increase in AT8 immunoreactivity was observed in ADLP^APP mice compared with age-matched ADLP^tau mice (Student’s t-test, n = 3–4 per group). Scale bar represents 200 μm. e Sarkosyl-insoluble tau fractions from 7 and 10 months ADLP mice hippocampus were analyzed by western blot analysis using human tau specific antibody (Tau13). f Each distinct size of sarkosyl-insoluble tau was quantified in 10 months old ADLP^tau and ADLP^APP mice (Chi-square test; n = 6 mice per genotype). g The CA1 pyramidal neurons of ADLP mice were stained with anti-NeuN antibody to determine degrees of neuronal loss. Scale bars represent 100 μm or 50 μm (enlarged figures). h Quantification of the number of CA1 neurons in 7- and 10-month-old ADLP mouse model (one-way ANOVA in each age of ADLP mouse model). i ADLP model mice showed memory impairment compared with wild-type mice, which examined by the Y-maze test (one-way ANOVA in each age of ADLP mouse model, n = 9–11 per group). Results are expressed as mean ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001

and the peptide samples were labeled with TMT reagents. The labeled samples were analyzed using Q Exactive mass spectrometer. The resulting 96 raw files (12 peptide fractions × 4 TMT-mix experimental sets × technical duplicates) were processed in Proteome Discoverer based on the SEQUEST-HT algorithm. In total, 9814 protein groups were identified from 125,683 unique peptides; among them, 7022 protein groups were successfully identified in all 36 samples (Fig. 2b and c). The expression levels of the mutated human proteins (APP, PSEN1, and tau) were uniquely identified in our dataset, indicating that our data expanded the hippocampal proteome to a great extent using a single type of sample on a more time-efficient platform (Additional file 2: Figure S4A). In addition, we compared the efficiency of protein identification with those reported in other proteomic studies of transgenic AD mouse models; our dataset contained the highest number of protein identifications (Additional file 2: Figure S4B).

Quality assessment of the proteomic data

The multiplexing capacities of the TMT-based workflow allowed us to characterize the quantitative variations within and between hippocampal samples. Our analysis of a non-homologous (i.e., ovalbumin) spiked-in standard for inter- and intrabatch normalization revealed a coefficient of variation of 6.3% during repetitive MS analysis (Additional file 2: Figure S5A). Although the intensity variations among non-normalized biological replicates showed a suitable reproducibility, we observed a slight improvement of reproducibility when the normalization was performed using ovalbumin (Additional file 2: Figure S5B and C). Thus, the reporter ion intensities of proteins were initially normalized with respect to the intensity of ovalbumin.

To investigate the quantitative reproducibility (technical and experimental set-to-set variations), we used protein abundances in pooled sample channels (131) to perform cross-correlation examinations. Based on the results, the duplicate MS analysis showed excellent consistency, with an average R² correlation value of 0.994 (Additional file 2: Figure S5D). The mean R² value of the quantified protein abundance between the TMT experimental sets was excellent at 0.992 (range, 0.988–0.995) (Additional file 2: Figure S5E). Furthermore, the results indicated good correlation among the biological replicates, with a mean Pearson’s correlation value of 0.991 (Additional file 2: Figure S5F). Thus, the observed differences in protein expression reflected the molecular diversity among the mouse histotypes, not the effect of our TMT-based strategy [49].
Fig. 2 (See legend on next page)
Proteome alterations in ADLP hippocampus

To resolve the regulated proteins in each ADLP mouse model, we quantitatively analyzed the protein expression in a ratiometric manner (see Methods). Based on the fold-change in the normalized protein abundance, we identified 63, 178, and 245 proteins that were significantly regulated in 4-, 7-, and 10-month-old ADLP APP/PS1 mice, respectively (Student’s t-test, p-value <0.05, fold-change >1.25). In comparison, 3, 42, and 4 proteins were significantly regulated in 4-, 7-, and 10-month-old ADLP APT mice, respectively, whereas 62, 131, and 311 proteins were significantly regulated in 4-, 7-, and 10-month-old ADLP APT mice, respectively. The scatter plots showed that more proteins were up-regulated during the progression of amyloid pathology than during that of NFT pathology. Interestingly, the overall tendencies of the significantly regulated proteins were similar in ADLP APP/PS1 and ADLP APT mice, indicating that the amyloid pathology exerted stronger effects on the overall proteome than did the NFT pathology (Fig. 3). The proteins with significant change in each model are summarized in Additional file 5: Table S3.

To identify the specific protein changes under the various AD pathologies (Aβ, Tau, or both Aβ and Tau), we used ANOVA to investigate the global diversity. Our ANOVA indicated that 1094 protein groups were differentially expressed proteins (DEPs) among the ADLP mouse models (FDR cut-off <0.05; Additional file 6: Table S4). Hierarchical clustering divided these proteins into three groups, including up-regulated proteins (cluster 2; 681 proteins) and down-regulated proteins (cluster 3; 121 proteins). ADLP Tau mice had relatively few DEPs, whereas ADLP APP/PS1 and ADLP APT mice shared DEPs that showed drastic age-related perturbations (Fig. 4a). This suggests that tau itself is insufficient to drive the hippocampal proteome regulation during the stage up to 10 months of AD pathogenesis.

We subsequently investigated how these DEPs affected downstream pathways in an unbiased manner. The ingenuity pathway analysis (IPA) platform identified five canonical pathway categories (nervous system signaling, immune response, cell cycle/apoptosis, cancer/disease signaling, and intracellular signaling pathways) that were significantly enriched by the DEPs (p-value <0.05; activation Z-score >1; Fig. 4b). The primary enriched pathway categories were nervous system signaling and the immune response, which are known to be associated with AD pathogenesis. While the signaling pathways of the nervous system tend to become inactive with age, most identified immune response pathways were predicted to be activated as the disease progressed. The exact Z-scores and p-values for the IPA results are listed in Additional file 7: Table S5.

Identifying molecular and functional signatures associated with pathology of ADLP APT mice

We next investigated the age-dependent dysregulation of biological functions in ADLP APT mice. We identified 732 proteins that were differentially expressed with respect to ADLP WT mice (DEPs APT, ANOVA FDR cut-off <0.05; Additional file 8: Table S6). Our hierarchical clustering analysis divided the DEPs APT into five clusters, including gradually down-regulated proteins (cluster 2; 79 proteins) and gradually up-regulated proteins (cluster 5; 499 proteins; Fig. 5a). Gene ontology (GO) enrichment analysis revealed that the proteins in cluster 2 were particularly involved in synapses and cytoskeleton binding, suggesting that the AD pathologies gradually damage the synaptic plasticity in the hippocampus of ADLP APT mice (Fig. 5a, right panel, and Fig. 5b). The proteins of cluster 5 were primarily assigned to the inflammatory and degradation systems, such as leukocyte-mediated immunity, phagocytosis, endosomes, and lysosomes, indicating that AD pathologies could activate both inflammation and degradation systems to remove Aβ plaques and NFTs (Fig. 5c and Additional file 9: Table S7).

To define biological activities that contribute to the pathological phenotypes of ADLP APT mice, we performed bioinformatics analysis using IPA to identify upstream regulators as well as downstream functions of DEPs APT (Additional file 10: Table S8). The IPA predicted 25 relevant upstream regulators to modulate DEPs APT. In addition, DEPs APT were assigned to 27 downstream biological functions associated with the immune response and the nervous system (p-value <0.01; Fig. 6a). To further explore the predictive biological pathways significantly altered in ADLP APT mice, we investigated relevant canonical pathways (p-value <0.01; Additional file 10: Table S8). Among these, one of the most dysregulated immune response-related pathways was the leukocyte extravasation signaling pathway. Adhesion-related proteins, which are mainly expressed in peripheral immune cells and endothelial cells, were differentially expressed in 10-month-old ADLP APT mice.
We speculate that changes in endothelial cells are likely to reflect the AD pathology in the brain parenchyma and that such changes recruit peripheral immune cells into the brain parenchyma. Among the canonical pathways related to the nervous system, synaptic long-term potentiation (LTP) was a representative pathway altered in ADLP<sup>APT</sup> mice (Fig. 6c). Post-synaptic proteins of this pathway were mainly dysregulated by the extracellular amyloid pathology and the intracellular NFT pathology. Thus, the biological pathway alterations seen
Protein quantitation overview and comparative pathway enrichment analysis of ADLP model mice.

Hierarchical clustering of differentially expressed proteins (DEPs) across different transgenic types and ages (ANOVA FDR < 0.05). Protein expression profiles were largely clustered into three patterns: the decreasing pattern (Cluster 2) and the increasing pattern (Cluster 3) of the mouse models with human APP (ADLP APP/PS1, ADLP APT) are prominent. The right panel shows the Z-normalized protein abundance according to the mouse samples as profile plots. Canonical pathway enrichment for DEPs. The categories of nervous system signaling and immune response were overrepresented (~50%) and are highlighted. The significant pathways (Fisher's exact test p-value < 0.05) were deduced using Ingenuity Pathway Analysis (IPA) and their predictive activation/inhibition status is represented as the Z-score. *APP/PS1 = ADLP APP/PS1, Tau = ADLP Tau, APT = ADLP APT mice.
Fig. 5 (See legend on next page.)
in ADLP\textsuperscript{APT} mice are consistent with the pathological changes seen in the nervous system of patients with AD.

**Network analysis of unique proteome changes in ADLP\textsuperscript{APT} mice**

To identify ADLP\textsuperscript{APT} mouse-specific protein changes, we investigated the exclusive DEPs\textsuperscript{APT} in 4- (early) and 10-month-old (late) ADLP\textsuperscript{APT} mice (Fig. 7a and Additional file 11: Table S9). Using the STRING database, we organized a protein–protein interaction map of the exclusive DEPs\textsuperscript{APT} representing expression changes by age and the GO categories into which these proteins could be classified. Our GO analysis revealed that the exclusive DEPs\textsuperscript{APT} were classified in the categories related to the immune response (e.g., cell–cell adhesion and inflammatory responses) and neuronal functions (e.g., syntaxin binding, lysosomes, and neuron projection development) (Fig. 7b). Subsequently, we examined the correlation of exclusive DEPs\textsuperscript{APT} with 2 AD-causative molecules, App and Mapt, in an effort to depict how the amyloid and NFT pathologies interconnect with one another. The constructed App–Mapt network showed the direct or indirect connections of proteins with App and Mapt, suggesting that these molecular targets could influence specific pathologies of ADLP\textsuperscript{APT} mice (Fig. 7c). The expression of Abca1, Ptprc, and Hcls1 that form a protein–protein interaction bridge between App and Mapt were further validated by the western blot analysis (Fig. 7d). The expression levels of exclusive DEPs\textsuperscript{APT} in the other transgenic mice are presented in Additional file 2: Figure S6.

**Comparison of ADLP\textsuperscript{APT} mouse proteome with human AD proteome**

We ultimately aimed to construct a mouse model that fully recapitulates the molecular and functional features of AD. To investigate how the ADLP\textsuperscript{APT} mouse model reflects AD, we performed a comparative analysis with the proteome data of a recently published human AD study [50]. This group performed proteomic analysis using brain tissues from patients with AD, elucidating molecular features and protein networks. Our dataset encompassed approximately 92% of the proteins identified in the human AD proteomics study. The expression patterns of DEPs common to both studies (Student’s t-test p-value <0.05) overlapped by 41 and 25% in up-regulated and down-regulated proteins, respectively (Additional file 2: Figure S4C). Although the numerical correlation between the two data was low ($R^2 < 0.3$), the commonly significant proteins have been classified into functional categories related to AD. Particularly, 10 up-regulated proteins, such as Clu, Mao-b, Cyp46a1, and Vps26a, were deduced to have significant associations with AD (Additional file 12: Table S10). These results implicated that our mouse model adequately reflects the known molecular characteristics of AD and can thus be further utilized in the researches to discover new drug targets. Furthermore, the other proteins in the categories related to neurodegeneration may play a significant role in future studies. The results of the comparative analysis with human proteomic data are listed in Additional file 4: Table S2.

**Discussion**

The amyloid cascade hypothesis claims that Aβ is the initial trigger for further pathological changes, including tau hyper-phosphorylation and NFT formation, which accelerates AD progression [51]. To investigate the prominent role of Aβ in causing AD pathogenesis, we developed ADLP\textsuperscript{APT} mice, a novel animal model of AD that develops more robust amyloid and NFT pathologies than other available animal models. This model showed no aggravation of the amyloid pathology beyond that seen in ADLP\textsuperscript{APP/PS1} mice, indicating that the development of the NFT pathology does not affect the amyloid pathology. However, based on sarkosyl-insoluble tau fractionation for the aggregated form of tau and immunostaining for phosphorylated tau, the accelerated NFT pathology appeared in the hippocampus of ADLP\textsuperscript{APT} mice starting from 7 months of age in the absence of any observable difference in the transcription/expression of the human MAPT gene. Abnormal tau phosphorylation is a crucial event that triggers tau aggregation in AD brains [52]. Various kinases have been suggested to be involved in tau phosphorylation process. Among external stimuli to activate kinases, the role of Aβ for activating tau kinases has been reported in a downstream of Aβ toxicity [53]. In order to investigate whether increased kinase expression levels are causative factors for accelerating NFT pathology, we have confirmed the expression levels of three kinases (Csnk1d, Gsk3-beta, and Rps6ka1) involved in tau phosphorylation by western
Notably, no differences were observed in the kinase levels between wild-type and ADLPAPT mice with the disease progression. In addition to western blot analysis, the MS results also indicated no changes in kinase expression levels. As for the kinases not checked by western blot analysis (e.g. Cdk5, Src, Fyn and Abl1), they were not changed among all groups, as confirmed by our hippocampal proteome data. These results support that accelerated NFT pathology in ADLPAPT mice hippocampus is not accompanied by changes of tau kinases (Additional file 2: Figure S7). Previous studies have hypothesized that Aβ is the initiator of the NFT pathology [53, 57, 58]. Indeed, Aβ affects tau kinase activity or localization resulting in tau toxicity in
neurons. Moreover, the amyloid pathology in the cortex accelerates tau propagation process throughout the entorhinal cortex and aggravates the NFT pathology [57]. Because the underlying molecular mechanisms of the pathophysiological interaction between Aβ and tau are unclear, ADLP APT mice may enable further exploration of the Aβ-tau axis hypothesis on the progression of AD pathogenesis.

To further investigate the biological functions altered in the hippocampus of ADLP APT mice, we first constructed a hippocampal proteomic database of ADLP animal models using LC-MS. Notably, as the disease progressed, the protein expression profiles of ADLP Tau mice resembled those of wild type mice, indicating that the relatively slow NFT pathology had a small impact on the proteomic changes during the early stage of the

Fig. 7 Exclusive DEPs in ADLP APT mice and their related functional networks. a Proteins that were significantly altered in early or late ADLP APT mice (Student’s t-test p-value <0.05 and fold-change >1.25) but not in the other models (fold change <1.25) were selected as exclusive DEPs APT. b The biological functions derived from the GO analysis are shown along with their networking with associated proteins. c The knowledge database-derived protein network of App, Mapt, and exclusive DEPs APT. The fold-change values of individual protein nodes were visualized in color circles (blue: down-regulation, red: up-regulation). The color of the inner circle is the fold-change of the 4-month ADLP APT mouse, and the color of the outer circle represents that of the 10-month ADLP APT mouse. d Three proteins that form bridge between App and Mapt (Hcls1, Ptprc, and Abca1) were validated by western blot analysis. Representative western blot images are shown herein. The expression levels of the proteins are quantified compared with wild type mice (n = 3 per genotype for 4 and 10 months; Student’s t test). Results are expressed as the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
blood vessels and in the brain parenchyma of patients with dementia who develop tauopathy resulted from P301L mutation within the tau gene [61]. Previously, using two-photon microscopy, we have observed that neutrophils can extravasate from blood vessels into the brain parenchyma where amyloid plaques are present. In the brain parenchyma, the neutrophils adhere to amyloid plaques and phagocyte them [62]. Moreover, Zenaro E et al. have reported that the number of neutrophils adhere both inside blood vessels and in the brain parenchyma of patients with AD [63]. Depleting temporarily neutrophils at the early stage of disease progression in a transgenic mouse model of AD prevented memory impairments, suggesting that the infiltration of neutrophils contributes to the pathogenesis of AD. The up-regulation of the proteins involved in the leukocyte extravasation signaling in ADLP APT mice suggested that peripheral immune cells may interact with capillary endothelial cells for infiltration into the brain parenchyma.

When glial or peripheral immune cells detect extracellular amyloid plaques, they clear the amyloid deposits through phagocytosis [64]. In addition to extracellular amyloid plaques, when neurons host NFTs in the cytosol, they activate the degradation system including the autophagy-lysosome system to eliminate them [65, 66]. Consistent with these, the GO analysis revealed that the gradually upregulated DEPs of ADLP APT included many components of the degradation system (cluster 5, Fig. 5c). In addition, constituents of the endosome category were upregulated, demonstrating that the endocytic pathway via endocytosis was also activated in ADLP APT mice. Thus, although we could not clarify which cell types exhibited these protein changes, the results of the GO analysis of upregulated proteins collectively suggested that AD pathologies trigger a sequential response that moves from phagocytosis to degradation via the endocytic pathway.

Several nervous system signaling pathways appear to differ between ADLP APP/PS1 and ADLP APT mice. The two canonical pathways, cAMP response element-binding protein (CREB) signaling in neurons and synaptic LTP, were inactivated only in the 10-month-old ADLP APT mice (Fig. 6c). It is established that CREB signaling contributes to cognitive functions by modulating synaptic plasticity [67]. Proteins associated with LTP processes are increased by CREB signaling in the hippocampus; in AD, alterations in Ca^{2+} signaling lead to decreased CREB signaling and altered LTP [68]. Similarly, our ADLP APT mice showed downregulated CREB signaling and LTP compared with ADLP Tau and ADLP APP/PS1 mice. Thus, these two pathways are predicted to be disturbed simultaneously by both Aβ accumulation and NFTs. This suggests that ADLP APT mice have pathological symptoms similar to those of patients with AD [69].

To investigate the putative mechanisms underlying the molecular pathogenesis of ADLP APT mice, we sorted exclusive DEPs in ADLP APT to identify unique molecular alterations. The protein–protein interaction map of exclusive DEPs APT showed their direct or indirect interactions with each other and categories that each protein belongs to. We further investigated the molecular interactions of exclusive DEPs APT with App and Mapt (Fig. 7c). The generated App–Mapt network contained 15 components that have been reported to interact among each other, although little is known about their relevance to the Aβ-tau axis. For example, Ptprc is a well-known microglia marker that has a strong
association with Aβ oligomerization [70]. However, to our knowledge, the regulation of this protein in the hippocampus of AD mouse models is not well studied. In contrast, Abca1, a risk factor for AD, has been reported to play a significant role in Aβ clearance [71]. In addition, Hcls1 is a leukocyte-specific actin-binding protein involved in immune response mechanisms [72]. We found that these proteins are involved in immune responses in the hippocampus, forming an interaction bridge on our App-Map network. Furthermore, western blot analysis clearly confirmed the regulation of these proteins in ADLP APT mice. Since our App-Map protein network provides information on DEPs affected by both Aβ and tau, it may help researchers to identify novel molecular mechanisms in the Aβ-tau axis.

To verify whether ADLP APT mice and its proteome data could be applied to further AD researches, we performed a comparative analysis with existing AD proteome datasets. First, the proteome data of ADLP APT mice were compared with that of human AD brain generated by Seyfried and Levey [50]. As a result, approximately 92% of the identified proteins were overlapped with the human AD proteome and approximately 30% of significantly regulated proteins in human AD data showed identical expression patterns with those of ADLP APT mice (p-value < 0.05 in both studies, respectively). We also assessed the commonality between our mouse model and the previously established 3xTg AD mouse model [73–75]. Notably, the comparative analysis indicated that our DEPs were largely different from those of other studies of 3xTg AD mice (data not shown). Most of our DEPs were neither detected nor showed consistent expression patterns in other studies. This may be attributed to the fact that the results of other studies were generated from 2-DIGE analysis [73] or the vesicular proteome isolated from the forebrain [74]. To the best of our knowledge, this study is the first to perform proteomic analysis in hippocampi developing both amyloid and NFT pathologies. These results indicate that the pathological relevance of our mouse model for AD research is valid at the protein level and in severe hippocampal pathologies.

Conclusion

We herein described a novel mouse model for AD, namely ADLP APT mouse. This model exhibited a rapid progression of AD pathology, including neuronal death and an accelerated NFT pathology that are particularly accelerated by the amyloid pathology. Thus, this model recapitulated the progression of human AD. Using LC-MS with 10-plex TMT isobaric labeling, we further constructed a hippocampal proteome database of ADLP mouse models. We used this database to understand the biological functions that were altered in each transgenic mouse model. This proteomic database also offers extensive resource of various molecular phenotypes in response to amyloid and NFT pathologies. Based on the bioinformatics analysis, we propose potential molecular targets for mechanistic studies and therapeutic development based on the proteomic database generated from our novel animal model.

Additional files

| Additional file 1: Supplemental Methods. (DOCX 31 kb) |
| Additional file 2: Figure S1. Pathological characterization of a novel animal model of Alzheimer’s disease. Figure S2. Activated neuroinflammation in a novel animal model of Alzheimer’s disease. Figure S3. TMT-based protein quantification strategy. Figure S4. The comparative analysis between ADLP APT and other AD proteome datasets. Figure S5. The quality assessment of MS analysis. Figure S6. The expression levels of exclusive DEPs in other ADLP mice. Figure S7. Longitudinal expression changes of kinases involved in phosphorylation of tau protein. (PPTX 2370 kb) |
| Additional file 3: Table S1. All identified protein groups. (XLSX 1130 kb) |
| Additional file 4: Table S2. All quantified protein groups. (XLSX 4540 kb) |
| Additional file 5: Table S3. Significantly regulated proteins from t-test. (XLSX 170 kb) |
| Additional file 6: Table S4. DEPs from ANOVA test. (XLSX 391 kb) |
| Additional file 7: Table S5. Canonical pathways enriched by IPA analysis. (XLSX 18 kb) |
| Additional file 8: Table S6. DEPs in ADLP APT mice. DEPs in ADLP APT mice. (XLSX 195 kb) |
| Additional file 9: Table S7. GO analysis results in ADLP APT mice. (XLSX 41 kb) |
| Additional file 10: Table S8. IPA analysis results in ADLP APT mice. (XLSX 53 kb) |
| Additional file 11: Table S9. Exclusive DEPs in ADLP APT. (XLSX 31 kb) |
| Additional file 12: Table S10. GO analysis of commonly significant proteins in human and ADLP APT. (XLSX 13 kb) |

Abbreviations

ADLP: Alzheimer’s disease-like-pathology; APP: Amyloid precursor protein; Aβ: Amyloid beta; BPRP: Basic pH reverse phase; DEPs: Differentially expressed proteins; GO: Gene ontology; IPA: Ingenuity pathway analysis; LC-MS: Liquid chromatography - Mass spectrometry; LTP: Long term potentiation; NFT: Neurofibrillary tangle; PPI: Protein-protein interaction; TMT: Tandem mass tag

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The MS proteomics data in this paper have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [76]; dataset identifier PXD006214.

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Authors’ contributions

DK, JP, DH participated equally in the overall work. DK, JP, DH, YK, and IM-J designed the research and experiments. DKK performed the immunohistochemistry, sarkosyl-insoluble tau fractionation, RT-PCR, and behavioral tests and wrote the manuscript. JP performed proteomics experiment and wrote the manuscript. DH contributed to the proteomics experiments and revised the manuscript. JW contributed to design sarkosyl-insoluble tau fractionation experiment and carry out the immunohistochemistry. AK contributed to behavioral tests and prepare for bioinformatics experiment. JW contributed to analyze the MS-based proteomics data. YK and IM-J revised manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Animals were treated and maintained as per the Helsinki Treaty, the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985), and the Animal Care and Use Guidelines of Seoul National University, Seoul, Korea. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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