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Amphiphysin-1 protein level changes associated with tau-mediated neurodegeneration

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Abstract

Tauopathies are a family of neurodegenerative diseases that have the pathological hallmark of intraneuronal accumulation of filaments composed of hyperphosphorylated tau proteins that tend to aggregate in an ultrastructure known as neurofibrillary tangles. The identification of mutations on the *tau* gene in familial cases of tauopathies underscores the pathological role of the tau protein. However, the molecular process that underlines tau-mediated neurodegeneration is not understood. Here, a proteomics approach was used to identify proteins that may be affected during the course of tau-mediated neurodegeneration in the tauopathy mouse model JNPL3. The JNPL3 mice express human tau proteins bearing a P301L mutation, which mimics the neurodegenerative process observed in humans with tauopathy. The results showed that the protein amphiphysin-1 (AMPH1) is significantly reduced in terminally ill JNPL3 mice. Specifically, the AMPH1 protein level is reduced in brain regions known to accumulate aggregates of hyperphosphorylated tau proteins. The AMPH1 protein reduction was validated in Alzheimer's disease cases. Taken together, the results suggest that the reduction of the AMPH1 protein level is a molecular event associated with the progression of tau-mediated neurodegeneration.

Keywords

Alzheimer's disease; amphiphysin; calpain; neurodegeneration; tau; tauopathy

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Conflicts of interest

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Introduction

Last year, a series of genome-wide association studies identified specific genes associated with late-onset Alzheimer's disease [1–4]. The genes identified could be classified into three main cellular pathways, namely, cholesterol metabolism, immune system function, and the synaptic function/cell membrane process [1]. Previous studies have shown that these pathways are affected in Alzheimer's disease and other tauopathies [1]. However, the pathological role of most of the identified genes is poorly understood. Therefore, the identification of proteins affected by tau-mediated neurodegeneration will contribute toward the identification of pathology-associated biomarkers and validation of molecular networks in neurodegeneration.

During the course of tau-mediated neurodegeneration, a neuronal response may trigger changes in protein levels and/or aberrant protein–protein interactions, leading to the activation of signaling molecules that disrupt the normal neuronal function and, consequently, induce neuronal death. The identification of these proteome changes may provide a better understanding of the mechanisms associated with neural death in tauopathies. We used, a proteomic approach to identify proteome changes associated with tau-mediated neurodegeneration in the tauopathy mouse model JNPL3 in a brain region-specific manner and validated them in Alzheimer's disease brain.

Materials and methods

Animal model and human tissue

Transgenic JNPL3 and nontransgenic age-matched mice were bred and genotyped as described previously [5]. This transgenic mouse model expresses the human tau isoform 0N4R with the mutation P301L (hTauP301L), under the control of the mouse prion promoter, commonly found in frontotemporal dementia and parkinsonism linked to chromosome 17 [5]. The JNPL3 mice develop tau pathology in an age-dependent and brain region-specific manner, specifically in the diencephalon, basal telencephalon, brain stem, cerebellar nuclei, and spinal cord [5]. Terminally ill JNPL3 mice are those animals with severe motor and behavioral disturbances characterized by hind leg paralysis and significant weight loss. Nontransgenic age-matched animals were used as control. A protocol (403–2006) for the use of animal models was approved by the institutional IACUC.

Human frontal cortex from normal aging (average age 74.5; $n=17$) and Alzheimer's disease (average age 75.4; $n=17$) were obtained from both EMORY and UPENN Pathology Core Facilities. The samples selected were confirmed pathologically as Alzheimer's disease cases with abundant senile plaque pathology and Braak and Braak stage of IV or more for neurofibrillary pathology. Age-matched cases without a history of dementia and no detectable tau pathology were used as controls.

Brain protein lysate preparation

The cortical and subcortical brain regions and the spinal cord were obtained from JNPL3 and nontransgenic mice. Human frontal cortices from normal aging and Alzheimer's disease were also used. Briefly, the dissected brain regions and spinal cord were homogenized in five volumes of buffer A (20mM Tris base, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM phenylmethylsulfonyl fluoride, 5mM sodium pyrophosphate, 30mM β -glycerol phosphate, and 30mM sodium fluoride) and the protein extract was centrifuged at 14 000 rpm (21 000g) for 10 min. The supernatant was transferred to a clean tube and used for western blot analyses. To determine the protein concentration, the Bradford assay was carried out by detecting changes in absorbance using a

spectrophotometer (DU730 Beckman Coulter; Beckman Coulter Puerto Rico, Caguas, Puerto Rico, USA).

2D-GE and tandem mass spectrometry

Two-dimensional gel electrophoresis (2D-GE) was carried out according to BioRad's 2-D electrophoresis for proteomics manual. Briefly, 50 µg of protein lysate was subjected to isoelectric focusing for 12 h at 50 V. A low-voltage (250 V at 20°C for 15 min) step was applied, followed by voltage increase (8000 V for an 11 cm strip) and current not exceeding 50 µA/strip at 20°C. The focusing was run for a total of 20 000 V h at 20°C. Then, the IPG strips (ReadyStrips; BioRad, Hercules, California, USA) were equilibrated for 10 min at room temperature. A second equilibration step was carried out for 10 min at room temperature. The IPG strips were washed once in 1× TGS (25mM Tris base, 192mM glycine, 0.1% SDS) running buffer and laid on the top of a criterion precast gradient gel (4–20% Tris HCl 1.0mm) (BioRad; cat# 163-2111). Proteins were visualized by silver staining using the SilverQuest Staining Kit (cat# LC6070; Life Technologies, San Diego, California, USA).

In-gel trypsin digestion and tandem mass spectrometry analysis were carried out on protein spots detected by silver staining of terminally ill JNPL3 mice and nontransgenic age-matched controls, as described previously [6]. The peptides were identified by correlating MS–MS spectra with sequences from the NCBI mouse databases using SEQUEST (Thermo Scientific Inc., Waltham, Massachusetts, USA) search algorithms, using the following database search filters parameters XCorr higher than 1.5 (+1), 2.0 (+2), or 2.5 (+3); Delta score >0.1; 10 or more b and y ions; MS2 intensity of $>5 \times 10^{-4}$; and peptide probability $>E \times 10^{-2}$.

Sarkosyl-insoluble tau differential fractionation

Seven hundred microgram of total protein from mice and 400 µg from human brain postnuclear lysates were centrifuged at 1 56 705g at 4°C for 15 min. Then, the resulting pellet was resuspended in 500 µl of buffer B (800mM NaCl, 10mM Tris base pH 7.4, 1mM EGTA, and 10% sucrose). After centrifugation at 1 56 705g at 4°C for another 15 min, the supernatant was transferred to a clean tube and aqueous sodium *N*-lauroyl sarcosine (sarkosyl detergent; Shelton Scientific, Shelton, Connecticut, USA) was added to a final concentration of 1% v/v. The reaction was incubated at 37°C for 1 h and centrifuged at 1 56 705g at 4°C for 30 min. The pellet (P3) was resuspended in 20 µl 2 × SDS loading buffer (125mM Tris-HCl pH6.8, 10% SDS, 10% BME, 0.004% bromophenol blue). Then, the levels of sarkosyl insoluble tau present in 5 µl of the resuspended P3 fraction were evaluated by western blot using Tau-13 antibodies (1 : 50 000).

Antibodies and western blot analysis

Anti-amphiphsin (1 : 1:000; Novus Biologicals, LLC, Littleton, Colorado, USA) and human specific anti-tau antibody Tau13 (1 : 50 000; Convance, Madison, Wisconsin, USA) were commercially acquired. The secondary antibodies used were peroxidase-conjugated goat anti-rabbit (1 : 2000), goat anti-mouse (1 : 2000) antibodies, and rabbit anti-goat (1 : 2000) (Chemicon, Temecula, California, USA). Thirty microgram of total protein lysate was used from the brain region or spinal cord homogenates. The SDS-PAGE-resolved proteins were transferred onto a pure nitrocellulose membrane (0.45 µm; BioRad). The membrane was blocked using a 5% dry-milk solution in 1 × TTBS (2.5mM Tris base, 15mM NaCl, 30mM KCl, 0.02% Tween 20 detergent). The nitrocellulose membrane was incubated with a predetermined concentration of primary antibody overnight at 4°C. The membrane was washed three times with 1 × TTBS and incubated in secondary antibody for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence reactions detected on

an X-ray film (cat. #4741008379; Thermo Scientific Inc.) or a chemidoc system (cat. #95-0310-01, UVP; BioRad).

Statistical analysis

Relative optic density was expressed as a ratio of either AMPH and GAPDH (loading control) using a densitometer, for western blots in films (GS-800; BioRad) and Quantity One program (System ID: 3DoF758E). All *P*-values for column graphs were obtained using one-tailed student's *t*-test (**P*<0.05, ***P*<0.01, ****P*<0.001). Column graphs were prepared using GraphPad PRISM [Prism Mac Serial number(s): GPM4-034396-RJD-2023; La Jolla, California, USA]. Error bars represent the standard error of the mean ($\pm$ SEM).

Results

The AMPH1 protein level is reduced in JNPL3 mouse brain

Figure 1a shows proteins resolved by 2D-GE from the brainstem of nontransgenic and terminally ill JNPL3 mice. Several differences were found when nontransgenic and JNPL3 extracts were compared. Particularly, a protein spot detected at ~125 kDa in the nontransgenic brainstem sample (Fig. 1a, circle) was reduced in the brainstem sample of the JNPL3 mouse (Fig. 1a, circle). The difference in protein abundance could not be explained by loading error as the intensities of other protein spots in the gel were similar in both the nontransgenic and the JNPL3 samples (Fig. 1a, arrow-head). This observation indicates that the protein(s) represented in this spot was affected by tau-mediated neurodegeneration.

Bottom-up tandem mass spectrometry analysis did not identify a protein in the JNPL3 mouse sample. However, the protein AMPH1 was identified in the nontransgenic sample. Western blot analysis with antibodies against AMPH1 was carried out to validate the tandem mass spectrometry results. Equal protein concentrations from nontransgenic and JNPL3 were resolved in a SDS-PAGE and transferred to a nitrocellulose membrane (Fig. 1b; Ponceau S). As expected, human tau proteins were detected in the JNPL3 mouse, but not in nontransgenic mice (Fig. 1b). Moreover, the 64 kDa species that represents hyperphosphorylated tau in the JNPL3 mouse was detected (Fig. 1b; arrow) [5]. Consistent with the 2D-GE and tandem mass spectrometry data, a reduction in the AMPH1 protein level was found in the JNPL3 mouse sample in comparison with the nontransgenic mouse control (Fig. 1b; asterisk). These results indicate that the AMPH1 protein level is reduced in subcortical brain regions known to accumulate tau pathology in JNPL3 mice.

The AMPH1 protein level reduction is brain region specific

The cortical and subcortical brain regions and the spinal cord were dissected from terminally ill JNPL3 and nontransgenic age-matched controls and subjected to western blot analysis (Fig. 2). The protein level was corrected using GAPDH as a loading control (Fig. 2). The AMPH1 protein level is significantly reduced in the spinal cord in comparison with nontransgenic control mice (Fig. 2, *P*=0.0008). In the subcortical and cortical brain regions of JNPL3, no significant reduction in AMPH1 protein was detected in comparison with nontransgenic controls (Fig. 2). However, JNPL3 mice showed a significant reduction in the AMPH1 protein level in the subcortical region in comparison with the level detected in their cortical region (Fig. 2, *P*=0.0067). In contrast, there was no significant difference in the AMPH1 protein level in nontransgenic mice's cortical and subcortical brain regions (Fig. 2). The reduction in the AMPH1 protein level was not found in young (3 months) JNPL3 mice, which do not show accumulation of pathological tau (data not shown). In addition, the accumulation of sarkosyl insoluble tau (a hallmark of pathological tau) was detected in the subcortical brain regions and the spinal cord (data not shown). These results indicate that in

JNPL3, the reduction in AMPH1 occurred in brain regions that are susceptible to age-dependent accumulation of aggregated tau.

AMPH1 protein level reduction in the frontal cortex of Alzheimer's disease cases

If the reduction in AMPH1 detected in the tauopathy mouse model JNPL3 is associated with the pathological process, then the AMPH1 protein level could also be affected in human tauopathy cases. Frontal cortex samples from Alzheimer's disease and normal-aging control cases were subjected to differential fractionation. Then, the AMPH1 protein level was determined for both Alzheimer's disease and normal-aging controls from two different brain banks (Fig. 3). The result showed that the AMPH1 protein was significantly reduced in the frontal cortex of Alzheimer's disease cases, in comparison with normal-aging controls (Fig. 3; $P=0.0069$). We observed reduced AMPH1 expression in both frontal and temporal cortices from Alzheimer's disease cases. This reduction was greater in the frontal cortex than the temporal cortex. This may be because of the normal variability of pathological involvement among different brain regions or selective vulnerability. Nevertheless, these results validate the reduction in the AMPH1 protein level detected in terminally ill JNPL3 mice, indicating that a reduction in the AMPH1 protein is a molecular event associated with neurodegeneration and not induced solely by the overexpression of human mutant (P301L) tau protein in a mouse model.

Discussion

AMPH1 is an abundant protein in nerve terminals and involved in the recruitment of dynamin to sites of clathrin-mediated endocytosis [2,7]. The recruitment of both proteins to endocytic buds regulates the last step in synaptic vesicle endocytosis, an essential neurophysiologic component in synaptic transmission [2]. Dynamin 1 and 2 have also been genetically and biochemically associated with Alzheimer disease [8]. AMPH1 also interacts with BIN1 (AMPH2) to mediate endocytic recycling [3]. BIN1 (AMPH2) has been implicated in Alzheimer's disease [1]. In addition to BIN1, PICALM has also been associated with Alzheimer's disease [1]. PICALM is involved in the early stages of clathrin-mediated endocytosis, promoting the formation of the clathrin cage and determining the amount of membrane to be endocytosed [9]. Interestingly, both cdk5 and calpain are physiological regulators of AMPH1 function [2]. These regulatory proteins have been shown to be aberrantly active during neurodegeneration [10]. Thus, overactivation of regulatory proteins may lead to the depletion of the molecular machinery that mediates endocytosis, affecting the physiological function of neurons. Consistently, AMPH1-knockout mice showed defects in synaptic vesicle recycling, high propensity to seizures, cognitive impairment, and increased mortality, indicating that AMPH1 is an essential protein for neuronal function [11]. On the basis of all these results, it is tempting to speculate that AMPH1 protein reduction is an event that contributes toward synaptic dysfunction in tauopathies.

Conclusion

The AMPH1 protein level is reduced in the tauopathy mouse model JNPL3 in a brain region-specific manner. This result was validated in the frontal cortex of Alzheimer's disease cases. Further experiments are required to delineate the signaling mechanisms that lead to reductions in the AMPH1 protein level in the progression of tau-mediated neurodegeneration.

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Fig. 1. Amphiphysin-1 (AMPH1) protein level is reduced in the JNPL3 mouse brain. (a) Two-dimensional gel electrophoresis of brainstem protein extracts from nontransgenic and JNPL3 mice. The black circle shows the same region on both gels. The arrowheads point to reference proteins indicating similar protein abundance in both gels. The ranges for pH and molecular weight are shown. (b) Western blot analysis was used to validate the identification of the protein AMPH1. Ponceau S stain shows equal loading on both nontransgenic and JNPL3 samples. Anti-AMPH1 antibodies labeled a protein band of the corresponding molecular weight (*). Tau13 antibodies specific to human tau proteins were used to confirm the sample from the transgenic JNPL3 mouse. The opened arrow indicates the hyperphosphorylated tau (64 kDa) form.

Fig. 2.

The reduction in the amphiphysin-1 (AMPH1) protein level is brain region specific. Densitometry analysis was used to quantify the difference in the AMPH1 protein level. The AMPH1 protein level was corrected for loading error using the level of GAPDH protein per sample. The white and grey bars indicate the AMPH1 level in nontransgenic and JNPL3 mice, respectively. The number of mice used in the statistical analysis is indicated on the graph. Error bars are represented as $\pm$ SEM (** $P=0.0067$; *** $P=0.0008$). OD, optic density

Fig. 3.

Amphiphysin-1 (AMPH1) protein level reduction in Alzheimer's disease (AD) brain. Densitometry analysis of the results obtained from western blot analyses was carried out. The white and gray bars indicate the AMPH1/GAPDH ratio plotted as the mean value obtained from normal-aging and AD cases, respectively. Error bars are presented as $\pm$ SEM (** $P=0.0069$).