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Ezrin Expression is Increased During Disease Progression in a Tauopathy Mouse Model and Alzheimer's Disease

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Abstract

Background—The lack of diagnostic tools and disease-modifying treatments against Alzheimer's disease (AD) and related disorders, collectively known as tauopathies, has led to a socioeconomic burden of epidemic proportion. Proteomics approaches can be used to identify novel proteome changes that could help us understand the pathogenesis of tau-related pathological hallmarks and/or cellular stress responses associated with tauopathy. These studies, however, need to be conducted taking into consideration brain region specificity and stage of neurodegeneration in order to provide insights about the pathological role of the identified proteins.

Methods—We used a tauopathy mouse model (JNPL3) that expresses human tau bearing a P301L mutation and develops motor impairment, the severity of which correlates with the increased accumulation of pathological tau. Tissue was dissected from asymptomatic and severely motor impaired JNPL3 mice as well as non-transgenic littermate controls and subjected to two-

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CONFLICT OF INTEREST

The authors read the BioMed Central's guidance on competing interests and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experiments were conducted in accordance with approved protocol #01–15–020–00 from Michigan State University IACUC.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are basis of this research.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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dimensional gel electrophoresis. Differentially abundant protein spots were identified by tandem mass spectrometry. Postmortem mild cognitive impairment (MCI), AD and normal aging controls were used to validate the pathological significance of the identified protein.

Results—Ezrin was identified as a protein that is upregulated in tau-mediated neurodegeneration. We demonstrate that Ezrin protein abundance increased in JNPL3 mice preceded motor impairment and was sustained in severely motor impaired mice. Ezrin expression was also increased in the temporal cortex of MCI and AD patients.

Conclusion—The results demonstrate that increased Ezrin protein abundance changes are associated with the early stages of neurodegeneration in tauopathy models and human disease. Understanding the role of Ezrin in tauopathies such as AD may provide new insights for targeting tau-mediated neurodegeneration.

Keywords

Alzheimer's disease; Ezrin; ERM; tau; neurodegeneration; biomarkers; tauopathy

1. INTRODUCTION

An important conundrum related to the pathophysiology of Alzheimer's disease (AD) and related neurodegenerative disorders is deciphering between neuroprotective processes that become activated in response to the accumulation of toxic molecules and neurodegenerative signals that mediate neuronal dysfunction and, subsequently, cell death that leads to clinical presentation years later [1]. Hence, the identification and characterization of proteome changes during neurodegeneration will improve our understanding of these differential processes in AD and related disorders and aid in the development of disease-modifying treatments. Proteomics approaches have indeed contributed to the identification of novel changes associated with neurodegeneration [2]. However, due to the heterogeneity of human postmortem tissue and differential overexpression of human proteins in animal models, it is important to define the identified proteome change based on brain region specificity and stage of neurodegeneration. We argue that a comparison of defined clinical or neurodegeneration stages will facilitate the identification of proteome changes associated with different stages of the pathophysiology of AD and related disorders. Based on this experimental philosophy, we established a strategy that takes advantage of a tauopathy mouse model and available human postmortem brain tissues, from different cohorts, to identify proteome changes associated with tau-mediated neurodegeneration.

The JNPL3 tauopathy mouse model expresses the human tau (htau) protein bearing the P301L mutation commonly found in frontotemporal dementia and parkinsonism-linked to chromosome 17 (FTDP-17) [3]. Aggregated tau proteins are detected in specific brain regions, namely the diencephalon, brain stem, cerebellar nuclei, hippocampus and basal ganglia, and spinal cord despite the ubiquitous neuronal expression of the mutant htau protein in all brain regions [3]. Additionally, JNPL3 mice develop an age-dependent motor impairment phenotype associated with increased accumulation of an htau protein band at an apparent molecular weight of 64kDa directly [3–6]. A Motor Impairment Score (MIS) system (ranging from 0 to 30) that rates motor dysfunction in JNPL3 mice based on their

performance on tail hang, rope hang and righting reflex tests [7] is used to classify JNPL3 mice as normal or with mild, moderate or severe motor impairment, allowing us to group JNPL3 mice based on their stage of neurodegeneration [8]. Importantly, proteome changes identified in JNPL3 mice have been validated in post-mortem AD brain tissue, demonstrating that tau-mediated neurodegeneration in this tauopathy mouse model is relevant to the pathological process that leads to human disease [4, 5, 8–10].

Here, we show that Ezrin, a member of the ezrin/radixin/moesin (ERM) family of actin interacting proteins, is upregulated in JNPL3 mice before motor impairment is detected and that its protein levels remain increased in mice with severe motor impairment. The upregulation of Ezrin was validated in the temporal cortex of mild cognitive impairment (MCI) and AD subjects, validating that changes in Ezrin protein abundance are associated with neurodegenerative processes involved in the onset of cognitive impairment and AD pathophysiology. Future work directed to decipher Ezrin's pathological role will help to determine the therapeutic benefit of targeting its biological activity.

2. MATERIALS AND METHODS

2.1. Antibodies

The primary antibodies used in this study were: rabbit polyclonal anti-Ezrin (Abcam: ab41672, 2µg/mL), mouse monoclonal anti-Ezrin (SIGMA: E8897–100uL, 1/1000), anti-phospho(T567)-Ezrin (Invitrogen, PA5–37763; 1/250), GAPDH (Cell Signaling, 5174S; 1/1000), anti-Tau13 (Ab-cam: ab19030, 1/10,000), anti-β tubulin (Cell Signaling, 5335S; 1/500). Secondary antibodies used were the Li-COR IRDye series: goat-anti-rabbit 680 (926–68021), goat-anti-rabbit 800 (926–32211), goat-anti-mouse 680 (926–68020), goat-anti-mouse 800 (926–32210), at a 1/2000 dilution.

2.2. Human Postmortem Tissue

Human samples were derived from two different cohorts (Table 1). The AD and corresponding cognitive normal aging (NA) samples were obtained from the Emory AD Center Neuropathology Core and MCI and NA samples are from the Rush Religious Orders Study. The AD cohort from Emory had a Braak stage of VI, whereas the MCI cohort had a Braak stage of III or IV. The average of the MMSE score for MCI cases was 25.5 while NA cases had an average MMSE score of 27.5. The Global Cognitive Score, a composite z-score of neuropsychological testing performance, was –0.441 and 0.080 for MCI and NA, respectively. The Braak stage of NA cases was I or II. Tissue samples from temporal cortex were excised, avoiding blood vessels and white matter. The tissue samples were homogenized in 5 volumes of RIPA buffer (Thermo Scientific, cat# 89901), using a tissue grinder. The homogenate was centrifuge at $18,407 \times g$ (14,000 rpm; Eppendorf 5424R) at 4°C for 10 min. The supernatant was transferred to a clean tube and total protein concentration was estimated using Pierce BCA protein assay (Thermo Fisher, cat# 23225).

2.3. Tauopathy Mouse Model

The transgenic (Tg) JNPL3 tauopathy mouse model and non-transgenic (NTg) control animals were originally obtained from Mayo Clinic Jacksonville (kindly provided by Drs.

Michael Hutton and Jada Lewis). This tauopathy mouse model expresses the human tau isoform 0N4R bearing the P301L mutation, commonly found in familial cases of FTDP-17, under the control of the prion promoter. The JNPL3 mouse colony is maintained as hemizygous for htau_{P301L} and inbred on the SW genetic background. The motor impairment of NTg and Tg mice was determined before euthanization. Motor Impairment Score (MIS) system is based on the performance on three tasks, namely tail hang, rope hang and righting reflex tests. Mice with a motor impairment score equal to or below 10 are catalogued as normal, whereas mice with a score of 12 or above are catalogued as motor impaired. Motor impaired mice are sub-catalogued in three groups: mild (12–17), moderate (18–23) and severe (24–30) [7]. MIS scores were used to classify mice based on the level of the neurodegeneration phenotype. The age of all mice used in this study fluctuate between 8 and 15 months and both sexes were used. Asymptomatic and motor-impaired Tg mice were age-matched both within-group and to NTg control mice. Moreover, mice from both sexes were used in this study. Since the motor impairment correlates primarily to the level of neurodegeneration in the spinal cord, we used spinal cord tissue in our experiments. The spinal cord was homogenized in 5 volumes of RIPA Buffer and prepared as described for the human tissue. Protein concentration was estimated using Pierce BCA protein assay. All experiments were conducted in accordance with Michigan State University approved protocol #01–15-020–00.

2.4. Two-dimensional Gel Electrophoresis

Isoelectric focusing (IEF) – 100µg of protein extract was precipitated using 13.5% TCA (final concentration). After incubating on ice for 15 min, the samples were centrifuged at $18,407 \times g$ (14,000 rpm; Eppendorf 5424R) at 4°C for 10 min. The resulting pellet was washed four times with 500µL of 1:1 ethanol:ethyl acetate and centrifuged at $18,407 \times g$ at 4°C for 5 min each time. Rehydration buffer (8M UREA, 2% CHAPS, 20 mM DTT, 0.2% (w/v) ampholytes, 0.001% bromophenol blue) was prepared immediately prior to separation by IEF. The protein pellets were resuspended in 200µL of rehydration buffer and shake for 2hrs at room temperature. The samples were loaded in the IEF tray, making sure to cover the entire well. IPG strips 11 cm, pH gradient 3–10 (BioRad, cat# 163–2014) were thawed prior to the IEF run. The plastic cover of the IPG strip was removed and the IPG strip was placed with the gel facing the protein extract. A rehydration step at 50 volts for 45 min was run before the IPG strip was covered with a thin layer of mineral oil. The IEF (BioRad, Protean i12 IEF-cell) was set for 5 step run (300V, Linear, 2 hrs; 500V, Linear, 2hrs; 1000V, Linear, 2hrs; 8000V, Linear, 8hrs; 8000V, Rapid, 10hrs) at a sustained 50µAMP. Prior to running the second dimension, IPG strips were equilibrated. The first equilibration step was required to saturate the IPG strips with SDS and the reducing agent. Briefly, the IPG strips were washed in Equilibration Buffer I (6M Urea, 2% SDS, 0.375 mM Tris-HCl pH 8.8, 20% glycerol, 130 mM DTT) for 10 minutes at room temperature. A second equilibration step was performed to prevent protein re-oxidation during electrophoresis and to alkylate residual DTT in order to minimize vertical streaking. The IPG strips were then washed in Equilibration Buffer II (6M Urea, 2% SDS, 0.375 mM Tris-HCl pH 8.8, 20% glycerol, 135mM Iodoacetamide) for 10 minutes at room temperature. Prior to SDS-PAGE, IPG strips were washed once in 1X TGS (25mM Tris Base, 192mM Glycine, 0.1% SDS) running buffer. The IPG strip was then laid onto the back of the Criterion pre-cast gel (4–20% Criterion-gel 1.0mm), secured in

place with a layer of agarose solution (BioRad cat# 163–2111). After polymerization of the agarose, the gels were run for 1 hr at 200 volts. Following the SDS-PAGE, gels stained with Sypro Ruby following manufacturer's recommendations (BioRad cat# 170–3125).

2.5. In-gel Trypsin Digestion and Tandem Mass Spectrometry Analysis

Sypro Ruby stained gels were visualized using Gel Doc EZ imager, Image Lab software, (BioRad). The intensity of protein spots was quantified using the quantitation tool in Image Lab. Selected protein spots (3 fold difference between Tg and NTg samples) were excised and sent for in-gel digestion and tandem mass spectrometry analysis at the MSU Proteomics Facility. Briefly, the dehydration of excised protein spots was conducted using 100% acetonitrile and 10mM DTT in 100mM ammonium bicarbonate (pH 8.0) for 45min at 56°C [11]. The samples were then treated with 50mM iodoacetamide in 100mM ammonium bicarbonate for 20min [11]. After washing with ammonium bicarbonate, the samples were incubated with trypsin (0.01µg/µL in 50mM ammonium bicarbonate) at 37°C overnight [11]. Peptides were extracted from the gel by water bath sonication in a solution of 60% ACN/ 1%TCA and vacuum dried to 2µL [11]. The samples were reconstituted in 2% acetonitrile/ 0.1% TFA to 25µL. From this, 5µL were automatically injected by a Thermo (www.thermo.com) EASYnLC 1000 onto a Thermo Acclaim 0.1 × 20mm C18 Peptide nanotrap and washed with buffer A for 5min. Bound peptides were then eluted onto a Thermo Acclaim RSLC 0.075mm × 150mm C18 column over 35min with a gradient of 5%B to 25%B in 24min, ramping to 100%B at 25min and held at 100%B for the duration of the run (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% Acetonitrile/0.1% Formic Acid) at a constant flow rate of 0.3µL/min. Tandem mass spectra were extracted by Scaffold (version Scaffold_4.4.1.1, Proteome Software Inc., Portland, OR). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.0) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot was set up to search the UP_mouse_crap_201601 database assuming the digestion enzyme strict trypsin. X! Tandem was set up to search a subset of the UP_mouse_crap_201601 database also assuming strict trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 5.0 PPM. Carbamidomethyl of cysteine was specified in Mascot and X! Tandem as a fixed modification. Deamidated of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications. Glu->pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, Gln->pyro-Glu of the N-terminus, deamidated of asparagine and glutamine and oxidation of methionine were specified in X! Tandem as variable modifications. The scaffold was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 99.0% probability. Peptide Probabilities from X! Tandem were assigned by the Peptide Prophet algorithm with Scaffold delta-mass correction [12–13]. Peptide Probabilities from Mascot were assigned by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [11–12]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone

were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

2.6. Western Blot

Samples of equivalent protein concentration were denatured with 2x SDS loading buffer (125 mM Tris-HCl pH6.8, 10% SDS, 10% BME, .004% bromophenol blue). Human and mouse samples (50µg of total protein lysate) were resolved in 4–20% or 10% SDS-PAGE and transferred to a pure nitrocellulose membrane (.45µm BIO-RAD). The membrane was blocked using 5% dry-milk solution in 1x TTBS (2.5mM Tris-Base, 15mM NaCl, 30mM KCL, 0.02% Tween 20 detergent) for 1 hr at room temperature. The nitrocellulose membrane was incubated with a predetermined concentration of primary antibody overnight at 4°C. After incubation with the corresponding secondary antibody for 1 hr at room temperature, protein bands were visualized in Li-COR Odyssey Imaging System, using Image Studio Software (ver 5.2). Image Studio was also used to quantify the protein signal obtained. The values were exported to Excel and then to GraphPad Prism 6 (Ver 6.05, 2014) for statistical analysis. When the combination of primary antibodies allowed, anti-GAPDH (loading control) was co-incubated with other primary antibodies; otherwise, the membrane was re-probed to account for loading variations.

2.7. Microarray Analysis

In a separate ongoing study, Ezrin was identified among transcripts upregulated in MCI and AD. For the present study, the generated microarray dataset was queried for changes in Ezrin's transcript levels. A full report of the microarray analysis data is being prepared in a separate manuscript (Counts SE, manuscript in preparation). Briefly, frozen temporal cortex (BA20) tissue from a separate cohort of NA, MCI, and mild/moderate AD cases (n = 12/group) representing both genders was acquired postmortem from participants in the Rush Religious Orders Study. For mRNA preparation, 40–50 mg of frozen tissue was cut on a platform in dry ice and RNA was extracted from the samples using a total RNA isolation kit (Ambion). Tissue was lysed using a TissueLyser (Qiagen) set to 20Hz in 10× volume of lysis/binding buffer and RNA was extracted using acid-phenol/chloroform. Total RNA was quantified by Nanodrop spectrophotometry and RNA quality was assessed on an Agilent Bioanalyzer (RIN cut-off = 7). Double-stranded cDNA was prepared and labeled with Cy3 (One-color DNA Labeling Kit, NimbleGen). Labeled cDNA (4 µg) was hybridized to NimbleGen 12 × 135K human arrays for 18 hrs at 42°C and analyzed on a GenePix 4200A scanner. Probe intensity levels were quantified with RMA preprocessing (NimbleScan v2.5, Roche). Raw data were imported into BioDiscovery Nexus Expression 3 software for normalization, false discovery rate correction, and quantitative analysis. Here, we only showed the statistical analysis of Ezrin's transcript in NA, MCI and AD cases.

2.8. Statistics

Power analysis (G*Power; Version 3.1.9.2, 2014) was used to determine the number of samples at an actual power = 0.876, using the following parameters: α err prob = 0.05, 1- β err prob = 0.85, effect size d = 2.0, two tails. For statistical analysis, the corrected protein band intensity values (using GAPDH as the loading control) were exported into GraphPad Prism 6. One-way ANOVA (with Holm-Sidak's multiple comparisons test was used for the

comparison of protein levels derived from NTg, Tg- asymptomatic and Tg-severe mice and microarray data. For all other statistical analyses comparing two groups, a two-tailed, unpaired t-test with Welch's correction (which does not assume equal standard deviations) was used. The results are illustrated in a scatter dot plot format showing means with standard deviation.

3. RESULTS

3.1. Increased Abundance of Ezrin Protein in JNPL3 Mice

To identify changes in protein abundance caused by tau-mediated neurodegeneration in tissue known to be affected by pathological tau, protein extract from spinal cord tissue from severe motor impaired Tg JNPL3 mice and unimpaired NTg controls were resolved by two-dimensional gel electrophoresis (Fig. 1A). The resolved proteins were visualized using the fluorescence dye SYPRO Ruby (Suppl. Fig. 1). (Fig. 1A) illustrates a protein spot (arrow) that is clearly detectable in Tg mouse protein extracts but not in age-matched NTg control extracts. This differentially abundant protein spot was excised and subjected to tandem mass spectrometry analysis (Fig. 1B). The protein spot was identified as Ezrin, a member of the Ezrin/Radixin/Moesin (ERM) family of actin-binding proteins. Although ERM protein sequences are similar, only one of the identified peptides was identical in both Ezrin and Radixin. Thus, the results suggest that Ezrin protein abundance is increased in tissue affected by tau-mediated neurodegeneration in JNPL3 mice.

3.2. Ezrin Protein Levels Increase Before Detectable Motor Impairment in JNPL3 Mice

To validate changes in Ezrin protein abundance in JNPL3 mice, we homogenized spinal cord tissue from Tg mice with or without motor impairment due to the progression of tau-mediated neurodegeneration and NTg mice as control. Two different anti-Ezrin antibodies were tested for cross-reactivity using a mouse and human protein lysate (Suppl. Fig. 2A). Both antibodies recognized a protein band at 80kDa, which corresponds to the expected migration of Ezrin by SDS-PAGE (Suppl. Fig. 2). Moreover, secondary antibodies alone did not detect a protein band at the expected molecular weight of Ezrin proteins (Suppl. Fig. 2C–D). The rabbit polyclonal anti-Ezrin antibody was used in all subsequent experiments, including the analyses of human brain tissue. Ezrin protein abundance was increased in JNPL3 mice with severe motor impairment (Tg-Severe; Average MIS = 25) in comparison with age-matched NTg mice (Fig. 2A). This result confirms that Ezrin protein level is increased in tissue vulnerable to pathological tau as illustrated by the accumulation of 64kDa tau species (Fig. 2A). To further understand the role of changes in Ezrin's protein level in tauopathy, we sought to determine the level of Ezrin before the onset of the motor impairment phenotype in JNPL3 mice. We found that Ezrin protein level is increased in asymptomatic (AS) Tg mice (Tg-AS; Average MIS = 6), indicating that Ezrin is upregulated before the onset of motor impairment and accumulation of 64kDa tau species in these mice (Fig. 2A, Tg-AS). These results indicate that the increase in Ezrin protein levels takes place before the onset of motor impairment and persist through the progression of tau-mediated neurodegeneration in JNPL3 mice.

3.3. Upregulation of Ezrin Protein in Alzheimer's Disease

To determine if changes in Ezrin protein abundance detected in JNPL3 mice are relevant to human tauopathy, we conducted western blot analysis of post-mortem temporal cortex protein extracts derived from normal-aging (NA) and AD cases (Fig. 3). The selected AD cases were at Braak stage VI, indicating severe accumulation of pathological tau (Table 1). The results demonstrated that Ezrin protein level is significantly higher in AD cases, in comparison to normal aging. This result indicates that Ezrin changes in protein abundance are associated with neurodegeneration in both a tauopathy mouse model and humans. Moreover, we detected an increase in Ezrin protein abundance in the cortex of other tauopathies, such as FTDP-17 and PSP (Suppl. Fig. 3), suggesting that Ezrin's upregulation is associated with tau-mediated neurodegeneration.

3.4. Ezrin Upregulation in Mild Cognitive Impairment

To determine if changes in Ezrin protein level are associated with cognitive impairment as an early event in the neurodegeneration process, we assessed the protein level of Ezrin in the temporal cortex of MCI cases in comparison to normal-aging cases (Fig. 4). The results indicate that Ezrin protein levels are significantly higher in MCI cases, confirming that Ezrin protein changes take place before the onset of the phenotypic or clinical presentation due to neurodegeneration in mice and humans, respectively (compare Fig. 2 and 4). The increase in Ezrin protein abundance could be explained by upregulation of gene expression, the stability of mRNA or decreased protein turnover. Interestingly, in a separate ongoing study, a microarray dataset was queried to determine changes in cortical Ezrin mRNA levels. Ezrin's expression was found upregulated by ~1.5–1.6-fold in MCI and AD in comparison to NA (Fig. 5). Thus, Ezrin's gene expression may be regulated in the course of cognitive impairment and AD, leading to the detected increase in protein abundance.

4. DISCUSSION

Ezrin is a member of the ezrin-radixin-moesin (ERM) F-actin binding protein family that plays an important role in the organization and maintenance of the cell cortex, anchors plasma membrane proteins to the actin cytoskeleton and serves as a scaffold protein to regulate cell signaling [14–19]. The present results indicate that Ezrin protein levels are altered in the JNPL3 tauopathy mouse model. Notably, the increased abundance in total Ezrin protein levels was detected in the spinal cord, an area highly vulnerable to tau-mediated neurodegeneration in these mice, even before the onset of motor impairment and persisted in those mice that were severely impaired, suggesting that Ezrin may play an important role in the pathophysiology associated with the progressive accumulation of toxic tau species.

Ezrin protein levels were also altered in the temporal cortex of MCI and AD subjects in comparison to normal aging controls. This result suggests that the increase in Ezrin protein abundance observed in JNPL3 mice is relevant to molecular mechanisms that also take place in humans during the progression of AD. Moreover, we detected differences in Ezrin protein abundance in other tauopathies, such as FTDP-17 and PSP (Suppl. Fig. 3). In an independent ongoing study, microarray analysis of frontal cortex tissue from normal aging, MCI and AD identified Ezrin as an upregulated transcript in MCI and AD. It is important to mention that

for the proteomics and microarray analyses conducted in this study we used tissue from different brain banks to broaden the representation of disease and normal aging cases. The results suggest that the increase in Ezrin protein abundance could be explained by either upregulation of gene expression or stability of mRNA in response to the pathological process associated with neurodegeneration. Ezrin upregulation could be directly associated with tau toxicity or it may represent a stress response mechanism in response to neuronal dysfunction in neurodegenerative diseases. Thus, further studies directed to understand the regulation and function of Ezrin will provide insights about its role in tauopathies and other neurodegenerative diseases.

It is plausible to hypothesize that during the early stages of neurodegeneration there is significant upregulation of Ezrin to mediate a neuroprotective role in response to the accumulation of toxic tau molecules. Ezrin's function is regulated by phosphorylation at different sites [18–19]. Ezrin phosphorylation induces its transition from a dormant cytosolic localization to an active membrane-actin associated form [18]. In addition, Ezrin mediates signaling in response to activation of receptors such as EGF receptor and regulates the cell survival PI3-kinase/Akt pathway [19]. In neurons, Ezrin plays a role in neurite outgrowth and axonal pathfinding [20–22]. Additionally, Ezrin is associated with neuronal responses to injury and was detected in the CSF of both rat models and humans with severe traumatic brain injury, indicating that this protein may play an important role in response to neuronal stress [15].

In regard to tauopathies, Ezrin's known physiological roles provide new avenues to dissect molecular mechanisms that become activated in response to the accumulation of toxic tau species. For example, Ezrin's role in activating PI 3-kinase and Akt [19] may promote Akt-mediated inhibition of GSK-3 β , a primary kinase involved in tau phosphorylation [23]. Interestingly, netrin-1 mediates the activation of Ezrin through RhoA/Rho kinase [24] and the overexpression of netrin-1 was found to reduce the production of amyloid- β and ameliorate the phenotype of Alzheimer transgenic mice (PDAPP) [25]. Additionally, Rho kinases are known to modulate the activity of Ezrin in cell survival [25]. Taken together, Ezrin could play a neuroprotective role during the early stages of neurodegeneration. An alternative hypothesis is based on previous studies that implicated Ezrin in the regulation of phagocytosis, endocytosis, plasma membrane dynamics and cytoskeleton organization [16, 18]. In the context of pathological tau propagation, Ezrin activation could also be involved in mediating the necessary cytoskeleton and plasma membrane dynamics to facilitate the release and/or up-take of tau proteins to and from the extracellular space. Thus, understanding Ezrin's upregulation in neurodegeneration could help disentangle molecular events associated with either neuroprotection or neurodegeneration.

Although the detected increase in Ezrin protein levels in a tauopathy mouse model was validated in MCI and AD, it is important to put the implications of this study into context. Firstly, transgenic JNPL3 mice model the pathology observed in tauopathies such as FTDP17 and PSP, but not AD. Thus, the detected protein changes are induced by the pathological accumulation of the human tau^{P301L} mutant protein in the absence of A β peptide aggregates. Secondly, the validation in human tissue of altered proteins identified in mouse models of neurodegeneration is an important step to determine if the detected

molecular event is conserved or specific to the biology of the mouse model used in the study. However, in those cases where the molecular event is validated in human tissue, further studies are required to determine the pathological role of the identified altered proteins. For example, the use of induced pluripotent stem cells from inherited and sporadic AD cases is a powerful tool to ascertain the putative pathological role of the identified biomarkers. Thirdly, the use of postmortem AD brain tissue from different cohorts should be a standard practice to validate results obtained in animal models. In this study, we used tissue from two different brain banks. We detected increases in Ezrin protein level using tissue from both cohorts. Nevertheless, the tissue used follows specific selection criteria based on clinically confirmed cases of specific age group, Braak stages and postmortem interval. Thus, the results presented in this study are within the limitations of the established selection criteria and number of cases.

CONCLUSION

The data presented in this study demonstrated that Ezrin becomes upregulated in asymptomatic JNPL3 mice and in MCI cases. This upregulation persists in terminally ill JNPL3 mice and AD cases. Further studies on the functional role of Ezrin in AD and other tauopathies may lead to novel insights into disease pathogenesis, and potentially useful as a pathological biomarker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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All authors made significant intellectual and technical contributions to the study. IEV conducted experiments, evaluate data, revised the literature and wrote the manuscript. AU, CSW and JSB conducted experiments and prepared figures. SEC conducted experiments, evaluate data and proofread the manuscript. The authors acknowledge the technical contributions of Jeriel Keeney and Marie Wallich. The work was supported, in part, by MSU Institutional Startup funds and NIH grants P30AG053760 and P01AG014449.

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Fig. (1). Ezrin protein levels is increased in JNPL3 mice.

A) Representative image of excised protein spots (arrows) from Non-transgenic (NTg) and Transgenic (Tg) spinal cord protein extract resolved by two-dimensional gel electrophoresis

B) List of Ezrin peptides identified by tandem mass spectrometry.

Fig. (2). Ezrin protein level increase before detectable motor impairment in JNPL3 mice.

A) Western blot analysis of spinal cord protein extract from asymptomatic (Tg-AS) and severe (Tg-Severe) motor impaired transgenic JNPL3 mice. Non-transgenic (NTg) mice were used as control. The identified proteins are indicated. **B)** Statistical analysis: One-way ANOVA (with Holm-Sidak's multiple comparisons test). (** $p=0.001$).

Fig. (3). Ezrin protein level in AD temporal cortex.

A) Western blot analysis of temporal cortex protein extract from normal aging (NA) and Alzheimer's disease (AD) cases. The identified proteins are indicated. **B)** Statistical analysis: Unpaired t test with Welch's correction (parametric; two-tailed). (*p=0.01).

Fig. (4). Ezrin protein level is increased in MCI.

A) Western blot analysis of temporal cortex protein extract from normal aging (NA) and Mild-cognitive impairment (MCI) cases. The identified proteins are indicated. **B)** Western Blot Statistical analysis: Unpaired t test with Welch's correction was performed.

Fig. (5). Ezrin transcript levels are increased in MCI and AD.

Ezrin transcript was found significantly upregulated in a microarray analysis of temporal cortex from mild-cognitive impairment (MCI) and Alzheimer's disease (AD) in comparison to normal aging (NA) cases. Statistics: One-way ANOVA (with Holm-Sidak's multiple comparisons test) (* $p=0.01$).

Table 1. List of subjects used in this study. Demographic and pathological description of cases used in this study.

Case	EMORY Brain Bank		Braak
	Expired age (y)	PMI (h)	
NA	76	3.5	IV
NA	92	15.5	III
NA	78	11.5	II
NA	74	7.0	II
NA	70	4.5	I
NA	68	11.0	0
Avg.	75.7	8.6	2
AD	72	7.0	VI
AD	64	4.5	VI
AD	77	12.0	VI
AD	58	6.0	VI
AD	78	8.0	VI
AD	75	12.0	VI
Avg.	70.7	8.2	6
Case	Rush Religious Order Study		Braak
	Expired age (y)	PMI (h)	
NA	92	4.9	II
NA	84	10.7	II
NA	91	7.9	II
NA	87	6.2	I
NA	91	6.5	I
NA	87	7.5	I
NA	89	4.4	II
NA	89	17.0	I
Avg.	88.7	8.1	1.5
MCI	83	7.6	III

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MCI	90	4.3	III
MCI	93	3.5	IV
MCI	90	5.4	IV
MCI	89	4.4	IV
MCI	82	6.6	IV
MCI	91	4.3	III
MCI	85	3.3	III
Avg.	87.9	4.9	3.5

Key: NA, normal aging; AD, Alzheimer's disease; MCI, mild cognitive impairment; Avg., average; PMI, postmortem interval.