Association of Retromer Deficiency and Tau Pathology in Down Syndrome

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Retromer deficiency is reported in Down syndrome and correlates with amyloidosis, however, its association with tau neuropathology remains unclear. Down syndrome and control brain tissues were evaluated for phosphorylated tau, tau modulators, and cathepsin-D activity. Several kinases and phosphatase PP2A were unchanged, but tau phosphorylation was elevated, and cathepsin-D activity decreased in aged patients with Down syndrome. Retromer proteins positively associated with soluble tau, whereas pathogenic tau negatively correlated with retromer proteins and cathepsin-D activity. Retromer deficiency and consequent reduction of cathepsin-D activity may contribute to pathogenic tau accumulation, thus, retromer represents a viable therapeutic target against tau pathology in Down syndrome.

ANN NEUROL 2022;91:561–567

Dysfunction of the endosomal-sorting system known as the retromer complex has emerged as a common pathology in various neurodegenerative diseases, including Alzheimer’s Disease (AD). 1, 2 Mechanistically, retromer system dysfunction can influence amyloid beta production by increasing the interaction of the retromer cargo amyloid precursor protein (APP) with beta-secretase. 1 However, recent studies have also implicated the retromer complex in the development of tau pathology, both through regulation of tau phosphorylation and degradation via lysosomes. 3–5 Furthermore, targeting the retromer system has shown promise as an AD therapeutic, because enhancing or restoring retromer function ameliorates the AD-like phenotype in animal models. 6, 7 We recently reported that, similarly to human AD, the levels of retromer recognition core proteins are decreased in subjects with Down syndrome, and that this deficiency inversely correlates with the development of the amyloidotic phenotype associated with AD-Down syndrome. 8 As a follow-up to our previous report, in this paper, we investigate the association between the retromer complex system and tau pathology in Down syndrome. Herein, we report that levels of retromer recognition core proteins positively associate with total soluble tau, and negatively with phosphorylated and insoluble tau protein. These associations appear to be independent of kinases and phosphatases, as these proteins are unchanged, but may rely on the availability of protease activity of the retromer cargo cathepsin-D, which we found significantly decreased in patients with Down syndrome. These findings indicate that the development of tau pathology coincides with the deficiency of retromer recognition core proteins during the evolution of the syndrome and support the hypothesis that this deficiency could contribute to the accumulation of pathogenic tau that occurs in Down syndrome.

Methods

Human Brain Samples

Post-mortem cortex and hippocampus tissues from AD, Down syndrome, and matched control subjects were obtained from repositories of the National Institutes of Health (NIH) Neurobiobank. Postmortem tissues from patients with Down syndrome and matched controls were previously used to examine retromer proteins in Down syndrome and detailed subject information was described. 8 Information for AD and matched control subjects is provided in Supplementary Table S1. The protocol for the study did not require institutional review board approval because it involves the analysis of existing tissue samples without identifiers.

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Additional supporting information can be found in the online version of this article.

Received Nov 30, 2021, and in revised form Jan 17, 2022. Accepted for publication Feb 10, 2022.

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.26321.

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**Immunoblot Analysis**

Brain homogenates were extracted and subjected to immunoblot analysis, as previously described. The following primary antibodies were used: HT7 (1:200, Invitrogen; MN1000), AT8 (1:200, Invitrogen; MN1020), PHF1 (1:200, gift from P. Davies), PHF13 (1:100, Cell Signaling Technologies; 9672), GSK3α/β (1:200, Cell Signaling Technologies; 5676), phospho-GSK3α/β (1:100, Cell Signaling Technologies; 9331), p38 (1:200, Cell Signaling Technologies; 8690), phospho-p38 (1:100, Cell Signaling Technologies; 4511), CDK5 (1:200, Cell Signaling Technologies; 12134), p25 (1:200, Cell Signaling Technologies; 2680), cathepsin-D (1:200, Santa Cruz; SC-377124), and GAPDH (1:500, Cell Signaling Technologies; 2118). GAPDH was used as an internal loading control. An internal control sample was used and samples from each group were included within gels to allow for inter-blot analysis and comparison between groups.

**Cathepsin-D Activity Assay**

Cathepsin-D activity was measured via a fluorometric assay following the manufacturer’s instructions (Abcam, ab65302). Fluorescence was measured using a fluorescence microplate reader at Ex/Em = 328/460 nm, and relative fluorescence normalized by the protein concentration of each sample.

**Data Analysis and Statistical Methods**

Descriptive summary data were expressed as counts and percentages for categorical variables and mean ± SD/SEM and/or median (range) for continuous variables. Continuous variables that are skewed (eg, tau measurements) were

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**FIGURE 1**: Phosphorylated and insoluble tau proteins are increased in cortices and hippocampi of aged subjects with Down syndrome. (A) Representative immunoblots of tau epitopes in cortices of young (age 15–40 years) and aged (age 40–65 years) subjects with Down syndrome and matched control (CTR) subjects. (B) Representative immunoblots of tau epitopes in hippocampi from young and aged subjects with Down syndrome and matched control subjects. (C–H) Densitometry analysis of immunoblots shown in panels A and B. (C. HT7, D. MC1, E. AT8, F. PHF1, G. PHF13, H. Insoluble [Ins] HT7). For the analysis of C to G, young CTR CX n = 7, aged CTR CX n = 16, young Down syndrome CX n = 10, aged Down syndrome CX n = 18, young CTR HC n = 5, aged CTR HC n = 14, young Down syndrome HC n = 4, aged Down syndrome HC n = 10. For the analysis of H, young CTR CX n = 7, aged CTR CX n = 7, young Down syndrome CX n = 6, aged Down syndrome CX n = 18, young CTR HC n = 5, aged CTR HC n = 14, young Down syndrome HC n = 4, and aged Down syndrome HC n = 10. Values represent mean ± standard error of the mean, *p < 0.05. [Color figure can be viewed at www.annalsofneurology.org]
transformed using the log function when the normality assumption is violated on the original scale. The Spearman correlation coefficient was used for correlation analyses between the tau measurements and retromer protein levels. Pairwise group comparisons of retromer proteins and tau measurements between age (<40 vs ≥40), brain region (cortex vs hippocampus), and type of subjects (Down syndrome vs controls) subgroups were performed under the framework of the multivariable mixed-effects regression model approach for each variable of interest to take into account the potential correlation between the observations of the two brain regions from the same subjects included in the study. All interaction terms among the age, brain region, and type of subjects were included in the model to account for possible heterogeneous effects across different subgroups. Multiple comparison adjusted p values and simultaneous 95% confidence intervals for the estimated group differences were derived via the Tukey–Kramer method from the multivariable mixed-effects regression model. Any p values <0.05 were considered statistically significant. SAS version 9.4 (SAS Institute Inc., Cary, NC) and Graphpad Prism for Windows version 7.00 were used for all the data analyses.

**Results**

To assess the relationship between retromer complex system and tau pathology in Down syndrome, we examined tau proteins in cortices and hippocampi from young (age = 15–30 years), and aged (age = 40–65 years) subjects with Down syndrome and controls (Fig 1). Total tau (HT7) did not differ among groups, and although there were reductions in the pathological conformation of tau detected by MC1 in the hippocampus compared to cortex, MC1 immunoreactivity did not differ significantly between control and Down syndrome groups. We also examined phospho-epitopes of tau recognized by the specific antibodies AT8, PHF1, and PHF13. Although all

![FIGURE 2: Regulators of tau pathology: kinases, phosphatases, and cathepsin-D. (A) Representative immunoblots of tau kinases and phosphatases in cortices of young (age 15–40 years) and aged (age 40–65 years) subjects with Down syndrome and matched control (CTR) subjects. (B–J) Densitometry analysis of immunoblots shown in panel A. (B. CDK5, C. p25, D. GSK3α, E. GSK3β, F. phospho-GSK3α, G. phospho-GSK3β, H. p38, I. phospho-p38, J. PP2A). Young CTR CX n = 11, aged CTR CX n = 14, young Down syndrome CX n = 9, aged Down syndrome CX n = 14. (K) Representative immunoblot of mature cathepsin D (CTSD) in cortices of young and aged subjects with Down syndrome and matched control subjects. (L) Densitometry analysis of immunoblots shown in panel K. Young CTR CX n = 11, aged CTR CX n = 12, young Down syndrome CX n = 8, and aged Down syndrome CX n = 11. (M) Cathepsin D activity in cortices of young and aged subjects with Down syndrome and matched control subjects. Young CTR CX n = 6, aged CTR CX n = 13, young Down syndrome CX n = 6, and aged Down syndrome CX n = 12. Values represent mean ± standard error of the mean, *p < 0.05. [Color figure can be viewed at www.annalsofneurology.org]
comparisons did not reach statistical significance due to high variability, each phospho-epitope was elevated in the cortex and hippocampus of aged Down syndrome individuals compared to all other groups, with PHF1 significantly increased in aged Down syndrome hippocampus compared to all controls and young Down syndrome subjects. Last, as a measure of more advanced tau pathology, we examined the insoluble fraction of tau protein. Although insoluble tau failed to fit our regression model due to the skewed data distribution, it was dramatically increased in both hippocampi and cortices of aged subjects with Down syndrome (see Fig 1).

Having confirmed that phosphorylated and insoluble tau were elevated in this Down syndrome cohort, we next examined the cortical levels of several tau kinases (CDK5, p25, GSK3α/β, phospho-GSK3α/β, p38, and phospho-p38) and the phosphatase PP2A as potential mechanisms of phospho-tau regulation in Down syndrome (Fig 2A–J). Protein levels of these kinases and the phosphatase did not significantly differ between groups. To explore potential clearance mechanisms of pathological tau, we next examined the protease cathepsin-D, as it is known to be involved in lysosomal degradation of tau and is regulated via the retromer complex system. Total protein levels of cathepsin-D did not differ among groups; however, cathepsin-D activity was significantly decreased in the cortices of aged patients with Down syndrome (Fig 2K–M). To determine whether this finding was specific to AD-Down syndrome, we next analyzed cathepsin-D activity levels in cortices of patients with AD and matched controls. No significant differences in cathepsin-D activity were observed between control and AD brain cortices (Supplementary Fig S1).

Using previously published data on retromer proteins in this cohort,8 we analyzed the relationship between tau and retromer recognition core proteins (VPS35, VPS26, and VPS29), the cation-independent mannose-6-phosphate receptor (CIMPR) retromer cargo receptor, and its cargo cathepsin-D (Table). Interestingly, retromer recognition core proteins significantly and positively correlated with total soluble tau but had statistically significant inverse correlations with the levels of two tau phospho-epitopes: PHF1 and PHF13. Furthermore, activity of cathepsin-D, and protein levels of its receptor, CIMPR, showed significant, negative correlations with PHF1, PHF13, and insoluble tau.

**Discussion**

Despite differences from a clinicopathological point of view, several neurodegenerative diseases share a common feature, which is characterized by the progressive

| TABLE. Spearman Correlations Between Tau Measurements and Retromer and CTSD Protein Levels, and CTSD Activity in Cortices of Down syndrome and Matched Control Subjectsa |
|-------------|---------|---------|---------|-----------|---------|---------|---------|---------|
|             | n       | VPS35   | VPS26   | VPS29   | CIMPR   | n       | M. CTSD | n       | CTSD activity |
| HT7         | 53      | 0.41    | 0.34    | 0.35    | 0.27    | 42      | 0.33    | 37      | 0.08 |
|             |         | 0.002   | 0.014   | 0.011   | 0.055   |         | 0.032   |         | 0.64 |
| AT8         | 51      | -0.11   | -0.08   | -0.18   | 0.03    | 42      | 0.45    | 37      | -0.21 |
|             |         | 0.44    | 0.59    | 0.21    | 0.81    |         | 0.003   |         | 0.22 |
| PHF1        | 49      | -0.44   | -0.21   | -0.53   | -0.33   | 42      | 0.41    | 37      | -0.39 |
|             |         | 0.001   | 0.14    | <0.0001 | 0.019   |         | 0.008   |         | 0.017 |
| PHF13       | 50      | -0.39   | -0.25   | -0.46   | -0.28   | 42      | 0.43    | 37      | -0.28 |
|             |         | 0.005   | 0.075   | 0.0008  | 0.045   |         | 0.005   |         | 0.092 |
| MC1         | 53      | 0.18    | 0.26    | 0.18    | 0.13    | 42      | 0.53    | 37      | 0.05 |
|             |         | 0.19    | 0.063   | 0.19    | 0.36    |         | 0.003   |         | 0.77 |
| Insoluble HT7 | 37    | -0.24   | -0.16   | -0.31   | -0.48   | 29      | 0.30    | 26      | -0.57 |
|             |         | 0.14    | 0.35    | 0.062   | 0.002   |         | 0.11    |         | 0.002 |

*aTable entry: First line = Spearman correlation coefficient, second line = p value. CIMPR = cation-independent mannose 6-phosphate receptor; CTSD = cathepsin-D; M. CTSD = mature cathepsin-D.
accumulation of unwanted proteinaceous material resulting in altered protein homeostasis. When internalized cargoes from the plasma membrane first enter the cell they reside in early endosomes, from which they are sorted and trafficked throughout the cell or shipped to the lysosome for degradation. Trafficking of cargoes from endosomes can be mediated by several sorting complexes, such as retromer or retriever, Rab GTPases, and sorting nexins in association with numerous accessory proteins. Defects in this endocytic pathway were first associated with AD neuropathology when the accumulation of enlarged early endosomes was first described in pyramidal neurons in human postmortem brain tissue from a cohort of patients with AD. Furthermore, genetic association studies revealed variants of several endosomal pathway proteins were associated with elevated AD risk, suggesting that endocytic pathway dysfunction could be an upstream event in AD pathogenesis.

The retromer complex is an endosomal system responsible for the sorting of cargoes from endosomes to the cell surface, trans-Golgi, or lysosome for degradation. As such, retromer has emerged as a key player in protein trafficking and homeostasis, and its dysfunction is implicated in several neurodegenerative diseases. We recently reported that protein levels of the retromer recognition core, VPS35, VPS26, and VPS29, are decreased and negatively correlate with the amount of brain amyloidosis in patients with Down syndrome. In the present study, we extend this analysis to include tau pathology in the same cohort. After first confirming that aged subjects with Down syndrome accumulate more phosphorylated and insoluble tau than matched controls, in search for a mechanism responsible for it, we evaluated known modulators of pathogenic tau. Examination of kinases and phosphatases known to play an active role in these post-translational modifications of tau protein revealed no significant changes among the different groups. However, cortices of aged patients with Down syndrome showed decreased activity of the lysosomal protease and retromer cargo protein, cathepsin-D. Interestingly, we did not observe similar decreases in cathepsin-D activity in cortices of patients with AD, demonstrating that this finding is unique to AD-Down syndrome and not merely secondary to AD pathology, neuronal injury, or cellular stress responses. These observations are consistent with previous reports showing that cathepsin-D activity is unaltered in the brains of patients with AD, yet is decreased in cellular models of Down syndrome, revealing a divergence in potential mechanisms underlying AD and AD-Down syndrome pathogenesis. Nevertheless, numerous studies have demonstrated the involvement of cathepsin-D in pathological tau clearance, thus, this observation provides a potential mechanistic link between the retromer recognition core deficiency in Down syndrome and tau pathology we found in the current study.

As a continuation of our previous study associating retromer deficiency with Aβ pathology in Down syndrome, we applied a regression analysis to our previously reported retromer recognition core proteins data with the current evaluation of tau protein, both soluble and pathological (ie, phosphorylated isoforms and insoluble fraction), cathepsin-D protein, and activity. Total levels of soluble tau (HT7) positively associated with retromer recognition core proteins, whereas the same proteins showed significant negative correlations with specific phospho-epitopes of tau: PHF1 and PHF13. We did not observe negative correlations between retromer core proteins and the tau epitopes recognized by the antibodies MC1 and AT8. Protein levels of soluble MC1 were not elevated in aged patients with Down syndrome within this cohort, possibly due to decreased solubility of this conformation of tau. Furthermore, previous analysis of the evolution of tau pathology in Down syndrome has demonstrated that AT8 accumulation is an early event in AD-Down syndrome that stabilizes during disease progression, whereas PHF1 accumulation continues to increase with advancing neuropathology. Thus, although we found that AT8 was elevated in aged patients with Down syndrome, the degree of AT8 accumulation was diminished compared to the other phospho-epitopes examined, possibly explaining the specificity of our findings. Activity of cathepsin-D, and protein level of its receptor, CIMPR, negatively and significantly associated with phospho-tau epitope PHF1, but showed the strongest inverse correlations with insoluble tau. This observation is consistent with the concept that this protease is involved in the clearing of pathological tau aggregates. It also suggests that individuals with high expression of retromer recognition core proteins retain the physiological, soluble species of tau, whereas retromer depletion promotes the accumulation of the pathogenic forms of tau via the reduction of cathepsin-D activity.

Even in aged individuals, incidence of dementia fails to reach 100% within the Down syndrome population despite increased APP gene dosage, implying that factors outside of HSA21 modulate dementia risk. Based on our findings, we propose that retromer complex insufficiency may allow for the genetic risk conferred by increased HSA21 gene dosage to translate into AD pathogenesis through two distinct mechanisms. First, retromer depletion directly influences the amyloidogenic phenotype found in Down syndrome via increased production of Aβ.
secondary to APP retention in endosomes. Second, efficacy of lysosomal degradation is also significantly impaired due to decreased trafficking and activity of cathepsin-D, resulting in decreased tau clearance and accumulation of phosphorylated tau and insoluble tau aggregates. Although our study focuses on classical AD proteinopathies, recent work detailing five distinct molecular subtypes of AD reveals that pathways involved in synaptic integrity may be most influential in determining the clinical features of AD.\textsuperscript{23} We propose that retromer dysfunction fits into this emerging theory of AD pathogenesis. The cluster of downregulated genes within the ATP6V1A subtype includes VPS35, the backbone of the retromer cargo recognition core.\textsuperscript{23} The molecular signature of this subtype suggests that endo-lysosomal system dysfunction may contribute to the accumulation of classical plaques and tangles through lysosomal acidification defects and misrouting of proteases, while also influencing synaptic function via regulation of synaptic proteins and presynaptic vesicle release.\textsuperscript{23,24} We hypothesize that retromer dysfunction in Down syndrome may yield similar pathogenetic mechanisms, given the role of the retromer complex in trafficking synaptic proteins and sorting of lysosomal proteases,\textsuperscript{1,3} but the genetic determinants of AD-Down syndrome residing on HSA21 likely cause some deviation from these molecular subtypes. 

Because of the established role of the retromer system in these pathways central to AD pathogenesis, it is likely that the altered retromer system expression levels contribute to the enhanced AD-like neuropathology that develops in patients with Down syndrome. Given that we found low expression of retromer recognition core proteins in the younger subset of patients, we propose that reduced retromer expression bestows high risk for the development of more robust tau and amyloid pathology in Down syndrome, and that the retromer represents a novel and viable therapeutic target in the treatment of both neuropathologies in Down syndrome.

Acknowledgments

The authors would like to thank all the patients and families who by generously donating the brain tissues made this research possible. We are also grateful to the brain bank repositories that provided us the tissues. Domenico Praticò is the Scott Richards North Star Charitable Foundation Chair for Alzheimer’s disease research. The work presented in this article was in part funded by grants from the National Institutes of Health (AG055707 and AG056689).

Author Contributions

M.C. and D.P. contributed to the conception and design of the study. M.C., T.S., and D.Y. contributed to the acquisition and analysis of data. M.C. and D.P. contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

The authors have no conflicting financial interests to disclose.

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