VPS35 regulates tau phosphorylation and neuropathology in tauopathy

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Abstract

The vacuolar protein sorting 35 (VPS35) is a major component of the retromer recognition core complex which regulates intracellular protein sorting and trafficking. Deficiency in VPS35 by altering APP/β metabolism has been linked to late-onset Alzheimer’s disease. Here we report that VPS35 is significantly reduced in Progressive Supra-nuclear Palsy and Pick’s disease, two distinct primary tauopathies. In vitro studies show that overexpression of VPS35 leads to a reduction of pathological tau in neuronal cells, whereas genetic silencing of VPS35 results in its accumulation. Mechanistically the availability of active cathepsin D mediates the effect of VPS35 on pathological tau accumulation. Moreover, in a relevant transgenic mouse model of tauopathy, down-regulation of VPS35 results in an exacerbation of motor and learning impairments as well as accumulation of pathological tau and loss of synaptic integrity. Taken together, our data identify VPS35 as a novel critical player in tau metabolism and neuropathology, and a new therapeutic target for human tauopathies.

Keywords

VPS35; tau protein; tau phosphorylation; tauopathy; transgenic mice

INTRODUCTION

Neurodegenerative diseases are characterized by aberrant accumulation of proteins within neurons and glial cells leading to a loss of cellular protein homeostasis. Proper sorting
and trafficking of intracellular proteins is critical for maintaining this delicate balance. Therefore, interconnected pathways, such as the endosomal-lysosomal system, are in place to preserve efficient transport of protein cargos for recycling or degradation. In recent years, the endosomal-lysosomal network, particularly the dysfunction of this system, has gained considerable attention in the neurodegeneration field and has been linked to several diseases, such as Alzheimer’s disease (AD) [1,2]. A key regulator in endosomal protein sorting is the retromer complex, an evolutionarily conserved multimeric system responsible for retrograde transport of cargo from the endosome to the trans-Golgi network (TGN) [3]. The retromer complex consists of two major components including a vacuolar protein sorting (VPS) trimer, VPS35/VPS26/VPS29, also known as the retromer recognition core, and a membrane targeting part containing different and specific sorting nexins proteins [4–6]. Among the various cargo proteins sorted by the retromer complex are the cation-independent mannose-6-phosphate receptor (CIMPR), which transports hydrolases, such as cathepsin D (CTSD) from the TGN to the endosome for proper maturation and ultimate delivery to the lysosome system, and the sortilin-related receptor, also known as SorLA, which binds to the amyloid β precursor protein (APP) [7, 8].

Data in the literature consistently show that the development of retromer dysfunction-dependent neuropathology is secondary to a partial “loss of function”. Thus, deficiency in the complex function resulting from down-regulation of VPS35 has been reported in hippocampi of AD patients; and genetic studies found that its variants increase the risk of developing AD [9,10]. Interestingly, several studies have shown a link between a deficit of VPS35 and amyloidogenesis, such that early deficiency in this sorting system promotes β-secretase-1 (BACE-1) activity in the endosomes, enhancing pathogenic cleavage of APP and Aβ formation [11]. VPS35 genetic reduction results in an increase of Aβ levels, cognitive impairments, and synaptic dysfunction in a mouse model of AD-like brain amyloidosis [12]. Moreover, VPS35 and the other components of the retromer recognition core display age-dependent, decreased protein levels in the brains of the Tg2576 mice, a model of brain amyloidosis [13].

While more studies are needed to dissect the precise mechanisms whereby VPS35 deficits may initiate and/or control amyloidogenesis in vivo, no study has investigated so far a direct role for VPS35 in the development of the second most common pathological feature of AD and related tauopathies: abnormal tau phosphorylation and neuropathology.

MATERIALS and METHODS

Human samples

Frozen human postmortem frontal cortex and hippocampal brain tissues were obtained from patients with a clinical diagnosis of progressive supranuclear palsy (PSP) (9 females and 5 males) or Pick’s disease (3 females 7 and males) along with normal age-matched controls (4 females and 6 males). Tissue samples were provided by the NIH NeuroBioBank with informed consent under approval by the appropriate institutional review board at each of the following brain banks: UCLA Brain Bank (WLA VA Medical Center), University of Maryland Brain and Tissue Bank, and Harvard Brain Tissue Resource Center. Information regarding tissue donor neuropathological criteria including age, sex, and post mortem
interval (PMI) is described in Supplementary Table 1. Postmortem diagnostic evaluation was performed in accordance with standard histopathological criteria.

**AAV1/2 vector construct**

Purified AAV2/1 vectors expressing VPS35 shRNA (AAV1-GFP-U6-mVPS35-shRNA) or scramble (AAV1-GFP-U6-scrmb-shRNA) under neuronal-specific promoter synapsin 1 were purchased from a commercial vendor (Vector Biolabs).

**Animals and Injection of AAV2/1 to neonatal mice**

This study was approved by the Temple Institutional Animal Care and Usage Committee, in accordance with the US National Institutes of Health guidelines. The P301S mice (PS19 line) expressing human mutant microtubule-associated protein tau, MAPT, driven by the mouse prion protein (Prnp) promoter were used for these studies [14]. Both male and female mice were used. The AAV2/1 injection procedures were performed as described previously [15,16]. Briefly, two microliters of AAV2/1-VPS35 shRNA or AAV2/1-scramble shRNA (1.3×10^{13} genome particles/ml) were bilaterally injected into the cerebral ventricle of newborn mice using a 5μl Hamilton syringe. A total of thirty-three pups were used; seventeen were injected with AAV2/1-VPS shRNA (8 wildtype P301S−/−, 9 P301S+/−; Females=10, Males=7) and sixteen were injected with scramble vector (8 wildtype, 8 P301S Females=7, Males=9). All animals were housed on a 12-h light/dark cycle in a pathogen-free environment and given regular chow and water, *ad libitum*. Animals underwent behavioral testing at age 10 months. Two weeks later mice were sacrificed for tissue processing. Upon euthanasia, mice were perfused with ice-cold 0.9% PBS containing EDTA (2mmol/L), pH 7.4. Brains were extracted, gently rinsed in cold 0.9% PBS and immediately dissected into two hemispheres. One half was stored at -80°C for biochemistry analyses and the other half was fixed in 4% paraformaldehyde diluted in PBS, pH 7.4, for immunohistochemistry.

**Behavioral Tests**

All animals were always handled for at least 3–4 consecutive days before testing, and were tested in random order by an experimenter who was unaware of the genotype/treatment.

**Y-maze**

The Y-maze apparatus consisted of three arms 32 cm (long) 610 cm (wide) with 26-cm walls (San Diego Instruments, San Diego, CA) and testing was performed as previously described [17–19]. Briefly, each mouse was placed in the center of the Y-maze and allowed to explore freely for five minutes to measure spontaneous alternating behavior. The sequence and total number of entries were video-recorded. An entry into an arm was considered valid if all four paws entered the arm. An alternation was defined as three consecutive entries into three different arms (1, 2, 3, or 2, 3, 1, etc.). Percentage of alternation was calculated using the following formula: total alternation number/total number of entries−2) × 100.

**Rotarod**

Mice were tested on Rotarod as previously described [20]. Briefly, a Rotarod instrument with automatic timers and falling sensors (Omnitech Electronics, Columbus, OH, USA) was...
used and testing was performed on four consecutive days. The mice were placed individually on a 30 mm diameter rotating cylinder suspended above a cage floor. The length of time the mice managed to remain on the rod was automatically recorded. The mice underwent six trials per day and the maximal observation time for each trial was 90 s. During the training phase (day 1–3), the speed of the rotation was increased gradually from 0 to 15 r.p.m. during the first 15 s and held constant at that rate for the rest of the trial (75 s). During the test (day 4), the speed of rotation was accelerated gradually from 0 to 90 r.p.m. during the 90 s of the trial.

**Morris Water Maze**

To perform the Morris Water Maze (MWM), we used a white circular plastic tank (122 cm in diameter, walls 76 cm high), filled with water maintained at 22±2 °C and made opaque by the addition of a nontoxic white paint, as previously described [17,21]. Mice were trained on four consecutive days to find a Plexiglas platform submerged in water from four different starting points. If they failed to find the platform within 60 s, they were manually guided to the platform and allowed to remain there for 15 s. Mice were trained to reach a training criterion of 20 s (escape latency). Mice were assessed in the probe trial, which consisted of a free swim lasting for 60 s without the platform, 24 h after the last training session. Animals’ performances were monitored using Any-Maze™ Video Tracking System (Stoelting Co., Wood Dale, IL) which provided data for the acquisition parameters (latency to find the platform and distance swam and) and the probe trial parameters (number of entries in the target platform zone of the platform and time in quadrants).

**Quantitative real-time RT-PCR**

RNA from PSP, Pick’s, and healthy control human brain frontal cortex and hippocampus was extracted and purified using the RNeasy mini-kit (Qiagen, Germantown, MD), and used as previously described [18,19]. Briefly, 1 μg of total RNA was used to synthesize cDNA in a 20 μl reaction using the RT2 First Strand Kit for reverse transcriptase-PCR (Super Array Bioscience). Human VPS35 and VPS26b genes were amplified by using the corresponding primers obtained from Super Array Bioscience. β-Actin was used as an internal control gene to normalize for the amount of RNA. Quantitative real-time RT-PCR was performed by using Eppendorf® ep realplex thermal cyclers (Eppendorf, Hauppauge, NY). Two microliters of cDNA was added to 25 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Each sample was run in duplicate, and analysis of relative gene expression was done by using the 2−ΔΔCt method [22]. Briefly, the relative change in gene expression was calculated by subtracting the threshold cycle (ΔCt) of the target genes (VPS35, VPS26b, VPS29) from the internal control gene (β-actin). Based on the fact that the amount of cDNA doubles in each PCR cycle (assuming a PCR efficiency of 100%), the final fold-change in gene expression was calculated by using the following formula: relative change = 2−ΔΔCt.

**Western Blot Analyses**

RIPA extracts from human and mouse brain homogenates were used for Western Blot analyses as previously described [16,23]. Briefly, samples were electrophoresed on 10% Bis–Tris gels or 3–8% Tris–acetate gel (Bio-Rad, Richmond, CA), transferred onto
nitrocellulose membranes (Bio-Rad), and then incubated overnight at 4°C with the appropriate primary antibodies; anti-VPS35 [dilution: 1:300] (Abcam, Cambridge), anti-VPS26 [dilution: 1:200] (Abcam), anti-VPS26b [dilution: 1:200] (Proteintech, Rosemont, IL), anti-VPS29 [dilution: 1:400] (Abcam), anti-CTSD [dilution: 1:200] (Novus Biologicals, Littleton, CO), anti-CIMPR [dilution: 1:200] (Abcam), anti-HT7 [1:200] (Thermo, Waltham, MA), anti-MC-1 [1:100] (Dr. Peter Davies), anti-AT8 [1:100] (Thermo), anti-AT180 [dilution: 1:200] (Thermo), anti-AT270 [1:200] (Thermo), anti-PHF1 [1:100] (Santa Cruz), anti-PHF13 [1:100] (Thermo), anti-SYP [1:300] (Santa Cruz), anti-PSD95 [1:200] (Thermo), anti-GFAP [1:200] (Santa Cruz), anti-IBA-11 [1:100] (Thermo), anti-HSP90 [dilution: 1:500] (Abcam), anti-Rab5 [dilution: 1:200] (Abcam), anti-LAMP2 [dilution: 1:200] (Abcam), anti-GAPDH [dilution: 1:1000] (Cell signaling, Danvers, MA) and anti-Beta actin [1:500] (Santa Cruz). After three washings with T-TBS (pH 7.4), membranes were incubated with IRDye 800CW-labeled secondary antibodies (LI-COR Bioscience, Lincoln, NE) at room temperature for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience, Lincoln, Nebraska). β-Actin or GAPDH was always used as internal loading control.

Sarkosyl Insolubility Assay

The assay for insoluble tau was performed as previously described [18]. Briefly, ultracentrifugation and sarkosyl extraction (30 min in 1% sarkosyl) was used to obtain soluble and insoluble fractions of tau from brain homogenates. Insoluble fractions were washed one time with 1% sarkosyl, then immunoblotted with the HT7 antibody.

Subcellular Fractionation

Cellular fractionation was performed to isolate purified cytosol, endosomes, and lysosomes according to the protocol described by Miura et.al, 2014 [24] (Figure 5A). Briefly, cells (1 × 10^8) were transfected with either VPS35 siRNA or control. Cells were harvested after 72 h incubation in ice-cold fractionation buffer (10 mM Tris/acetic acid pH 7.0 and 250 mM sucrose) and homogenate was cleared by three successive centrifugation steps (500 ×g for 2 min, 1000 ×g for 2 min and 2000 ×g for 2 min). The supernatant was centrifuged at 4000 ×g for 2 min to obtain the plasma membrane and nuclei in a pellet. The supernatant was then ultracentrifuged at 100,000 ×g for 2 min to pellet the mitochondria, endosomes, and lysosomes. Lysosomes were isolated from the previous fraction by a 10-min osmotic lysis using 5 times the pellet volume of cold distilled water. This fraction was then centrifuged again at 100,000 ×g for 2 min to obtain lysosomes in the supernatant and endosomes/lysosomes in the final pellet. Pellets were resuspended in solubilization buffer (RIPA) and subjected to western blotting as described above.

Immunohistochemistry

Immunostaining was performed as previously described [19–21]. Briefly, serial coronal sections were mounted on 3-aminopropyl triethoxysilane (APES)-coated slides. Every eighth section from the habenular to the posterior commissure (8–10 sections per animal) was examined with unbiased stereological principles. The sections used for testing HT7, AT8, AT270, PHF13, synaptophysin, PSD95, GFAP, and IBA-1 were deparaffinized, hydrated, rinsed with phosphate-buffered saline, and pretreated with citric acid (10 mm)
for 5 min for antigen retrieval, then with 3% H₂O₂ in methanol for 30 min to eliminate endogenous peroxidase activity and with blocking solution (2% normal serum in Tris buffer, pH 7.6). The sections were incubated with appropriate primary antibody overnight at 4°C then with secondary antibody at room temperature and developed using the avidin–biotin complex method (Vector Laboratories, Burlingame, CA, USA) with 3,3-diaminobenzidine (DAB) as chromogen.

**Cell line and treatment**

Neuro-2A neuroblastoma (N2A) cells stably expressing human tau (N2A-Htau) were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL streptomycin (Mediatech, Herdon, VA) and 100 mg/mL Hygromycin (Invitrogen, Carlsbad, CA) at 37°C in the presence of 5% CO₂. For knockdown experiments, cells were cultured to 70% confluence in six-well plates and then transfected with 100nM control siRNA or VPS35 siRNA (Thermo Fisher/Ambion, Waltham, MA) by using Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and as previously described [16]. After 72 h treatment, supernatants were collected and cells were harvested in lytic buffer for biochemistry analyses. For TPT experiments, cells were grown under the same conditions as stated above after which, 25 µM or 50 µM was added to cells for 48 h. Cells were subsequently collected and used for western blot analyses. For overexpression studies, cells were cultured to 70% confluence in six-well plates and then transfected with 5ug VPS35 plasmid or empty vector by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions and as previously described [16]. After 48 h treatment, supernatants were collected and cells were harvested in lytic buffer for biochemistry analyses. For some experiments, transfected cells were collected after 48 h and used to measure CTSD activity, using a fluorometric Assay Kit, according to the manufacturer’s instructions (Abcam). Briefly, cell lysates were centrifuged at 10,000 x g for 10 minutes at 4°C and the supernatant were used. Cathepsin D activity was determined at the 48hr timepoint by cleavage of the fluorescence peptide substrate [DnP-DR-MCA, GKPIFFRLK(DnP)-DR substrate peptide labeled with MCA]. For pepstatin A experiments, cells were pre-treated with 100µM pepstatin A (Sigma) 24 h before plasmid overexpression to inhibit CTSD activity.

**Immunofluorescence**

Cell immunostaining was performed as previously described with slight modification [25,26]. Briefly, N2A–Htau cells were plated on Matrigel-coated 35 x 10 mm No 1.5 glass-bottom dishes (Eppendorf) and grown to 60% confluence prior to VPS35 siRNA transfection. After a 72 h incubation, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 20 min at RT. After washing several times with PBS and permeabilizing with 0.2% Triton X-100 in PBS, cells were incubated in blocking solution (5% normal donkey serum in PBS) for 1 h at RT followed by an O/N incubation at 4°C with a combination of primary antibodies prepared in 2% blocking solution in PBS against VPS35 (1:250, Abcam), Tau13 (1:200, Biolegend), PHF13 (1:20, Thermo), AT270 (1:100, Thermo), and MC-1 (1:20, provided by Peter Davies). After several washings with PBS, cells were incubated for 1 h at RT with secondary Alexa Fluor 568-conjugated antibody against VPS35 and 647-conjugated antibody against Tau13, AT270, PHF13 or MC-1 (all at
Cells were then stained with DAPI, washed with PBS and subsequently held at 4 °C in the dark until imaging.

Confocal Microscopy

Stained cells were maintained in PBS and imaged under a PLAN APO Lambda 60x oil objective (NA 1.40) on an Eclipse Ti2 microscope using a Galvano scanner and a GaAsP multi-unit detector on the Nikon A1R Resonant Scanning Confocal System. Using sequential engagement of the 405, 561 and 640 laser lines and a pinhole size of 23 µm, multiple 5 × 5 2-D stitched FOVs were collected from each condition at a format size of 512 × 512, with a pixel resolution of 0.41µm, to determine ROIs comprised of a minimum of multiple Tau-expressing neurons. Laser power and gain for each laser line was standardized for each condition and its respective control(s). VPS35 was captured using a TRITC filter (BP 595/50) and Tau13, MC-1, and Phospho-Tau were captured using a Cy5 filter (BP 700/75). Upon ROI determination per condition, multiple 25 µm z-stacks, with a step size of 0.25 µm each, were collected at an image resolution of 1024 × 1024 pixels and a pixel resolution of 0.21 µm. Captured z-stacks were subsequently deconvolved in automatic mode and 3-D rendered using Nikon’s NIS-Elements AR 5.02.00 software interface. 3-D renderings were thresholded against the Cy5 channel (pseudo-colored purple) to capture only tau, MC-1 or phosho-Tau expressing cells and subsequently filtered by size to exclude background and non-cellular artifacts. Cy5 channel mean fluorescence intensity was determined using several Z-stacks from each group (CTR siRNA and VPS35 siRNA) and results were normalized to percent of control. For co-localization determination, Tau/MC-1/P-Tau+ cells were further thresholded to delineate a final ROI that displayed TRITC (VPS35) positivity, thereby excluding all TRITC+ signals found in non-Tau expressing cells.

Statistical Analysis

All the data are expressed as mean ± standard error of the mean and represented with individual data values. All statistical analysis were determined following Gaussian distribution with α=0.05. Comparisons between two groups were made using an unpaired two-tailed t-test. Comparisons between more than two groups were made using a one-way ANOVA with Bonferroni’s multiple comparisons test. The p-values for each comparison are listed in each figure legend with p<0.05 considered statistically significant. All statistical tests were performed using GraphPad Prism 8.0.1.

RESULTS

VPS35 is down-regulated in two human tauopathies

We assessed protein levels of VPS35 and the other two components of the retromer recognition core, VPS26b and VPS29, in human post-mortem brain tissues from patients with Progressive Supra-nuclear Palsy (PSP), one of the most common forms of primary tauopathy, and age-matched healthy controls. In contrast to controls, both frontal cortices and hippocampi from PSP patients showed a significant decrease in the levels of all three retromer recognition core proteins as measured by western blot analyses (Figure 1A-D; supplementary Table). We confirmed this observation also in a separate cohort of patients with a diagnosis of Pick’s disease, a distinct human primary tauopathy (Figure 1E-H;
supplementary table). Since we observed a change in protein levels for VPS35, VPS26b and VPS29 next we performed RT-PCR to determine relative expression of mRNA levels for these proteins in the same samples. In contrast to changes in protein level, no differences between PSP and healthy matched controls were detected for mRNA levels of the three core proteins (Supplementary Figure 1A, B).

**VPS35 modulates tau conformational change and phosphorylation in vitro**

Having observed a significant reduction in VPS35 levels in two distinct human primary tauopathies, next we determined whether VPS35 expression levels could directly affect tau and its phosphorylation status by implementing an *in vitro* cell model. To this end, we utilized N2A-Htau cell line, which express all six isoforms of the human tau protein, and silenced VPS35 gene expression with a specific siRNA. First, we confirmed this treatment resulted in a significant down-regulation of VPS35 protein levels together with a significant reduction in VPS26, but not significant changes in VPS29 (Figure 2A, B). Under this experimental condition, we observed a significant increase in phosphorylated tau at specific epitopes recognized by the antibodies AT270 and PHF13, and a higher immunoreactivity to the antibody MC-1, which recognizes pathological, conformational changes of tau (Figure 2C, D). By contrast, no significant changes were observed for phosphorylated tau at the epitopes recognized by the antibodies AT8 and AT180 (Figure 2C, D). Next, to investigate whether VPS35 silencing affected also tau intracellular localization we performed subcellular fractionation to isolate cytosol, endosome, and lysosome fractions following VPS35 silencing. Compared to controls, VPS35 downregulation resulted in a significant increase in pathological tau as measured by the MC-1 immunoreactivity, and phosphorylated tau recognized by the antibody PHF13 in the cytosol as well as the endosomal fractions, but not in the lysosome. (Figure 2F, G). By contrast, no significant changes were observed for the immunoreactivity of phosphorylated tau recognized by the antibody AT270 in any of these cellular fractions (Figure 2F, G).

Given our western blot findings that silencing VPS35 in neuronal cells results in conformational and phosphorylated tau accumulation, we utilized immunocytochemistry to determine mean fluorescence intensity of total, phosphorylated, and pathological tau after VPS35 silencing using confocal microscopy. Using 3D rendering of z-stacks, we determined mean fluorescence intensity of each tau signal from cells with or without VPS35 genetic silencing. Compared to control, VPS35 silenced cells had a significant increase in mean fluorescence intensity for MC-1 and PHF13 (Figure 3A, B), while there were no changes in intensity for Tau13 or AT270. Additionally, we aimed to determine if VPS35 and tau isoforms colocalize in our cell line. To control for discrepancies in cell size, we used NIS elements software to threshold for object volume and size in the Cy5 channel for each tau antibody. After applying a volume and size threshold to VPS35 signal as well, we calculated the fraction of colocalization as mean object intensity of VPS35 divided by mean object intensity of tau within specific ROIs. In this way, we were able to calculate the amount of co-localization between VPS35 and Tau13, AT270, PHF13, or MC-1. As shown in figure 3C shows that VPS35 partially co-localizes with total, phosphorylated and pathological tau to a similar degree.
**VPS35 overexpression reduces accumulation of pathological tau**

Next, we over-expressed VPS35 and investigated its effect on tau phosphorylation in N2A-Htau cells. First, we confirmed the efficiency of the VPS35 overexpression system using western blot and found a significant ~50% increase in its protein levels, which was associated with an elevation in VPS26 levels, but no changes in levels of VPS29 (Figure 4A, B). While we observed that the steady state protein levels of CIMPR and CTSD were unchanged between the two groups, we found a significant increase in CTSD enzymatic activity in lysates from cells over-expressing VPS35 (Figure 4A-C). To investigate the effect of VPS35 overexpression on tau, we assayed protein levels of total and pathological tau under this experimental condition. As shown in figure 4D, compared with controls, we observed a significant decrease in total tau (HT7), pathological tau (MC-1), and phosphorylated tau as recognized by the antibody AT270 in cells expressing higher levels of VPS35. By contrast, no changes were observed when the immunoreactivity for phosphorylated tau recognized by the antibodies AT8 and PHF13 were assayed (Figure 4D, E). To further support these findings, neuronal cells were treated with TPT-260, a pharmacological chaperone which has been reported to stabilize and increase the levels of VPS35 [13,27]. Cells were incubated for 48 hours with TPT-260 (25 μM and 50 μM) and lysates collected for western blot analysis. As shown in figure 4F, cells treated with the highest concentration of the drug had a significant increase in steady state levels of VPS35 and VPS26, but no changes were observed for VPS29. Moreover, we observed that while there were no differences between control cells for CIMPR levels, the high concentration of the drug induced a significant increase in mature CTSD levels (Figure 4F, G). In association with these changes we observed that levels of phosphorylated tau, as recognized by the antibodies AT270 and PHF13 as well as pathological tau, as recognized by the antibody MC-1, were also significantly reduced (Figure 4 H, I). By contrast, the treatment did not affect phosphorylated tau at the epitopes recognized by the antibodies AT8 and AT180 (Figure 4 H, I).

**The effect of VPS35 on tau is dependent on cathepsin D activity**

Given that the retromer complex traffics CTSD, a known degradative protease, and that genetic manipulation of VPS35 in vitro was associated with corresponding changes in the available enzyme’s activity, we next investigated the functional role of this protease in the VPS35-dependent effect on the accumulation of pathological tau. Cells over-expressing VPS35 were pretreated with pepstatin A, a specific inhibitor of CTSD activity, or vehicle and changes in tau were assessed by western blot analysis. Neuronal cells treated with pepstatin A alone but not over-expressing VPS35 showed no changes in either VPS35 levels or any forms of tau (total, pathological, phosphorylated) (Figure 5A, B); however, CTSD activity in these cells was decreased (Figure 5C) as expected. VPS35 overexpression lead to a decrease in pathological tau measured by MC-1 immunoreactivity as well as phosphorylated tau as measured by AT270, but no significant changes were observed for total tau levels (Figure 5A, B). Interestingly, in the presence of pepstatin A although cells had elevated levels of VPS35 the decrease in MC-1 and phosphorylated tau levels was abolished, suggesting that CTSD activity was necessary for the VPS35-dependent effect on tau pathological changes (Figure 5A-C).
Down-regulation of VPS35 exacerbates motor and learning impairments in P301S mice

The data accumulated so far provide support for a direct role of VPS35 in modulating tau pathologic changes in neuronal cells, to make this observation physiologically relevant, next, we sought to investigate whether genetic down-regulation of VPS35 in vivo would alter the onset and development of the phenotype of a tau transgenic mouse model, P301S mice. To this end, newborn WT and P301S mice were administered with intraventricular injections of either AAV-VPS35 shRNA or AAV-control shRNA and followed until 9–10 months of age, when the mice were tested on three behavioral paradigms. First, mice were tested on Y-maze to assess working memory. No differences in general locomotor activity as measured by the total number of arm entries in the maze were observed among the different groups (Figure 6A). Conversely, P301S mice receiving AAV-VPS35 shRNA had lower numbers of arm alternations compared to P301S mice receiving control vector (Figure 6B). No significant differences were observed between WT groups receiving empty vector or AAV-VPS35 shRNA (Figure 6A, B). Mice were also tested in the Rotarod paradigm to assess their motor learning ability. First, across groups, mice did not show any baseline motor issues, as they spent comparable time on the rod during the training phase. While mice across groups did not display significant differences during the training days (Figure 6C), in the probe test on Day 4, P301S mice receiving AAV-VPS35 shRNA showed decreased motor function compared to both WT groups (Figure 6D). Lastly, mice were assessed on spatial learning and memory via the Morris water maze test. During the training phase over four consecutive days, we observed no differences in performance among the different groups (Figure 6E); however, during test day, P301S mice had a lower number of platform crosses and this effect was exacerbated in the P301S-AAV-VPS35 shRNA mice group (Figure 6F). We observed no significant changes across groups in the latency to reach the platform or the time spent in the platform quadrant (Figure 6G, H). No significant differences were observed among the different groups when males and females were analyzed separately (Supplemental figure 2).

Genetic downregulation of VPS35 worsens tau neuropathology in P301S mice

Following behavioral studies, mice were euthanized and brain tissues subjected to biochemical analyses. First, we wanted to confirm the efficacy of our AAV-VPS35 shRNA intraventricular injection approach in these mice. As expected, we observed a significant reduction in the steady state protein levels of VPS35, which was associated with a similar reduction in the levels of VPS29 (Figure 7A, B). Moreover, we observed that compared with control group, P301S mice receiving AAV-shVPS35 had a significant reduction in the levels of mature CTSD, and CIMPR (Figure 7A, B). As shown in Figure 7C, D, levels of total tau, as recognized by the antibody HT7, as well as levels of phosphorylated tau at several epitopes were significantly increased in P301S mice with VPS35 downregulation. Additionally, we found that compared to brains from P301S controls, the mice receiving VPS35 shRNA had elevated levels of insoluble tau fraction (Figure 7C, D). Confirming the Western blot data, histochemical staining showed elevated phosphorylated tau and HT7 immunoreactivities in the brains of P301S-AAV-VPS35 shRNA mice compared to P301S controls (Figure 7E).
VPS35 genetic downregulation affects synaptic pathology and neuroinflammation

Since deficits in cognition as well as tau phosphorylation often correlate with alterations in synaptic integrity, we investigated whether biomarkers of synaptic integrity were affected by VPS35 gene downregulation. Compared to P301S controls, mice receiving AAV-VPS35 shRNA displayed significant reductions in the steady state levels of synaptophysin and PSD-95, pre-synaptic and post-synaptic markers, respectively, as assayed by both western blot and immunohistochemistry (Figure 7F-H). We also measured neuroinflammatory markers and found that compared with controls, P301S mice with VPS35 down-regulation had a significant elevation in levels of the glial fibrillary acidic protein (GFAP), a marker of astrocyte activation (Figure 7F-H). By contrast, we observed no changes in microgliosis as recognized by ionized calcium-binding adapter molecule 1 (IBA1). Immunohistochemical analyses confirmed these findings, showing an increase in immunoreactivity for GFAP but not for IBA-1 (Figure 7H).

DISCUSSION

VPS35 is the major component of the recognition core of the retromer complex system, which normally controls transport and sorting pathways for several cargo proteins out of the endosomes. It is the single most critical protein of the whole retromer assembly since knocking it down is sufficient to cause dysfunction of the entire complex. Several studies have shown that low levels of VPS35 affect the formation of the complex by influencing expression of the other two core proteins (VPS26 and VPS29) suggesting a general destabilization of the entire complex system [28]. Additionally, down-regulation of VPS35 or mutations in its cytoplasmic domains results in disruption of the retromer cargo interaction also leading to dysfunction of the complex, which ultimately results in accumulation of cargo proteins into the endosomes. While in recent years abundant literature has clearly established a biologic link between VPS35 and APP/Aβ peptides, no data have been presented so far in support of a direct interaction between VPS35 and tau, another important pathological player in AD pathogenesis.

Here we provide new experimental evidence implicating VPS35 in the regulation of tau phosphorylation levels with functional implications for the pathogenesis of AD and related tauopathies. Progressive accumulation of hyper-phosphorylated and pathological conformation-changed tau inside neurons represents a central pathogenic event in AD and related tauopathies, and several studies have shown that the amount of tau pathology better correlates with neuronal dysfunction and cognitive impairments than Aβ load [29,30]. Among the possible causes of the accumulation of pathological tau, a dysfunction in its degradation pathways has gained increasing support. Tau degradation can occur via two major mechanisms: the autophagy-lysosome and the ubiquitin-proteasome systems [31,32] and blockade of either of them promotes tau accumulation and neuropathology [33,34]. Interestingly, in recent years a third pathway has emerged as potentially relevant for the pathogenesis of AD and other neurodegenerative diseases: the endosome retromer-dependent system. Nevertheless, its role in regulating tau phosphorylation and metabolism and importantly its functional relevance in the pathogenesis of tauopathy is unknown.
In the current work, we implemented both *in vivo* and *in vitro* experimental approaches to demonstrate a critical role for this sorting pathway, in particular for VPS35, in regulating tau phosphorylation and neuropathology.

First, we show that VPS35 and the other two components of the retromer recognition core are down-regulated in two distinct primary tauopathies, PSP and Pick’s disease. Having observed these changes, we asked whether VPS35 is directly involved in the metabolism of tau and its phosphorylation, and ultimately, if VPS35 can modulate the development of classical tau neuropathology.

Here we present evidence that, in neuronal cells, downregulation of VPS35 alone resulted in an alteration of the other two components of the recognition core, VPS26 and VPS29, and this was associated with a significant increase in phosphorylated tau at specific epitopes, and accumulation of pathological tau inside the cells. By performing cell fractionation analysis we demonstrated that indeed under this condition there was a significant accumulation of both MC-1 and PHF13 immunoreactivities not only at the cytosol but most importantly at the endosomal level. Further confirming these findings, immune-cytochemical studies showed that VPS35 silencing resulted in increased fluorescence intensity for pathological and phosphorylated tau (i.e., MC-1 and PHF13). It is of interest to note that while we were able to show similar changes for PHF13 and MC-1 immunoreactivity after VPS35 down-regulation in the whole cell, fractionation, and co-localization studies, we did not observe the same changes for AT270 in the immunofluorescence experiments. While we do not have an explanation for this slight difference, we believe that this finding underscore the challenge of implementing different methods to gain further support for any obtained results.

By using an opposite approach in which we up-regulated VPS35 steady state levels in the same cells, we demonstrated that phosphorylated and pathological tau were significantly reduced, and this effect was associated with an increase in the activity of CTSD. Importantly, these findings were reproduced by using a pharmacological approach in which, we treated our cells with pharmacological chaperone, TPT-260, a known agent to stabilize the retromer complex [27]. Under this condition, we observed a significant increase in the steady state levels of VPS35, as well as the mature form of CTSD, which then resulted in a significant reduction in tau phosphorylation and its pathological levels. Since we observed that manipulation of VPS35 levels and the subsequent changes in pathological tau were coincidental with alteration in the availability of CTSD activity, an important protease previously involved in AD pathogenesis [35, 36], next we explored its involvement in the VPS35-dependent effect on tau. Previous work has shown that CTSD is one of the various cargo proteins sorted by the retromer complex system [37]. Indeed normal level of the retromer recognition core components, the necessary condition for a proper sorting function of the retromer as a complex, is fundamental for the transport of this hydrolase from the endosomes to the TGN from where after final maturation is properly delivered to the lysosome system [38]. Herein, we demonstrate that selective pharmacological inhibition of CSTD activity is sufficient to neutralize the effect that VPS35 has on pathological tau. While previous reports have indicated that CTSD is implicated in tau degradation [39,35], our work is the first to directly link retromer dysfunction with alteration of tau phosphorylation and pathological tau accumulation via this protease.
Moreover, the fact that we were able to reverse VPS35 deficiency by using a small pharmacological chaperone is significant from a translational point of view. Thus, we believe that these newly described molecules [27] deserve a much closer look as viable therapeutic agents against AD, related tauopathies and other neurodegenerative diseases where VPS35 dysfunction have been well documented [40].

Most notably, we also show that in vivo genetic downregulation of VPS35 in the CNS results in a worsening of the phenotype in a relevant mouse model of human tauopathy, the P301S transgenic mice [14]. Under this condition, we observed that mice with reduced levels of VPS35 had a further reduction in the motor abilities as well as learning and memory skills together with a significant increase in tau phosphorylation and neuropathology, disruption of synaptic integrity and increased neuroinflammatory responses. While we did not observe any changes in the classical kinases and phosphates involved in the major post-translational modifications of tau, we observed that mice with VPS35 downregulation had a significant increase in the steady state levels of total tau. Taken together these findings support the idea that reduction in retromer complex function does not directly influence these modifications, but most likely leads to an increased time spent by tau and its phosphorylated isoforms in the endosomal system.

In summary, our current findings identify VPS35 as an important and critical new regulator of tau phosphorylation and proteostasis, and support its direct involvement in the pathogenesis of tauopathies. Considering the data in the literature and our most recent paper showing that a gain in function of VPS35 rescue the phenotype of an AD mouse model with amyloid plaques and tau tangles [41], it is evident that this pathway by modulating independently both Aβ and tau pathology should be considered highly relevant in the design of future therapeutic interventions.

As it becomes increasingly evident that endosomal sorting and trafficking dysfunction is a common cellular event in many neurodegenerative diseases, our work provides new insights into a previously unexplored research area involving VPS35 as a novel and viable therapeutic target for reducing both Aβ and tau pathology not only in AD, but also in many other related neurodegenerative conditions all characterized by an altered proteostasis.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Retromer core components are down-regulated in postmortem human PSP and Pick’s disease brains.

A. Representative Western blot analyses for VPS35, VPS26b, and VPS29 in frontal cortex homogenates from PSP patients and age-matched controls. B. Densitometric analyses of the immunoreactivities to the antibodies shown in panel A (*p=0.0193 (VPS35); ***p=0.005; *p=0.0304 (VPS29); N=14 PSP, N=10 age-matched controls). C. Representative Western blot analyses for VPS35, VPS26b, and VPS29 in hippocampus homogenates from PSP patients and age-matched controls. D. Densitometric analyses of the immunoreactivities to the antibodies shown in panel C (**p=0.0062; *p=0.0287 (VPS26b); *p=0.0167 (VPS29); N=12 PSP, N=10 age-matched controls) E. Representative Western blot analyses for VPS35, VPS26b, and VPS29 in frontal cortex homogenates from Pick’s disease patients and age-matched controls. F. Densitometric analyses of the immunoreactivities to the antibodies shown in panel E (*p=0.0124; ***p=0.0004 (VPS26b); ***p=0.0003 (VPS29) G. Representative Western blot analyses for VPS35, VPS26b, and VPS29 in hippocampus homogenates from Pick’s disease patients and age-matched controls. H. Densitometric analyses of the immunoreactivities to the antibodies shown in panel G (*p=0.0175 (VPS35); *p=0.0218 (VPS26b); **p=0.0012; N=10 Pick’s, N=9 age-matched controls). All values are expressed as mean ± SEM.
Figure 2. VPS35 silencing promotes accumulation of pathological tau.
N2A Htau cells were transfected with VPS35 siRNA or controls for 72 hrs, then supernatants and cell lysates were harvested for biochemistry. **A.** Representative Western blot analysis for retromer core, VPS35, VPS26, and VPS29, in cells lysates transfected with 100nM VPS35 siRNA, siRNA or empty vector control. **B.** Densitometric analyses of the immunoreactivity to the antibodies shown in panel A (One-way ANOVA F(2,15) = (from left to right) 25.8 ***p=0.0002; 8.179 **p=0.004; 0.6038 p=0.5595). **C.** Representative Western blot analysis for total tau (HT7), pathological tau (MC-1), and phosphorylated tau at residues S202/T205 (AT8), Thr231 (AT180), T181 (AT270), and Ser396 (PHF13) **D.** Densitometric analyses of the immunoreactivity to the antibodies shown in panel C (One-way ANOVA F(2,15) = (from left to right) 0.0969 p=0.9089; 5.16 *p=0.0196; 0.283 p=0.7573; 0.0855 p=0.9184; 3.789 *p=0.0466; 8.426 **p=0.0035), Results are mean ± SEM (N=6 per group, 3 individual experiments). **E.** Subcellular fractionation schematic: N2A cells were transfected with VPS35 siRNA or control siRNA and cell lysates were separated into cytosol, endosome, and lysosome fractions using high-speed centrifugation (P=pellet, SUP=supernatant). **F.** Representative immunoreactivity for HT7, MC-1, AT270, and PHF13 in cytosol, endosome, and lysosome fractions of neuronal cells transfected with 100nM VPS35 siRNA or control siRNA. Protein markers for cytosol (HSP90), endosome (Rab5), and lysosome (LAMP2) were used to assess the efficiency of the fractionation and for normalization. **G.** Densitometric analyses of the immunoreactivity to the antibodies shown in the previous panel (two-tailed T test: HT7 (from left to right) p=0.2184, p=0.5453, p=0.6943. MC-1 (from left to right) *p=0.0282, *p=0.0184, p=0.7031. PHF13 (from left to right) *p=0.0262, *p=0.0171, p=0.3951. AT270 (from left to right) p=0.8914, p=0.3651, p=0.8539 All results are mean ± SEM (N=5 per group, 5 individual experiments).
Figure 3. Silencing VPS35 increases fluorescence immunoreactivity of phosphorylated and pathological tau.

A. Representative confocal microscopy images of VPS35 silenced and control cells for Tau13, AT270, MC-1, PHF13 (Cy5 channel pseudo-colored purple), VPS35 (TRITC: red), nuclear stain DAPI (blue); (Scale bar = 10µm).

B. Mean fluorescence intensity for total tau (Tau13), pathological tau (MC-1) and phosphorylated tau AT270 and PHF13 in VPS35 silenced cells versus control. (N=6 z-stacks per group; two-tailed T-test (from left to right) p=0.2705, p=0.6217, **p=0.0010, *p=0.0424).

C. Fraction of colocalization between tau isoforms and VPS35 [N=4 (Tau13), N=5 (AT270), N=6 (MC-1), N=6 (PHF13)]; one-way ANOVA F(3.17)=1.230, p=0.3295.
Figure 4. Genetic overexpression and pharmacological stabilization of VPS35 reduces pathological tau.

N2A-Htau cells were transfected with either VPS35 plasmid, GFP plasmid (5ug), or empty vector for 48 hrs then supernatants and cell lysates were harvested for biochemistry. A. Representative Western blot analysis for VPS35, VPS26, and VPS29 as well as CTSD and CIMPR protein in cells lysates transfected with VPS35 plasmid, GFP plasmid, or empty vector for 48hrs. B. Densitometric analyses of the immunoreactivity to the antibodies shown in panel A (From left to right one-way ANOVA F(2,15)=9.936 **p=0.0018, 5.852 *p=0.0132; 0.5013 p=0.6115; 0.0995 p=0.9059; 0.469 p=0.6344; 0.5662 p=0.5794). Results are mean ± SEM (N=6 per group, 3 individual experiments). C. Cathepsin D activity following 48 hr transfection with VPS35 plasmid, GFP plasmid, or empty vector. Data represent the mean ± SEM (One-way ANOVA F(2,24)=5.428 *p=0.0114; N=9 per group, 3 individual experiments run in triplicate. D. Representative Western blot analysis for total tau (HT7), pathological tau (MC-1), and phosphorylated tau AT8, AT270, and PHF13. E. Densitometric analyses of the immunoreactivity to the antibodies shown in panel D (From left to right one-way ANOVA F(2,15)=4.356 *p=0.0322, 4.445 *p=0.0305; 0.1951 p=0.8248; 4.513 *p=0.0292; 0.5339 p=0.5970). Results are mean ± SEM (N=6 per group, 3 individual experiments). Cells were treated with pharmacological chaperone, TPT-260, 48 hrs F. Representative Western blot analysis for VPS35, VPS26, VPS29, CTSD, and CIMPR following 48hr TPT treatment at 25 µM and 50 µM. G. Densitometric analyses of the immunoreactivity to the antibodies shown in panel F (From left to right one-way ANOVA F(2,15)=8.758 **p=0.0030, 5.108 *p=0.0203; 0.1872 p=0.8312; 0.1704 p=0.8449; 4.460 *p=0.0302; 0.0609 p=0.9412). H. Representative Western blot analysis for total tau (HT7), pathological tau (MC-1), and phosphorylated tau AT8, AT180, AT270, and PHF13. I. Densitometric analyses of the immunoreactivity to the antibodies shown in panel H (From left to right one-way ANOVA F(2,15)=0.1383 p=0.8720, 10.40 **p=0.0015; 0.1466 p=0.8648; 0.2148 p=0.8091; 6.354 *p=0.0103; 3.914 *p=0.0429). All results are mean ± SEM (N=6 per group, 3 individual experiments).
Figure 5: Effect of VPS35 on tau pathology is dependent on cathepsin D activity.
N2A cells were pre-incubated with 100 μM pepstatin A, or vehicle (DMSO) for 24hrs prior to transfection with VPS35 plasmid, GFP plasmid, or empty vector. Cell lysates were collected at the 48hr timepoint for either Western blot analyses or the CTSD activity assay. A. Representative Western blot analysis for VPS35, total tau (HT7), pathological tau (MC-1), and phosphorylated tau AT8, AT270, and PHF13 in cells under the above experimental conditions. B. Densitometric analyses of the immunoreactivity to the antibodies shown in panel A (From left to right one-way ANOVA F(5,30)=6.253 *p=0.0186 empty vs VPS35 *p=0.0487 empty vs VPS35 pepA; 0.7990 p=0.5582; 4.025 *p=0.0215 empty vs. VPS35, p=0.0482 VPS35 vs. VPS35 pepA; 3.572 p*=0.0370 empty vs. VPS35; 0.1622 p=0.9745. Results are mean ± SEM (N=6 per group, 3 individual experiments). C. Cathepsin D activity was determined in cell lysates under the experimental conditions described above. Data represent the mean ± SEM (F (5,30)=9.355 *p=0.0202; N=6 per group, 3 individual experiments run in duplicate.)
Figure 6: VPS35 genetic downregulation exacerbates cognitive and motor deficits in P301S mice.

A. Total number of arm entries for WT and P301S mice injected with either VPS35-AAV-shRNA or empty vector tested on Y-maze at 9–10 months of age. B. Percentage of alternations for each of the above group of mice (F (3,25) = 5.587 **p=0.0045).

C. Rotarod training phase over three consecutive days. D. Probe trial for the rotarod, measuring seconds to fall (F(3,25) = 7.164 *p=0.0473, **p=0.0072, ***p=0.0006).

E. Training phase of Morris water maze (MWM) as measured by latency to reach the platform zone over four consecutive days for WT-control, WT-VPS35, P301S-control, and P301S-VPS35.

F-H. During the probe trial, the following paradigms were measured: number of platform crosses for each group (F (3,28)=4.42 *p=0.0430, **p=0.0058, ****p<0.0001), latency to platform, and time spent in platform zone for the four groups. Values are expressed as mean ± SEM.
Figure 7. VPS35 genetic downregulation modulates tau phosphorylation and neuropathology of P301S mice.

A. Representative Western blot analyses for VPS35, VPS26b, VPS29, CTSD, and CIMPR in brain cortex homogenates from P301S and P301S-VPS35 mice. B. Densitometric analyses of the immunoreactivities to the antibodies shown in panel A (Two tailed T-test from left to right ***p<0.00052, p=0.3233, **p=0.0013, *p=0.0012, ***p=0.0006; Results are mean ± SEM, n=6 per group). C. Representative Western blot analyses for total soluble tau (HT7), phosphorylated tau (AT8, AT180, AT270, PHF-1, and PHF13) and total insoluble tau (HT7) in brain cortex homogenates from P301S and P301S-VPS35 mice. D. Densitometric analyses of the immunoreactivities to the antibodies shown in panel C (Two tailed T-test from left to right: *p<0.0369, p=0.0372, *p=0.0124, **p=0.0097, *p=0.0364, ***p=0.024, *p=0.0145; n=6 per group). E. Representative images of immunohistochemical staining of the hippocampus (CA1 region) of P301S and P301S-VPS35 mice for HT7, AT8, AT270 and PHF13 antibodies. (Scale bar = 50μm). F. Representative Western blot analyses for synaptophysin (SYP), postsynaptic density protein 95 (PSD95), glial fibrillary acidic protein (GFAP), and ionized calcium-binding adapter molecule 1 (IBA1) in brain cortex homogenates from P301S and P301S-VPS35 mice. G. Densitometric analyses of the immunoreactivities shown in the previous panel (Two-tailed T-test from left to right: ***p=0.0002, **p=0.0041, ***p=0.0004, p=0.8426; n=6 per group). H. Representative images of immunohistochemical staining of the hippocampus (CA1 region) of P301S and P301S-VPS35 mice for SYP, PSD95, GFAP and IBA1 antibodies. Results are mean ± SEM.