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Original Article

Retrograde trafficking of VMAT2 and its role in protein stability in non-neuronal cells

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Abstract

Increasing evidence suggests that the impaired neuroprotection of vesicular monoamine transporter 2 (VMAT2) contributes to the pathogenesis of Parkinson's disease. That has been linked to aberrant subcellular retrograde trafficking as strongly indicated by recent genomic studies on familial Parkinson's diseases. However, whether VMAT2 function is regulated by retrograde trafficking is unknown. By using biochemistry and cell biology approaches, we have shown that VMAT2 was stringently localized to the trans-Golgi network and underwent retrograde trafficking in non-neuronal cells. The transporter also interacted with the key component of retromer, Vps35, biochemically and subcellularly. Using specific siRNA, we further showed that Vps35 depletion altered subcellular localization of VMAT2. Moreover, siRNA-mediated Vps35 knockdown also decreased the stability of VMAT2 as demonstrated by the reduced half-life. Thus, our work suggested that altered vesicular trafficking of VMAT2 may play a vital role in neuroprotection of the transporter as well as in the pathogenesis of Parkinson's disease.

Keywords: Parkinson's disease, VMAT2, Vps35, retrograde trafficking

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease which commonly occurs in the elderly population. Its pathological changes are mainly embodied in the dopaminergic neuronal loss in the substantia nigra of midbrain. Clinical manifestations are locomotor ataxia, such as rest tremor, bradykinesia, rigidity and postural instability[1-2]. Previous studies on PD pathogenesis have been primarily focused on the finding of exogenous neurotoxin and related mechanisms leading to neuronal loss[3-4], whereas the endogenous protective mechanism is poorly investigated. Our laboratory previously found that vesicular monoamine transporters (VMATs, SLC18A family) can protect dopaminergic neurons from the toxicity of neurotoxin MPP^+[5-6]. Thus, it has been hypothesized that attenuated expression or function of VMAT2, the brain isoform of VMATs, is associated with altered detoxification capacity of the transporter and exacerbation of the cell oxidative state. Although there is increasing evidence supporting such reduced neuroprotection of VMAT2 in PD pathogenesis[7], limited molecular and cellular mechanistic studies have been reported. Recently, works on the mechanism of the vesicular positioning and the membrane trafficking of

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the transport protein itself provide a physiologic basis for understanding its cellular mechanisms of neuroprotection and potential dysfunction.

VMAT2 facilitates a vesicular package of the newly synthesized and recycled monoamine transmitters, like dopamine, into secretory vesicles such as synaptic vesicles (SVs) and large dense core vesicles (LDCVs)[8–10]. Since monoamine transmitters are often readily oxidative in the cytoplasm and may produce toxicity against cells, it is of physiologic significance that VMAT2 can protect neurons by transporting these oxidation-prone chemicals into the vesicles, away from sensitive organelles such as mitochondria[11]. Thus, the precise vesicular membrane localization of VMAT2 is vital for maintaining such functions as synaptic transmission and detoxification as well. VMAT2 contains 12 transmembrane domains, and its membrane trafficking and the correct vesicular localization are determined by the cytosolic C-terminal sequence[12-13]. For instance, the sorting of VMAT2 to the newly formed secretory vesicles occurs at the *trans*-Golgi network (TGN), and this process may be determined by the acidic patch sequence at the distal end of the C terminus[14]. In addition, upon exocytosis VMAT2 requires its dileucine motif for internalization from plasma membrane to be sorted to endosomal compartments[15]. However, how the intracellular membrane trafficking of VMAT2 is regulated remains largely elusive. Importantly, many PD related gene mutations may contribute to aberrant membrane trafficking, especially endosome-to-TGN retrograde trafficking[16–18], whereas the specific affected cargo protein is still unknown.

It is well established that internalized membrane proteins often encounter three fates at the endosomal compartments[19]. First, they recycle back to the plasma membrane through recycling endosomes. Secondly, they undergo degradation through the endo-lysosomal pathway. Thirdly, they may traffic back to the TGN for reuse through a conserved retrograde trafficking pathway which is mediated by the retromer. Retromer complex is composed of two subcomplexes: one is the cargo-selective Vps trimer, including Vps26, Vps29 and Vps35, and the other is sorting nexin (SNX) dimer, containing SNX1/2 and SNX5/6, which presumably senses membrane curvature changes[20]. Recently, it has been reported that Vps35 has a close relationship with the pathogenesis of neurodegenerative diseases, such as PD[16,18] and Alzheimer's disease (AD)[21-22]. Moreover, the mutant $Vps35^{D620N}$ has been indicated as gain-of-function form involved in PD pathogenesis[16,23]. Nonetheless, the functional specificity of Vps35 within neuronal cells is far from clear and its potential cargo proteins in the dopaminergic cells are poorly investigated.

Here, we report that Vps35 specifically interacted with VMAT2 based on biochemical and cell biology analysis. The depletion of Vps35 by its specific siRNA disturbed VMAT2 subcellular localization at the TGN. Moreover, depletion of Vps35 altered the protein stability of VMAT2, suggesting that retrograde trafficking plays a vital role in subcellular localization and functional regulation of VMAT2.

Materials and methods

Cell culture and transfection

All cells were cultured under a standard protocol described by ATCC and in DMEM supplemented with 10% cosmic calf serum (CCS, HyClone) at 37°C and 5% CO_2. CHO stable transformants of rat 3Flag-VMAT2 were prepared according to the protocol described previously in our laboratory[9].

For transient overexpression, Lipofectamine 2000 (Life Technologies) were used and transfected cells were harvested and analyzed 24-48 hours later. For siRNA, transfection was also performed using Lipofectamine 2000 according to the manufacturer's protocol. For immunoblotting assays on protein expression analysis, siRNA knockdown cells were incubated with 1.25 nmol/L of each siRNA for 24 hours.

Antibodies

The following antibodies were used in the study: mouse anti-Flag M2, mouse anti-EEA1 and polyclonal anti-IL2α (Sigma), mouse anti-HA.11 (Covance), polyclonal sheep anti-TGN46 (AbD Serotec), and polyclonal rabbit anti-CI-M6PR and mouse anti-LAMP1 (Abcam). Secondary donkey anti-rabbit IgG Alexa Fluor488, goat anti-mouse IgG Alexa Fluor568, goat anti-rabbit IgG Alexa Fluor568 and goat anti-sheep IgG Alexa Flour 488 were purchased from Life Technologies.

Plasmids, oligonucleotides and reagents

The cDNA fragments in both plasmids of pcDNA3.1-3Flag-VMAT2 and pcDNA3.1-3HA-Vps35 were generated by proof-reading PCR and then inserted to corresponding vectors. The pcDNA3.1-Tac-M was described previously[15]. The following reagents were purchased from Sigma-Aldrich: cycloheximide (CHX), NP-40 and saponin.

Immunoprecipitation and protein half-life analysis

Cells were washed with phosphate buffered saline

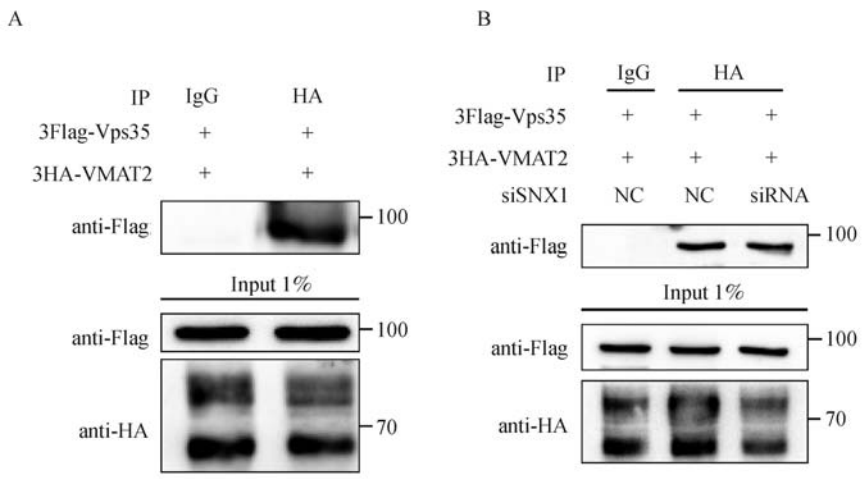

Fig. 1 **Vps35 specifically interacts with VMAT2.** A. COS7 cells overexpressing 3Flag-Vps35 and 3HA-VMAT2 were lysed and immunoprecipitated with anti-HA, and analyzed by Western blotting. Input and bound proteins were analyzed by immunoblotting with antibodies against HA and Flag, respectively. The data indicated that Vps35, but not nonspecific IgG, interacts with VMAT2. B. COS7 cells overexpressing both 3Flag-Vps35 and 3HA-VMAT2 and cotransfected with SNX1 specific siRNA were lysed and immunoprecipitated with anti-HA. Input and bound proteins were analyzed by blotting with antibodies against HA and Flag, respectively. The data suggested that reduced SNX1 had little influence on the interaction between Vps35 and VMAT2.

(PBS) and lysed in NP-40 lysis buffer (0.5% NP-40, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.0 and 5 mmol/L EDTA) supplemented with 1 mmol/L PMSF and 0.1 mmol/L leupeptin for 10 minutes on ice. Cell lysate was centrifuged at 1,600 *g* and the supernatants were then precleared by incubation for 60 minutes at 4°C with 30 μL protein A/G agarose beads (Thermo Scientific Pierce) and centrifugation at 8,000 g for 5 minutes. The precleared lysates were incubated for 2 hours at 4°C with 30μL protein A/G agarose beads bound to polyclonal antibody to tagged protein. After immunoprecipitation, the beads were washed 4 times with wash buffer (0.5% NP-40, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.0 and 5 mmol/L EDTA) and then underwent SDS-PAGE analysis.

For half-life detection, VMAT2 stable transformants were transiently transfected with Vps35 specific siRNA. After 24 hours, cells were treated with 100 μg/mL CHX and samples were collected at the time course of 0, 2, 4, 6, 8 and 10 hours. Expression levels of VMAT2 were then analyzed by immunoblotting. Prestained protein standard marker (Thermo Scientific) (at. 26616) was used for side-labelling Western blot. All the Western blotting analyses were performed utilizing the Tanon 5200 Multi Autoimaging System (Tanon, Shanghai).

Immunofluorescence

For double immunostaining, cells were plated onto glass coverslips coated with poly-D-lysine and Matrigel (Collaborative Research) as previously described[9], and fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.2. After permeabilization and blocking in PBS containing 2% bovine serum albumin, 1% fish skin gelatin, and 0.02% saponin for 1 hour, the cells were incubated with primary antibody in the same buffer for 1.5 hours at room temperature, washed 3 times for 10 minutes each in the same buffer, incubated an additional 1 hour at room temperature with the appropriate secondary antibody at an adequate dilution in the same buffer, and washed again 3 times for 5 minutes each.

RNA interference experiment

The siRNA oligonucleotides were obtained from GenePharma (Shanghai) and resuspended in double

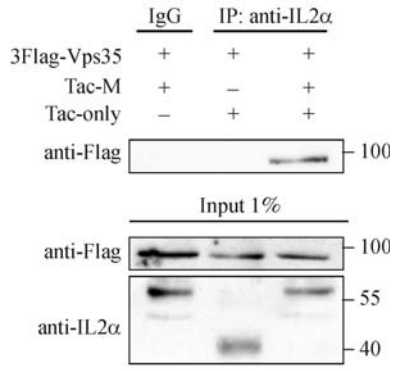

Fig. 2 **VMAT2 C-terminus is required and sufficient for the interaction between VMAT2 and Vps35.** COS7 cells overexpressing 3Flag-Vps35 and Tac-M or Tac-only were lysed for co-immunoprecipitation with IL-2α polyclonal antibody. Input and bound proteins were analyzed by immunoblotting with antibodies against Tac and Flag, respectively. The data suggested that VMAT2 C-terminus is required and sufficient for the interaction.

distilled water according to the manufacturer's instructions. The sequences used for SNX1 siRNA interference were5′-CACUCUCAGAAUGGCUUCATT-3′ and 5′-GUAGCCUACAAAGUUACAATT-3′. The sequences for Vps35 knockdown study were 5′-GCGUUUGUUUCUUCGAAAUTT-3′, 5′-GGAGCAAGUUGUGAAUUGUTT-3′ and 5′-GCCUAAAGAAGGCACUAAATT-3′. siRNA transfection was performed using Lipofectamine 2000 according to the manufacturer's protocol (Life Technology). The cells were harvested 48 h after transfectionand analyzed by PAGE with Western blotting or other assays as specified in each experiment.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 5.0, GraphPad Software). For quantitative analysis of immunoblots, the expression level of VMAT2 in half-life studies were quantified by densitometry of the bands between two treatment groups and statistical significance was determined by unpaired t-test as indicated. Results are expressed as the mean ± SD if not indicated otherwise.

Results

Vps35 specifically interacts with VMAT2

Genetic and biochemical analyses have proved Vps35p as the cargo recognition component of the retromer[24]. To determine whether Vps35 regulated the membrane trafficking of VMAT2 through specific interaction, we performed immunoprecipitation on COS7 cells co-expressing both Vps35 and VMAT2. Transient transfected cells were lysed in lysis buffer

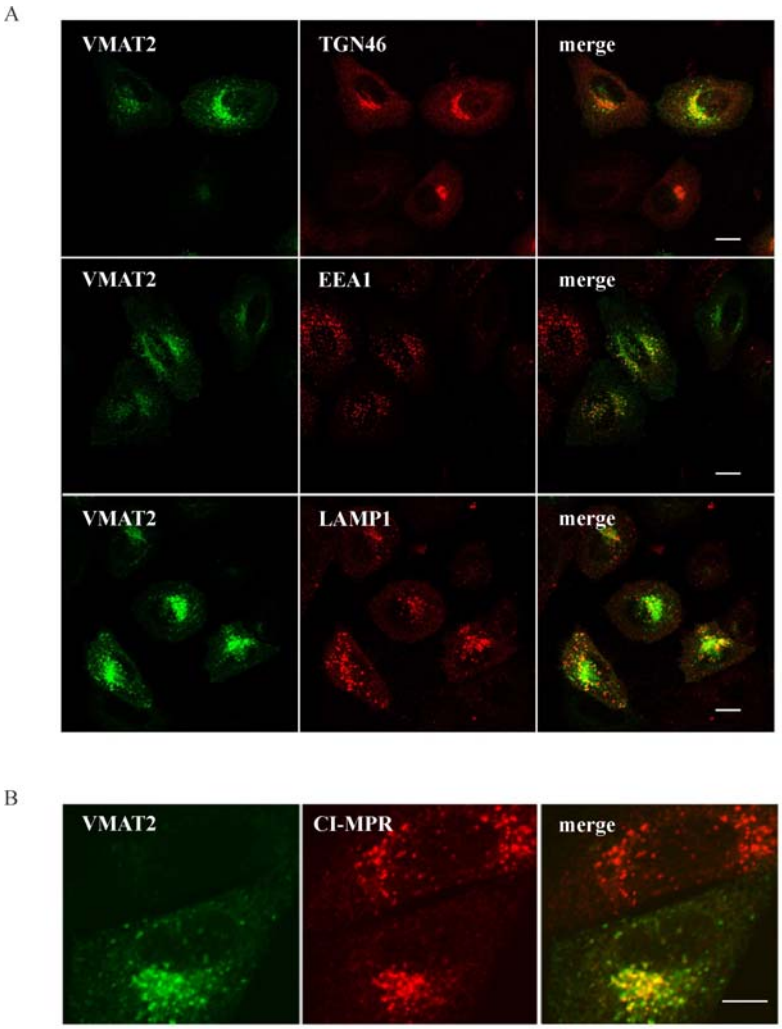

Fig. 3 **Localization of VMAT2 to TGN A.** HeLa cells transiently transfected with plasmids of 3Flag-VMAT2 were immunostained for organelle markers TGN46, EEA1 and LAMP1 followed by confocal microscopic analysis. Pictures showed that VMAT2 was significantly colocalized with TGN46 and partial colocalized with EEA1 and LAMP1. Scale bar, 10 μm. B. HeLa cells expressing 3Flag-VMAT2 were double immunostained with Flag antibodies and endogenous CI-MPR followed by confocal microscopic analysis. The data showed that two proteins were significantly co-localized perinuclearly. Scale bar, 10 μm.

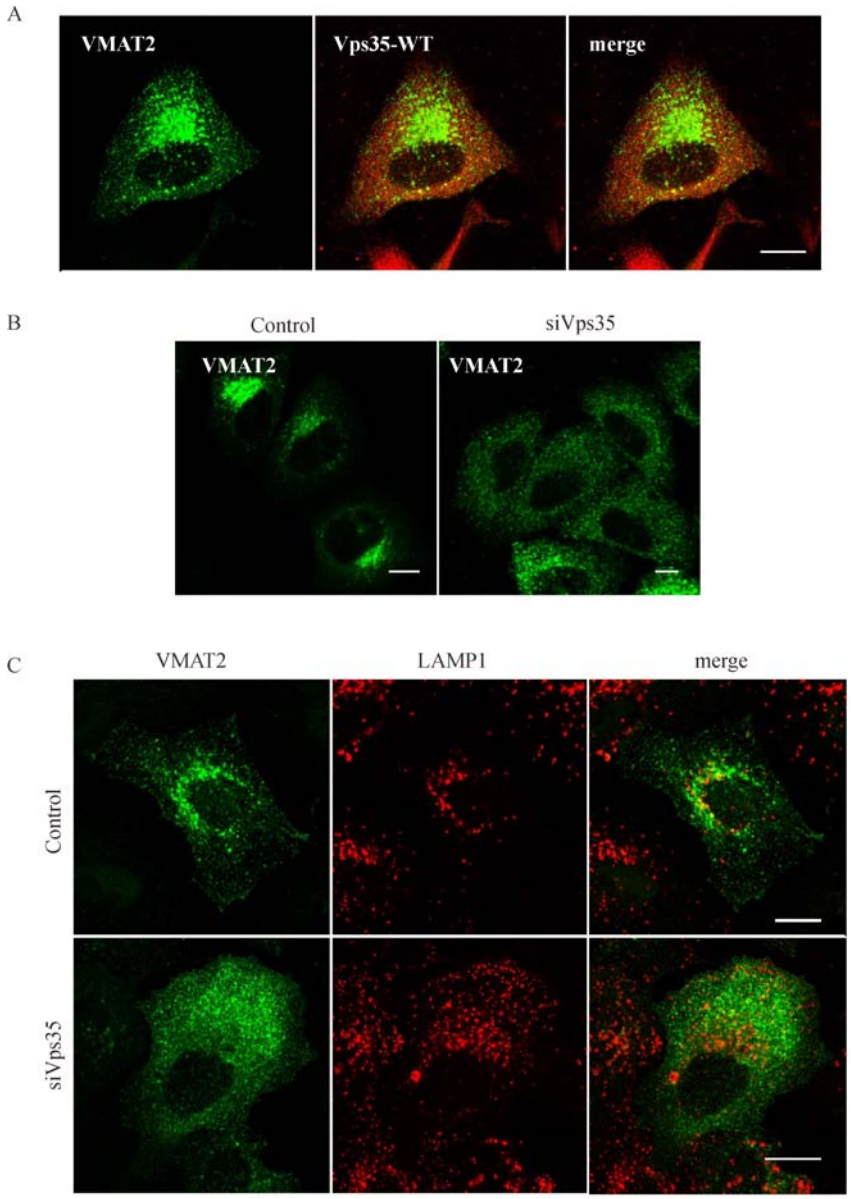

Fig. 4 **Reduced expression of Vps35 alters the subcellular localization of VMAT2.** A. HeLa cells were transiently transfected with two plasmids, 3Flag-VMAT2 and 3HA-Vps35, followed by double immunostaining for confocal microscopic analysis. The data showed that wild type Vps35 did not alter the perinuclear staining pattern of VMAT2. Scale bar, 10 μm. B and C. HeLa cells expressing 3Flag-VMAT2 were transiently transfected with Vps35 specific siRNA and scramble siRNA, and were stained with Flag antibody (B, green), and with LAMP1 conjunction (C, red), and examined by confocal microscopy. The data showed that Vps35 depletion led to mis-targeting of VMAT2 from perinuclear pattern to diffused pattern and increased colocalization with lysosome. Scale bar, 10 μm.

containing 0.5% NP-40 and then HA-tagged VMAT2 was pull down as the bait protein, followed by immunoblotting to determine whether Vps35 can be detected as a binding partner. As shown in ***Fig. 1A***, Vps35 was co-immunoprecipitated with overexpressed VMAT2 but not with IgG in the control group, suggesting a specific interaction between the two proteins. Importantly, double immunofluorescence study also showed Vps35 and VMAT2 were partially co-localized in HeLa cells (***Fig. 4A***). Interestingly, when another retromer component, SNX1, was depleted via the specific siRNA-mediated knockdown, the interaction between VMAT2 and Vps35 overexpressed in the transient transfected COS7 cells was not significantly altered (***Fig. 1B***), indicating that SNX1 was not required for this functional interaction. Thus, these results suggested that Vps35 specifically interacted with VMAT2.

Vps35 binds to the C-terminus of VMAT2

To determine how the two proteins interact, we further analyzed domains required for this binding. We

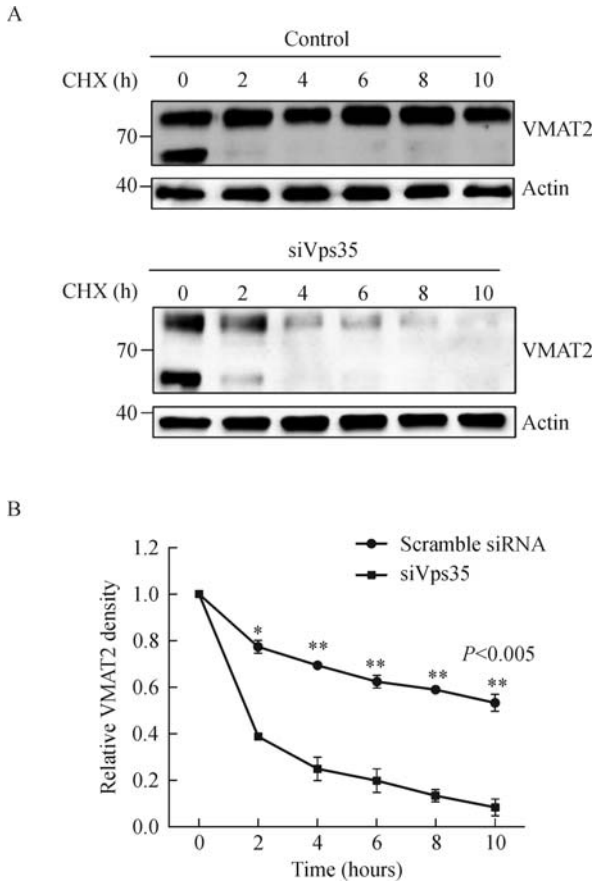

Fig. 5 **Reduced expression of Vps35 alters the half-life of VMAT2.** A. CHO stable transformants of rat 3Flag-VMAT2 were transiently transfected with Vps35 specific siRNA. After 24 hours, cells were treated with 100 μg/mL CHX and collected samples at the indicated time points (0, 2, 4, 6, 8, and 10 hours). Proteins were analyzed by blotting. Actin is used the loading control. The data suggested that Vps35 depletion caused shorter half-life of VMAT2 compared with that in control group. B. The arbitrary densitometry value in the control group and siRNA group was measured using imaging analysis software Image J. Data are presented as mean ± SD, $n = 3$. $^{**}P < 0.005$, unpaired t-test.

used a chimeric protein, Tac-M (the construct was made by fusing an unrelated plasma membrane protein called Tac with the C-terminus of VMAT2) to demonstrate the sufficiency of the C-terminus of the transporter for the interaction. Again both Flag-Vps35 and Tac-M were overexpressed for co-immunoprecipitation study. As shown in ***Fig. 2***, Vps35 interacted with Tac-M but not Tac-only, suggesting that the C-terminus of VMAT2 was required and sufficient for the interaction.

Vps35 regulates retrograde trafficking of VMAT2

The interactions described above prompted us to examine potential involvement of Vps35 in subcellular vesicular trafficking of VMAT2. We first determined whether VMAT2 is subcellularly localized to retrograde trafficking related organelles such as TGN. Immunofluorescence assay showed that VMAT2 was significantly colocalized with TGN marker, TGN46, and partially colocalized with endosome (EEA1) and lysosome (LAMP1) (***Fig. 3A***). Importantly, VMAT2 showed extensive colocalization with endogenous cation-independent mannose 6-phosphate receptor (CI-M6PR), a classical retrograde trafficking cargo as well as a well-known marker for TGN (***Fig. 3B***). Thus, these results clearly indicated that VMAT2 served as a target of retromer, and Vps35 may be involved in regulating its retrograde trafficking. To further investigate such possibility, we first showed VMAT2 and Vps35 colocalized in overexpressed cells (***Fig. 4A***). We then determined whether VMAT2 subcellular localization was altered by Vps35 depletion. As shown in immunofluorescent staining in ***Fig. 4B***, Vps35 depletion led to mis-targeting of VMAT2 from a perinuclear pattern to a diffused pattern. More importantly, Vps35 KD cells showed increased co-localization of VMAT2 and LAMP1, suggesting that Vps35 regulated the TGN targeting of VMAT2, possibly keeping it away from lysosome mediated degradation (***Fig. 4C***).

Vps35 depletion decreases the half-life of VMAT2

To determine whether Vps35 plays a role in protecting VMAT2 from degradation, the half-life of VMAT2 in Vps35 KD cells was tested. We transfected Vps35 specific siRNA into a CHO stable transformant expressing Flag-VMAT2 cells followed by collecting the samples at an interval of 2 hours after the blocking of protein synthesis with cycloheximide. The half-life of wildtype VMAT2 is approximately 18-20 hours according to our previous study. Vps35 knockdown caused a significantly decreased half-life compared with the control group, with a dramatica reduction of the VMAT2 level to less than half of the beginning in 2 hours (***Fig. 5A,B***). Of note, as shown in ***Fig. 5A***, VMAT2 has mature and immature forms, and the immature form will transform to the mature form over time, nearly 2 hours based on our experiments. These data indicated that Vps35 mediated VMAT2 entering retrograde trafficking pathway for reuse, leading to an increased 'using life' of VMAT2. In conclusion, Vps35 mediated retrograde trafficking may be important in vesicular targeting of VMAT2, keeping it away from lysosomal degradation while maintaining VMAT2 stability in check.

Discussion

In this paper, we investigated whether retromer complex regulated the membrane trafficking of

VMAT2 using biochemical and cell biology methods. By interaction with VMAT2, Vps35 not only determined retrograde trafficking related subcellular localization at TGN but was also involved in the protein stability of the transporter. The study demonstrated that VMAT2 underwent stringent retrograde trafficking in non-neuronal cells and may be subject to subcellular regulation of retromer complex in neurons.

It has been established that VMATs requires specific mechanism for targeting to secretory vesicle[25]. Newly synthesized transport proteins may be *en route* cytoplasm membrane to be recruited to synaptic vesicle, which depends on a stringent internalization signal dileucine motif[26]. In non-neuronal cells, internalized VMAT2 is localized to the endosomal compartment to be functional in transport activity mediated detoxification[5]. However, VMATs may also contain sorting information for retrograde trafficking. Our previous work indicated that VMAT1 appeared at perinuclear localization in CHO transformants[27]. The work reported here confirmed such intracellular organelle association as TGN for VMAT2 with much more relevant markers (***Fig. 3A***). Furthermore, series data supports that such localization can be regulated by retromer protein Vps35. Thus, this retrograde trafficking related subcellular phenomenon may suggest a molecular mechanism for regulating vesicular targeting and function such as neuroprotection of VMAT2 in neuron.

As a key component of the extremely conserved retromer complex, Vps35 often serves as a cargo recognition protein[28]. Its interaction with VMAT2 provides a regulatory connection to the transporter in retrograde trafficking. With little influence from overexpressed SNX1, another component of retromer, we may conclude that the interaction is rather specific. However, the current detection assay heavily relies on an overexpression system for both proteins in COS7 cells. Without detailed interaction on endogenous proteins or other physiologic measurement on isolated proteins, it is still hard to rule out the possibility of other protein(s) involved in the retromer-mediated binding. Moreover, recent studies suggested that mammalian cells seem to have more regulatory crosstalk with other cytosolic machinery such as WASH complex and sorting nexins other than classical SNX subcomplex[29,30]. Thus, how VMAT2 is regulated on its retrograde trafficking and the nature of its regulatory machinery in neurons may still be the motivation of further study. Importantly, recent reports on gain of function Vps35 mutant in familiar PD suggest a role of altered membrane trafficking in PD pathogenesis. Our work here may provide an insight in potential mechanism underlying PD pathogenesis.

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