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VPS35 regulates cell surface recycling and signaling of dopamine receptor D1

Chen Wang[a,b], **Mengxi Niu**[a], **Zehua Zhou**[a], **Xiaoyuan Zheng**[a], **Lingzhi Zhang**[a], **Ye Tian**[a], **Xiaojun Yu**[a], **Guojun Bu**[a,c], **Huaxi Xu**[a,d], **Qilin Ma**[b,*], and **Yun-wu Zhang**[a,*]

[a]Fujian Provincial Key Laboratory of Neurodegenerative Disease and Aging Research, Institute of Neuroscience, Collaborative Innovation Center for Brain Science, College of Medicine, Xiamen University, Xiamen, Fujian 361102, China

[b]Department of Neurology, The First Affiliated Hospital of Xiamen University, Xiamen, Fujian 361003, China

[c]Department of Neuroscience, Mayo Clinic, Jacksonville, FL 32224, USA

[d]Degenerative Diseases Program, Sanford-Burnham-Prebys Medical Discovery Institute, La Jolla, CA 92037, USA

Abstract

Vacuolar protein sorting-associated protein 35 (VPS35) is a retromer complex component regulating membrane protein trafficking and retrieval. Mutations or dysfunction of VPS35 have been linked to Parkinson's disease (PD), which is pathologically characterized by the loss of dopamine neurons in brain substantia nigra region. Dopamine plays a key role in regulating various brain physiological functions by binding to its receptors and triggering their endocytosis and signaling pathways. However, it is unclear whether there is a link between VPS35 and dopamine signaling in PD. Herein, we found that VPS35 interacted with dopamine receptor D1 (DRD1). Notably, overexpression and downregulation of VPS35 increased and decreased steady-state cell surface levels of DRD1 and phosphorylation of CREB and ERK that are important dopamine signaling effectors, respectively. In addition, overexpression of VPS35 promoted cell surface recycling of endocytic DRD1. Furthermore, downregulation of VPS35 abolished dopamine-induced CREB/ERK phosphorylation. More importantly, although the PD-associated VPS35 mutant VPS35(D620N) still interacted with DRD1, its expression did not affect cell surface recycling of DRD1 and phosphorylation of CREB/ERK, nor rescue the reduction of CREB/ERK phosphorylation caused by VPS35 downregulation. These results demonstrate that VPS35 regulates DRD1 trafficking and DRD1-mediated dopamine signaling pathway, and that the

*Corresponding author at: Fujian Provincial Key Laboratory of Neurodegenerative Disease and Aging Research, Institute of Neuroscience, Collaborative Innovation Center for Brain Science, College of Medicine, Xiamen University, Xiamen, Fujian 361102, China. Tel./fax: +86 592 2188528. yunzhang@xmu.edu.cn (Y.-w. Zhang), or Department of Neurology, The First Affiliated Hospital of Xiamen University, Xiamen, Fujian 361003, China. Tel./fax: +86 592 2139812. qilinma@yeah.net (Q. Ma).

Disclosure statement

None of the authors has a conflict of interest to declare in relation to the present research.

PD-associated VPS35(D620N) mutant loses such functions, providing a novel molecular mechanism underlying PD pathogenesis.

Keywords

dopamine; dopamine receptor D1; dopamine signaling; Parkinson's disease; Trafficking; Vacuolar protein sorting-associated protein 35

1. Introduction

Parkinson's disease (PD) is a common progressive neurodegenerative disease characterized by resting tremor, rigidity, bradykinesia and postural gait instability. A major pathology of PD is the loss of dopamine neurons in the brain substantia nigra region (Wood-Kaczmar, et al., 2006). Dopamine is an essential neurotransmitter and regulates various physiological functions of normal brain such as locomotion, emotion and behavior (Bromberg-Martin, et al., 2010, Cools, 2011). Dopamine receptors are G-protein-coupled receptors and grouped into two classes as D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors, based on their functional properties to stimulate adenylyl cyclase (AC) via $G_{s/olf}$ or to inhibit AC via $G_{i/o}$, respectively. Activation of dopamine receptor D1 (DRD1) can promote cyclic AMP production and PKA activity, inducing the phosphorylation of ERK and CREB that are important for neuronal functions (Calabresi, et al., 2014,Hasbi, et al., 2011). Dopamine signaling pathways have been found to be impaired in PD (Narayanan, et al., 2013), but underlying mechanisms remain largely elusive.

The pathogenesis of PD may be attributed to comprehensive factors such as aging, environmental toxins and genetic variations. Mutations in multiple genes have been identified in PD patients, including α-synuclein, parkin, Pink1, LRRK2, DJ-1, UCH-L1, etc. Additional risk genes associated with PD are also being rapidly discovered by using advanced technical strategies such as next generation sequencing and genome-wide association studies [see reviews in (Gasser, 2009,Verstraeten, et al., 2015)]. Recently, mutations in the gene encoding vacuolar protein sorting-35 (VPS35) were found in autosomal dominant PD patients, with the D620N being a common mutant (Vilarino-Guell, et al., 2011,Zimprich, et al., 2011). VPS35 is a key subunit of the retromer complex that also includes VPS29 and VPS26. The VPS35/retromer orchestrates the retrieval of membrane proteins from endosome-to-cell surface and endosome-to-Golgi (Bonifacino and Hurley, 2008,Burd and Cullen, 2014). VPS35 protein levels were also found to be decreased in the substantia nigra of PD patients (MacLeod, et al., 2013). Several recent studies have suggested that VPS35 mutations or deficiency can cause dopamine neuron loss by impairing mitochondrial fusion and function through promoting mitochondrial E3 ubiquitin ligase 1-mediated mitofusion 2 (MFN2) degradation (Tang, et al., 2015b), and/or through enhancing turnover of the mitochondrial dynamin-like protein 1 (DLP1) complexes (Wang, et al., 2016). VPS35 deficiency or mutation also results in impaired endosome-to-Golgi retrieval of lysosome-associated membrane glycoprotein 2a (Lamp2a) and accelerated Lamp2a degradation, while Lamp2a is critical for α-synuclein degradation (Tang, et al., 2015a). Furthermore, VPS35 interacts genetically with another PD-associated gene EIF4G1

(Chartier-Harlin, et al., 2011); and they converge on α-synuclein pathology in PD (Dhungel, et al., 2015). Moreover, VPS35 can interact with AMPA receptor subunits and its deficiency or mutation impairs AMPA receptor trafficking and reduces dendritic spine maturation (Munsie, et al., 2015,Tian, et al., 2015). Nevertheless, how exactly VPS35 mutation or deficiency contributes to PD has yet to be fully determined.

In the present study, we found that VPS35 could interact with DRD1 and regulate DRD1 cell surface recycling, as well as DRD1-mediated dopamine signaling. Intriguingly, we showed that although the PD-associated VPS35(D620N) mutant could still interact with DRD1, it lost the function on regulating DRD1 trafficking and signaling. These findings suggest that mutation or deficiency in VPS35 may interfere with normal dopamine signaling pathways, establishing a novel mechanism underlying PD pathogenesis.

2. Methods

2.1. Cell culture and manipulation

Mouse neuroblastoma N2a cells and Human embryonic kidney 293T (HEK 293T) cells were maintained as previously described (Feng, et al., 2015). Dopaminergic neurons were isolated from the ventral midbrain of wild-type C57BL/6 mice at embryonic days 17-18 (E17-18) and cultured in Neurobasal (Gibco) medium supplemented with B27 (Gibco), GlutaMAX-1, penicillin (100 units/ml), and streptomycin (100 μg/ml), following a published protocol (Lin, et al., 2012). Animal procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Xiamen University.

For cell transfection, mammalian expression vectors were transiently transfected into N2a and HEK 293T cells with the TurboFect Transfection Reagent (Thermo Scientific) for 24-72 h, and into mouse dopaminergic neurons with the Lipofectamine 2000 Reagent (Invitrogen) at DIV4-6 for 24 h, following the manufacturers' protocols.

For dopamine treatments, cells were treated with dopamine (Sigma-Aldrich) at 50 μM (dissolved in DMEM) for 36 h.

2.2. Western blot and antibodies

After treatments, cells were lysed in TNEN buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, and 1% Nonidet P 40, pH 8.0), supplemented with protease inhibitors and phosphatase inhibitors. Protein lysates were separated on SDS-polyacrylamide gels, electrophoretically transferred onto PVDF membrane, and immunoblotted with indicated antibodies. Antibodies used were: anti-VPS35 from Millipore and also kindly provided by Dr. Wen-Cheng Xiong at Georgia Regents University, USA; anti-GFP and anti-HA from Abmart; anti-DRD1 and anti-myc from Santa Cruz Biotechnology; anti-CREB, anti-phosphorylated CREB, anti-ERK, and anti-phosphorylated ERK from Cell Signaling Technology. The Ab14 antibody against PS1-NTF has been reported previously (Thinakaran, et al., 1996).

2.3. DNA constructs

The human DRD1 protein-encoding sequence was amplified by polymerase chain reaction (PCR) using a sense primer (5′-CGGAATTCATGAGGACTCTGAACACCTCTG-3′) and an antisense primer (5′-CCTCGAGTCAGGTTGGGTGCTGACCGTTTTGT-3′) and subcloned into the pCMV-HA vector (Clontech) at *EcoR*I and *Xho*I sites to generate the DRD1-HA vector. It was also subcloned into the mCherry-C1 vector (Clontech) at *EcoR*I and *Xho*I sites to generate the mCherry-DRD1 vector. The human VPS35 protein-encoding sequence was amplified using a sense primer (5′-CCGCTCGAGATGCCTACAACACAGCAGTCCC-3′) and an antisense primer (5′-CGGGAATTCTTAAAGGATGAGACCTTCATAAATT-3′) and subcloned into the pEGFP-C3 vector (Clontech) at *Xho*I and *EcoR*I sites to generate the pEGFP-VPS35 vector, and subcloned into the pGEX-4T-1 vector (Clontech) at *Xho*I and *EcoR*I sites to generate the GST-VPS35 vector. The VPS35-myc vector was kindly provided by Dr. Wanjin Hong of Institute of Molecular and Cell Biology, Singapore. The VPS35(D620N)-myc vector was generated through site-directed mutagenesis to convert Asp620 to Asn. For shRNA expressing vector generation, a scrambled control sequence (5′-GATCATTGCACGTCCTAAT-3′), two sequences targeting mouse VPS35 (shRNA1: 5′-CATGCCTCCAATATGCTTGG-3′; and shRNA2: 5′-GACTACGTCGATAAAGTTCT-3′), two known sequences targeting human VPS35 (shRNA1: 5′-TCAGAGGATGTTGTATCTTTACAAGTCTC-3′; and shRNA2: 5′-GCTTCACACTGCCACCTTTGGTATTTGCA-3′) (Belenkaya et al., 2008) and a known sequence targeting mouse DRD1 (5′-CAGCTCTCCAAACGCCTTTT-3′) (Li, et al., 2013) were synthesized and subcloned into a pAAV-U6-EGFP vector (Cell Biolabs).

2.4. Co-immunoprecipitation

Equal protein amounts of cell lysates were incubated with normal mouse IgG (Santa Cruz Biotechnology) or indicated antibodies, together with Trueblot IP^{TM} beads (eBioscience), at 4°C overnight. Immunoprecipitated proteins were analyzed by Western blot with indicated antibodies.

2.5. GST pull-down assay

The GST-VPS35 vector and the control pGEX-4T-1 vector were transformed into BL21 *E.coli*. Transformants were grown to exponential phase and then induced with 1mM IPTG at 25°C overnight. Bacteria were pelleted in lysis buffer and lysed by sonication. Lysates were centrifuged at 15,000 rpm for 30 min at 4°C after adding PMSF to the final concentration of 1 mM. Supernatants were transferred into a fresh tube and incubated with 20μl glutathione sepharose beads overnight at 4°C with mixing. After washing with 1× PBS, Glutathione sepharose beads were incubated with N2a cell lysates overnight at 4°C. Affinity precipitated proteins were analyzed by Western blot with indicated antibodies.

2.6. Real-Time PCR

Treated cells were subjected to total RNA extraction using TRIzol reagent (Invitrogen) and reverse transcription using ReverTra Ace qPCR RT Kit (TOYOBO). Quantitative real-time PCR was executed using ABI 7500 Fast Real-Time PCR System (Life technologies) with

FastStart Universal SYBR Green Master (ROX) (Roche). Primers for mouse DRD1 were as follows: DRD1-forward (5′-ATGGCTCCTAACACTTCTACCA-3′) and DRD1-reverse (5′-GGGTATTCCCTAAGAGAGTGGAC-3′).

2.7. Adeno-associated virus (AAV) packaging and infection

shRNA expressing vectors containing a scrambled control sequence or a mouse VPS35-targeting sequence (shRNA1) were co-transfected with helper vectors (Cell Bioabs) into HEK 293T cells. Cells were harvested 48-72 h later and subjected to several freeze-thaw cycles. Cell debris were removed by centrifugation and AAVs in the supernatant were further concentrated and purified with an AAV Purification Kit (Cell Biolabs). Mouse dopaminergic neurons cultured in 6-well plates for 10-12 d were infected with AAVs (10 μl/well, titer 2×10^8 $TCID_{50}$/ml) for 72 h.

2.8. Immunofluorescent assays

Cells were grown on coverslips. After treatments, cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, incubated with primary antibodies and then with fluorescence-conjugated secondary antibodies, stained with DAPI, and observed under a confocal microscope (Olympus FV1000). Quantitation of VPS35 and DRD1 colocalization, indicated by Pearson's coefficient and overlap coefficient, were carried out following the manufacturer's Instructions.

2.9. Cell surface protein biotinylation, endocytic and Recycling assays

These assays were carried out following previously described protocols (Feng, et al., 2015,Wang, et al., 2013). Briefly, cells were labeled with EZ-Link™ Sulfo-NHS-SS-Biotin (Thermo Scientific) at 4°C for cell surface protein biotinylation. For the endocytic assay, after cell surface biotinylation, cells were incubated at 37°C to allow protein endocytosis for indicated time periods, and then treated with glutathione at 4°C to cleave biotin on surface proteins, so that only internalized proteins were biotinylated. For the recycling assay, after cell surface biotinylation and allowing protein endocytosis at 37°C for 30 min, cells were treated with glutathione at 4°C to cleave biotin on surface proteins, and then incubated at 37°C again for indicated time periods. Cells were treated with glutathione at 4°C again to cleave biotin on proteins recycling back to cell surface. Hence the residual biotinylated proteins were not recycled yet. In all assays, biotinylated proteins were affinity precipitated by Streptavidin Agarose Resin (Thermo Scientific) and detected by Western blot.

2.10. Statistical analyses

Protein band intensity was quantified by densitometry using ImageJ. Data were analyzed using Prism 5.0 software (GraphPad). Results were expressed as means±standard error of the mean (SEM). Statistical significance was assessed by paired or unpaired t test and $p<0.05$ was considered to be statistically significant.

3. Results

3.1. VPS35 interacts with DRD1

DRD1 is subjected to dynamic trafficking process when mediating dopamine-induced signaling pathways (Calabresi, et al., 2014,Hasbi, et al., 2011). VPS35 is important for regulating endosome-to-Golgi and endosome-to-cell surface retrieval of membrane proteins (Bonifacino and Hurley, 2008,Burd and Cullen, 2014). To determine whether VPS35 also regulates DRD1 trafficking, we first explored potential interaction between the two. When we co-expressed DRD1-HA and pEGFP-VPS35 vectors in HEK293T cells, we found that anti-HA and anti-GFP antibodies immunoprecipitated pEGFP-VPS35 and DRD1-HA, respectively (Fig. 1A). In addition, in the mouse midbrain lysates, we also found that endogenous VPS35 and DRD1 could be immunoprecipitated by anti-DRD1 and anti-VPS35 antibodies, respectively (Fig. 1B). To confirm physical interaction between the two, we carried out GST pull-down assay and found that recombinant purified GST-VPS35 protein immobilized on glutathione sepharose beads could pull down DRD1 as well as the known VPS35-interacting protein APP (Vieira, et al., 2010) in N2a cell lysates (Fig. 1C). Furthermore, when mCherry-DRD1 and pEGFP-VPS35 vectors were co-transfected into HEK293T cells (Fig. 1D), immunofluorescence results showed co-localization between the two. Together, these results demonstrate that VPS35 can interact with DRD1.

3.2. VPS35 affects cell surface levels of DRD1

After determining the interaction between the two, we asked whether VPS35 affects cell surface distribution of DRD1. To investigate this, cell surface protein biotinylation assays were carried out. In HEK293T cells expressing exogenous DRD1, we found that overexpression of VPS35 dramatically increased steady-state cell surface levels of DRD1 without affecting total levels of DRD1 (Fig. 2A), whereas shRNA-mediated downregulation of VPS35 had an opposite effect (Fig. 2B). Consistently, in N2a cells expressing endogenous DRD1, we also found that overexpression (Fig. 2C) and downregulation (Fig. 2D) of VPS35 increased and decreased steady-state cell surface levels of DRD1, respectively. In addition, we studied and found that overexpression (Fig. 2E) and downregulation (Fig. 2F) of VPS35 had no effect on DRD1 mRNA expression. These results indicate that VPS35 may affect DRD1 trafficking. In contrast, although it was recently found that VPS35 can interact with PS1-NTF (Ueda, et al., 2016), we found that modulation of VPS35 did not affect steady-state cell surface levels of PS1-NTF (Fig. 2A-D).

3.3. VPS35 regulates cell surface recycling of endocytic DRD1

Since VPS35 is important for orchestrating the retrieval of membrane proteins from endosome-to-cell surface (Bonifacino and Hurley, 2008,Burd and Cullen, 2014), we next explored whether VPS35 regulates DRD1 endocytosis and recycling. During the endocytosis assay, we found that in both HEK293T cells co-expressing DRD1 with control vector and with VPS35, endocytic (biotinylated) DRD1 levels reached the maximum at 5 min after the initiation of endocytosis (Fig. 3A). However, although at this time point endocytic (biotinylated) DRD1 levels in cells co-expressing VPS35 were dramatically higher than those in cells co-expressing control vector, there was no difference between the two cells when endocytic (biotinylated) DRD1 levels were normalized to respective steady-state cell

surface DRD1 levels (Fig. 3A). These results indicate that VPS35 overexpression does not affect DRD1 endocytosis. In addition, we found that overexpression of VPS35 did not affect PS1-NTF endocytosis (Fig. 3A). During the recycling assay, when endocytic (biotinylated) proteins were allowed to recycle, we found that as time went by (5-45 min), co-expression of VPS35 markedly reduced levels of DRD1 remaining endocytic (biotinylated) but not those of PS1-NTF (Fig. 3B), indicating an accelerated rate of DRD1 but not PS1-NTF recycling to cell surface. Taken together, these results suggest that VPS35 can regulate cell surface recycling of DRD1.

3.4. VPS35 regulates DRD1-mediated dopamine signaling

Activation of DRD1 by dopamine can stimulate the AC/cAMP/PKA pathway, promoting the phosphorylation of CREB and ERK. When VPS35 was transiently overexpressed in N2a cells, we found that levels of phosphorylated CREB and phosphorylated ERK were dramatically increased, while downregulation of VPS35 had an opposite effect (Fig. 4A).

When we downregulated DRD1 levels in N2a cells, we found a significant increase in CREB phosphorylation and a trend of increase in ERK phosphorylation (Fig. 4B). These results are consistent with previous reports (El-Ghundi, et al., 2010,Santini, et al., 2012). However, VPS35 overexpression did not further promote the phosphorylation of CREB and ERK in DRD1-downregulated cells (Fig. 4B), suggesting that the effect of VPS35 on CREB/ERK phosphorylation is depended on DRD1.

Dopamine treatments significantly promoted the phosphorylation of CREB and ERK in N2a cells, reflecting the activation of DRD1-mediated signaling (Fig. 4C). Interestingly, when VPS35 was downregulated, dopamine treatments no longer promoted CREB/ERK phosphorylation (Fig. 4C). These results indicate that dopamine-DRD1 mediated signaling pathway requires the involvement of VPS35.

3.5. D620N mutation abolishes the regulatory effects of VPS35 on DRD1 trafficking and signaling

Several mutations including D620N in VPS35 has been identified to be associated with PD (Vilarino-Guell, et al., 2011,Zimprich, et al., 2011). By carrying out co-immunoprecipitation assay, we found that VPS35(D620N) mutant still interacted with DRD1 (Fig. 5A). However, overexpression of VPS35(D620N) mutant could not increase steady-state cell surface levels of DRD1 as wild-type VPS35 did (Fig. 5B). could not affect the endocytic rate of DRD1 (Fig. 5C), and even reduced cell surface recycling of DRD1 to some extent (Fig. 5D). Moreover, overexpression VPS35(D620N) mutant could not promote CREB/ERK phosphorylation (Fig. 5E) and rescue the reduction of CREB/ERK phosphorylation in VPS35-downregulated cells (Fig. 5F). These results suggest that PD-associated mutations such as D620N in VPS35 interfere with its regulation on DRD1 trafficking and downstream signaling pathways.

3.6. VPS35 affects DRD1 cell surface levels and signaling in dopaminergic neurons

We also infected mouse dopaminergic neurons with adeno-associated virus (AAV) containing mouse VPS35 shRNA or mock control shRNA. Consistent with the results found

in N2a cells, we found that downregulation of VPS35 dramatically decreased steady-state cell surface levels of DRD1 and the phosphorylation of CREB and ERK (Fig. 6A). Moreover, we found that when mCherry-DRD1 and pEGFP-VPS35 vectors were co-transfected into mouse dopaminergic neurons, there was a colocalization between the two (Fig. 6B). Together, these results further confirm the role of VPS35 in regulating DRD1 trafficking and signaling in dopaminergic neurons,

4. Discussion

It has been established that DRD1 undergoes rapid endocytosis via clathrin-coated pits after dopamine activation; and internalized DRD1 can recycle rapidly and efficiently to cell surface (Ariano, et al., 1997,Dumartin, et al., 1998,Vickery and von Zastrow, 1999). A number of proteins regulating DRD1 trafficking have been identified, such as DRiP78 (Bermak, et al., 2001), COPI (Bermak, et al., 2002) and SNX5 (Villar, et al., 2013). In the present study, we demonstrate that cell surface recycling of DRD1 can also be regulated by another important trafficking factor, VPS35, which interacts with DRD1.

As a major subunit of the retromer complex, VPS35 is important for membrane protein trafficking (Burd and Cullen, 2014). Multiple VPS35/retromer cargos have been identified so far, among which some are related to neuronal functions and neurodegenerative diseases, such as APP (Vieira, et al., 2010), BACE1 (Wen, et al., 2011), and AMPA receptors (Munsie, et al., 2015,Tian, et al., 2015). Herein, we found that overexpression of VPS35 could promote DRD1 recycling to cell surface, consequently leading to increased steady-state levels of cell surface DRD1.

Moreover, we found that the phosphorylation of CREB and ERK, two important effectors downstream the dopamine-DRD1 signaling, was increased and decreased upon overexpression and downregulation of VPS35, respectively. Because the effects of VPS35 on CREB/ERK phosphorylation were diminished when DRD1 levels were downregulated, VPS35 may participate in the dopamine-DRD1 signaling pathway. Indeed, downregulation of VPS35 could abolish dopamine-induced CREB/ERK phosphorylation, indicating the importance of VPS35 in mediating dopamine-induced DRD1 signaling, probably through regulating DRD1 trafficking.

Mutations or dysfunction of VPS35 have been reported to be a risk factor for PD (MacLeod, et al., 2013,Vilarino-Guell, et al., 2011,Zimprich, et al., 2011). Here we found that the PD-associated mutation D620N could abolish the effect of VPS35 on regulating DRD1 endocytosis/recycling and CREB/ERK phosphorylation, suggesting that VPS35 mutations may interfere with its regulation on DRD1 trafficking, therefore affecting the dopamine-DRD1 signaling pathway that is impaired in PD.

There are conflicting reports on the role of VPS35 pathogenic mutations in neurodegeneration. Although many papers have shown that VPS35 D620N mutation confers partial loss of function (e.g. Malik, et al., 2015,Munsie, et al., 2015), studies suggesting that VPS35 D620N mutation can lead to neurodegeneration in a gain-of-function mechanism have also been reported (e.g. Tsika, et al., 2014). Herein, we found that although there were

trends of reduction in DRD1 cell surface levels (Fig. 5B) and in CREB/ERK phosphorylation (Fig. 5E) upon overexpression of the VPS35(D620N) mutant, these reductions were not significantly different. Therefore, although we can not completely exclude a dominant negative role of VPS35 D620N mutation, it is more likely that VPS35 D620N mutation confers loss of function in affecting dopamine signaling.

In addition to our present results, several recent mechanistic studies have suggested that VPS35 mutations or deficiency can also cause dopamine neuron loss by impairing mitochondrial dynamics through promoting MFN2 degradation (Tang, et al., 2015b) and/or DLP1 complex turnover (Wang, et al., 2016), enhance α-synuclein toxicity through interfering with EIF4G1 (Dhungel, et al., 2015) and/or Lamp2a (Tang, et al., 2015a), and disrupt synaptic function through impairing AMPA receptor trafficking (Munsie, et al., 2015,Tian, et al., 2015). Together ours and these results indicate that VPS35 mutations or deficiency may cause deficits in multiple molecular pathways, and a combination of these deficits leads to PD pathogenesis.

5. Conclusion

VPS35 is a major component of the retromer complex that is crucial for membrane protein trafficking. Mutations or deficiency in VPS35 have been found to be associated with Parkinson's disease (PD) that has impaired dopamine signaling, yet the underlying mechanism has not been clearly defined. We demonstrate that VPS35 can interact with dopamine receptor D1 (DRD1), regulate DRD1 recycling to cell surface, and modulate CREB and ERK phosphorylation that are downstream of DRD1 signaling. More importantly, we find that the ability of VPS35 to regulate DRD1 trafficking and signaling is lost in the PD-associated VPS35(D620N) mutant. These results suggest that VPS35 mutations or deficiency may interfere with normal dopamine signaling pathways, thus contributing to PD pathogenesis.

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- VPS35 interacts with DRD1
- VPS35 regulates DRD1 recycling to cell surface
- VPS35 modulates dopamine-DRD1 signaling
- PD-associated VPS35(D620N) mutant loses the function to regulate DRD1

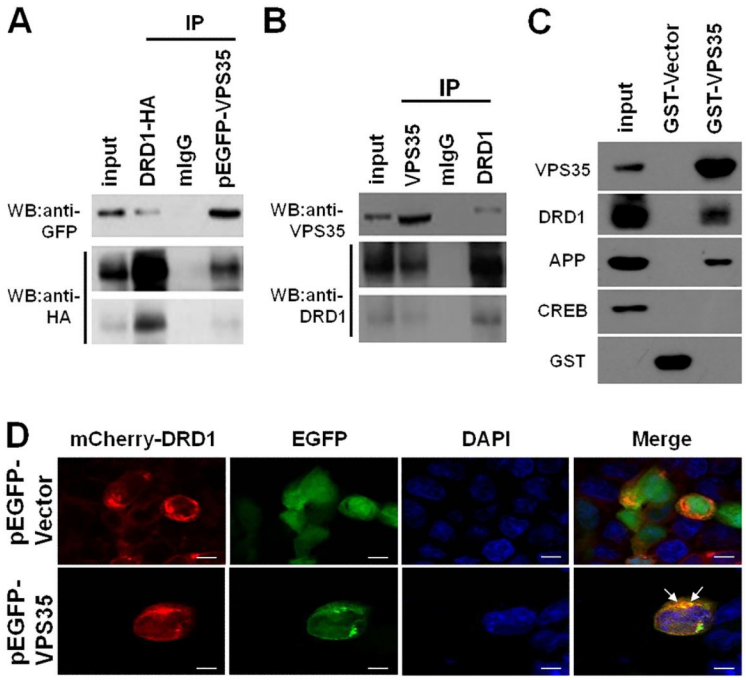

Fig. 1.

VPS35 interacts with DRD1. (A) pEGFP-VPS35 and DRD1-HA vectors were co-transfected into HEK293T cells for 24 h. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody, anti-GFP antibody or mouse IgG (mIgG). VPS35 and DRD1 in immunoprecipitated proteins were immunoblotted with anti-GFP and anti-HA antibodies, respectively. Five percent of cell lysates were used as input. (B) Midbrain lysates of wild-type C57BL/6 mice were subjected to IP with anti-VPS35 antibody, anti-DRD1 antibody, or mIgG. Immunoprecipitated proteins were assayed for VPS35 and DRD1 by Western blot. Five percent of cell lysates were used as input. (C) GST and GST-VPS35 recombinant proteins were immobilized on glutathione sepharose beads and incubated with N2a cell lysates. Affinity precipitated proteins were assayed by Western blot. (D) mCherry-DRD1 (in red) vector was co-transfected with pEGFP-VPS35 (in green) or pEGFP control vectors into HEK293T cells for 24 h. The nuclei were stained with DAPI (shown in blue). Immunofluorescence was observed under the Olympus FV1000 confocal microscope. Colocalization of mCherry-DRD1 and pEGFP-VPS35 was indicated by white arrows (in yellow). Scale bars: 10 μm.

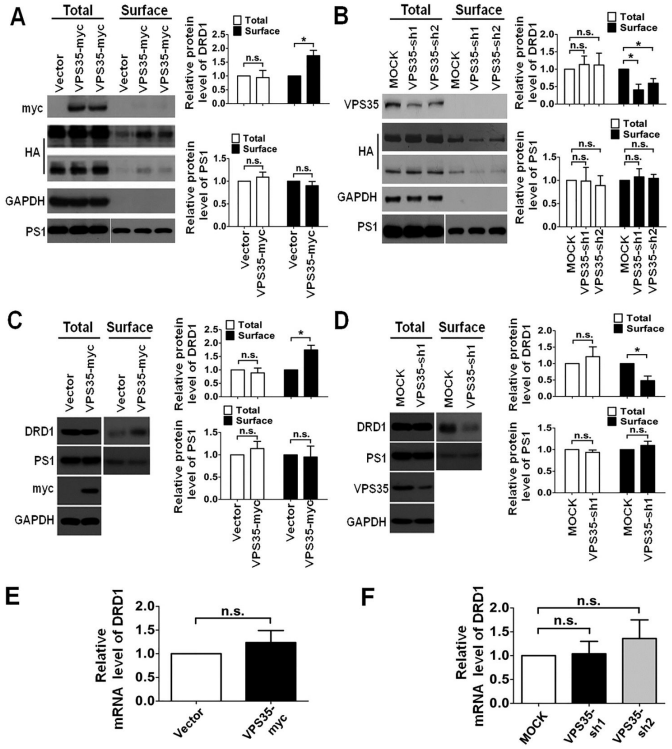

Fig. 2.

VPS35 affects cell surface levels of DRD1. (A,B) HEK293T cells were first transfected with HA-DRD1. After equal splitting, cells were either transfected (A) with VPS35-myc and control vectors for 24 h, or (B) with VPS35 shRNA (sh1 and sh2) and mock control shRNA vectors for 72 h. Cells were subjected to surface biotinylation. After lysing and affinity precipitation, biotinylated proteins were analyzed by Western blot for HA-DRD1 and PS1-NTF. (C-F) N2a cells were transfected (C,D) with VPS35-myc and control vectors for 24 h, or (D,E) with VPS35 shRNA (sh1 and/or sh2) and mock control shRNA vectors for 72 h. (C,D) After surface biotinylation, biotinylated proteins were analyzed by Western blot for endogenous DRD1 and PS1-NTF. Protein levels were quantified by densitometry, and compared to those of controls (set as one arbitrary units). (E,F) Alternatively, cells were subjected to RNA extraction, reverse transcription and quantitative real-time PCR to determine DRD1 levels for comparison. n=3, *p<0.05, n.s.: not significant.

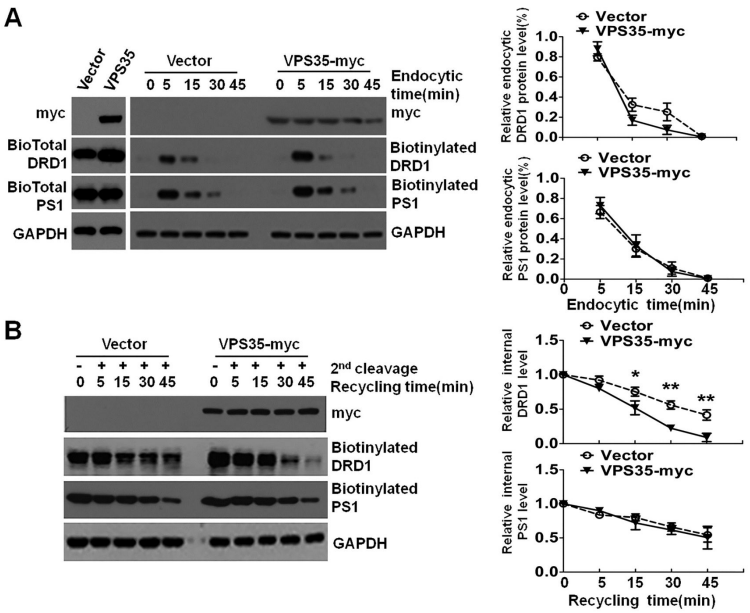

Fig. 3.

VPS35 affects cell surface recycling of DRD1. HEK293T cells were transfected with DRD1-HA vector, split equally, and then transfected with VPS35-myc and control vectors for 24 h. Cells were equally split again and biotinylated for surface proteins at 4°C. (A) Biotinylated cells were either lysed directly for determining total biotinylated (BioTotal) DRD1-HA and PS1-NTF (left panels of gel images), or incubated at 37°C for indicated time periods and then treated with glutathione at 4°C to cleave biotin on remaining surface proteins (right panels of gel images). (B) Alternatively, biotinylated cells were incubated at 37°C for 30 min, treated with glutathione at 4°C to cleave biotin on remaining surface proteins, incubated for indicated time periods, and then treated with glutathione at 4°C again to cleave biotin on proteins recycling to cell surface. After lysing and affinity precipitation, biotinylated DRD1-HA and PS1-NTF were analyzed by Western blot. Protein levels were quantified by densitometry and normalized for comparison (set as one arbitrary units). n=3, *p<0.05, **p<0.01.

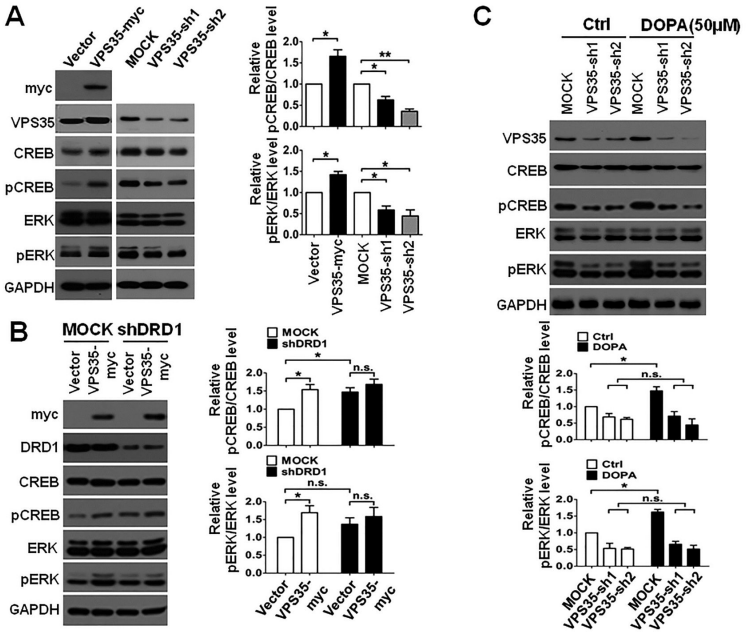

Fig. 4.

VPS35 regulates DRD1-mediated dopamine signal pathway. (A) N2a cells were transfected with VPS35-myc and control vectors for 24 h (left panels) or two mouse VPS35 shRNA (sh1 and sh2) and mock control shRNA vectors for 72 h (right panels). (B) N2a cells were transfected with DRD1 shRNA (shDRD1) and mock control shRNA vectors for 72 h. After equal splitting, cells were transfected with VPS35-myc and control vectors for 24 h. (C) N2a cells were transfected with mouse VPS35 shRNA (sh1 and sh2) and mock control shRNA vectors for 72 h. Cells were then treated with 50 μM dopamine (DOPA) or vehicle control (Ctrl) for another 36 h. After treatments, cells were lysed and equal protein amounts of cell lysates were subjected to Western blot analyses for indicated proteins. Protein levels were quantified by densitometry. Relative pCREB/CREB and pERK/ERK levels were calculated and compared to those of controls (set as one arbitrary units), n=3, *p<0.05, **p<0.01; n.s.: not significant.

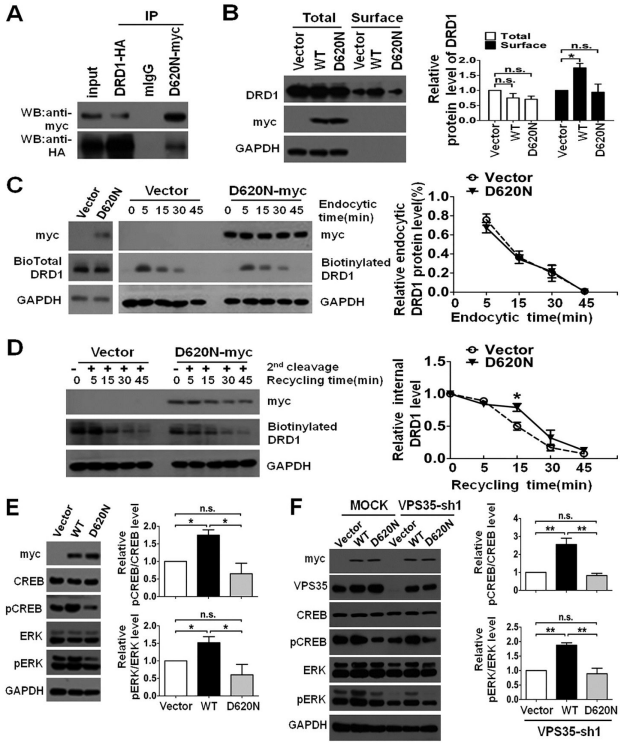

Fig. 5.

The D620N mutation in VPS35 impairs its regulation on DRD1 trafficking and downstream signaling. (A) VPS35(D620N)-myc and DRD1-HA vectors were co-transfected into HEK293T cells for 24 h. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody, anti-myc antibody or mouse IgG (mIgG). VPS35(D620N) and DRD1 in immunoprecipitated proteins were immunoblotted with anti-myc and anti-HA antibodies, respectively. (B) N2a cells were transfected with control, wild-type (WT) VPS35-myc and VPS35(D620N) vectors for 24 h, and then subjected to surface biotinylation. Affinity precipitated biotinylated proteins, as well as proteins in total lysates were analyzed by Western blot. (C,D) HEK293T cells were transfected with DRD1-HA vector and split equally. After second transfection with VPS35(D620N)-myc and control vectors for 24 h, cells were equally split again and biotinylated for surface proteins at 4°C. Biotinylated cells were incubated at 37°C for indicated time periods and then treated with glutathione at 4°C to cleave biotin on remaining surface proteins (C), or incubated at 37°C for 30 min, treated with glutathione at 4°C to cleave biotin on remaining surface proteins, incubated for indicated time periods, and then treated with glutathione at 4°C again to cleave biotin on proteins recycling back to cell surface (D). After lysing and affinity precipitation, biotinylated DRD1-HA was analyzed by Western blot with anti-HA antibody. (E) N2a cells were transfected with control, WT VPS35-myc and VPS35(D620N) vectors for 24 h. Cell lysates were assayed for indicated proteins by Western blot. (F) N2a cells were transfected with VPS35 shRNA1 (sh1) and mock shRNA vectors for 60 h. After equal splitting, cells were transfected with control, WT VPS35-myc and VPS35(D620N) vectors for another 24 h. Cell lysates were assayed for indicated proteins by Western blot. Protein levels were

quantified by densitometry for comparison to those of controls (set as one arbitrary units), n=3, *p<0.05, **p<0.01; n.s.: not significant.

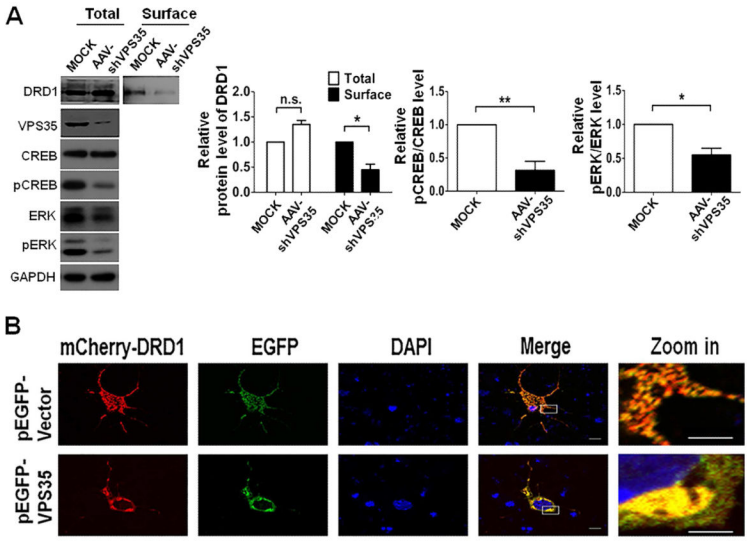

Fig. 6.

VPS35 regulates DRD1 trafficking and signaling in dopaminergic neurons. (A) Mouse dopaminergic neurons were infected with AAVs containing VPS35 shRNA1 (shVPS35) or mock shRNA for 72h. Neurons were then subjected to surface biotinylation. Biotinylated endogenous DRD1 was analyzed by Western blot. Total lysates were also assayed for indicated proteins. Protein levels were quantified by densitometry for comparison to those of controls (set as one arbitrary units), n=3, *p<0.05, **p<0.01; n.s.: not significant. (B) mCherry-DRD1 (in red) vector was co-transfected with pEGFP-VPS35 (in green) or pEGFP control vectors into mouse dopaminergic neurons for 24 h. The nuclei were stained with DAPI (shown in blue). Immunofluorescence was observed under the Olympus FV1000 confocal microscope. Scale bars: 5 μm for Zoom in images and 10 μm for all other images.