Vps35 Deficiency Impairs Cdk5/p35 Degradation and Promotes the Hyperphosphorylation of Tau Protein in Retinal Ganglion Cells

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Received: July 6, 2019
Accepted: November 16, 2019
Published: January 29, 2020

Citation: Gao L, Xiao H, Ai L-Q-Y, et al. Vps35 deficiency impairs Cdk5/p35 degradation and promotes the hyperphosphorylation of tau protein in retinal ganglion cells. *Invest Ophthalmol Vis Sci.* 2020;61(1):1. https://doi.org/10.1167/iovs.61.1.1

Purpose. Vacuolar protein sorting 35 (Vps35) mutations and protein dysfunction have been linked to the hyperphosphorylation and accumulation of tau protein in a number of central neurodegenerative disorders. The aims of the present study were to investigate the mechanism underlying the tau hyperphosphorylation caused by Vps35 deficiency.

Methods. The cells used in this study were primary retinal ganglion cells (RGCs). The rat retinal glutamate excitotoxicity model was used in vivo. Fresh retinal tissues or eyeballs were collected at different time points. The expression and interactions of Vps35, Cdk5/p35, tau hyperphosphorylation, LAMP1, EEA1 and UBE1 in RGCs were studied by immunofluorescence staining, Western blotting, and immunoprecipitation.

Results. The downregulation and overexpression of Vps35 increased and decreased the expression of p35 and tau hyperphosphorylation, respectively. More important, roscovitine, a Cdk5 inhibitor, could effectively decrease the hyperphosphorylated tau level induced by Vps35 deficiency. Furthermore, this study confirmed that the inhibition of Vps35 could increase the activity of Cdk5/p35 by affecting the lysosomal degradation of p35 and lead to the degeneration of RGCs.

Conclusions. These findings demonstrate the possibility that Cdk5/p35 acts as a "cargo" of Vps35 and provide new insights into the pathogenesis of RGC degeneration caused by hyperphosphorylated tau protein. Vps35 is a potential target for basic research and clinical treatment of RGC degeneration in many ocular diseases such as glaucoma.

Keywords: vacuolar protein sorting 35 (Vps35), cyclin-dependent kinase 5 (Cdk5), retinal ganglion cells (RGCs), degeneration

Vacuolar protein sorting 35 (Vps35) functions as a core subunit of the retromer. The Vps35/retromer orchestrates the endosome-to-cell surface, endosome-to-Golgi, and mitochondria-to-peroxidase retrieval of membrane proteins, which plays an important role in neurodegenerative diseases, such as Alzheimer's disease[1,2] and Parkinson's disease.[3–5] The hyperphosphorylation of tau protein is known to be pivotally involved in the etiopathogenesis of neurodegenerative diseases.[6] The retina, known as the "peripheral brain," is considered to be a part of the central nervous system. Several clinical studies have reported that tau hyperphosphorylation (p-tau) and its isoforms increased in the optic nerve, retina, and vitreous cavity of glaucoma patients,[7,8] suggesting a common pathologic mechanism of glaucoma and central neurodegeneration: the abnormal phosphorylation of tau protein.

To date, the function of the retromer complex is not completely understood, especially in the eyes. Our previous study has shown that p-tau in the retina increased with age in Vps35$^{+/m}$ mice, while the expression was weak in wild-type mice.[9] The changes in the retina caused by glutamate excitotoxicity[10] and Vps35 deficiency[11] are consistent with the degeneration of RGCs in glaucoma, which suggests that Vps35 deficiency leads to the degeneration of RGCs and an increase in p-tau levels in the retina.

Cdk5 has been considered a key kinase that contributes to tauopathy. It plays an important role in cell architecture and in the migration and differentiation of neurons.[12] Excessive Cdk5 activity has been implicated in neuronal loss of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.[13] Moreover, the elevation of p-NR2A^{S1232} by Cdk5/p35 contributes to RGC apoptosis in experimental glaucoma rats.[14] The hyperphosphorylation of tau, mediated by Cdk5, leads to the abnormal microtubule structure of neurons, and thereby apoptosis of neurons.

Table 1. Antibodies Used for Immunofluorescence

Antibodies	Cat No.	Dilution
Abcam		
Rabbit anti-tau antibody	ab32057	1 to 300
Rabbit anti-tau phospho s396	ab109390	1 to 200
Rabbit anti-tau phospho s404	ab131338	1 to 200
Goat anti-Vps35	ab10099	1 to 200
Rabbit anti-Vps35	ab157220	1 to 250
Rabbit anti-LAMP1	ab24170	1 to 200
Goat anti-mouse IgG H&L (Cy3)	ab97035	1 to 300
Donkey anti-goat IgG H&L (Cy3)	ab6949	1 to 500
Goat anti-rabbit IgG H&L (Alexa Fluor 488)	ab150077	1 to 300
Donkey anti-rabbit IgG H&L (Alexa Fluor 488)	ab150073	1 to 300
Rabbit anti-beta III tubulin	ab18207	1 to 500
Mouse anti-CD90/Thy1	ab225	1 to 300
Goat anti-GFP	ab6662	1 to 300
Cell Signaling Technology		
Rabbit anti-p35/25 (C64B10) mAb	#2680	1 to 200
Rabbit anti-UBE1a/b	#4891	1 to 25
Rabbit anti-EEA1 (C45B10) mAb	#3288	1 to 100
Santa Cruz Biotechnology		
Mouse anti-p35 (4G11)	sc-293184	1 to 200
Mouse anti-Cdk5 (J-3)	sc-6247	1 to 200

The aim of the present study was to investigate the mechanism involved in the hyperphosphorylation of tau protein in the retina caused by Vps35 deficiency.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 180 to 200 g and P1-P3 mice were purchased from the Laboratory Animal Center of Daping Hospital of Army Medical University. All adult animal experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The retinal neurodegeneration model was achieved by intravitreal injection of 3 µL containing 50 nmol of glutamate (Solarbio, Beijing, China) into the right eye with a 30-gauge needle (Hamilton Company, Reno, NV, USA). The control group received the same volume of 0.1 mol/L of sterile PBS (HyClone Laboratories, Logan, UT, USA). The intravitreal injection was performed as described previously.[10] The animals were killed with an intraperitoneal injection of an overdose of 10% chloral hydrate at various time points after intravitreal injection. At least three animals were used for each experimental condition. The eyes were enucleated and processed for further analysis.

Immunofluorescence

Sections were prepared for immunofluorescence according to standard techniques. Briefly, specimens were permeabilized with 0.1% Triton X-100 (Solarbio) and blocked with 10% goat serum for 1 hour. Subsequently, the specimens were incubated with primary antibodies overnight at 4°C. In addition, after rinsing with PBS, the specimens were incubated with secondary antibodies for 2 hours at room temperature (Table 1). Images were obtained with an SP-8 confocal microscope (Leica, Germany) at a 200× magnification and were processed with Leica LAS AF Lite software and ImageJ.

Table 2. Primers for RT-PCR

Name	Sequence
Vps35	5'-GAAATCGTCTCTCAGGACCAGG-3'
	5'-CGCAGAAGGTGAATAAATCGG-3'
CDK5	5'-GCCCGAGCCTTTGGTATCC-3'
	5'-GGTCATCCACATCATTGCCG-3'
p35	5'-AGTGAGCCCCTACCCTTCCTT-3'
	5'-GCTGAGCATTGGTTCTCTTGGT-3'
actin	5'-GCAGGAGTACGATGAGTCCG-3'
	5'-ACGCAGCTCAGTAACAGTCC-3'

RGC Culture and Transfection

For the RGC culture, the retinae were dissected from P1-P3 mice and placed in prechilled Dulbecco's Modified Eagle Medium. The cells of retinae were dissociated by 0.25% trypsin, purified by an RGC-specific anti-CD90/Thy1 antibody panning method as previously reported.[15] Dissociated retinal cells were resuspended in PBS and incubated in the selection plate for 2 hours, which sequestered Thy1-positive cells from the supernatant. The supernatant containing Thy1-negative cells was discarded and Thy1-positive cells were detached using 0.25% trypsin and cultured in neurobasal medium (90% neurobasal-A, 2% B27, 10% FBS, 0.1% glutamine, 0.1% penicillin and streptomycin). One-half of the medium was changed every 3 days. The ratio of Brn-3a positive RGCs in primary culture is about 80% (Supplementary Fig. S1). The primary culture RGCs were transfected with Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) at days in vitro 7 to 8 according to the manufacturer's instructions. For overexpression of Vps35, RGCs were transfected with pcDNA3.1-Flag-Vps35 plasmid DNA (kindly provided by Professor Wen-Cheng Xiong) (Supplementary Table S1). pcDNA3.1-GFP was used as negative control. For downregulation of Vps35, RGCs were transfected with Vps35 siRNA (sc-63219, Santa). Scramble control siRNA (GenePharma Company, Shanghai, China) (Supplementary Table S1) was used as negative control. Forty-eight hours later, the culture medium was discarded and the mRNA or protein was extracted for further experiments. The transfection rate of primary culture is about 20% (Supplementary Fig. S2).

Real-Time Analysis of mRNA Expression

Total mRNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA). mRNA was reverse transcribed into cDNA using a Transcript or First-Strand cDNA Synthesis Kit (Roche Life Science, Basel, Switzerland) according to the manufacturer's directions. The SYBR Green real-time PCR method was used to compare gene expression. The primers are shown in Table 2. Changes in mRNA expression were normalized to that of actin and were calculated using the $2^{-\Delta\Delta CT}$ method as previously described.[16]

Western Blot Assay

Cells and tissues were homogenized in RIPA buffer (Thermo Fisher Scientific) with a protease inhibitor cocktail (100×)

Table 3. Antibodies Used for Immunoblotting

Antibodies	Cat No.	Dilution
Abcam		
Rabbit anti-tau antibody	ab32057	1 to 5000
Rabbit anti-tau phospho s396	ab109390	1 to 10,000
Rabbit anti-tau phospho s404	ab131338	1 to 1000
Rabbit anti-LAMP1 antibody	ab24170	1 to 700
Rabbit anti-Vps35 antibody	ab157220	1 to 10,000
Mouse anti-GAPDH antibody	ab8245	1 to 1000
Goat anti-mouse IgG H&L (HRP)	ab6789	1 to 5000
Goat anti-rabbit IgG H&L (HRP)	ab6721	1 to 5000
Cell Signaling Technology		
Rabbit anti-p35/25 (C64B10) mAb	#2680	1 to 1000
Rabbit anti-UBE1a/b antibody	#4891	1 to 1000
Rabbit anti-EEA1 (C45B10) mAb	#3288	1 to 1000
Santa Cruz Biotechnology		
Mouse anti-Cdk5 (J-3)	sc-6247	1 to 200
GenScript		
Mouse anti-Flag	A00187	1 to 1000

(Thermo Fisher Scientific) as previously described.[10] The proteins were electrophoresed in 8%, 10%, or 12% SDS-PAGE. The separated proteins were transferred to PVDF membranes (Merck Millipore Ltd., Burlington, MA, USA), blocked in 5% BSA (Solarbio) for 1 hour and probed with the appropriate antibodies (Table 3). Images were taken with a gel imaging system (Aplegen, Gel Company, Inc, San Francisco, CA, USA). The protein bands were recorded and analyzed with Lab Works. The protein signal intensity was normalized to that of GAPDH.

Coimmunoprecipitation

To test the possible correlation between Vps35 and p35 or LAMP1 and p35, coimmunoprecipitation experiments were performed by using a co-IP kit (Pierce Biotechnology, Waltham, MA, USA) following the manufacturer's instructions. Briefly, the retinae of rats from different groups were homogenized in modified RIPA buffer on ice. After centrifugation at 13,000 rpm for 10 minutes, equal amounts of protein of approximately 300 µg were immunoprecipitated with anti-p35/25 (C64B10) mAb (#2680, Cell Signaling Technology, Danvers, MA, USA, 1:50) overnight on a carousel at 4°C. Subsequently, the mixture was allowed to bind to protein G-agarose beads (Roche Life Science) and rotated for approximately 5 hours at 4°C. The bead pellet was washed three times with RIPA buffer. The protein complexes were eluted with SDS sample buffer by boiling for 5 minutes, followed by immunoblot analysis with the appropriate antibodies.

Statistical Analysis

At least three independent experiments were performed at least in triplicate for each assay. Statistical analysis was performed with GraphPad Prism 6. The data are presented as mean ± SEM. A *t*-test was used to compare the two groups. One-way ANOVA and Tukey's multiple comparisons test were used for comparisons of the different groups. Significance was defined as a P value of less than 0.05.

Results

Vps35 Expression was Downregulated, While the Expression of p35, p-tau s396, and s404 Was Increased in an Excitotoxic Model Induced by Glutamate In Vivo and In Vitro

We explored a rat retinal neurodegeneration model by an intravitreal injection of 50 nmol of glutamate.[10] Immunofluorescence was used to detect the distribution of Vps35, p35, Cdk5, and p-tau s396 in the retina. We found that Vps35, Cdk5, and p35 were mainly expressed in the ganglion cell layer (GCL) and the inner nuclear layer (Figs. 1A–C). Vps35 and p35 were colocalized in the GCL. In comparison with the control group, the signal of Vps35 decreased and the signal of p35 increased 1 week after intravitreal injection of 50 nmol of glutamate (Figs. 1A and B). P-tau s396 was mainly expressed in the nerve fiber layer, GCL, inner plexiform layer, and few in the outer plexiform layer. Vps35 and p-tau s396 were colocalized in the GCL (Fig .1D). In vitro, the signals of Vps35, Cdk5, p35, and p-tau s396 were detected in primary RGCs by immunofluorescence (Figs. 1E–J), and Vps35 and p35 were colocalized in the cytoplasm and neurites of RGCs (Fig. 1G).

Consistent with the results of immunofluorescence, 14 days after the intravitreal injection of 50 nmol of glutamate, the relative mRNA and protein expression of Vps35 decreased significantly ($P < 0.05$) (Figs. 2A–C), whereas the protein expression of p35, CdK5, p-tau s396, and s404 increased with time (p25 was not detected, as shown in Fig. 2A, indicating that p35 was not truncated into p25 by calpain), and there were significant differences between the experimental group and the control group ($P < 0.05$) (Figs. 2A and B).

The relative expression of the Vps35 protein in RGCs treated with 25 µmol/L glutamate for 24 hours decreased, whereas that of p35 and p-tau s396 increased, and these results were significantly different from those in the control group ($P < 0.05$). The protein expression of Cdk5 increased slightly compared with that in the control group, but there was no significant difference ($P > 0.05$) (Figs. 2D and E).

Vps35 Alters the Activity of Cdk5/p35 and the Phosphorylation of Tau Protein

Our previous study confirmed that Vps35 deficiency led to neurodegenerative changes in RGCs.[11] To determine whether the depletion of Vps35 affects the activity of Cdk5/p35 and the phosphorylation of tau protein, we downregulated Vps35 in primary culture RGCs by the transfection of Vps35 siRNA. The results show that Vps35-specific siRNA could significantly inhibit Vps35 mRNA and protein expression compared with the control condition ($P < 0.05$). Downregulation of Vps35 could statistically enhance the levels of Cdk5/p35 ($P < 0.05$) and resulted in an increase in p-tau s396 ($P < 0.05$) (Figs. 3A, B, and E).

Additionally, we upregulated Vps35 in primary cultured RGCs by transfecting pcDNA3.1-Flag-Vps35 plasmid DNA. The results show that, compared with the control, the Vps35-specific plasmid could significantly upregulate the Vps35 mRNA and protein levels in RGCs ($P < 0.05$). Upregulation of Vps35 considerably decreased the levels of Cdk5/p35 ($P < 0.05$), accompanied by a decrease in p-tau s396 levels ($P < 0.05$) (Figs. 3C, D, and F).

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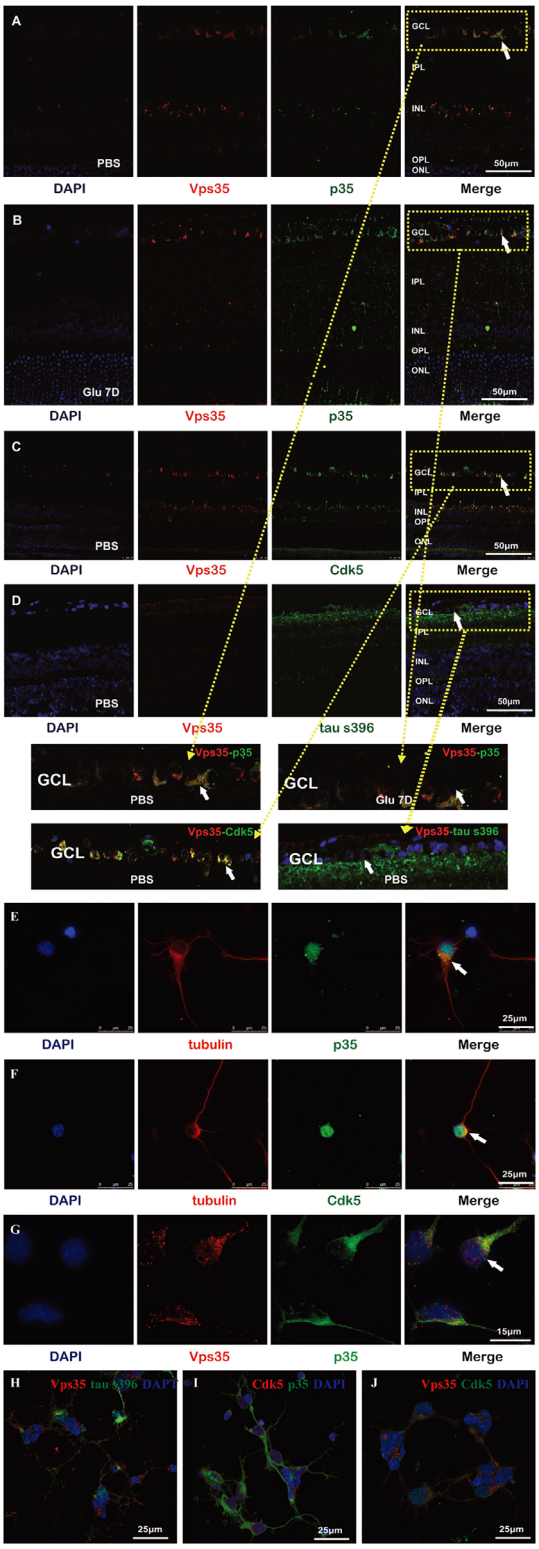

Figure 1. The expression of Vps35, p35, CDK5, and tau s396 in retina and primary cultured RGCs. (**A**, **B**) Vps35 and p35 were colocalized in the GCL and the signals of Vps35 decreased while the signals of p35 increased 1 week after intravitreal injection of 50 nmol glutamate. (**C**, **D**) Vps35 and p35, and Vps35 and tau s396 colocalized in the GCL. (**E**, **F**) The expression of p35 and Cdk5 in primary cultured RGCs. (**G**, **H**) The colocalization of Vps35 and p35, Vps35 and tau s396, p35 and Cdk5, and Vps35 and Cdk5 in primary cultured RGCs.

To examine whether Cdk5 takes part in tau phosphorylation in cultured RGCs, roscovitine, an inhibitor of Cdk5, was added to RGCs cultured in vitro at a final concentration of 25 μmol/L. Dimethylsulfoxide was added to the control group. After 24 hours, protein of RGCs was extracted for WB analysis. In agreement with the results of previous studies,[17] 25 μmol/L of roscovitine significantly inhibited Cdk5 ($P < 0.05$), resulting in a decrease in the p35 and p-tau s396 levels ($P < 0.05$) (Figs. 3G and H).

Vps35 and Cdk5 Contribute to the Increased p-tau s396 Caused by Glutamate Excitotoxity and Cdk5 Is an Important Kinase of p-tau Caused by Vps35 Deficiency

For further exploring the role of Vps35 and Cdk5 in p-tau s396 caused by glutamate, we detected p-tau s396 with Vps35 overexpression plasmid and rosocovitine in primary culture RGCs dealt with glutamate. The results show that Vps35 overexpression plasmid and roscovitine could significantly reduce the increase of p-tau s396 caused by glutamate ($P < 0.05$) (Figs. 4A and B).

To determine whether Cdk5 takes part in the increase of p-tau s396 level caused by Vps35 deficiency, RGCs were transfected with Vps35 siRNA by Lipofectamine 3000. After 24 hours, roscovitine was added to one of the groups. The protein extracted from RGCs was collected 24 hours later, and the levels of p-tau s396 and total tau were detected by WB. The results show that compared with the control, the deficiency of Vps35 led to a significant increase in p-tau s396 levels ($P < 0.05$); however, the inhibition of Cdk5 could significantly reduce the increase in p-tau s396 levels caused by Vps35-specific siRNA ($P < 0.05$) (Figs. 4C and D), suggesting that Cdk5 is an important kinase for the hyperphosphorylation of tau protein caused by Vps35 deficiency.

p35 Interacts with Vps35 In Vivo and In Vitro

We verified that Vps35 and p35 were colocalized in the cytoplasm of RGCs in vivo and in vitro by immunofluorescence (Figs. 5A and B). The interaction between Vps35 and p35 by immunoprecipitation (IP) was obvious 1 week after the intravitreal injection of 50 nmol of glutamate and weakened 2 weeks after the intravitreal injection (Fig. 5D). It is suggested that Vps35 may be directly involved in the recycling of p35.

Cdk5 was first purified as one of the two tau protein kinases. The kinase activity of Cdk5 is mainly determined by the available protein amounts of p35 in neurons, which is mainly degraded by the ubiquitin–proteasome system. Thus, the major factor that regulates Cdk5 activity is the degradation of p35. The interaction of Cdk5 and p35 in the retina was confirmed by immunofluorescence and IP in our study (Figs. 5C and D).

Deficiency of Vps35 Leads to a Decrease in LAMP1 Levels

The expression of LAMP1 and EEA1 in the retina significantly decreased 1 week after the intravitreal injection of 50 nmol of glutamate compared with vehicle ($P < 0.01$), as seen by immunoblotting. However, there was no significant change in UBE1 ($P > 0.05$) (Figs. 6A and B), which suggested

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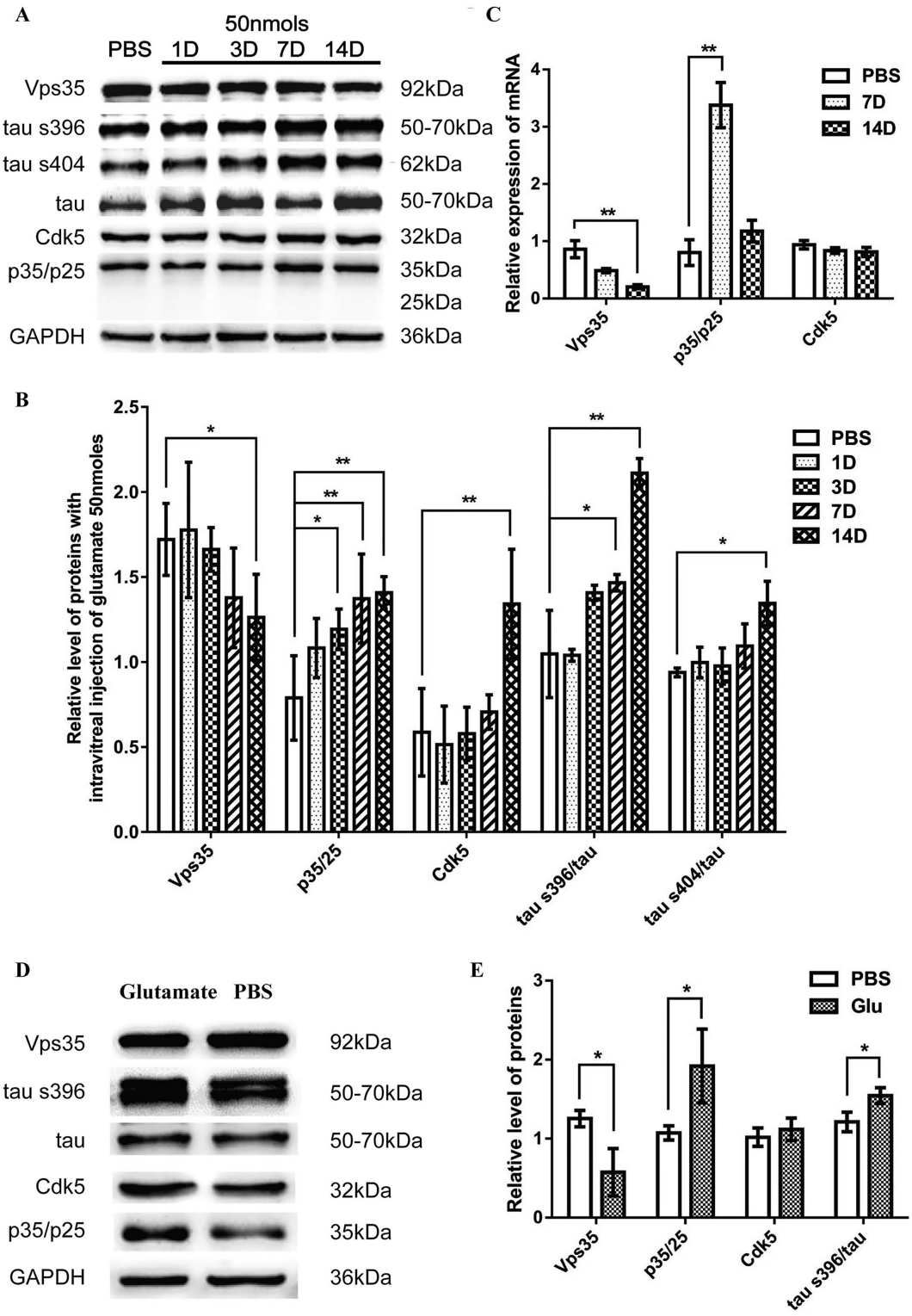

Figure 2. The relative mRNA and protein expression of Vps35 decreased, while the expression of p35, p-tau s396 increased with glutamate excitotoxity both in vivo (especially 7D and 14D after intravitreal injection of glutamate 50 nmol) (**A–C**) and in vitro (**D**, **E**). $^{**}P < 0.01$; $^{*}P < 0.05$.

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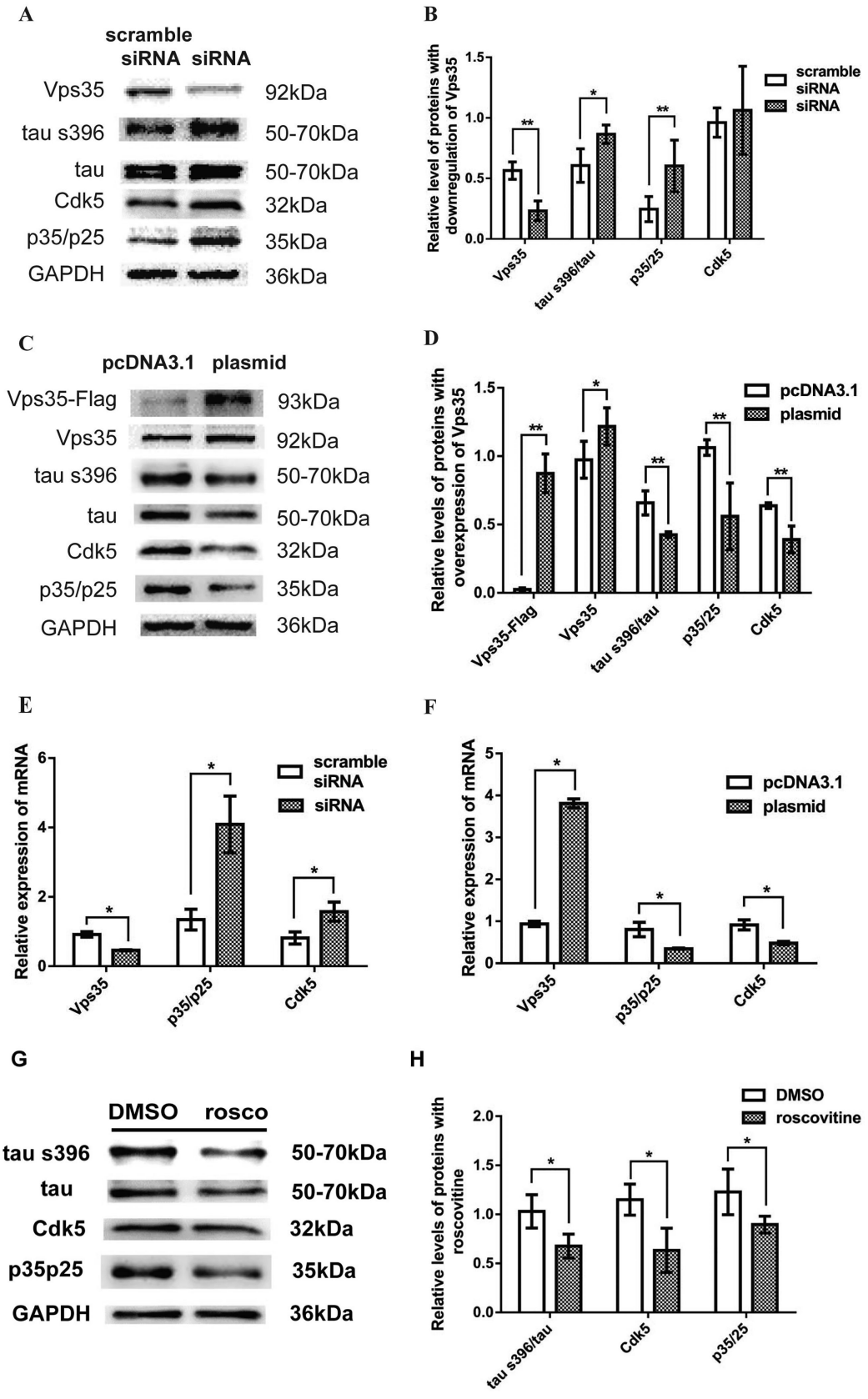

Figure 3. Vps35 alters the activity of Cdk5/p35 and the phosphorylation of tau protein. (**A**, **B**, **E**) Downregulation of Vps35 in primary cultured RGC could statistically enhance the levels of Cdk5/p35 and tau s396. (**C**, **D**, **F**) Overexpression of Vps35 in primary cultured RGC could considerably decrease the levels of Cdk5/p35 and tau s396. (**G**, **H**) Roscovitine significantly inhibited Cdk5 ($P < 0.05$), resulting in a decrease in the p35 and p-tau s396 levels in primary cultured RGCs. $^{**}P < 0.01$; $^{*}P < 0.05$. DMSO, dimethylsulfoxide.

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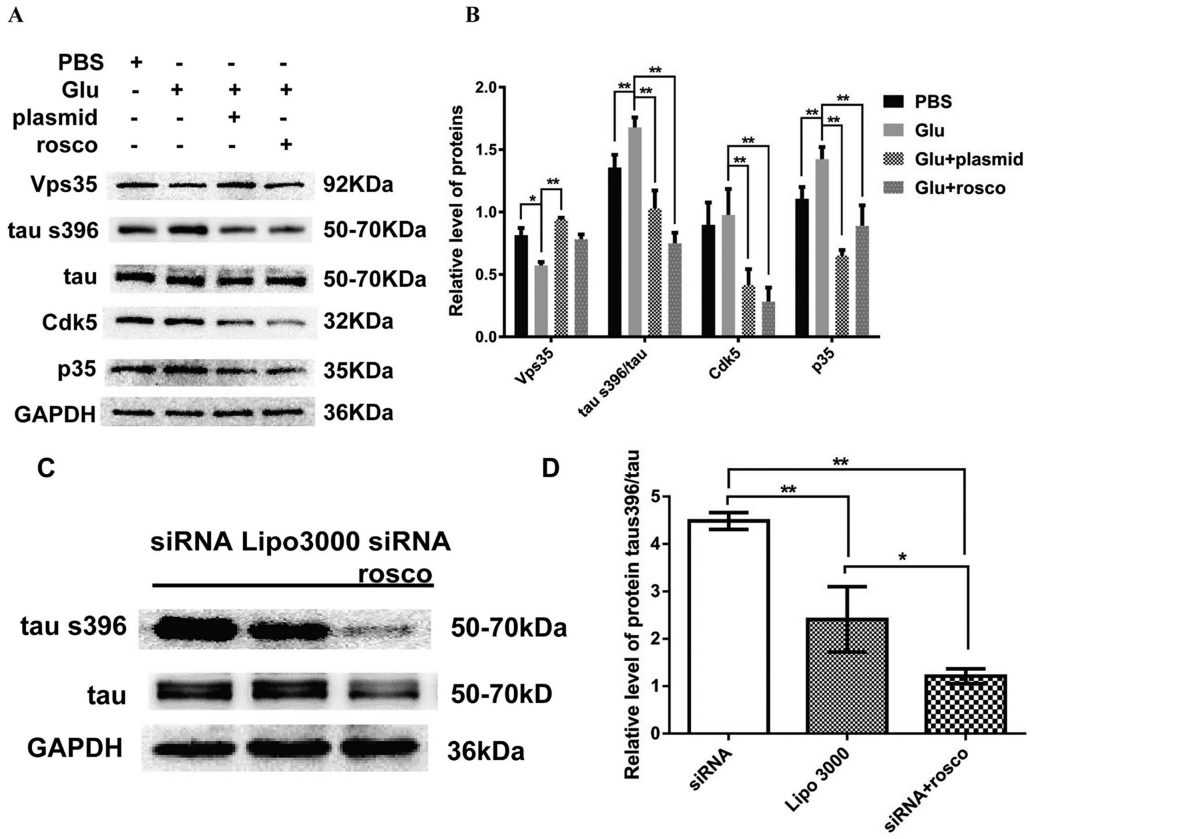

Figure 4. Vps35 and Cdk5 contribute to the increased p-tau s396 caused by glutamate excitotoxity and Cdk5 is an important kinase of p-tau caused by Vps35 deficiency. (**A**, **B**) Overexpression of Vps35 and inhibition of Cdk5 could significantly reduce the increase in p-tau s396 caused by glutamate excitotoxity in primary RGCs. (**C**, **D**) Inhibition of Cdk5 could significantly reduce the increase in p-tau s396 caused by Vps35-specific siRNA. $^{**}P < 0.01$; $^{*}P < 0.05$.

a lysosomal dysfunction in retinal excitotoxicity induced by the intravitreal injection of 50 nmol of glutamate.

To confirm the effect of Vps35 on lysosomes and proteasomes, primary cultured RGCs were transfected with Vps35-specific siRNA by Lipofectamine 3000. Cycloheximide (30 µg/mL) was added 24 hours later to inhibit protein synthesis. The relative protein levels of UBE1 and LAMP1 were detected by immunoblotting. Our results show that the inhibition of Vps35 resulted in a decrease in LAMP1 levels ($P < 0.05$), whereas UBE1 levels did not change significantly ($P > 0.05$) (Figs. 6C and D), which confirmed that Vps35 could regulate the expression of LAMP1 and affect the degradation ability of lysosomes.

There Was a Direct Interaction between Vps35 and LAMP1 and between LAMP1 and p35

To further explore the mechanism by which Vps35 affects the degradation of p35, we used immunofluorescence and IP to confirm their interaction. Vps35 and p35, Vps35 and LAMP1, p35 and LAMP1, and p35 and EEA1 were colocalized in RGCs both in vivo and in vitro, which was verified by immunofluorescence (Figs. 7A–F). Bands at the locations corresponding to Vps35 (92 kDa) and LAMP1 (120 kDa) were detected in the IP derived using the antibody against p35 (Fig. 5A and Fig. 7G), suggesting a specific interaction between p35 and Vps35 and between LAMP1 and p35. A similar result was observed when Vps35 was detected in the IP with the LAMP1 antibody. The interaction of p35 and LAMP1 decreased 2 weeks after intravitreal injection (Fig. 7G). One possibility is that the lack of Vps35 caused by glutamate excitotoxity may lead to a decrease in Cdk5/p35 transported to the lysosome for degradation, resulting in an increase in Cdk5/p35 activity and the expression level of the p-tau protein. This finding suggests the possibility that p35 is a cargo of Vps35. Additionally, Vps35 deficiency might decrease the degradation ability of lysosomes, leading to an increase in p35 levels.

Discussion

In this study, we first showed that Vps35 decreased, whereas p35, p-tau s396 increased in glutamate-induced excitotoxic model. Second, we found that Vps35 alters the activity of Cdk5/p35 and the phosphorylation of tau protein. Third, we demonstrated that Vps35 and Cdk5 contribute to the increased p-tau s396 caused by glutamate excitotoxity and Cdk5 is an important kinase of p-tau caused by Vps35 deficiency. Finally, we showed that Vps35 deficiency might cause decreased p35 degradation by lysosomes. Cumulatively, these data suggest that the possibility that Cdk5/p35 acts as a cargo of Vps35 and might contribute to the pathogenesis of retinal neurodegeneration.

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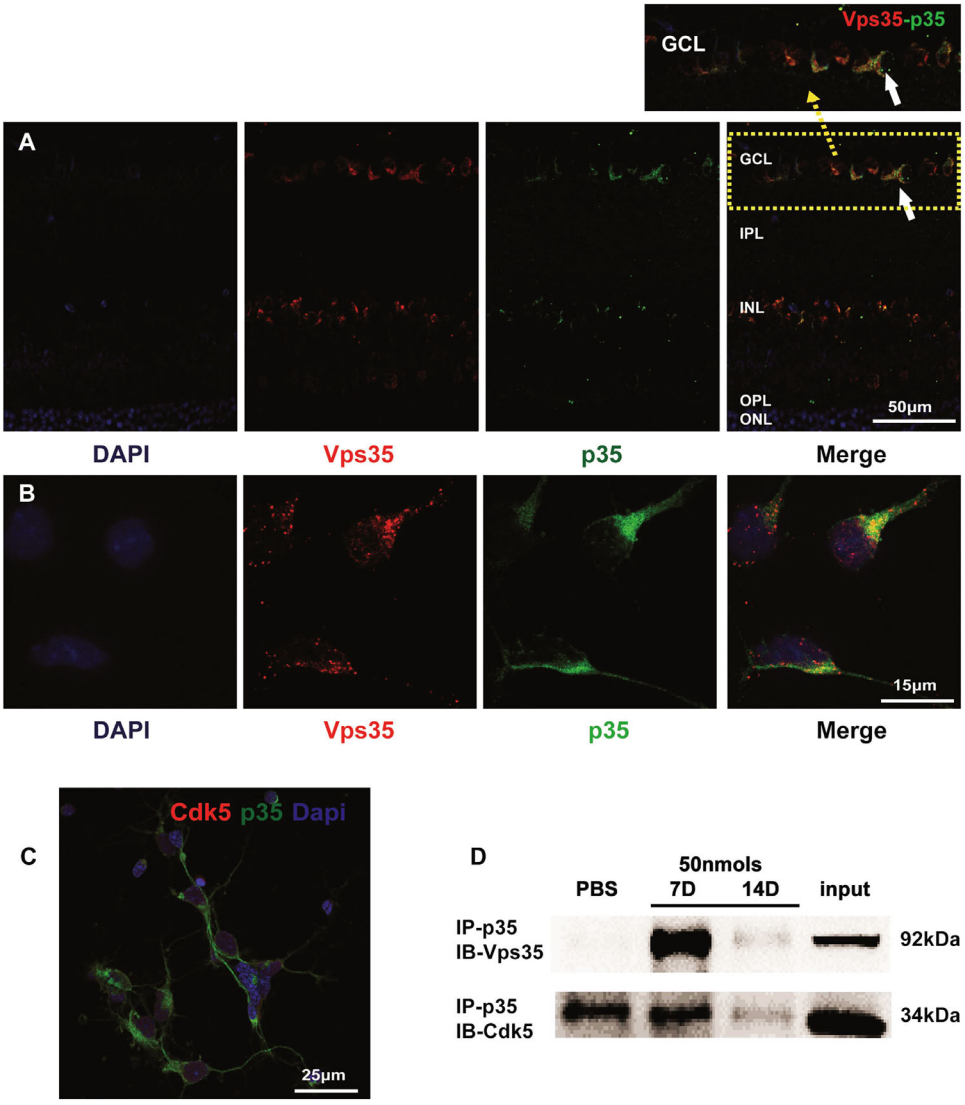

Figure 5. Vp35 interacts with p35 in vivo and in vitro. (**A**, **B**) Vps35 and p35 were colocalized in the cytoplasm of RGCs in vivo and in vitro by immunofluorescence. (**C**) The colocalization of Cdk5 and p35 in primary cultured RGCs. (**D**) The interaction between Vps35 and p35 by IP was obvious 1 week after the intravitreal injection of 50 nmol glutamate. Cdk5 interacted with p35 both in control group and one week after the intravitreal injection of 50 nmol of glutamate.

The excitatory amino acids have been regarded as a final common pathway for neurologic disorders.[18] Increased p-tau s396 levels were detected in the rat retina after intravitreal injection of 50 nmol of glutamate and in primary cultured RGCs stimulated by glutamate. Moreover, in primary cultured RGCs, the inhibition of Vps35 led to an increase in p-tau s396, whereas a decrease p-tau s396 by the upregulation of Vps35, indicating that Vps35 is involved in the regulation of the abnormal phosphorylation of tau protein. The phosphorylation state of tau alters its intrinsic functions and binding affinity to microtubules. The abnormal phosphorylation of tau protein plays an important role in RGC degeneration.[19]

Glycogen synthetic kinase-3β (GSK-3β)[20] and cyclin-dependent kinase 5 (Cdk5)[13] are the two main kinases of tau. GSK-3β is the most frequently reported kinase of tau, which is localized in Müller cells in the synaptic layers and within the inner segments of photoreceptor cells.[21] Our previous study demonstrated that GSK-3β was downregulated in the retinas of Vps35$^{+/m}$ mice and that GSK-3β colocalized with glial cells but not with RGCs (unpublished data), suggesting that GSK-3β might not be involved in the upregulation of p-tau induced by Vps35 deficiency or needs to be further explored.

Cdk5, a proline-directed serine/threonine kinase, plays multiple roles in neural development and synaptic plasticity by phosphorylating numerous synaptic substrates. Cdk5/p35 has different responsibilities in physiologic and pathologic conditions. In our study, both Cdk5/p35 activity and p-tau s396 levels increased 14 days after the intravitreal injection of 50 nmol of glutamate, suggesting the relevance of Cdk5/p35 and p-tau s396 in the mechanisms of RGC degeneration caused by glutamate, which agrees with the results by Chen et al.[14] that roscovitine, a Cdk5 inhibitor, can effectively ameliorate apoptosis of RGCs induced by high intraocular pressure.

In our glutamate-induced excitotoxic retinal neurodegeneration model, the increase in the p35 level (D7) seemed

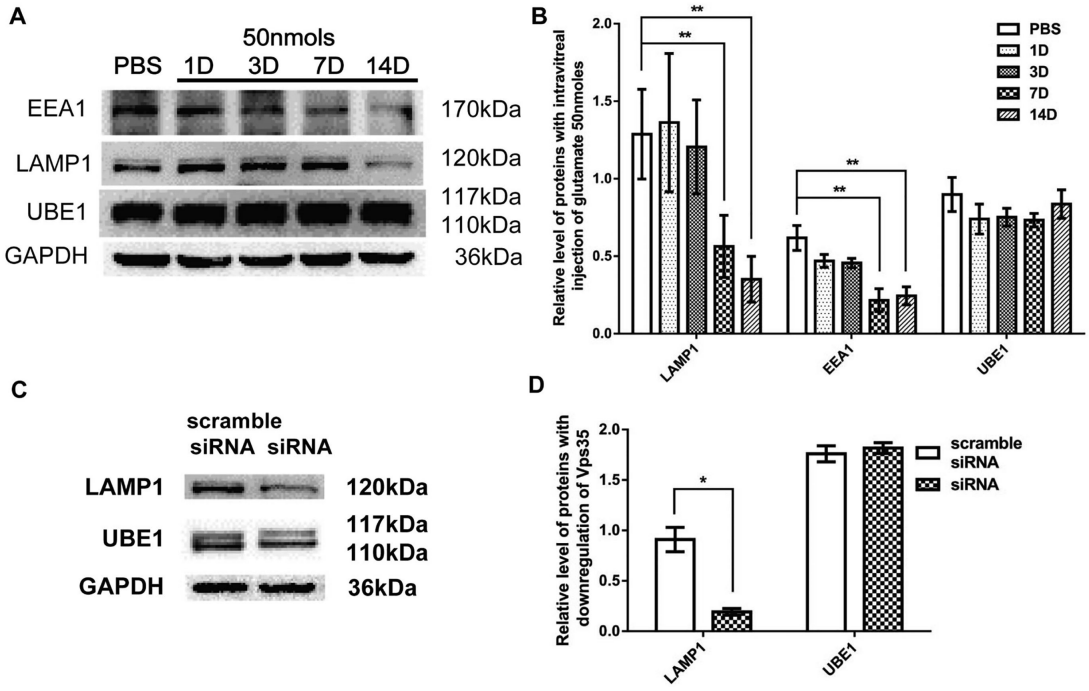

Figure 6. Deficiency of Vps35 leads to a decrease in LAMP1 of retina. (**A**, **B**) The expression of LAMP1 and EEA1 in the retina significantly decreased, while p35 increased, 1 week after the intravitreal injection of 50 nmol of glutamate. (**C**, **D**) The inhibition of Vps35 resulted in a decrease in LAMP1 in primary RGCs. $^{**}P < 0.01$; $^{*}P < 0.05$.

to precede that of Cdk5 (D14), which suggests that the increased expression of p35 may be a cause leading to the upregulation of Cdk5 expression in glutamate-induced retinal excitotoxicity. In vitro, we found that the inhibition of Vps35 could cause the upregulation of p35 in primary culture RGCs. Thus, Vps35 deficiency may be one of the mechanisms underlying the upregulation of p35 by glutamate-induced retinal excitotoxicity. One possibility for activated Cdk5 is that Cdk5 in the cytoplasm is very unstable and easily degraded by ubiquitination, but because its ubiquitination site is located in the p35 binding area, the ubiquitination of Cdk5 was blocked in the presence of high levels of p35.[22] Therefore, high levels of p35 can increase the expression of Cdk5 in the cytoplasm and can increase the activity of Cdk5/p35. In agreement with the results of the optic nerve transection[23] and the glaucoma model,[14] p25 was not detected in vitro or in vivo in our study, suggesting that p35 was not truncated into p25 by calpain. One possibility is that such conversion occurs transiently; thus, the p25 level may be too low to be detected. Another possibility is that the increased Cdk5 activity suppresses both the degradation of p35 and the calpain-mediated cleavage of p35.[24] In this context, it was indeed reported that Cdk5 could be activated without p25, and the activation of Cdk5/p35 was related to cell death in different pathologic conditions.[25] The interaction between Cdk5 and p35 was confirmed in our experiments both in vivo and in vitro. The interaction of Cdk5/p35 decreased 2 weeks after the intravitreal injection, which may be related to the phosphorylation of p35 or the feedback of Cdk5/p35 itself.

In addition, we found that Vps35 and p35 colocalized in RGCs by immunofluorescence. Furthermore, by IP, the results suggest that there was a direct interaction between Vps35 and p35 1 week after the intravitreal injection. Along with the decrease in Vps35 caused by glutamate, the binding of Vps35 and p35 decreased two weeks after the intravitreal injection, which suggests that Vps35 may be directly involved in the recycling of p35.

Endosome–lysosome and ubiquitin–proteasome are the two main pathways of protein degradation,[26] and the two pathways are synergistic in cells. Some proteins need to be modified by polyubiquitination before they can be recognized and transported to lysosomes for degradation. Studies have shown that the stability of p35 may depend not only on the ubiquitin–proteasome pathway, but also on the endosome–lysosome pathway[27] and the phosphorylation at Thr138 in p35 by Cdk5 stimulates its degradation.[28] Our results confirmed that the deficiency of Vps35 resulted in a decrease in LAMP1 and EEA1, although there was no obvious change in UBE1; this finding indicates that there was little possibility of p35 degradation by the ubiquitination pathway. The interactions between Vps35 and LAMP1 and between p35 and LAMP1 were confirmed by immunofluorescence and IP. This evidence suggests that Vps35 deficiency decreases the degradation of Cdk5/p35 transported to lysosomes, which results in activation of Cdk5/p35 and an increase of p-tau, leading to the degeneration and apoptosis of RGCs. Additionally, it was reported that autophagy is involved in the degradation of overexpressed tau and hyperphosphorylated tau and that the inhibition of autophagy enhances tau aggregation and cytotoxicity,[29] which may be another explanation for p-tau accumulation caused by the dysfunction of the lysosome when there is Vps35 deficiency.[30]

In conclusion, our findings demonstrate the possibility that Cdk5/p35 acts as a cargo of Vps35 and provide new insights into the pathogenesis of RGC degeneration caused by hyperphosphorylated tau protein. Vps35 constitutes a

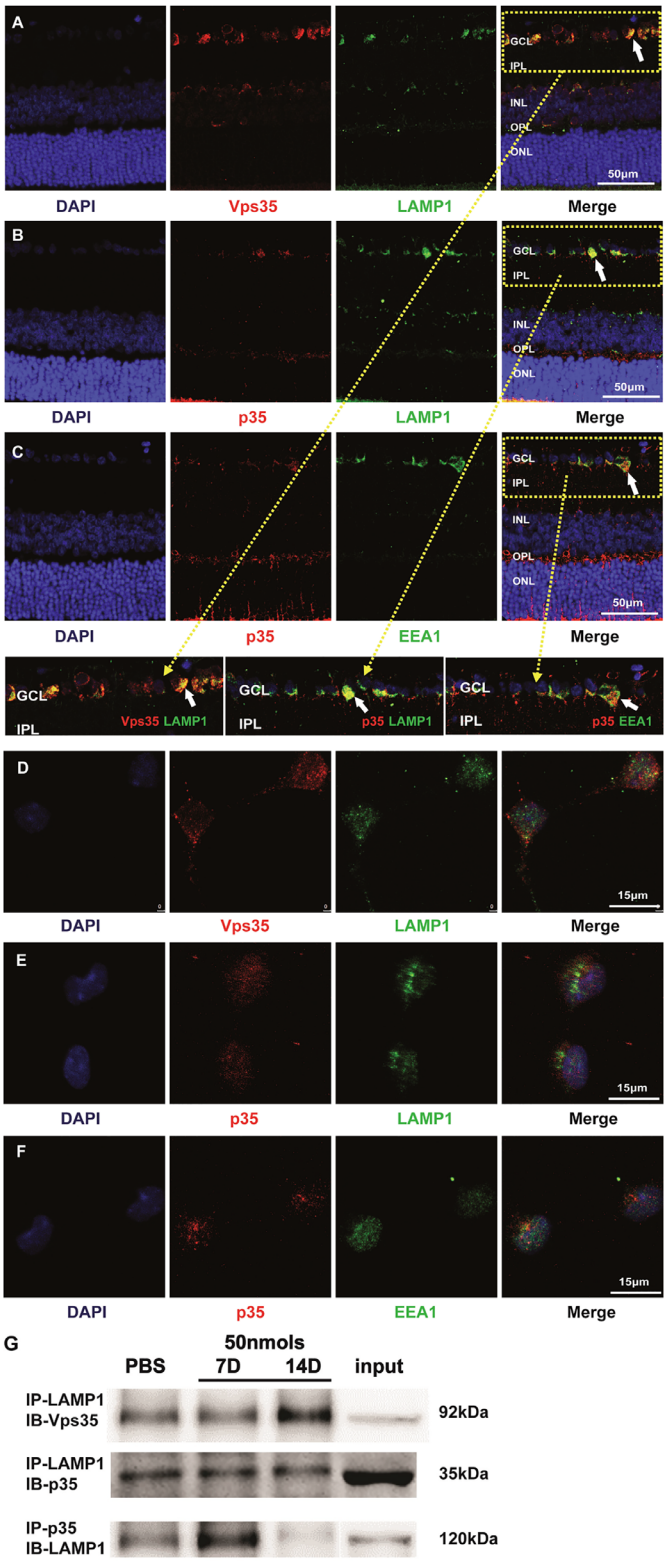

Figure 7. There was a direct interaction between Vps35 and LAMP1 and between LAMP1 and p35. (**A–C**) The colocalization of Vps35 and LAMP1, p35 and LAMP1, and p35 and EEA1 in the GCL of retina. (**D–F**) The colocalization of Vps35 and LAMP1, p35 and LAMP1, and p35 and EEA1 in primary cultured RGC. (**G**) The coimmunoprecipitation showed a direct interaction of Vps35 and LAMP1, and LAMP1 and p35, with protein extracted from rat retinae.

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potential target for basic research and clinical treatment of RGC degeneration in many ocular diseases such as glaucoma.

Acknowledgments

The authors thank Jiaxing Zhou, Xi Chen, Zhizhong Huang, and Zhuohang Zhang for assistance.

Supported by the National Natural Science Foundation of China (No. 81400398).

Disclosure: **L. Gao**, None; **H. Xiao**, None; **L.Q.Y. Ai**, None; **C.L. Chen**, None; **S. Lin**, None; **Y.G. Zhou**, None; **J. Ye**, None; **W. Liu**, None

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