VPS35 regulates developing mouse hippocampal neuronal morphogenesis by promoting retrograde trafficking of BACE1

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Introduction

Retromer, a protein complex initially identified in the yeast, is essential for retrograde transport of numerous membrane proteins from the endosomes to the trans-Golgi network (TGN) (Seaman, 2005; Bonifacino and Hurley, 2008). It contains two sub-protein complexes: one for the cargo-selection, and the other one for membrane deformation. The cargo-selective complex is a trimer of vacuolar protein sorting (Vps) proteins VPS35, VPS29, and VPS26 that sorts cargos into tubules for retrieval to the Golgi apparatus. The membrane deformation sub-complex consists of sorting nexin (SNX) dimers (Vps5p and Vps17p in yeast, and sortin nexins 1/3 or 5/6 in vertebrates) (van Weering et al., 2010; Seaman, 2005; Bonifacino and Hurley, 2008). A growing list of retromer cargos has been identified, including cation independent mannose 6-phosphate receptors (CI-MRP) (Arighi et al., 2004), mannose 6-phosphate receptors (CI-MRP) (Arighi et al., 2004), and mannose 6-phosphate receptors (CI-MRP) (Arighi et al., 2004). Genetic mutations of SorLA, a family of retromer cargo of retromer, and Vps35 are identified in late-onset AD (Rogaeva et al., 2007; Willnow et al., 2011), and PD patients (Vilarino-Güell et al., 2011; Zimprich et al., 2011), respectively. These observations suggest that dysfunction of retromer complex may be a general risk factor for a growing list of neurodegenerative disorders. This view is further supported by recent studies using genetically mutant mouse models of retromer (Muhammad et al., 2008; Wen et al., 2011). Both Vps35 and Vps26 heterozygotes exhibit an increase in the production of amyloid β peptide (Aβ) (Muhammad et al., 2008; Wen et al., 2011), a 40–42 amino acid peptide derived from β- and γ-secretase cleavage of APP that is believed to be a major culprit of AD. In addition, Vps35 heterozygotes in Tg2576 mouse model of AD show earlier onset and enhanced AD-like neuropathology (Wen et al., 2011). Further mechanistic cellular studies suggest that loss of retromer function may alter the

Summary

VPS35, a major component of the retromer, plays an important role in the selective endosome-to-Golgi retrieval of membrane proteins. Dysfunction of retromer is a risk factor for neurodegenerative disorders, but its function in developing mouse brain remains poorly understood. Here we provide evidence for VPS35 promoting dendritic growth and maturation, and axonal protein transport in developing mouse hippocampal neurons. Embryonic hippocampal CA1 neurons suppressing Vps35 expression by in utero electroporation of its micro RNAs displayed shortened apical dendrites, reduced dendritic spines, and swollen commissural axons in the neonatal stage, those deficits reflecting a defective protein transport/trafficking in developing mouse neurons. Further mechanistic studies showed that Vps35 depletion in neurons resulted in an impaired retrograde trafficking of BACE1 (β1-secretase) and altered BACE1 distribution. Suppression of BACE1 expression in CA1 neurons partially rescued both dendritic and axonal deficits induced by Vps35-deficiency. These results thus demonstrate that BACE1 acts as a critical cargo of retromer in vitro and in vivo, and suggest that VPS35 plays an essential role in regulating apical dendritic maturation and in preventing axonal spheroid formation in developing hippocampal neurons.

Key words: VPS35, BACE1, Neural development

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trafficking of its cargos, including SorLA, APP, and BACE1, resulting in an increased Aβ production and enhanced AD neuropathology (Vieira et al., 2010; Willnow et al., 2010; Finan et al., 2011; Wen et al., 2011). However, the exact mechanism remains elusive.

In order to study retromer’s function, we have generated Vps35 mutant mouse, as VPS35 is a major component of retromer cargo recognition complex and is responsible for cargo recognition and the complex assembly (Seaman, 2005; Bonifacino and Hurley, 2008; McGough and Cullen, 2011). While hemizygous deletion of Vps35 gene in Tg2576 mouse model of AD leads to an earlier-onset AD-like phenotypes, homozygous died early during embryonic development (<E10, before neurogenesis) (Wen et al., 2011), preventing us from using this genetic model to address VPS35’s function during mouse development. We thus used the RNA interference (RNAi) technology and the in utero electroporation assay to address this issue. Here, we showed that expression of miRNAs that suppress Vps35 expression in developing mouse CA1 neurons results in shortened apical dendrites, reduced dendritic spines, and swollen axons. These results suggest a role for VPS35/retromer in dendritic arborization or maturation and in preventing axonal spheroid formation during neonatal hippocampal development. We further investigated the underlying mechanisms and found that Vps35 depletion in hippocampal neurons resulted in an impaired retrograde trafficking of BACE1 and altered BACE1 distribution. Suppression of BACE1 expression rescued Vps35 deficiency induced defects, suggesting a role of BACE1 in contributing to the Vps35 deficiency induced phenotypes during development. These results thus demonstrate a critical role for VPS35 in developing hippocampal neurons and yield insights into further mechanisms of retromer regulated AD pathogenesis in mature neurons.

Results
Shortened apical dendrites and swollen axons in Vps35 deficient CA1 neurons

To investigate possible functions of VPS35 in hippocampal neurons, we first examined VPS35’s expression in developing and adult mouse hippocampus by taking advantage of the Vps35+/m mouse, in which the LacZ gene was “knocked-in” in the intron of the Vps35 gene, thus, LacZ expression is controlled by the promoter of the Vps35 gene (Wen et al., 2011). The β-gal activity was weakly and diffusely distributed in the hippocampal region of E15.5 mouse embryos, and became highly restricted to CA1–3 regions of the hippocampus in neonatal stage [e.g., postnatal day 10 (P10)] (Fig. 1A). The expression appeared to be peaked at the neonatal stage (P10–P15) of the hippocampus (Fig. 1A), and this view was also supported by the Western blot analysis (Fig. 1B). As P10–P15 is a critical time-window for the establishment of axonal–dendritic sorting, synaptogenesis, and circuitry of hippocampal neurons, the peak level of VPS35 expression at P10–P15 thus implicates VPS35 in these events.

We next examined VPS35’s function in developing mouse CA1 neurons by use of the RNA interference (RNAi) technology and an in utero electroporation assay (supplementary material Fig. S1A–C). Several miRNA-Vps35 (miR-Vps35) constructs targeting different exons of Vps35 were generated, and miR-Vps35-1 and miR-Vps35-3 showed high and medial efficiency in knocking down Vps35 expression in HEK 293 cells, respectively, determined by Western blot assay (supplementary material Fig. S1D). The in utero electroporation of miR-Vps35-1 into the progenitor cells of CA1 pyramidal neurons in mouse hippocampus at E15.5 also markedly suppressed endogenous Vps35 expression (supplementary material Fig. S1E). At P10, the majority of miR-Vps35 transfected neurons had migrated to pyramidal cell layer of hippocampal CA1 region, however, a mild but significant migration defect was observed in miR-Vps35-1 neurons; ~13% of neurons were mislocated out of pyramidal cell layer as compared to ~5% in control (supplementary material Fig. S2). This migration defect was not observed in miR-Vps35-3 neurons (~5% mis-distribution), suggesting that the migration defect happens when VPS35 protein level was largely reduced. In addition, the apical dendrites of miR-Vps35-1 neurons were much shorter as compared to that of control neurons, which formed apical dendritic tufts in the superficial region of CA1 (Fig. 2A,B). The miR-Vps35-3 apical dendrites also displayed a similar but less severe phenotype as compared to that of miR-Vps35-1 (Fig. 2B,C), suggesting a Vps35 dose-dependency. The shortened apical dendrite phenotype developed initially at P7, a stage when control apical dendrites have not fully arborized (supplementary material Fig. S2). The loss of apical dendritic tufts in miR-Vps35-1 expressing CA1 neurons was not corrected at later stages of the development (e.g., P14 and P25) (Fig. 2C). Moreover, a reduced dendritic spine density with an increased spine head size was also observed in the Vps35 deficient CA1 neurons (Fig. 2D–F). These morphological dendritic defects suggest the importance of VPS35/retromer in promoting CA1 dendritic growth or maturation in developing CA1 neurons, and reveal a role for VPS35 in keeping healthy dendritic spine structure, which is critical for synapse formation and function.
We then asked if CA1 neuronal axonal outgrowth and morphology were affected by suppression of Vps35 expression. Hippocampal commissures (HCC), which contain most axons from CA1 neurons, were examined, and no significant defect was observed in viewing axonal length/outgrowth between the control and miR-Vps35 expressing neurons (data not shown). However, in comparing with the control miRNA expressing axons in the HCC, a marked increase of axonal swellings or spheroid formation (viewed by GFP with area size $10 \mu m^2$) was observed in Vps35-deficient axons (Fig. 3A–C). The axonal swelling sizes were measured, and a statistical analysis was performed (Fig. 3D). The spatial distribution pattern of axonal swellings was quantified and illustrated, and each dot represents a spheroid with size $> 10 \mu m^2$. Data were from 6 brains at P10.
spheroid formation appeared to be more severe in the distal axons or in HCC after crossing midline as compared with that before crossing (Fig. 3D), and more obvious in miR-Vps35-1 expressing neurons as compared with that of miR-Vps35-3 (Fig. 3B,C). The axonal spheroid phenotype was not confined to HCC, but also present in callosal axons (CC) from cortical pyramidal neurons (data not shown).

Taken together, loss of VPS35 expression in hippocampal neurons caused apical dendrite growth defects, spine malformation, and swollen commissural axons, particularly in distal regions of dendrites and axons, during hippocampal development.

Defective retrograde trafficking of BACE1 in Vps35 deficient hippocampal neurons

The axonal swellings in Vps35 deficient neurons may reflect in a defective protein transport or trafficking. We thus examined if retromer cargos, including APP, SorLA, and BACE1, or subcellular organelles (early and late endosomes) were “trapped” in the spheroids due to Vps35 deficiency. Interestingly, APP and BACE1, but not SorLA, were identified in the spheroids (Fig. 4A,B,D). Also detected in the spheroids was the late endosome/early lysosome marker, LAMP1, but not early endosome marker, EEA1 (Fig. 4A,D). The absence of SorLA and EEA1 signals in the spheroids was not due to the inefficiency of the antibodies, as these antibodies recognized SorLA and EEA1 proteins as punctae patterns in CA1 soma and dendritic, particular basal dendritic compartments (Fig. 4B). Together, these results implicate that BACE1 and APP, possibly in the late endosomes, may require VPS35/retromer for their retrograde trafficking.

We further tested this view by examining BACE1’s distribution and trafficking in Vps35 deficient neurons in culture and in vivo using BACE1-mCherry. BACE1-mCherry was predominantly distributed at the peri-nuclear vesicles, close to Golgi apparatus in the soma, and dendritic compartments of control neurons in culture (Fig. 5A–C). In contrast, the BACE1 punctae in Vps35 depleted neurons were enlarged in size, and no...
longer confined to the Golgi apparatus (Fig. 5A–C), suggesting the necessity of VPS35 in enriching BACE1 localization to the Golgi apparatus and proximal dendrites in neurons. Also observed was GFP “aggregates” or spheroid-like punctae in Vps35 deficient neurites (Fig. 5D,E). Further support for Vps35 regulating BACE1 trafficking in vivo was the observation of altered BACE1-mCherry distribution in Vps35 deficient mouse CA1 neurons by in utero electroporation (Fig. 6). It was largely distributed in the major apical dendrites of the control CA1 neurons, with peak level at the apical side of proximal dendrites, where Golgi or TGN is located (Fig. 6A,B) (data not shown). However, in CA1 neurons expressing miR-Vps35-1, BACE1-mCherry was distributed in both apical and basal dendrites without the enrichment at the proximal apical dendrites (Fig. 6B,C). Many BACE1-puncta were shifted to the basal side (Fig. 6C) and enlarged in size (Fig. 6D), in addition to be detected in the axonal spheroids in Vps35 deficient CA1 neurons (Fig. 6E). These results suggest that VPS35 may promote endosome-to-Golgi retrograde trafficking of BACE1 not only in primary rat hippocampal neurons, but also in mouse CA1 neurons, not only in dendrites, but also in axons.

To further test if BACE1’s retrograde trafficking is affected in Vps35 deficient neurons, we viewed BACE1-mCherry’s movement in control and Vps35 depleted neurons by time-lapse imaging analysis. BACE1-mCherry labeled vesicles exhibited both active anterograde and retrograde movement along neurites in the control neurons (Fig. 7A–D). In contrast, Vps35 depletion resulted in a defective retrograde movement of BACE1-mCherry towards the soma, without obvious effect on its anterograde movement (Fig. 7A–D). Consequently, the ratio of BACE1-mCherry vesicle in the stationary phase was increased in the Vps35 deficient neurons (Fig. 7E). These results thus demonstrate that Vps35 depletion impaired retrograde trafficking/transport of BACE1 in neurons.

Rescue of Vps35 deficiency induced dendritic and axonal deficits in CA1 neurons suppressing BACE-1 expression

We next asked if BACE1 contributes to Vps35 deficiency induced CA1 neuropathology. To this end, we examined whether suppressing BACE1 can rescue Vps35 deficiency induced dendritic and/or axonal phenotypes. The plasmids encoding miRNA-Bace1 (miR-Bace1) were generated, and the miR-Bace1-1 suppressed BACE1 expression specifically and efficiently (supplementary material Fig. S3A). This plasmid was thus co-electroporated with miR-Vps35-1 in mouse embryos (E15.5), and their apical dendrites and axons at P10 were evaluated in comparison with that expressing miR-Vps35-1 with the control (Fig. 8A). Remarkably, both distal dendritic loss and axonal spheroid formation were greatly rescued when miR-Bace1-1 was co-expressed (Fig. 8A–C). This rescue effect was
not due to the inhibition of miR-Vps35-1’s suppressing activity on Vps35 expression, as Vps35’s expression in CA1 neurons was also markedly reduced by both electroporations (miR-Vps35+control vs. miR-Vps35-1+miR-Bace1) (supplementary material Fig. S3B). These results suggest that the morphological changes in Vps35 deficient CA1 neurons are BACE1-dependent.

Discussion
In this paper, we showed that loss of Vps35 expression in developing mouse hippocampal neurons results in developmental defects, including shorter apical dendrites, reduced dendritic spines, and increased swollen axons. We further showed that BACE1, a critical cargo of retromer in CA1 neurons, contributes to Vps35 deficiency induced CA1 neuropathology during development. These observations thus establish an important role for VPS35 in promoting retrograde transport of BACE1 in developing hippocampal neurons, leading to a working model depicted in Fig. 8D, and providing insights into the pathogenesis of neurodegenerative disorders in adults.

Using in utero electroporation of miRNA to suppress Vps35 expression in embryonic CA1 neurons in a wild type background, we are able to assess the cell autonomous function of Vps35/retromer during mouse hippocampal development. The morphological phenotypes due to Vps35 depletion in developing CA1 neurons suggest a dependence of VPS35/retromer in hippocampal neuron morphogenesis in vivo. In carefully examining the phenotypes, it was revealed that the loss of apical dendritic tuft was restricted to the distal, but not proximal, regions of Vps35 deficient CA1 neurons, and the axonal swollen were more severe in HCC after midline crossing (Fig. 3D). These observations thus suggest that VPS35/retromer may have a more important role in the distal regions of dendrites and axons, implicating a neuronal regional dependence of VPS35. The regional dependence between proximal and distal dendrites of CA1 neurons is also reported in adult rat hippocampal slices, in which L-LTD (longer-lasting forms of long term depression) is induced in the distal, but not proximal, regions of CA1 neurons (Parvez et al., 2010). In addition, the cytoplasmic dynein heavy chain 1, a motor moving toward the minus ends of microtubules, also functions in a regional dependent manner (Hirokawa et al., 2010). It is essential for retrograde transport of cargos in distal dendrites and axons, but not in proximal dendrites, where dynein conveys cargos to both the periphery and soma regions (Hirokawa et al., 2010) (Fig. 8D). This regional dependent dynein-mediated transport is believed due to the mixed polarity of the microtubules in the proximal dendrites, but highly polarized microtubules in the distal dendrites and axons (Conde and Cáceres, 2009; Hirokawa et al., 2010). In light of these observations, we speculate that the regional dependent phenotypes in Vps35 deficient CA1 neurons may reflect in its function in promoting dynein mediated retrograde transport of cargos (Fig. 8D). Further support for this view are observations that SNX5/6, a subcomponent of retromer, interacts with not only BACE1 (Okada et al., 2010), but also p150Glued component of dynactin, an activator of dynein motor complex (Hong et al., 2009; Wassmer et al., 2009), and that impaired retrograde, but not anterograde, movement of BACE1 was observed in Vps35 deficient neurons (Fig. 7). These results thus reveal a molecular link underlying VPS35/retromer regulating dynein mediated retrograde transport (Fig. 8D).

BACE1, an essential membrane proteinase for APP metabolism, is involved in AD pathogenesis (Vassar and Kandalepas, 2011). It is believed to be a cargo of retromer based on cell culture studies

Fig. 6. Altered BACE1 distribution in Vps35 deficient mouse CA1 neurons. Co-electroporation of BACE1-mCherry with control miRNA or miR-Vps35-1 was performed at E15.5 embryos in utero and brain slices were examined at P10. (A) Representative images showing BACE1-mCherry distribution in the proximal apical dendrites of the CA1 neurons. Inserts of enlarged images showing basal shift of BACE1-mCherry signal in VPS35 deficient neurons. Scale bar: 5 μm. (B) Sample images at higher magnification showing BACE1-mCherry aggregation and basally shifted redistribution. Scale bar: 2.5 μm. (C) Quantification analysis of BACE1-mCherry distribution in miR-VPS35-1 neurons and control neurons. Basal shift index (BSI, see Materials and Methods) was introduced to judge the degree of BACE1-mCherry redistribution from apical to basal side of the neuron. Bars showing average BSI (Control: 46.8; miR-VPS35-1: 87.5; n = 54 from 3 brains for each group). (D) Quantification analysis of BACE1-mCherry aggregation in miR-VPS35-1 neurons and control neurons. The size of BACE1-mCherry puncta was measured (see Materials and Methods) (n = 300 from 3 brains for each group). The bars showing average size of BACE1-mCherry puncta (Control: 0.57 μm²; miR-VPS35-1: 1.29 μm²). (E) Representative images of BACE1-mCherry distribution in control and Vps35 deficient CA1 axons in HCC region. Scale bar: 50 μm.
Fig. 7. Defective BACE1-mCherry retrograde trafficking in primary hippocampal neurons expressing miR-Vps35-1. (A) Representative images showing distribution patterns of BACE1-labeled vesicles in control and miR-Vps35-1 expressing hippocampal neurons. Neurons were co-transfected with BACE1-mCherry with control and miR-Vps35-1 at DIV5 and followed by time-lapse imaging analysis 48 hours after transfection. Scale bar: 2 μm. (B) Representative kymographs showing the mobility of BACE1 positive vesicles/endosomes during 15-min recordings in control and miR-Vps35 expressing neurons. Vertical lines represent stationary BACE1-vesicles; oblique lines or curves to the right represent anterograde movements and lines to the left indicate retrograde transport. (C–E) Relative mobility (anterograde, retrograde, and stationary) of BACE1-vesicles in control and miR-Vps35-1 expressing neurons. Data were quantified from the total number of 17 BACE1-vesicles in neurons from >3 experiments, as indicated in parentheses. Error bars: S.D. *P<0.01.
Our current work supports this notion and provides further evidence for BACE1 as a cargo of retromer not only in neuronal culture but also in mouse. Our studies also suggest that the BACE1 contributes to the Vps35 deficiency induced phenotypes, as suppressing BACE1 expression in CA1 neurons rescued the phenotypes (Fig. 8), revealing the importance to control BACE1 trafficking and distribution by retromer.

In addition to a defective retrograde trafficking of BACE1, Vps35 depletion in CA1 neurons also results in a loss of dendritic spine. Dendritic spines are small actin-rich protrusions that form the postsynaptic part of most excitatory synapses (Hoogenraad and Akhmanova, 2010). They are highly dynamic structures and play crucial roles in synaptic functions during learning and memory (Hoogenraad and Akhmanova, 2010; Svitkina et al., 2010). It is unclear how VPS35 regulates dendritic spine dynamics. The decreased dendritic spines may be due to a defective membrane protein trafficking and/or actin remodeling in Vps35 deficient CA1 neurons. In fact, retromer/VPS35 is implicated in actin remodeling as it interacts with WASH protein complex (Seaman, 2007; Gomez and Billadeau, 2009; Harbour et al., 2012), an important complex for actin remodeling, receptor endocytosis, and tubulin cross talk. Thus, it is conceivable that loss of Vps35 function may results in an impaired WASH1 mediated actin remodeling in dendrites, leading to a reduction in dendritic spine density but increase in the spine head size. However, this speculation requires further investigation.

It is worth noting that VPS35 deficiency induced dendritic and axonal phenotypes in developing brain resemble to the morphological changes observed in neurodegenerative diseases in adult. However, we did not observe obvious neuronal loss even at 6-month-old Vps35+/m mice (data not shown), suggesting that ~50% reduction in VPS35 expression alone in neurons does not result in neurodegeneration in young animals. Due to the limitation of our in vivo knock-down approach, which does not allow longer term (e.g., > 40 days after electroporation) expression of the miRNAs, we are unable to address whether vPS35 deficiency causes neuronal degeneration in older mice.

Fig. 8. Rescue of Vps35 deficiency induced axonal spheroids and apical dendritic arborization defect by suppressing BACE1 expression. Co-electroporation of miR-VPS35-1 with control construct or miR-BACE1 was performed at E15.5 in utero and brains were examined at P10. (A) Effects of miR-BACE1 on miR-VPS35 induced axon- and dendrite-defects. Upper panels, similar distribution pattern and cell density of electroporated cells between miR-VPS35-1+control and miR-VPS35-1+miR-BACE1 shown in low magnification. Middle panels, apical dendrites in the distal region were much longer in miR-VPS35-1+miR-BACE1 than in miR-VPS35-1+control. Lower panels, spheroids in miR-VPS35-1+miR-BACE1 expressing axons in HCC and CC regions were greatly reduced compared to that in miR-VPS35-1+control. Scale bar: 50 μm. (B) Quantification of apical dendrite growth by measuring normalized length of apical dendrites. miR-VPS35-1+miR-BACE1 expression dendrites were significantly longer than control miR-VPS35-1 dendrites (*P<0.01). (C) Quantification of axon spheroid formation by measuring the size of swellings in commissural axons (n=300 from 3 brains for each group). Bars showing average size of selected swellings (miR-VPS35-1+control: 9.16 μm²; miR-VPS35-1+miR-BACE1: 5.36 μm²). The percentage of spheroids (<10 μm²) is 29% for miR-VPS35-1+control and 5% for miR-VPS35-1+miR-BACE1. (D) Schematic illustration of a working model for VPS35 containing retromer in promoting retrograde transport of BACE1.
This will again need more systematic studies by employing strategies such as conditional knock-out of Vps35. Nevertheless, the molecular mechanisms for VPS35 deficiency induced deficits in developing hippocampus may facilitate the pathogenic dissection of mechanisms underlying Vps35-loss associated neurodegeneration in mature neurons.

In summary, the data presented in this manuscript suggest an important function of VPS35 in dendritic growth or maturation and in preventing axonal spheroid formation during mouse hippocampal development. These functions may be due to reтрomer regulation of dynactin/dynein mediated retrograde transport of membrane proteins, such as BACE1. It remains an open question whether the early developmental defects induced by VPS35 deficiency may partially contribute to the evolution of neurodegeneration by VPS35 mutation observed in human genetic diseases including AD and PD.

Materials and Methods

Reagents and animals

Rabbit polyclonal anti-Vps35 antibody was generated using the antigen of GST-VPS35D1 fusion protein as described (Small et al., 2005b; Wen et al., 2011). Vps35 mutant mice were generated by injection of mutant embryonic stem (ES) cells obtained from Bay Genomics as described previously (Wen et al., 2011). The wild type pregnant mice in CD1 background were used for in utero electroporation. All experimental procedures were approved by the Animal Subjects Committee at the Georgia Health Sciences University, according to US National Institutes of Health guidelines.

Expression plasmids

The miRNA-Vps35 or miRNA-BACE1 expression vectors were initially generated by the BLOCK-it Poll miRNA expression System (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction (Wen et al., 2011). Oligonucleotide sequences for miRNA constructs were below.

miR-VPS35-1: 5’-GCTGTAAGGTCCTACACATTTACGCTGGTGCCACTGACGAA-GGTGTAAGGTCACGTTGATTA3’ (sense);

5’-CCTGTAAGGTCCTACACATTTACGCTGGTGCCACTGACGAA-GGTGTAAGGTCACGTTGATTA3’ (antisense);

miR-VPS35-3: 5’-GCTGTAAGGTCCTACACATTTACGCTGGTGCCACTGACGAA-GGTGTAAGGTCACGTTGATTA3’ (sense);

5’-CCTGTAAGGTCCTACACATTTACGCTGGTGCCACTGACGAA-GGTGTAAGGTCACGTTGATTA3’ (antisense);

miR-BACE1: 5’-TGCTGAATGTTGGCACGCACAGTGACGTTTTGGCCACTGACGAA-GGTGTAAGGTCACGTTGATTA3’ (sense);

5’-TGCTGAATGTTGGCACGCACAGTGACGTTTTGGCCACTGACGAA-GGTGTAAGGTCACGTTGATTA3’ (antisense);

Targeting sequence: GAGGTTAAGGTCACGTTGATTA (antisense).

For analysis of BACE1-mCherry puncta size, 5 neurons from one brain were examined. The size and distance of each spheroid was plotted. For analysis of BACE1-mCherry puncta size, 5 neurons from one brain were examined. The size and distance of each spheroid was plotted.

Immunofluorescence staining and confocal imaging analysis

For immunofluorescence staining of brain slices, age-matched electroporated littermates were perfused transcardially with 4% paraformaldehyde in PBS and brains were post-fixed at 4°C for 24 hours. Floating slices (50 μm in thickness) were incubated with indicated 1st and 2nd antibodies as described previously (Wen et al., 2011). The immunostained slices were then incubated with the PBS for an hour before imaging. For immunofluorescence staining analysis of transected neurons, primary cultured neurons on the coverslips were fixed with 4% paraformaldehyde and 4% sucrose at room temperature for 45 min, followed by 0.1% Triton X-100 for 8 min, and then subjected to immunostaining analysis using indicated antibodies as described previously (Zhu et al., 2007). Confocal images were obtained using Nikon C1 confocal system (for brain slices) or Zeiss LSM 510 (for culture neurons).

Image acquisition and data analysis

All the measurements on the brain sections were performed at the approximate level of bregma −2.18. For analysis of apical dendritic length of CA1 pyramidal neurons, the total area of EGF labeled apical dendrites from the exit point of pyramidal cell layer to the distal end of apical dendrites were measured and normalized to the total area from the exit point of pyramidal cell layer to the pia surface (the boundary between CA1 and dentate gyrus). One normalized value of apical dendrite length was obtained from one brain and a total of 3–4 brains were measured for each group. For analysis of spine morphology, a total of about 2 mm dendrites from 3 brains for each group were examined. Spine density was calculated as the average number of spines per 20 μm dendrites length. The sizes of 50 largest spines from each section (one section from one brain) were identified and their head sizes were measured. 300 values of each group were pooled together for comparison between groups. For analysis of axonal spheroid formation in commissural axons, the sizes of 100 largest swellings from each section (one section from each brain) were measured and a total of 3 brains were examined for each group. 300 values of each group were pooled together for comparison between groups. For analysis of distribution pattern of axonal spheroids, the sizes of axonal swellings (> 10 μm)2 and their distances relative to the brain midline were measured and a total of 6 brains electroporated with miVPS35-1 were examined. The size and distance of each spheroid was plotted.

Cell culture, transient transfection, and kymographs

HEK293 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, and 100 units/mL of penicillin G and streptomycin (Gibco). Primary rat E18 hippocampal neurons were cultured as previously described (Zhu et al., 2007). Calcium phosphate method was used for transfection of HEK293 cells. Forty-eight hours following transfection, cells were lysed in modified RIPA immunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% NP40, 0.25% sodium-deoxycholate, protease inhibitors). Lysates and medium were subjected to immunoblotting analyses. Neurons were transfected with various constructs at DIV3 using the calcium phosphate method followed by immunocytochemistry or time-lapse imaging analysis 48 hours after transfection as described previously (Zhu et al., 2010).
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