VPS35 haploinsufficiency increases Alzheimer’s disease neuropathology

Lei Wen,1,2 Fu-Lei Tang,1,2 Yan Hong,1,2 Shi-Wen Luo,1,2 Chun-Lei Wang,1,2 Wanxia He,3 Chengyong Shen,1,2 Ji-Ung Jung,1,2 Fei Xiong,1,2 Dae-hoon Lee,1,2 Quan-Guang Zhang,1,2 Darrell Brann,1,2 Tae-Wan Kim,4,5 Riqiang Yan,3 Lin Mei,1,2 and Wen-Cheng Xiong1,2

1Institute of Molecular Medicine and Genetics and 2Department of Neurology, Medical College of Georgia, Georgia Health Sciences University, Augusta, GA 30912
2Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195
3Department of Pathology and Cell Biology and 4Taub Institute for Research on Alzheimer’s Disease and the Aging Brain, Columbia University Medical Center, New York, NY 10032

VPS35, a major component of the retromer complex, is important for endosome-to-Golgi retrieval of membrane proteins. Although implicated in Alzheimer’s disease (AD), how VPS35 regulates AD-associated pathology is unknown. In this paper, we show that hemizygous deletion of Vps35 in the Tg2576 mouse model of AD led to earlier-onset AD-like phenotypes, including cognitive memory deficits, defective long-term potentiation, and impaired postsynaptic glutamatergic neurotransmission in young adult age. These deficits correlated well with an increase of β-amyloid peptide (Aβ) level in the mutant hippocampus. We further demonstrate that VPS35 is predominantly expressed in pyramidal neurons of young adult hippocampus and interacts with BACE1, a protease responsible for Aβ production. Loss of VPS35 function in the mouse hippocampus increased BACE1 activity. Suppression of VPS35 expression in culture decreased BACE1 trans-Golgi localization but enriched it in endosomes. These results demonstrate an essential role for VPS35 in suppression of AD neuropathology and in inhibition of BACE1 activation and Aβ production by promoting BACE1 endosome-to-Golgi retrieval.

Introduction

Aβ, a 40–42 peptide released from amyloid precursor protein (APP) after the sequential cleavages by β- and γ-secretases, is recognized as a major culprit of Alzheimer’s disease (AD), the most common dementia in people >65 yr of age. Cortical and cerebral vascular Aβ deposition is a pathological hallmark in AD, in addition to neurofibrillary tangles, chronic inflammation, and neuronal loss (Tanzi and Bertram, 2005). Mutations in the genes of APP and presenilin, an essential component of γ-secretase, are found in the early-onset AD. Many risk factors or genes associated with the late-onset AD also affect APP protein metabolism and Aβ production (Tanzi and Bertram, 2005). Multiple forms of Aβ have been reported, but the prefibrillar and/or soluble oligomers of Aβ, not Aβ deposits, have been recognized to be early and key intermediates in AD-related synaptic dysfunction (Walsh et al., 2005; Smith et al., 2007; Stefani and Liguri, 2009). Mouse brain slices exposed to soluble Aβ oligomers exhibit synaptic depression, decrease of the number of spine protrusions or synaptic contacts, and impaired long-term potentiation (LTP), a form of synaptic plasticity (Roselli et al., 2005; Townsend et al., 2006; Nimmrich et al., 2008; Selkoe, 2008; Li et al., 2009). In addition, synaptic function is disrupted, and synapses are lost in AD patients and in animal models even before amyloid plaques are detected (Selkoe, 2002a, 2008; Ashe and Zahs, 2010). Collectively, these lines of evidence from pathological, genetic, biochemical, and physiological studies have indicated a crucial role of Aβ in the etiology of AD.

Extensive efforts have been invested to the understanding of Aβ production. β-secretase, also called BACE1, is essential...
for this event (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999, 2009; Yan et al., 1999; Tanzi and Bertram, 2005; Bertram and Tanzi, 2008). BACE1 activity is increased in postmortem brains of AD patients and in brains of AD mouse models (Fukumoto et al., 2002; Yang et al., 2003). Genetic ablation of BACE1 in AD animals abolishes Aβ production and rescues AD-like phenotypes (Luo et al., 2001; Ohno et al., 2004; Laird et al., 2005). BACE1 is a type I transmembrane aspartyl protease that has a broad substrate profile, including sialyltransferase, P-selectin glycoprotein ligand 1, sodium channel, and APP-like proteins, in addition to APP (Vassar et al., 2009). An in vitro assay for this event has suggested that the optimal pH of BACE1-mediated APP cleavage is pH ~5.0, suggesting that an acidic intracellular compartment (e.g., endosome) may be more favorable for BACE1 activation (He et al., 2003). After the cleavage by BACE1, γ-secretase—a multimeric complex that contains presenilins, nicastrin, aph-1, and pen2—cleaves the membrane-anchored C-terminal fragment of APP to release Aβ40/42 (Selkoe and Wolfe, 2007). Genetic mutations surrounding the γ-cleavage sites in APP can alter the ratio of Aβ40 versus more toxic and amyloidogenic Aβ42 (Selkoe and Wolfe, 2007). Mutations in presenilin genes in the early-onset AD patients may cause a gain of function of γ-secretase, leading to the increase in Aβ42 to Aβ40 ratio (Selkoe, 2002b; Sisodia and St George-Hyslop, 2002). Finally, APP can also be cleaved by α-secretases, including ADAM10, that switch APP processing to a nonamyloidogenic pathway and thus reduce the Aβ40/42 production (Esler and Wolfe, 2001).

The retromer contains two subprotein complexes: the cargo-selective subcomplex and membrane deformation subcomplex (Seaman, 2005; Bonifacino and Hurley, 2008). 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 subcomplex, consisting of sorting nexin dimers (Vps5p and Vps17p) in yeast and sortin nexins 1/3 or 5/6 in vertebrates, deforms the donor membrane into a tubular profile (Seaman, 2005; Bonifacino and Hurley, 2008; van Weering et al., 2010). The retromer selectively mediates retrograde transport of multiple transmembrane proteins from the endosomes to the TGN. They include mannose 6-phosphate receptors (Ariyih et al., 2004), wntless (a receptor for Wnt morphogens; Belenkya et al., 2008; Eaton, 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008), Cdc1 (a phagocytic receptor; Chen et al., 2010), and VPS10 family proteins, such as VPS10 in yeast (Iwaki et al., 2006), sortlin, and sortlin-related receptor (SorL1 or SorCS1) in vertebrates (Canuel et al., 2008; Kim et al., 2010; Okada et al., 2010). Recent studies have suggested that SorL1, also called SorLA and LR11, is an APP-binding protein and is involved in the pathogenesis of late-onset AD (Rogaeva et al., 2007; Willnow et al., 2010). In addition, the cargo-selective retromer proteins VPS35 and VPS26 are decreased in the postmortem hippocampus of AD patients (Small et al., 2005). Moreover, VPS26 heterozygote mice show increased levels of endogenous Aβ and impaired LTP (Muhammad et al., 2008). These observations suggest that the retromer may be involved in the AD pathogenesis. However, the underlying mechanisms remain elusive.

To elucidate functions of the retromer in AD pathogenesis, we crossed Tg2576 mice, a well-established AD mouse model that expresses the Swedish mutant form of human APP (APPSwe) under the control of prion promoter (Chapman et al., 1999; Rockenstein et al., 2007), with Vps35 mutant mice. Vps35 homozygous mutant mice died during early embryonic development, but heterozygotes can survive into adulthood with relatively normal behavior, synaptic plasticity, and life span. Remarkably, Vps35 heterozygotes;Tg2576 mice demonstrated an earlier onset of AD-like neuropathology, including cognitive deficits, impaired glutamatergic transmission, and synaptic plasticity, and more Aβ-positive plaques detected in the brain. These earlier-onset AD-like phenotypes in the double mutant mice suggest that reduced VPS35 levels may exacerbate Aβ toxicity. We have investigated underlying mechanisms, and our results suggest that VPS35 is required for the endosome-to-Golgi retrieval of BACE1.

**Results**

**Genetic enhancement and earlier onset of Tg2576-associated cognitive deficit by loss of VPS35 function**

Defective spatial learning and memory are known to be early symptoms of AD (Middeld et al., 2006; Bizon et al., 2007; Good and Hale, 2007; Reed et al., 2010). They are also observed in 8-mo or older Tg2576 mice that express APPswe (Hsiao et al., 1996; Chapman et al., 1999; Rockenstein et al., 2007). To study the potential role of VPS35 in AD pathology, we generated Vps35-deficient mice by using gene-trapping embryonic stem (ES) cells from Bay Genomics (see Materials and methods; Fig. S1 A). Homozygotes, named Vps35−/−, were lethal at an early embryonic stage (under embryonic day 10 [E10]), but heterozygotes, named Vps35+/−, whose expression of Vps35 was ~50% of that in wild type (WT; Fig. S1 B), survived to adulthood. We crossed Vps35−/− mice with Tg2576 mice to generate Vps35−/−;Tg2576 mice (Fig. S1 B) and subjected them and control littermates (WT, Tg2576+/+) to the Morris water maze test to assess their spatial learning and memory (Morris, 1991). In this assay, 3- and 6-mo-old mice were dropped into a pool and allowed to swim to locate a submerged platform to escape swimming. Tg2576 and Vps35−/− mice had normal spatial learning and memory response as compared with the age-matched WT control in viewing their learning the location of the submerged platform with repeated testing (Fig. 1). However, the double mutant (Tg2576−/−;Vps35−/−) mice were impaired in this task, exhibiting increases of latencies in finding the hidden platform (Fig. 1, A and B) and in pathway length (Fig. 1, C and D) as compared with the other three age-matched groups. In addition, Tg2576−/−;Vps35−/− mutant mice performed worse than all other groups in probe trials, with less frequency to probe the hidden platform (Fig. 1, E and F). Note that no significant difference was observed in velocity among the four groups (Fig. 1, G and H). Intriguingly, deficits of Tg2576−/−;Vps35−/− mice in latency and distance and platform probe frequency appeared to be severer in 6-mo-old Tg2576−/−;Vps35−/− mice compared with those in 3-mo-old (Fig. 1, A–F). Moreover, double mutant mice at 1–2 mo
Figure 1. Impaired spatial learning and memory by the Morris water maze test in young adult Tg2576+/Vps35+/mice. (A–H) 3 (A, C, E, and G)- and 6 (B, D, F, and H)-mo-old Tg2576+/Vps35+/m mice showed increases of swimming latency (A and B) and total distance (C and D) in the training sessions but decreases of the platform probe frequency (E and F) in the probe tests relative to theagematched Tg2576+/+, Vps35+/+, and nontransgenic (WT) mice. Although Tg2576+/+, Vps35+/+, showed slightly higher velocity at day 1–3 sessions, repeated measures ANOVA analysis of the total five sessions showed no significant change of the velocity among the four groups in both 3- and 6-mo-old mice (G and H). Comparing the performance between 3- and 6-mo-old Tg2576+/+;Vps35+/+ mice, a trend toward longer latency and distance was noted in the 6-mo-old mice, but the difference was not significant (P > 0.05, using repeated measures ANOVA). However, the platform probe frequency was significantly reduced in 6-mo-old Tg2576+/+;Vps35+/+ mice as compared with that in 3-mo-old (P < 0.05). Data shown are mean ± SEM, and n = 6–10 for each of the genotypes in 3-mo-old mice, whereas n = 5–11 for each of the genotypes in 6-mo-old mice. *, P < 0.05, with a significant difference from the Tg2576+/+, Vps35+/+, and WT control mice by repeated measures ANOVA; #, P < 0.05; ##, P < 0.01, with a significant difference by oneway ANOVA followed by the least significant difference test.

old did not exhibit significant impairment (unpublished data). Thus, these results demonstrate an impaired spatial learning and memory in the double mutant mice at the age of 3 and 6 mo old, suggesting an earlier onset of Tg2576-associated cognitive deficit by loss of VPS35 function.
both AMPA and NMDA receptor-mediated mEPSCs in the double mutant CA1 neurons (Fig. 3, A, B, E, and F). This may be a result of a defective presynaptic glutamate release or reduction of the number of synapses. To further examine whether this is a result of a presynaptic defect, paired-pulse facilitation was measured. The ratio of paired-pulse facilitation was not significantly different among the four groups of mice (Fig. 3 H), suggesting that the decreased frequencies of both currents in the double mutant CA1 neurons may be caused by a reduction of the number of postsynaptic glutamatergic neurons/synapses. Consistent with the results in mEPSC recordings, both amplitudes of evoked AMPA receptor-mediated and NMDA receptor-mediated EPSCs were lower in CA1 neurons from the double mutant slices as compared with the other three groups (Fig. 3 G), confirming the deficits of the glutamatergic synaptic transmission at the SC-CA1 synapses of young adult Tg2576+/−;Vps35+/m mice.
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Figure 3. Deficit in glutamatergic synaptic transmission in hippocampal CA1 pyramidal neurons of young adult Tg2576+/−;Vps35+/− mice. (A and B) Representative traces of AMPA (A) and NMDA (B) receptor-mediated mEPSCs. All mEPSCs were recorded at a holding potential of −65 mV. NMDA receptor-mediated mEPSCs were recorded in Mg2+-free aCSF (see Materials and methods). Bars, 200 ms, 40 pA. (C and D) Cumulative distribution plots (left) and mean amplitude of AMPA receptor-mediated mEPSCs (C, right) and NMDA receptor-mediated mEPSCs (D, right). (E and F) Cumulative frequency plots of the interevent interval (left) and quantitative analysis of the frequency of AMPA receptor-mediated mEPSCs (E, right) and NMDA receptor-mediated mEPSCs (F, right). (G, bottom) Representative traces of eEPSCs (NMDA [right] and AMPA [left]) and quantitative analysis of evoked AMPA and NMDA receptor-mediated EPSC amplitude to stimulus intensity. AMPA and NMDA receptor-mediated EPSCs were recorded at a holding potential of −65 mV and +40 mV, respectively. (Top) From left to right, the stimulus intensities for each trace were 2, 5, 10, and 30 µA (saturating stimulus). (H) Paired-pulse ratio analysis. Data were obtained from whole-cell recordings of CA1 pyramidal neurons from the four indicated groups of mice at the age of 2 mo old, including WT, Tg2576+/−; Vps35+/−, and Tg2576+/−;Vps35+/− mice. (C–H) Data shown as mean ± SEM were analyzed by one-way ANOVA followed by Dunnett’s test (for averaged mEPSC amplitude and frequency and paired-pulse ratio), Kolmogorov–Smirnov test (for mEPSC cumulative histograms), and repeated measures ANOVA (for eEPSC amplitude). *, P < 0.05; **, P < 0.01; n = 9–16.

Note that Tg2576+/− and Vps35+/− mutants also showed reductions of the amplitudes and frequencies of both AMPA and NMDA receptor-mediated mEPSCs and the amplitudes of both evoked AMPA and NMDA receptor-mediated EPSCs as compared with the WT control (Fig. 3, A–G), but these reductions were not statistically significant. We also examined GABAergic neurotransmission in the four groups of mice by evaluating the miniature and evoked inhibitory postsynaptic currents (mIPSCs and

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Interestingly, there are more, larger plaques in both 6- and 12-mo-old Tg2576+/−;Vps35+/m brains in comparison with those in Tg2576 brain samples at respective ages (Fig. 4, A and B). These results demonstrate that Aβ deposits occurred in higher frequency and in younger Vps35+/m;Tg2576+/- mice than that in Tg2576 mice.

To investigate mechanisms of how Aβ deposits were increased when VPS35 levels were reduced, we measured Aβ40 levels in hippocampus and cerebral cortex. Tg2576 mice express the Swedish mutant form of human APP695 (Hsiao et al., 1996; Chapman et al., 1999; Rockenstein et al., 2007). ELISA analysis of human Aβ40 detected Aβ in homogenates of hippocampus and cortex from Tg2576 mice, which is in agreement with previous studies (Hsiao et al., 1996; Chapman et al., 1999; Rockenstein et al., 2007). No human Aβ40 was detected in WT or Vps35+/m mice (unpublished data). Remarkably, Aβ40 levels were significantly higher in 2-mo-old Tg2576+/-;Vps35+/m mice (compared with WT) and Tg2576+/-;Vps35+/m mice (compared with Tg2576+/-). (C) Hippocampus and cortex from WT and indicated mutant mice were homogenized with guanidine HCl extraction buffer, and the homogenates were analyzed by ELISAs for human (C) and mouse (D) Aβ1–40. Data, shown as mean ± SEM, were analyzed by Student’s t test. *, P < 0.01. n = 4 for each of the genotypes.

Figure 4. Increase of Aβ deposits and Aβ40 in the hippocampus and cortex by loss of VPS35 function. (A and B) Increase of Aβ deposits in 6-mo or older Tg2576+/-;Vps35+/-m brain as compared with that of Tg2576+/- by immunohistochemical staining analysis using anti-6E10 antibody. Images are shown in A, and quantification data are presented in B (mean ± SEM; n = 4 for each of the genotypes). *, P < 0.01, with a significant difference from the control (Tg2576+/-) by Student’s t test. (C) Increase of human Aβ40 levels in 2-mo-old Tg2576+/-;Vps35+/-m hippocampus and cortex as compared with that of Tg2576+/- mice. (D) Increase of mouse Aβ40 levels in 2-mo-old hippocampus and cortex of Vps35+/-m mice (compared with WT) and Tg2576+/-;Vps35+/-m mice (compared with Tg2576+/-). (A and B) Aβ deposits or plaques were undetectable in brain sections of Vps35+/-;Tg2576+/- mice or the other three genotypes at the age of 2–4 mo old (unpublished data). No Aβ deposit was observed in brain samples of WT or Vps35+/-m mice as old as 12 mo (Fig. 4, A and B). Aβ deposits were rare in 6-mo-old Tg2576+/- brain but increased in number and size in 12-mo-old samples (Fig. 4, A and B), which is in agreement with a previous study (Hsiao et al., 1996). Interestingly, there are more, larger Aβ plaques in both 6- and 12-mo-old Tg2576+/-;Vps35+/-m brains in comparison with those in Tg2576 brain samples at respective ages (Fig. 4, A and B). These results demonstrate that Aβ deposits occurred in higher frequency and in younger Vps35+/-;Tg2576+/- mice than that in Tg2576 mice.

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when VPS35 levels were reduced, suggesting that VPS35 negatively regulates Aβ40 production. To test this hypothesis further, we examine the effect of Vps35 mutation on Aβ40 production from endogenous APP on WT and Tg2576 background. Indeed, ELISA analyses of mouse Aβ40 revealed an increase in the hippocampus and cerebral cortex of 2-mo-old Vps35+/m compared with WT (Fig. 4 D). Moreover, mouse Aβ40 was also higher in the 2-mo-old Tg2576+/−;Vps35+/m hippocampus and cerebral cortex than that of Tg2576 samples (Fig. 4 D). Elevated mouse Aβ40 level was also detected in the hippocampus and cortex of 4–6-mo-old Vps35+/m mice (compared with the WT controls) and Tg2576+/−;Vps35+/m mutant mice (compared with the Tg2576+/− mice; unpublished data). These results corroborate that VPS35 negatively regulates Aβ40 production. In addition to Aβ40, levels of human and mouse Aβ42 were also increased by Vps35+/m mutation (Fig. S3 A and not depicted). However, the ratio of Aβ40/Aβ42 was not changed (Fig. S3 B), implicating that changes in VPS35 levels may not modulate the specificity of the γ-secretase cleavage of APP. Together, these data suggest that VPS35 negatively regulates Aβ production and support the view that increased Aβ levels may be a key detrimental factor for the cognitive and LTP deficits associated with the AD mice.

Expression of VPS35 in hippocampal and layer IV–V cortical pyramidal neurons and interaction of VPS35 with BACE1

We further determined in what cells VPS35 is expressed in the brain by taking advantage of the Vps35+/m mice. Indeed, ELISA analyses of mouse Aβ40 revealed an increase in the hippocampus and cerebral cortex of 2-mo-old Vps35+/m compared with WT (Fig. 4 D). Moreover, mouse Aβ40 was also higher in the 2-mo-old Tg2576+/−;Vps35+/m hippocampus and cerebral cortex than that of Tg2576 samples (Fig. 4 D). Elevated mouse Aβ40 level was also detected in the hippocampus and cortex of 4–6-mo-old Vps35+/m mice (compared with the WT controls) and Tg2576+/−;Vps35+/m mutant mice (compared with the Tg2576+/− mice; unpublished data). These results corroborate that VPS35 negatively regulates Aβ40 production. In addition to Aβ40, levels of human and mouse Aβ42 were also increased by Vps35+/m mutation (Fig. S3 A and not depicted). However, the ratio of Aβ40/Aβ42 was not changed (Fig. S3 B), implicating that changes in VPS35 levels may not modulate the specificity of the γ-secretase cleavage of APP. Together, these data suggest that VPS35 negatively regulates Aβ production and support the view that increased Aβ levels may be a key detrimental factor for the cognitive and LTP deficits associated with the AD mice.

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These results support an interaction between VPS35 and BACE1 in vitro and in vivo and reveal a BACE1-binding site in VPS35 (the amino acids of 390–606; Fig. 6 F).

Increase of BACE1 in endosomal compartments in Vps35-deficient NLT cells and neurons

To investigate the potential function of VPS35–BACE1 interaction, we examined whether BACE1 subcellular distribution is regulated by VPS35. To this end, we generated small hairpin RNAs (shRNAs) and microRNAs (miRNAs) to suppress VPS35 expression. Transfection of shRNA-Vps35 or miRNA-Vps35, but not control (a scrambled miRNA), inhibited expression of cotransfected Flag-Vps35 in HEK293 cells (Fig. S5 A) and endogenous VPS35 in NLT cells (Fig. S5 B). HA-tagged BACE1 was present in perinuclear vesicles, close to Golgi apparatus, in control NLT cells (Fig. 7 A). In cells whose VPS35 expression was suppressed, BACE1 punctae were enlarged in size and no longer confined to the Golgi apparatus (Fig. 7 A). Instead, BACE1 became colocalized with LAMP1, a marker of late endosomes/lysosomes (Fig. 7, A and C), suggesting that VPS35 is critical for BACE1 to be localized in the Golgi apparatus, and, when VPS35 levels were lower, BACE1 is relocated. To further explore the effects of lower VPS35 expression, we examined the distribution of BACE1 in other regions of WT and Vps35−/− mice. In contrast, the levels of VPS35 were low in interneurons, i.e., neurons labeled by parvalbumin in WT mice or GFP in GAD67-GFP mice (Fig. 5, C and D). Subcellularly, VPS35 appeared to be enriched in dendrites that were labeled by MAP2, a dendritic marker in both the cortex and the CA1–3 regions of the hippocampus (Fig. 5 D).

$\beta_{A42}$ production requires BACE1. Thus, we determined whether VPS35 associates with BACE1 in neurons by coimmunostaining analysis. BACE1 and VPS35 were colocalized largely at perinuclear compartments in NLT cells, a cell line derived from GnRH neurons, and in mouse cortical neurons (Fig. 6, A and B). These results implicate a potential interaction between VPS35 and BACE1 in neurons. We further tested this view by coimmunoprecipitation analyses of mouse brain lysates, in which VPS35 was detected in BACE1-immunoprecipitated complexes (Fig. 6 C). We then mapped the interaction domain by in vitro GST pull-down assay and coimmunoprecipitation analysis of lysates from HEK293 cells expressing BACE1 and mutant VPS35. BACE1 displayed seemingly greater binding affinity for full-length VPS35 (VPS35FL) as compared with that of VPS351-390 in in vitro GST pull-down assays (Fig. 6, D and F). In coimmunoprecipitation analysis, however, VPS351-606 showed stronger association than that of the VPS35FL (Fig. 6, E and F).
study this phenomenon, we coexpressed various markers for early, late, and recycling endosomes (GFP-Rab5, Rab7, and Rab11, respectively). As shown in Fig. 7 (B and C), suppression of VPS35 expression increased BACE1 localization in both Rab5- and Rab7-positive endosomal compartments but reduced BACE1 in Rab11-labeled recycling endosomes. Similar effects were observed in NLT cells expressing miRNA-Vps35 (unpublished data). In agreement with this, BACE1-Rab7 colocalization was increased in neurons of Vps35<sup>+/−</sup> mice (Fig. 7, D and E). Collectively, these results demonstrate that VPS35 is required for the retrieval of BACE1 from endosomes to the Golgi apparatus in both NLT and hippocampal neurons (Fig. 7 F).
Increased BACE1-mediated cleavage of APP and BACE1 activity in VPS35 mutant mice

The aforementioned results demonstrate that VPS35 regulates BACE1 subcellular distribution. To determine whether these effects have any functional consequences, we measured BACE1 activity in Vps35+/m hippocampus and cortex. BACE1 activity was first determined by Western blot analysis of BACE1-mediated cleavage fragments (βCTF or C99 and sAPPβ) of APP (Fig. 8 A). Using 6E10, a monoclonal antibody that specifically recognizes the N-terminal 1–16 amino acid residues of human C99 and β40/42, βCTF or C99 was not detected in WT or Vps35+/m but was in Tg2576 hippocampus and cortex (Fig. 8 B). Interestingly, C99 levels were increased in Tg2576+/−;Vps35+/m (Fig. 8, B–D), suggesting an increased activity of BACE1 when Vps35 was deficient. To test this hypothesis further, samples were analyzed by Western blots using a polyclonal antibody that specifically recognizes the sAPPβ and the 6687 polyclonal antibody that recognizes both C99 and C83 (an α-secretase cleavage product; Fig. 8 A). Again, levels of sAPPβ and C99 were increased in Tg2576+/−;Vps35+/m hippocampus compared with Tg2576 alone (Fig. 8, B–F). However, C83 levels were not consistently altered by Vps35 mutation (Fig. 8, C and E), suggesting that Vps35 mutation had no or weak effect, if there is any, on α-secretase activity. Together, these results indicate that loss of Vps35 promotes BACE1-mediated cleavage of APP or APPswe, thereby increasing Aβ production.

We then measured the BACE1 activity directly by an in vitro assay in both hippocampus and cortex from the four different groups at the age of 2 mo old (Fig. 8 G). Approximately 25 and 10% increases of BACE1 activity were found in Tg2576+/− hippocampus and cortex, respectively (compared with the WT control; Fig. 8 G), which is in line with previous studies (Fukumoto et al., 2002; Yang et al., 2003). Interestingly, Vps35+/m hippocampus and cortex exhibited significant increases of BACE1 activity by ~50 and 20%, respectively, as compared with that of WT controls (Fig. 8 G). The double mutant showed a further increase of BACE1 activity in both hippocampus and cortex as compared with the single mutant mice (Fig. 8 G). Note that the increase of BACE1 activity in the mutant hippocampus was more dramatic as compared with...
that in the cortex (Fig. 8 G). Thus, these results support the notion that loss of VPS35 function leads to an increase of BACE1 activity and thus Aβ production.

**Discussion**

VPS35, a major component in the cargo recognition complex of retromer, plays an important role in endosome-to-Golgi retrieval of multiple membrane proteins. Although retromer is implicated in the pathogenesis of late-onset AD, the function of VPS35 in AD pathology and the underlying molecular mechanisms remain largely unclear. In this study, we showed that VPS35 regulates BACE1 cellular trafficking, and loss of Vps35 function leads to an increase of BACE1 activity and Aβ levels (Figs. 4–8). Tg2576 mice with reduced VPS35 expression exhibit an earlier onset of AD-like phenotypes, including cognitive memory deficits, reduced LTP, and decreased glutamatergic neurotransmission in 2-mo-old mice (Figs. 1–3). We also demonstrated that knocking down VPS35 expression in cultured cells increased BACE1 localization to the endosomal compartments where the BACE1 cleavage of APP is favored, providing a mechanism underlying VPS35/retromer regulation of BACE1 activation (Fig. 7 F). Moreover, we demonstrated that VPS35 is highly expressed in pyramidal neurons in young adult CA1–3 hippocampus, interacts with BACE1, and regulates BACE1 distribution. Collectively, our work provides strong evidence for VPS35 to be a crucial component in BACE1 trafficking, APP metabolism, and hippocampal neuronal synaptic function and establishes a novel mouse model in which impaired retromer function leads to altered BACE1 activation and Aβ production, thus facilitating AD disease progression in an age-dependent manner.

Many animal models have been developed for the study of pathological features of AD, including Tg2576, a transgenic line expressing Swedish mutant APP. Tg2576 mice normally develop AD-like phenotypes, including cognitive deficits and Aβ deposits, at the age of at least 10 mo or older (Chapman et al., 1999; Middei et al., 2006; Bizon et al., 2007). Remarkably, the double mutant mice (Vps35+/m;Tg2576+/−) develop AD-like phenotypes at the age of 2/3 mo old, featured with cognitive memory deficit, synaptic plasticity (LTP) impairments, decrease of glutamatergic transmissions, and increase of Aβ levels (Figs. 1–4). These observations demonstrate that VPS35 plays an important role in the control of the early onset of AD neuropathology, contributing to the growing body of evidence for the retromer-sorting pathway in the pathogenesis of AD.

The double mutant mice (Vps35+/m;Tg2576+/−) display impairments in NMDA and AMPA receptor-mediated synaptic transmission and synaptic plasticity (LTP) but not GABAergic transmission. These defects may be caused by a loss of excitatory synapses and/or a decrease of glutamatergic synapse activity, which contribute to the progressive cognitive decline in the mutant mice. Our data corroborate with the view that the decreased NMDA and AMPA currents may be induced by the increase of soluble Aβ oligomers, but not Aβ deposits, in the double mutant brain (Nimmrich et al., 2008; Selkoe, 2008), as these deficits occur as early as 2 mo old, whereas Aβ deposition is detectable at the age of 6 mo and older. On the other hand, it cannot be excluded in an Aβ-independent mechanism that may also contribute to the loss of synapses and synaptic activity in the double mutant hippocampus. Note that the hippocampus of Vps35+/− in the WT background also exhibits an increase of Aβ levels, a decrease of synaptic activity (both NMDA and AMPA currents), and a loss of synapses (decrease of frequency of mEPSCs) but without obvious defects on cognitive learning and memory or LTP (Figs. 1–4). These observations suggest that it may need to reach a threshold level of Aβ (as does the double mutant but not Vps35+/m) or an Aβ-independent factor to induce the loss of synaptic plasticity and cognitive deficit. It is possible that loss of Vps35 function may predispose neurons to a greater Aβ toxicity and may facilitate disease progression in an age-dependent manner.

Genetic, biochemical, animal, and human studies have all pointed to Aβ peptide to be a major culprit in AD pathogenesis (Tanzi and Bertram, 2005; Selkoe, 2008). Identification of mutations in APP and presenilins in the early-onset AD provides a strong genetic support for the amyloid hypothesis (Hardy and Selkoe, 2002). Many risky factors associated with the late-onset AD also affect APP metabolism and increase Aβ production, which include ApoE4 and SorL1 (Rogaeva et al., 2007; Willnow et al., 2010). Interestingly, SorL1 is a Vps10 family member in the retromer complex that interacts with APP and VPS35, and the interaction of SorL1 with VPS35 is necessary for SorL1 to control APP metabolism (Lane et al., 2010). Our results, in line with these observations, support the view for the dysfunction of VPS35-containing retromer as a risk factor for AD. This view is also supported by the observation that VPS35 is decreased in the postmortem hippocampus of AD patients (Small et al., 2005). Of interest to note is that loss of function of SorCS1, a SorL1 family protein and a risk factor for type 2 diabetes, also leads to an increase of Aβ and a decrease of VPS35 levels (Lane et al., 2010), raising a possibility for the involvement of Vps35 retromer in regulating insulin sensitivity in addition to APP metabolism. Also of interest to note is that mutation in Vps35 gene has recently been identified in patients of late-onset Parkinson’s disease (PD; Vilarinho-Güell et al., 2011; Zimprich et al., 2011), indicating the involvement of Vps35 in the pathogenesis of PD. Thus, these observations together with our results support the view for the impairment of Vps35 retromer as a general risky factor for a growing number of chronic degenerative disorders, including AD and PD.

How does retromer regulate Aβ production? Our data suggest that BACE1 activation in the Vps35+/− hippocampus may be essential for this event. VPS35 is codistributed with BACE1 in hippocampus (not depicted) and in cultured cells (Fig. 6). Loss of VPS35 function in mouse hippocampus increases BACE1 activity (Fig. 8). Depletion of Vps35 expression in culture increases BACE1 localization to the endosomal compartments (Fig. 7), an acidic environment favoring BACE1 activation. Although our experiments have pointed to the importance of endosomal BACE1 cleavage of APP, it remains to be further investigated whether the activity of Golgi/endosome-associated BACE1 is altered by loss of Vps35 function, in which subcellularly Aβ is produced in Vps35-deficient neurons, and whether APP and APPswe protein trafficking is differentially regulated.
by VPS35. It is likely that, in addition to BACE1 activation, altered APP metabolism also contributes to the elevated $\Delta B$ levels in Vps35-deficient neurons. The observation that SorL1 interaction with VPS35 is required for SorL1 to control APP metabolism (Willnow et al., 2010) supports this view. The increased $\Delta B$ levels in both Vps35$^{+/+}$ and Vps35$^{+/+}$, Tg2576 mutant mice (Fig. 4, C and D) suggest a role for VPS35 not only in regulating WT APP, but also APPswe, metabolism. However, mechanisms underlying VPS35 regulation of APPswe and WT APP may be different, as it is reported that APPswe exhibits a much higher affinity to BACE1 as compared with the WT APP, and APPswe may be cleaved by BACE1 at the TGN, whereas WT APP may be cleaved at the endosome compartments (Haass et al., 1995; Selkoe et al., 1996; Thinakaran and Koo, 2008). Collectively, the data presented in this manuscript suggest an important function of VPS35 in suppression of BACE1 activation and $\Delta B$ production, thus preventing AD-associated memory cognitive deficits and impairment of glutamatergic synaptic activity.

Materials and methods

Reagents and animals
Rabbit polyclonal anti-VPS35 antibody was generated using the antigen of GST-VPS35D1 fusion protein, as previously described (Small et al., 2005). Rabbit polyclonal anti-APP6678 (Invitrogen) and anti-BACE1 (Millipore) antibodies and monoclonal anti-APP (6E10; Covance), BACE1 (Millipore), CaMKII (Clone 6G9; Millipore), parvalbumin (PV235; Swant, Inc.), and MAP2 (Millipore) antibodies were used. Other chemicals and reagents used in this study were of analytical grade.

Vps35 mutant mice were generated by injection of mutant ES cells obtained from Bay Genomics. In brief, ES cells that contain the Vps35 gene disrupted by gene trapping using the pGT1TMpt vector (Fig. S1 A) were obtained from Bay Genomics. ES cell clones were transferred into mouse blastocysts. Chimeras generated from ES cells were crossed to C57/BL6 mice for more than six generations, and the resulting heterozygous animals were crossed to Tg2576 mice (purchased from Taconic) to produce four different genotyped mice (WT, Tg2576+/−, Vps35+/−, and Vps35−/−). Mice were maintained on a standard rodent diet (Teklad 52335; Harlan Laboratories, Inc.), Control litters (WT, Vps35+/+, and Tg2576+/+) and Tg2576+/− mice were processed in parallel for each experiment. Tg2576 and Vps35 mutations were confirmed by genotyping using PCR (Fig. S1 B). All experimental procedures were approved by the Animal Subjects Committee at the Georgia Health Sciences University, according to National Institutes of Health guidelines.

Expression vectors
The cDNAs encoding Vps35 were amplified by PCR and subcloned into mammalian expression vectors downstream of a signal peptide and a Flag epitope tag (MDYKDDDDKGP) and under control of the cytomegalovirus gene and deposited by R. Pagano (Mayo Clinic, Rochester, MN). The au-
tority of all constructs was verified by DNA sequencing.

Behavior tests
Behavioral performance was assessed. For whole-cell patch-clamp recordings, stratum pyramidale of CA1 neurons were visualized using an infrared differential interference contrast microscope with a 40x water immersion lens (Axioskop 2 Fs Plus; Carl Zeiss) and an infrared-sensitive charge-coupled device camera. All experiments were conducted on neurons located within the cell dense band of CA1 region and bearing pyramidal-shaped cell bodies with single apical dendrites. Whole-cell patch-clamp recordings were performed using an amplifier (Axon Multiclamp 700B) and digitized by pClamp software (version 9.2; Molecular Devices). Both mEPSCs and mIPSCs were recorded at a holding potential of −65 mV. For mEPSC recording, glass pipettes were filled with the solution containing 124 mM NaCl, 2.5 mM KCl, 1.3 mM NaH2PO4, 10 mM MgSO4, 5.0 mM CaCl2, 26 mM NaHCO3, 10 mM glucose, and 120 mM sucrose. Slices were allowed to recover for 30 min at 34°C and then at room temperature (25 ± 1°C) for an additional 2–8 h in artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 2.5 mM KCl, 1.3 mM NaH2PO4, 2.0 mM MgSO4, 2.0 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose. Slices were transferred to the recording chamber and superfused with aCSF [2 mL/min] at 34°C. All solutions were saturated with 95% O2/5% CO2 (volume/volume). IEPSPs were evoked in the CA1 stratum radiatum or in the stratum moleculare of the DG by stimulating the SCs/commissural pathway (for CA1) or the perforant pathway (for DG) with a two-centro-
idal bipolar stimulating electrode (25-mm pole separation; FHC, Inc) and recorded by the Axon Multiclamp 700B amplifier (Molecular Devices) with aCSF-filled glass pipettes (2–5 MΩ). Stimuli consisted of monophasic 100-μs pulses of constant currents (with intensity adjusted to produce 25% of the maximum response) at a frequency of 0.0167 Hz. The strength of synaptic transmission was determined by measuring the initial (10–60% rising phase) slope of IEPSPs. LTP in the CA1 area was induced by one train of 100-Hz stimuli with the same intensity of the test stimulus, and a cut was made between CA1 and CA3 in hippocampal slices to prevent the propagation of epileptiform activity. For LTP experiments in the DG, slices were incubated in 20 μM bicuculline to block GABA-mediated inhibition, and LTP was induced by the theta burst stimulation (six pulses at 250 Hz, with bursts delivered at a frequency of 5 Hz, and six trains of bursts delivered six times, with 20 s between trains). Before hidden-platform training, mice were given one pretraining trial in which no platform was placed in the pool, and mice had to swim in the pool for 60 s. Mice that had less motivation for swimming were excluded from the experiment. The day after pretraining, mice were trained in the circular water maze. For hidden-platform training, the escape platform (10 cm in diameter) was submerged 1.0 cm beneath the water surface. The platform location remained the same throughout hidden-platform training, but the drop location varied semirandomly between trials. Mice received one training session for a consecutive 5 d. Each session consisted of four trials with a 1-min intertrial interval. The maximum time allowed per trial in this task was 120 s. If a mouse did not find the platform, it was gently guided to it and allowed to sit on it for 30 s before being removed to its retaining cage. The mean latency and distance moved were calculated for each mouse by analyzing the latency during the first 3 s of each trial. For probe trials, the platform was removed, and mice were allowed to swim for 60 s before they were removed. The drop location for probe trials was 180° from where the platform was located during hidden-platform training. The swimming behavior was monitored by camera (VV-BP334; Panasonic), and images and swimming paths were stored in a computer and analyzed automatically by using EthoVision software (version 7.0; Noldus Information Technology).

The spontaneous locomotor activity in an open field was measured as previously described (Wen et al., 2010). In brief, transverse hippocampal slices (0.40 mm) were prepared from WT and mutation littermates using a Vibratome (VT1000S; Leica) in an ice-cold solution containing 64 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 10 mM MgSO4, 0.5 mM CaCl2, 26 mM NaHCO3, 10 mM glucose, and 120 mM sucrose. Slices were allowed to recover for 30 min at 34°C and then at room temperature (25 ± 1°C) for an additional 2–8 h in artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 2.0 mM MgSO4, 2.0 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose. Slices were transferred to the recording chamber and superfused with aCSF [2 mL/min] at 34°C. All solutions were saturated with 95% O2/5% CO2 (volume/volume). IEPSPs were evoked in the CA1 stratum radiatum or in the stratum moleculare of the DG by stimulating the SCs/commissural pathway (for CA1) or the perforant pathway (for DG) with a two-centro-nidal bipolar stimulating electrode (25-mm pole separation; FHC, Inc) and recorded by the Axon Multiclamp 700B amplifier (Molecular Devices) with aCSF-filled glass pipettes (2–5 MΩ). Stimuli consisted of monophasic 100-μs pulses of constant currents (with intensity adjusted to produce 25% of the maximum response) at a frequency of 0.0167 Hz. The strength of synaptic transmission was determined by measuring the initial (10–60% rising phase) slope of IEPSPs. LTP in the CA1 area was induced by one train of 100-Hz stimuli with the same intensity of the test stimulus, and a cut was made between CA1 and CA3 in hippocampal slices to prevent the propagation of epileptiform activity. For LTP experiments in the DG, slices were incubated in 20 μM bicuculline to block GABA-mediated inhibition, and LTP was induced by the theta burst stimulation (six pulses at 250 Hz, with bursts delivered at a frequency of 5 Hz, and six trains of bursts delivered six times, with 20 s between trains).
KCI (final concentration) for mIPSC recording. NDMA receptor-mediated mEPSCs were recorded in a modified aCSF [0 mM MgSO₄, 5 mM KCl, and 1.6 mM CaCl₂]. For evoked EPSC (eEPSC) and eIPSC recording, Kgluconate was substituted with 120 mM Cs₂CH₃SO₃, and 5 mM lidocaine N-methylthylglycine [QX-314] was added in the pipette. The resistance of pipettes was 3–5 MΩ. AMPA and NDMA receptor-mediated EPSCs were recorded at a holding potential of −65 mV and +40 mV, respectively; eIPSCs were recorded at a holding potential of −65 mV. 1 µM tetrodotoxin was included in the perfusion solution for mEPSC and mIPSC recording. For mEPSC and eEPSC recordings, 20 µM bicuculline was used to block GABA receptors and, in some cases, 100 U/ml penicillin G and streptomycin (Invitrogen). Mouse corti-nerve terminals and HEK293 cells were maintained in DME supplemented with 10% FCS and 100 U/ml penicillin G and streptomycin (Invitrogen). Mouse cortical neuron cultures were prepared at a density of 25,000 cells per cm² on polyornithine-coated coverslips from cortical tissues of E18–19 mouse embryos, as previously described (Xie et al., 2005). Neurons were grown overnight in plating medium (5% FBS and 1× B27 supplemented with 100× glutamine in Neurobasal medium [Invitrogen]). Starting at day in vitro 2 (DIV2), cultures were maintained in conditioned medium with half-fed changes of neuronal feed (1× B27 in Neurobasal medium) every 3 d. For transfection, NLT cells were plated at a density of 10⁴ cells per 10-cm culture dish and allowed to grow for 12 h before transfection using Lipofectamine 36. 36 h after transfection, cells were subjected to immunostaining analysis. The calcium phosphate method was used for transfection of HEK293 cells. 48 h after transfection, cells were lysed in modified radiimunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% NP-40, 0.25% sodium deoxycholate, and protease inhibi- tors). Lysates and medium were subjected to immunoblotting analyses. Neurons were transfected with various constructs at either DIV4 or 7 using the calcium phosphate method following by immunocytochemistry 96 h after transfection, as previously described (Zhu et al., 2007).

**Statistical analysis**

All data were expressed as mean ± SEM. All data were analyzed by one-way or repeated measures analysis of variance [ANOVA; SPSS16] followed by the least significant difference or Dunnnett’s test for post hoc comparisons or an independent sample t test unless otherwise specified. The signifi-cance level was set at P < 0.05.

**Online supplemental material**

Fig. S1 demonstrates the generation of Vps35^+/m and Tg2576+/+;Vps35^+/m mutant mice. Fig. S2 depicts the normal inhibitory synaptic transmission in hippocampal CA1 pyramidal neurons of Tg2576+/+;Vps35^+/m mice. Fig. S3 shows an increase of Aβ42 in young adult Tg2576+/+;Vps35^+/m hippocam-pus without a change of the ratio of Aβ40 versus Aβ42 in the mutant mice. Fig. S4 demonstrates an age-dependent reduction of Vps35 expression in CA1 and cortical neurons. Fig. S5 shows the suppression of Vps35 expression by both shRNA and miRNA-Vps35. Online supplemental material is avail-able at http://www.jcb.org/cgi/content/full/jcb.201105109/DC1.

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