Increased Microglial Activity, Impaired Adult Hippocampal Neurogenesis, and Depressive-like Behavior in Microglial VPS35-Depleted Mice

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Vacuolar sorting protein 35 (VPS35) is a critical component of retromer, which is essential for selective endosome-to-Golgi retrieval of membrane proteins. VPS35 deficiency is implicated in neurodegenerative disease pathology, including Alzheimer's disease (AD). However, exactly how VPS35 loss promotes AD pathogenesis remains largely unclear. VPS35 is expressed in various types of cells in the brain, including neurons and microglia. Whereas neuronal VPS35 plays a critical role in preventing neurodegeneration, the role of microglial VPS35 is largely unknown. Here we provide evidence for microglial VPS35's function in preventing microglial activation and promoting adult hippocampal neurogenesis. VPS35 is expressed in microglia in various regions of the mouse brain, with a unique distribution pattern in a brain region-dependent manner. Conditional knocking out of VPS35 in microglia of male mice results in regionally increased microglial density and activity in the subgranular zone of the hippocampal dentate gyrus (DG), accompanied by elevated neural progenitor proliferation, but decreased neuronal differentiation. Additionally, newborn neurons in the mutant DG show impaired dendritic morphology and reduced dendritic spine density. When examining the behavioral phenotypes of these animals, microglial VPS35-depleted mice display depression-like behavior and impairment in long-term recognition memory. At the cellular level, VPS35-depleted microglia have grossly enlarged vacuolar structures with increased phagocytic activity toward postsynaptic marker PSD95, which may underlie the loss of dendritic spines observed in the mutant DG. Together, these findings identify an important role of microglial VPS35 in suppressing microglial activation and promoting hippocampal neurogenesis, which are both processes involved in AD pathogenesis.

Key words: Alzheimer's disease; hippocampus; microglia; neurogenesis; retromer; VPS35

Significance Statement

The findings presented here provide the first *in vivo* evidence that Vacuolar sorting protein 35 (VPS35)/retromer is essential for regulating microglial function and that when microglial retromer mechanics are disrupted, the surrounding brain tissue can be affected in a neurodegenerative manner. These findings present a novel, microglial-specific role of VPS35 and raise multiple questions regarding the mechanisms underlying our observations. These findings also have myriad implications for the field of retromer research and the role of retromer dysfunction in neurodegenerative pathophysiology. Furthermore, they implicate a pivotal role of microglia in the regulation of adult hippocampal neurogenesis and the survival/integration of newborn neurons in the adult hippocampus.

Introduction

Alzheimer's disease (AD) is a severely debilitating neurodegenerative condition characterized by abnormal accumulation of neurotoxic amyloid β peptides and tau protein, accompanied by severe hippocampal neuronal loss. While AD pathophysiology

has fundamentally been identified, the underlying mechanisms that contribute to initial disease onset and perpetuate disease progression remain open to speculation.

A multitude of genes have been identified as susceptibility genes in AD pathology, including vacuolar protein sorting-associated protein 35 (VPS35; Small et al., 2005). VPS35 is an essential member of

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the cargo recognition module of retromer (Nothwehr et al., 1999; Hierro et al., 2007), a multimeric protein complex that facilitates intracellular retrograde trafficking of select transmembrane proteins (Seaman et al., 1998). Molecular participants in the neurodegenerative cascade have been identified as retromer cargos, and when retromer is made dysfunctional, functional activity of retromer cargos can be impaired or altered, affecting a multitude of pathological consequences (Small and Petsko, 2015).

VPS35 is a ubiquitously expressed protein, with expression level varying by cell type (e.g., neurons and glial cells) and region throughout the CNS (Wen et al., 2011; Wang et al., 2012; Lucin et al., 2013; Liu et al., 2014). Much attention has been given to the role of neuronal VPS35, where VPS35/retromer has been shown to participate in the mediation of crucial cellular processes, such as the regulation of amyloid precursor protein trafficking and proteolytic processing (Nielsen et al., 2007; Muhammad et al., 2008; Wen et al., 2011; Ueda et al., 2016), the trafficking of neurotransmitter receptors (Munsie et al., 2015; Tian et al., 2015), mitochondrial fusion/fission dynamics (Tang et al., 2015b; Wang et al., 2016), and the establishment and regulation of neuronal function (Prasad and Clark, 2006; Bonifacino, 2014; Vergés, 2016). Little is known regarding the function of microglial VPS35 in the pathogenesis of AD.

Microglial VPS35 is downregulated in the brains of AD patients (Lucin et al., 2013). *In vitro* studies have shown that microglial VPS35 deficiency can reduce the presentation of microglial phagocytic receptors at the cellular membrane, impairing the phagocytic capacity of microglia (Lucin et al., 2013). More recently, *in vitro* deletion of VPS35 from the BV2 microglial cell line has been shown to exacerbate the microglial inflammatory response (Yin et al., 2016). While these observations implicate microglial VPS35 in AD pathogenesis, exactly how the cellular functions of microglia are affected by VPS35 deficiency and how VPS35-deficient microglia might affect surrounding neural tissue *in vivo* remain poorly understood.

Here, we used a Cre-lox inducible mouse model of microglial VPS35 depletion (VPS35^{CX3CR1}) and found a regional-specific increase in hippocampal microglial density in conjunction with exacerbated hippocampal microglial activity. Hippocampal phenotype analysis of the adult VPS35^{CX3CR1} mouse revealed aberrant neurogenesis and a neurodegenerative-like deficit of newborn hippocampal DG neurons. VPS35^{CX3CR1} mice also exhibited a depressive behavioral phenotype and impaired long-term recognition memory. *In vitro* microglial-neuronal cocultures revealed, along with enlarged vacuolar structures, exacerbated microglial phagocytic activity in VPS35-depleted microglia. In aggregate, our results suggest that microglial VPS35 loss of function contributes to upregulated microglial activity *in vivo* in a manner that may affect neurodegeneration more severely in the hippocampus, contributing to AD-relevant pathology and implicating a novel microglial-specific function of VPS35 in maintaining hippocampal homeostasis and adult neurogenesis.

Materials and Methods

Animals

Both Rosa26^{tdTomato} (Ai9) and CX3CR1^{Cre-ER} mice on C57BL/6 background were purchased from the Jackson Laboratory (stock no. 00790, RRID:IMSR_JAX:007905, and stock no. 021160, RRID:IMSR_JAX:021160, respectively). VPS35^{lox/lox} mice, generated as described previously (Tang et al., 2015a), were backcrossed onto C57BL/6J background for >6 generations. VPS35^{lox/lox} mice were crossed with CX3CR1^{Cre-ER} mice to generate VPS35^{lox/lox};CX3CR1^{Cre-ER/+} mice (labeled in this text as VPS35^{CX3CR1-CreER}). Mice were group-housed under constant

12 h light/dark conditions and fed a diet of standard rodent chow. All experimental procedures were approved by the Animal Subjects Committee at Augusta University and Case Western Reserve University according to U.S. National Institutes of Health guidelines.

Experimental design and statistical analysis

At postnatal day (P) 15, VPS35^{CX3CR1-CreER} mice were administered 75 mg/kg of tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich) intraperitoneally daily for 5 consecutive days to induce Cre recombination via the CX3CR1 promoter, resulting in VPS35^{CX3CR1/+} mice (labeled in this text as VPS35^{CX3CR1}). Unless otherwise noted in the text, data reported are those recorded from age-matched littermate control mice that were vehicle-injected (corn oil) VPS35^{CX3CR1-CreER} (labeled in this text as Ctrl).

All experimental results were also confirmed in tamoxifen-injected controls (CX3CR1^{Cre-ER/+} or VPS35^{lox/lox} mice) to eliminate any potential effects of tamoxifen alone upon our observations. Male mice were used for all *in vivo* experiments to exclude any potential differential effects of the female estrous cycle.

The number of animals varied per experiment and is noted in the corresponding figure legend. For immunohistochemical analyses, a ≥7 brain sections/animal were evaluated (40 μm sections unless otherwise noted; every fourth or sixth section was used). Number of cells analyzed varied by experiment and is indicated in the corresponding figure legend. Experimental details specific to behavioral testing are included in Behavioral analyses below.

Statistical analyses were performed using Prism 5 (GraphPad Software). All values are expressed as mean ± SEM. The test was considered significant when $p < 0.05$. For all analyses, the following apply: *, significant $p < 0.05$; ns, not significant $p > 0.05$. Exact p value for each analysis is indicated in the corresponding figure legend as well as the number of animals/cells used. Normally distributed data were analyzed by ANOVA followed by Bonferroni or Fisher's least significant difference *post hoc* tests as necessary. Student's t test was used to compare pairs of means.

5-bromo-2'-deoxyuridine labeling of dividing cells

Every 3 h over a 12 h timespan, 50 mg/kg of bromodeoxyuridine (BrdU; EMD Millipore) in solution was administered, resulting in a total of four injections. Brain tissue was removed either 24 h or 7 d following the initial BrdU injection, as indicated in the text.

Stereotaxic injection of retroviral vector

CAG-GFP retrovirus was generated in which the expression of enhanced GFP is driven by the compound promoter CAG (which contains the cytomegalovirus enhancer chicken β -actin promoter and a large synthetic intron). Retrovirus was produced as described previously by Zhao et al. (2006). Resulting titer was $\sim 3 \times 10^8$ cfu/ml. Mice were placed under anesthesia (isoflurane; Sigma-Aldrich, catalog #792632) and stereotaxic injections of 2 μl of CAG-GFP retrovirus were placed into the right dentate gyrus (DG) of mice immediately following tamoxifen or corn oil treatment (at P20). Stereotaxic coordinates of injection were as follows: anteroposterior, −2.0 mm; lateral, 1.5 mm; ventral, 1.7 mm relative to bregma. One month following administration of the retrovirus, mice were perfused with PBS, followed by perfusion with 4% PFA. Brain tissue was removed and fixed overnight in 2% PFA and 100 μm sections were cut by the Leica vibratome system for immunohistochemical analysis.

Behavior analyses

Groups of male mice were prepared for behavioral analysis by daily handling by the investigator 2 weeks before behavioral assessment. Behavioral analyses were performed in a dedicated behavioral facility. Animals were relocated to the facility daily ≥1 h before the onset of testing to allow for acclimation. All behavioral assessments were initiated ≤2 h before the onset of the animals' active cycle to ensure alertness. Mice had ≥2 d of resting time between tests to decrease carryover effects from prior tests. The order of tests occurred as follows: open-field test, Y-maze, novel-object recognition, tail-suspension test, forced swim test, sucrose preference. Arenas were cleaned with 70% ethanol between each animal. Unless otherwise noted, all behavioral trials were recorded on digital video and manually assessed in a blind manner.

Open-field test. The open-field test was performed in an arena measuring 50 cm². Mice, placed in the center of the open field, were allowed to explore the arena undisturbed for 10 min. Video analysis and data acquisition were obtained with Noldus tracking software (EthoVision XT, 7.0) to analyze total distance and mean velocity.

Y-maze. Spatial memory was analyzed with the Y-maze test, conducted using a symmetrical Y-maze with 35-cm-long arms and 8-cm-tall walls. Each arm contained differently shaped markers upon the opposing face. For each trial, the mouse was placed in the starting arm for 30 s, after which the animal was allowed to explore the maze. Upon entering an arm, the animal was blocked off within that arm for 60 s and then returned to the starting arm for the next trial. Animals were subjected to five trials, with each arm entry manually recorded by the investigator. Percentage alternation was reported as number of trials in which the animal alternated arm entry divided by total number of trials.

Novel-object recognition. Recognition memory was assessed using the novel-object recognition paradigm. Each animal was placed in a 25 × 50 cm arena, with objects placed at each end of the arena. All objects used were fixed on a stationary base. Location in the arena varied pseudorandomly across trials. At time-point zero, animals were individually placed into arenas containing two identical objects on opposing sides of the cage. Following 10 min of investigation, animals were removed and returned to home cages. After each time point, one object was replaced with a novel object and animals were allowed 5 min of investigation. All trials were videotaped and exploratory intervals were manually scored by a blind observer. Investigation of an object was defined as time spent with nose directed at the object at a distance of ≤3 cm from the object for the duration of the recording. Recognition index is reported as time spent investigating novel object divided by the sum of the time spent investigating novel object plus time spent investigating familiar object (total time of exploratory behavior).

Tail-suspension test. The tail-suspension test was performed using a specially manufactured tail-suspension box (55 × 60 cm) sectioned into four 15 cm compartments. Within each compartment, a small, plastic bar hung from the base. The tail of each animal was taped to this bar and animals were suspended for 6 min. Percentage immobility is reported as time spent immobile divided by total time, where immobility was gauged as a lack of escape-driven activity.

Forced-swim test. Clear, plastic cylinders, each with a radius of 30 cm, were filled with room-temperature water and partitioned so that animals were unable to observe animals in the neighboring apparatus. Mice were placed into the water for 6 min, after which they were removed, dried, and returned to a cage warmed on a heating mat. The last 4 min of testing time were scored and percentage immobility was reported as time spent immobile divided by total time scored (4 min) where mobility was defined as “any movement other than those necessary to balance the body and keep the head above the water” (Cryan et al., 2002).

Sucrose-preference testing. Mice were individually housed in cages containing two dual bearing sipper tubes filled with water for 3 d to habituate mice to the presence of two water sources. Following the acclimation period, one tube was filled with 2% sucrose while the other contained water only. Tube levels were measured daily for 4 d and rotated to prevent location bias. Sucrose preference is reported as a percentage of the volume of sucrose intake over the total volume of fluid intake averaged over the testing period.

Histology, immunohistochemistry, and immunofluorescence

For immunostaining analysis, male mice were perfused with PBS followed by perfusion with 4% PFA. Brain tissue was removed and fixed overnight in 2% PFA. Forty micrometer sections were cut using the Leica vibratome system and blocked with blocking solution containing 0.1% Triton X-100 for permeabilization. Before blocking, sections to be analyzed for BrdU immunostaining were first incubated in 2N HCl at room temperature for 1 h, followed by one 5 min rinse and one 10 min rinse with 0.1 M sodium borate buffer, pH 8.5. Sections were incubated at 4°C overnight with primary antibody as follows: GFP (Aves Labs, GFP-1020, RRID:AB_10000240; 1:1000), IBA1 (Abcam, ab5076, RRID:AB_2224402; 1:300), doublecortin (DCX; Santa Cruz Biotechnology, SC-8066, RRID:AB_2088494; 1:200), BrdU (Sigma-Aldrich, B-2531, RRID:AB_476793;

1:200), VPS35 (Abcam, ab10099, RRID:AB_296841; 1:150), CD16/32 (Abcam, ab24187, RRID:AB_2294040; 1:200), Ki67 (Dako, M724001-2, RRID:AB_2631211; 1:200), Olig2 (Abcam, ab33427, RRID:AB_776906; 1:500), Tuj1 (Covance, MMS-435P, RRID:AB_2313773; 1:750), Tmem119 (Abcam, ab209064, RRID:AB_2728083; 1:100). Sections were incubated for 1 h at room temperature the following day using the appropriate secondary antibody at 1:500 (Thermo Fisher Scientific, Alexa Fluor conjugates). Slides were mounted using Prolong Diamond Antifade mounting media with or without DAPI (Thermo Fisher Scientific) and confocal images were obtained using a Nikon A1R MP+ multiphoton confocal microscope with 20× or oil-immersion 60× objective with sequential-acquisition setting. Quantitative analyses were performed using ImageJ software as a measure of mean optical density.

Stereological analysis of cellular density

Stereological estimation for cell count density was obtained manually in a blind manner as follows. Regional images of every fourth section of brain tissue from each animal were obtained by Nikon NS Elements imaging software, large-image acquisition. Images were analyzed and total cell counts by regional area were obtained manually. Cellular density was calculated based upon total section volume (cells/mm³).

Microglial morphological analysis

Random images of GFP+ cells in the region of interest were obtained using an oil-immersion 60× objective lens on a Nikon A1R MP+ multiphoton confocal microscope.

Calculation of microglial soma volume

Nd2 files were converted into .ser files using Reconstruct software (<https://synapseweb.clm.utexas.edu>) and individual cell somas were traced at each step of the series. Only cell somas fully included within the section were included in this analysis. Each Reconstruct series was scaled to size and the volume of each object traced (i.e., total soma) was calculated by the software.

Calculation of total process length

Cells identified were those in which the cell soma was centered in the tissue section and all processes were included in the image stack. Using ImageJ, the image stack was merged and scaled before the threshold was applied. This image was loaded using the ImageJ plugin, NeuronJ, and the microglial processes were traced to enable calculation of total process length.

Western blotting analysis

Cells and tissue were homogenized in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with protease inhibitors (Roche Applied Science). After a 20 min incubation on ice, protein extracts were clarified by centrifugation at 12,000 × g for 20 min at 4°C and protein concentrations were determined by BCA Protein Assay Kit (Pierce Biotechnology). For Western blot analysis, 20–40 μg of lysate per lane was separated by Bis-Tris SDS-PAGE gel. After transfer to nitrocellulose membranes, the membranes were immunoblotted with the following antibodies: rabbit anti-VPS35 (1:10,000; gift from Dr. K.-W. Kim, Columbia University), IBA1 (1:10,000; Abcam, ab5076, RRID:AB_2224402), MHCII (1:10,000; Thermo Fisher Scientific, 14-5321, RRID:AB_467560), IL-6 (1:500; Santa Cruz Biotechnology, sc-1265, RRID:AB_2127470). For semiquantitative analysis, protein bands were detected by the Odyssey Infrared Imaging System (Li-Cor) and analyzed using ImageJ software.

Isolation of microglia for protein analysis

Microglial cell lysates were prepared from adult brain tissue following isolation with the Miltenyi Biotec MACS (magnetic activated cell sorting) cell separation system with minor modifications to the manufacturer's protocol. Briefly, brains from VPS35^{CX3CR1} mice or corn oil-treated controls were dissected and tissue was dissociated in DMEM (EMD Millipore) using the Neural Tissue Dissociation Kit—Postnatal (Miltenyi) with the gentleMACS Octo dissociator with heaters using program 37C_ABDK_1. The dissociated tissue was filtered through a 100 μm mesh filter and rinsed with Dulbecco's PBS. Cellular debris were removed with Debris Removal Solution (Miltenyi). Microglia were isolated from the final single-cell suspension using the MACS technology with

Figure 1. Microglial VPS35 expression. **A**, Representative images of *in vivo* microglial VPS35 expression in various brain regions [CA1 (hippocampus), striatum, Ec-Ctx] as exhibited by IBA-1 coimmunofluorescence. Scale bar, 10 μ m. **B**, Representative image of primary cultured microglial VPS35 expression by coimmunostaining analysis. Primary microglia cultures were obtained from C57BL/6J mice and immunostained with primary antibodies for IBA1 and VPS35. Scale bar, 10 μ m. **C**, Representative image of primary cultured cortical neuronal VPS35 expression by coimmunostaining analysis. Cortical neuronal cultures were obtained from C57BL/6J mice and immunostained with primary antibodies for Tuj1 and VPS35. Arrow, Neuron; arrowhead, non-neuronal cell (likely a glial cell). Scale bar, 20 μ m. **D**, Western blot analysis of microglial VPS35 expression. Soluble lysates from primary microglia and neuronal cultures from C57BL/6J mice were subjected to Western blot analysis. **E**, VPS35 mRNA levels in microglia and cortex from mice at indicated age. Data were adapted from http://web.stanford.edu/group/barres_lab/brainseq2/brainseq2.html. FPKM, Fragments per kilobase of transcript per million mapped reads.

anti-cluster of differentiation molecule 11b (CD11b) MicroBeads (Miltenyi).

Isolation of primary microglia

Brain tissue from adult VPS35^{CX3CR1} mice or corn oil-treated controls was dissociated using the Miltenyi Neural Tissue Dissociation Kit as described above. Following MicroBead isolation, cells were counted and resuspended in microglia culture medium [DMEM, 10% fetal bovine serum (Sigma-Aldrich), 1% penicillin-streptomycin (Sigma-Aldrich) plus 10 ng/ml GM-CSF (Sigma-Aldrich)]. Cells were plated to poly-D-lysine (Sigma-Aldrich)-coated coverslips in a 24-well plate at a density of 1×10^5 cells suspended in 50 μ l of medium. Cells were allowed to rest 30 min at 37°C, after which 450 μ l of medium was added to each well. Cells were maintained in 5% CO₂ incubator at 37°C, replacing 50% of the culture medium every 24 h. Primary cell cultures were used for experimental analyses following 7 d *in vitro* (DIV).

Microglial–neuronal coculture and transfection

Neuronal tissue was dissociated from cortices of P0 C57BL/6J mice using the Miltenyi Neural Tissue Dissociation Kit as described above, replacing DMEM with Neurobasal-A. Dissociated tissue was resuspended in neuronal growth medium (Neurobasal-A plus $1 \times B27$, 2 mM GlutaMAX-1, and 1% penicillin/streptomycin) and plated onto poly-D-lysine (Sigma-Aldrich)-coated coverslips in a 12-well plate at a density of 1×10^5 cells per well. Cells were maintained in 5% CO₂ incubator at 37°C, replacing 50% of the growth medium every 24 h. At 14 DIV, neurons were overlaid with primary microglia (isolated as described above and resuspended in neuronal growth medium) at density of 2×10^4 microglia/well (to obtain a 1:5 microglia/neuron ratio) and were cocultured for an additional 5 DIV. Cocultures were then fixed and stained for analysis.

Neuronal cultures obtained as described above were subjected to transient transfection with mCherry (mCh) construct at 4 DIV using the calcium phosphate-mediated gene transfer method, as described previ-

ously (Jiang and Chen, 2006; Zhu et al., 2007). At 7 DIV, transfected neurons were overlaid with primary microglia as described above for an additional 5 DIV. Cocultures were then fixed and stained for analysis.

Results

Microglial VPS35 expression

To investigate how VPS35 deficiency promotes the pathogenesis of neurodegenerative disorders, including AD and Parkinson's disease (PD), we first sought to determine the cell types that express VPS35. Coimmunofluorescence staining analysis in young-adult mouse brain sections showed abundant VPS35 expression not only in neurons (Fig. 1C) (both glutamatergic and dopaminergic neurons; Wen et al., 2011; Tang et al., 2015b; Wang et al., 2016), but also in IBA microglia in various brain regions, including the striatum, entorhinal cortex (Ec-Ctx), and hippocampus (Fig. 1A). Microglial VPS35 expression was further verified by the coimmunostaining analysis of VPS35 with IBA (ionized calcium binding adaptor) in primary cultured microglia (Fig. 1B) and by Western blot analysis of microglial cell lysates (Fig. 1D). Notice that a slightly higher molecular weight of microglial VPS35 than that of neuronal VPS35 was detected by Western blot analysis (Fig. 1D), suggesting a different isoform/splice variant or a post-transcriptional modification of microglial VPS35 from that in neurons. Also notice that gene expression profiling studies of VPS35 in microglial cells and cortices of mice at various ages showed variable expression levels of microglial VPS35 throughout the murine lifespan (Fig. 1E), supporting the view for microglial VPS35 expression in both the developing and adult brain. Note that microglial VPS35 expression was higher in mice at the neonatal age (P7–P21) compared with that of mice at

Figure 2. Characterization of microglial Cre activity in the CX3CR1^{Cre-ER/+} mouse. **A**, Microglia-specific Cre expression in the CX3CR1^{Cre-ER/+} mouse line. Top, Schematic of the gene encoding Cre-ER, followed by IRES-EYFP element as expressed in the CX3CR1^{Cre-ER/+} mouse. Bottom, Representative images of microglia-specific Cre expression following tamoxifen injection in CX3CR1^{Cre-ER/+} mice. Scale bar, 20 μ m. **B**, Generation of the CX3CR1^{Cre-ER/+};Ai9 mouse line by crossing the CX3CR1^{Cre-ER/+} mouse with the Rosa26^{tdTomato} reporter line (Ai9). Seventy-five milligrams per kilogram tamoxifen was administered intraperitoneally to P15 animals over 5 d, and tissue was analyzed $\sim$ 30 d following the final tamoxifen injection. **C**, Representative images depicting GFP coimmunofluorescence with tdTomato in the hippocampus and spleen in CX3CR1^{Cre-ER/+};Ai9 mouse. Scale bar, 50 μ m. **D**, Quantification of GFP:tdTomato coexpression throughout the CNS and the spleen, which shows high GFP + :tdTomato + reporting throughout the CNS with low coexpression in the spleen.

a young-adult age (P60; Fig. 1E). Also microglial VPS35 expression increased in response to an LPS stimulus (Fig. 1E), implicating VPS35 in the LPS-stimulated microglial response.

Increased microglial density and activity and altered microglial morphology in the adult hippocampus of microglial VPS35-depleted mice

To determine whether microglial VPS35 plays a role *in vivo*, we generated VPS35^{flox/flox};CX3CR1^{Cre-ER/+} mice by crossing mice possessing the floxed VPS35 allele (VPS35^{flox/flox}) with CX3CR1^{Cre-ER/+} mice. The CX3CR1^{Cre-ER} mouse line was chosen for the following reasons: (1) it expresses Cre-ER under the control of the CX3CR1 promoter expressed in macrophages/monocytes/microglial cells

(Parkhurst et al., 2013); and (2) the CX3CR1 promoter in the CX3CR1^{Cre-ER} mouse line is tagged with enhanced yellow fluorescent protein (EYFP; (Fig. 2A), which can be used to identify all CX3CR1-expressing cells, including microglia and macrophages (Parkhurst et al., 2013). To confirm microglial-specific Cre-ER expression, we crossed CX3CR1^{Cre-ER} with the Rosa26^{tdTomato} reporter line (Fig. 2B). Tamoxifen was administered over 5 d to CX3CR1^{Cre-ER};Rosa26^{tdTomato} mice, starting at P15. This age was chosen for tamoxifen administration since murine microglia do not attain a mature phenotype until $\sim$ P14 (Hirasawa et al., 2005; Bennett et al., 2016) and because VPS35 is highly expressed in microglia at this age (Fig. 1E). Thirty days following the final tamoxifen injection, the CX3CR1-driven Cre remained active in

Figure 3. Generation of the VPS35^{CX3CR1} mouse. **A**, Diagram outlining the treatment strategy implemented to generate VPS35^{CX3CR1} mice. VPS35^{flox/flox} female mice were crossed with male CX3CR1^{Cre-ER/Cre-ER} mice followed by subsequent backcrossing with female VPS35^{flox/flox} mice to generate male VPS35^{flox/flox};CX3CR1^{Cre-ER/+} (VPS35^{CX3CR1-Cre-ER}) mice, subsequently treated with tamoxifen or corn oil (controls) at P15–P19 to generate VPS35^{CX3CR1} mice (labeled throughout text as VPS35^{CX3CR1}). Unless otherwise noted in the text, control data reported are from male littermate-matched vehicle-treated VPS35^{CX3CR1-Cre-ER} mice, denoted as CX3CR1^{Cre-ER}. **B**, Primary microglia isolated from adult mice (P45–P60) and immunostained for VPS35 show depletion of VPS35 from VPS35^{CX3CR1} microglia. Scale bar, 10 μ m. **C**, Microglia isolated from the brains of adult VPS35^{CX3CR1} mice and vehicle-treated controls were immediately lysed and analyzed for protein levels of VPS35 via Western blot to exhibit microglia-specific depletion of VPS35 in the CNS of VPS35^{CX3CR1} mice. **D**, Weight loss of VPS35^{CX3CR1} mice compared with vehicle-treated (CX3CR1^{Cre-ER}) and tamoxifen-treated (Tam VPS35^{flox/flox}) controls.

microglia, as evidenced by tdTomato⁺ immunofluorescence in the brain, but little in peripheral macrophages/monocytes in the spleen (Fig. 2C,D), due to the high turnover rate of peripheral macrophages/monocytes versus the slow turnover rate of microglia, which was in accord with the literature (Parkhurst et al., 2013).

Using the same protocol of tamoxifen treatment (intraperitoneal injection at P15, examined at P50) of VPS35^{flox/flox};CX3CR1^{Cre-ER/+} mice (referred to in this text as VPS35^{CX3CR1} mice; Fig. 3A), VPS35 was depleted in primary microglia from adult VPS35^{CX3CR1} mice treated with tamoxifen, but not corn oil, as confirmed by immunofluorescent staining (Fig. 3B) and West-

ern blot analyses (Fig. 3C). We thus chose the above protocol of tamoxifen treatment (Fig. 3A) to study VPS35's function in adult murine microglia *in vivo*. Notice that the body weight was slightly lower in VPS35^{CX3CR1} mice than that of control mice treated with tamoxifen (Fig. 3D), implicating an *in vivo* function for microglial VPS35.

We then examined the cell density and morphological features of GFP⁺ cells in the brains of VPS35^{CX3CR1} mice treated with tamoxifen. As a control, CX3CR1^{Cre-ER/+} mice (without the floxed VPS35 allele) treated with tamoxifen were used to ensure any tamoxifen-specific effects on microglial activity were accounted for during our initial assessments. Total GFP⁺ cell density by re-

Figure 4. Regional-specific increase in microglial density following VPS35 depletion. **A–E**, Microglial VPS35 loss of function affects a regional-specific increase in microglial density. **A**, Representative images from the prefrontal cortex (PFC), hippocampus (HC), molecular layer (ML), granular cell layer (GCL), subgranular zone (SGZ), and Ec-Ctx. **B**, Representative images of GFP immunofluorescence in the DG. White boxes represent the region shown in **C**. Scale bar, 100 μ m. **C**, Enlarged images of GFP⁺ cells in the SGZ suggest altered morphology of VPS35^{CX3CR1} microglia. Scale bar, 50 μ m. **D**, Statistical analysis of microglial density by region. Ctrl, CX3CR1^{Cre-ER/+}; VPS35, VPS35^{CX3CR1} (Student's unpaired *t* test, *n* = 4; PFC: *p* = 0.20; HC: *p* < 0.0001; Ec-Ctx: *p* = 0.0023; SN: *p* = 0.035). **E**, Statistical analysis of hippocampal microglial density by hippocampal region. Ctrl, CX3CR1^{Cre-ER/+}; VPS35, VPS35^{CX3CR1}. GCL, Granular cell layer. (Student's unpaired *t* test, *n* = 4; CA1: *p* = 0.071; CA2: *p* = 0.058, CA3: *p* = 0.009; DG: *p* = 0.001; SGZ: *p* = 0.02; GCL: *p* = 0.06). For all analyses, statistical significance (**p* ≤ 0.05).

gional volume was calculated, revealing a regional-specific increase of cell density in VPS35^{CX3CR1} mice, compared with that of controls (Fig. 4A,D). Most notably, the hippocampus and Ec-Ctx, vulnerable regions in the AD brain, showed significant increases

in GFP⁺ cell density (Fig. 4B–D), with a notable increase in the subgranular zone (SGZ) of the DG (Fig. 4C,E). These GFP⁺ cells are primarily microglia, because >90% of GFP⁺ cells expressed TMEM119 (transmembrane protein 119; Fig. 5A,B), a

Figure 5. Infiltration of peripheral macrophages and altered SGZ microglial morphology following VPS35 depletion. **A**, Representative images of GFP:TMEM119 coimmunofluorescence in the DG. Scale bar, 50 μ m. **B**, Statistical analysis of GFP⁺ and TMEM119⁺ cells indicates >90% of GFP⁺ cells express TMEM119. **C**, Statistical analysis of increased GFP⁺:TMEM119⁺ cell density in the DGs of VPS35^{CX3CR1} mice (Student's unpaired *t* test, *n* = 5, *p* = 0.0002). **D**, Statistical analysis of increased GFP⁺:TMEM119⁻ cell density in the DGs of VPS35^{CX3CR1} mice implicates an infiltration of peripheral macrophages (Student's unpaired *t* test, *n* = 5, *p* = 0.03). **E**, Representative images of SGZ microglial morphology and 3D visualizations of soma volume as calculated using Reconstruct software. Scale bar, 141.52 μ m. **F**, Statistical analysis of increased microglia soma volume in SGZ microglia of VPS35^{CX3CR1} mice. Ctrl, CX3CR1^{Cre-ER/+}; VPS35, VPS35^{CX3CR1} (Student's unpaired *t* test, *n* = 14–31, sampled from 3 animals per group, with ≥ 4 microglia per animal analyzed, *p* < 0.0001). **G**, Statistical analysis of increased total microglia process length in SGZs of VPS35^{CX3CR1} mice. Ctrl, CX3CR1^{Cre-ER/+}; VPS35, VPS35^{CX3CR1} (Student's unpaired *t* test, *n* = 13–23, sampled from 3 animals per group, with ≥ 4 microglia per animal analyzed, *p* < 0.014). For all analyses, statistical significance (**p* $\leq$ 0.05).

marker for microglia that is undetectable in macrophages (Ben-nett et al., 2016; Satoh et al., 2016). Interestingly, the cell densities of both GFP:TMEM119 (Fig. 5C) and GFP:TMEM119 microglial cells were higher in the VPS35^{CX3CR1} hippocampi than in those of controls (Fig. 5A,D), implicating an increase of peripheral macrophage infiltration. GFP⁺ cellular morphology appeared to be altered in the VPS35^{CX3CR1} SGZ (Fig. 4C), so morphological features of SGZ GFP⁺ cells were analyzed by measuring soma volume and total length of cell processes. VPS35^{CX3CR1} SGZ microglia displayed increased soma volume (Fig. 5E,F), as well as increased length of cell processes (Fig. 5E,G).

Increased microglial density and altered microglial morphology are often associated with increased microglial activation. We thus assessed microglial activity using markers known to be upregulated in concurrence with microglial activation (e.g., IBA1, CD16/32). There were significant increases in hippocampal IBA1 and CD16/32 [CD16: low-affinity IgG Fc receptor III (FcR III); CD32: FcR II; a marker upregulated under M1 proinflammatory conditions] immunofluorescence in VPS35^{CX3CR1} mice (Fig. 6A–D), both of which were also significantly upregulated in the SGZ of the VPS35^{CX3CR1} DG (Fig. 6B,D). Hippocampal microglial activity was also assessed by probing hippocampal tissue lysates for IBA1 and CD11b (cluster of differentiation molecule 11b/CR3A/ITGAM, a molecule shown to upregulate in concurrence with microglial proinflammatory conditions). Western blot analysis showed increased relative density of both IBA1 and CD11b in VPS35^{CX3CR1} hippocampal tissue (Fig. 6E–G). These results thus support the idea that microglial activation is upregulated in the VPS35^{CX3CR1} brain in a regional-specific manner (e.g., hippocampus and SGZ).

Elevated microglial differentiation following microglial VPS35 loss of function

To further analyze potential regulation of microglial density in VPS35^{CX3CR1} mice, we wondered whether loss of microglial VPS35 results in elevated microglial proliferation, thus increasing microglial density. To test this view, VPS35^{CX3CR1} mice and CX3CR1^{Cre-ER} littermate controls were administered BrdU (intraperitoneally, 4 times over 12 h) and killed 24 h after the commencement of BrdU treatment (Fig. 7A). Hippocampal BrdU⁺:GFP⁺ cells were quantified, however, with no observable difference in the density of BrdU⁺:GFP⁺ cells in the DGs of VPS35^{CX3CR1} mice compared with controls (Fig. 7B,C), excluding microglial proliferation as the possible underlying mechanism.

We then examined microglial differentiation/migration in control and VPS35^{CX3CR1} mice. To this end, mice were administered BrdU and killed 7 d later (Fig. 7D). BrdU⁺:GFP⁺ cells indicated new microglial cells that had either differentiated or migrated from surrounding regions since the initial BrdU injection. The hippocampi of CX3CR1^{Cre-ER} controls often contained no or few BrdU:GFP cells. By contrast, BrdU:GFP cells were frequently observed in the DGs of VPS35^{CX3CR1} mice (Fig. 7E–G), suggesting that increased microglial density might be a result of increased microglial differentiation and/or migration. To determine whether other glial cell types (astrocytes and oligodendrocytes) were affected in a similar manner, we examined additional glial markers for BrdU coimmunofluorescence, observing no significant differences (Fig. 7H–J).

Increased neural stem cell proliferation, but reduced neuronal differentiation, in microglial VPS35-depleted DGs

Following the initial BrdU analyses, we found it interesting that VPS35^{CX3CR1} mice showed significantly higher numbers of BrdU⁺ cells in the SGZ, a primary site of adult hippocampal neurogenesis, at both 1 and 7 d following BrdU injections (Fig. 8A–F), implicating both elevated neural stem/progenitor cell (NSC/NPC) proliferation and survival. We thus further assessed for additional indices of hippocampal neurogenesis in VPS35^{CX3CR1} mice. Ki67 is a proliferative marker that can be used to identify dividing cells throughout the cell cycle with the exception of G0 and early G1 phases (Zhang and Jiao, 2015). Ki67:BrdU coimmunofluorescence and Ki67 quantification indicated increased Ki67⁺ cell density in the VPS35^{CX3CR1} SGZ, compared with that of controls (Fig. 8G,H), supporting the notion of increased NSC/NPC proliferation. The ratio of Ki67⁺:BrdU⁺ cells to total BrdU⁺ cells also increased in the VPS35^{CX3CR1} SGZ (Fig. 8I), suggesting impaired cell-cycle exit in these NSC/NPCs. These results suggest that microglial VPS35 loss of function disturbs hippocampal neurogenesis by increasing NSC/NPC proliferation as well as disrupting cell-cycle exit.

To determine whether microglial VPS35 loss of function affects hippocampal neuronal differentiation, mice underwent BrdU treatment (Fig. 7D) and hippocampi were immunostained for DCX, a marker specifically expressed by cells of a neuronally determined lineage (late neuroblasts and immature neurons; Fig. 9A,B). Despite an increased number of BrdU⁺ cells in the SGZ of VPS35^{CX3CR1} mice, the percentage of BrdU⁺ cells expressing DCX was significantly lower in VPS35^{CX3CR1} hippocampi (Fig. 9B), suggesting reduced neuronal differentiation in the VPS35^{CX3CR1} hippocampus. Together, these unanticipated results suggest a model in which microglial VPS35 loss of function disrupts adult hippocampal neurogenesis, increasing the presence of NSC/NPCs while differentially reducing neuronal differentiation and arresting cell-cycle exit (Fig. 9G).

Reduced newborn neurons and their dendritic morphogenesis in microglial VPS35-depleted DGs

Given that neuronal differentiation was reduced in VPS35^{CX3CR1} hippocampi, we examined whether there existed an overall reduction in newborn neurons following microglial VPS35 depletion by quantifying total DCX immunofluorescence (Fig. 9C–F). In support of this speculation, DCX quantification revealed a significant reduction of newborn neurons in the VPS35^{CX3CR1} hippocampus (Fig. 9C,D). Reduced DCX levels in the hippocampi of VPS35^{CX3CR1} mice were confirmed by Western blot analysis (Fig. 9E,F). Interestingly, the overall complexity of DCX⁺ neuronal processes in VPS35^{CX3CR1} hippocampi appeared to be diminished (Fig. 9C).

To further assess the effects of microglial VPS35 depletion on newborn neurons in the adult DG, a retroviral-mediated labeling strategy was implemented to visualize dividing cells and their progeny. DGs of VPS35^{CX3CR1} mice were stereotactically injected with a GFP-labeled retrovirus (Fig. 10A). One month following retrovirus injection, mice were killed and morphological features of GFP-expressing neurons in the DGs were examined. GFP⁺ neurons in the DGs of VPS35^{CX3CR1} mice exhibited significant reductions in dendritic process length and complexity (Fig. 10B–E) and reduced density of dendritic spines (Figs. 10F,G). These results provide additional evidence supporting a model of disturbed adult hippocampal neurogenesis and neuronal differentiation, implicating a novel role of microglial VPS35 in the regulation of newborn neuronal maturation in the adult CNS, in

Figure 6. Upregulated hippocampal microglial activity following microglial VPS35 depletion. **A**, Representative images of IBA1 immunofluorescence in the hippocampus following microglial VPS35 loss of function. **B**, Statistical analysis of hippocampal IBA1 fluorescent optical density [Student's unpaired *t* test, *n* = 3, hippocampus (HC): *p* = 0.012; SGZ: *p* = 0.046]. **C**, Representative images of CD16/32 immunofluorescence in the hippocampus following microglial VPS35 loss of function. **D**, Statistical analysis of hippocampal CD16/32 fluorescent optical density (Student's unpaired *t* test, *n* = 3, HC: *p* = 0.014; SGZ: *p* = 0.022). **E–G**, Hippocampal tissue was solubilized and subjected to Western blot analysis, confirming an increase in IBA1 and identifying increased CD11b, both indicative of upregulated microglial activity (Student's unpaired *t* test, *n* = 3, IBA1: *p* = 0.044; CD11b: *p* = 0.0009). For all analyses, statistical significance (**p* < 0.05).

Figure 7. Increased microglial survival and/or migration in the VPS35^{CX3CR1} hippocampus. **A–C**, Analysis of hippocampal microglial proliferation. **A**, Schematic of BrdU treatment for analysis of proliferation. **C**, Representative images of GFP:BrdU costaining. **B**, Statistical analysis of BrdU+ proliferative microglia shows no difference in microglial proliferation following microglial VPS35 loss of function (Student's unpaired t test, $n = 3$, $p = 0.3528$). **D–F**, Analysis of hippocampal microglial differentiation and survival. **D**, Schematic of BrdU treatment for analysis of NPC differentiation. **E**, Representative images of GFP:BrdU costaining show increased GFP+ :BrdU+ costaining in the hippocampus of VPS35^{CX3CR1} mice. **F**, Increased density of BrdU+ differentiated microglia in the DGs of VPS35^{CX3CR1} mice suggests increased hippocampal microglial survival or differentiation. (Student's unpaired t test, $n = 3$, $p = 0.0036$). **G–J**, Statistical analysis of glial differentiation based upon percentage of BrdU+ microglia (**G**; $p = 0.0193$), oligodendrocytes (**H**; $p = 0.3158$), or astrocytes (**I**; $p = 0.4325$; **J**; $p = 0.3337$) out of total BrdU+ cells 7 d following BrdU treatment (Student's unpaired t test, $n = 2–3$). For all analyses, statistical significance (* $p \leq 0.05$).

which microglial VPS35 deficiency causes neurodegenerative morphological deficits upon hippocampal newborn neurons before synaptic integration.

Increased microglial engulfment of postsynaptic elements following microglial VPS35 loss of function

To understand how microglial VPS35 loss of function results in impaired newborn neuronal dendritic morphogenesis and spine

formation, we performed microglial–neuronal cocultures. Adult primary microglia were first isolated from control and VPS35^{CX3CR1} cortices of P50 mice. Notice that VPS35^{CX3CR1} microglia were GFP positive and displayed abnormally high numbers of grossly enlarged LAMP1⁺ vacuolar-like structures, compared with microglia of controls (Fig. 11A,B). Adult primary microglia were then cocultured with primary neurons transfected with mCh plasmid (Fig. 11C). Five days following addition of microglia to

Figure 8. Aberrant hippocampal neurogenesis following microglial VPS35 loss of function, characterized by elevated neural progenitor proliferation and arrested cell-cycle exit. **A**, Animals were treated with BrdU and analyzed 24 h following treatment to assess BrdU+ cells for proliferation. **B, C**, Increased BrdU density in the SGZs of VPS35^{CX3CR1} mice 24 h following BrdU treatment (Student's unpaired *t* test, $n = 3$, $p = 0.0001$) suggests increased proliferation of hippocampal NSCs following microglial VPS35 loss of function. **D**, Animals were treated with BrdU and analyzed 7 d following treatment for survival of differentiated NPCs. **E, F**, Increased BrdU density in the SGZs of VPS35^{CX3CR1} mice 7 d following BrdU treatment (Student's unpaired *t* test, $n = 3$, $p = 0.0127$) suggests an increased survival rate of hippocampal NPCs following microglial VPS35 loss of function. **G, H**, Ki67 immunofluorescence displays a significant increase in overall Ki67+ cells, suggesting an increase in Ki67+ -proliferating NSCs following microglial VPS35 loss of function. **I**, Ki67+ :BrdU+ cells were quantified 1 week following BrdU treatment to assess cell-cycle exit. A significant increase in total percentage of BrdU+ cells which are Ki67+ was noted, suggesting BrdU+ cells may be increased due to a failure to exit the cell cycle (Student's unpaired *t* test, $n = 3$, $p < 0.0324$). For all analyses, statistical significance (* $p \leq 0.05$).

the transfected neuronal culture, microglia were observed for mCh fluorescence. VPS35^{CX3CR1} microglia were found to exhibit a significant increase in mCh fluorescence (Fig. 11D,E). The increased mCh fluorescence was deduced to result from either an

increase in microglial engulfment of mCh and/or an impairment in mCh degradation. To further test this view, we examined the specific neuronal presynaptic and postsynaptic markers synapsin-1 and postsynaptic density 95 (PSD95) in microglial–

Figure 9. A decrease of neuronal differentiation and a reduction of immature neurons in VPS35^{CX3CR1} DGs. **A**, Representative images of BrdU:DCX coimmunofluorescence 1 week following BrdU treatment. Scale bar, 50 μ m. **B**, Statistical analysis of percentage of BrdU+ cells expressing DCX 7 d following BrdU treatment (as a percentage of total BrdU+ cells) indicates decreased neuronal differentiation following microglial VPS35 loss of function (Student's unpaired *t* test, $n = 3$, $p = 0.0034$). **C**, Representative images of hippocampal DCX immunofluorescence and 60 $\times$ zoomed images, demonstrating the reduced levels of DCX and DCX+ processes following microglial VPS35 depletion. **D**, Statistical analysis of DG DCX fluorescent optical density (Student's unpaired *t* test, $n = 3$, $p = 0.0207$). **E**, Hippocampal tissue was solubilized and subjected to Western blot analysis, confirming reduced levels of DCX in VPS35^{CX3CR1} mice (Student's unpaired *t* test, $n = 3$, $p = 0.033$). **F**, Schematic depicting the model in which microglial VPS35 loss of function affects aberrant hippocampal neurogenesis, during which an increase is observed in proliferating NSCs, concurrent with a decrease in immature neurons and arrested cell-cycle exit. For all analyses, statistical significance ($*p \leq 0.05$).

neuronal cocultures (Fig. 11F). A significantly higher PSD95 fluorescence, but not synapsin-1 fluorescence, was detected in VPS35^{CX3CR1} microglia (Fig. 11G–I), suggesting an increased uptake of postsynaptic, but not presynaptic, elements by

VPS35^{CX3CR1} microglia. We then tested this view in the brains of control and VPS35^{CX3CR1} mice by coimmunostaining analysis using antibodies against PSD95 and GFP. Indeed, VPS35^{CX3CR1} microglia contained more PSD95 fluorescence than those of con-

Figure 10. Neurodegenerative-like morphology of newborn neurons in VPS35^{CX3CR1} DGs. **A**, Schematic of timeline for stereotaxic injection of a GFP-expressing retroviral vector into the DG to selectively label dividing cells. **B**, Representative images of GFP+ neurons and tracings. Scale bar, 20 μ m. **F, G**, GFP+ neurons in DGs of VPS35^{CX3CR1} mice exhibited decreased dendritic spine density (Student's *t* test, $n = 10$ secondary branches from 2 animals per group, $p < 0.05$). **C**, Sholl analysis indicates decreased number of intersections in GFP+ neurons in the DGs of VPS35^{CX3CR1} mice. **D, E**, GFP+ neurons in VPS35^{CX3CR1} mice were found to have decreased total length of processes ($p = 0.0028$) and total number of branches ($p = 0.027$; Student's *t* test, $n = 20$ neurons from 2 animals per group). **F, G**, GFP+ neurons in DGs of VPS35^{CX3CR1} mice exhibited decreased dendritic spine density. Scale bar, 2.5 μ m (Student's unpaired *t* test, $n = 10$ secondary branches from 2 animals per group, $p = 0.0023$). For all analyses, statistical significance (* $p \leq 0.05$).

trols (Fig. 11J,K). This signifies a potential direct targeting of postsynaptic material by VPS35^{CX3CR1} microglia, which potentially is a mechanism through which microglial VPS35 loss of function contributes to the observed *in vivo* phenotype of hippocampal newborn neuronal spine/synapse deficit.

When considered together, these data (Figs. 8–11) suggest that the reduction of DCX+ neurons and the degenerative morphology of newborn neurons observed in the VPS35^{CX3CR1} hippocampus could be due to a combination of both defective neuronal differentiation and exacerbated pruning of postsynaptic elements.

Figure 11. Increased microglial engulfment of postsynaptic elements following microglial VPS35 loss of function. **A, B**, Primary microglia isolated from adult VPS35^{CX3CR1} mice exhibit grossly enlarged vacuolar structures. Scale bar, 10 μ m (Student's unpaired *t* test, $n = 7$ –10 microglia per group, $p = 0.0059$). **C–E**, VPS35^{CX3CR1} primary microglia cocultured with primary cortical neurons transfected with mCh construct exhibit increased uptake of mCh+ neuronal debris. Scale bar, 10 μ m (Student's unpaired *t* test, $n = 8$ microglia per group, (Figure legend continues.)

Impaired long-term memory and depression-like behavior following microglial VPS35 depletion

To determine whether microglial VPS35 depletion functionally affects VPS35^{CX3CR1} mouse behavior, a behavioral panel was performed to assess behavioral phenotype (Fig. 12A). An open-field test was first carried out to analyze locomotor activity and assess any proclivity for anxiety. No difference between control and VPS35^{CX3CR1} mice was observed in distance traveled, velocity, or time spent in the center (Fig. 12B–E), suggesting normal locomotor activity without obvious anxiety in the mutant. Mice were then subjected to the Y-maze test to assess spatial recognition (Fig. 12F). Again, no difference in number of alternations was observed (Fig. 12G), indicating that spatial recognition in the mutant mice was comparable to that of controls. We further assessed recognition memory by the novel-object recognition paradigm at 30 min, 120 min, and 48 h to assess both short-term and long-term memory (Fig. 12H). A slight reduction (not significant) in recognition index ($p = 0.06$) was observed after 30 and 120 min following the training session (Fig. 12I). A significant reduction in recognition was observed after 48 h, suggesting impaired long-term memory following microglial VPS35 depletion (Fig. 12I).

Finally, we performed sucrose-preference, forced-swim, and tail-suspension tests to measure depressive behavior (Fig. 12J, L). Decreased sucrose preference in VPS35^{CX3CR1} mice suggests a reduced drive for pleasure-seeking activity (Fig. 12J). The time that VPS35^{CX3CR1} mice spent immobile during tail suspension was significantly increased (Fig. 12K), suggesting a tendency of VPS35^{CX3CR1} mice for depressive behavior. While there was no significant difference in time spent immobile during the forced-swim test, VPS35^{CX3CR1} mice did exhibit a trend toward increased immobility (Fig. 12L). Together, these results demonstrate depression-like behavior and impaired long-term memory in VPS35^{CX3CR1} mice.

Discussion

The roles of dysfunctional microglia and associated neuroinflammation have been highly implicated in neurodegenerative disease pathology, but evidence regarding the role, if any, that microglial VPS35 might play in the pathogenesis of AD is limited. To address this issue, we developed an *in vivo* mouse model of microglial-specific VPS35 loss of function to assess microglial VPS35's function in the adult CNS and how its dysregulation might contribute to neurodegenerative disease pathology. Based upon this model, our findings provide a novel, microglial-specific role for VPS35, in which microglial VPS35 is essential for not only preventing microglial activation *in vivo*, but also for maintaining adult hippocampal neurogenesis and promoting the development of newborn neurons in the hippocampus.

Using this *in vivo* mouse model of microglial VPS5 loss of function, our initial observations showed that loss of VPS35 from mature microglia *in vivo* increases microglial density in a regional-specific manner. Microglia have been shown to proliferate and

differentiate from CNS nestin-positive progenitor cells (Elmore et al., 2014), so while we saw no increase in microglial proliferation within the hippocampus, there remains the possibility that increased microglia in the hippocampus resulted from either increased differentiation of progenitor cells into microglia or migration of microglia from neighboring regions.

CX3CR1 is not a microglial-specific marker as it is also expressed by other myeloid cells, and peripheral macrophages have been observed to invade neuronal tissue under pathological conditions. Infiltration of peripheral macrophages has been shown to contribute to elevated levels of microglial activity as well as to neurodegenerative disease pathology (for review, see Rezai-Zadeh et al., 2009; Sevenich, 2018). Our observed increase of GFP⁺:Tmem119⁺ cell density (in addition to an increased density of GFP⁺:Tmem119⁺ cells) suggests the possibility of an increase of peripheral macrophages (or other cells of myeloid origin) in the VPS35^{CX3CR1} hippocampus in addition to increased microglial cell density.

Microglial retromer loss of function was also shown to up-regulate microglial activity within the hippocampus in a manner consistent with reports of proinflammatory activity. It would be beneficial for future studies to further investigate potential mechanisms underlying our observations by determining the inflammatory profile of microglia following VPS35 depletion and how increased activity affects the overall function and nature of VPS35-depleted microglia. Such investigations could help identify potential mechanistic players that might contribute to the observed phenotype of VPS35^{CX3CR1} mice.

Disturbances to the local pool of hippocampal microglia co-occurred with disrupted hippocampal neurogenesis, as observed by an increase of proliferating NSCs/NPCs, impaired cell-cycle exit, a decrease in immature neurons, abnormal dendritic morphology of newborn neurons, and aberrant microglial engulfment of synaptic material. It is of interest to note that recent advances implicate a role for microglia in adult hippocampal neurogenesis, a phenomenon that occurs primarily in the SGZ and that can be impaired by dysfunctional microglia and elevated proinflammatory factors (Ekdahl et al., 2003; Monje et al., 2003; Sierra et al., 2010, 2013). While microglia have been identified as contributors to the regulation of adult hippocampal neurogenesis, the full extent of this regulation and its mechanisms remain to be elucidated. Our findings implicate a regulatory role of microglia during hippocampal neurogenesis. The exact nature of this novel role of microglia and the mechanisms through which VPS35-deficient microglia disturb hippocampal neurogenesis will require further investigation.

It is important to note that disturbed hippocampal neurogenesis has been implicated as an early event in the course of AD pathogenesis (Jin et al., 2004; Ziabreva et al., 2006). Also, in AD mouse models, impaired hippocampal neurogenesis compromises hippocampal function and contributes to cognitive deficits (Rodríguez et al., 2008; Demars et al., 2010; Hamilton et al., 2010; Lazarov and Marr, 2010, 2013). While reports regarding the exact nature and extent of disrupted adult hippocampal neurogenesis in AD vary, the overall consensus is that such events play a role in the early stages of AD progression. This study presents a behavioral phenotype of VPS35^{CX3CR1} mice concurrent with the literature describing behavioral impairments that co-occur with impaired hippocampal neurogenesis. Depressive behavior is a classic hallmark of disrupted hippocampal neurogenesis (Sahay and Hen, 2007; Mateus-Pinheiro et al., 2013) and long-term memory has shown to be reliant upon hippocampal neurogenesis, with deficiencies

(Figure legend continued.) $p = 0.0187$). F–I, VPS35^{CX3CR1} primary microglia cocultured with primary cortical neurons exhibit increased engulfment of PSD95 + postsynaptic material ($p = 0.0323$), without any significant uptake of synapsin + presynaptic material ($p = 0.4965$). Scale bar, 10 μm (Student's unpaired t test, $n = 9$ microglia per group). J, K, VPS35^{CX3CR1} hippocampal microglia display increased engulfment of PSD95 + postsynaptic material *in vivo* as exhibited by high-resolution confocal microscopy analysis of GFP:PSD95 coimmunofluorescence. Scale bar, 50 μm (Student's unpaired t test, $n = 7$, $p = 0.0002$). For all analyses, statistical significance (* $p \leq 0.05$).

Figure 12. Behavioral phenotypes of the VPS35^{CX3CR1} mouse. **A**, Outline of behavioral analysis experimental design. **B–E**, Open-field test locomotor activity (**C**, **D**) and anxiety-related behavior (**E**) are not altered in VPS35^{CX3CR1} mice as gauged by total distance ($p = 0.285$), mean velocity ($p = 0.3695$), and time spent in the center ($p = 0.8$). **F**, **G**, No significant difference was observed in Y-maze performance ($p = 0.5586$), suggesting that spatial memory is not impaired in VPS35^{CX3CR1} mice. **H**, **I**, Novel-object recognition suggests a slight impairment in VPS35^{CX3CR1} recognition memory as indicated by a p value of 0.06 and a recognition index of 50% 30 and 120 min following initial exposure and a significant impairment in long-term memory (48 h after exposure, $p = 0.0023$). **J–L**, Depressive model behavioral testing revealed tendency toward depressive behavior in VPS35^{CX3CR1} mice. **J**, Sucrose preference is significantly lower in VPS35^{CX3CR1} mice ($p = 0.0019$) than in controls, suggesting a reduced tendency VPS35^{CX3CR1} pleasure-seeking behavior. **K**, Time spent immobile during tail suspension is significantly higher in VPS35^{CX3CR1} mice ($p = 0.0275$). **L**, Forced-swim test shows a trend toward increased time spent immobile by VPS35^{CX3CR1} mice, but not significantly ($p = 0.1882$). All tests were statistically analyzed by Student's t test, except for novel-object recognition, which was analyzed by one-way ANOVA ($n = 8$). For all analyses, statistical significance ($*p \leq 0.05$).

observed following impaired hippocampal neurogenesis (Bruehl-Jungerman et al., 2005; Sahay et al., 2011; Pan et al., 2012).

AD pathogenesis first begins to manifest in the Ec-Ctx, and Ec-Ctx VPS35 levels are downregulated in AD compared with VPS35 levels in the DG (Small et al., 2005). As our data indicated increased microglial density in the Ec-Ctx, it can be hypothesized that VPS35^{CX3CR1} microglial activity in this region would be similar to observations made in the hippocampus and might affect Ec-Ctx dendritic morphology. Ec-Ctx stimulation has been shown to promote the proliferation and neurogenesis of newborn neurons in the DG (Stone et al., 2011), so it is feasible the neurogenic abnormalities observed in VPS35^{CX3CR1} mice could be attributed to functional impairment of the Ec-Ctx. Additionally, newborn hippocampal neurons in the DG integrate into the hippocampal circuitry to send axonal projections to hippocampal CA3 neurons (Markakis and Gage, 1999), so it follows that hippocampal circuitry might be adversely regulated in VPS35^{CX3CR1} mice. Further investigations into the functional consequences of microglial VPS35 loss of function should explore the functionality of both Ec-Ctx and hippocampal circuitry.

In concurrence with previous reports of microglial VPS35 regulation of phagocytic receptors (Lucin et al., 2013; Yin et al., 2016), our studies implicate microglial VPS35's function in regulating microglial phagocytic activity. It is important to note here the relevance of these findings, as microglial depletion of C9orf72 [a regulator of endosomal trafficking (Farg et al., 2014)] results in similar enlargement of LAMP1⁺ vesicles, as observed in VPS35^{CX3CR1} microglia (O'Rourke et al., 2016), and microglial depletion of TDP-43 [also a regulator of endosomal trafficking (Schwenk et al., 2016)] leads to increased levels of lysosomal markers, which coincides with excessive synaptic pruning (Paolicelli et al., 2017). In addition to enlarged LAMP1⁺ vacuolar-like structures, our data suggest that VPS35-depleted microglia exhibit upregulated engulfment of postsynaptic processes, which is possibly one mechanism through which microglial VPS35 loss of function acts to affect the neurodegenerative phenotype observed in the VPS35^{CX3CR1} hippocampus. It is important to note that our results do not exclude the possibility that cocultured VPS35-deficient microglia may take up neuronal material as a result of neuronal death via indirect consequences of microglial VPS35 loss of function (as opposed to direct phagocytosis of live neuronal processes). It is also possible that the increased mCh and PSD95 in VPS35 mutant microglia are due to impaired degradation of these proteins in the mutant microglial lysosomes. During embryonic development and in the developing postnatal brain, microglia participate in synaptic pruning via complement receptor-mediated phagocytosis of synaptic elements, with recent evidence suggesting microglia-mediated synaptic pruning continues throughout adulthood via similar mechanisms (Stevens et al., 2007; Tremblay et al., 2010; Paolicelli et al., 2011; Schafer et al., 2012). Due to the mounting evidence that complement receptor-mediated phagocytosis plays a role in synaptic pruning and integration of adult neuronal processes, it will be of interest to address the role of microglial VPS35 in the regulation of synaptic pruning and to address the issue of how microglial VPS35 loss of function might contribute to disease pathology through the phagocytic activity of microglia upon dendritic processes.

The findings of this study suggest a region-specific effect of microglial VPS35 depletion, as well as the potential for an age-specific effect. Further studies should investigate the consequences of microglial VPS35 depletion in an age-specific and regional-specific manner. Of primary interest is the substantia nigra (SN), in which we also observed increased microglial density. Since dysfunction

of VPS35 is implicated in PD, it is necessary to also investigate whether VPS35-deficient microglia affect functional and/or morphological changes in this region. Neurogenesis has been documented in the SN (Zhao et al., 2003; Yoshimi et al., 2005), so investigations into whether SN neurogenesis and its functional results are altered in VPS35^{CX3CR1} mice would be intriguing. Unreported data from VPS35^{CX3CR1} mice suggested that the SN might also present a neurodegenerative morphological phenotype (examined via Golgi staining of SN neurons), so mechanisms underlying microglial VPS35-mediated regulation of this region could contribute to our understanding of VPS35's role in PD pathology.

The findings presented here provide the first *in vivo* evidence that VPS35/retromer is essential for maintaining microglial homeostasis, and when microglial retromer mechanics are disrupted, the effects can be observed not only in microglia themselves, but in surrounding neurons and critical neurogenic processes. Uncovering the molecular mechanisms underlying disease pathology is the first step toward disease prevention and discovery of cures. Retromer and VPS35 have been implicated as molecular mediators in neurodegenerative pathology, but the exact mechanisms through which retromer dysfunction imparts disease pathology have yet to be elucidated. Identification of cell-specific mechanisms underlying disease pathology is an important step for improving the experimental models used to investigate disease. By further identifying retromer-associated mediators of the neurodegenerative pathway, we hope to establish new methods for early detection of disease onset, preventative measures to counter pathological mechanisms, and therapeutic options to target the retromer pathway.

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