ISSN (Online) 2287-3406
Journal of Life Science **2020** Vol. 30. No. 8. 708~712 DOI : https://doi.org/10.5352/JLS.2020.30.8.708

- Note -

Deletion of the VPS26b-VPS29-VPS35 Retromer Complex Results in Learning Disabilities and Neurodegeneration

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Received April 17, 2020 /Revised June 3, 2020 /Accepted August 14, 2020

Vacuolar protein sorting (VPS) 26b is a newly discovered member of the retromer complex; it is encoded by a single-copy gene located on mouse chromosome 9, and the complex has been reported as being composed of proteins VPS26, VPS29, and VPS35. We have previously shown that mice lacking VPS26b exhibited no significant body size or health issues. Although retromer components are widely expressed in mouse tissue, their roles have not yet been completely elucidated. The current study investigates whether the VPS26b-associated retromer complex can be used as a neurodegeneration model. Previously, we observed a significant reduction in VPS35 and VPS29 in the brain cells of in VPS26b-deficient mice as well as an absence of the VPS26b-VPS29-VPS35 retromer complex despite the normal presence of VPS26a-VPS29-VPS35. Recent studies have suggested that low levels of VPS35 can lead to Alzheimer's disease-like phenotypes including cognitive memory deficits. In this study, we successfully demonstrate an association between the absence of the VPS26b-VPS29-VPS35 retromer complex, reduced cell density in the CA3 region of the hippocampus, and learning disability in VPS26b knock-out mice. The results also indicate that the VPS26b-associated retromer complex affects neurodegenerative disorders and learning processes.

Key words : Alzheimer's disease, learning deficits, neurodegeneration, vacuolar protein sorting-associated protein (VPS)

Introduction

Alzheimer's disease (AD), a neurodegenerative condition associated with hippocampal neuronal loss, is characterized by abnormal accumulation of neurotoxic amyloid beta (Aβ 42) and tau proteins [6]. Numerous susceptibility genes have been identified in AD pathology, including vacuolar protein sorting-associated protein (VPS) 35, 29 and 26[2].

Retromer, a protein complex of VPS26, VPS29, and VPS35 molecules, plays a critical role in endosomal trafficking; moreover, retromer dysfunction has been linked to a growing number of neurological disorders [9]. The process of intracellular trafficking and recycling is crucial for maintenance of intracellular homeostasis, which is partly achieved through the retromer complex. The retromer complex plays a primary role in sorting out the cargoes from endosomes back to the cell surface for reuse, to the trans-Golgi network (TGN) or alternatively to specialized endomembrane compartments away from lysosomal degradation [2]. Normally, there is a coordinated relationship between these pathways, however, a defect such as haplo-insufficiency or mutation in one or several units of retromer may lead to various pathologies [9]. VPS35, the main factor for endosome-to-Golgi retrieval of membrane proteins, is a ubiquitously expressed protein including neurons and glial cells. Dysfunction of VPS35 is implicated in the pathogenesis of Alzheimer's disease (AD) as well as Parkinson's disease (PD) due to mutations in the *VPS35* gene that have been identified in the late-onset PD and AD patients [11]. Thus, it is of considerable interest to investigate how VPS35 deficiency contributes to neurodegeneration. The representative hypothesis is that VPS35 expression in pyramidal neurons is critical to prevent AD-relevant neuropathology. Kerr *et al*., have shown that VPS26 is present as two distinct subtypes (VPS26a and VPS26b) that share approximately 70% identity and are localized on mouse chromosomes 10 and 9, and human chromosomes 10 and 11, respectively [3].

Mouse VPS26b is recently shown to be expressed predominantly in the brain. Unlike VPS26a, which is localized in the endosome, VPS26b is primarily located in the plasma membrane, suggesting different role(s) for the two isoforms

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in intra-cellular sorting and/or transportation of various proteins [4]. We previously showed that deficiency of VPS26b reduces expression of VPS29 and VPS35 due to absence of the VPS26b – VPS29 – VPS35 retromer complex, and increases the expression of sortilin, which is a protein regulated by the retromer complex [5]. In mice, deletion of the *VPS26a* gene is embryonically lethal, and heterogeneous mice exhibit hippocampal dysfunction due to Aβ42 accumulation [7, 10]. To investigate the role of the VPS26b in brain, we disrupted retromer function by selectively deleting *VPS26b* gene using gene-targeting system in the mouse.

Materials and Methods

Mouse care

All animal care and experiments were conducted in accordance with the guidelines for the Care and Use of Animals in Deagu Catholic University (IACUC-2015-038).

Preparation of protein extracts

The mouse brain hippocampal regions were homogenized in a lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 1% Triton X-100 (TX-100), 15 mM NaCl, and 1% protease inhibitor cocktail (Sigma – Aldrich) for the extraction of proteins. After centrifugation at 10,000 *g* for 10 min at 4℃, the proteins in the supernatant solution were analyzed [4].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis

Proteins were denatured by boiling for 3 min in the presence of 1% SDS and 1% 2-mercaptoethanol, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon- membranes (Millipore). The membranes were blocked with Tris-buffered saline Tween-20 (TBS-T) containing 2% skim milk, incubated with the primary antibody at room temperature for 2 hr, and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) for another 1.5 hr. The immunoreactive proteins were detected using an ECL Western blotting detection kit (Amersham Biosciences).

Histological analysis

The brain, liver, lung, kidney, and testis of 5-month-old *Vps26b*$^{-/-}$ mice were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, washed with PBS, and frozen in optimal cutting temperature (OCT) compound. Sections were prepared using a Leica microtome SM2000R; they were stained with hematoxylin/eosin as described previously [10] and were observed under an Olympus BX50 microscope (Tokyo, Japan).

Morris water maze test

The *Vps26b*$^{-/-}$ mice (generations back-crossed to C57BL/6:ICR, n=9) and wild-type mice (n=10) obtained from interbreeding of Vps26b-hetero male and female mice were used for all behavioral studies, and all experimental animals were allowed to adapt to the environment before subjecting them to the Morris water maze (MWM) test. In the MWM test, *Vps26b*$^{-/-}$ mice (4 male and 5 female mice) and wild-type (WT) mice (5 male and 5 female mice) were placed in a circular pool (diameter, 150 cm; height, 35 cm) filled with water made opaque with non-allergenic, water-soluble white paint. The water was maintained at a temperature of 22℃ ± 2℃. A platform that was 12 cm in diameter was hidden at a permanent location (the quadrant center) within the pool with its top surface submerged 1.5 cm below the water surface. A video camera was mounted on the ceiling above the pool, and it was connected to a video recorder and tracking device, permitting on- and off-line automated tracking of the path taken by the mouse. The mice were trained to get to the location of the platform for 3 consecutive days; each day, 3 trials lasting for a maximum time of 1 minute each were conducted. The latency time required to find the platform was recorded as a measure of spatial memory. On day 4, the mice were released in the quadrant opposite to the target and were forced to swim for 1 min in the pool without the platform. The time spent searching for the platform in the target zone (i.e., the previous location of the platform), was recorded as a measure of memory retention. The behavioral studies were carried out by well-trained experimenters blinded to the mouse genotypes.

Statistical analysis

The data results of the MWM spatial acquisition test were compared between groups and between days. For the spatial acquisition experiments, two-way repeated measures analysis of variance (ANOVA) tests were conducted to assess the effects of group (genotype + sex) and day. Data for male and female mice were pooled and analyzed together with one-way repeated ANOVA. When the ANOVA detected significant day effects, pairwise differences between the means for a given variable were evaluated using Tukey's *post hoc*

multiple comparison tests, with significance set at $p<0.05$. For reference memory experiments, the data were subjected to one-way ANOVA between 4 groups－WT-male, WT-female, knockout (KO)-male, and KO-female. In addition, data for male and female mice were pooled and comparisons were made between WT and KO mice by using the *t*-test. All statistical analyses were conducted using SPSS version 17.0.

Results and Discussion

VPS26b knock-out mice exhibit no histological changes

No histological changes were observed in the heart, lung, liver, kidney, and testis (data not shown). However, the absence of Vps26b molecule has halved the vps35 and vps29, despite no difference of the expression level for VPS26a in knock-out brain (Fig. 1). In addition, slightly lower cell density in the CA3 region of the hippocampus than those of wild-type mice (Fig. 2). Indeed, VPS26, 29 and 35 molecules that do not form retromer do not appear to exist alone [10]. Because, when the VPS35 knockdown, which constitutes the retromer, the VPS26 and VPS29 were reduced [1]. Thus, the absence of VPS26b may be natural for the components of vps26b related retromer to decrease. We previously have reported that there are VPS26a and VPs26b present in cells, each of which constitutes two types of retromer [4]. VPS26a is normally present in the VPS26b knock-out mouse, suggesting that the retromer with VPS26a is normally present.

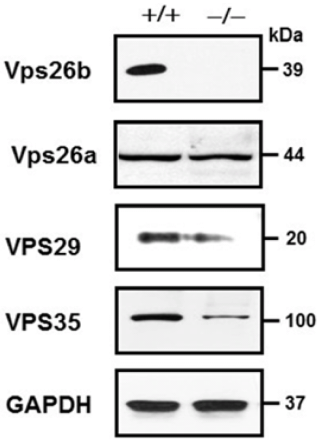

Fig. 1. Presence of retromer components in the hippocampal region of VPS26b$^{-/-}$ brain. Proteins in Triton X-100 extracts from the tissue were separated by SDS-PAGE under reducing conditions and subjected to Western blot analysis using antibodies against VPS26b, VPS26a, VPS29, VPS35 and GAPDH antibodies.

VPS26b knock-out mice have impaired learning disability partially

To assess whether the VPS26b-VPS29-VPS35 retromer deficiency affects hippocampal function, we used the Morris-water maze test (MWM) to test spatial learning and memory in 4 groups of mice (wild-type males, wild-type females, *VPS26b*$^{-/-}$ males and *VPS26b*$^{-/-}$ females) for 4 days. During each test, we determined the mean latency of each group to reach a hidden platform. A spatial acquisition task was performed on the first 3 days, followed by a reference memory task without the platform on the fourth day. The results of the spatial acquisition experiments used in the MWM test are shown in Fig. 3A and B. We did not find any statistically significant difference in the escape latencies of wild-type or VPS26b$^{-/-}$ mice to reach the hidden platform ($F(6, 339) = 0.825$, $p= 0.533$). However, 2-way ANOVA revealed a significant main effect of days with a continuous reduction in the time needed to reach the platform ($F(2, 4110) =10.01$, $p<0.001$) (Fig. 3A). Since ANOVA did not detect any significant effect of sex or genotype on the spatial acquisition task, we pooled the data from both sexes and analyzed with one-way repeated ANOVA (Fig. 2B). In this analysis, wild-type mice showed significant reduction in escape latencies during the first 3 days ($F(2, 3732) =9.667$, $p<0.001$); however,

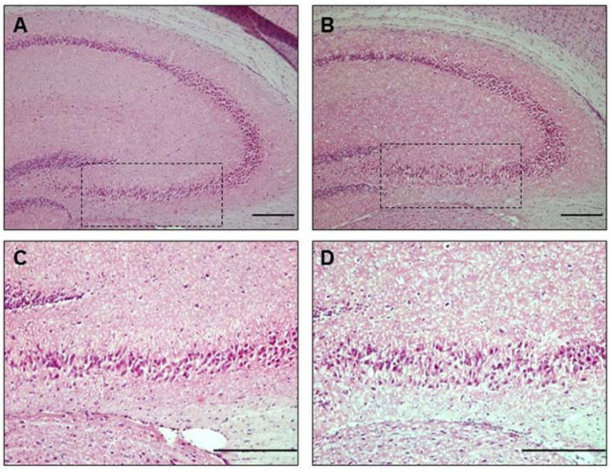

Fig. 2. Hippocampal sections from *VPS26b*$^{-/-}$ mice stained with hematoxylin and eosin (HE). HE stained coronal sections of the hippocampal formation were compared in wild-type (A and C), and *VPS26b*$^{-/-}$ mice (B and D). In the hippocampal formation, CA3 neurons were less densely packed in the *VPS26b*$^{-/-}$ mice than in the wild-type mice. The boxed regions in A and B are enlarged in C and D. Magnification 100′, scale bar 200 μm.

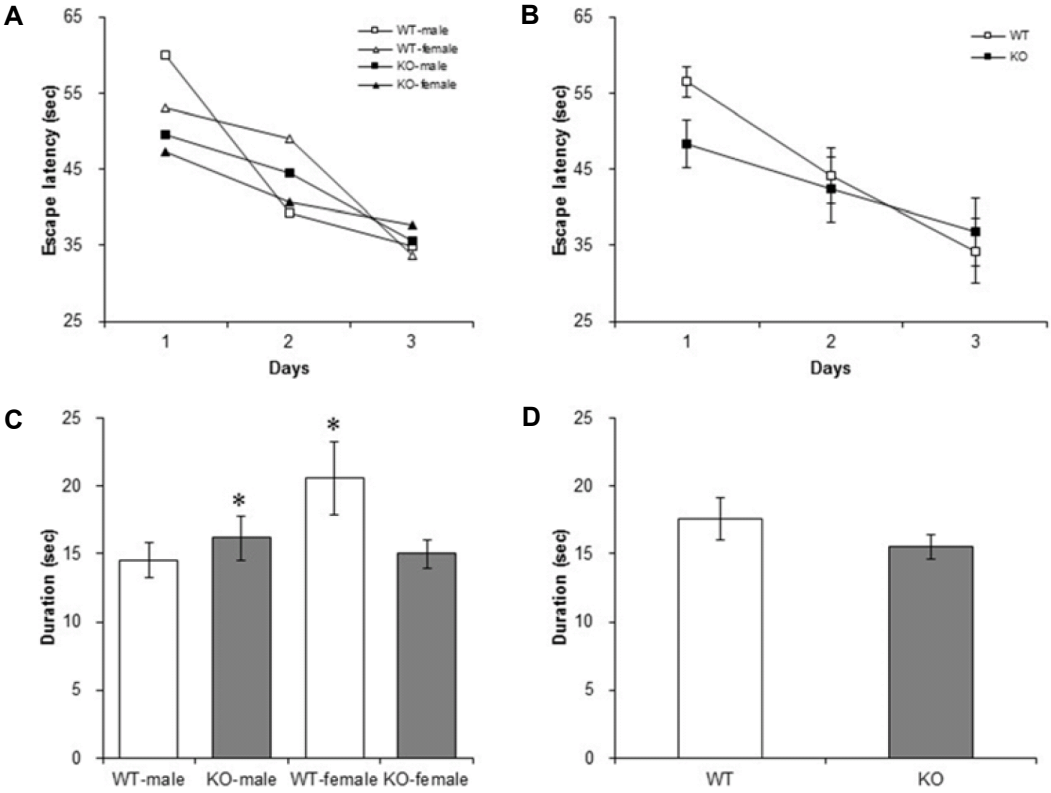

Fig. 3. Mean escape latencies for *VPS26*$^{+/-}$ and *VPS26b*$^{-/-}$ mice in Morris water maze task. Four groups of mice (*VPS26b*$^{+/+}$ males, *VPS26b*$^{+/+}$ females, *VPS26b*$^{-/-}$ males, and *VPS26b*$^{-/-}$ females) were trained with a spatial acquisition task 3 times a day for 3 consecutive days. On the fourth day, a probe test was conducted as a reference memory task. (A, B) Escape latencies to find the hidden platform during the training period. (C, D) Time spent in the target quadrant during the probe test. All tests were performed with *VPS26b*$^{+/+}$ (n=10) and *VPS26b*$^{-/-}$ (n=9) mice. Values are expressed as mean (SEM). Statistically significant differences (p<0.05) are indicated with an asterisk (*). Wild type group shows significant effect of daily learning ability compared with first trial day (*p<0.05).

there was no significant difference in *VPS26b*$^{-/-}$ mice (F(2.907) =2.112, p=0.136). Subsequently, we used one-way ANOVA to compare differences in the mean latencies between each day. There was a significant decrease in the escape latencies for the second and third day as compared with the first day (*p<0.005) in wild-type mice but not in the *VPS26b*$^{-/-}$ group. The results of the reference memory task during the probe test are shown in Fig. 2C and D. One-way ANOVA did not reveal any significant difference in the time spent in the target quadrant among the 4 groups of mice. In addition, no significant differences were found when we pooled the data for sexes and used Student's t-test to compare the wild-type and *VPS26b*$^{-/-}$ groups.

In this study, we successfully demonstrated an association between the absence of the VPS26b-VPS29-VPS35 retromer, reduced cell density in the CA3 region of the hippocampus, and learning disability in *VPS26b*$^{-/-}$ mice. However, *VPS26b*-deficient mice were born normally, and demonstrated learning disability despite no significant difference in Aβ accumulation. Although further studies are needed to elucidate the functions of the retromer complex in more detail, the VPS26b-VPS29-VPS35 retromer may be involved in learning processes. Finally, *VPS26b*$^{-/-}$ mice are a potentially useful tool for investigating the role of the retromer complex in the mammalian nervous system.

Acknowledgement

This work was supported by National Research Foundation of Korea Grant funded by the Korean Government (NRF-2020R111A3072358).

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : VPS26b-VPS29-VPS35 리트로머 복합체 결여가 마우스 뇌조직에 미치는 영향

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리트로머(retromer)는 VPS26, VPS29, VPS35 분자로 구성된 복합체로, 세포막에 존재하는 특정 단백질을 엔도솜에서 트렌스골지망으로 리사이클에 관여하는 단백질 복합체이다. 2000년대 초반 콜롬비아대학의 Scott A, Small 팀에 의해서, 알츠하이머 환자에서 리트로머 분자의 발현량이 저하된다는 것을 발견하였으며, 리트로머를 구성하는 VPS35 발현을 저하시킨 마우스를 이용한 Morris Water Maze (MWM) 실험에서 인지능력이 떨어진다는 것을 보고 하였다. 본 연구진은 리트로머를 구성하는 VPS26 분자에 대한 서브타입인 VPS26b를 발견하였고, 낙아웃 마우스를 제작하였다. VPS26b 낙아웃 마우스 뇌조직을 이용한 웨스턴 블롯 결과, 낙아웃 마우스 뇌조직에서 VPS29와 VPS35의 발현량의 50% 정도로 감소되는 것을 확인하였다. 또한, VPS29 낙아웃 마우스를 이용하여 MWM실험은 한 결과 인지능력이 저하되는 것을 확인하였으며 뇌조직의 해마 CA3영역의 세포 분포도가 정상 마우스에 비해 감소되는 것을 확인하였다. 결과적으로 이번 연구를 통하여 VPS26b 낙아웃 마우스는 뇌질환 연구에 대한 실험 동물로서 기초 자료를 제공할 수 있을 것임을 보여준다.