Vps18 deficiency inhibits dendritogenesis in Purkinje cells by blocking the lysosomal degradation of Lysyl Oxidase

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1. Introduction

The development of dendrites occupies a central position in the formation of the nervous system. Purkinje cells, an important group of neurons in the cerebellum, have very large, planar, and highly branched dendritic trees. This unique morphology makes it an ideal model system to study dendrite growth and differentiation [1,2]. In rodents, the development of Purkinje cell dendrites begins shortly before birth and completes at about 4 weeks after birth [3]. Extensive studies of Purkinje cells have made great contributions to our understanding of the dendrite development process. Recently, the accumulation of lysyl oxidase (Lox) was found to block Purkinje cell dendrite development through inhibiting the NF-\(\kappa\)B signaling pathway [4].

Previous studies illustrate the critical role of endocytosis system in dendrite development [5]. However, despite the report that synaptoctin VII-regulated exocytosis of lysosomes was critical for neurite outgrowth [6–9], little is known about the function of lysosomes in dendritogenesis. In a mouse model of Sandhoff disease, a kind of lysosome storage disease, the neurite outgrowth of the dorsal root ganglion neurons is normal [10]. Therefore, one fundamental question that remains unanswered is whether the degradative function of lysosomes is required for dendrite development.

The Vps18 protein is a subunit of class C Vps complex, which also includes Vps11, Vps16, and Vps33 [11]. Extensive researches in lower eukaryotic organisms have shown that Vps-C complex plays essential roles in late endosome and lysosome-related vesicle transport pathways. Vps-C complex dysfunction results in the loss of vacuole structure and accumulation of autophagosomes and late endosomes in yeast [11,12]. Deletion of Vps18 in yeast leads to the breakdown of the whole Vps-C complex, thus putting Vps18 in a central position [13]. Mutating Vps18 homolog, dor, in Drosophila causes the accumulation of exaggerated multivesicular structure in retinal cells, blockage of autophagosome–lysosome fusion in larval fat body, and promotion of tumor metastasis [14–16]. In mammalian cells, knockdown of Vps18 or treatment of anti-Vps18 antibody blocks autophagosome–lysosome and early endosome fusion [17,18]. Neural-specific deletion of Vps18 leads to severer neurodegeneration and disturbed neuronal migration in mice due to the blockage of multiple vesicle transport pathways to lysosome, including endocytosis, autophagy, and biosynthetic pathways [19]. However, the function of the Vps18 gene in dendrite development of neural cells is still unknown.

Here we report the critical function of Vps18 in the dendrite development of Purkinje cells. Dendritogenesis of Purkinje cells in Vps18\(^{-}\)/; Nestin-Cre mice is severely inhibited. However, the dendrites of cerebral cortical neurons are largely unaffected. Interestingly, despite server Purkinje cell loss in Vps18\(^{-}\)/; Pcp2-Cre mice, the dendrite of remaining Purkinje cells developed normally. Finally, we demonstrate that the Lox protein is degraded through lysosome pathway and accumulates in the cerebellum of Vps18\(^{-}\)/;
Nestin-Cre mice, which may cause the dendrite defect of Purkinje cells. Our results suggest for the first time a critical function of lysosome degradation pathway in dendrite development.

2. Materials and methods

2.1. Animals

Vps18<sup>Cre</sup>; Nestin-Cre mice, Vps18<sup>CreLOX</sup> and Vps18<sup>Cre/Cre</sup>, Pcp2-Cre mice were described by C. Peng et al. [19]. All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University.

2.2. Western blot

Proteins were extracted with radioimmune precipitation assay (RIPA) buffer and resolved on SDS–PAGE followed by Western blotting with antibodies against Lox (sc-66948; Santa Cruz), c-Myc (sc-40; Santa Cruz) or GAPDH (KC-5G4; KangChen) as loading control.

2.3. Immunofluorescent staining analyses

The treatment of mouse brains for frozen section was described by C. Peng et al. [19]. Sections were then collected at 30 μm and stained with calbindin antibody (C9848; Sigma). Fluorescence micrographs were acquired using a Zeiss LSM710 confocal microscope.

2.4. In utero electroporation

In utero electroporation was carried out following standard protocols [20]. Plasmids expressing EyFP were injected into the lateral ventricles of mouse embryos at E14. Five days after electroporation, embryos were collected for analysis.

2.5. Quantification of dendrites

To calculate the total dendrite length, node or end number, and dendrite density (sholl analysis) of neural cells, 4–12 fields of Purkinje cells in the cerebellum (lobules V–VIII) or cerebral cortical neurons in layer II/III were randomly imaged by confocal microscopy (Zeiss Axiosvert 100 M). Total dendrites were traced and analyzed by the Image J software with Neuron J plugin. Sholl analysis was performed as described by [4,21].

2.6. Behavioral tests

Behavioral tests were carried out on 6–11 months old Vps18<sup>Cre/Cre</sup>, Pcp2-Cre mice and littermate controls, which were backcrossed C57BL6 for six generations.

2.6.1. Footprint test

Footprint analysis was carried out according to the protocol described [22]. Briefly, mouse hind paws were dipped in nontoxic ink, and mice were allowed to walk down a tunnel lined with white paper. The footprint patterns were analyzed and measured for gait width and stride length.

2.6.2. Dowel test

Mice were place on a horizontal wooden rod (9 mm diameter) and the latency time to fall was recorded up to a maximum of 3 min.

2.6.3. Accelerating rotating rod test

The accelerating rotating rod test was carried out as described [23]. Briefly, mice were placed on the rod of a rotating rod apparatus (Tianhuan Instruments), which accelerated from 4 to 40 rpm in 5 min linearly, and the time the mice stayed on the rod was measured.

2.7. Lox cDNA

Plasmid expressing Myc tagged Lox is a kind gift from Y. Eugene Chin (Institute of Health Sciences, Chinese Academy of Sciences).

2.8. Statistical analysis

Unpaired t-test was used. A value of *P < 0.05, **P < 0.01 or ***P < 0.001 denoted statistical significance.

3. Results

3.1. Suppression of the development of Purkinje cell dendrites in Vps18<sup>Cre/Cre</sup>; Nestin-Cre mice

We have generated Vps18<sup>Cre/Cre</sup>, Nestin-Cre mice [referred to as Vps18 CKO (conditional knock out)], in which exon 3 and 4 of the Vps18 gene were specifically deleted in neural cells [19]. To understand the function of the lysosome degradation pathway in the dendrite development of neural cells, we evaluated the effects of Vps18 deficiency on dendrite development of Purkinje cells in Vps18 CKO and Vps18<sup>Cre/Cre</sup> (referred to as Ctrl) mice by immunofluorescent staining with Calbindin antibody. Our results revealed that the dendrite of Vps18 deficient Purkinje cells was shorter and less branched (Fig. 1A and B). Quantitative analysis showed that Purkinje cell dendrites in Vps18 CKO mice had a shorter total length and significantly reduced numbers of nodes and ends (Fig. 1C). Sholl analysis [24], which determines the number of crossings between dendrite and concentric circles, showed that the dendrite density of Purkinje cells in Vps18 CKO mice was greatly reduced compared with that of the control (Fig. 1D). These results indicate that Vps18 plays a critical role in the development of Purkinje cell dendrites.

3.2. Vps18 deficiency does not affect dendritic morphogenesis in the cerebral cortex

Given the morphological defects observed in Purkinje cells, we questioned whether the effect of Vps18 deficiency on dendritogenesis was Purkinje cell-specific or ubiquitous. We evaluated the dendritic development of cerebral cortical neurons by performing in utero electroporation with the plasmid pEYFP at E14 and analyzing dendritogenesis of EyFP-labeled neurons at E19. The dendrite morphology of cortical neurons in layer II/III of Vps18 CKO mice looked similar to that of control mice (Fig. 2A and B). Quantitative analyses showed that total node or end numbers and total dendrite lengths per cell in Vps18 CKO and control cerebral cortices were comparable (Fig. 2C), suggesting that Vps18 is not essential for dendrite development in cerebral cortex at least during embryonic stage.

3.3. Purkinje cell dendrite developed normally in Vps18<sup>Cre/Cre</sup>; Pcp2-Cre mice

Development of Purkinje cell dendrites begins shortly before birth and completes at about 4 weeks after birth [3]. Severe impairment of the development of Purkinje cell dendrites in Vps18 CKO mice at P10 suggests that Vps18 is required for early stage den-
dritogenesis, but it provides no clue whether Vps18 also functions in late stage development and maintenance of Purkinje cell dendrites. Because Vps18 CKO mice died by postnatal day 12 [19], we addressed the question using Vps18F/F; Pcp2-Cre mice. Unlike Vps18 CKO mice, Vps18F/F; Pcp2-Cre mice can live up to at least 12 months (unpublished observation). However, the mice displayed abnormal behavior as early as 3 months old. We carried out behavior tests on 6 to 11 month old Vps18F/F; Pcp2-Cre mice and littermate controls. Our results showed that Vps18F/F; Pcp2-Cre mice displayed a severe impairment of performance on the rotarod apparatus and static rod, compared to littermate controls (Fig. 3A and B). Analysis of the footprint pattern showed that both the stride length and gait width were also shorter for Vps18F/F; Pcp2-Cre mice (Fig. 3C and D). These results demonstrate an impairment of the balance and coordination ability of Vps18F/F; Pcp2-Cre mice, indicating the disturbance of Purkinje cell functions, which may result from loss and/or dendrite defects of Purkinje cells in Vps18F/F; Pcp2-Cre mice.

We next assessed the dendrite development of Purkinje cells in Vps18F/F; Pcp2-Cre mice at the age of 3 months by immunofluorescent staining with anti-calbindin. Surprisingly, we found that the dendrite of the remaining Purkinje cells looked quite normal (Fig. 3E and F), although most Purkinje cells were lost in the mutant mice [19]. Quantitative analyses showed that the total note and end numbers and total length of Purkinje cell dendrites in Vps18F/F; Pcp2-Cre mice were all similar to that in littermate controls (Fig. 3G and H). These results indicate that Vps18 has little if any function in late stage development or maintenance of Purkinje cell dendrites. However, we cannot completely rule out other possibilities currently (see Discussion).

3.4. Lox protein accumulates in the Vps18 CKO cerebellum

Since Vps18 plays a critical role in the lysosome degradation pathway [11,19], the defects of Purkinje cell dendrite in Vps18 CKO mice may be caused by disturbed degradation of proteins that are important for dendrite development. Recent research has demonstrated that the accumulation of Lox in Purkinje cells impedes...
Fig. 2. Vps18 deficiency does not inhibit dendritogenesis of the cerebral cortical neurons. (A and B) Representative images of control or Vps18 deficient cortical neurons in layer II/III transfected with plasmid expressing EYFP (A) and traced with Image J (B). (C) Quantitative analysis of total node or end number and total dendrite length per cell in the cerebral cortices of E19 Vps18 CKO and control mice. ns, no significance. Values represent the means ± SEM. (n = 4; Scale bar: 50 μm).

Fig. 3. The effects of Vps18 deficiency on behavior and Purkinje cell dendrite development of Vps18F/F; Pcp2-Cre mice. (A) Poor performance of Vps18F/F; Pcp2-Cre mice on the rotating rod. Vps18F/F; Pcp2-Cre mice and littermate controls were tested for endurance to stay on the rotating rod with four trials per day for four consecutive days. (B) Static rod performance of Vps18F/F; Pcp2-Cre mice and littermate controls. (C and D) Abnormal gait in Vps18F/F; Pcp2-Cre mice. Representative footprint patterns of the Vps18F/F; Pcp2-Cre mice and littermate controls (C). Statistics of stride length and gait width (D). (E and F) Images of Purkinje cells from 3 months old Vps18F/F; Pcp2-Cre and littermate control mice stained with anti-calbindin (E) and traced with Image J (F). (G) Quantitative analysis of total node or end number and total dendrite length per field of Purkinje cells in the Vps18F/F; Pcp2-Cre and control mice. (H) Sholl analysis showed total intersection numbers of Purkinje cell dendrites with each concentric circle (10 μm, 20 μm, 280 μm) and peak values representing areas with high dendritic density, ns, no significance. *P value < 0.05, **P value < 0.001. Values represent the means ± SEM. (For all behavior tests n = 8; for quantitative analysis of dendrites n = 6; Scale bar: 50 μm.)
dendrite development [4]. Therefore, we hypothesized that the blockage of lysosome function resulted from Vps18 deficiency might lead to accumulation of Lox and then interfere with Purkinje cell arborization. To test this hypothesis, we first examined if Lox could be degraded through the lysosome. 293T cells were transfected with myc-tagged human Lox gene or RFP (control) were treated with lysosome inhibitors, chloroquine and monensine, at indicated concentrations for 24 h and analyzed by Western blot with anti-myc antibody. (B) Western blot analysis showed the restricted expression of the Lox protein in P10 mouse cerebellum and the accumulation in Vps18 CKO cerebellums.

Fig. 4. Accumulation of Lox in the Vps18 CKO cerebellum. (A) The Lox protein was degraded through the lysosome. 293T cells transfected with myc-tagged human Lox gene or RFP (control) were treated with lysosome inhibitors, chloroquine and monensine, at indicated concentrations for 24 h and analyzed by Western blot with anti-myc antibody. (B) Western blot analysis showed the restricted expression of the Lox protein in P10 mouse cerebellum and the accumulation in Vps18 CKO cerebellums.

4. Discussion

Although the cellular and molecular mechanisms of dendrite development in neural cells have been extensively studied, the function of lysosomes in this process remains largely unknown. Particularly, it is still not clear whether the degradative function of lysosomes plays a role in dendrite development. Here, we report the characterization of the dendrite development in Purkinje and cerebral cortical neurons in the Vps18 CKO mice. We found that dendritogenesis was impaired in the Purkinje cells of the Vps18 CKO mice but appeared normal in cerebral cortical neurons at least during the embryonic stage. This difference may be caused by the differential expression of Lox in cerebral cortex and cerebellum since our result showed that the expression level of Lox was much higher in the latter.

Although Vps18 deletion in Vps18CKO; Pcp2-Cre mice lead to severe loss of Purkinje cells at the age of 3 months [19] and complete loss at the age of 10 months (data not shown), we surprisingly found that it had no obvious effect on the dendrite morphology of Purkinje cells in Vps18CKO; Pcp2-Cre mice. There are several possible explanations for this result. The first explanation is that Vps18 is required for early stage dendritogenesis but not for the late stage development or maintenance of dendrite. The second explanation is that the protein level of Lox is not accumulated high enough to impair the dendrite development and maintenance in Vps18CKO; Pcp2-Cre mice because (1) Vps18 is deleted much later (starting from P6) in Vps18CKO; Pcp2-Cre mice than what happens in the Vps18 CKO mice (starting from E11); and (2) Lox expression decreases with CNS development and maturation [4]. The third explanation is that the Vps18 gene is not completely deleted in the Purkinje cells of 3 months old mutant mice although a previous study has demonstrated that in Pcp2-Cre mice the Cre-mediated recombination is fully established 2–3 weeks after birth [27]. Further extensive experiments are needed to distinguish these possibilities.

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References


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