Dynamin-Related Protein 1 Deficiency Leads to Receptor-Interacting Protein Kinase 3—Mediated Necrototic Neurodegeneration

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Mitochondria are dynamic organelles that divide and fuse to modulate their number and shape. We have previously reported that the loss of dynamin-related protein 1 (Drp1), which mediates mitochondrial division, leads to the degeneration of cerebellar Purkinje cells in mice. Because Drp1 has been shown to be important for apoptosis and necroptosis, it is puzzling how Purkinje neurons die in the absence of Drp1. In this study, we tested whether neurodegeneration involves necrotic cell death by generating Purkinje cell-specific Drp1-knockout (KO) mice that lack the receptor-interacting protein kinase 3 (Rip3), which regulates necroptosis. We found that the loss of Rip3 significantly delays the degeneration of Drp1-KO Purkinje neurons. In addition, before neurodegeneration, mitochondrial tubules elongate because of unopposed fusion and subsequently become large spheres as a result of oxidative damage. Surprisingly, Rip3 loss also helps Drp1-KO Purkinje cells maintain the elongated morphology of the mitochondrial tubules. These data suggest that Rip3 plays a role in neurodegeneration and mitochondrial morphology in the absence of mitochondrial division. (Am J Pathol 2016, 186: 2798–2802; http://dx.doi.org/10.1016/j.ajpath.2016.06.025)
Drp1, exhibit normal mitochondrial morphology in the same Drp1-KO cerebellum.

Drp1 is required for the maintenance of post-mitotic Purkinje neurons. To circumvent the developmental effects and study the role of Drp1 in post-mitotic neurons, we deleted Drp1 in post-mitotic Purkinje neurons after the completion of development using Cre recombinase, which is expressed from the Purkinje cell cell-specific L7 promoter. Drp1-KO Purkinje neurons produce excessively elongated mitochondrial tubules because of a lack of division; these long tubules then unexpectedly collapse into large spherical structures because of the accumulation of oxidative damage from decreased mitophagy. These mitochondrial defects result in respiratory deficiencies and the progressive degeneration of Drp1-KO Purkinje neurons.

It has been shown that decreasing mitochondrial size and remodeling of the mitochondrial outer membrane facilitates BAX oligomerization and cytochrome c release from mitochondria during apoptosis. During degeneration, there were no signs of apoptotic cell death in Drp1-KO Purkinje neurons, consistent with the role of Drp1 in apoptosis as a proapoptotic factor. Interestingly, in addition to apoptosis, recent studies have suggested that Drp1-mediated mitochondrial division is also important for necroptosis that is mediated by the protein complex containing receptor-interacting protein kinase 1 and 3 (Rip1 and Rip3, respectively). Mitochondrial division is stimulated via the dephosphorylation of Drp1 by a protein phosphatase, PGAM5, which functions as a downstream effector of the Rip1-Rip3 complex. The critical roles of Drp1 in both apoptosis and necroptosis prompted us to ask how neurons die when Drp1 is absent. In this study, we genetically blocked necroptosis by taking advantage of Rip3 whole-body KO mice to reveal the mechanism explaining how neurodegeneration results from a lack of Drp1-mediated mitochondrial division.

Materials and Methods

Animals

All of the work with animals was conducted according to guidelines established by the Johns Hopkins University Committee on Animal Care and Use. The Drp1flox/flox and Rip3flox/flox mice have been described previously. The L7-Cre mice were obtained from the Jackson Laboratory (Bar Harbor, ME). By breeding Rip3flox/flox::L7-Cre mice and Rip3flox/flox::Drp1flox/flox mice, we generated littermate control (Drp1flox/flox), Rip3-KO (Rip3flox/flox), L7-Drp1-KO (L7-Cre::Drp1flox/flox), and double-KO (Rip3flox/flox::L7-Cre::Drp1flox/flox) mice. The Drp1flox/flox mice are phenotypically wild type.

Immunofluorescence Microscopy

Immunofluorescence microscopy of cerebellar Purkinje cells was performed as previously described with some modifications. The mice were fixed by perfusing ice-cold 4% paraformaldehyde in phosphate-buffered saline. The brain was dissected, fixed in 4% paraformaldehyde in phosphate-buffered saline for 2 hours at 4°C, incubated in phosphate-buffered saline containing 30% sucrose overnight, and frozen in optimal cutting temperature compound (VWR,
Figure 2  Rip3 deficiency decreases the formation of large spherical mitochondria in Drp1-KO Purkinje neurons. A: Mitochondrial morphology. Sagittal sections of the cerebellum in the control, Rip3-KO, L7-Drp1-KO, and double-KO mice were stained using antibodies against carbonic anhydrase 8 (Car8) and a mitochondrial protein, pyruvate dehydrogenase (PDH), at an age of 3 months. B: Quantification of mitochondrial morphology. The number of soma of the Purkinje cells (PCs) that contained tubular mitochondria was determined and normalized relative to the length of the Purkinje cell layer. C: The percentage of Purkinje cells that contained tubular mitochondria was calculated by dividing the number of cells that contained tubular mitochondria by the number of surviving Purkinje cells. The t-test was performed to statistically analyze the difference between the L7-Drp1-KO and double-KO mice. Values represent means ± SEM (B and C). n = 7 (B and C, animals for each genotype). ***P < 0.001. Scale bar = 10 μm (A).
Rangor, PA). The frozen sections were cut, washed in phosphate-buffered saline, and blocked in 10% donkey or sheep serum. The sections were then incubated with antibodies to carboxic anhydrase 8 (Car8-Rb-A1330; Frontier Institute, Hokkaido, Japan) and pyruvate dehydrogenase (ab110333; Abcam, Cambridge, MA), followed by fluorescently labeled secondary antibodies (Alexa 488 anti-rabbit IgG A21206 and Alexa 568 anti-mouse IgG A10037; Invitrogen, Carlsbad, CA). We examined the samples using a Zeiss LSM780 FCS laser scanning confocal microscope equipped with a 10× (0.4 numerical aperture) objective and a Zeiss LSM510-Meta laser scanning confocal microscope equipped with a 100× (1.3 numerical aperture) objective.

Results

We generated control, Rip3-KO (Rip3fl/fl), L7-Drp1-KO (L7-Drp1fl/lox), and double-KO (Rip3fl/fl::L7-Drp1fl/lox) mice by breeding. Cerebella of mice at an age of 3 months were fixed, dissected, and sectioned around the median line. Cerebellar sections were subjected to immunofluorescence microscopy using antibodies against the Purkinje cell marker carboxic anhydrase 8, and soma were counted to quantify the number of Purkinje cells. Consistent with our previous studies, approximately 65% of the Purkinje cells were lost in the L7-Drp1-KO mice compared with the control mice (Figure 1, A and B). In contrast to the L7-Drp1-KO mice, the Rip3-KO mice contained a normal number of Purkinje neurons (Figure 1, A and B). In double-KO mice, we found a significant increase in the number of Purkinje cells compared with the L7-Drp1-KO mice (Figure 1, A and B). These data suggest that a significant population of Drp1-KO Purkinje cells die via a necrotic mechanism involving Rip3 at 3 months.

To further determine the effect of the loss of Rip3 on Drp1-KO Purkinje cells at a later time, we analyzed double-KO mice at 5 months. We found that the number of Purkinje cells had further decreased at 5 months compared to 3 months in double-KO mice (Supplemental Figure S1). Therefore, Rip3 loss delays the degeneration of Drp1-KO Purkinje cells but does not prevent it. We speculate that other cell death mechanisms, such as apoptosis, may be induced after a prolonged absence of necroptosis to eliminate Purkinje cells that contain dysfunctional mitochondria.

This suppression of neurodegeneration via Rip3 loss is interesting for two reasons. First, it has been shown that oxidative stress induces Rip3-mediated necroptosis. Second, we have previously shown that feeding L7-Drp1-KO mice an antioxidant, coenzyme Q10, partially rescues the degeneration of Purkinje cells. The rescuing effect of depleting Rip3 on the number of Drp1-KO Purkinje neurons appears to be similar to that of feeding the mice coenzyme Q10. It is possible that the accumulation of reactive oxygen species caused by Drp1 deficiency stimulates necroptosis via Rip3.

The effect of antioxidants is not limited to neurodegeneration; it also affects mitochondrial shape. As described above, the loss of Drp1 leads to the formation of large spherical mitochondria after mitochondrial elongation. Mitochondrial elongation directly results from continuous fusion over decreased mitochondrial division, and the second morphological transformation into spheres is mediated by oxidative stress, although its exact molecular mechanisms currently remain unknown. Sphere formation can be suppressed via antioxidant treatment in Drp1-KO Purkinje cells.

To determine the impact of the loss of Rip3 on mitochondrial morphology in Purkinje cells, cerebellar sections were stained using antibodies to a mitochondrial protein, pyruvate dehydrogenase, and carboxic anhydrase 8. Similar to antioxidants, Rip3 deletion also significantly increased the number of Purkinje neurons that contained nonspherical mitochondria in double-KO mice (Figure 2, A and B). This suppression of sphere formation is not simply because of the increased number of surviving Purkinje neurons, because the percentage of Purkinje neurons with nonspherical mitochondria is significantly increased in double-KO mice (Figure 2C). Therefore, Rip3 appears to mediate the morphological transformation of mitochondria from elongated tubules into large spheres induced by oxidative stress when mitochondrial division is inhibited.

Discussion

In this study, we revealed that post-mitotic Purkinje neurons degenerate via necrotic cell death in response to the loss of mitochondrial division. Our data suggest that the necroptotic mechanism can both still function in the absence of Drp1 and significantly contribute to neurodegeneration in vivo. Because Drp1-KO Purkinje neurons die in the absence of Rip3, it is important to determine the precise cell-death mechanisms that account for the remaining cell death. Because Purkinje cells undergo necroptosis without Drp1, these cells may also undergo apoptosis in the absence of Drp1. We are planning to address this key question in our future studies.

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Supplemental Data

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References


