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p62/sequestosome-1 knockout delays neurodegeneration induced by Drp1 loss

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ABSTRACT

Purkinje neurons, one of the largest neurons in the brain, are critical for controlling body movements, and the dysfunction and degeneration of these cells cause ataxia. Purkinje neurons require a very efficient energy supply from mitochondria because of their large size and extensive dendritic arbors. We have previously shown that mitochondrial division mediated by dynamin-related protein 1 (Drp1) is critical for the development and survival of Purkinje neurons. Drp1 deficiency has been associated with one of the major types of ataxia: autosomal recessive spastic ataxia of Charlevoix Saguenay. Using post-mitotic Purkinje neuron-specific Drp1 knockout (KO) in mice, we investigated the molecular mechanisms that mediate the progressive degeneration of Drp1-KO Purkinje neurons *in vivo*. In these Purkinje neurons, p62/sequestosome-1, a multi-functional adaptor protein that balances apoptotic cell death and cell survival, was recruited to large mitochondria resulting from unopposed fusion in the absence of mitochondrial division. To test the role of p62 in Drp1-deficient neurodegeneration, we created mice lacking both Drp1 and p62 and found that the additional loss of p62 significantly extended the survival of Purkinje neurons lacking Drp1. These results provide insights into the neurodegenerative mechanisms of mitochondrial ataxia and a critical foundation for therapeutic interventions for this disease.

1. Introduction

Although it has been over a century since the significance of the cerebellum for motor control was first identified, ataxia caused by cerebellar impairment remains an incurable disease (Bird, 1993; Fine et al., 2002; Schmahmann, 2004). Cerebellar impairment causes ataxia characterized by an impaired gait, difficulty coordinating one's extremities, disordered eye movement, poor articulation and tremor. The basic deficit common to the motor incapacity is the impairment of the rhythm, rate and force of contraction (Diener and Dichgans, 1992; Marsden and Harris, 2011). When the motor syndrome is fully manifest, it is a severely disabling condition. However, the mechanisms of pathogenesis and therapeutic strategies for ataxia remain elusive.

Cerebellar Purkinje neurons are one of the most important neurons for motor control, and their dysfunction leads to impaired motor coordination and locomotor learning (Kapfhammer, 2004; Tyrrell and Willshaw, 1992; Zhang et al., 2010). As the largest neurons in the central nervous system, Purkinje neurons require a very efficient energy supply through mitochondrial oxidative phosphorylation due to their highly arborized dendritic trees and their lengthy projection distances (Herndon, 1963; Phillips et al., 2016). Highly active mitochondrial oxidative phosphorylation in Purkinje neurons generates large amounts of toxic reactive oxygen species (ROS) as byproducts. One victim of ROS is the mitochondrion itself (Bhatti et al., 2017; Tonnies and Trushina, 2017); ROS impair mitochondrial components, such as mitochondrial membranes, proteins and DNA, rendering Purkinje neurons highly vulnerable to mitochondrial damage (Batlevi and La Spada, 2011; Federico et al., 2012; Itoh et al., 2013; Stadtman, 2006). In fact, studies have shown that mitochondrial health is significantly correlated with the pathogenesis of ataxia, which is termed mitochondrial ataxia (Batlevi and La Spada, 2011; Chrysostomou et al., 2016; Di Bella et al., 2010; Itoh et al., 2013; Lax et al., 2012; Mori et al., 2000; Vedanarayanan, 2003; Zeviani et al., 2012). Therefore, elucidating the mechanisms of Purkinje neuron death caused by mitochondrial damage will provide major insights into therapeutic strategies for ataxia.

Post-mitotic Purkinje neurons must maintain mitochondrial

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homeostasis throughout their lifespan. A key mechanism for the maintenance of mitochondria is the autophagic degradation of damaged mitochondria, which is termed mitophagy (Roy et al., 2015; Shirihai et al., 2015; Youle and van der Bliek, 2012). This degradation process depends on mitochondrial division, which makes mitochondria smaller and facilitates the efficient engulfment of mitochondria by autophagosomes, which eventually fuse with lysosomes for degradation.

Dynamin-related protein 1 (Drp1) mediates mitochondrial division as a critical mechano-chemical enzyme together with another GTPase, dynamin-2 (Adachi et al., 2016; Bui and Shaw, 2013; Lee et al., 2016; Roy et al., 2015; Tamura et al., 2011). During mitochondrial division, Drp1 assembles as oligomeric complexes on the mitochondrial outer membrane. These complexes wrap around mitochondria and constrict to initiate the division of the mitochondria. Importantly, studies have suggested that Drp1-mediated mitochondrial division is compromised autosomal recessive spastic ataxia of Charlevoix Saguenay, one of the major forms of human mitochondrial ataxia that is associated with increased mitochondrial size, mitochondrial dysfunction and Purkinje neuron degeneration (Bouchard et al., 1978; Bradshaw et al., 2016; Girard et al., 2012).

By generating Drp1-knockout (KO) mice, we demonstrated that cerebellar Purkinje neurons, which strongly express Drp1, are highly sensitive to Drp1 loss (Kageyama et al., 2012, 2014; Wakabayashi et al., 2009; Yamada et al., 2016). The conditional KO of Drp1 in post-mitotic Purkinje neurons using the Cre recombinase under the control of the L7 promoter (Barski et al., 2000) caused progressive Purkinje neuron degeneration and ataxia with decreased motor coordination in mice (Kageyama et al., 2012, 2014). Mitochondria in Drp1-KO Purkinje neurons dramatically increased their size due to unopposed fusion in the absence of division, showed decreased mitophagy and became defective in oxidative phosphorylation (Kageyama et al., 2012, 2014; Yamada et al., 2016). These studies have demonstrated that Purkinje-neuron-specific Drp1-KO mice are an excellent model for studying mitochondrial ataxia.

We have recently shown that post-mitotic Drp1-KO Purkinje neurons degenerate through necroptotic cell death (Yamada et al., 2016). The KO of receptor-interacting protein kinase 3 (Rip3) that is involved in necroptosis significantly delays the death of Drp1-KO Purkinje neurons. However, Rip3 loss did not completely block the neurodegeneration. These data suggest that neurodegeneration caused by Drp1 deficiency is mediated by multiple cell death pathways.

In this study, we investigated the role of p62/sequestosome-1, a scaffold protein that controls cell death and survival (Katsuragi et al., 2015; Manley et al., 2013), in Drp1-deficient neurodegeneration because we observed the recruitment of p62 to mitochondria in cultured Purkinje neurons in the absence of Drp1. We knocked out p62 in Drp1-Purkinje neurons in mice and analyzed the survival of Purkinje neurons and the mitochondrial morphology. Our results revealed that p62 KO significantly slows the degeneration of Purkinje neurons. However, in contrast to Rip3 KO, p62 did not interfere with ROS-induced morphological transformation of mitochondria in response to the loss of Drp1-mediated mitochondrial division. These data suggest that p62 and Rip3 act at different times during the degeneration of Purkinje neurons caused by Drp1 deficiency.

2. Materials and methods

2.1. Animals

All of the work with animals was conducted according to guidelines established by the Johns Hopkins University Committee on Animal Care and Use. $Drp1^{flox/flox}$ and $p62^{-/-}$ mice have been described previously (Komatsu et al., 2007; Wakabayashi et al., 2009). The :*L7-Cre* mice were obtained from the Jackson Laboratory (Barski et al., 2000). By breeding $p62^{+/-}$::*L7-Cre*$^{+/-}$::$Drp1^{flox/flox}$ mice and $p62^{+/-}$::$Drp1^{flox/flox}$ mice, we generated littermate control ($Drp1^{flox/flox}$), L7-Drp1-KO (*L7-Cre*$^{+/-}$::$Drp1^{flox/flox}$), p62-KO ($p62^{-/-}$) and L7-Drp1p62-KO (*L7-Cre*$^{+/-}$::$Drp1^{flox/flox}$::$p62^{-/-}$) mice. The $Drp1^{flox/flox}$ mice were phenotypically wildtype (Kageyama et al., 2012).

2.2. Antibodies

We used the following primary antibodies: Car8 (Car8-Rb-Af330, Frontier Institute, Hokkaido, Japan), PDH (ab110333, Abcam, MA, USA) and p62 (GP-62C, Progen, Heidelberg, Germany). We purchased the following secondary antibodies from Invitrogen (CA, USA): Alexa 488 anti-Rabbit IgG (A21206), Alexa 568 anti-guinia pig IgG (A11073) and Alexa 647 anti-mouse IgG (A31571).

2.3. Confocal immunofluorescence microscopy

We performed immunofluorescence microscopy of cerebellar Purkinje neurons as previously described (Kageyama et al., 2012) with some modifications (Kageyama et al., 2014; Yamada et al., 2016). The mice were anesthetized by intraperitoneal injection of Avertin and fixed by cardiac perfusion of ice-cold 4% paraformaldehyde in PBS as previously described (Kageyama et al., 2014; Yamada et al., 2016). The brain of each mouse was dissected, fixed in 4% paraformaldehyde in PBS for 2 h at 4 °C, incubated in PBS containing 30% sucrose overnight and frozen in OCT compound (Tissue-Tek, Torrance, CA). The frozen sections were cut, washed in PBS, and blocked in 10% donkey or sheep serum. The sections were then incubated with primary antibodies, followed by fluorescently labeled secondary antibodies. We examined the samples using a Zeiss LSM800 laser scanning confocal microscope equipped with a 10× (0.4 NA) objective and a Zeiss LSM780 FCS laser scanning confocal microscope equipped with a 100× (1.3 NA) objective.

2.4. Statistical analysis

To statistically analyze data, P-values were determined using ANOVA followed by Tukey *post hoc* test. $^{*}p < 0.05$, $^{***}p < 0.001$.

3. Results

3.1. p62 is recruited to mitochondria in Purkinje neurons in L7-Drp1-KO mice

We have previously shown that p62 is recruited to mitochondria in cultured Drp1-KO Purkinje neurons *in vitro* (Kageyama et al., 2012). To test whether this mitochondrial accumulation of p62 also occurs *in vivo*, we produced control mice ($Drp1^{flox/flox}$), L7-Drp1-KO mice ($L7\text{-}Drp1^{flox/flox}$), p62-KO mice ($p62^{-/-}$) and L7-Drp1p62-KO mice (*L7-Cre*$^{+/-}$::$Drp1^{flox/flox}$::$p62^{-/-}$) by breeding. We fixed these mice at an age of 3 months by cardiac perfusion of 4% paraformaldehyde, dissected the brain and cut frozen sections around the median line. The brain sections were immunostained using primary antibodies against the Purkinje neuron marker carbonic anhydrase 8 (Car8), p62 and a mitochondrial protein, pyruvate dehydrogenase (PDH), and fluorescently labeled secondary antibodies. Using confocal microscopy, we found that mitochondria become large spheres in Drp1KO Purkinje neurons, consistent with our previous studies (Kageyama et al., 2014). Upon loss of Drp1,

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