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Effects of neuronal PIK3C3/Vps34 deletion on autophagy and beyond

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PIK3C3/Vps34 plays important roles in the endocytic and autophagic pathways, both of which are essential for maintaining neuronal integrity. However, it is unclear how inactivating PIK3C3 may affect neuronal endosomal versus autophagic processes in vivo. We generated a conditional null allele of the *Pik3c3* gene in mouse, and specifically deleted it in postmitotic sensory neurons. Subsequent analyses reveal several interesting and surprising findings.

Key words: PIK3C3/Vps34, ATG7, sensory neurons, neurodegeneration, autophagy, abnormal endosome

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PIK3C3 (commonly known as Vps34) is the class III phosphatidylinositol 3-kinase (PtdIns3K) that specifically catalyzes the formation of phosphatidylinositol-3-phosphate (PtdIns3P). It is the only PtdIns3K that is conserved from lower eukaryotes to mammals, and represents the most ancient form of PtdIns3Ks. Studies in invertebrate organisms as well as mammalian cell lines show that PIK3C3/Vps34 regulates multiple aspects of both the endocytic and the autophagic pathways. On one hand, PIK3C3 is important for the progression of early endosome to late endosome, and the biogenesis of multivesicular bodies. On the other hand, PIK3C3 is critical for the initiation of autophagosome formation. A chemical inhibitor of PIK3C3, 3-MA, has been commonly used as a specific inhibitor for autophagy. The distinct functions of PIK3C3 are thought to be carried out by at least two different PIK3C3 complexes. In yeast, complex I (Vps34, Vps15, Atg6 and Atg14) is involved in autophagy, whereas complex II (Vps34, Vps15, Atg6 and Vps38) functions in the vacuolar protein sorting process. In mammals, the homologue of complex I (PIK3C3, p150, Beclin 1 and Atg14L) activates autophagy, whereas the homologue of complex II (PIK3C3, p150, Beclin 1 and UVRAG/Vps38) regulates endocytic trafficking.

To characterize the in vivo function of PIK3C3 in mammals, we generated a conditional allele of the *Pik3c3* gene in mouse and specifically deleted it in postmitotic sensory neurons (*Pik3c3*-cKO mouse). We focused our analyses on sensory neurons because *Pik3c3* is most abundantly expressed in these neurons. Detailed analyses of the sensory ganglia in the knockout mice reveal rapid but differential neurodegenerations of different types of sensory neurons within a few days after birth. Large-diameter myelinated mechanosensory and proprioceptive neurons undergo fast degeneration, whereas mutant small-diameter unmyelinated nociceptive neurons degenerate slower and survive longer.

Interestingly, the large-diameter *Pik3c3*-deleted neurons rapidly accumulate ubiquitin-positive aggregates as well as numerous enlarged vesicles, which are likely abnormal endosomes. The accumulation of enlarged vesicles not only sequesters the cellular membrane source, but also could create trafficking jams that block the transport of prosurvival signals and/or material and organelles, and thus may underlie the rapid demise of large neurons. By contrast, the small-diameter *Pik3c3*-deleted neurons contain a limited number of vacuoles but gradually build up lysosome-like organelles. The marked increase of lysosomes seems to be more tolerable by neurons, but the mechanism underlying this phenotype is unclear. It could represent a protective and homeostatic response of neurons challenged with stress and insults to their endomembrane system. Alternatively, since sorting of many lysosomal proteins requires PtdIns3P, this

phenotype may also result from a build-up of nonfunctional lysosomes as was the case in cathepsin B and L knockout mice. It is also unclear why two types of sensory neurons respond differently to a universal insult. One speculative explanation is that the large-diameter neurons are constantly activated under normal physiological conditions by touch and body movement and thus they contain more active endocytic and membrane trafficking processes; whereas small-diameter pain-sensing neurons are normally not activated and have less endocytic events. These differences might allow the two types of neurons to respond differently to PIK3C3 deletion.

We further show that the fast and differential degeneration phenotypes in the *Pik3c3*-cKO mice are caused primarily by a disruption in the endosomal but not the autophagic pathway. This is validated by comparing the neuronal phenotypes of *Pik3c3*-cKO mice with those of *Atg7*-cKO mice, in which the autophagy-specific gene *Atg7* is deleted using the same sensory neuron-specific cre driver. Disrupting autophagy leads to a slow degeneration of all types of sensory neurons over a period of several months, and formation of very large intracellular inclusion bodies in all sensory neurons. No increase of lysosomes or accumulation of enlarged vesicles is observed. The completely distinct phenotypes observed in *Atg7*-cKO versus *Pik3c3*-cKO mice suggest that inactivation of PIK3C3 primarily disrupts the endosomal pathway rather than inhibiting autophagy (at least in neurons). It calls into attention that care needs to be taken to interpret the results of using PIK3C3 inhibitors such as 3-MA as autophagy-specific inhibitors.

The most surprising finding is the existence and activation of a noncanonical, PIK3C3-independent macroautophagy pathway in small-diameter *Pik3c3*-mutant neurons. Although PIK3C3 is traditionally viewed as indispensable for autophagy initiation, several recent studies suggest a possible PIK3C3-independent autophagy pathway in various cell lines and in Drosophila. We show that this noncanonical autophagy pathway can occur in sensory neurons in vivo using three different assays: crossing *Pik3c3*-cKO mice to the GFP-LC3 reporter line, western blot analyses of LC3 isoforms, and performing autophagy flux experiments. Interestingly, analyses of *Pik3c3/Atg7* double-mutant neurons indicate that this alternative autophagosome initiation pathway still requires ATG7 and hence the conventional conjugation systems. Therefore, this noncanonical autophagy is distinct from the newly reported ATG5/ATG7-independent but PIK3C3-dependent autophagy. We speculate that activation of this PIK3C3-independent autophagy in small-diameter mutant neurons is part of the reason for their longer survival period.

The molecular mechanism underlying the PIK3C3-independent autophagosome formation is unknown. It is possible that PtdIns3P can be generated at a low level on the membrane of pre-autophagosomes/phagophores by salvage pathways using other lipid kinases or phosphatases. Alternatively, other mechanisms may direct the formation of the crescent-shaped double membrane structures. For instance, asymmetric insertion into the membrane of proteins with amphipathic helices can induce membrane curvature; BAR domain-containing proteins can also detect and facilitate the formation of curved membrane structures. Thus, these types of proteins might potentially be recruited to nucleate the formation of pre-autophagosomes in the absence of PIK3C3. Finally, the role of this PIK3C3-independent autophagy under normal physiological conditions in vivo needs to be explored.