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Related Commentary, page 2273 Research article

Autophagy is essential for mouse sense of balance

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Autophagy is an evolutionarily conserved process that is essential for cellular homeostasis and organismal viability in eukaryotes. However, the extent of its functions in higher-order processes of organismal physiology and behavior is still unknown. Here, we report that autophagy is essential for the maintenance of balance in mice and that its deficiency leads to severe balance disorders. We generated mice deficient in autophagin-1 protease (Atg4b) and showed that they had substantial systemic reduction of autophagic activity. Autophagy reduction occurred through defective proteolytic processing of the autophagosome component LC3 and its paralogs, which compromised the rate of autophagosome maturation. Despite their viability, *Atg4b*-null mice showed unusual patterns of behavior that are common features of inner ear pathologies. Consistent with this, *Atg4b*-null mice showed defects in the development of otoconia, organic calcium carbonate crystals essential for sense of balance (equilibrioception). Furthermore, these abnormalities were exacerbated in *Atg5*$^{-/-}$ mice, which completely lack the ability to perform autophagy, confirming that autophagic activity is necessary for otoconial biogenesis. Autophagy deficiency also led to impaired secretion and assembly of otoconial core proteins, thus hampering otoconial development. Taken together, these results describe an essential role for autophagy in inner ear development and equilibrioception and open new possibilities for understanding and treating human balance disorders, which are of growing relevance among the elderly population.

Introduction

Autophagy is a degradative process in which portions of cytoplasm are engulfed by a double-membrane vesicle called the "autophagosome." Once autophagy is completed, the autophagosome fuses with a lysosome, and its content and inner membrane are degraded by hydrolases and recycled (1). Genetic studies on yeast have identified more than 20 autophagy-specific (*Atg*) genes that are required for autophagosome formation (2). Among yeast *Atg* genes, *Atg8* encodes a protein that forms part of a ubiquitin-like conjugation system essential for autophagy execution (3). Atg8 protein is synthesized as a cytoplasmic precursor, which is cleaved after a Gly residue by the cysteine proteinase Atg4 (4). This initial proteolytic processing is required for the subsequent conjugation of Atg8 with membrane-bound phosphatidylethanolamine (PE), which is in turn essential for autophagosome completion (5). The complex Atg8-PE is also deconjugated by the protease Atg4, facilitating the release of Atg8 from membranes. This modification system is conserved in higher eukaryotes including mammals (6). We previously identified and cloned the 4 human orthologs of the yeast proteinase Atg4 (7), and parallel studies have revealed that there are at least 6 orthologs of yeast Atg8 in mammals (microtubule-associated protein 1 light chain 3α [LC3A], microtubule-associated protein 1 light chain 3β [LC3B], microtubule-associated protein 1 light chain 3γ [LC3C], GABARAP-like 2 [GATE-16], developed this array of closely related enzymes, contrasting with other essential autophagy genes such as *Atg3*, *Atg5*, or *Atg7* for which a single ortholog is present in the mammalian genome.

To get insights into the in vivo roles of this complex system, we generated mutant mice deficient in autophagy-related 4B (*Atg4b*$^{-/-}$ mice). Although they were viable, these mice exhibited a clear reduction of basal- and starvation-induced autophagic flux in all tissues, which is caused by a deficit in the initial proteolytic cleavage of Atg8 murine orthologs. This finding indicates that autophagin-1 (Atg4b) has a major functional role in the context of autophagy in mammals. In addition, *Atg4b*$^{-/-}$ mice showed a balance-related behavioral phenotype that is linked to profound inner ear developmental defects. We also report that these abnormalities were exacerbated in *Atg5*$^{-/-}$ neonates that were totally autophagy impaired, confirming that autophagic activity is essential for otoconial biogenesis. Finally, we analyzed the molecular mechanisms underlying these abnormalities and found that autophagy deficiency impairs the secretion and assembly of otoconial core proteins into vestibular lumen, resulting in the otoconial development defects and the behavioral balance disorders exhibited by *Atg4b*$^{-/-}$ mice.

Results

Generation, development, and growth of Atg4b mutant mice. To address the in vivo role of Atg4b cysteine proteinase, we decided to gener-