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Autophagy dysregulation in cell culture and animals models of spinal muscular atrophy

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ABSTRACT

Abnormal autophagy has become a central thread linking neurodegenerative diseases, particularly of the motor neuron. One such disease is spinal muscular atrophy (SMA), a genetic neuromuscular disorder caused by mutations in the SMN1 gene resulting in low levels of Survival Motor Neuron (SMN) protein. Despite knowing the causal protein, the exact intracellular processes that are involved in the selective loss of motor neurons remain unclear. Autophagy induction can be helpful or harmful depending on the situation, and we sought to understand the state of the autophagic response in SMA. We show that cell culture and animal models demonstrate induction of autophagy accompanied by attenuated autophagic flux, resulting in the accumulation of autophagosomes and their associated cargo. Expression of the SMN-binding protein a-COP, a known modulator of autophagic flux, can ameliorate this autophagic traffic jam.

Introduction

Spinal muscular atrophy (SMA) is a devastating inherited disorder caused by the loss of the SMN1 gene responsible for production of the Survival Motor Neuron (SMN) protein (Clermont et al., 1995; Rodrigues et al., 1995). Low levels of this protein lead to dysfunction and degeneration of alpha motor neurons, muscle wasting and eventually death in the more severe forms. Understanding the processes by which these motor neurons fail and the timing of that failure increases our understanding of the disease state. Such information may educate therapeutic decisions. One cellular process, macroautophagy, has come to the forefront in motor neuron diseases as a common pathology present in degenerating motor neurons. Further study of the onset of autophagy and the dynamics of the autophagic pathway in SMA will allow for the potential therapeutic intervention, as pharmacological manipulation of this system is well understood.

Macroautophagy, hereafter referred to as autophagy, is responsible for the degradation of cytoplasmic proteins and organelles by incorporation into a double-membrane vesicle that is transported to the lysosome for degradation (Moreau et al., 2010). This process appears to be dysregulated in a number of neurodegenerative conditions; typically becoming hyper-activated in an attempt to preserve cellular function or remove damaged cellular material such as at the axon terminal following injury. In other cases such as Alzheimer's disease, Huntington's disease or aging, the normal autophagic flux slows, resulting in a failure to clear the cell of obsolete proteins and organelles (Menzies et al., 2006). Tissue-specific knockout experiments in mice have highlighted the importance of this pathway in neurons, demonstrating that loss of critical genes involved in the execution of the autophagic pathway in the central nervous system results in profound neurodegenerative disease (Komatsu et al., 2006; Nishiyama et al., 2007). Induction of autophagy has been reported in numerous models of neurodegenerative disease, and may be a component of the cellular response to depleted SMN levels (Son et al., 2012). A recent report describes the accumulation of autophagosomes in motor neuron cultures following depletion of SMN and in motor neurons cultures from SMA mice (Garcera et al., 2013). However, this study did not examine the dynamics of the autophagic pathway in SMA model cells. Previous work in cultured cells has shown that depletion of the SMN binding partner α-COP impaired autophagy resulting in an accumulation of autophagosomes that failed to complete fusion with the lysosome (Razi et al., 2009). Autophagosome accumulation can be a hallmark of induction of autophagy in response to general cellular stress but can also result from ineffective intracellular trafficking and slow delivery of autophagic vesicles to the lysosomal compartment. In fact, a recent publication demonstrated that SMN-depleted NSC-34 cells, a motor neuron-like cell line, showed impaired microtubule assembly and accumulation of mitochondria, both signs that normal intracellular trafficking is disturbed in the absence of SMN (Wen et al., 2010).

In this report, we demonstrate that autophagic vesicles are present both in an inducible NSC-34 cell culture model of SMA and in fibroblasts

Abbreviations: SMA, spinal muscular atrophy; SMN, Survival Motor Neuron; LC3, microtubule-associated protein 1A/1B-light chain 3; GFP, green fluorescent protein; RFP, red fluorescent protein; ALS, amyotrophic lateral sclerosis; α-COP, coatomer vesicle protein alpha.

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isolated from SMA patients. Using live-cell imaging, we find that cells display a reduced autophagic flux when challenged following SMN depletion. A nearly identical phenotype is seen in SH-SY5Y cells following knockdown of the SMN binding partner α-COP, and expression of α-COP reduced the number of autophagosomes in SMN-depleted NSC-34 cells. Finally, analysis of spinal cord lysates from a severe mouse model of SMA shows accumulation of LC3-II protein as well as an increase in p62 protein levels, indicating that dysregulated autophagy is a component of SMA disease pathology in these animals.

Results

SMN depleted NSC-34 cells accumulate autophagic puncta

We selected the motor neuron-like hybrid cell line NSC-34 to model cellular pathology in SMA. As we previously reported, we used a clonal line (NSC-34 #4–#56) expressing both the reverse Tet transactivator and a Tet-responsive construct containing shRNA specific to murine SMN (Custer et al., 2013). Following the addition of doxycycline, there is a robust knockdown of SMN to an average of 47.4 +/− 10.9% of normal within 48 h (Fig. 1C). The cell line quickly recovers normal levels of SMN protein following removal of the doxycycline. For comparison and to determine any off-target effects of doxycycline, we maintain the NSC-34 clone #4 which expresses only the rTta plasmid but not the shRNA. This cell line is hereafter referred to as #4. These tools allowed us to perform tightly controlled experiments to analyze the consequences of SMN depletion. When viewed with light microscopy, we observed that following SMN depletion, these cells became heavily vacuolated compared to control cultures (not shown), and we hypothesized that the cells were undergoing autophagy. To evaluate this, we transfected doxycycline-treated and control cultures with GFP–LC3. Prior to incorporation into autophagosomes, GFP–LC3 displays diffuse cytoplasmic staining, however after LC3I is lipidated to form LC3II and is incorporated in the autophagic vesicle membrane, it appears as discrete puncta. Following fixation and fluorescent microscopy, the number of puncta per cell was determined using the ImageJ GFP–LC3 macro that was created and characterized for the quantitative assessment of autophagic puncta in fluorescent micrographs (Chu et al., 2009; Dagda et al., 2008). We could clearly visualize that SMN-depleted cultures had an increase in GFP–LC3-positive puncta compared to control cultures in which LC3 staining was mostly diffuse (Fig. 1A, quantified in Fig. 1B). Puncta counts from 3 separate experiments showed a significant increase in the number of LC3 puncta per cell in SMN-depleted cultures by Student's t-test (54 +/− 14 puncta per cell compared to 9 +/− 6 in control cultures, $p < 0.01$). These changes are specific to SMN knockdown as doxycycline-treated cultures from NSC-34 clone #4, which expresses the rTta but not the SMN shRNA, show no significant increase in the number of GFP positive puncta per cell (Fig. 1B). LC3 conversion can also be visualized by Western blot as LC3-II migrates faster than LC3I. Western blots of doxycycline-treated cultures show increased LC3-II compared to untreated controls. These levels return to normal upon removal of doxycycline and resumption of normal SMN protein production (Fig. 1C). Quantification of Western blots from 3 separate knockdowns followed by a 48 hour doxycycline washout shows that on average, p62 increased to 2.4 fold +/− 0.19 compared to controls ($p < 0.01$ by Student's t-test) and LC3-II increased to 1.46 fold +/− 0.08 compared to controls ($p < 0.05$) (Fig. 1D).

Reduced autophagic flux in SMN-depleted NSC-34 cells

The accumulation of LC3 positive puncta and the increase in LC3II protein indicate induction of autophagy. However, we were interested

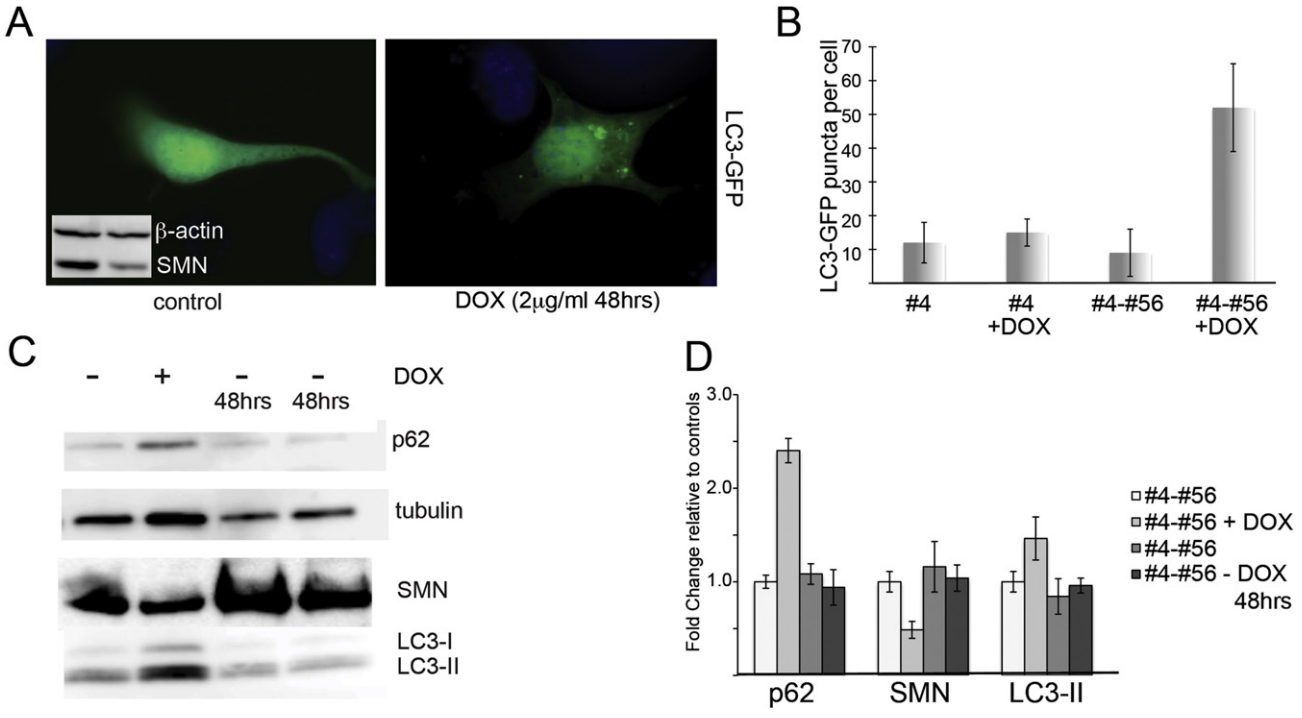

Fig. 1. SMN knockdown in NSC-34 cells induces autophagy. Clonal NSC-34 cells expressing a doxycycline-inducible shRNA against murine SMN (clone #4–#56) were treated with doxycycline (2 μg/ml) for 48 h to deplete SMN levels to 47.4 +/− 10.9% relative to controls (inset in A shows Western blot of SMN levels following 48 h knockdown) and then transfected with GFP–LC3 to visualize autophagosomes. A) Control cultures show mostly diffuse LC3–GFP fluorescence with very few autophagic puncta per cell compared with Dox-treated cultures, which accumulate numerous GFP-positive autophagic puncta. B) Quantification of the number of LC3–GFP puncta per cell shows that SMN-depleted cells have a statistically significant increase in puncta by Student's t-test compared to control cultures ($p < 0.05$). No increase in autophagic puncta was observed when cells from clone #4, which express only the rTta plasmid, were treated with doxycycline. C) Western blotting for LC3 shows that following SMN knockdown, the ration of LC3-II/LC3-I increases. Removal of doxycycline for 48 h allows SMN levels and LC3 ratios to return to normal. D) Quantitative analysis of repeated Western blots shows a significant increase in LC3-II protein following SMN depletion ($p < 0.05$ by Student's t-test).