● **PERSPECTIVE**

Modification of autophagy-lysosomal pathway as a neuroprotective treatment for spinal cord injury

Spinal cord injury (SCI) is one of the most common causes of long-term disability among young adults world-wide. In the United States, 12,000–20,000 new cases are reported annually and approximately half a million people currently live with SCI. Unfortunately, beyond surgery for immobilization of the spine and prolonged rehabilitation there are no effective treatments to improve functional outcomes after SCI. This is at least in part due to the complex and heterogeneous nature of injury after spinal cord trauma. The physical impact during SCI results in direct mechanical damage to some cells and tissues (primary injury). Primary injury also sets off a cascade of widespread, progressive biochemical changes leading to further neuronal and glial cell death, neuroinflammation and glial scar formation (secondary injury) (Beattie et al., 2002). Secondary injury can occur over hours, days and months after the initial impact, and may involve cells distant from but functionally connected to the injury site. Thus secondary injury can further intensify and spread tissue damage and functional deficits. Blocking or attenuating it could significantly limit incapacitation consequent to injury.

Macroautophagy (hereafter called autophagy) is a lysosome-dependent cellular catabolic process important for degradation of proteins, protein aggregates and other cellular components (Mizushima et al., 2008). Autophagy is initiated by formation of double membrane vesicles (autophagosomes), which sequester cytoplasmic constituents including damaged organelles and toxic protein aggregates. Autophagosomes fuse with lysosomes, thus allowing degradation of cargo by lysosomal proteases. The resulting amino acids, lipids and nucleic acids are then re-used by the cell as building blocks or source of energy. The progress of cargo through the autophagy system, leading to its delivery and degradation in lysosomes is termed autophagy flux. Autophagy flux is important for homeostasis and quality control in all cells, but is especially crucial in terminally differentiated cells such as neurons and oligodendrocytes. Neural tissue-specific inhibition of autophagy in mice causes neurodegeneration and leads to abnormal motor function and reflexes. Impaired autophagy is also implicated in neurodegenerative disorders such as Parkinson's, Alzheimer's and lysosomal storage diseases such as Gaucher's and Niemann-Pick. Conversely, increasing efficiency of autophagic flux can improve outcomes in animal models of neurodegenerative and lysosomal storage diseases and has been proposed as a potential therapeutic approach (Mizushima et al., 2008; Bove et al., 2011).

Increased markers of autophagy have been observed after SCI but its function remained controversial, with both beneficial and detrimental roles proposed (Lipinski et al., 2015). This controversy is due to the fact that although usually it is neuroprotective, under certain circumstances pathologically increased autophagy can contribute to cell death. This may occur particularly when autophagic degradation is impaired, for example due to lysosomal dysfunction. Under those circumstances autophagy flux is not able to proceed to completion, leading to pathological accumulation of dysfunctional autophagosomes. Therefore, flux is a crucial parameter that can radically alter the function of autophagy. Neither autophagy flux nor lysosomal function has been previously assessed after SCI.

In a recent study, we investigated the mechanisms and function of autophagy following moderate contusive SCI in a rat model (Liu et al., 2015). Consistent with previous studies, we observed accumulation of autophagosomes starting within 24 hours after injury. However, levels and activity of upstream mediators and regulators of autophagy remained unchanged. This suggests that increased initiation is unlikely to account for the increase in the number of autophagosomes. Instead, we observed a pronounced accumulation of autophagy substrates such as the SQSTM1/p62 protein. SQSTM1 is an adaptor protein directing ubiquitinated cargo to

autophagosomes. Since SQSTM1 is degraded by autophagy along with its cargo, when autophagy flux is high, levels of SQSTM1 protein decrease. Conversely, decrease in autophagy flux leads to SQSTM1 protein accumulation. Therefore, increase in levels of SQSTM1 in the injured spinal cord indicates that autophagosome accumulation after SCI is not due to increased initiation of autophagy, but rather to inhibition of autophagy flux.

Autophagic degradation is dependent on lysosomal proteases. We observed a decrease in the number of cathepsin D (CTSD) positive lysosomes and decreased autophagosome-lysosome fusion near the injury site after SCI. Therefore, decrease in autophagy flux after injury likely reflects disruption of lysosomal function, which prevents autophagosomes and their cargo from being degraded (**Figure 1**). Lysosomal function abnormalities underlie autophagy defects observed in lysosomal storage diseases and have been reported in neurodegenerative diseases (Mizushima et al., 2008; Bove et al., 2011). It appears that a similar mechanism may occur after SCI, with impairment of lysosomal function contributing to defects in autophagic clearance. Together our data for the first time identify the cellular mechanism leading to accumulation of autophagosomes after SCI.

We also investigated cell type specificity of autophagy after SCI. At 24 hours after injury pathological accumulation of autophagosomes was most prominent in ventral horn motor neurons (Liu et al., 2015). Relatively fewer autophagosomes were detected in dorsal sensory neurons, despite their proximity to the impact site. Therefore, motor neurons may be particularly sensitive to perturbation of autophagy flux after SCI.

Autophagy is essential for neuronal cell function and survival and its inhibition can lead to neurodegeneration (Mizushima et al., 2008). The mechanisms may include pathological accumulation of autophagic cargo such as toxic protein aggregates and defective organelles. Accumulated autophagosomes themselves may also serve a pathologic function, for example as sites of amyloid β generation or platforms for assembly of pro-death signaling complexes. Autophagy is also up-regulated, and often plays a protective function, in response to cell injury. For example, it can mitigate the effects of homeostasis perturbation in the endoplasmic reticulum (ER stress). Conversely, defects in autophagy flux can potentiate ER stress and increase ER stress-induced apoptosis (Boyce et al., 2011). Since we observed accumulation of autophagosomes in ventral horn neurons at a time when autophagy flux is blocked, we hypothesized that it may contribute to neuronal cell death. Consistently, we observed pronounced association of defects in autophagy with signs of neuronal cell death. In particular, motor neurons with blocked autophagy displayed markers of ER stress and ER stress induced apoptosis. Activation of ER stress and its contribution to secondary injury after SCI have been previously reported (Ohri et al., 2013). However, the mechanisms contributing to the perturbation of ER homeostasis after SCI remain unknown. Our data suggest that disruption of autophagy after SCI may exacerbate ER stress and contribute to ER stress induced neuronal cell death (**Figure 1**). Thus our analysis indicates inhibition of neuronal autophagy flux as part of the acute secondary injury mechanism.

In addition to neurons, we observed accumulation of autophagosomes in white matter microglia/macrophages and oligodendrocytes (Liu et al., 2015). In the white matter the total number of LC3 positive cells was highest near the injury site and peaked between 3–7 days after SCI. Based on morphology, only the most activated amoeboid microglia/macrophages accumulated autophagosomes after SCI. Recent data indicate that in cancer-associated macrophages, autophagy can regulate inflammatory responses *via* the NFκB pathway (White et al., 2010). It is therefore possible that autophagy may also contribute to the regulation of neuroinflammatory processes after SCI. In the oligodendrocyte lineage, we observed accumulation of autophagosomes both in mature oligodendrocytes and in NG2 positive precursors. Since oligodendrocyte cell death is prevalent after SCI (Beattie et al., 2002), similarly to neurons pathological accumulation of autophagosomes could contribute to oligodendrocyte cell death after injury. However, in a myelin mutant rat model autophagy can promote oligodendrocyte precursor survival and myelin development (Smith et al., 2013) and it is possible that it may play a similar function after SCI.
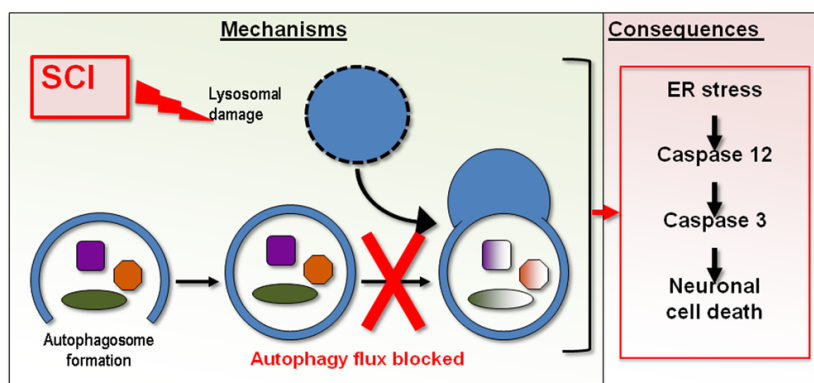
Figure 1 Mechanisms and consequences of inhibition of autopahgy flux after spinal cord injury (SCI).
We propose that SCI causes lysosomal membrane permeabilization and consequent decrease in lysosomal function. This in turn prevents autophagosomal-lysosomla fusion, leading to pathological accumulation of autophagosomes and inhibition of autophagy flux. In neurons, inhibition of autophagy flux causes exacerbation of endoplasmic reticulum (ER) stress and activation of ER stress-induced apoptosis.

Accumulation of SQSTM1 spontaneously resolved by day 7 after SCI (Liu et al., 2015). This correlated with increased expression of CTSD and other lysosomal genes. Therefore, increased lysosomal biogenesis and consequently improved lysosomal function may eventually allow restoration of autophagy flux. This should return autophagy to its normal neuroprotective function and could contribute to the spontaneous locomotor recovery usually observed after SCI. Increasing lysosomal biogenesis has been shown to augment autophagy flux and improve outcomes in animal models of neurodegenerative diseases. We hypothesize that this approach may also be beneficial after SCI.

Lysosomal biogenesis can be activated by the transcription factor EB (TFEB), which is negatively regulated by mTOR (Pena-Llopis et al., 2011). It has been previously demonstrated that treatment with Rapamycin, an mTOR inhibitor and a well-known inducer of autophagy, can attenuate functional deficits after SCI (Lipinski et al., 2015). Our preliminary data indicate that Rapamycin can increase number of lysosomes in the CNS and stimulate autophagy flux after SCI (Lipinski and Wu, unpublished). This supports the idea that increasing lysosomal biogenesis and autophagy-lysosomal function may represent a potential novel treatment after SCI. However, Rapamycin is known to affect other cellular pathways and functions in addition to autophagy. Therefore, it remains to be confirmed that neuroprotective function of Rapamycin after SCI is mediated *via* restoration of the autophagy-lysosomal pathway.

Additionally, mTOR activity plays a vital role in processes necessary for recovery after SCI. This includes function of the mTOR complex I (mTORC1) in oligodendrocyte differentiation and myelination. mTORC1 is needed for proper myelination in all areas of the CNS, but the deleterious effects of its inhibition are most pronounced in the spinal cord (Bercury et al., 2014; Wahl et al., 2014). Additionally, mTOR has been reported to promote compensatory neuronal sprouting important for recovery after nerve injury (Park et al., 2008; Lee et al., 2014). Therefore, inhibition of mTOR may not be appropriate as SCI treatment. Instead, drugs able to promote lysosomal biogenesis and autophagy flux *via* mTOR-independent pathways may offer best therapeutic benefits.

Our recent data for the first time identify both the cellular mechanisms and the function of autophagy after SCI. They indicate that lysosomal damage leads to inhibition of autophagy flux soon after SCI, resulting in pathological accumulation of autophagosomes. In ventral horn motor neurons, this likely leads to exacerbation of ER stress and potentiation of ER stress induced apoptosis. Much additional future work remains, including functional studies in autophagy deficient mice, as well as investigation of the molecular mechanisms leading to both, inhibition and restoration of autophagy flux after SCI. Nevertheless, our current data clearly identify inhibition of autophagy after SCI as part of acute secondary injury and suggest restoration of autophagy flux as a potential neuroprotective strategy. Because of the unique importance of oligodendrocyte differentiation and axonal sprouting for recovery after SCI, this may require development of novel drugs able to increase lysosomal biogenesis and autophagy flux without mTOR inhibition.

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