ULK1 as a novel therapeutic target in neurodegeneration

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Axonal degeneration is an early and key pathophysiological feature of many traumatic and neurodegenerative disorders of the central nervous system (CNS), such as spinal cord injury (SCI), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). As the regenerative capacity of injured axons is severely restricted in the CNS, axonal degeneration frequently results in the irreversible loss of neuronal connections causing progressive neurological deficits and clinical disability. A better understanding of the mechanisms of axon degeneration is therefore hoped to unravel new therapeutic avenues to combat neurodegeneration (Lingor et al., 2012).

Axonal degeneration is a fine-tuned cellular cascade of events leading to axonal self-destruction. In one of our previous studies, we investigated its detailed mechanisms after axonal injury using an optic nerve crush model (Knoferle et al., 2010). We observed rapid and progressive axonal fragmentation of retinal ganglion cell axons spanning up to 500 µm in length on both sides of the lesion site within few hours after injury. Mechanistically, axonal lesion led to rapid influx of calcium resulting in the activation of calcium-dependent proteases and macroautophagy (here: autophagy). Inhibition of autophagy using the unspecific autophagy blocker 3-methyladenine attenuated acute axonal degeneration (AAD) after optic nerve crush, indicating a detrimental role of autophagy activation post-injury. In keeping with our results, several other publications reported traumatic brain injury-induced autophagy induction, the suppression of which resulted in beneficial effects. For example, downregulation of the key autophagy-related gene 7 (*Atg7*) was shown to be protective in models of traumatic neurodegeneration (Yang et al., 2013). However, contrary to these findings, a number of studies demonstrated cytoprotective properties of autophagy activation in models of traumatic brain injury and acute axonal injury, for instance by applying the autophagy-inducing drug rapamycin after optic nerve axotomy (Rodriguez-Muela et al., 2012). Thus, the detailed role of autophagy during axonal degeneration is context-dependent and warrants a more thorough investigation.

More recently, we studied in detail the involvement of autophagy after axonal injury and assessed the regulation of key autophagic proteins in degenerating axons using a dorsal hemisection model of SCI (Ribas et al., 2015). We found a pronounced accumulation of autophagosomes and autophagic proteins such as uncoordinated 51-like kinase 1 (ULK1), ATG5, and ATG7 in axonal degeneration bulbs after injury. The expression levels of ATG5, ATG7, and ULK1 all peaked within 24 hours after lesion, reinforcing an early and key involvement of autophagy in axonal degenerative mechanisms. Particularly, ULK1 accumulated strongly in close vicinity to the lesion site. ULK1 is a serine/threonine kinase governing autophagy activation by integrating input from various indicators of the cellular nutritional status, such as AMP-activated protein kinase and mammalian target of rapamycin (mTOR). ULK1 activation triggers downstream effector mechanisms that ultimately result in the formation of autophagosomes enabling autophagic degradation of cellular cargo upon fusion with lysosomes. Our findings therefore raised the possibility that ULK1-dependent autophagy might be a crucial executing mechanism of axonal degeneration. Intriguingly, ULK1 had been one of the strongest hits in an earlier screen for kinases that regulated axon growth *in vitro*, where siRNA-mediated ULK1 inhibition resulted in a markedly increased neurite outgrowth and regeneration of primary cerebellar granule neurons and dopaminergic neurons after scratch lesion (Loh et al., 2008). Based on these findings and the aforementioned result of attenuated AAD after unspecific autophagy inhibition, we hypothesized that inhibition of the early autophagy-related ULK1 protein might elicit a protective effect against axonal degeneration.

To test this hypothesis in our most recent study (Vahsen et al., 2020), we employed an adeno-associated viral vector expressing a dominant-negative form of ULK1 (ULK1.DN) to inhibit ULK1 function and investigated its effects on axonal degeneration *in vitro* and *in vivo*. Indeed, primary cortical neurons transduced with ULK1.DN showed decreased formation of degeneration bulbs up to 6 hours after selective axonal transection *in vitro*, indicating an axon-protective effect of ULK1 inhibition. In line with this, we also demonstrated attenuated axon fragmentation of transduced retinal ganglion cells over 6 hours after crush lesion in vivo, confirming that ULK1 inhibition protects from AAD. Additionally, we investigated more chronic effects of ULK1 inhibition in a rat model of SCI. Axons of the rubrospinal tract transduced with ULK1.DN showed reduced axon degeneration even one week after axonal lesion, demonstrating that ULK1 inhibition also mediates long-term axon protection. Together, we uncovered that ULK1.DN-mediated ULK1 inhibition protects three neuronal subtypes from lesion-induced axon degeneration, in both acute and chronic paradigms. Previous literature on the role of ULK1 in axonal degeneration is scarce, except for some conflicting evidence describing that *Ulk*1 knock-out leads to perinatal death and conditional *Ulk1* knock-out induces axon guidance defects (Joo et al., 2016; Wang et al., 2018). However, in contrast to knock-out models, transduction with ULK1.DN reduced endogenous ULK1 levels only by approximately 50% in our study. Bearing in mind the crucial importance of ULK1 in cell homeostasis, our data therefore emphasize that a moderate degree of ULK1 inhibition is vital for therapeutically beneficial effects.

We then aimed to understand the mechanisms underlying the protective effects of ULK1.DN-mediated ULK1 inhibition. Based on our initial hypothesis that ULK1 inhibition could protect from autophagy induction after axonal injury, we first investigated the effects of ULK1 inhibition on autophagy. Expectedly, transduction with ULK1.DN led to a significant reduction in autophagy *in vitro* and *in vivo*, confirming that ULK1 inhibition exerts axon-protective effects via decreased autophagy. However, the extent of autophagy reduction in ULK1.DN-transduced cells was rather mild and seemed to not fully correlate with its pronounced axon-protective effects. As ULK1 interacts with a vast number of molecular targets, we chose to analyze the molecular changes after transduction with ULK1.DN in an unbiased manner and performed a proteomic analysis of transduced cortical neurons.

Surprisingly, we found a distinct modulation of splicing and translation-associated proteins in neurons transduced with ULK1.DN. A regulation of splicing-associated proteins was rather unexpected, as – to our best knowledge – a role of ULK1 in splicing had not been described thus far. The vast evidence implicating splicing dysregulation in neurodegeneration, for example in ALS, prompted us to characterize this effect of ULK1 inhibition on splicing in more detail. Therefore, we additionally performed a differential exon expression analysis of neurons transduced with ULK1.DN. Indeed, 36 of the sequenced genes showed differential exon usage after transduction with ULK1.DN, with key roles in axon extension, neurite regeneration, and mitochondrial transport along microtubules. Of particular interest, we found increased exon usage for the motor protein *Kif1b*, the reduced expression of which was previously implicated in different models of axonal degeneration. As ULK1 had already been connected to kinesin-dependent axonal transport (Toda et al., 2008), we speculated that ULK1 inhibition might exert additional axon-protective effects through a splicing-mediated modulation of axonal transport. Another interesting gene with differential splicing by ULK1.DN was *Ddit3*, which had previously been shown to mediate endoplasmic reticulum (ER) stress after SCI (Penas et al., 2007). We found reduced exon expression of *Ddit3* after transduction with ULK1.DN, which might indicate that ULK1.DN additionally attenuates axonal degeneration by protecting from ER stress. Our data therefore lead to a model, in which ULK1 inhibition – in addition to its effects on autophagy – results in the differential splicing of degeneration-associated genes, which could lead to axon-protective effects via modulation of axonal transport and ER stress.

Additionally, we investigated the ULK1.DN-mediated upregulation of proteins involved in translation outlined by our proteomic analysis in more detail. Mutual inhibition between ULK1 and the translational master regulator mTOR represents a well-known fine-tuning mechanism between the catabolic process autophagy and protein biosynthesis. We thus analyzed the expression of phosphorylated (active) mTOR and its molecular target phosphorylated ribosomal protein S6, both of which revealed significantly higher expression in neurons transduced with ULK1.DN. Dysregulated translation has been demonstrated in a number of axonal degenerative disorders. For instance, inhibited intra-axonal translation was shown in a FUS-model of ALS (Lopez-Erauskin et al., 2018). Enhanced translation thus represents another mechanism by which ULK1.DN elicits axon-protective effects, possibly by stimulating the local axonal synthesis of axon-protective molecules.

Irrespective of different etiologies, the pathophysiological mechanisms of axon degeneration in traumatic and degenerative disorders of the CNS have many commonalities. Therefore, we hypothesized that ULK1.DN-mediated ULK1 inhibition might not only protect from lesion-induced axonal degeneration but also exert axon-protective effects in models of neurodegenerative disorders. As autophagy dysregulation is discussed to contribute to the pathogenesis of PD, we evaluated the effects of ULK1.DN in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Balke et al., 2020).

We injected ULK1.DN into the substantia nigra pars compacta, induced degeneration of dopaminergic neurons and their projections by intraperitoneal administration of MPTP, and assessed potential neuroprotective effects of ULK1 inhibition two and six weeks after toxin administration. We found significantly higher numbers of tyrosine hydroxylase (TH)-positive and Nissl-positive cells as well as an increased density of nigrostriatal projections in the midbrain of ULK1.DN-injected animals two weeks after MPTP treatment, demonstrating that ULK1.DN prevents dopaminergic nigral neurons from acute MPTP-induced neurodegeneration. However, these immunohistochemical findings were not correlated with changes in the high-performance liquid chromatography assessment of striatal neurotransmitters or improvements

in motor behavior analyses. Yet, 6 weeks after MPTP administration, we observed improved motor performance in the cylinder rearing test in ULK1.DN-injected animals, whereas no effects were visible in immunohistochemical analyses at this time point. ULK1.DN therefore elicits neuroprotective effects also in the MPTP model of PD, but it remains to be determined why ULK1 inhibition seems to exert diverging effects on histological and behavioral analyses at acute and chronic timepoints in this paradigm. We speculated that extra-striatal effects of ULK1.DN, which we did not assess in our analyses, have to play a relevant role. Mechanistically, we confirmed elevated mTOR activity in the midbrain of ULK1.DN-transduced animals treated with MPTP. We also observed increased expression of the indirect autophagy marker p62 after transduction with ULK1.DN in the MPTP model, but the levels of LC3 were not altered, indicating only a moderate inhibitory effect on autophagy. These results thus reinforce our observation that autophagy inhibition alone does not fully explain the beneficial effects of ULK1.DN, with activated translation being an important molecular mediator of its neuro-protective effects.

Finally, we confirmed some of the key findings of ULK1.DN-mediated ULK1 inhibition using the small-molecule ULK1 inhibitor SBI-0206965 (Vahsen et al., 2020). Indeed, treatment of retinal ganglion cells with SBI-0206965 attenuated AAD for up to 6 hours after optic nerve crush lesion in a dose-dependent manner. Corresponding to our results with ULK1.DN-transduced neurons, SBI-0206965 administration resulted in reduced autophagy and increased mTOR activity. Particularly with respect to the translational applicability of our results with ULK1.DN, this is a promising finding, as treatment with SBI-0206965 would be a therapeutically more easily feasible strategy than adeno-associated viral-mediated ULK1 inhibition. In future studies, it will be crucial to apply SBI-0206965 in different models of traumatic and degenerative axonal degeneration to extend our preliminary findings regarding its promising translational potential and evaluate its side effects and dose-response kinetics.

Taken together, we have revealed a key role of ULK1 in axonal degeneration across multiple axonal populations in the CNS. ULK1.DN-mediated ULK1 inhibition attenuates AAD *in vitro* and *in vivo*, elicits protective effects on long-term axonal degeneration after SCI, and even provides protection from neurodegeneration in the MPTP mouse model of PD. Mechanistically, ULK1.DN decreases autophagy, enhances mTOR-dependent translation, and mediates the differential splicing of degeneration-associated genes such as *Kif1b* and *Ddit3*. We propose that the combination of reduced autophagy, increased translation, and modulated splicing is crucial for the axon-protective effects of ULK1.DN-mediated ULK1 inhibition (**Figure 1**). Correspondingly, we have confirmed some of our key findings by administering the small-molecule ULK1 inhibitor SBI-0206965, which provides protection against AAD via diminished autophagy and increased mTOR-dependent translation. These results demonstrate that ULK1 inhibition represents a putative therapeutic approach in traumatic and neurodegenerative disorders of the CNS. Our findings additionally pave the way for future studies that characterize the translational applicability of SBI-0206965-mediated ULK1 inhibition in axonal degeneration. We envisage that additional studies confirming neuroprotective effects of ULK1 inhibition could lay the groundwork for a therapeutic modulation of ULK1 in human disease.

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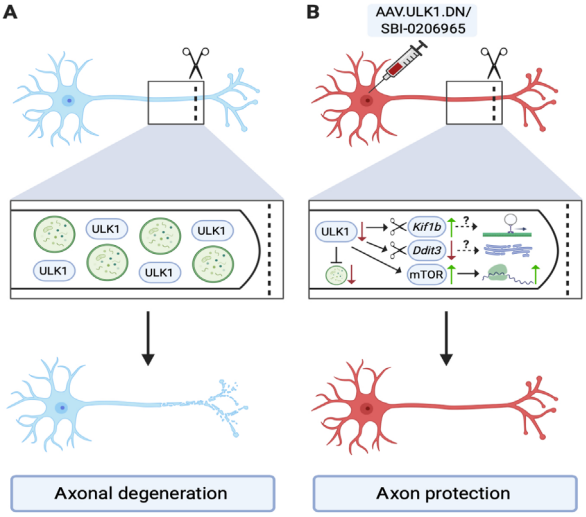

Figure 1 | **Schematic overview of the molecular mechanisms of axon protection after ULK1 inhibition.** (A) ULK1-mediated autophagy induction occurs early during axonal degeneration after axonal lesion, suggesting that ULK1-dependent autophagy represents a detrimental mechanism executing axonal degeneration (Ribas et al., 2015). (B) Inhibition of ULK1 via AAV-mediated expression of ULK1.DN or administration of the small-molecule ULK1 inhibitor SBI-0206965 protects axons from degeneration in different models of axonal injury *in vitro* and *in vivo* (Balke et al., 2020; Vahsen et al., 2020). ULK1 inhibition reduces autophagy, increases mTOR-dependent translation, possibly leading to local synthesis of axon-protective molecules, and mediates differential splicing of the degeneration-associated genes *Kif1b* and *Ddit3*, which might modulate axonal transport and ER stress. We propose that reduced autophagy, increased translation, and modulated splicing act in concert to mediate the axon-protective effects of ULK1 inhibition. Figure was created with BioRender.com.

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