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A DLK-dependent axon self-destruction program promotes Wallerian degeneration

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Abstract

Axon degeneration underlies many common neurological disorders, but the signaling pathways that orchestrate axon degeneration are unknown. We demonstrate that the dual leucine kinase (DLK) promotes degeneration of severed axons in *Drosophila* and mice, and its target JNK promotes degeneration locally in axons as they commit to degenerate. This pathway also promotes degeneration after chemotherapy exposure, and thus may be a component of a general axon self-destruction program.

Axons degenerate in a range of neurological disorders including mechanical injury, chemotherapy-induced neuropathy, hereditary neuropathies, glaucoma, diabetes, and neurodegenerative diseases such as Alzheimer's Disease and Parkinson's Disease¹. Degenerating axons follow a stereotyped pathological progression that was first described in the 1850's for the breakdown of the distal segments of severed axons and termed Wallerian degeneration². This degeneration likely results from an active self-destruction program rather than passive deterioration² since it is delayed by over-expression of the chimeric Wallerian degeneration slow³ (Wld^s) protein and its component nicotinamide mononucleotide adenylyltransferase^{4, 5}, by preventing Ca²⁺ influx⁶, by blocking protein degradation⁷, and by disrupting the phagocytic clearance of axon fragments⁸. A variety of insults trigger axon degeneration^{2, 9}, so identifying the components of the hypothesized axon self-destruction program could have broad clinical value. To date, however, no loss-of-function mutations have been identified that disrupt the internal axon breakdown machinery, and the internal signaling pathways that orchestrate axon breakdown in injury and disease remain unknown.

Candidate components of axon breakdown pathways should be present in axons and activated by diverse cellular insults. One such candidate is DLK, a mitogen-activated protein kinase kinase (MAP3K)¹⁰. One of DLK's downstream targets, the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK), is activated following axonal injury¹¹. We tested the hypothesis that DLK promotes axon degeneration using a well-established *Drosophila* olfactory receptor neuron (ORN) axotomy model^{12, 8}. We expressed green fluorescent protein (GFP) in ORNs to visualize their axons, which extend from cell bodies in the antennae into the antennal lobes of the brain and across a midline commissure (Fig. 1a). To sever ORN axons and induce degeneration, we removed the antennae from wildtype flies and mutants lacking the *Drosophila* ortholog of DLK, *wallenda* (*wnd*)¹³. Most wildtype axons

degenerated within twenty-four hours (Fig. 1b), while *wnd* mutant axons were significantly preserved (Fig. 1c). Wnd is therefore required for normal axon degeneration in *Drosophila*.

Wnd could act within neurons to promote breakdown after injury or within surrounding cells to promote axon clearance. To distinguish between these possibilities, we expressed Wnd in the GFP-expressing subpopulation of ORNs in *wnd* mutant flies. Such Wnd expression was not sufficient to induce degeneration in the absence of injury. However, we found that the Wnd-expressing axons of these otherwise *wnd* mutant flies were not preserved twenty-four hours after axotomy (Fig. 1d). Thus, Wnd functions in an internal neuronal pathway that promotes injury-induced axon degeneration. Wnd may selectively promote injury-induced axon degeneration, as we found no defects in the developmental pruning of mushroom body gamma-lobe axons (data not shown).

To determine if DLK promotes Wallerian degeneration in mammals, we used dorsal root ganglion (DRG) cultures from littermate wildtype (Fig. 2a–c) and DLK-deficient (Fig. 2d–f) embryos¹⁴ (Supplementary Fig. 1). We severed DRG axons to induce degeneration and evaluated degeneration of the distal axon segment. Twenty-four hours after severing, wildtype axons distal to the transection deteriorated into axon fragments, while DLK-deficient axons remained continuous (Fig. 2b,e). To quantify the extent of axon fragmentation, we measured the fraction of total axonal area occupied by axon fragments (degeneration index, DI), and we found that DLK-deficient axons were significantly preserved (Fig. 2b,e). This delay in fragmentation persisted for forty-eight hours (Supplementary Fig. 2). Since non-neuronal cells are eliminated in this DRG culture system, DLK must operate within mammalian neurons to promote axon breakdown. Neuronal DLK therefore promotes axon fragmentation after injury in flies and mice.

To determine if DLK promotes degeneration in response to multiple insults, we assessed the response of DLK-deficient DRG axons to vincristine, a chemotherapeutic drug that induces axon degeneration *in vitro*, and whose dose-limiting side effects in patients include neuropathy¹⁵. We found that DLK-deficient axons were significantly protected from vincristine-induced fragmentation (Fig. 2c,f), suggesting that DLK operates in a general axon breakdown program.

To determine if disrupting DLK protects injured axons *in vivo*, we transected the sciatic nerves of littermate wildtype and DLK-deficient adult mice (Fig. 2g–i). Fifty-two hours post-transection, wildtype axons degenerated, whereas DLK-deficient axons were significantly preserved (Fig. 2h,i). Electron microscopy revealed that preserved axon profiles contain mitochondria and a cytoskeleton (Fig. 2l and Supplementary Fig. 3). Thus, normal Wallerian degeneration *in vivo* in adult mice requires DLK.

DLK is a MAP3K that can activate JNK and p38 via intermediary MAP2Ks¹⁰. To determine whether either downstream kinase promotes axon degeneration, we inhibited JNK and p38 in the DRG axotomy model using wildtype cultures. Inhibition of JNK, but not p38, protected transected axons from fragmentation (Fig. 3a–e), and a significant delay in fragmentation persisted for over forty-eight hours (Supplementary Fig. 2). Thus JNK, like DLK, acts within neurons to promote axon degeneration.

Axon degeneration is hypothesized to comprise at least three distinct phases – competence to degenerate, much of which is determined transcriptionally before axotomy; commitment to degenerate, which occurs in the substantial delay period between injury and axon fragmentation; and the execution phase, when axons fragment¹. If JNK's primary role were to promote competence to degenerate, then JNK activity should be required prior to axotomy. This is not the case: applying the JNK inhibitor twenty-four hours prior to axotomy and then removing it just before axotomy was not protective (Fig. 3f). In contrast, JNK inhibition started

concurrently with axotomy was protective (Fig. 3g). Thus, JNK promotes axon fragmentation after the competence period and acts within the severed distal axon segment.

JNK could commit axons to degenerate during the delay between injury and breakdown, or it could operate during the subsequent execution phase of axon breakdown. To test whether JNK activity is required during the execution phase, we added the JNK inhibitor three hours after axotomy, which is approximately nine hours before the onset of fragmentation. This treatment schedule spans the transition from the proposed commitment phase to the execution phase and the entire execution phase itself. Continuous JNK inhibition beginning three hours post-axotomy did not delay axon fragmentation (Fig. 3h). Therefore, JNK activity is not required during the execution phase of axon fragmentation. Rather, JNK activity is required during the early response to injury that commits the axon to breakdown hours later.

Converging lines of evidence suggest that there is a general internal axon self-destruction program, but its molecular components are unknown. We now show that the MAP3K DLK and its downstream MAPK JNK are important elements of such a program. Disrupting this pathway delays axon fragmentation in response to both axotomy and the neurotoxic chemotherapeutic agent vincristine. Thus, a common self-destruction program may promote axon breakdown in response to diverse insults, and so may be targetable in multiple clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Neuronal Wnd promotes axon degeneration in *Drosophila*

(a) GFP expressed in a subpopulation of ORNs using Or47b-Gal4 to visualize ORN axons. Before severing, the commissure shown in the boxed region is present in all genotypes. Higher power images of the commissural region taken twenty-four hours after axotomy show that (b) no axons remained in the commissure of most wildtype flies (14 of 46 flies had a commissure) and (c) axons are significantly preserved in the commissure of most *wnd* mutant flies (33 of 49 flies had a commissure). This reflects a delay of degeneration as remaining commissures were thinner than non-axotomized commissures, and *wnd* mutant flies reached wildtype levels of degeneration after forty-eight hours (data not shown). (d) When Wnd was exclusively expressed in the GFP expressing subpopulation of ORNs of otherwise *wnd* mutant flies, no axons were detected in the commissure of most flies (3 out of 20 flies had a commissure). Thus, Wnd promotes degeneration cell-autonomously. (e) Fraction of flies of each genotype with and without an ORN commissure. (Axons were significantly preserved in genotype c compared to both genotype b and genotype d, $p < 0.005$, and axons in genotype

d were not preserved compared to genotype **b**, $p > 0.25$, χ^2 , two degrees of freedom). Scale bar = (**a**) 25 μm and (**b–d**) 10 μm .

Fig. 2. Normal axon degeneration in mice requires DLK *in vitro* and *in vivo*

Phase contrast images of DRG axons from (a–c) littermate wildtype and (d–f) DLK mutant embryos. (b,e) Twenty-four hours after axotomy, DLK-deficient axons had a $65\% \pm 3.2\%$ (s.e.m) DI decrease relative to controls ($p < 0.001$, Student's t-test). (c,f) The chemotherapeutic drug vincristine induced $59\% \pm 6.3\%$ (s.e.m) less DI in DLK mutants relative to littermate controls after 48 hours ($p < 0.002$, Student's t-test). (g–l) *In vivo* sciatic nerve transections in adult mice. Sciatic nerve cross-sections distal to the transection site (g–i) stained with Toluidine blue or (j–l) imaged by EM. (h,i) Fifty-two hours post-axotomy, axons are significantly preserved in DLK-deficient nerves ($208\% \pm 22\%$ (s.e.m) axon profiles per μm^2 compared to wildtype nerves, $p < 0.007$, Student's t-test, $n = 4$ wildtype animals and 5 DLK mutant animals).

(j–l) EM shows that these profiles contain axons with mitochondria and a cytoskeletal network (also see Supplementary Fig. 3). Phase contrast scale bar = 20 μm . Toluidine blue scale bar = 20 μm . EM scale bar = 2 μm . All mouse experiments were approved by the Washington University School of Medicine Animal Studies Committee.

Fig. 3. JNK promotes Wallerian degeneration and is critical in the first three hours after axotomy Wildtype DRG axons distal to the axotomy twenty-four hours post-axotomy. **(a)** Shaded areas designate the drug treatment period. ↓P38 refers to the P38 inhibitor (SB203580, 20 μ M) and ↓JNK refers to the JNK inhibitor (SP600125, 15 μ M). **(b)** DI of conditions c–h. **(c–h)** Phase contrast images of axotomized wildtype DRG axons with the indicated drug treatment. Conditions e and g are significantly less degenerated than c, d, f, and h ($p < 0.001$, ANOVA and post-hoc Tukey test). Error bars are s.e.m. Scale bar = 20 μ m.