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Spatial and Temporal Dynamics of Neurite Regrowth

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Abstract

Injury to mature neurites triggers a series of events that have both growth promoting and inhibitory roles. Recent evidence from a variety of experimental models has revealed new neuronal re-growth modulators. The action of these modulators must be precisely regulated both in time and space, and involves multiple cellular processes including retrograde signaling and local translation in the injured neurite. New genetic techniques, in combination with pharmacological approaches, have served to advance mechanistic dissection of neuronal response to injury. Better understanding of the spatio-temporal cues would greatly aid in the development of effective regenerative therapies.

Keywords

neurite injury; neurite regrowth; microtubule dynamics; localized RNA; DLK kinase; retrograde signaling

Introduction

Neurite outgrowth following injury is an important step in repairing neuronal damage. The extent of outgrowth is long known to depend on extrinsic and intrinsic signaling pathways [1,2]. Recent research has revealed new signaling pathways that may help increase the intrinsic neuronal growth potential. An insult to neurites generates injury signals that must be relayed to the cell body. Concomitant with this process is the initiation of growth from the injured neurite. Cytoskeletal structure undergoes major remodeling immediately after injury, and persistent growth of neurites is dependent on microtubule dynamics. Regulators of mRNA can play important roles in coordinating local signaling and trafficking of the injury signals. Among the new pathways, the MAPKKK dual-leucine zipper kinase (DLK) has emerged as a master regulator of injury-triggered stress responses. In this minireview, we summarize the recent insight into the regulation of microtubule outgrowth during regeneration. We then look into the formation and transport of the immediate response at the injury site and highlight new work that elucidates the mechanism of action of DLK. We

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conclude this review by discussing the relevance of spatio-temporal dynamics in neuronal response to injury.

Insights into microtubule remodeling in neurite regrowth

The regenerative failure of CNS neurons post injury has long been attributed to the inability of these axons to generate and maintain growth cone-like structures. Indeed, the formation of dystrophic ends in brain and spinal cord neurons was observed by Cajal a century ago, when he found that transected axons extend for short distances before they stop and form swollen and stunted ends, which he termed “axon retraction bulbs” [3]. Only recently has the underlying cause been directly linked to the failure in modulating microtubule (MT) dynamics in the injured axon [4,5].

Recent pharmacological therapies have provided strong evidence for enhanced regrowth through modulation of MT dynamics, highlighting the importance of cytoskeletal remodeling following neurite injury. Taxol, an MT stabilizing and anti-cancer drug, can prevent retraction bulb formation and promote axon regeneration in CNS injury models [6•, 7•]. Taxol also inhibits glial scar formation, thereby ameliorating the inhibitory environment, which is long known to contribute to CNS regenerative failure [6•]. Caltubin, a novel calcium binding protein isolated in water snails, can bind to α -tubulin in mammalian cells and can prevent axon retraction after injury, potentially by modulating α -tubulin levels at the growth cone [8•]. Caltubin has no clear mammalian homologs, but its effectiveness in promoting axon outgrowth in PC12 cells and mouse cortical neurons suggests potential therapeutic strategy.

An exciting series of recent work has revealed multiple regulatory pathways that modulate MT dynamics in a variety of model organisms. GFP-tagged MT plus-end binding proteins provide valuable tools for *in vivo* visualization of growing MT polymers [9–12]. Chen *et al.* showed that in *Drosophila* larval sensory neurons the cytoskeletal changes elicited by axonal injury are not restricted to the injured axon [9•]. Severing the axon appears to activate a JNK dependent pathway that increases MT growth in the dendrites, resulting in increased dendritic stability. The increase in the number of growing microtubules in the dendrites requires the MT nucleation protein α -tubulin, yet regeneration from the severed axon is normal with reduced levels of α -tubulin [9•]. In this model, injury triggers two parallel pathways: one to regenerate from the injured neurite and another to protect the remaining neurites from further damage.

Several studies have shown MT dynamics are essential for axon regeneration in *C. elegans* [10–12]. Loss of an MT plus end binding protein EBP-1 results in the formation of expanded but immotile growth cones and inhibits regrowth of touch neurons [10•]. Axotomy triggers local up-regulation of growing MTs near the injury site, which steadies into a growth phase with reduced catastrophe frequency, correlating with the formation and extension of a growth cone-like structure [11•,13]. Touch neuron axons fail to initiate regeneration in a dominant α -tubulin mutant, although the axons that manage to initiate regeneration do so to the normal extent [12]. These studies highlight the importance of growth cone initiation in axon regeneration and suggest that growth cone initiation and axon extension may require different cytoskeletal regulation.

Microtubules are characterized by their “dynamic instability”, a behavior marked with rapid switches between persistent growth and shrinkage. MT catastrophe factors typically accumulate at the ends of MTs and mediate their depolymerization [14]. Current studies have revealed roles for numerous MT catastrophe factors in neurite regrowth [10•,11•,15]. The N-terminus of EFA-6, a putative MT catastrophe factor, is necessary and sufficient to limit MT growth near the cell cortex of *C.elegans* embryonic cells [16]. Chen *et al.* showed that

EFA-6 acts cell autonomously to inhibit regrowth in *C. elegans* touch neurons [10••]. In regrowing axons, overexpression of EFA-6 N-terminus reduces the number of growing MTs, while loss of *efa-6* increases them. The regenerative block caused by EFA-6 overexpression could be overcome by taxol, supporting a primary role of EFA-6 in MT destabilization. *C. elegans* kinesin-13, a MT depolymerizing protein [17], maintains steady state MT number in uninjured touch axons, and inhibits growing MTs after injury [11•]. In *Drosophila* sensory axons, normal gene dosage of MT severing protein Spastin is required for regeneration [15]. Loss of one copy of spastin blocks regeneration, and overexpression significantly reduces regeneration length. The differential activity of these MT destabilizing proteins on neurite regrowth may reflect the timing of their action. Both EFA-6 and Kinesin-13 reduce MT dynamics in early stages of regrowth to inhibit growth cone formation [10••,11•]. On the other hand, spastin is probably required to maintain persistent MT growth after the initial remodeling process [15], perhaps by generating local seeds that act as substrates for new MT polymerization [18] (Figure 1).

Tubulins and MTs have long been known to undergo post-translational modifications (PTMs), which can influence cellular growth and transport properties by regulating MT interactions with MT associated proteins (MAPs) [19,20]. MT-PTMs including 2 modification, polyglutamylation, tyrosination, and acetylation, are catalyzed by a variety of enzymes that are widely expressed in vertebrates and invertebrates [21]. Specific MT-PTMs can also affect motor protein affinity and stability in the nervous system. In *C. elegans*, carboxypeptidases CCPP-1 and CCPP-6 [22,23], which catalyze the 2 modification of tubulin, act cell autonomously to promote regrowth, while a putative tubulin polyglutamylase TTLL-5 inhibits initial growth cone formation [11•]. 2 modifications, which are associated with stabilized MTs, are enriched in the mammalian brain [21,24], suggesting the importance of MT stability in neurites. Tubulin deacetylation and tyrosination, both of which have been associated with unstable microtubules [20], are upregulated in a mouse model of PNS injury [25] (Figure 1). MT detyrosination suppresses kinesin-13 activity *in vitro* [26], and tubulin polyglutamylation promotes spastin mediated MT severing [27]. Taken together, these studies highlight the importance of precise modulation of the levels of tubulin PTMs in enhancing regrowth ability after injury.

Advances in understanding local signals and retrograde trafficking after injury

Since injury generally occurs at a distance from the cell body, a long-standing notion is that generation of injury signals and retrograde transport are necessary for a regenerative response [28,29]. Recent studies provide strong evidence for this model [30–32]. Importins, comprised of α and β subunits, retrogradely transport nuclear localization signal (NLS)-bearing cargo proteins [33,34]. Interestingly, Importin β mRNA containing a longer 3' UTR is localized to the sciatic nerve axon [30••]. In response to injury, Importin β is locally translated, leading to heterodimer formation with Importin α to facilitate retrograde transport [35]. To specifically address the axonal role of Importin β Perry *et al.* created a knockout mouse lacking only the axonal Importin β mRNA isoform. These mice showed a decreased response to axon injury and delayed behavioral recovery, which was attributed to the lack of local translation of Importin β . They also observed decreased transcription of genes that are normally upregulated after injury, supporting a crucial role of axonal Importin β for regeneration [30••].

What might be the cargos for retrograde transport? Using previously collected phosphoproteomic and microarray data, Ben-Yakkov *et al.* identified several transcription factors that are retrogradely transported from the axon [36•,37]. They show that retrograde transport of phosphorylated STAT3 is essential for neuronal survival post sciatic crush

injury [36•]. Injecting STAT3 NLS peptides into the sciatic nerve competed with nuclear import of phosphorylated STAT3 and dynein mediated retrograde transport of pSTAT3, resulting in decreased neuronal survival after injury [36•]. Additionally, an independent study has recently linked phospho-STAT3 retrograde transport with the MAP kinase DLK [31••](discussed later). Together, these results stress the importance of the spatial regulation of the transport machinery during the injury response (Figure 2).

Localization and regulation of RNA in neurite regrowth

Mechanisms involving localized mRNAs endow spatial and temporal regulation in neurons. The development of compartmentalized axonal culture chambers has greatly aided the identification of localized mRNAs [38]. Since the ability of the neuron to regenerate decreases with age, Gumy *et al.* undertook a comparative analysis of mRNAs localized to axons of DRG neurons in embryonic and adult stages [39]. A striking difference between the two is the enrichment of cytoskeletal and transport mRNAs in embryonic axons[39•]. In contrast, inflammatory and immune related mRNAs are present in adult axons prior to injury and later are enriched in injured neurons, along with mRNAs associated with protein synthesis, mitochondrial function and cell death[39•]. The reduced presence of cytoskeletal and transport mRNAs in adult axons may underly the age related decline in regeneration. Further studies of these differentially expressed mRNAs can provide insight into the functional relevance of RNA in neurite regeneration.

In another genome wide screen for mRNAs localized to growth cones, Feltrin *et al.* has uncovered a link between local translation of MKK7, a kinase in the JNK signaling cascades that have previously been suggested as regulators of MT dynamics [40,41], and MT bundling in neurite extension [42•]. Translation of MKK7 mRNA at the growth cone requires its 3' UTR. Knockdown of MKK7 decreased neurite elongation and created disorganized MTs. They further showed that pMKK7 acts downstream of DLK. This study suggests that local translation of MKK7 at the growth cone would provide tight spatial control of MT dynamics through localized DLK and JNK signaling.

Supporting the broad roles of localized mRNAs in regulating growth and dynamics of neurites, diverse RNA regulators are emerging as key players in regeneration. One RNA binding protein, hnRNP K, has been shown to be necessary for recovery from optic nerve injury by possibly affecting the loading of target mRNAs into polysomes for translation [43]. A series of recent studies have begun to reveal roles of specific microRNAs in the regenerative response of axons and dendrites in *Drosophila* and zebrafish [44,45], as well as an age-dependent decline in regeneration in *C. elegans* [46]. In cultured *Lymnaea* neurons, local translation of Caltubulin (discussed previously) was essential for its growth promoting effects[8]. These studies suggest neurons precisely regulate the localization and quantity of specific mRNAs through various levels of regulation during regeneration.

Pleiotropic roles of a MAP kinase DLK in stress response and injury

The dual leucine zipper kinase (DLK) is a neuronally expressed MAPKKK that has emerged as a signaling hub in neurite regeneration and degeneration, stress-induced gene expression and neuronal death[47–53]. Several recent studies reported a rapid elevation of DLK post-transcriptionally following axonal injury [31••,53–56]. In flies and mice, DLK activation results in JNK phosphorylation[54–56] and an upregulation of p-cJun in the soma after injury[31••]. However, Xiong *et al.* observed that while Wallenda, the *Drosophila* DLK homolog, was not required for synaptic or axonal degeneration after injury in motoneurons, inhibition of JNK prevented axonal degeneration, implying additional independent regulators of JNK in promoting degeneration of the distal stump [56]. The *C.elegans* DLK homolog, DLK-1, promotes axon regeneration through a p38 MAPK pathway [48,53], and

can also coordinate with the MAPKKK MLK-1, leading to crosstalk between the downstream p38 and JNK pathways [57].

In mammals, recent studies have begun to uncover the complex roles of DLK in axon regeneration, degeneration and neuronal death. In a high-throughput approach, Welsbie *et al.* screened 623 kinases and found that knockdown of DLK or its known mammalian substrate, MKK7, protected primary RGCs from neuronal cell death[55••]. Using a conditional deletion allele of *dlk* or a small molecule inhibitor, Tozasertib, they found that the survival rate of RGCs in an optic nerve crush injury model could be significantly improved by inhibiting DLK signaling. Watkins *et al.* showed that RGC survival was dramatically improved when DLK was deleted from the retina before an optic nerve crush[54••]. Deletion of DLK, despite protecting RGCs from degeneration, substantially reduced the number of regrowing axons even when PTEN was deleted to enhance intrinsic growth potential[54••, 58]. Microarray analysis of the differentially expressed genes in DLK depleted injured RGCs showed upregulation of both pro-apoptotic genes (CHOP, Puma, Bim) and pro-regenerative genes (APTF3, Sprr1a, Klf6) 3 days after injury[54••]. These studies suggest that DLK initiates the injury response of a neurite by activating both regenerative and degenerative processes, allowing the cell to adopt either pathway based on other intrinsic and extrinsic cues. The DLK pathway also plays a crucial role in enhancing intrinsic growth potential after a PNS preconditioned lesion, where previously injured neurons are transected again to test for regeneration capacity[59]. Shin *et al.* showed that DLK knockout mice do not respond to a pre-conditioning lesion in a sciatic nerve crush injury model[31••]. They also showed that DLK regulates the retrograde transport of activated transcription factors in response to injury, including the transcription factor STAT3. This study and similar work in *Drosophila*[56] support the idea that DLK potentially activates a pro-regenerative signal in the neuron following injury, which transiently stabilizes the axon against further damage.

Previous evidence indicates that timely activation of DLK is critical for the injury response[48], with emerging reports supporting posttranscriptional mechanisms [55]. Recent work in *C.elegans* has uncovered a mechanism for DLK activation. Yan and Jin showed that DLK-1 is expressed as two isoforms, DLK-1L (for Long) and DLK-1S (for Short), which can form homo- and hetero- dimers with each other through the leucine zipper domain[60••]. Their analysis reveals a model whereby DLK-1L and DLK-1S are bound to one another in the uninjured state, which inactivates DLK-1. In response to axotomy, calcium influx can dissociate DLK-1L from DLK-1S, which then activates DLK-1 signaling. Such tight regulation of DLK/DLK-1 in the neuron may facilitate its ability to activate a wide variety of downstream targets in response to neuronal stresses like axonal injury (Figure 2).

Perspectives

The cellular response to neurite injury is regulated by a variety of signaling pathways. The new findings combining live imaging and molecular genetics have provided a closer glimpse of the immediate changes at the injury site. To initiate regenerative growth from the injury site, MT dynamics at the growth cone must be precisely modulated by a variety of MAPs and MT-PTMs (Figure 1). The recent work reviewed here suggests that optimal regrowth is achieved by a balance of MT stability and catastrophe rather than stability alone. Cellular damage as well as calcium influx likely trigger DLK activation. Proper localization of injury-related mRNA and nuclear import proteins along the axon then plays a key role for retrograde transport of the injury signal for regeneration and recovery (Figure 2). The ubiquitous role of DLK in injury suggests that it can provide the neuron with the flexibility to respond to different cues. DLK possibly acts as a sensor for the injured neuron and allows it to adopt an apoptotic or regenerative response depending on other available signals. Furthermore, DLK is potentially a switch that is temporally determined by the condition of

the neuron. Given the different temporal effects of RNA localization and MT dynamics on regeneration, it is important to implement therapeutic strategies at precise time points. Finally, integrating what we know about the spatio-temporal intrinsic response with knowledge of extrinsic factors that also affect regenerative ability[61], will provide insight into effective therapy for neurite injury.

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Highlights

1. We discuss MT modulators, retrograde transport, RNA and DLK roles in neurite regrowth
2. MT stability and neurite regrowth are intricately linked
3. DLK promotes both degenerative and regenerative responses after injury
4. We propose that spatio-temporal regulation is essential for regeneration

Figure 1. Summary of new modulators of MT cytoskeletal remodeling and their effects on MT dynamics and neurite regrowth
EFA-6 and Kinesin-13 are MT-catastrophe factors that decrease MT stability (red arrow) and regrowth [10•,11•]. Spastin reduces MT stability but increases regrowth (green arrow) [15]. MT-PTMs like CCPPs and TTLLs, have similar effects on MT stability and regrowth [11•] however HDAC5, a MT deacetylase, decreases MT stability while increasing regrowth [25].

Figure 2. Summary of recent studies on spatial-temporal regulation of neurite regrowth

1) After neurite injury, influx of calcium activates DLK signaling at the injury site [60••]. 2) Locally translated Importin α binds to Importin β and together with dynein retrogradely transports injury signals to the nucleus [30••,35]. 3) DLK promotes retrograde transport of phosphorylated STAT3, but not activation of STAT3, to the nucleus [31••]. 4) Initiation of growth cone formation at the proximal stump, and possibly concurrent degeneration of the distal end. 5) Transcriptional programs for apoptosis and regeneration are activated in the nucleus, likely in response to DLK activation and other injury signals [54••].