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Cytoskeleton dynamics in axon regeneration

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Recent years have seen cytoskeleton dynamics emerging as a key player in axon regeneration. The cytoskeleton, in particular microtubules and actin, ensures the growth of neuronal processes and maintains the singular, highly polarized shape of neurons. Following injury, adult central axons are tipped by a dystrophic structure, the retraction bulb, which prevents their regeneration. Abnormal cytoskeleton dynamics are responsible for the formation of this growth-incompetent structure but pharmacologically modulating cytoskeleton dynamics of injured axons can transform this structure into a growth-competent growth cone. The cytoskeleton also drives the migration of scar-forming cells after an injury. Targeting its dynamics modifies the composition of the inhibitory environment formed by scar tissue and renders it more permissive for regenerating axons. Hence, cytoskeleton dynamics represent an appealing target to promote axon regeneration. As some of cytoskeleton-targeting drugs are used in the clinics for other purposes, they hold the promise to be used as a basis for a regenerative therapy after a spinal cord injury.

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Introduction

After an injury in the adult mammalian central nervous system (CNS), axons fail to regenerate [1]. This is fundamentally different in the peripheral nervous system (PNS) and in the embryonic CNS, where lesioned axons regrow [2,3]. Two major events hamper regeneration in the adult CNS. First, inhibitory molecules secreted by oligodendrocytes and scar-forming cells block axon regrowth [4–7]. Second, central axons lose their intrinsic growth ability upon maturation [8]. Among the variety of intrinsic processes preventing axon regeneration, pathological cytoskeleton dynamics have emerged as a major impediment to CNS axon regeneration.

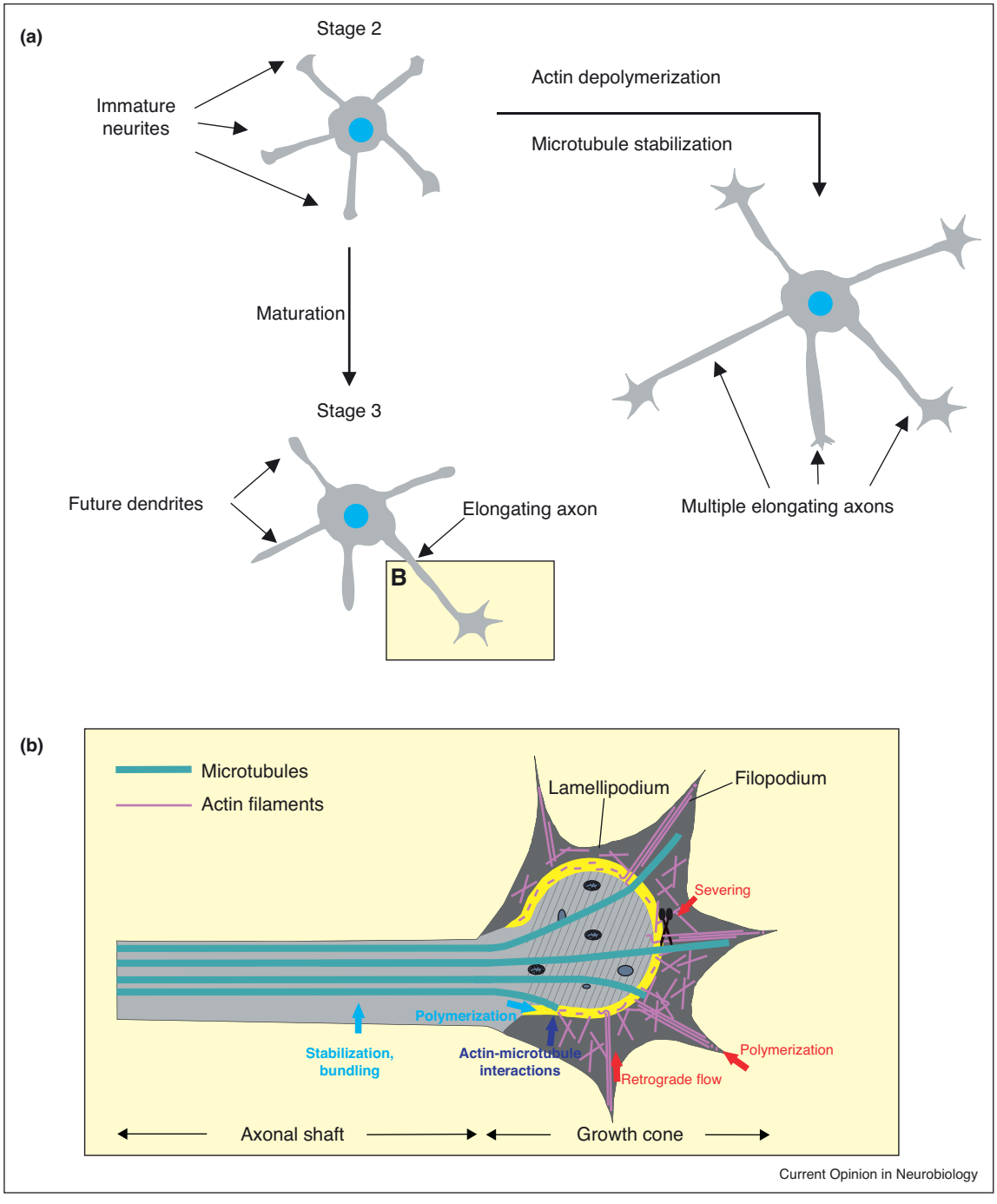
During development, the cytoskeleton creates and maintains the shape of neurons and non-neuronal cells. Due to their dynamics, microtubules and actin filaments control the establishment of neuronal polarity [9–12]. In this review, dynamics of the cytoskeleton refer to any modification of its stability and include events such as polymerization, depolymerization, severing or bundling. Notably, a stereotypical spatial organization and dynamics of the cytoskeleton at the tip of the axon, within the growth cone, ensure the elongation and steering of developing and adult injured peripheral axons. The inability of adult central neurons to re-form a growth cone following axotomy represents a major obstacle to axon regrowth [13].

In this review, we first outline the main stages leading to axon elongation during development and describe the organization of the axonal cytoskeleton within the growth cone of elongating axons in comparison to the retraction bulb of growth-incompetent axons. We then summarize evidence demonstrating that manipulation of cytoskeleton dynamics can restitute the intrinsic regenerative ability of adult neurons and discuss the major signaling pathways that underlie this cytoskeleton disorganization. We also discuss the role of cytoskeleton-mediated axonal transport in injured central neurons. Finally, we highlight evidence demonstrating that modulating cytoskeleton dynamics affects both the migration of cells toward the lesion site and the release of inhibitory proteins by scar-forming cells, thus modifying scar tissue composition. Together, targeting cytoskeleton dynamics represents a powerful and potentially clinically translatable strategy to enhance regeneration following injury.

Axon growth during development

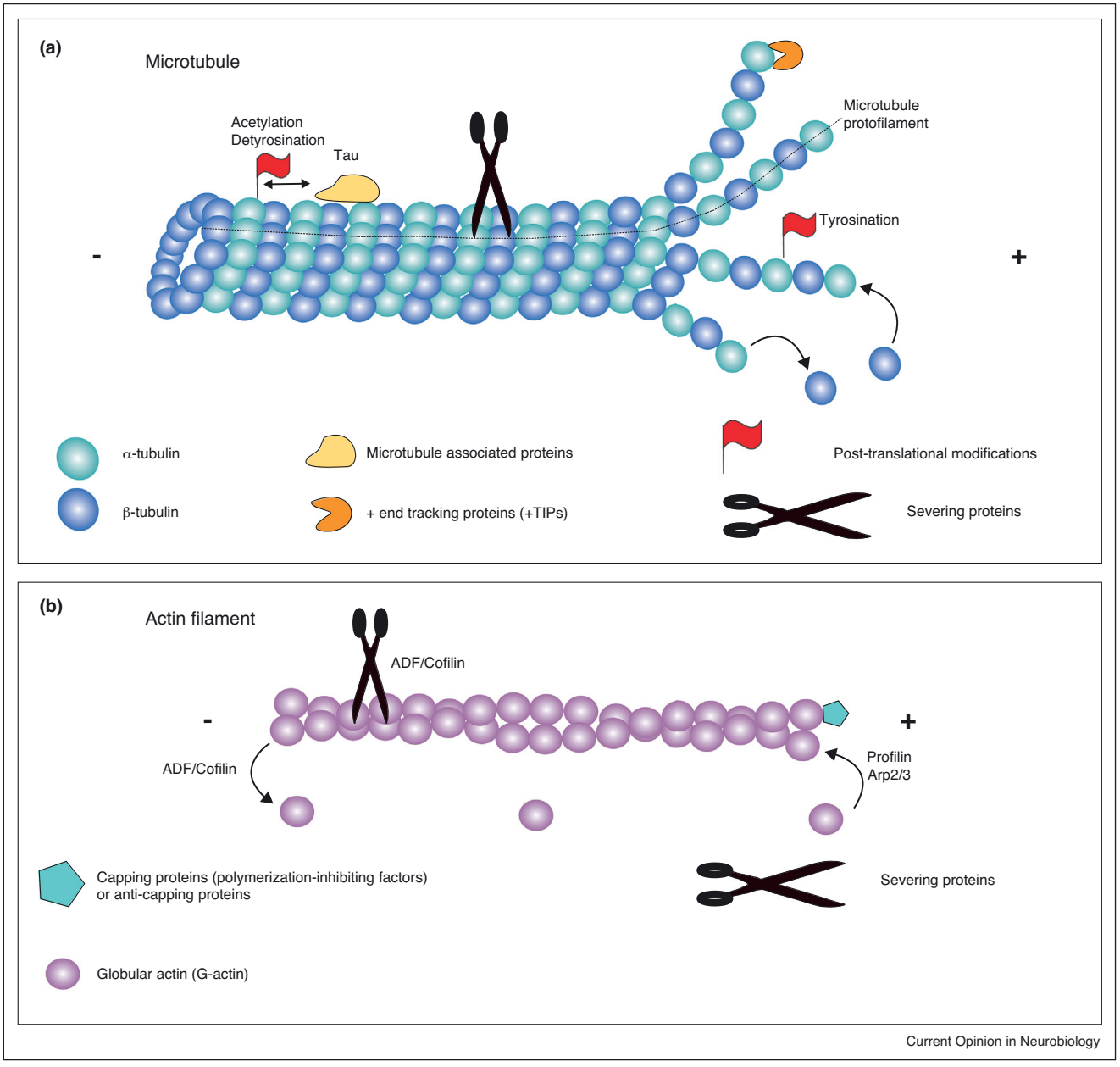
Cytoskeleton dynamics within the growth cone ensures the growth and steering of developing axons. Apprehending how the cytoskeleton organizes within developing axons and how its dynamics leads to axonal elongation (Figure 1) is essential to understand the mechanisms underlying the intrinsic inability of adult CNS neurons to regrow their axons and provide a therapeutic strategy overcoming this regeneration failure. The growth cone contains an actin-rich peripheral domain (P-domain) that contains filopodia, bundled parallel actin filaments, and lamellipodia, that form a branched actin network in between filopodia. The central domain (C-domain) of the growth cone contains microtubules that project with their polymerizing end toward the P-domain. Developing axonal shafts display more stable microtubules whereas

Figure 1



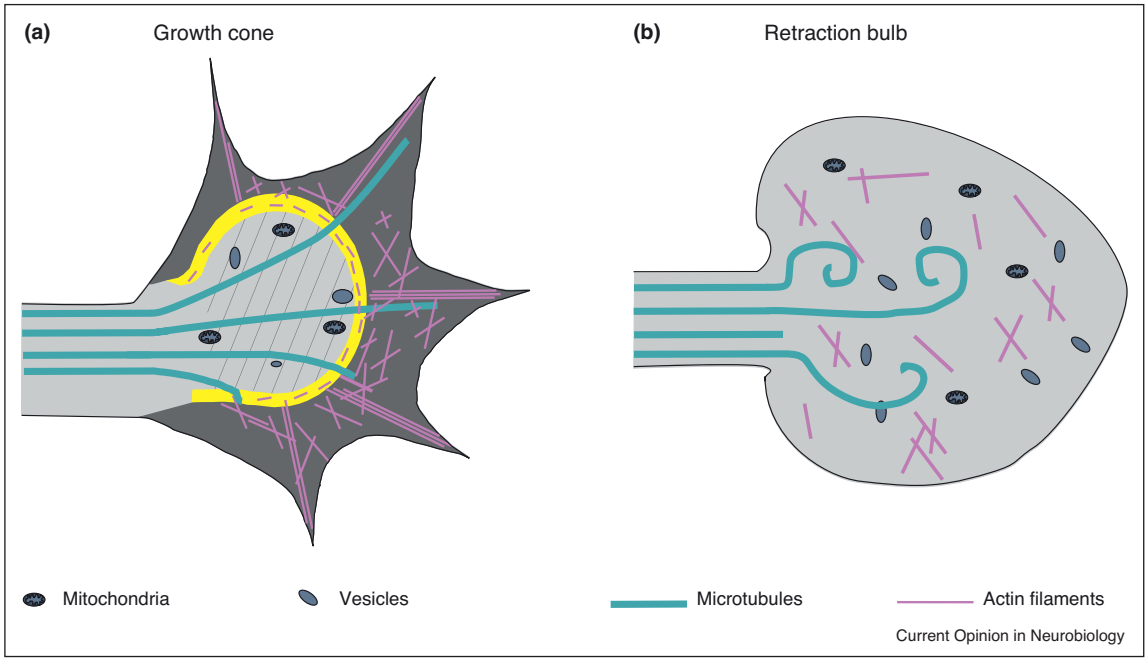
Neuronal polarization and axonal elongation. **(a)** Shortly after plating, neurons form four to five neurites of about 15 μm length (stage 2). Neurons acquire polarity when one of the neurites rapidly extends: this dynamic neurite differentiates into an axon while the remaining minor neurites give rise to dendrites (stage 3). The polarity of developing neurons can be manipulated by modulating the cytoskeleton dynamics: low doses of the microtubule-stabilizing agent taxol and application of the actin-depolymerizing drug cytochalasin D both lead to the formation of several axons. **(b)** The axonal shaft of elongating axons contains stable and tightly bundled microtubules whereas their growth cone contains a dynamic cytoskeleton. In the center of growth cones (grey stripped area), dynamic microtubules protrude from the axonal shaft. More peripherally (dark grey area), long bundles of actin radiate outward, giving rise to the filopodia. Meshes of actin filaments, the lamellipodia, intertwine these radial actin bundles. Interactions between actin and microtubules mostly occur in the transition domain (yellow area). Elongation takes place when polymerizing microtubules protrude in the peripheral domain along filopodial actin. Red and blue arrows represent the main cytoskeletal dynamics leading to axon elongation, respectively for actin and microtubules.

Figure 2



Actin and microtubule dynamics. **(a)** Microtubules are 25 nm-thick hollow cylinders composed of 13 protofilaments, each of them arising from the longitudinal polymerization of α-β-tubulin heterodimers. Microtubules are polarized: they present a minus end (−) on the soma side and a plus end (+), where most of the catastrophes occur, facing the axonal tip. Two main families of proteins, the microtubule-associated proteins (MAPs) and the plus-end tracking proteins (+TIPs), influence microtubule dynamics. The MAPs, for example, Tau, stabilize microtubule bundles and antagonize severing proteins. At the plus end of microtubules, TIPs proteins such as end-binding proteins control microtubule growth and catastrophe events. Post-translational modifications of α-tubulin further influence both the intrinsic stability of microtubules as well as their affinity to microtubule-binding proteins and thus enable a tighter control of MT dynamics. Please note that tyrosination/detyrosination face the outside of the microtubule whereas acetylation occurs in the lumen of microtubules. **(b)** Actin filaments are thin (≈8 nm-thick) helical double-strand filaments composed of G-actin (globular) monomers. Like microtubules, actin filaments are polarized: plus ends (+), or barbed ends, face the leading edge of the growth cone while minus ends (−), or pointed ends, point toward the cell body. G-actin monomers are incorporated to the plus-ends of actin filaments.

Figure 3



Cytoskeletal organization in growth cones versus retraction bulbs. **(a)** Growth cones display three distinct regions. The center (C-domain, grey stripped area) contains microtubules which emerge from the axonal shaft. In the periphery (P-domain, dark grey area), a dense network of actin filaments controls the progression of microtubule toward the axon tip and excludes vesicles and organelles from the P-domain. This obstacle occurs at the border between the C-domains and the P-domains, the transition domain (T-domain, yellow arc). **(b)** Regeneration failure is associated with the formation of a retraction bulb at the tip of the injured axon. The separation between C-domain, T-domain and P-domain is lost. In addition, microtubules are depolymerized to a large extent. The remaining ones are disorganized and do not reach the axon tip.

microtubules extending into the central domain (C-domain) of the growth cone undergo dynamic events (Figures 1b and 2a). Notably, detyrosination and acetylation are two posttranslational modifications correlating with the age of microtubules and are mostly found in the axonal shaft. Tyrosination is used as a marker for dynamic microtubules and is found on microtubules protruding into the growth cone. Protruding microtubules are restrained by the actin filaments in the transition domain (T-domain) that have formed actin arcs mediated by the motor protein myosin II.

The process of axon elongation is divided into three steps [14,15]. In a first phase, the 'protrusion', actin filaments polymerize their barbed-end (or plus-end) at the leading edge of the growth cone (Figures 1b and 2b), thereby triggering the elongation of filopodia and lamellipodia [10,16–18]. At their minus-end, actin filaments undergo cofilin-mediated depolymerization [19]. This leads to treadmilling of the actin filaments, which is observed as retrograde flow [19]. Depolymerization of actin filaments provides a pass through which polymerizing microtubules can protrude and elongate into the former peripheral domain [20•,21]. This second step is called 'engorgement'. The transition from polymerization to stabilization of microtubules within the proximal growth cone enables the formation of a newly generated neurite shaft. This is the 'consolidation'. Repeated cycles of these three phases lead to axon elongation. Together, the growth cone of elongating neurons provides an environment in which polymerizing microtubules can protrude and thus lead to axon elongation. In fact, these mechanisms enable the neuron to polarize during development. On the one hand, microtubules are more stable in the future axon shaft than in the shaft of the non-growing minor neurites. Moderate stabilization of microtubules by taxol enables the microtubules to polymerize and to extend, which transform the non-growing neurites into growing axons [9]. On the other hand, the axon growth cone contains actin filaments that are more dynamic and less stable compared to the non-growing minor neurites' growth cones. Actin destabilization is sufficient to transform non-growing neurites into growing axons [11]. Could the reactivation of these mechanisms induce axon regeneration in the adult nervous system?

Anatomy of retraction bulbs

By contrast to developing neurons [10,22] or to axotomized peripheral neurons [23], adult injured central neurons do not display a growth cone following axon injury [24,25]. Instead, injured mature CNS neurons form a dystrophic bulb, the so-called retraction bulb [26,27] (Figure 3). Retraction bulbs are heterogeneous oval structures about four times larger than the axon right after

axotomy and continue to increase in size overtime [26]. In cell culture, they lack filopodia but display lamellipodia-like structures [27]. Surprisingly, although these atrophic ends fail to elongate the axon, they are dynamic, with their lamellipodia undulating back and forth [27]. The growth-restrictive intracellular mechanisms associated with these structures are still unclear. This is because *in vitro* models in which injured central neurons generate a retraction bulb comparable to the *in vivo* situation are relatively recent [26–28]. These studies highlight the aberrant cytoskeleton organization in these atrophic structures. Whereas growth cones display the aforementioned microtubule-rich central domain relatively segregated from an actin-rich peripheral domain, the two cytoskeleton components largely overlap in retraction bulbs [27]. Instead of forming the parallel and tight bundles typical in growing axons, microtubules disassemble and are disoriented in retraction bulbs [26]. Like growth cones, retraction bulbs present dynamic, polymerizing microtubules. However they are restricted to the center of the bulb [26]. These observations raise the question whether modulating cytoskeleton dynamics could represent an efficient strategy to transform retraction bulbs into growth cones. If so, which differences in the expression and activation profiles of cytoskeleton-associated proteins and which upstream events preclude injured adult central neurons from forming a growth-competent growth cone? Gaining insight into these questions could enable the development of efficient therapeutic strategies to transform retraction bulbs into growth cones and ultimately to overcome the growth failure of injured CNS neurons.

From retraction bulbs to growth cones: modifying the microtubule cytoskeleton dynamics

Treating dorsal root ganglia (DRG) neurons with the microtubule-depolymerizing drug nocodazole disperses microtubules within the bulb and transforms the axon tip into a retraction bulb-like structure similar to the one found after CNS injury [26]. This finding supports the hypothesis that pathological microtubule dynamics cause microtubule disorganization and lack of regenerative capacity of mature injured neurons. Conversely, enhancing microtubule polymerization at the axon tip by administering the microtubule-stabilizing agent epothilone B reduces retraction bulb formation and boosts axon regeneration of central neurons following spinal cord injury [29••]. Accordingly, destabilizing microtubules by application of low doses of nocodazole abolishes epothilone B-dependent microtubule protrusion within the growth cone as well as axon elongation [29••]. Similar to epothilone B, the microtubule-stabilizing drug taxol shifts microtubule polymerization toward the axon tip [9] and improves growth cone formation in adult injured CNS neurons [30,31]. Hence, these data provide evidence that an abnormal microtubule dynamics reduces the ability of adult central neurons to re-form a growth cone and that controlling microtubule dynamics could efficiently enhance the regenerative capacity of injured adult neurons. Interestingly, growth cone formation is observed only with low doses of either taxol or epothilone, indicating that moderate stability of microtubules is required for efficient axon regrowth. In the last years, effort has been made in unraveling the mechanisms underlying the abnormal cytoskeleton organization and dynamics observed in retraction bulbs. As mentioned before, tubulin acetylation is commonly used as a marker for microtubule longevity and protects microtubules against breakage [32•,33•]. In injured adult peripheral neurons — but not in central neurons — HDAC5 promotes the deacetylation of axonal microtubules in a growing gradient from cell body to lesion site [34]. This posttranslational modification is triggered by Ca^{2+} release at the injury site and is necessary for axonal regrowth [34]. It should be noted that only physiological levels of HDAC5 are required for axon regrowth but both inhibition and overexpression of this enzyme impair axonal repair [34] indicating that fine local and quantitative control of microtubule dynamics is required for the axon to efficiently regenerate. This finding further suggests that peripheral axons require less stable microtubules than central axons, a hypothesis that could be explained by an environment more permissive in the PNS than in the CNS [34]. It should be noted, however, that HDAC5 targets other proteins than microtubules [35,36]. We will now discuss the role of actin filaments in axon regeneration.

From retraction bulbs to growth cones: the actin dynamics

Although the microtubule reorganization following axotomy has been well described [26,29••,30,37–39], there is still relatively little known about the role of actin dynamics in axon regeneration. *In vitro*, injury is followed by a change in actin filaments at the tip of central neurons [40••]. This effect can be prevented by overexpressing the doublecortin-like kinases 2 (DCLK2), a protein promoting axon regeneration in adult central neurons [40••]. However, which modality of actin is necessary for the axon to regenerate remains to be investigated. In fact, it is even unclear whether neurons extend their axon by the growth cone 'pulling' the axon or if axon growth occurs more through an amiboid-type of movement. Cytoskeleton reorganization can be further achieved by targeting the cytoskeleton-associated protein nonmuscle myosin II [41]. In adult DRG neurons, the specific inhibitor of nonmuscle ATPase activity blebbistatin results in drastic actin reorganization, including decreased actin filament-positive areas and increased filopodia formation, and improves the protrusion of microtubules into the peripheral domain [42]. The inhibitor also promotes axon regeneration, an effect which can be abolished treating blebbistatin-treated cells with low doses of nocodazole [42]. These data underline the role of actin in axon regeneration and

illustrate how fine modifications of its dynamics allow adult injured neurons to upscale their intrinsic regenerative ability. It is expected that by studying regeneration paradigms, the role of actin dynamics in axon regeneration will be better understood. For example, DRG neurons form a growth cone and regenerate their central axon coursing in the CNS when the axon in the PNS was injured beforehand, a phenomenon called 'conditioning' [43–45]. Since part of the conditioning induced regeneration might be attributed to recapitulating a developmental growth program [2,46••] and actin dynamics are instrumental for neurite formation [19], it might be possible that conditioning fundamentally affects actin regulating proteins to drive regenerative growth. This hypothesis, however, requires rigorous testing in the future.

The cytoskeleton, intracellular trafficking and axon regeneration

The cytoskeleton ensures the active transport of proteins, vesicles and organelles along the axonal shaft besides promoting the forward movement of the axon tip and constituting the backbone of neurons. After a first phase of Ca^{2+}-dependent retrograde signaling [13,47], a cytoskeleton-based retrograde transport is believed to convey the 'injury signal' to the nucleus and activate pro-survival and pro-regenerative programs [48–50]. Axotomy of the peripheral branch of DRG neurons leads to increased local transcription of the protein importin β, which in turn enables dynein-mediated retrograde transport of nuclear localization signal-bearing proteins, invoking regeneration [49]. Translation of the pro-regenerative transcription factor STAT3 is also locally increased following peripheral nerve injury and retrogradely transported to the soma along microtubules by the motor protein dynein [48]. Together, these data demonstrate that retrograde transport is necessary for axon regeneration. Conversely, the increase in retraction bulb size over time together with the observation that retraction bulbs display a higher density of mitochondria and small vesicles in comparison to growth cones [26] indicate that retraction bulbs might be associated with a deficient retrograde transport. The mechanisms initiating retrograde injury signaling are beginning to be elucidated. Injury-dependent activation of the enzyme tubulin-tyrosine ligase (TTL) promotes the tyrosination of α-tubulin in peripheral neurons and thereby promotes minus-end directed transport [34,51•]. Notably, TTL knockdown delays the activation of the pro-regenerative transcription factor c-JUN and significantly prevents the regeneration of DRG neurons [51•]. In this context, it is interesting to note that chronic treatment of the sciatic nerve with the microtubule-depolymerizing drug colchicine recapitulates a conditioning lesion [52]. Thus, microtubules have certainly a dual function in axon regeneration. Besides their role in supporting axon growth they provide the transport roads for retrograde signals back to the nucleus. Injured central neurons fail to sustain expression of pro-regenerative transcription factors [53] and reactivation of these factors restore their regenerative ability [54,55].

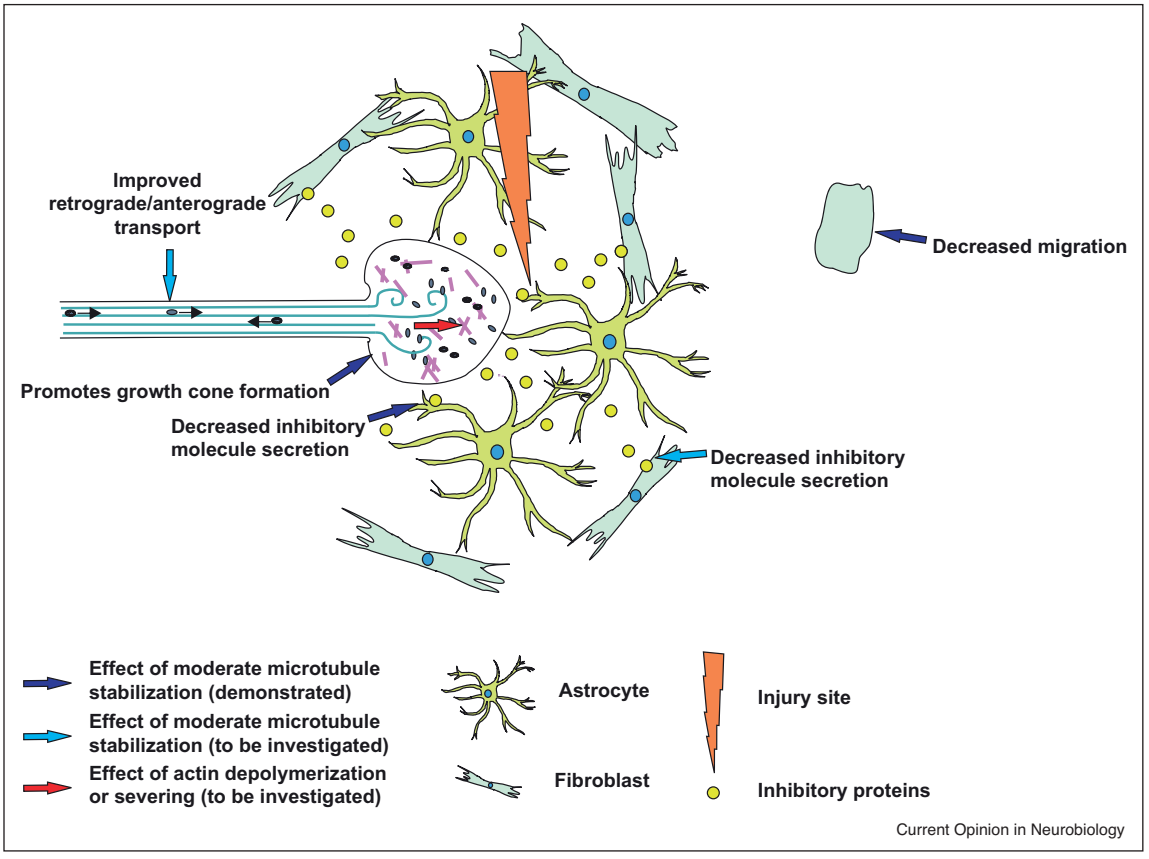
Axon regeneration further requires the anterograde transport of organelles (e.g. mitochondria) and material (e.g. actin and tubulin) at the lesioned axonal tip. In Aplysia growth cones, microtubules rapidly depolymerize following axotomy before reorganizing into two distinct pools of opposite polarity [38]. This reorganization allows the formation of two vesicle-rich traps: a plus-end trap capturing anterogradely transported vesicles and a distal trap concentrating retrogradely transported vesicles [38]. The density of anterogradely transported Golgi-derived vesicles increases due to this microtubule reorganization and is necessary for the extension of growth cones after axotomy, indicating the importance of microtubule-directed anterograde transport for axon regeneration [38]. Moreover, in retraction bulbs, repolymerized microtubules fail to point their end toward the axon tip [47]. A direct correlation between transportation rate and regeneration capacity in injured central axons support the hypothesis that anterograde transport represents a limiting factor of regeneration in CNS neurons [56,57,58••]. In support of this hypothesis, the pro-regenerative action of DCLK requires the anterograde transport activity of their microtubule-binding domain [40••]. Conversely, inhibiting the motor kinesin 5, a protein which restrains transport of short microtubules along the axons [59], improves *in vivo* axon regeneration when neurons grow within a permissive graft [60]. Together, these data indicate that adjusting microtubule stability might improve anterograde transport to the growth cone to supply elements necessary for axon regrowth.

Cytoskeleton dynamics and the formation of a scar tissue

Fibrotic and glial scar tissue contains inhibitory molecules and constitutes a major environmental obstacle to axon elongation [4,5]. Besides promoting the intrinsic axon elongation of neurons by stabilizing their axonal microtubules, taxol hampers the formation of fibrotic scar tissue and decreases chondroitin sulfate proteoglycan (CPGS) deposition [30,31]. Following injury, an increase in TGF-β promotes the production of inhibitory proteins by astrocytes [61]. Mothers against decapentaplegic homolog 2 (Smad2), a downstream effector of TGF-β, binds to microtubules through kinesin-1 and transduces the signal to the nucleus [62]. Thereby, enhancing microtubule stability with taxol significantly alters kinesin1-dependent intracellular transport and impedes the translocation of Smad2 from microtubule to the nucleus [30]. Hence, taxol-dependent inhibition of TGF-β/Smad2 signaling pathway results in a significant reduction of inhibitory proteins release by astrocytes in the lesion site [30].

Taxol injection further compromises the upregulation of laminin, fibronectin and collagen IV [30], three extracellular

Figure 4



Manipulating microtubule and actin dynamics to achieve axonal regeneration. The presence of microtubule and actin in neurons as well as in scar-forming cells and their role in a variety of cellular processes including intracellular transport, proliferation and migration place these two intracellular constituents as promising therapeutic targets.

matrix proteins released by fibroblasts in response to injury [63]. Immunostaining experiments have confirmed that impediment in fibroblasts migration underlies this effect [30]. Similar to taxol, epothilone B and D decrease the expression of inhibitory fibrotic molecules [29••,37]. In fact, besides promoting the intrinsic growth ability of neurons, epothilone B stabilizes the whole microtubule network in fibroblasts and hence abolishes the polarization necessary for the migration of these cells toward the site of injury, thus allowing the reduction of the fibrotic scar tissue [29••,64]. The antithetic effect of epothilone B on microtubule polymerization in fibroblasts versus neurons is due to the neuron-specific expression of Tau, a microtubule-associated protein regulating microtubule polymerization, bundling and binding to microtubule-stabilizing proteins [29••]. It is likely that taxol decreases the fibrotic tissue through a similar mode of action as taxol and epothilones bind to the same pocket of β-tubulin [65].

Together, these data demonstrate that cytoskeleton dynamics impact axon regeneration not only by boosting the intrinsic regenerative ability of injured adult neurons but also by modifying cellular migration toward the lesion site and secretion of inhibitory extracellular signals by these scar tissue cells (Figure 4).

RhoA signaling: linking extracellular inhibitory signals to the cytoskeleton?

Although cytoskeleton dynamics appears to be a major player in controlling axon regeneration, we still understand relatively little about how the various growth inhibitory signaling pathways act onto the cytoskeleton. Interestingly, various inhibitory signaling cascades hampering axon regeneration appear to be mediated through RhoA signaling. RhoA is activated in response to a variety of inhibitory cues including CSPGs [66], myelin-associated glycoprotein (MAG) [67] or Nogo [68] and controls the stability of actin cytoskeleton [69]. Overexpression studies and the usage of bacterial enzymes deciphered that the major downstream effector of RhoA is the kinase ROCK. ROCK, in turn, phosphorylates and activates the actin-binding protein profilin. Another major target of Rho A is the LIM-kinase 1. This kinase inactivates the major depolymerizing protein enzyme cofilin and therefore

improves actin filaments stability [70,71]. ROCK also phosphorylates the myosin light chain, which in turn increases the actomyosin contractility and thus reduces neurite extension [72]. Thus, these *in vitro* studies suggest that RhoA may be crucial to translate extracellular inhibitory cues into intracellular cytoskeletal changes. Consistently, the Rho GTPase inhibitor C3 improves axonal regeneration in cultured adult retinal neurons and after spinal cord injury *in vivo* [73,74] and the inhibitor is presently in the phase of clinical trials for the treatment of acute spinal cord injury [75]. However, the physiological role of RhoA in axon regeneration is unclear. Moreover, the physiological effectors downstream of RhoA remain to be identified. The analysis of a RhoA knockout mice will facilitate our understanding of this pathway and the role of cytoskeleton therein substantially.

Conclusion

Central neurons fail to regenerate following injury but efficient treatments do not exist. This is because targets which both boost the intrinsic regenerative capacity of neurons and hamper the formation of an inhibitory scar tissue were not available until recently. Modifying the cytoskeleton dynamics restores a growth cone in non-regenerating neurons and enhances axonal transport. Recently, it has been highlighted that targeting the cytoskeleton further decreases formation of inhibitory surroundings by reducing the migration of fibroblasts toward the lesion site and preventing the release of inhibitory proteins by astrocytes and fibroblasts. Thus, cytoskeleton dynamics appear as an optimal target to promote axon regeneration. In fact, treating spinal cord injured rats with taxol, epothilone B or epothilone D leads to functional recovery [29••,30,37,76]. In the future, understanding the molecular players leading to pro-regenerative cytoskeletal rearrangements will be crucial to translate these findings into efficient clinical treatments.

Conflict of interest statement

H. Witte, A. Ertürk, F. Hellal and F. Bradke filed a patent on the use of microtubule-stabilizing compounds for the treatment of lesions of CNS axons (European Patent no. 1858498).

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- • of special interest
- •• of outstanding interest

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