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INVITED REVIEW

Inhibition of kinesin-5 improves regeneration of injured axons by a novel microtubule-based mechanism

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Abstract

Microtubules have been identified as a powerful target for augmenting regeneration of injured adult axons in the central nervous system. Drugs that stabilize microtubules have shown some promise, but there are concerns that abnormally stabilizing microtubules may have only limited benefits for regeneration, while at the same time may be detrimental to the normal work that microtubules perform for the axon. Kinesin-5 (also called kif11 or Eg5), a molecular motor protein best known for its crucial role in mitosis, acts as a brake on microtubule movements by other motor proteins in the axon. Drugs that inhibit kinesin-5, originally developed to treat cancer, result in greater mobility of microtubules in the axon and an overall shift in the forces on the microtubule array. As a result, the axon grows faster, retracts less, and more readily enters environments that are inhibitory to axonal regeneration. Thus, drugs that inhibit kinesin-5 offer a novel microtubule-based means to boost axonal regeneration without the concerns that accompany abnormal stabilization of the microtubule array. Even so, inhibiting kinesin-5 is not without its own caveats, such as potential problems with navigation of the regenerating axon to its target, as well as morphological effects on dendrites that could affect learning and memory if the drugs reach the brain.

***Key Words:** microtubule; axon; kinesin-5; Eg5; regeneration; monastrol; molecular motor protein*

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Injured adult axons can regenerate, but their regenerative capacity is quite limited, especially in the central nervous system (CNS). Injured axons in the adult CNS tend to retract and degenerate rather than regrow (Liu et al., 2011). This is because the injured axon encounters physical and chemical obstacles such as scar tissue and injury-associated inhibitory molecules, because the adult CNS environment is normally rich in factors such as myelin that are not conducive to regeneration, and because the intrinsic growth potential of the adult axon does not match that of a juvenile axon (Mar et al., 2014). In recent years, there has been a focus on microtubules as among the most promising targets for augmenting the capacity of injured adult axons to regenerate. Microtubules, hollow polymeric filaments of tubulin subunits, provide structural support for the axon and act as a substrate for many of the molecular motor proteins responsible for intracellular transport. Microtubules are intrinsically polar structures, each with a plus end favored for assembly over the minus end. Molecular motor proteins convey cargoes such as membranous organelles along the lattice of the microtubule, specifically toward its plus or minus end, depending on the particular motor protein.

In the axon, the microtubules are aligned into a paraxial array with the plus ends of the microtubules directed away from the cell body, thus establishing the directionality with which different motors convey their cargoes (Baas and Lin, 2011). Microtubules gather together and funnel into the hillock region of the axon and then splay apart again at sites of branch formation and within the growth cone at the tip of the elongating axon (Baas and Buster, 2004). Each microtubule in the axon consists of a stable domain that resists depolymerization, with most stable domains giving rise to a more dynamic labile domain that assembles from the plus end of the stable domain (Baas and Black, 1990). Microtubules are relevant to axonal growth and regeneration for reasons related to all of these factors. The dynamic properties of microtubules are critically important, especially in the distal tip of the axon, for the capacity of the axon to form a viable growth cone, to turn properly in response to external cues, and to grow with the vitality needed for proper development (Conde and Caceres, 2009).

Evidence for the importance of microtubules for axonal regeneration has come from studies suggesting that taxol, a microtubule-stabilizing drug commonly used for cancer

therapy, can positively impact the regeneration of injured axons in the adult CNS (Hellal et al., 2011; Sengottuvel et al., 2011). However, these studies are not without controversy, as other studies suggest that the key to axonal regeneration is virtually the opposite of what taxol does. These other studies suggest that the key to axonal regeneration is transforming the predominantly stable microtubules in the adult axon into a more labile/dynamic population, especially in the distal area of the axon (Bradke et al., 2012). Interestingly, recent work has shown the importance of the status of post-translational tubulin modifications, as it appears that axons regenerate better in the peripheral nervous system (PNS) because the microtubules in the damaged region of the axon become less post-translationally acetylated (Cho and Cavalli, 2012). Such a reduction in microtubule acetylation does not occur in the injured CNS, suggesting that tubulin modifications that accompany microtubule stability negatively impact the capacity of the axon to regenerate. Taxol increases microtubule acetylation, further indicating that whatever taxol's positive effects may be, they are not due to recapitulating the developmental mechanisms of axonal growth. Collectively, these observations implicate microtubules, but do not recommend with clarity what should be done in terms of treatment to best augment axonal regeneration.

Recent studies revisiting the taxol work suggest that taxol's positive effects may not have been as robust as once believed, and may have been due mainly to the drug's impact on non-neuronal cells relevant to regeneration (Popovich et al., 2014). In terms of potential effects on the axon itself, taxol may seem to positively affect regeneration in simpler laboratory assays because stabilization of microtubules prevents axonal retraction, and because stabilized microtubules may enable the tip of the regenerating axon to push through normally inhibitory environments. Such effects, while perhaps contributing to axonal regeneration in the short-term, do not reflect how axonal growth and retraction are normally regulated or how the dynamic growth cone of the axon functions during development. A better therapeutic approach may be to exploit the normal mechanisms by which the microtubule array is regulated during development (Baas and Ahmad, 2013).

Adult axons have a higher proportion of stable microtubule mass than developing axons. This presumably means that the labile domains are relatively longer in the case of microtubules in developing axons compared to adult axons. During development, a variety of microtubule-related proteins contribute to regulating axonal growth and navigation. Some of these proteins regulate the proportion of the microtubule mass that is stable or labile. Optimally, we would like to identify molecules that can be manipulated to enable CNS axons to grow faster and to enable them to ignore/overcome inhibitory molecules associated with the CNS and the injury site. We have recently argued that a potentially powerful approach would be to add microtubule mass to the axon to promote its growth, and specifically to add labile microtubule mass (Baas and Ahmad, 2013; Baas, 2014). The idea is to shift the microtubule array to a ratio of labile to stable more similar to that which exists during development. Knocking down or inhibiting proteins that normally tamp back the expansion of the labile domains could theoretically accomplish this. At present, this remains our favorite idea, but the results of such an approach remain to be seen. We cannot dismiss the possibility that this strategy might encounter unexpected negative repercussions of so drastically changing the microtubule content of the axon.

What other microtubule-related approaches could be taken? In a set of recently published studies, we have taken an entirely different approach that theoretically should have no effect on either microtubule levels or microtubule stability in the axon (Lin et al., 2011; Xu et al., 2015). This approach seeks to augment axonal growth by affecting certain motor-driven forces on the microtubules. To understand this approach, it is helpful to think of the axonal microtubule array not as a static architectural structure, but rather as a machine with moving parts (Baas and Ahmad, 2001). A number of different molecular motor proteins impose forces on the microtubules, but in this case not to move small cargoes along the microtubules, but rather to transport short microtubules along longer microtubules (or along actin filaments). Such transport of short microtubules is important as these short microtubules convey tubulin down the axon for the expansion of the microtubule array (Baas et al., 2006). Some short microtubules elongate into longer ones, while other short microtubules depolymerize to yield their subunits for the elongation of neighboring microtubules. A greater fraction of the short mobile microtubules moves anterogradely, but many of them move retrogradely (He et al., 2005). The directionality of the movement relative to the polarity of each short microtubule is believed to underlie the mechanism by which the nearly uniform polarity orientation of axonal microtubules is achieved and preserved against potential corruption (Baas and Mozgova, 2012). In this view, the retrogradely moving microtubules are reverse-oriented, and their movement back into the cell body represents a clearing mechanism.

The motor-driven forces that drive and regulate the transport of short microtubules are not selective for short microtubules, and also impinge upon longer microtubules. When such forces act between two microtubules, both of which are too long to move in a concerted fashion, the result is like an isometric exercise. A panoply of such forces throughout the microtubule array serves to integrate the microtubules into a functional unit. A shift in the balance of such forces can influence whether the axon grows, retracts, or pauses (Baas et al., 2006). Regulation of such forces can also control, regionally, whether microtubules invade one side of the growth cone or the other, which influences the directionality of the turning of the growth cone. The various molecular motors that contribute in this fashion harbor a great deal of potential as targets for augmenting axonal regeneration after injury. In theory, shifting the balance of these motor forces can speed axonal growth, reduce axonal retraction and also

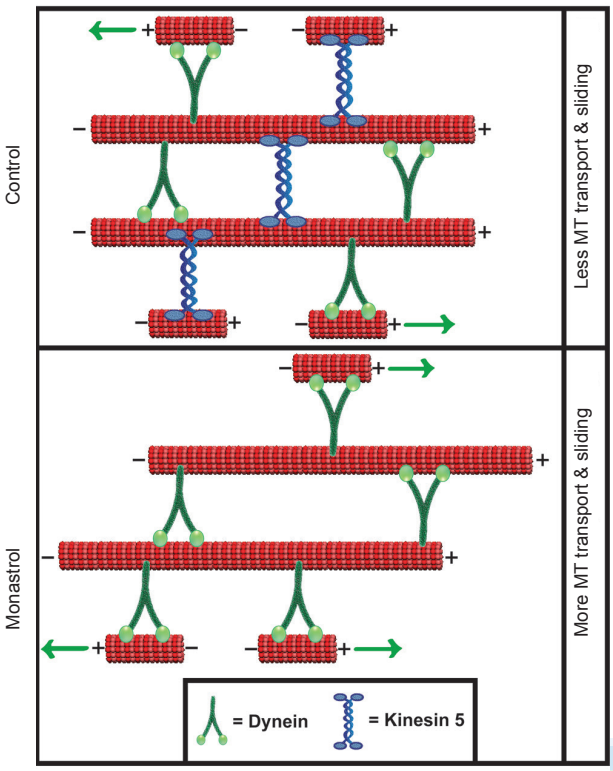

Figure 1 Kinesin-5 is a brake on microtubule movements in the axon.
Shown is a schematic model for how molecular motors regulate microtubule movements in the axon. In the axon, long microtubules are directed with their plus ends distal to the cell body. Cytoplasmic dynein transports short microtubules in both directions in the axon, with the short microtubules moving either forward or backward depending on their polarity orientation (Baas and Mozgova, 2012). Kinesin-5 acts as a brake that impedes microtubule transport and sliding. When kinesin-5 is pharmacologically inhibited by monastrol, a greater number of short microtubules become mobile. The balance of forces on the longer microtubules changes as well, enabling greater invasiveness of the long microtubules into the growth cone.

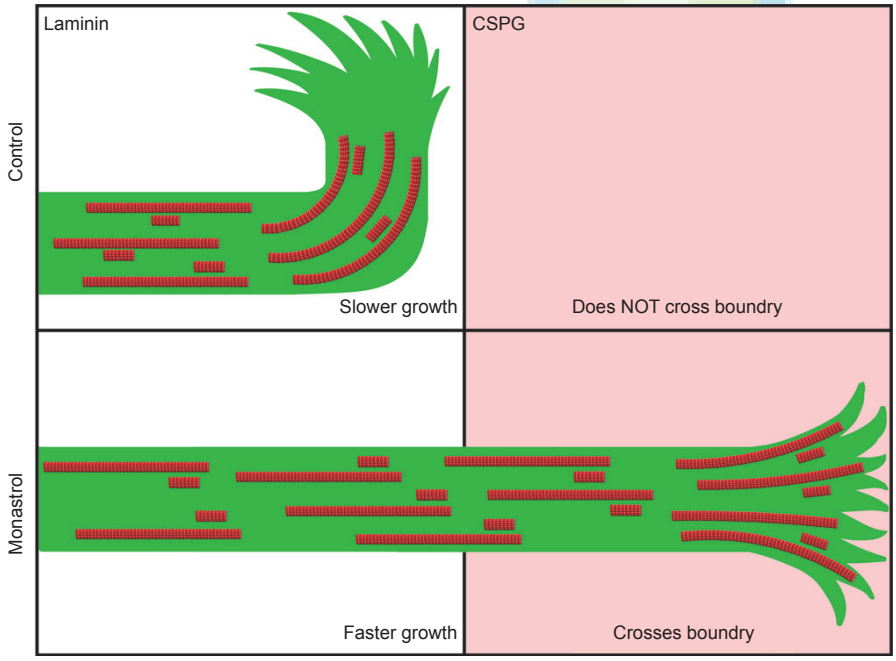

Figure 2 Kinesin-5 inhibition enables injured adult axons to regenerate better.
Kinesin-5 inhibition by monastrol results in a faster growing axon, due to greater mobility within the microtubule array, as well as an enhanced capacity of the axon to cross over a boundary onto a chondroitin sulfate proteoglycans (CSPG) (inhibitory) substrate. Crossing onto CSPGs is enhanced if the monastrol treatment is coupled with an enzyme treatment that partially digests the CSPGs.

potentially assist regenerating axons in entering less favorable environments as opposed to turning away from them.

There is good evidence that cytoplasmic dynein is a principal motor for transporting microtubules in the axon (Ahmad et al., 1998, 2006; He et al., 2005), and potentially could do most or all of the work in transporting microtubules in both directions in the axon (Baas and Mozgova, 2012). Several years ago, we sought to test whether kinesin-5 might be another relevant motor in the axon. Kinesin-5 is also called kif11, Eg5, KSP, or bimC, and should not be mistaken for kif5, which is actually kinesin-1. Kinesin-5 is most famously known as a mitotic motor protein, and exists in cells as homotetramers with four motor domains projected outward. As such, kinesin-5 is suited to slide oppositely oriented microtubules relative to one another. When we first started studying this motor in neurons, the question arose as to exactly what kinesin-5 would theoretically do in a microtubule array consisting predominantly of microtubules of the same

polarity orientation. Fortunately, our studies were assisted by the availability of drugs such as monastrol that are highly specific to kinesin-5, designed by the cancer community to inhibit cell division.

Using monastrol and also RNA interference to deplete kinesin-5 from cultured neurons, we found that inhibition of kinesin-5 causes juvenile axons to grow notably faster, to retract less, and to ignore cues that normally cause growth cones to turn (Myers and Baas, 2007; Nadar et al., 2008). We found that inhibition of kinesin-5 causes an increase in the number of short microtubules moving in both directions within the axon. We also observed effects on the longer microtubule indicative of stronger dynein-driven forces, as evidenced by deeper and less discriminate invasion of microtubules into the growth cone (Nadar et al., 2008, 2012). Thus the effects were dramatic, but the mechanism was somewhat baffling. The simplest interpretation was that kinesin-5 acts as a brake that attenuates the capacity of other motors, chiefly cytoplasmic dynein, to generate movements of microtubules in the axon (Myers and Baas, 2007; Kahn et al., 2015). This is illustrated schematically in **Figure 1**.

Additional studies as well as insights from the mitosis field have led us to a mechanistic answer for how kinesin-5 functions in neurons. Kinesin-5 is a very slow motor, and effectively limits the rate that any other motor could move microtubules. A partner protein called TPX2 creates drag on kinesin-5 that slows it even further, enabling it to act as a brake. In terms of growth cone turning, kinesin-5 is localized to microtubules on the side of the growth cone opposite to the direction of turning, so that the microtubules have greater penetration in the direction of turning (Nadar et al., 2008, 2011). These activities of kinesin-5 are regulated in part by phosphorylation of kinesin-5 by CDK5 at a site important for microtubule interaction, and in part by a preference for kinesin-5 to interact with microtubules that are not rich in post-translationally detyrosinated tubulin (Kahn et al., 2015). Regulation at the level of TPX2 probably also plays a role, but we have not yet studied this aspect of kinesin-5's regulation in neurons.

On the basis of the effects observed in the juvenile axons, we reasoned that inhibition of kinesin-5 might offer a powerful means for augmenting injured adult axons to regenerate. In theory, such inhibition would make the axon grow faster, retract less, and overcome inhibitory obstacles that would otherwise cause the axon to turn away. Moreover, the idea is appealing because kinesin-5 drugs are available that have already been put through clinical trials for use on human patients in treating cancer. The potential stickler, however, is whether or not there are sufficient levels of kinesin-5 in adult neurons such that inhibition would elicit any effect at all. Our early *in situ* hybridization analyses on rodents indicate that kinesin-5 expression is barely detectable in the adult CNS and PNS relative to development (Ferhat et al., 1998). In more studies aimed at detecting kinesin-5 protein rather than mRNA, we established that adult neurons do express detectable levels of kinesin-5, with higher levels in CNS than PNS (Lin et al., 2011). In studies on adult dorsal root ganglion (DRG) neurons in culture, we found that inhibition of kinesin-5 results in faster growing axons and an increase in the number of short mobile microtubules in the axon (Lin et al., 2011). The response was not as strong as with juvenile neurons, but was otherwise the same. Kinesin-5 inhibition assisted axons in crossing onto an inhibitory substrate of chondroitin sulfate proteoglycans (CSPGs), but the effect was not as robust as with myosin inhibition (Yu et al., 2012). These effects, shown schematically in **Figure 2**, were stronger when combined with chondroitinase ABC (ChABC), which is an enzyme that partially digests the CSPGs. Another research group recently reported that monastrol assisted the towing of axons with micro-tweezers together with a microfluidic device on a CSPG substrate (Kilinc et al., 2014). This effect was even stronger than that achieved with a RhoA kinase inhibitor.

Eager to test whether kinesin-5 inhibition could assist axonal regeneration *in vivo*, we collaborated with the laboratory of Dr. Veronica Tom (Xu et al., 2015). For these studies, we used a peripheral nerve graft, which provides a permissive environment for the regrowth of injured CNS axons as well as a path to circumvent the glial scar. The axons grow robustly through the graft, but the problem for the axons is emerging from the favorable PNS environment within the graft to the less favorable CNS environment of the spinal cord. For these studies, rats received complete thoracic level 7 (T7) transections and PNGs and were treated intrathecally with ChABC and either monastrol or DMSO vehicle. Just as with our cell culture work on adult DRG neurons, combining ChABC with monastrol significantly enhanced axonal regeneration in the *in vivo* regime. However, addition of monastrol to the regime resulted in no additional improvements in function or enhanced c-Fos induction (an indicator of integration of the regenerating axons) upon stimulation of the spinal cord rostral to the transection. This might be because the boost in axonal growth provided by monastrol is insufficient within itself to lead to integration of the regenerating axons with physiologically relevant targets, or because the monastrol is actually detrimental to appropriate path-finding of the regenerating axons. If the latter is the case, future *in vivo* studies may benefit from reducing the window of time during which the experimental site is exposed to the drug so that the drug can be cleared once the beneficial boost in axonal growth has occurred.

Despite the lack of functional recovery in the first *in vivo* study, we are encouraged that drugs that inhibit kinesin-5 can be powerful components of a multi-tiered strategy to enhance nerve regeneration in human patients. Moreover, we are encouraged by the proof-of-principle that regeneration of the axon can be improved by altering the balance of motor-driven forces that impinge on its microtubule array. Kinesin-12 (also called kif15) is another so-called "mitotic" motor whose inhibition in cultured neurons dramatically increases microtubule transport and axonal growth in cultured neurons, and even more dramatically than inhibition

of kinesin-5 (Liu et al., 2010). While there are currently no drugs against kinesin-12, mitotic motor proteins are favored targets of the cancer community, and such drugs may become available in the near future.

Finally, a note of caution is due. While manipulating microtubule-related proteins is a more subtle approach than applying a microtubule-stabilizing drug such as taxol, each microtubule-related protein presumably has its own work to do. Prolonged inhibition of any microtubule-related protein, even if the effects on nerve regeneration are positive, could also have negative consequences, especially if the inhibition reaches other nervous tissue, such as the brain. Recent studies have shown that kinesin-5 is inhibited by beta amyloid during Alzheimer's disease, and that this inhibition may contribute to the degeneration of neurons in the brain (Ari et al., 2014). We have recently shown that monastrol alters dendritic morphology (Kahn et al., 2015), indicating that kinesin-5 might play a role in the plasticity of the dendritic arbor, which is important for learning and memory. We remain hopeful that exposure of the drug to injury sites for limited windows of time could be optimized to limit any potential negative consequences and to capitalize on the positive effects of kinesin-5 inhibition on nerve regeneration.

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