

● PERSPECTIVE

Acetylation as a mechanism that regulates axonal regeneration

The capacity for adult axons to regenerate after injury is diminished compared with developing axons. In the case of central nervous system (CNS) axons, injury causes a total failure to regenerate. This failure is due to both the intrinsic developmental decrease in growth capacity and the extrinsic inhibitory environment formed because of the injury. One way to re-invigorate mature axons into a regrowth state is to induce regenerative gene expression in the nucleus to increase the intrinsic growth state of the neuron. One potential mechanism is through changes in epigenetic factors. Another possible method is to alter posttranslational modifications in axonal and growth cone microtubules of the axonal cytoplasm (Trakhtenberg and Goldberg, 2012; Cho and Cavalli, 2014). An increasing number of studies have turned to epigenetic manipulation using pharmacological inhibitors to try and enhance axonal growth. Numerous reversible posttranslational modifications are considered important for axonal growth and regeneration but such modifications are regulated by families of enzymes which affect system wide changes in the body, sometimes shuttling between the nucleus and the cytoplasm as well as between the cell body and axon. Two families of enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs), act antagonistically to affect acetylation and de-acetylation respectively on many proteins throughout the body. Acetylation in neurons is critical for many physiological events such as cell division, cell growth and proliferation. This paper will focus on the potential implications of acetylation homeostasis on axonal growth.

Histone acetylation: Histones act as structural support for DNA chromatin to wrap around nucleosomes. Acetylation of the N-terminal histone tail is gated by HATs and HDACs. While histone acetylation is known to upregulate gene expression, histone de-acetylation is associated with suppression of gene expression. Regulation of HATs and HDACs in neurons is important for preventing neurological disease progression, brain function as well as axon and dendrite outgrowth (Graff and Tsai, 2013). Thus, HDAC inhibitors and HAT activators have traditionally been implemented to improve brain function in many animal models.

It has been suggested that HAT activation can improve regenerative gene expression in damaged axons. The nuclear-specific HAT proteins p300 and p300/CBP-associated factor (PCAF) have both been shown to promote axonal regeneration in DRG neurons and spinal cord neurons in overexpression studies (Gaub et al., 2011; Puttagunta et al., 2014). Interestingly it was found that Trichostatin A (TSA) (Graff and Tsai), a commonly used non-specific HDAC inhibitor had the same effect on axonal outgrowth as overexpression of HATs. Others have shown that Tubacin, a more specific inhibitor of HDAC6 can improve axon growth in DRG neurons (Rivieccio et al., 2009) but even this compound

is known to inhibit other HDACs. In contrast, our laboratory has shown that TSA and Tubacin inhibit DRG axonal growth and that in cultures treated with the inhibitors there is a reduction of axons crossing over an inhibitory chondroitin sulfate proteoglycan (CSPG) border substrate. Interestingly we found that HAT inhibitors, anacardic acid and cyclopentylidene-[4-(4-chlorophenyl)thiazol-2-yl]hydrazide (CPTH2), can promote axonal growth (Lin et al., 2015). Our studies looking at a range of concentrations of TSA showed that increasing concentrations could inhibit axonal growth in adult DRG neurons, while at lower concentrations there were no changes in axon lengths. Furthermore, when Tubacin and TSA were applied only to the cell body or the axonal compartments of neurons grown in microfluidic chambers (**Figure 1**), there was no improvement in axonal growth over inhibitory CSPG substrates. This suggests that the HDACs targeted by these inhibitors, whether in the cell body or the axon, do not have an effect on axonal regeneration. The fact that HAT inhibitors could promote axonal growth and axonal crossing of CSPGs suggests that there could be acetyl transferases other than p300 or PCAF that are detrimental to axonal growth or that certain genes upregulated by HATs could hinder regeneration. Taken together, this data suggests that inhibition of HDACs and HATs using non-specific inhibitors cannot be used to conclusively show any mechanism of axonal regeneration.

A better approach would be to express specific HATs or HDACs to look at whether axonal growth can be improved. There are several families of HDAC proteins which are subdivided according to their cellular localizations. Class I and IIa HDACs predominantly function in the nucleus, deacetylating histone proteins and Class IIb HDACs mostly function in the cytoplasm (Cho and Cavalli, 2014). In the nucleus, HDACs generally repress gene expression by forming complexes with transcription factors, methyl transferases and with HATs. Recent studies have also shown that HDAC5 is transported out of the nucleus after axonal injury through a calcium dependent retrograde injury signaling cascade (Cho and Cavalli, 2012). Furthermore, this cytoplasmic HDAC5 is phosphorylated and increases towards the distal end of severed axons correlating with a decrease in tubulin acetylation. Knockdown of HDAC5 reduces the rate of axonal regeneration after axotomy *in vitro* while overexpression enhances growth cone dynamics. This demonstrates that HDAC5 has a function in the cytoplasm that could promote axonal growth. Future studies should make full use of microfluidic chambers, which separate axonal and cell body compartments, in order to understand the specific localization of acetylation changes (**Figure 1**). Such studies would also benefit from genome wide analysis of changes in neuronal gene expression after injury employing methods such as CHIP analysis.

Microtubule acetylation: In neurons microtubules form an essential part of the cytoskeleton affecting neuronal development, polarization, growth and repair. Microtubule properties are dependent on a variety of posttranslational modifications and are also subject to a “tubulin code” (Song and Brady, 2014). Microtubule acetylation is important for a variety of processes including kinesin-based axonal transport

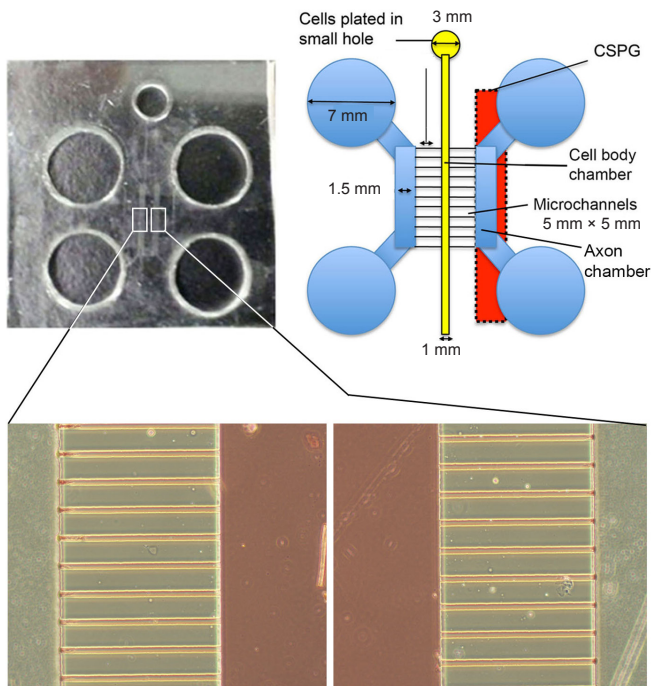
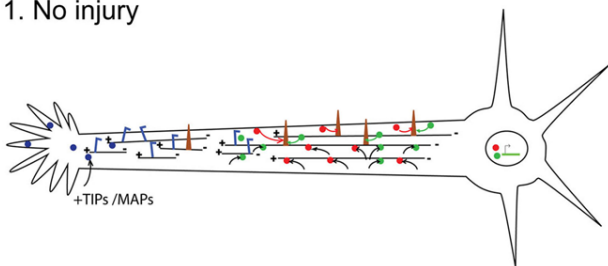


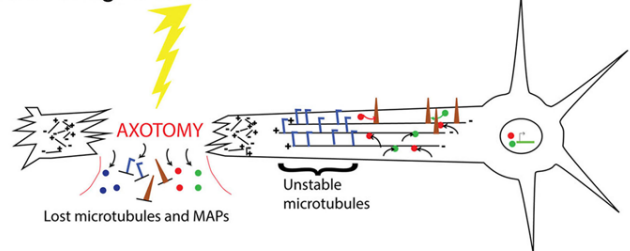
Figure 1 Microfluidic chambers can be used to study compartmental localization of the effects of acetylation inhibitors and other neuronal changes in adult axons.

Microfluidic chambers can be cast by pouring silicone elastomer into a prefabricated microfluidic template and holes can be punched to mark the chambers. Image below shows magnification of microfluidic channels on both sides of the central chamber. Microfluidic chambers are placed over a chondroitin sulfate proteoglycan border stripe before neurons are plated into a small hole and allowed to occupy the central chamber (cell body chamber). Neurons can be grown for several days until axons reach the chondroitin sulfate proteoglycan (CSPG) border stripe.

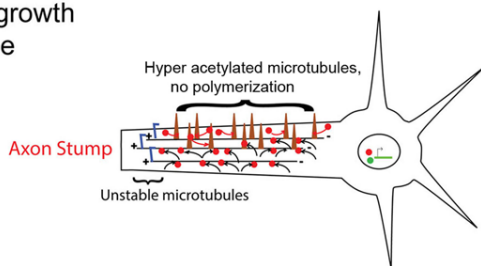
1. No injury



2. Damaged axon



3. No axon growth after damage



4. To improve recovery

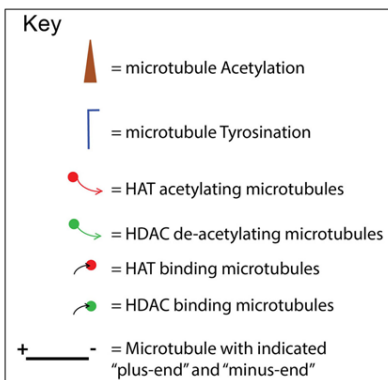
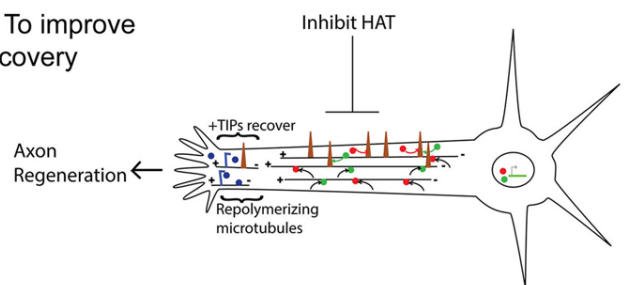


Figure 2 Predicted microtubule changes which occur during axonal regeneration.

1. In adult neurons, stable microtubule arrays are arranged with more acetylation at the proximal end of the axon and more tyrosination distally. HDACs and TATs reversibly regulate acetylation on microtubules and on histones in the nucleus. HDACs and TATs also bind to microtubules themselves and can stabilize them. Other MAPs, such as +TIPs, aid polymerization of microtubules at the distal end of the axon and in the growth cone.

2. Axotomy disrupts the microtubule array causing loss of many MAPs, and an unstable pool of microtubules is initially created in the distal stump of the axon. Meanwhile, HDACs and HATs are exported out of the nucleus.

3. Over a longer period of time, the remaining microtubule array becomes hyper acetylated along with an accumulation of TATs. Hyper acetylated microtubules are also prone to severing by katanin, which can disrupt axonal transport and axonal growth.

4. To augment recovery, one can overexpress HDACs in the axon or overexpress the catalytically inactive α TAT1. This would allow microtubules to become less acetylated and also allow more +TIPs to bind. The result would be re-polymerization of microtubules, potentially aiding axonal regeneration.

HDACs: Histone deacetylases; HAT: histone acetyl transferase; +TIPs: microtubule plus-end tracking proteins; MAPs: microtubule associated proteins.

of neurotrophins, clathrin-mediated receptor endocytosis and cell division control. More labile pools of microtubules are shorter in length and are less modified, often associated with tyrosination, whereas more stable microtubule pools are longer, more modified and associated with acetylation. Microtubules are highly dynamic in nature, undergoing cycles of polymerization and catastrophe at the “plus ends” and are coordinated by microtubule associated proteins, such as end binding proteins (+TIPs) (Figure 2). Since developing neurons with growing axons have a larger pool of labile microtubules, especially towards the growth cones, it stands to reason that for axonal regeneration to occur, microtubules should also be dynamic and less modified and less acetylated.

Although HATs can also be exported from the nucleus into the cytoplasm, it remains a matter of controversy whether HATs have a function in regulating axonal growth. One recently discovered acetyl transferase, known as α -tubulin acetyl transferase 1 (α TAT1), or MEC-17, specifically binds to and acetylates tubulin. Mice with targeted deletion of α TAT1 show decreased microtubule acetylation across all tissues and reduced sperm motility but no other cellular changes are known to be affected. One reason for the lack of changes observed after disruption of α TAT1 may be that microtubule acetylation itself does not cause microtubule stability but is rather a marker of long-lived microtubules (Song and Brady, 2014). Over-expression of α TAT1 in cultured mouse DRG neurons and NIH3T3 cells increases microtubule acetylation (Kalebic et al., 2013). However, overexpression of the catalytically inactivated α TAT1, which does not affect its binding affinity to tubulin, results in not only less acetylated tubulin but also a more labile pool of microtubules with less resistance to nocodazole-induced depolymerization (Kalebic et al., 2013). This could suggest a role for catalytically inactive α TAT1 in promoting microtubule dynamics and a role, which may be useful, in the promotion of axonal growth.

Microtubule de-acetylation is regulated predominantly by HDAC6 but also to some extent HDAC5 and the sirtuins. Knockdown of HDAC6 and SIRT2 increases the pool of hyperacetylated microtubules while overexpression of HDAC6 has the opposite effect. Depletion of HDAC6 does not appear to affect general cell organization or other functions. However acetylation does make microtubules more prone to severing by Katanin, a microtubule cleavage protein which becomes ineffective when microtubule polymers are bound by tau (Sudo and Baas, 2010).

Targeting HDACs and TATs in the axon for regeneration:

For a long time experiments that focused on promoting axonal regeneration by enhancing regenerative gene expression changes in CNS axons have failed to show significant results. Many recent studies have employed tools to manipulate epigenetic changes in the nucleus, such as increasing histone acetylation, in an attempt to stimulate regeneration. However, posttranslational changes also occur in the axonal cytoplasm and changes that are possible in the cytoskeleton have often been overlooked. We propose that increasing microtubule dynamics will enhance the growth potential of damaged axons, particularly in adult neurons after spinal cord injury.

Our model for promoting axonal growth is to produce a more labile pool of microtubules in damaged axons. One way this might be done is by over-expressing axonal HDACs and catalytically inactive α TAT1 (Figure 2). By reducing tubulin acetylation with HDACs, microtubules will be less susceptible to severing enzymes leaving longer microtubule polymers to hold a rigid structure. By increasing the content of catalytically inactive α TAT1, microtubules will be more dynamic and enter into growth phases more frequently. Future studies will need to identify which specific HDACs or other TATs are involved in axonal growth and whether they are functioning in the axonal cytoplasm in vivo to enhance neuronal recovery after injury.

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