Neuron
Previews

Inside Out: Core Network of Transcription Factors Drives Axon Regeneration

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http://dx.doi.org/10.1016/j.neuron.2016.02.022

In this issue, Chandran et al. (2016) pursue a multi-level bioinformatics approach combined with wet bench validation to identify gene networks associated with the regenerative state of injured adult sensory neurons. A small molecular compound, ambroxol, mimics aspects of the identified gene expression patterns and promotes axon regeneration in the injured adult mouse CNS, demonstrating feasibility of in silico-based methods to identify compounds that promote neuronal growth following CNS injury.

In higher vertebrates, including humans, the regenerative capacity of neurons in the injured adult central nervous system (CNS) is extremely limited. Accounts of spinal cord injury (SCI) and its treatment attempts date back to ancient times. The Greek physician Hippocrates of Kos (~460–377 B.C.), considered the father of medicine and orthopedics, quite accurately noted: "There are no treatment options for spinal cord injury that resulted in paralysis, and unfortunately, those patients suffering from such injuries were destined to die." While post-injury survival and surgical options for SCI patients have dramatically improved in recent years, moderate to severe CNS injury remains a serious medical challenge, with limited treatment options and a poor prognosis for complete recovery.

In the spinal cord, traumatic injury of neural tissue typically results in an interruption of vital ascending and descending fiber tracts, causing a range of functional deficits. The long-term goal of SCI research is to develop strategies to ameliorate these deficits and improve, or fully restore, function. One key step toward accomplishing this ambitious goal is to re-establish neuronal innervation interrupted by SCI. Severed CNS axons typically show a modest and transient injury response that does not result in long-distance axonal regeneration. This stands in marked contrast to peripheral nervous system (PNS) injury, where sensory and motor axons can and often do regenerate over long distances, supporting substantial anatomical regeneration and functional recovery (Abe and Cavalli, 2008). This dichotomy between PNS and CNS regeneration is, at least in part, the result of the growth-inhibitory nature of injured CNS tissue, first demonstrated by an elegant series of nerve transplantation experiments (Aguayo et al., 1978). Subsequent studies revealed that CNS myelin formed by mature oligodendrocytes contains many factors that potently inhibit growth and sprouting of severed axons (Winzeler et al., 2011; Fawcett et al., 2012; Schwab and Strittmatter, 2014). To make matters worse, CNS axons are faced with additional obstacles; within days following injury, a glial scar composed of reactive astrocytes, microglia, and meningeal fibroblasts that migrate into the lesion site forms around the injury. Not only does scar tissue form a physical barrier to axonal regeneration, scar-associated molecules also function as chemical inhibitors that block axonal growth (Bradbury et al., 2002; Busch and Silver, 2007).

Many of these growth-inhibitory molecules are either missing or greatly reduced in the injured PNS, providing clues for why axon regeneration in the CNS is more limited than in the PNS. While environmental (or extrinsic) constraints limit axonal growth, adult neurons, even when presented with a growth-permissive substrate, show greatly reduced axonal growth compared to their developing counterparts (Goldberg et al., 2002). In other words, as neurons mature, cell-intrinsic programs that drive rapid axon extension during development go dormant and are incompletely activated following injury. Recent efforts to activate neuron-intrinsic growth programs have met with success and achieved impressive axonal regeneration in the injured optic nerve (Park et al., 2008; Moore et al., 2009; Benowitz et al., 2015) and spinal cord (Liu et al., 2010). Activation of the mTOR pathway combined with elevated Jak/STAT signaling leads to more impressive axonal regeneration than either treatment alone (Sun et al., 2011; Benowitz et al., 2015). This suggests that multiple parallel pathways must be activated to achieve robust regeneration. The underlying gene networks that enable injured CNS neurons to extend long axons, however, remain poorly understood. In this issue of *Neuron*, Chandran and colleagues (2016) use an in silico approach combined with wet bench validation to discover transcriptional networks and their driver or "hub" genes associated with axon outgrowth in the PNS that are not recapitulated in the CNS. They go on to show that pharmacological activation of core elements of this transcriptional network is sufficient to elicit axonal regeneration in the injured adult mouse optic nerve.

Sensory neurons in dorsal root ganglia (DRG) feature two long axons, one projecting peripherally and the other centrally to innervate the spinal cord or brainstem. Sensory signals originating from the lower limb travel through the sciatic nerve to the spinal cord, and they then extend into the medial part of the dorsal column where they travel rostrally to the gracile nucleus in the medulla oblongata. Severed DRG

axons in the dorsal column do not regenerate spontaneously. However, a conditioning injury (CI) to the peripheral branch of DRG neurons, such as sciatic nerve crush injury, prior to dorsal column injury greatly enhances the regenerative capacity of the central branch (Richardson and Issa, 1984). This seminal observation has been exploited by many investigators to uncover molecules, signaling pathways, and transcription factors that are regulated by CI, commonly referred to as regeneration-associated genes (RAGs) (Qiu et al., 2002; Omura et al., 2015; Kwon et al., 2015; Niemi et al., 2016). Overexpression of a single RAG alone is not sufficient to elicit significant neuronal growth, suggesting that combined activation of multiple RAGs may be needed for robust regeneration to occur.

Over the past 10+ years, a large number of studies have examined how CI regulates gene expression in whole DRGs, including longitudinal studies examining transcriptional changes at different post-CI time points. Taking advantage of these existing datasets, gathered from a total of 382 microarrays, Chandran et al. (2016) performed weighted gene co-expression network analysis and identified gene networks and key signaling pathways regulated by CI. Combined with consensus network analysis, Chandran et al. (2016) identified hub genes and 14 co-expression modules preserved across independent nerve injury datasets, representing pathways associated with nerve regeneration. Focusing on significant module trait relationships, five regeneration-associated gene modules were identified, two modules with genes that are upregulated by CI and three with genes that are downregulated. Regulation of all five modules was found to be conserved in an independent dataset of peripheral nerve injury, providing confidence in this initial observation (Costigan et al., 2002).

Perhaps the most difficult issue posed by this kind of database mining is prioritizing which of the many genes or gene networks associated with regeneration (or any biological process under investigation) should be selected for further experimentation. Faced with this challenge, Chandran et al. (2016) decided to examine each module for the presence of genes associated with axon regeneration, using PubMed as a reference followed by a Gene Ontology enrichment analysis to further annotate module function. As a step toward validation, the top 50 hub genes that represent the most central genes in all five regeneration modules identified above were compared to microarray datasets generated from injured CNS neurons lesioned by cervical SCI. Network relationships for two of the core modules were not preserved in CNS datasets, indicating that core PNS injury-related co-expression networks identified in DRG neurons are not preserved in injured neurons that fail to regenerate. To experimentally validate network-based predications about gene products associated with neuronal regeneration, 16 previously unidentified candidate RAGs were picked and assayed in primary neurons. In a first set of experiments, candidate RAGs were overexpressed in adult DRG neurons, and neurite outgrowth was quantified. Strikingly, 10 of the 16 RAGs tested showed a significant increase in neurite length and/or number. The top four genes (*Fxyd5*, *Gfpt1*, *Smagp*, and *Tacstd2*) were selected for shRNA-based loss-of-function studies in dissociated DRG neurons primed for enhanced neurite outgrowth by re-plating. For all genes examined, neurite outgrowth was significantly reduced compared to control shRNA-transduced DRG neurons, indicating their functional contribution to CI elicited growth effects. Therefore, Chandran et al. (2016) have identified several novel RAGs in DRG neurons that will need to be examined further, including functional regeneration studies in injured PNS and CNS neurons in vivo.

Once regeneration gene modules are identified, a central question is understanding how they are regulated, since this could provide insight into how they might be globally controlled in the service of promoting regeneration. To this end, the authors chose to focus on the identification of transcription factors (TFs) that regulate co-expression of gene networks associated with axon regeneration. This was approached by scanning canonical promoter sequences in each RAG co-expression module for TF binding site enrichment. The scan identified a total of 62 significantly enriched TFs predicted to bind to promoters of regeneration genes examined, 39 of which had previously been confirmed by chromatin immunoprecipitation experiments. Strikingly, out of the five regeneration-associated modules originally identified, the two upregulated modules showed enrichment for TFs previously associated with axonal growth and neuronal injury response, including JUN, FOS, ATF3, EGR1, KLF4, STATs, SMAD, SP1, and SP2. While this finding provides confidence in this approach, it does not reveal insights into potential signaling pathways that may be controlled by the identified gene networks.

To address this question, Chandran et al. (2016) used an in silico method to determine the protein-protein interaction (PPI) network in all five regeneration-associated modules. A relatively small network of interactions, consisting of 280 nodes and 496 edges, was identified. Interestingly, an enrichment in signaling pathways already implicated in neuronal regeneration was noted, including neurotrophin, MAP-kinase, TGFβ, chemokine, and Jak-STAT signaling pathways (Abe and Cavalli, 2008). Thus, PPI not only provides independent validation of the relationships inferred by RNA co-expression, but it also reveals specific signaling pathways that may be targeted for therapeutic intervention following nervous system injury.

Interestingly, there was a remarkable correspondence between TF binding site enrichment analysis in the RNA transcripts in co-expression modules and the hub genes identified in the PPI network. Moreover, many genes belonging to the enriched signaling pathways were also enriched for transcription factor binding sites of TFs in the PPI network. Collectively, these findings suggest that coordinate regulation of several regeneration-associated pathways may be necessary to achieve substantial neuronal growth and regeneration following injury. This also indicates that the identified TFs connect regeneration pathways, and their coordinate regulation is necessary for regeneration. This prediction was confirmed by bioinformatics removal of these TFs, resulting in disconnection of the network, leaving the distinct pathways unlinked. Importantly, the coordinate upregulation of these TFs is observed only in injured PNS neurons but not in injured CNS neurons, suggesting that differences in intrinsic gene expression patterns underlie the very different regenerative