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CNS repair and axon regeneration: Using genetic variation to determine mechanisms

Andrea Tedeschi[1,*], **Takao Omura**[2,*], and **Michael Costigan**[3]

[1]German Center for Neurodegenerative Diseases (DZNE), 53175 Bonn, Germany

[2]Department of Orthopedic Surgery, Hamamatsu University School of Medicine, Hamamatsu, Japan

[3]FM Kirby Neurobiology Center, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA

Abstract

The importance of genetic diversity in biological investigation has been recognized since the pioneering studies of Gregor Johann Mendel and Charles Darwin. Research in this area has been greatly informed recently by the publication of genomes from multiple species. Genes regulate and create every part and process in a living organism, react with the environment to create each living form and morph and mutate to determine the history and future of each species. The regenerative capacity of neurons differs profoundly between animal lineages and within the mammalian central and peripheral nervous systems. Here, we discuss research that suggests that genetic background contributes to the ability of injured axons to regenerate in the mammalian central nervous system (CNS), by controlling the regulation of specific signaling cascades. We detail the methods used to identify these pathways, which include among others Activin signaling and other TGF-β superfamily members. We discuss the potential of altering these pathways in patients with CNS damage and outline strategies to promote regeneration and repair by combinatorial manipulation of neuron-intrinsic and extrinsic determinants.

Keywords

genetic background; congenics; collaborative cross; CAST/Ei; CNS regeneration; Activin/TGF-β signaling; neuron-intrinsic and extrinsic factors

Correspondence should be addressed to: Andrea Tedeschi (andrea.tedeschi@dzne.de); Takao Omura (t.omura@hama-med.ac.jp); Michael Costigan (michael.costigan@childrens.harvard.edu).

*These authors have contributed equally.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Introduction

Since the experiments and theories of Moravian monk Gregor Johann Mendel (Mendel, 1866) and English naturalist and geologist Charles Darwin (Darwin, 1859) in the mid-nineteenth century, scientists have studied the role of genetic variation in the morphological and functional differences between species and different lineages within taxa. With the development of DNA sequencing technology, scientists have begun to understand the contribution of genetic variation associated with complex traits and diseases. As sequencing technology has improved, the entire genomes of many complex organisms, including thousands of human individuals and multiple inbred mouse strains, have been fully sequenced and curated (Lander et al., 2001; Waterston et al., 2002). This remarkable milestone, one which has heralded a new genomic era (Guttmacher and Collins, 2003), allows direct comparison of genomic information across species that are separated by many millions of years of evolution or between sub-species separated by hundreds of thousands of years. In nature, differences in the genetic background can range from single nucleotide variants to large regions of the genome, deleted, duplicated or transposed. Variability in the genetic background ultimately leads to changes in body shape, behavior, disease resistance and many other complex traits including the ability to regenerate body parts such as the adult nervous system.

Over the past century, regeneration of the nervous system has attracted a longstanding interest. Many experiments have demonstrated that nervous system regeneration occurs spontaneously in a number of animal lineages including invertebrates, fish, amphibians and reptiles (Tanaka and Ferretti, 2009). In addition, injuries at perinatal age or within the mammalian peripheral nervous system (PNS) are generally followed by a certain degree of functional regeneration (Huebner and Strittmatter, 2009; Tanaka and Ferretti, 2009). In contrast, the adult mammalian CNS is regeneration-incapable. Such a regenerative failure, combined with the very limited sprouting ability of injured adult CNS neurons, contributes to poor functional recovery after a variety of trauma, including stroke and traumatic brain and spinal cord injury (SCI) (Case and Tessier-Lavigne, 2005). At later stages of CNS development, axon growth ability in mammals is suppressed to allow synapse formation and synaptic maintenance, both of which are essential for neuronal circuit formation and function.

Mammalian CNS neurons have likely lost their regenerative capacity in order to strengthen synaptic structure, to protect our thought processes and therefore character. As mammals became more complex, retaining intelligence likely became more important than the potential of regeneration following serious CNS injury. In order to promote nerve regeneration in patients, however, we now need to understand the molecular signaling cascades that enable successful regeneration in the PNS as well as in the CNS structures of lower organisms. Once these pathways are known it is hoped that we can focally apply them to promote regeneration in injured nerves and aid functional outcome by recreating the original synaptic connections, or at least a good proxy, to aid patients' lives following neuronal damage.

Mechanistically, both the non-permissive environment and reduced intrinsic growth capacity account for diminished axon regrowth and lack of functional recovery in the adult mammalian CNS (Blesch and Tuszynski, 2009; Fawcett et al., 2012; Liu et al., 2011; Silver and Miller, 2004; Silver et al., 2015). Myelin-derived inhibitors (e.g. Nogo, Myelin associated glycoprotein, and Oligodendrocyte-myelin glycoprotein), Chondroitin sulfate proteoglycans (CSPGs) and a dense fibrotic scar represent a barrier to axon growth in the adult CNS (Bradbury et al., 2002; Schwab and Strittmatter, 2014; Silver and Miller, 2004). Thus far, several strategies have been developed to effectively overcome extrinsic impediments by releasing intrinsic brakes to axon re-growth (Bei et al., 2016; Belin et al., 2015; Blackmore et al., 2012; de Lima et al., 2012; Moore et al., 2009; Neumann and Woolf, 1999). Such evidence supports the idea that the environment that adult mammalian CNS neurons reside can be manipulated to gain regenerative ability.

Axon growth inhibitors, as well as intrinsic neural growth pathways have therefore been identified, however the contribution of the genetic architecture in enabling central axonal regrowth is less well understood. As functional regeneration can occur in the CNS structures of multiple lower organisms and in the mammalian PNS, data suggest that it is genetic mechanisms, which can be unlocked, that determine this ability. Interestingly in mice, inter-strain variation in CNS axonal regeneration exists and in this regard, the expression of a few core genes strongly correlate with the ability to regenerate severed axons after CNS injury (Dimou et al., 2006; Ma et al., 2004; Omura et al., 2015). Here, we discuss evidence originating from multiple experimental mouse strains and models, each of which points to considerable genetic differences in neuroprotection, structural plasticity and axon regeneration after injury and how we can use these clues to develop regenerative strategies in patients.

Mouse models for studying axon regeneration

Among the different mammalian models used to study the genetic contribution to complex traits, mice have by far been the most applied model system (Rossant and McKerlie, 2001). The mouse and human genomes contain very similar numbers of protein coding genes, around 20,000 in both, and despite being separated in evolution by 90 million years around 15,000 of these are 1:1 orthologues with an average of 88% amino acid sequence homology between them, making mice a strong genetic model of humans (Church et al., 2009). The fact that mice are easy to reproduce, maintain and genetically manipulate adds to their predominance as the mammalian model of choice in genotype-phenotype association studies.

Over the past few decades, the use of mouse models has served as a powerful tool for studying axon regeneration after CNS injury. Classical inbred mouse strains are derived from four wild-derived subspecies comprising *Mus musculus castaneus, Mus musculus musculus, Mus musculus domesticus* and the hybrid *Mus musculus molossinus* (Japanese). The subspecies *Mus musculus musculus* (Near Eastern/Central Asian), *Mus musculus castaneus* (South East Asian) and *Mus musculus domesticus* (European) diverged from a common ancestor about one million years ago. In the 1700s, mouse fanciers in Japan and China inter-bred mainly the Asian *Mus musculus musculus* subspecies and domesticated

many varieties as pets, which were later exported to Victorian England and mixed with local *Mus musculus domesticus* derived varieties. In the early twentieth century, Abbie Lathrop from Granby, Massachusetts in the United States began mating programs using a limited number of founding 'fancy' varieties from England (Wade and Daly, 2005). This collection was inherited by Harvard University and subsequently by Jackson Laboratories giving rise to many of the modern laboratory strains. Consequently these classic inbred lines are a mix of *Mus musculus musculus* and *Mus musculus domesticus* and their genetic polymorphisms are rather constrained as they are derived from a limited pool of ancestors. Wild-derived inbred strains, such as CAST/Ei, have been domesticated relatively recently and bred homozygous by brother x sister mating's across tens of generations, this process results in newer fixed inbred strains with more distant genomic architecture's relative to classic lab mice (Figure 1). In setting up a phenotype-genotype screen it is clearly an advantage to include some wild-derived lines, however, even classic recombinant inbred lab lines alone have become a useful tool for system genetics (Wade and Daly, 2005).

Recently, a large collection of multi-parental recombinant mouse lines has been introduced, derived from a genetically diverse set of founder strains and these congenic inbred progeny lines make up the Collaborative Cross (CC) (Figure 2). The eight founder strains of the CC include three wild-derived strains to maximize diversity (Bogue et al., 2015). As each CC strain contains a mix of up to eight parental genomes these new lines are genetically diverse, but fixed, making them ideal for genomic analysis of complex traits (Churchill et al., 2004). Given that dense genotyping and haplotype reconstructions of the CC inbred strains are available, these data when combined with the founder genome sequences, represent a comprehensive map of stable, reproducible genomes (Morgan and Welsh, 2015). There are currently about 150 extant CC strains. Each strain can provide genetically identical mice allowing a researcher to screen the cohort to determine a spectrum of phenotypes across the population. By comparing the differing phenotypes between CC strains with their respective genomic construction, it is possible to determine the loci responsible for that trait, and be able to continue mechanistic work on those strains that maximize or minimize the phenotype of interest.

Even though the CC models the complexity of the human genome better than any mammalian model system yet devised, these methods are still limited by our genetic and behavioral divergence from mice and many other issues such as the vagaries of inbreeding. Therefore other genetic screening methods especially including human participants, such as association genetics or whole genome sequencing of patients with rare genic disorders, are also crucial.

As transgenic technologies improve, genetically modified mice offering multiple ways of studying gene function and axon regeneration have become available including Cre/loxP gene editing methods (Kuhn et al., 1995) and *in vivo* imaging systems ((Feng et al., 2000; Kerschensteiner et al., 2005)). Within the excitement of these new genetic tools, it is important to consider which background each of these is bred onto, as this can markedly alter the observed phenotype.

In order to examine nervous tissue regeneration and assess functional recovery after CNS injury in mice, several lesion paradigms have been developed. The optic nerve injury model has served as a powerful screening tool for CNS regenerative pathways for more than two decades (Benowitz et al., 2015). Despite their peripheral location, retinal ganglion cells (RGCs) and their projections in the optic nerve are part of the CNS. RGCs can be manipulated by genetic, viral-based and pharmacological approaches (Park et al., 2008; Yungher et al., 2015). In addition, the development of functional neuroimaging and electrophysiology techniques combined with information of the visual topographic map allows functional assessments of regenerating axons (Bei et al., 2016; de Lima et al., 2012; Li et al., 2015a). Mouse SCI ranges from contusion to compression to transection models, all of which aim to reproduce neurobehavioral and neuropathological features of human SCI (Liu et al., 2010; Lu et al., 2012; Neumann and Woolf, 1999; Blackmore et al., 2012; Fink et al., 2015; Wang et al., 2015)

Following CNS injury, the scarring and wound-healing process develops in a comparable manner in mice and other mammalian models, suggesting translation of promising findings. Indeed many important discoveries made in mouse models of CNS injury now await translation to large animals and non-human primates prior to embarking on clinical trials in humans (Kwon et al., 2015). Preferably promising mouse or other model animal leads would have convergent human genetic evidence backing them prior to early translational work in primates (Izpisua Belmonte et al., 2015).

The role of the genetic background on CNS repair and regeneration

Genetic determinants influencing the response to optic nerve injury

The failure of axonal regeneration within the adult mammalian CNS is partly due to the high propensity of the neurons to undergo apoptosis following injury. RGCs represent one such population of CNS neurons and optic nerve injury can cause considerable RGCs death in adult rodents (Berkelaar et al., 1994; Selles-Navarro et al., 2001). In adult mammals, loss of RGCs can vary from 50% to more than 90% (Berkelaar et al., 1994). Both the type and injury location play a crucial role in shaping the cell body response. (Berkelaar et al., 1994; Villegas-Perez et al., 1993).

Over the last decade, a number of studies have determined, at least in part, the contribution of genetic factors controlling intrinsic cell death programs in RGCs. Screening 15 inbred mouse strains has shown different susceptibilities to optic nerve crush injury between the lines; RGCs survival is greatest in DBA/2 mice and least in BALB/cBy (Li et al., 2007). Notably, the pattern of inheritance exhibited after crossing these two strains suggests that the cell death resistant phenotype can be inherited in a Mendelian fashion (Li et al., 2007). Genome wide mapping of the F2 generation has identified a dominant trait locus in a 25 centimorgan (cM) interval on chromosome 5 that associates with the resistance phenotype (Dietz et al., 2008). A total of 578 genes are associated with this genomic region and further analysis has identified 7 genes including Protocadherin 7, toll-like receptor 1 and 6 as potential regulators of the RGC cell death program after injury (Dietz et al., 2008).

Following optic nerve crush injury, RGCs of C57BL/6 mice are more susceptible to cell death than those of DBA/2. Transcriptome profiling in these two strains has identified 1580 differentially expressed genes following injury. Among these differences, expression changes in the Crystallin gene family likely represent part of the molecular signature modulating susceptibility of RGCs to cell death (Templeton et al., 2009). Detailed analysis of the regulatory regions of the Crystallin gene network has identified overrepresented transcription factor binding sites for members of the V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog (MAF) family. Indeed, chromatin immunoprecipitation confirms that Maf-A binds regulatory regions in several genes of the Crystallin family (Templeton et al., 2013). Interestingly, MAF mediated signaling has been identified as one of the principal transcriptional hubs 24 hours after ischemia-reperfusion injury in the retina (Andreeva et al., 2015). Given that several transcription modulators exhibit time- and context-dependent variability, defining the complete list of MAF activated and repressed target genes after optic nerve injury may be necessary to fully understand this regulatory network and how member proteins aid or inhibit neuronal survival. Defining and then overcoming injury induced neuronal death in the CNS is clearly an important hurdle to overcome before functional regeneration can take place, and natural differences between strains may well be key to this process.

Genetic determinants influencing the response to stroke

Cell death is not the only primary consequence of CNS injury. After stroke, brain architectural changes occur over the course of days and months (Carmichael, 2015). Time-dependent reorganization of neuronal circuits can promote functional recovery, but may also lead to uncontrolled neuron firing and detrimental alteration of brain function. Thus far, several studies have highlighted the presence of genetic determinants influencing neuronal vulnerability and the repair program after stroke. Studies showing that C57BL/6 developed greater brain damage after transient ischemia than SV/129 mice established that structural differences in vasculature in C57BL/6 mice prevents collateral blood flow following occlusion of the carotid and basilar arteries and this represents a risk factor in this strain (Fujii et al., 1997; Wellons et al., 2000). Again malformed vasculature makes BALB/c mice more sensitive to focal ischemia in a screen of BDF, CFW, and BALB/c lines (Barone et al., 1993). Further evidence for a genetic component for stroke-induced brain damage has been documented with BALB/c mice developing a larger injury site relative to C57BL/6 and 129X1/Sv (Majid et al., 2000). Congenic mapping combined with phenotypic analysis of a region of the mouse genome attributed to variation in vasculature architecture has found a 737 kilobase (kb) locus on chromosome 7 containing 28 genes (Sealock et al., 2014). Among others, the membrane trafficking Ran-binding protein 2 (*Rabep2*) has been identified as a candidate gene controlling collateral blood vessel growth. In addition to alterations in the native vasculature, other factors contribute to brain damage after stroke. A genome-wide association study performed in 33 inbred mouse strains has identified a 500 kb region of the genome associated with the size of brain damage (Du et al., 2015). Of 38 genes expressed within this region, angiopoietin-1 and ZBTB7C genes were linked by further analysis to the response to damage in the CNS (Du et al., 2015).

Blood vessels and axons share molecular mechanisms of guidance, growth and terminal arborization (Weinstein, 2005) (Carmeliet and Tessier-Lavigne, 2005), all of which are necessary steps to improve functional outcome after stroke. Indeed recent evidence has demonstrated the intimate connection between immune cells, newly formed blood vessels and axonal regrowth in the periphery (Parrinello et al., 2010) (Cattin et al., 2015). By using an inbred mouse neuronal phenotypic screen, we have recently found that axonal sprouting ability after stroke is controlled by genetic components (Omura et al., 2015). Quantitative mapping of cortical connections shows little degree of axonal sprouting in C57BL/6 at one month after stroke. In CAST/Ei mice, however, axons grow several millimeters promoting reorganization of motor, pre-motor and somatosensory areas. It is not known yet whether these newly formed connections are functional. Whether increased axonal sprouting in CAST/Ei mice parallel changes in neurogenesis, vascular reorganization, gliosis and microglial proliferation is not clear and deserves further investigation.

Genetic determinants influencing axon regeneration capacity

Since the ability of axons to regenerate is highly variable between different species or rodent lineages, genotype-regeneration phenotype association studies have recently attracted much interest (Bely and Nyberg, 2010; Tanaka and Ferretti, 2009). Differences in the regulation of gene function represents a major driving force in evolution, indeed in most cases the structure of ancestral proteins and therefore gene coding sequences do not change across taxa, instead it is the regulation of these proteins as well as divergence through duplication that is the engine of evolution. Protein function can be altered in many ways and altering the rates of mRNA transcription represents a primary control point. Consistent with this, recent evidence suggests that transposable elements common within our genomes represent an attractive route by which complex gene networks can be divergently modulated through evolution (Chuong et al., 2016). Here, cis-regulatory regions (transcription factor binding sites) move throughout the genome by hitching a ride on these endogenous retroviral mobile elements. Furthermore, these sequences can copy-paste one and other easily allowing for concerted development gene regulated gene networks such as the interferon-γ pro-inflammatory response of the innate immune system (Lynch, 2016). Thus, it is likely that specific gene expression patterns will control marked differences in regeneration ability across different species and mouse genetic backgrounds.

Nogo-A knockout (KO) mice on a 129X1Sv mixed background display two to four times more corticospinal tract regeneration than C57BL/6 Nogo-A KO mice following SCI (Dimou et al., 2006). Microarray analysis shows a number of genes differentially regulated in these strains (Dimou et al., 2006). Interestingly, many of these genes control neurite growth, synapse formation and inflammation/immune response. Neuronal injury is often accompanied by an inflammatory response with either positive or negative effects, promoting or impeding recovery respectively (Kroner et al., 2014; Popovich and Longbrake, 2008). Due to diminished negative effects in the chronic inflammatory response, 129X1Sv mice show enhanced axonal growth after spinal cord contusion when compared with C57BL/6 (Ma et al., 2004). Strikingly, a decrease in macrophage density is accompanied by an increase in astrocytic processes as well as peripheral and central axonal projections within the lesion site of 129X1Sv mice (Ma et al., 2004). As a result of CNS injury,

astrocytic response can range from reversible alterations in cellular morphology to long lasting changes in tissue anatomy such as scar formation (Anderson et al., 2014). The presence of glial bridges at the injury epicenter has been documented in several studies where successful CNS regeneration has been reported (Goldshmit et al., 2012; Lee et al., 2010; Liu et al., 2010). Although immature astrocytes are permissive for axon growth (Filous et al., 2010; Smith et al., 1990), lineage analysis has demonstrated that growth-permissive astrocyte bridges likely do not derive from ependymal stem cells within the spinal cord (Zukor et al., 2013).

Following SCI, tissue damage is cleared by microglia and macrophages and often, the glial scar surrounds a fluid-filled cavity that represents an additional barrier to axon regeneration (Basso et al., 1996; Beattie et al., 1997). Alternatively, macrophage-filled cysts persist when fluid-filled cavities are absent. By performing a mid-thoracic spinal contusion injury in four strains of mice including C57BL/6, C57BL/10, BALB/c and B10.PL, Kigerl *et al* show that intraparenchymal inflammation is greatest in C57BL/6 and least in BALB/c mice. Interestingly, mouse strains with exacerbated intra-spinal inflammation also develop intense fibrosis, further supporting the conclusion that neuroinflammation after SCI has a genetic component (Kigerl et al., 2006).

Following moderate SCI, locomotor analysis shows higher recovery in C57BL/10, B10.PL and C57BL/6x129S6 F1 than the BALB/c and C57BL/6 strains (Basso et al., 2006). After spinal contusion injury, MRL/Mp mice develop a more permissive lesion environment compared to C57BL/6. A reduction in both glial activation and recruitment of inflammatory cells promotes extensive axonal growth within the site of injury in MRL/Mp mice (Kostyk et al., 2008). Ultrastructural analysis highlights the presence of disorganized and loosely arranged cellular substrates at the site of injury in these mice. Similarly, another study has shown that astrocytic response and cavity formation are also reduced in MRL/Mp mice following dorsal hemisection injury (Thuret et al., 2012). Gene expression profiling reveals intrinsic differences in the injured MRL/Mp spinal cord when compared to C57BL/6. Expression of a number of genes including the interferon activated gene 202B and allograft inflammatory factor 1 gene is significantly increased in MRL/Mp mice (Thuret et al., 2012), potentially providing a cellular mechanism for the reduced CNS scarring present in this line.

Together, the observations above highlight the importance of the genetic background, and suggest the significance of strain-specific genetic factors controlling CNS repair and axonal regeneration among various recombinant inbred mouse strains. Screening for differences in axon growth capacity among these lines may facilitate the discovery of previously unknown regenerative pathways, accelerating progress towards potential therapies for CNS diseases.

Using pre-conditioned adult DRG sensory neurons of nine genetically diverse inbred mouse strains consisting of A/J, C3H/He, C57BL/6, DBA/2, 129S1/SvIm, NOD/Lt, NZO/HlLt, CAST/Ei, and WSB/Ei (Figure 1), which include the founder lines of the CC (Figure 2), we have recently demonstrated that differences in axon growth and regeneration capacity among these strains correlate with profound changes in gene expression (Omura et al., 2015). Indeed, when the top 20 differentially-regulated genes following pre-conditioning are compared among the different strains, only 10 genes are found in common between them;

these common genes and their functional partners, however, represent the core-conditioning response (Chandran et al., 2016) (Figure 3). Injury conditioning is where pre-injured DRG neurons are able to regenerate much quicker and stronger than their non-injured counterparts (Richardson and Issa, 1984); (Smith and Skene, 1997). This injury-induced program is present in most rodent strains so far examined (Ylera et al., 2009); (Omura et al., 2015). In rats, pre-conditioning injury allows the central axons of the pre-injured ascending sensory neurons to grow into and beyond the lesion following dorsal column SCI (Mills et al., 2007; Neumann and Woolf, 1999). In C57BL/6 mice this also occurs to some extent (Cafferty et al., 2004). In search of pathways that enable this preconditioning response, and therefore greater levels of CNS regeneration, we have identified the CAST/Ei mouse strain as capable of initiating an intrinsic growth program, which allows for high levels of axonal regrowth in a CNS environment both *in vitro* and *in vivo* (Omura et al., 2015). This regrowth is substantially superior to each of the other strains assayed including C57BL/6 – the most commonly used reference strain (Figure 2B). These data suggest that there is something specific to the CAST/Ei strain that allows long distance regeneration of injured neurons in the challenging CNS environment.

CAST/EiJ mice are extremely sensitive to infection with monkeypox virus (MPXV), and die when exposed to a 10,000-fold lower dose than BALB/c mice (Americo et al., 2010). Out of 38 mouse strains tested, CAST was by far the most sensitive to this virus (Americo et al., 2010) and further work has shown that C57BL/6 mice deficient in Stat1 signaling are also very sensitive to MPXV (Stabenow et al., 2010) suggesting lethality is associated with a deficient gamma interferon response in these mice. T-lymphocytes produce pro-inflammatory cytokines such as interferon-γ and participate in fibrosis and scarring (Wynn, 2004). Reactive T-lymphocytes exacerbate axonal injury and demyelination after SCI (Jones et al., 2004; Jones et al., 2002). Whether the scarring process after CNS injury is altered in CAST/Ei mice as a result of changes in interferon-γ response is not yet known.

In comparison with C57BL/6, conditioned CAST/Ei DRG neurons regenerate extensively with many axons regrowing long distances after SCI. Similarly, injury-primed RGCs robustly regenerate their axons after optic nerve crush injury (Omura et al., 2015). It is likely that many regeneration-associated mechanisms may be associated with novel regulatory elements in the genome of CAST/Ei mice relative to other inbred lab mice. As the founder strains CAST/Ei and most common laboratory mice diverged about a million years ago, CAST/Ei carry a high density of DNA polymorphisms distinct from C57BL/6 and other common lab mice (Figure 1). There are over 17.7 million single nucleotide polymorphisms, 2.6 million indels and 86,000 structural variants between seventeen mouse genomes, including four wild-type strains (CAST/Ei, WSB/Ei, PWK/Ph and SPRET/Ei) and most common lab mice (including A/J, C3H, C57BL/6, DBA, 129 and BALB/c) (Keane et al., 2011). In search of genes and molecular pathways accounting for this enhanced axonal growth in CAST/Ei mice, genome-wide microarray and RNA-seq expression profiling of the naïve and preconditioned DRG neurons has identified Activin transcript *Inhba* as the most strongly correlating with the axonal ability to overcome an inhibitory growth environment (Figure 4).

Although Activin sponsored Smad2 signaling is one of the potential mechanisms for the large increase in the intrinsic growth capacity of injured CAST/Ei axons in the CNS, heritability studies suggest that there is more than one mechanism contributing to this phenotype (Omura et al., 2015). Dissection of the molecular and genetic components controlling axon growth and regeneration in CAST/Ei mice will be an important direction for future investigations and one way to achieve this is by analyzing the CNS growth potential of injured neurons across the CC (Figure 2C). The advantage of this method is that it does not assume a change must be present at the transcriptional level as in (Omura et al., 2015) but rather screens the genomic structure of each CC-line for their phenotypic differences. When a phenotype of interest is only present in one founder member of the CC, as here, it is not necessary to screen all CC sub-strains because we assume at least some CAST/Ei genome should be present in any fixed progeny lines that will possess neurons with increased regenerative capacity. In such a case it is possible to reduce the number of CC-strains screened for the phenotype of interest (Crisman *et al* 2016, in press).

The discovery that the Activin signaling cascade explains much of the difference in axon regeneration ability between CAST/Ei and common laboratory mice poses new intriguing questions. The reason why such a remarkable strain difference is present in mammalian CNS axon regeneration is not clear. In addition understanding how selection pressure promoted these differences will be an important point to consider for future investigations. One may speculate that these signaling cascades, which are used in embryonic neural growth and in simpler life forms can be repurposed to produce effective CNS regeneration later. In mammals, however, this is not the case because the signals to turn the Activin or TGF-related cascades back on are securely switched off. It is possible therefore that CAST/Ei mice lack these dampening signals rather than developing a new growth system. After all CAST/Ei neurons still need the preconditioning signal to grow well in the CNS, so some of the active block on unconstrained growth remains. In this case it is also tempting to speculate that there does not need to be a reason for this essentially random event, and it will remain uncommon, unless evolutionary selection leads to its amplification.

Activin signaling in neural development, function and regeneration

Activins are the members of the transforming growth factor β (TGF-β) superfamily of morphogens, which comprise at least 42 members in humans and includes inhibins (INHB), TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factor (GDF), myostatin, Müllerian-inhibiting substance and others (Oshimori and Fuchs, 2012) (Figure 5). Activin/Nodal and TGF-β pathways share the downstream Smad effectors (Figure 6). Activins are formed by homodimers or heterodimers of Inhibin subunits (βa, βb, βc, βe), which are also held together by a disulphide bond. They exert their biological effects by interacting with two types of transmembrane receptors (types I and II), which have intrinsic serine/threonine kinase activities in their cytoplasmic domains. Type II Activin receptor (ActRII/IIB) binding by Activin leads to the recruitment, phosphorylation and activation of various type I Activin receptors (Activin receptor-like kinases, or ALKs, including ALK1-7), in particular ALK4, which is also known as ActRIB (Tsuchida et al., 2004). Activin and TGF-β signaling pathways are specifically mediated through Smad2 and Smad3 (R-Smads), Smad4 (Co-Smad) and Smad7 (I-Smad) (Shi and Massague, 2003). Smad2 and

Smad3 form a complex (Smad2/3) in the cytoplasm, which interacts with Smad4 after phosphorylation. Once inside the nucleus, Smads2/3/4 occupy genome regulatory elements together with master transcription factors to mediate cell-type-specific responses (Mullen et al., 2011) (Figure 6).

Activin is expressed during development, cell proliferation, differentiation and regeneration (Wijayarathna and de Kretser, 2016). In embryonic stem cells, Activin is necessary and sufficient to maintain the pluripotent status of the post-implantation epiblast (Vallier et al., 2004). Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm (Smith et al., 2008). In zebrafish, Activin is required for regenerative growth of the fin (Jazwinska et al., 2007). In leopard gecko, which can regenerate a tail after predatory loss, a marked increase in phosphorylated Smad2 expression and strong Activin upregulation is observed during the early stages of blastema formation, indicating a crucial role of this pathway in this regenerative response (Gilbert et al., 2013). In *Xenopus*, Smad2 specific TGF-β signaling is required for tail regeneration (Ho and Whitman, 2008). Activin exerts a significant role during wound healing and is strongly expressed after skin injury (Hubner et al., 1996). Moreover, it is known to increase the number of mature mast cells in mouse skin *in vivo* (Antsiferova et al., 2013). Planaria, flatworms of the class Turbellaria, retain an amazing ability to regenerate their head or tails following amputation. A recent study suggests that such remarkable regeneration program is centered on a follistatin homolog gene (Smed-follistatin), which is expressed after injury. Smed-follistatin likely initiates tissue regeneration via regulating Activin signaling following injuries that cause tissue loss (Gavino et al., 2013).

In the CNS, Activin exerts its neuroprotective effect by regulating basic fibroblast growth factor (Tretter et al., 2000). In serum response factor and megakaryoblastic leukemia-dependent manner, Activin promotes dendritic complexity by increasing the number and length of dendrites (Ishikawa et al., 2010). In Drosophila, mutations in Baboon, a TGF-β/Activin type I receptor, and the downstream transcriptional effector dSmad2 block neuronal remodeling in the larval mushroom bodies (Zheng et al., 2003). Knocking out dActivin around the mid-third instar stage interferes with this process, further supporting a role of Activin signaling pathway in neuronal remodeling (Zheng et al., 2003).

Consistent with these results, Activin significantly increases axonal elongation and neurite initiation in C57BL/6 cultured DRG sensory neurons after conditioning. Blocking Activin with SB-431542, a potent Activin receptor antagonist significantly reduces both axon elongation and initiation. Intra-ocular addition of Activin in zymosan-primed RGCs results in a four-fold increase in the number of regenerating axons following optic nerve injury demonstrating an important role for Activin in CNS regeneration (Omura et al., 2015). Preconditioning of the neurons was required in these studies to initiate Activin expression and downstream signaling possibly suggesting an embryonic or developmental role for this pathway, which has become unmasked in injured CAST/Ei neurons (Figures 2 and 4). Further studies will be required to determine all of the necessary factors needed to specifically activate mammalian neurons into a pro-CNS growth state, but the fact that pre-injured CAST/Ei neurons can grow robustly suggests that axonal growth in the mammalian adult CNS is possible (Omura et al., 2015). Moreover, recapitulation of embryonic

development paradigms, through enhancement of Activin signaling, could augment CNS regeneration without manipulating pathways which can interfere with tumor development such as phosphatase and tensin homolog (PTEN) (Park et al., 2011). Our in vitro study shows that although significant axonal growth and initiation can be achieved by addition of Activin in C57 sensory neurons, maximal axonal growth values were about half of the conditioned CAST neurons. Interestingly, the comparison of our *in vivo* optic nerve injury study also show that the number of regenerating axons in C57 with the administration of Activin was approximately half of the CAST values. These results indicate that Activin is likely to be contributing to roughly 50% of the difference between C57 and CAST in CNS axonal growth. Together, these findings suggest that extrinsic and intrinsic pathways likely impinge on Activin signaling modules affecting cell growth and differentiation. Molecular dissection of the role of Activin in controlling both neuron-intrinsic and extrinsic determinants requires further investigation.

A role for other TGF-β superfamily members in axonal regeneration

Members of the TGF-β superfamily promote intrinsic growth of injured CNS neurons (Krieglstein et al., 2011) (Figure 5). TGF promotes neurite outgrowth of DRG explants in a three dimensional culture of astrocytes (Fok-Seang et al., 1998). In a RGC line, TGF-β1 supports cell survival and stimulates neurite outgrowth through p38 mitogen-activated protein kinase (MAPK) (Walshe et al., 2011).

BMP signaling is developmentally regulated and governs axonal growth in DRG neurons. Selective transduction of BMP4 in the DRG by intrathecal delivery of adeno-associated viral (AAV) particles induces pSmad1 accumulation in the nucleus and promotes regeneration of ascending dorsal column axons after experimental SCI in mice (Parikh et al., 2011). BMP is also required for peripheral nerve regeneration. In primary mouse DRG cultures deficient for the BMP co-receptor repulsive guidance molecule b (RGMb), the neurites are significantly less and shorter, but this defect can be compensated by addition of BMP-2. Noggin, an endogenous inhibitor for BMP, attenuates the preconditioning effect both *in vitro* and *in vivo* (Ma et al., 2011).

The TGF-β superfamily member growth and differentiation factor 10 (GDF10) is induced after stroke in peri-infarct neurons in mice, non-human primates and humans. In a mouse stroke model, GDF10 promotes axonal sprouting in peri-infarct cortex and enhanced functional recovery (Li et al., 2015b). Importantly, GDF10 increases axonal outgrowth in primary mouse cortical neurons *in vitro* through TGF-β receptor and Smad2/3 signaling (Li et al., 2015b). GDF10 knockdown negatively impacts on axonal sprouting and behavioral recovery after stroke (Li et al., 2015b), thus supporting a causal link between increased GDF10 expression and improved motor recovery after brain injury. Transcriptome profiling of isolated cortical neurons from the peri-infarct area reveals an intriguing molecular program orchestrated by GDF10 targeting specific canonical pathways including axonal guidance molecules, PTEN and PI3 kinase signaling (Li et al., 2015b). A comprehensive transcriptome analysis in different model systems may provide further insights into mechanisms that regulate axonal sprouting and recovery upon GDF10 administration.

GDF15, another member of the TGF-β superfamily, is ubiquitously expressed in the CNS and is prominently upregulated in cerebral cortical and ischemic lesion paradigms (Schindowski et al., 2011). In dopaminergic neurons, spinal cord neurons, brainstem neurons, sensory neurons and the cerebellar granule neurons, GDF15 acts as a potent pro-survival factor (Strelau et al., 2000); (Strelau et al., 2009; Subramaniam et al., 2003).

Promoting axonal regeneration and CNS repair by manipulation of neuron-intrinsic and extrinsic factors

Promoting axonal regeneration is crucial for functional recovery after CNS injury. To be functionally capable, regenerating axons not only have to travel long distances to reach denervated targets but they also have to form new synapses and remyelinate. Despite the progress that has been made in understanding the cellular and molecular mechanisms of axonal regeneration, long distance functional regeneration still represents a remarkable challenge. Will we be able to achieve long distance functional regeneration? Even two segments (c.a. 2 cm) of spinal recovery will be a tremendous achievement for SCI patients with cervical injury. Motor functions in patients with a C4/5 injury are limited to shoulder movements and elbow flexion, while C5/6 injured patients can extend their wrist and pronate their forearm. Patients with injury at the C6/7 level have their triceps function, wrist flexion and finger extension, enabling them to perform push-ups on their wheelchair. Thus, a few centimeters of functional regeneration can significantly improve the quality of life of SCI patients. Manipulation of neuron-intrinsic and extrinsic factors represents a promising way to achieve this arduous goal.

Cell intrinsic strategies

Thus far, one of the best known targets for promoting cell intrinsic growth is PTEN, a negative regulator of the mammalian target of rapamycin (mTOR) pathway. Deletion of *Pten* activates the PI3K/mTOR pathway, which controls cell growth and size by regulating cap-dependent protein translation initiation (Lee et al., 1999; Ma and Blenis, 2009). *Pten* deletion promotes robust axon regeneration after optic nerve injury (Park et al., 2008), SCI (Liu et al., 2010; Zukor et al., 2013) and peripheral nerve injury (Christie et al., 2010). Deletion of the cytokine signaling 3 suppressor (SOCS3) supports axon regeneration via a gp130-dependent pathway (Smith et al., 2009). Interestingly, co-deletion of *Pten* and *Socs3* acts synergistically enabling long distance RGC regeneration (Sun et al., 2011). Regenerating RGC axons are able to form new synapses with neurons in the suprachiasmatic nucleus after injury (Li et al., 2015a; Sun et al., 2011). When combined with administration of voltage-gated potassium channel blockers to restore axonal conduction (Sun et al., 2010), such a genetic-based approach recovers visual function in injured mice (Bei et al., 2016).

The vast majority of RGCs undergo cell death after optic nerve injury (Bahr, 2000). Using quantitative proteomics analysis of intact and injured RGCs, a recent study has identified c-*myc* as a novel target for neural repair strategies. C-*myc* functions as a key regulator of cell cycle progression, apoptosis and cellular transformation. Forced expression of c-*myc* in RGCs before or after optic nerve injury can double the survival rate and allows significant increase in the number and length of the regenerating axons. C-*myc* overexpression

synergizes with *Pten/Socs3* deletion, promoting additional regeneration than *Pten/Socs3* deletion alone (Belin et al., 2015). Doublecortin-like kinases (DCLKs) are also shown to promote survival and axonal elongation of RGCs via stabilization of microtubule structures and prevention of destabilization of the F-actin in the injured axon stumps (Nawabi et al., 2015). To a similar extent as after *Pten* deletion, more than 50% of the RGCs overexpressing *Dclk2* can survive after optic nerve injury. When combining *Dclk2* overexpression and *Pten* deletion, RGCs survival further improves (up to 70%) (Nawabi et al., 2015).

Guidance molecules steer axons by regulating cytoskeletal dynamics in the CNS and genetic manipulation of these molecules can lead to enhanced regeneration. Roundabout-Like protein 1 (Robo1) is expressed in the growth cones and known as a receptor for Slit homolog (Slit). Robo1 functions as an intrinsic brake for the thalamocortical axons and disruption of either *Robo1* or *Slit1* accelerates the progression of thalamocortical axons *in vivo* (Mire et al., 2012). Manipulation of signaling pathways such as RhoA-ROCK-LIMK downstream of various extracellular stimuli influences regeneration. Specific knockdown of *Rock2* and *Limk1* equally enhances neurite outgrowth of RGCs on inhibitory substrates and both induces substantial neuronal regeneration over distances of more than 5mm, 28 days after rat optic nerve crush (Koch et al., 2014). Krüppel-like factor (Klf) 4 and 7 are in the same family of zinc finger transcription factors but contribute to axonal regeneration in opposite ways. While overexpression of *Klf4* in RGCs decreases axonal growth (Moore et al., 2009), overexpression of *Klf7* promotes corticospinal axon sprouting and regeneration in the injured spinal cord (Blackmore et al., 2012).

Together, these studies suggest that targeting one single pathway may not be sufficient to effectively promote long distance axon regeneration and functional recovery after injury. Further testing of different combinatorial approaches will be an important direction for future investigations. A critical point to consider is that several of these genetic strategies target tumor suppressor genes. As deletion of these genes is associated with the development of cancer, manipulation of these genes may not be safe for immediate clinical interventions. Thus, finding safer alternatives will be desirable. Such less invasive strategies, that manipulate specific regeneration pathways, could include the TGF-β related embryonic growth cue signaling systems discussed above.

Cell extrinsic strategies

Although many axons may survive initial trauma or eventually regenerate following a successful intervention, the death of neurons and glia impacts negatively on recovery of function. Transplantation of genetically engineered cells derived from embryonic stem cells or fetal nervous tissues represents a promising strategy to create more favorable conditions for axon regeneration and functional connectivity after injury (Lu et al., 2014; Ruff et al., 2012).

Loss of oligodendrocytes negatively impacts the action potential conduction and interventions to promote re-myelination include transplantation of myelin-producing cells. Embryonic stem cells can be genetically engineered to produce functional oligodendrocytes capable of myelinating axons *in vivo* (Cummings et al., 2005; Keirstead et al., 2005; Liu et al., 2000). Cell replacement strategies continuously develop. Recent evidence shows that

human platelet-derived growth factor-responsive neural precursors differentiate into mature oligodendrocytes capable of re-myelinating axons in the injured rat spinal cord (Plemel et al., 2011).

After severe SCI, transplanted multipotent neural progenitor cells can differentiate into neurons and glia. Several thousands of graft-derived axons are seen growing into the host grey and white matter tissue, serving as a neuronal relay across a complete spinal cord lesion (Lu et al., 2012). Such robust axonal growth of grafted cells has never been achieved in the adult CNS. Part of this success can be attributed to novel methods to suspend and deliver cells to the site of injury using 3-dimensional scaffolds, highlighting the importance of tissue engineering in regenerative medicine (Madigan et al., 2009). Global gene expression profiling in these cells may identify candidate genes to influence growth responsiveness. By promoting cell survival and maintaining the growth and regeneration of axons, neurotrophic factors play an important role in combinatorial approaches for spinal cord repair (McCall et al., 2012).

After fetal neural stem cells transplantation, it is common to find ectopic colonies in different areas of the nervous system (Steward et al., 2014). The fact that cells in these ectopic colonies continue to proliferate 2 months after cell grafting with formation of large cellular masses, represents a risk factor for physiological complications. Finding the right balance between conditions that promote survival, proliferation and axon outgrowth while avoiding formation of ectopic colonies may be necessary before clinical trials occur.

After SCI, inhibitory proteoglycan expression associated with the perineuronal net increases in denervated target neurons, thus creating an active barrier for target re-innervation (Massey et al., 2008). Removal of these inhibitory molecules with Chondroitinase ABC has proven to promote functional recovery in a variety of experimental SCI models (Alilain et al., 2011; Bradbury et al., 2002; Garcia-Alias et al., 2009; Wang et al., 2011). These studies also highlight the importance of combining treatments with target-specific rehabilitation programs. Transplantation of Schwann cells modified to secrete Chondroitinase ABC and neurotrophin promotes axon regeneration, myelination and functional recovery after spinal cord contusion injury in rats (Kanno et al., 2014). Recent observations suggest that the beneficial effects of Chondroitinase ABC are not limited to enhanced plasticity but it also modulates the immune response by altering activated macrophage polarization with beneficial effects on tissue repair after CNS injury (David and Kroner, 2011; Didangelos et al., 2014; Kigerl et al., 2009).

Although fibrosis is an important step during wound healing, it significantly inhibits axon regeneration after CNS injury (Soderblom et al., 2013). Therefore, ideal combinatorial treatments for CNS repair should also reduce fibrotic scarring. Recent studies show that moderate microtubule stabilization reduces fibrotic scarring by suppressing polarization and migration of scar-forming fibroblasts (Hellal et al., 2011; Ruschel et al., 2015). Implementation of these strategies, either alone or in combination with other treatments like blockade of myelin inhibitors and aiding intrinsic neuronal grow, is well positioned to leverage significant improvement of neurological outcome in SCI patients.

Conclusion

Despite recent progress in identifying intrinsic regenerative pathways, understanding the molecular and genetic mechanisms of neuronal regrowth after CNS injury represents a continuous challenge. Axon regeneration and neural repair are complex, multi-step processes. While the site of injury undergoes time dependent reorganization, nerve damage triggers molecular signals that need to travel from the site of injury back to the neuronal cell body to activate cellular growth programs (Abe and Cavalli, 2008). Retrograde transport of injury-activated factors leads to profound changes in the nuclear architecture regulating gene expression thus controlling the intrinsic neuronal regenerative response (Ben-Yaakov et al., 2012; Cho et al., 2013; Costigan et al., 2002; Finelli et al., 2013; Puttagunta et al., 2014; Stam et al., 2007). Each of these mechanisms needs to be considered as well as the environment, injured or not, that the regenerating axons grow within. This incredibly complex set of challenges will necessitate the continued use of experimental mammals to accurately model the systems at play. Mouse genetic lines tallied with evidence from other species, including lower organisms such as *Drosophila*, reptiles, amphibians and fish, represent our best hope of developing functional nerve regeneration strategies. With the development of high-throughput omics technologies, interrogation and visualization of complex profiling data across regenerating and non-regenerating model systems has allowed identification of regeneration-associated gene expression signatures. Screening of small compound libraries that induce a regeneration signature and corresponding cellular phenotype can identify promising drug leads in well-conceived screens. In addition, analysis of molecular interaction profiles may allow the design of novel multicomponent approaches.

In conclusion, the capacity to accurately integrate and deconvolute complex biological networks and gene expression data from growing and non-growing injured neurons represents an incredible source of opportunities for discovering novel molecular targets to promote CNS regeneration and repair strategies. We have never been more able to determine, understand and therefore successfully manipulate the complex signaling pathways required to sponsor true functional regeneration in the CNS. As even imperfect axonal growth and regeneration strategies will likely be of profound benefit to SCI patients, through collateral sprouting for instance, we must continue the quest to functionally repair the traumatically damaged adult CNS.

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Figure 1.

Genetic determinants control axon growth and regeneration capacity. Phylogenetic analysis of nine inbred mouse strains. The CAST/Ei strain is much less related to many of the standard inbred laboratory lines.

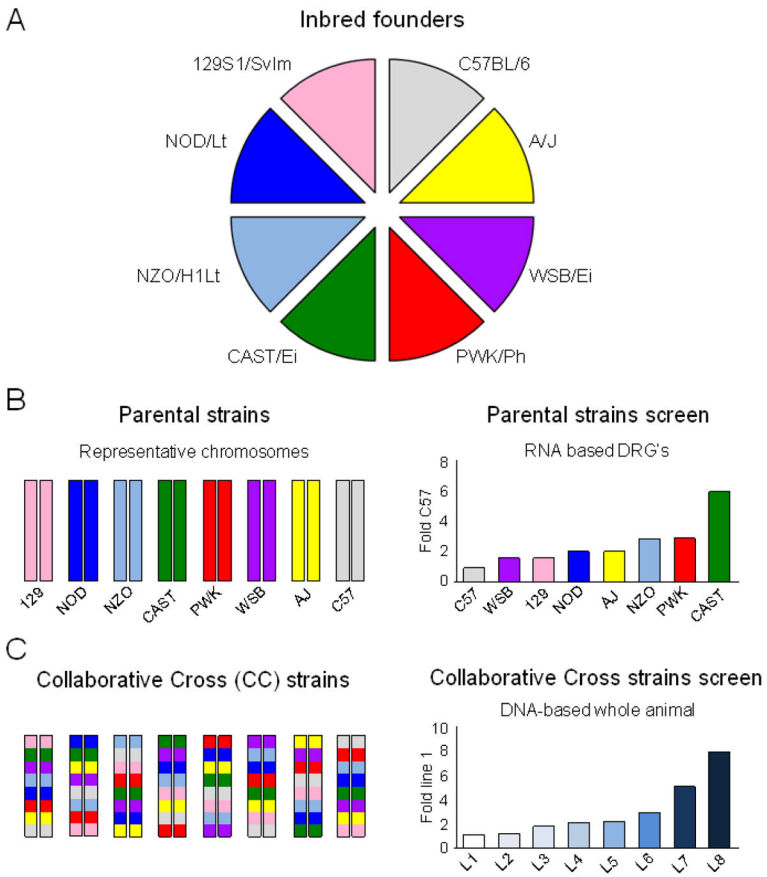

Figure 2.

The Collaborative Cross, a large panel of recombinant inbred lines. (**A**) The pie chart shows the eight parental strains used for generating the collaborative cross lines. (**B**) Parental strains are genetically diverse. (**C**) Founders genomes are inbred to generate recombinant inbred strains. Mixed color indicates the parental origin of genomic segments.

A

Core set of genes after preconditioning

NPY	Neuropeptide Y
GAL	Galanin
GPR151	G protein-coupled receptor 151
ATF3	Activating transcription factor 3
SPRR1A	Small proline-rich protein 1A
ECEL1	Endothelin converting enzyme-like 1
WFDC3	WAP four-disulfide core domain 3
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin)
KIF22	Kinesin family member 22
FLRT3	Fibronectin leucine rich transmembrane protein

B

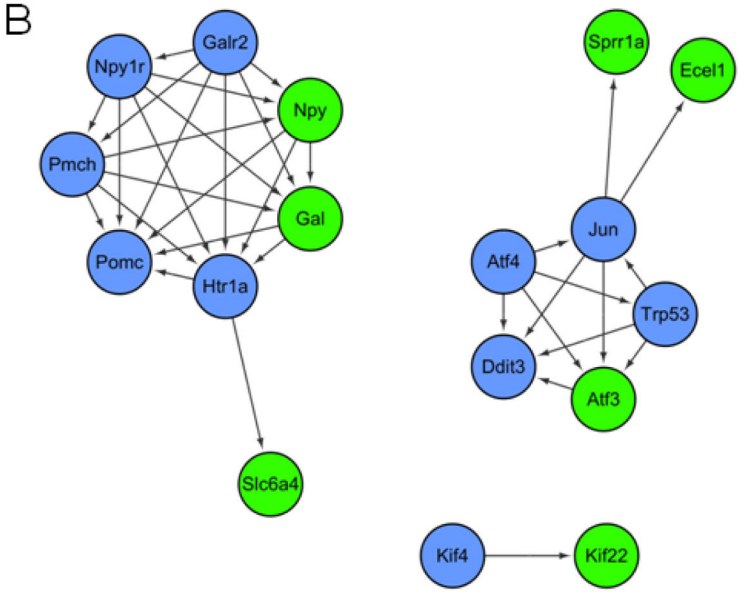

Figure 3.

Core set of genes whose expression increases in DRG after a peripheral nerve lesion. (**A**) List of the top ten genes shared among all nine inbred mouse strains shown in Figure 1. (**B**) The gene list in (A) was first submitted to STRING (Szklarczyk et al., 2015) to determine functional protein association network. Functional networks were then generated using Cytoscape V3.2.1 (Shannon et al., 2003). Gene circles represent core injury associated genes, blue circles are genes that are in close functional association with the core injury genes.

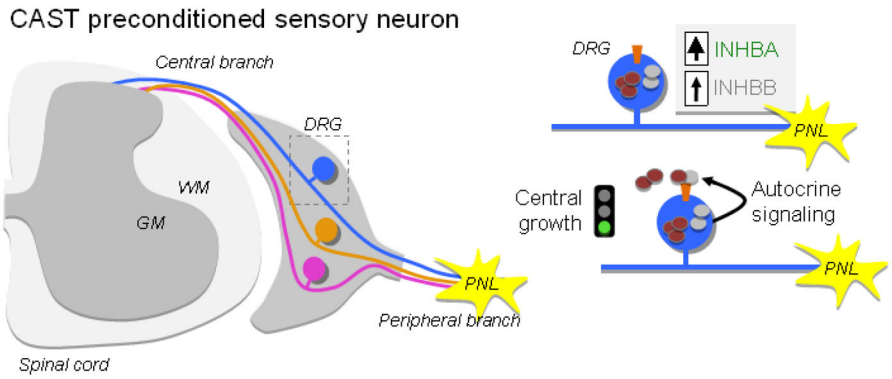

Figure 4.

Activin transcript *Inhba* strongly correlates with the ability to overcome axon growth inhibitors in CAST/Ei mice. Schematic showing the mechanism of action of Activin regulation after a conditioning lesion, Activin signaling and consequent axonal growth in a CNS environment. Regulation of *Inhba* is CAST/Ei specific, whereas regulation of *Inhbb* is part of the core regeneration response across strains. Both transcripts are required for strong axonal growth in the CNS.

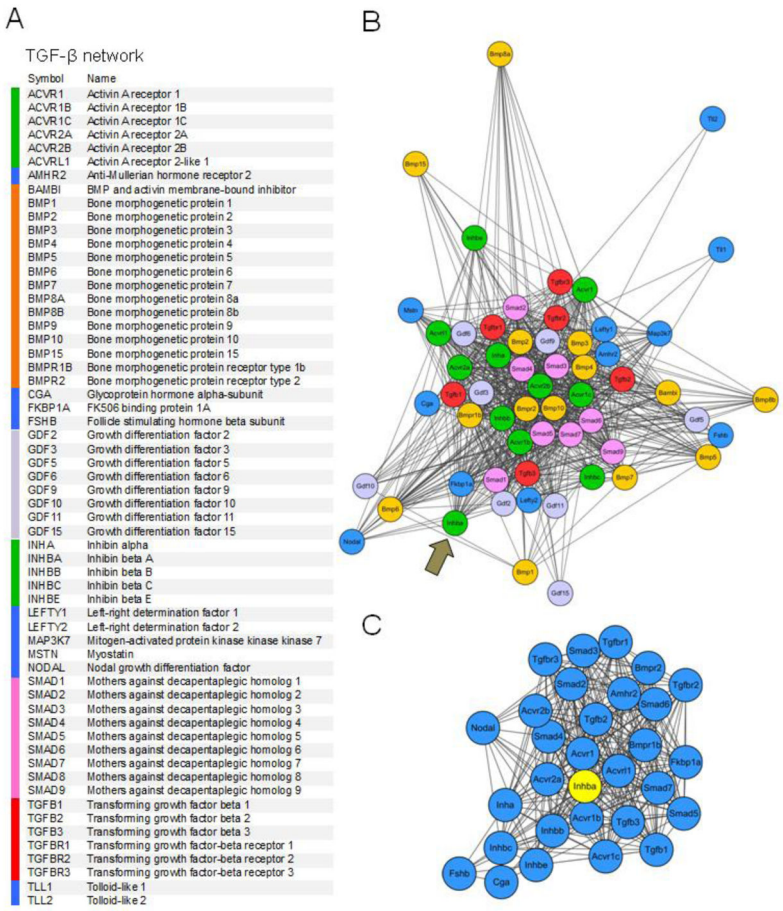

Figure 5.

The TGF-β network, a potent signaling pathway for multiple cellular functions. (**A**) List of the TGF-β pathway members. (**B**) The gene list in (A) was first submitted to STRING to determine functional protein association network. A functional network was then generated using Cytoscape. *Inhba* indicated by arrow. (**C**) Network showing first-order interactions between *Inhba*, the gene encoding for Activin A, and other members of the TGF-β pathway.

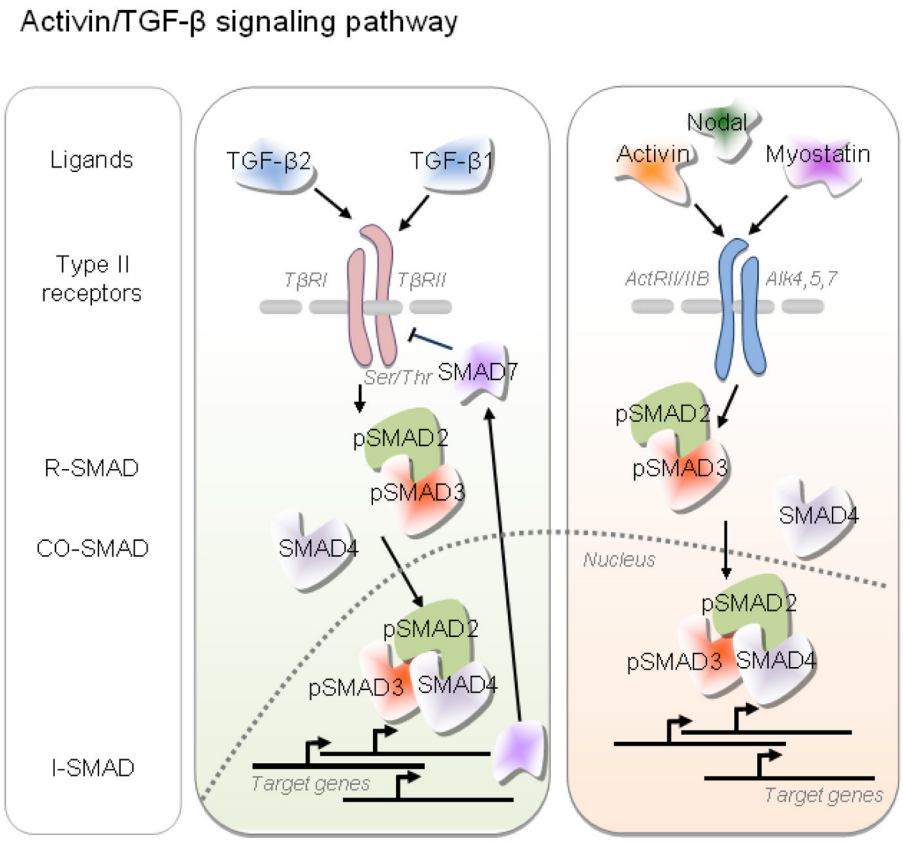

Figure 6.

Schematic representation of the Activin/TGF-β mediated Smad2/3 signaling pathway. This pathway is likely an integral part of intrinsic axonal growth in the CNS.