UNIVERSITY OF CALIFORNIA, SAN DIEGO

Mechanisms of Axonal Regeneration in the Central Nervous System

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

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2014

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University of California, San Diego

2014

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ACKNOWLEDGEMENTS

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Chapter 3, in its entirety, is currently being prepared for submission for publication of the material. Gunnar H.D. Poplawski, Tetsuhiro Ishikawa, Edward Wang, Qing Wang, Giovanni Coppola, W. Marie Campana and Mark Tuszynski. The dissertation author was the primary investigator and first author of this paper.

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PUBLICATIONS

Poplawski GH, Tranziska AK, Leshchyns'ka I, Meier ID, Streichert T, Sytnyk V, Schachner M. **L1CAM increases MAP2 expression via the MAPK pathway to promote neurite outgrowth**. Mol Cell Neurosci. 2012 Jun;50(2):169-78. doi: 10.1016/j.mcn.2012.03.010. Epub 2012 Apr 6. PubMed PMID: 22503709.

Dehmelt L, Poplawski G, Hwang E, Halpain S. **NeuriteQuant: an open source toolkit for high content screens of neuronal morphogenesis**. BMC Neurosci. 2011 Oct 11;12:100. doi: 10.1186/1471-2202-12-100. PubMed PMID: 21989414; PubMed Central PMCID: PMC3208608.

ABSTRACT OF THE DISSERTATION

Mechanisms of Axonal Regeneration in the Central Nervous System

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University of California, San Diego, 2014

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The regenerative capacity of central nervous system (CNS) axons after injury is severely impaired compared to axons of the peripheral nervous system (PNS). We hypothesized that mechanisms both intrinsic and extrinsic to the

neuron influence the ability of CNS axons to regenerate. To investigate this hypothesis we explored two model systems. In the first model system, we identified a regeneration transcriptome in injured corticospinal motor neurons that is associated with enhanced central axon regeneration after spinal cord injury. The genetic mechanisms identified in this model include cAMP-Erk-CREB, Huntingtin, NFE2L2, ephrin and semaphorin signaling, and provide a dataset for potential therapeutic intervention to improve axonal regeneration *in vivo* after spinal cord injury. In the second model, we tested the hypothesis that glial cells of the peripheral nerve, Schwann cells, are an essential mechanism contributing to central axonal regeneration after “conditioning” lesions, wherein injury to the peripheral branch of a dorsal root ganglion sensory neuron enhances regeneration of the central branch of the sensory neuron. The gene encoding Low-density lipoprotein Receptor-related Protein-1 (LRP1) was conditionally deleted in Schwann cells, impairing the survival and function of Schwann cells after injury; animals with Schwann cell-specific deletion of LRP1 exhibited a significant reduction in axon regeneration *in vitro* and a trend towards central sensory axon regeneration after conditioning lesions, confirming that glial cells exhibit an essential but partial role in supporting axonal regeneration. Overall, these studies identify novel molecular and cellular mechanisms that influence central axon regeneration, and suggest therapeutic approaches to improve neural repair after CNS injury.

1 INTRODUCTION

1.1 CURRENT STATE OF SPINAL CORD INJURY CASES

Each year thousands of people suffer traumatic, non-fatal spinal cord injury. Worldwide there are approximately 130,000 new spinal cord injury (SCI) cases each year. In the Unites States alone, there are on average 10,000 new spinal cord injury cases each year. As of today there are approximately 250,000 patients living with spinal cord injury in the United States, with an estimated annual economic burden of 7.7 billion dollars. (International Campaign for Cures of Spinal Cord Injury Paralysis, www.campaignforcure.org). SCI affects mainly young adults between the ages of 16 and 30, of whom 80% are male. The average age of patients living with spinal cord injury is currently 33.4 years. The cause of SCI is categorized into vehicular (63.5%), falls (28.5%), violence (primarily gunshot wounds) (14.3%), sports (9.2%) and other or unknown (11.4%). The most frequent neurological category since 2010 is incomplete tetraplegia (40.6%), followed by incomplete paraplegia (18.7%), complete paraplegia (18.0%) and complete tetraplegia (11.6%). (National Spinal Cord Injury Statistical Center, www.nscisc.uab.edu).

1.2 CLASSIFICATION OF SEVERITY

SCI injury patients are categorized by the American spinal injury Association impairment scale (AIS). There are 4 subcategories: sensorimotor complete (A), motor complete, sensory incomplete (B), motor and sensory incomplete (C and D), normal (E). During the first year following SCI, most patients convert from higher to lower categories of injury. However, about 80% of

ASI-A patients stay in the ASI-A category. Spontaneous recovery is more common for patients with less severe injuries in the ASI-C and ASI-D category. Rapid improvement occurs during the first six month after injury, and is essentially maximal after one year. (Fawcett et al., 2007)

1.3 PRIMARY AND SECONDARY DAMAGE

The primary damage is caused by impact to the spinal column. In most cases a fracture of the vertebra occurs, pressing inwards on the spinal cord. The immediate response to those injuries includes stabilization of the spinal column and decompression of the spinal cord by removing broken bones. This primary impact causes hemorrhage and ischemia that leads to cell death within a confined area, typically not more than one spinal segment away from the zone of impact. This cell death can however spread further in the rostral and caudal direction. Secondary damage occurs within hours after the impact, and often leads to an extension of the initial injury boundaries. Characteristics of secondary damage include the formation of fluid filled cysts and the retraction and dieback of axons from the lesion center. Inflammation and activation of microglia occurs within minutes after SCI, leading to secretion of pro-inflammatory cytokines and chemokines that further promote the secondary damage. However, the precise mechanisms involved in the formation secondary damage are not completely understood (Fehlings & Baptiste, 2005; Hurlbert, 2006; Rossignol et al., 2007).

1.4 EXTRINSIC FACTORS IN SPINAL CORD REGENERATION

Ramon y Cajal stated almost a century ago that axons of the CNS are unable to regenerate: “In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated”. Half a century later, Aguayo and colleagues demonstrated that axons of the CNS are indeed able to regenerate when provided with a permissive tissue graft derived from the peripheral nervous system (PNS) (Aguayo et al. 1981, David & Aguayo 1981; Richardson et al. 1984). These early observations suggest that the adult extrinsic environment within the CNS is inhibitory to CNS axon regeneration, but that some adult CNS axon themselves contain the intrinsic ability to regenerate. Driven by this hypothesis many researchers investigated the inhibitory nature of the adult CNS environment. Many extrinsic inhibitory cues and their downstream signaling pathways within the neurons have been subsequently identified (see Chapter 1.5). However, the expected regenerative benefit from blocking or removing these inhibitory extrinsic cues was not fully fulfilled, thus leading to the hypothesis that important neuron intrinsic mechanisms have to be active for successful regeneration of CNS axons.

The earlier experiments from Aguayo and colleagues only showed regeneration of a subset of axons (David & Aguayo 1981, Richardson et al. 1984) that retain the intrinsic ability to regenerate in the adult when provided with a permissive graft. Other CNS systems like the corticospinal tract (CST) failed to regenerate in this model, lacking the intrinsic ability for adult axon growth. However, if the spinal cord is injured during the developmental phase, before

CST axons have grown into the injury site, then CST axons circumvent the injury site and innervate appropriate targets caudal to the injury (Bregman et al. 1989). This time window can be extended if fetal spinal cord grafts are presented to injured CST axons (Bregman et al. 1989). This indicates that intrinsic embryonic growth mechanisms within the CST neurons are active during this developmental window and allow for axon regeneration, but are then lost in adulthood.

Further evidence of the importance of intrinsic growth mechanism comes from two studies grafting embryonic cortical tissue or spinal cord derived neural stem cells (NSCs) into the adult motor cortex or spinal cord, respectively (Gaillard et a., 2007; Lu et al., 2012). GFP-expressing embryonic cortical neurons that were grafted into the adult rat motor cortex projected axons to appropriate targets including the thalamus and spinal cord (Gaillard et al., 2007). Further, GFP-expressing embryonic spinal cord derived NSCs extended axons readily over long distances when grafted into the adult rat spinal cord. Axons extending from the NSC grafts completely neglected the inhibitory nature of the adult spinal cord, thus growing along white matter tracts as well as into the grey matter (Lu et al., 2012)

These examples show that intrinsic growth mechanisms active during embryonic development define the regenerative ability of injured axons, and that this intrinsic ability for growth has the ability to override inhibitory extrinsic mechanisms present in adult injured as well as intact CNS. The field of spinal cord injury now faces the challenging task of identifying intrinsic growth

mechanisms that can promote axon regeneration, and then manipulate these pathways for therapeutic intervention.

1.4.1 Extrinsic Manipulations Can Trigger Changes in Intrinsic Gene Expression

For adult CST axons to be able to regenerate, intrinsic growth mechanisms have to be reactivated. These intrinsic mechanisms are likely similar to the ones that promote axonal outgrowth during development of the nervous system. Reactivation of these early developmental intrinsic mechanisms can however be triggered by an appropriate extrinsic environment. This environmental niche has to be permissive for growth, potentially of embryonic nature. In the examples above (Chapter 1.4) either extrinsic or intrinsic mechanisms need to be supportive of growth. During embryonic development of the CST, both intrinsic mechanisms of the CST neurons as well as the extrinsic environment of the developing spinal cord are supportive of axon growth. Later in adulthood once CST development is complete and target regions have been innervated, both intrinsic and extrinsic mechanisms change and become inhibitory for axon regeneration.

Coumans et al (2001) reported that an extrinsic embryonic environment can activate growth of the adult CST. Grafted embryonic spinal cord tissue was able to stimulate the regeneration of adult CST axons following injury. This study did not get a lot of attention, as the amount of regenerating CST axons was relatively minor and the grafting paradigm included the osmotic delivery of NT3 or

BDNF making the interpretation of the findings more complex (Coumans et al. 2001).

We adapted the improved paradigm of grafting embryonic spinal cord derived neural stem cells (NSC) from Lu et al., 2012 and achieved significant and extensive CST axon regeneration into the NSC grafts (Kadoya et al., manuscript in preparation). The optimized grafting paradigm utilizes single cell suspensions of NSC embedded in a fibrin matrix instead of grafting whole tissue pieces of the embryonic spinal cord as in Coumans et al. 2001. This new method results in an increased area of contact between grafted cells and injured CST axons. The number of CST axons responding to the graft substantially exceeds reports of Coumans et al. Chapter 2 investigates the changes in intrinsic gene expression of adult CST neurons in response to extrinsic growth permissive embryonic NSC grafts.

1.5 EXTRINSIC NON-NEURONAL MECHANISMS IN REGENERATION

1.5.1 Prototypic Myelin Associated Inhibitors (MAI)

MAIs are growth inhibiting molecules that are expressed by glial cells in the CNS. These inhibitory molecules include MAG, OMgp, Nogo-A, netrin, ephrin and semaphorin (Liu et al., 2006; Yiu and He et al., 2006; Löw et al., 2008). After spinal cord injury these molecules are exposed to the injured axons due to mechanical damage to the myelin sheath and can inhibit axon regeneration via receptor binding both *in vitro* and *in vivo*.

Myelin-associated glycoprotein (**MAG**) belongs to the l-type lectin protein family of the Ig-superfamily. It is composed of 5 Ig-like domains, a single transmembrane domain and a short cytoplasmic tail (Filbin et al., 2003). The inhibitory function of MAG is developmentally regulated, transitioning from neurite outgrowth stimulation in embryonic neurons to neurite outgrowth inhibition in adult neurons *in vitro* (Johnson et al. 1989, DeBellard et al., 1996). The inhibitory function of MAG on neurite outgrowth has been well established, nevertheless MAG-deficient mice do not show increased corticospinal or optic nerve axon regeneration after injury *in vivo* (Bartsch et al., 1995).

Oligodendrocyte myelin glycoprotein (**OMgp**) is exclusively expressed by CNS glia and neurons (Habib et al. 1998; Lee et al. 2009). It belongs to the LRR family of leucine rich repeat proteins and is linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (Wang et al., 2002, Mikol and Stefansson, 1988). OMgp deficient mice show increased sprouting of serotonergic fibers but no increase in CST axon regeneration (Ji et al. 2008)

Nogo-A or RTN4a belongs to the reticulon family (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). Nogo-A contains two distinct inhibitory domains; Amino-Nogo and Nogo66 (Fournier et al. 2001; Oertle et al. 2003). Nogo-A deficient mice fail to show increased CST axon regeneration (Zheng et al. 2003; Lee et al. 2009). Although controversial, inhibition of Nogo-A by functional blocking antibodies has been reported to increase growth of serotonergic axons (Gonzenbach and Schwab, 2008) as well as regeneration and sprouting of CST axons (Schnell and Schwab, 1990; Bregman et al. 1995;

Thallmair et al. 1998). The debate concerning the benefits of Nogo-A neutralization is ongoing (Zheng et al. 2005; Lee et al. 2009). Despite this controversy, Nogo-A antibody blockade has progressed to human clinical trials in spinal cord injury that are currently in progress.

1.5.2 MAI Receptors and Mechanisms of Growth Inhibition

The role of myelin-associated inhibitors in CNS axonal regeneration is controversial. Nogo66 receptor 1(NgR1) was the first identified receptor for MAIs. It binds to Nogo66, MAG and OMgp (Fournier et al. 2001; Domeniconi et al. 2002; Liu et al. 2002; Wang et al. 2002). Nogo receptor 2 (NgR2) had only been shown to interact with MAG (Venkatesh et al. 2005). Postnatal DRG neurons lacking NgR1 were protected against cone collapse in response to Nogo66, MAG, or OMgp *in vitro* (Kim et al. 2004; Chivatakarn et al. 2007). NgR1 and 2 deficient DRG neurons showed a partial reduction in inhibitory response to MAG *in vitro* (Worter et al. 2009).

NgR1 can form receptor complexes with the co-receptors of the low affinity neurotrophin tyrosine receptor kinase family including P75, TROY and LINGO1. P75 and TROY deficiency has been shown to reduce inhibition by MAG, OMgp and Nogo-66 (Wang et al., 2002, Yamashita et al., 2002, Park et al., 2005, Shao et al., 2005).

Co-expression of NgR1, P75 or TROY and LINGO1 has been shown to be sufficient to stimulate responses to MAIs in non-neuronal cells by as assayed by activation of the downstream signal RhoA (Park et al., 2005, Shao et al., 2005). RhoA is an important downstream mediator of MAI induced neurite outgrowth

inhibition and growth cone collapse by regulating actin cytoskeleton dynamics (Niederost et al., 2002).

In 2008 Atwal and colleagues identified paired immunoglobulin-like receptor B (PirB), as yet another receptor binding Nogo-66, OMgp, and MAG. PirB is a member of the leukocyte immunoglobulin receptor (LIR) subfamily. Interference with PirB activity or expression levels via function blocking antibodies or gene knockout leads to reduced inhibition in response to myelin substrates. PirB also forms a receptor complex with NgR1. Ablation of both receptors leads to a drastic recovery of axonal outgrowth in response to myelin inhibition (Atwal et a., 2008).

Intrathecal administration of NgR1(310)-Fc, a soluble peptide that complexes with NgR1 ligands and thereby competes for ligand binding to the NGR1 neuronal cell surface receptor-complex, reportedly led to CST and raphespinal axon regeneration and modestly improved function outcomes after spinal cord injury. (Li et al. 2004; Wang et al.2006). This was contradicted by Steward and colleagues in 2007, who, significantly, demonstrated that the result was an artifact of erroneous axon labeling techniques. While NgR1(310)-Fc has been shown to overcome myelin inhibition in several studies *in vitro* (Fournier et al. 2002; He et al. 2003; Zheng et al. 2005; Peng et al. 2010), its role in influencing axonal regeneration *in vivo* is controversial.

The LDL receptor-related protein-1 (LRP1) was recently identified as another receptor for MAG which is partially responsible for MAG inhibition of cerebellar granule neurons *in vitro* (Stiles et al., 2013).

It is now clear that MAI signals through a variety of receptors that can also interact with each other. Combinatorial approaches seem necessary to completely overcome myelin inhibition by individually targeting each receptor. This could be archived by application of function blocking antibody cocktails inhibiting every single ligand receptor interaction. It is likely that not all MAI associated receptors have been identified, and new myelin binding receptors are still being discovered to date. Therefore, additional approaches are necessary to interfere with receptor ligand binding. For example, one could target common downstream mechanisms of MAI receptor signaling such as the RhoA-ROCK-LIMK pathways that regulate cytoskeletal dynamics.

1.5.3 The Role of Glia in Scarring and Regeneration

The glia “scar” is formed within days after spinal cord injury. It surrounds the lesion site and consists of reactive astrocytes, microglia, meningeal fibroblasts and extracellular matrix molecules such as chondroitin sulfate proteoglycans (CSPGs), and has been thought to act as an effective barrier for axon regeneration (Bradbury et al. 2002).

Host astrocytes of the injured spinal cord become reactive, characterized by hypertrophy and increased proliferation. The reactive astrocytes migrate to the lesion boundary and produce large amounts of glial fibrillary acid protein (GFAP) and form the major component of the glial scar (Sofroniew, 2005). GFAP immunolabeling is widely used to define the lesion boundary after spinal cord injury in mammals. Reactive astrocytes also start to secrete large amounts of CSPGs (McKeon et al. 1995; Davies et al. 1996; Fitch et al. 1999; Jones et al.

2002; Morgenstern et al. 2002; Jones et al. 2003), which are inhibitory to adult axon regeneration (Snow et al. 1990; Davies et al. 1999).

In contrast to its inhibitory function on axon growth, the glial scar has also been shown to be beneficial following spinal cord injury since it isolates the injury site and protects the surrounding host tissue from secondary damage and macrophage infiltration, thereby reducing tissue loss and functional deficits and aiding axonal regeneration (Faulkner et al., 2004). In addition, astrocytes have been shown to support axon growth by secreting growth promoting molecules like laminin (Liesi et al., 1984; Grimpe & Silver, 2002), hence regenerating axons are often associated with astrocytic processes infiltrating the cell matrix within the lesion site (Kawaja & Gage, 1991).

Beside the secretion of growth stimulatory ECM molecules like laminin, other ECM molecules can inhibit axon outgrowth by presenting a physical barrier for growth cone advancement, as well as activating repulsive signaling pathways that lead to growth cone collapse and axon retraction (Silver & Miller, 2004, Smith-Thomas et al., 1994). These molecules include pro-inflammatory molecules and cytotoxins that create a hostile environment for axon regeneration (Antony *et al.*, 2004; Ikeda & Murase, 2004).

1.5.4 Role of the Extracellular Matrix: Proteoglycans

Proteoglycans consist of a core protein that is covalently linked to glycosaminoglycan (GAG) side chains (Galtrey and Fawcett, 2007). The major sulfate PG in the CNS are lecticans (neurocan, versican, aggrecan and brevican), phosphacans and NG2, beside other PGs including hyaluronan and

tenascins (Carulli et al., 2005; Galtrey and Fawcett, 2007; Kwok et al., 2008). In particular, secretion of Chondroitin sulfate PGs (CSPGs) by astrocytes and microglia is highly upregulated following CNS injury, and CSPGs inhibit axonal growth in a variety of neurons *in vitro*.

The known receptors for sulfate PGs include protein tyrosine phosphatase σ (PTPσ), LAR phosphatase, NgR1 and NgR3 (Dickendesher et al., 2012; Fisher et al., 2011; Shen et al., 2009). PTPσ is a member of the receptor protein tyrosine phosphatases (RPTPs) of the leukocyte antigen related (LAR) subfamily. This family of receptors is associated with heparan sulfate PG (HSPG) binding (Aricescu et al. 2002; Fox and Zinn, 2005; Johnson et al. 2006), and was recently identified as a specific receptor for CSPGs that mediates neurite outgrowth inhibition *in vitro* (Shen et al. 2009) and was further implicated in CST axon regeneration *in vivo* (Fry et al. 2009).

Other molecules that facilitate neurite outgrowth inhibition by CSPGs include integrins, which normally bind the ECM molecule laminin (Afshari et al., 2010; Condic et al.1999; Tan et al., 2011). The axon guidance molecule Sema5A has also been shown to interact with both HSPG and CSPGs. Upon binding to CSPG, Sema5A turns from an attractive to a repulsive cue (Kantor et al., 2004).

Proteolytic cleavage of the GAG side chains from the core protein by chondroitinase ABC (ChABC) can almost completely neutralize the inhibitory effects of proteoglycans *in vitro* (McKeon et al. 1995; Zuo et al. 1998; Grimpe et al. 2005). *In vivo* delivery of ChABC extends experience dependent plasticity in the rat visual cortex (Pizzorusso et al. 2002) and enhances axonal growth after

injury (Moon et al. 2001; Bradbury et al. 2002; Tester and Howland 2008), although there has been surprisingly little confirmation of ChABC effects directly on either axonal regeneration or sprouting. Direct application of ChABC into sites of CST axon injury prevents atrophy of injured axons and activates growth promoting signaling pathways (Carter et al. 2008). Several studies have implicated an increase in regeneration of injured axons or collateral sprouting upon ChABC treatment (Bradbury et al., 2002, Crespo et al., 2007, Davies et al., 1999, Fawcett, 2006, Jefferson et al., 2011 and Smith-Thomas et al., 1995). Expression of ChABC in reactive astrocytes under the GFAP promoter has been shown to improve CST and ascending sensory fiber regeneration (Cafferty et al., 2007).

1.5.5 The Role of Neurotrophic Factors in Axon Regeneration

Neurotrophins are secreted molecules that promote axon outgrowth and neuronal survival. During development, gradients of neurotrophins guide axons to their specific targets and promote synapse formation. After establishment of neuronal circuitry neurotrophins are generally downregulated, thus in adulthood most of these gradients are lost and regeneration of injured axons becomes difficult. Areas of adult neurogenesis and functional plasticity like the SVZ and the dentate gyrus express neurotrophins even after the development of the nervous system is completed (Maisonpierre et al. 1990). Reestablishment of growth factor gradients to recapitulate developmental guidance pathways seems to be necessary to successfully guide injured axon during regeneration.

The classic neuronal growth factors are nerve growth factor (NGF), brain-derived neurotropic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Lee et al., 2001) whose expression patterns are spatially and temporally regulated. Neurotrophins bind to neurons and glial cells via tropomyosin receptor kinases (trk). The receptor-ligand interactions are highly specific to enable selective simultaneous axon guidance. NGF binds to trkA, BDNF and NT4/5 to trkB, and NT3 to trkC (Bibel & Barde, 2000). P75 has been described as a low affinity neurotrophic receptor that binds to all neurotrophins, with the potential to enhance high affinity neurotrophin receptor signaling via receptor complex formation.

In contrast to the PNS, where neurotropic factors are upregulated by Schwann cells to promote neuronal survival and axon regeneration (Meyer et al., 1992; Funakoshi et al., 1993), in the CNS growth factor levels stay constant after injury, possibly contributing reduced axon regeneration after injury compared to the PNS (Plunet et al., 2002).

If neurotropic factors are artificially provided to the CNS after injury they can promote neuronal survival and axon regeneration. BDNF delivery promoted survival of CST (Giehl & Tetzlaff, 1996; Giehl et al. 2001; Lu et al. 2001) and rubrospinal neurons (Kobayashi et al., 1997) and increased axon regeneration of serotonergic, rubrospinal, reticulospinal, coerulospinal, and large-diameter ascending sensory neurons (Lu et al. 2005; Liu et al. 1999; Jin et al. 2002). Interestingly, these studies demonstrated that BDNF was able to prevent CST neuron loss (neuroprotection) but failed to improve CST axon regeneration,

highlighting the refractory nature of this important system (Lu et al. 2001). NGF delivery can promote the survival of cholinergic neurons (Kromer, 1987) and increase axon regeneration of cholinergic motor neurons and ascending sensory neurons (Tuszynski *et al.*, 1996).

Combinatorial strategies providing the neurotrophic guidance molecule and the corresponding receptor have led to successful CST regeneration via trkB/BDNF expression (Hollis et al., 2009). Alto et al. 2009 demonstrated regeneration of sensory axons following an NT3 gradient that led to reinnervation and synapse formation on appropriate targets in the nucleus gracilis. Unfortunately these studies did not lead to a functional improvement due to myelination deficits of the newly formed circuitry.

1.5.6 Role of Schwann Cells in Peripheral Axon Regeneration

Peripheral nerve injury is followed by Wallerian degeneration of the distal part of the lesioned axon. Schwann cells in the distal region that lost contact with a functional axon dedifferentiate to a more immature phenotype and become reactive (Scherer et al., 1994) via Krox-20 and c-Jun signaling (Parkinson et al., 2008). The reactive Schwann cells proliferate and migrate to sites of lesioned axons to support their regeneration. When they come in contact with the regenerating axon they redifferentiate again into a mature phenotype (Jessen and Mirsky, 2008). Between 1 and 5 days after peripheral nerve injury Schwann cells proliferate and promote the process of Wallerian degeneration by the clearance of myelin debris in coordination with macrophages (Bradley and Asbury, 1970). The activation of p38 MAPK has been shown to be sufficient to

induce myelin breakdown and to promote the dedifferentiation of Schwann cells via the regulation of Krox 20 and c-Jun (Hossain et al., 2012 and Yang et al., 2012). C-Jun has recently been described as a global regulator of the injury response of Schwann cells (Arthur-Farraj et al., 2012).

To ensure their own survival and to promote axon regeneration, Schwann cells upregulate neurotrophic receptors and release neurotrophins, such as NGF, BDNF, NT4, glial cell-derived neurotrophic factor (GDNF) and insulin-like growth factor-1 (IGF-1). The secretion of BDNF by Schwann cells has been shown to be necessary for regeneration and myelination of regenerating axons following injury (Zhang et al. 2000; Song et al. 2008; Geremia et al. 2010). In addition Schwann cells secrete ECM molecules that promote and guide axon regeneration *in vitro* and *in vivo* such as collagen, fibronectin, laminin, tenascins and proteoglycans (Babington et al., 2005; Gardiner et al., 2007; Werner et al., 2000). Schwann cells also secrete cytokines, signaling molecules that trigger the inflammatory response and lead to the recruitment of macrophages to the injury site. Besides aiding the clearance of myelin and axonal debris, macrophages secrete additional growth factors and cytokines that facilitate peripheral nerve regeneration, such as IL6 and LIF (Barrette et al., 2010; Gordon et al., 2009)

After injury Schwann cells upregulate matrix metalloproteinases (MMPs) that promote the degradation of CSPGs, an action that is thought to facilitate regeneration (Ferguson and Muir, 2000; Zuo et al., 1998, 2002 Krekoski et al., 2001; Udina et al., 2010). In particular MMP-2 and MMP-9 increase in expression during Wallerian degeneration (Ferguson and Muir, 2000).

1.6 INTRINSIC NEURONAL MECHANSIMS IN REGENERATION

1.6.1 Transcriptional Regulation

Transcription factors are DNA binding proteins that can simultaneously regulate sets of genes that are involved in axon regeneration in response to an injury signal. Transcription factor expression is increased immediately after injury, where genes involved in neurotransmission and certain structural proteins are downregulated (Bareyre and Schwab, 2003). Increased transcriptional activity is an indicator of the initial regenerative response, since early inhibition of transcription leads to reduced regeneration (Smith and Skene, 1997).

The initially activated transcription factors regulate the expression of regeneration associated genes that are necessary for axon regeneration to occur (Bosse et al., 2001; Schmitt et al., 2003; Bosse et al., 2006). The general transcriptional response to injury is far greater in neurons when axons regenerate as compared to axons lacking regeneration. Thus, the altered gene expression of dorsal root ganglion neurons following peripheral branch injury is significantly greater than after central branch injury (Hoffman, 2010)

A single transcription factor can bind multiple locations on the genome and thereby regulate the expression of hundreds of genes. Transcription factors are themselves regulated by the level of their expression and by post-translational modifications in response to injury of already present transcription factors.

1.6.1.1 TP53

The transcriptional regulator TP53 is widely known as tumor suppressor gene with specific functions in inducing apoptosis and regulating cell cycle

progression and DNA repair (Vogelstein et al., 2000; Bargonetti and Manfredi, 2002; Miller et al., 2000; Morrison et al., 2003; Culmsee and Mattson, 2005). In the CNS, TP53 expression is more prominent during development when axons are actively extending (Gottlieb et al.,1997; Komarova et al., 1997). TP53 has been shown to have a positive benefit on axon regeneration *in vitro* and *in vivo* (Di Giovanni et al., 2005; Di Giovanni et al., 2006). TP53 regulates the expression of growth associated genes like Coronin1b, Rab13, and GAP-43 and axon guidance molecules including netrins, semaphorins, ephrins, and slits (Gottlieb et al.,1997; Komarova et al., 1997; Arakawa, 2005). Posttranslational modifications of TP53 like acetylation, phosphorylation, and ubiquitination are involved in the ability to regulate axon regeneration (Tedeschi and Di Giovanni, 2009; Lavin and Gueven, 2006).

1.6.1.2 c-Jun

c-Jun is part of the Jun family of transcription factors (c-Jun, JunB and JunD) that heterodimerize with other transcription factors of the Fos, basic leucine zipper domain (bZIP)-containing and activating transcription factor (ATF) families (Jochum et al., 2001). Both c-Jun and JunD upregulation has been associated with axon regeneration (Kenney and Kocsis, 1998; Herdegen and Zimmerman, 1994). c-Jun is expressed during development and declines after postnatal day 15 (Wilkinson et al., 1989; Bennett et al., 1997; Mellstrom et al., 1991, Herdegen et al., 1991; Raivich and Behrens, 2006) c-Jun expression is then again upregulated after axon injury (Herdegen and Waetzig, 2001; Raivich,

2008) and has been associated with both pro and anti-apoptotic mechanisms in CNS neurons following injury (Crocker et al., 2001; Lingor et al., 2005 ; Behrens et al., 1999; Herdegen et al., 1993, Hull and Bahr, 1994; Robinson, 1994; Robinson, 1995). C-Jun upregulation occurs in a variety of regenerating neurons (Herdegen et al., 1991; Jenkins and Hunt, 1991; Leah et al., 1991; Herdegen et al., 1997; Lindwall and Kanje, 2005). Activation of c-Jun has been linked to retrograde transport of the c-Jun N-terminal kinase (JNK) in regenerating peripheral axons (Lindwall and Kanje, 2005). Mice deficient in c-Jun show decreased regeneration and sprouting following facial nerve axotomy (Raivich et al., 2004), however transgenic mice overexpressing c-Jun have failed to show improved axon regeneration (Carulli et al., 2002). In addition to changes in expression levels, the post-translational activation of c-Jun by phosphorylation seems to be critical for axon regeneration (Dragunow et al., 2000; Herdegen et al., 1998, Lindwall et al., 2004).

1.6.1.3 ATF3

ATF3 (activated transcription factor 3) belongs to the family of ATF/CREB (cAMP-responsive element binding protein) bZIP transcription factors. The common denominator of these transcription factors is a bZIP DNA binding domain (Hai et al., 1999). They can heterodimerize with each other or other transcription factors from the Fos and Jun families (Hai et al., 1999; Hai and Hartman, 2001). ATF3 homodimerization leads in most cases to suppression of gene transcription including its own (Wolfgang et al., 2000). ATF3 expression is upregulated after injury (Chen et al., 1996; Francis et al., 2004; Tsujino et al.,

2000; Isacsson et al., 2005; Seijffers et al., 2006; Takeda et al., 2000; Mayumi-Matsuda et al., 1999) and associated with increased axon regeneration in both CNS and PNS (Campbell et al., 2005; Mason et al., 2003; Seijffers et al., 2006). ATF3 overexpression has been shown to increase axon regeneration in DRG neurons *in vitro* and *in vivo* and to increase expression of hsp27, Sprr1a, and c-Jun in DRGs of transgenic mice (Seijffers et al., 2007). ATF3 is postulated to synergistically interact with c-Jun since phosphorylated c-Jun colocalizes with neurons showing increased ATF3 expression after injury (Tsujino et al., 2000; Lindwall et al., 2004) and inhibition of JNK also leads to reduced ATF3 expression (Lindwall et al., 2004).

1.6.1.4 STAT3

STAT3 (signal transducers and activators of transcription) is one of seven members of the STAT family of transcription factors (Copeland et al., 1995). STATs are activated by extracellular signals such as growth factors and cytokines via the activation of associated receptor tyrosine kinase Janus kinases (JAKs) or non-receptor tyrosine kinases such as Src, leading to homo- and heterodimerization as well as tetramerization followed by translocation to the nucleus and DNA binding via consensus sis-inducible element, gamma activated sequence, or interferon stimulated regulating element sequences.(Vinkemeier et al., 1996; John et al., 1999; Zhang and Darnell, 2001; Levy and Darnell, 2002; Lim and Cao, 2006). STAT3 is expressed in neurons and glia from E14 till adulthood (De-Fraja et al., 1998; Gautron et al., 2006). Activation of STATs can be triggered by the growth factors such as CNTF, NGF, ILGF1 and PDGF as well

as IL-6, leukemia inhibitory factor (LIF) and granulocyte colony-stimulating factor, all of which have been implicated in neuroprotection (Dziennis and Alkayed, 2008). Deficiency or over activation of STAT3 leads to motor neuron path finding defects in zebra fish (Conway, 2006). Phosphorylation and upregulation of STAT3 is connected to peripheral facial (Schwaiger et al., 2000) and sensory axon regeneration (Qiu et al., 2005). STAT3s influence on sensory axon regeneration is mediated by JAK signaling leading to induction of GAP-43 expression (Qiu et al., 2005). Thus, JAK/STAT inhibitors lead to abolishment of the conditioning effect accompanied with reduced GAP-43 expression. Deletion of the upstream activators IL-6 and LIF have been shown to influence sensory axon regeneration in response to a conditioning injury (Cafferty et al., 2001; Cafferty et al., 2004). STAT3 phosphorylation is also involved in optic nerve regeneration *in vivo* and hippocampal axon outgrowth *in vitro* (Fischer et al., 2004; Ng et al., 2006).

1.6.1.5 SOX11

SOX11 is one of 20 members of the sex determining region Y (SRY)-box containing (SOX) gene family (Prior and Walter, 1996; Kamachi et al., 2000). Most SOX proteins have a c-terminal activation domain but rely on other DNA binding co-factors for the regulation of transcription (Kamachi et al., 2000). SOX11 has been connected to differentiation and development in the nervous system (Uwanogho et al., 1995; Hargrave et al., 1997; Rex et al., 1998; Hyodo-Miura et al., 2002). It is highly expressed in the developing DRG and RGC neurons, down regulated in adulthood, but then again highly expressed during

regeneration of these neurons. It shows the highest expression levels during axon regeneration (Hargrave et al., 1997; Tanabe et al., 2003; Jankowski et al., 2006; Wang et al., 2007; Veldman et al., 2007; Sun et al., 2005). In DRG neurons and Neuro2a cells, SOX11 expression highly correlates with neurite outgrowth and SOX11 knockdown has been shown to reduce neurite outgrowth but also increase apoptosis in DRG neurons *in vitro* and *in vivo* (Tanabe et al., 2003; Jankowski et al., 2006; Jankowski et al., 2009). SOX11 has been shown to affect expression of regeneration associated genes like MAP2, βIII-tubulin, Arpc3 and ATF3 (Bergsland et al., 2006; Jankowski et al., 2006; Jankowski et al., 2009). However in the CNS SOX11 knockdown did not lead to reduced axon regeneration (Veldman et al., 2007).

1.6.1.6 CREB

CREB (cAMP-responsive element binding protein), another member of the family of ATF/CREB bZIP transcription factors mediates cAMP signaling via PKA in the CNS (Hai and Hartman, 2001; Hannila and Filbin, 2008). CREB interacts with other CREB, ATF and CREM (cAMP-responsive element modulator) isoforms to bind CREs (cAMP-responsive element s) in the DNA (Walker et al., 1996; Walker and Habener, 1996; Lonze and Ginty, 2002). CREB deficiency results in reduced axonal outgrowth of DRG neurons *in vitro* and *in vivo* (Lonze et al., 2002). A dominant negative form or CREB lead to decreased neurite outgrowth in cortical neurons and the inability to respond positively to cAMP or neurotrophins *in vitro* (Redmond et al., 2002). A constitutively active form of CREB increased regeneration of central sensory axons by a factor of 3 (Gao et

al., 2004). CREB positively regulates, beside many other molecules, the expression of arginase I and BDNF, which have both been shown to promote sensory and motor axon regeneration (Deng et al., 2009; Gao et al., 2004; Shieh et al., 1998; Tao et al., 1998; Finkbeiner, 2000; Mayr and Montminy, 2001; Impey et al., 2004). CREB activity is regulated by phosphorylation by several kinases (PKA, PKC, CAMKII, CAMKIV, AKT, MAPKAP K2) and phosphatases (PP1 and PP2A) (Riccio et al., 1997; Watson et al., 1999; Watson et al., 2001; Lonze et al., 2002; Arthur et al., 2004; Riccio et al., 2006; Spencer et al., 2008; Mayr and Montminy, 2001; Sun et al., 1994)

1.6.1.7 NFATc

NFATc is one of the five member of the NFAT family of transcription factors, that act as signal integrators, since their function is the direction of STAT3, c-Jun, CREB and ATF3 to specific DNA binding sites. NFATs cannot regulate transcription on their own (Graef et al., 2001; Manukyan et al., 2009). NFATc1-4 is activated by an increase in intracellular calcium via dephosphorylation through calcineurin, which reveals the NLS, targeting it to the Nucleus (Graef et al., 1999; Coghlan et al., 1995; Miyazaki et al., 1996; Lai et al., 1998; Sun et al., 1998; Lin et al., 1999; Fuentes et al., 2000; Rothermel et al., 2000). Further regulation occurs by several kinases like GSK-3, DYRK1A or 2 (dual-specificity tyrosine-phosphorylation-regulated kinase, JNK, p38 MAPK, and casein kinase in combination with MEKK1 (Beals et al., 1997; Neal and Clipstone, 2001; Sheridan et al., 2002; Gwack et al., 2006; Chow et al., 1997; Gomez del Arco et al., 2000; Zhu et al., 1998). NFAT based transcription in

neurons can be triggered by NGF, BDNF and substance P (Graef et al., 2003; Groth and Mermelstein, 2003; Seybold et al., 2006; Groth et al., 2007) as well as synaptic activity and depolarization (Graef et al., 1999). It is expressed throughout the brain and spinal cord, increasing in expression from E10 till adulthood (Plyte et al., 2001; Wilkins et al., 2004; Seybold et al., 2006; Groth et al., 2007, Nguyen et al., 2009). NFATc2, c3 and c4 deficient mice showed defects in sensory axon projection as well as reduced axon outgrowth in response to netrin *in vitro* (Graef et al., 2003).

1.6.1.8 NFκB

NFκB (Nuclear Transcription factor kappa-B) family transcription factors are dimers made up of 5 possible subunits (Hoffmann et al., 2006). NFκB is inhibited from translocating to the nucleus by a family of inhibitor proteins (IKBs) (Perkins, 2007; Vallabhapurapu and Karin, 2009) and by other proteins like IL-4, and IL-10, TGFβ, GSK-3β and glucocorticoids. NFκB can be activated by a variety of signals including cytokines, growth factors, neurotransmitters and electrical activity (Kaltschmidt et al., 2005; Mattson, 2005). The expression of NFκB in the brain and DRGs begins during mid-embryonic development and prolongs into adulthood (Schmidt-Ullrich et al., 1996; Bhakar et al., 2002). After sciatic nerve injury, NFκB is upregulated in DRGs and spinal cord. (Ma and Bisby, 1998; Fernyhough et al.,2005; Pollock et al., 2005). It has been shown to be retrogradely transported and can thereby transmit signals from the injury site to the nucleus (Wellmann et al., 2001). NFκB controls synaptic plastic, learning and memory as well as cell survival in the brain and can be activated by

traumatic brain injuries as wells as seizures and stroke (Mattson, 2005; Romano et al., 2006). NFκB inhibition has been associated with increased neurite outgrowth of sensory (Gutierrez et al., 2005) and other neurons (Sole et al., 2004; Gallagher et al.,2007) but not of cultured sympathetic neurons (Gutierrez et al., 2008). This difference in regulation of axon regeneration seems to be dependent on the activity of IKKβ, which blocks the activity of NFκB in the specific neuron type (Gutierrez et al., 2008). NFκB transcription has been shown to regulate BDNF, glutamate receptor subunits (Mattson, 2005) and cell adhesion molecules like NCAM (Simpson and Morris, 2000), β1-Integrin (Wang et al., 2003) and tenascin C (Mettouchi et al., 1997). NFκB interacts with many other transcription factors like ATF, CREB, Jun, fos, STAT3 and TP53 (Perkins, 2007; Schumm et al., 2006). This indicates that NFκB acts more like a general mediator of transcription and not necessarily as a direct regulator of axon regeneration.

1.6.1.9 SnoN

SnoN is a member of the ski/sno/dac gene family of transcription factors that homo – or heterodimerize (Cohen et al., 1999; Luo, 2004 Pot and Bonni, 2008). SnoN has been shown to be involved in proliferation and differentiation (Pot and Bonni, 2008) by acting as a repressor for TGFβ (transforming growth factor β) signaling (Luo, 2004). Activation of TGFβ is achieved by SnoN degradation via ubiquitination mediated by the SMAD specific E3 ubiquitin protein ligase 2 (Smurf2), E3 ubiquitin ligases APC and Arkadia (Bonni et al., 2001; Stroschein et al., 2001; Wan et al., 2001; Levy et al., 2007; Nagano et al., 2007). In cerebellar granule neurons (CGN) SnoN regulates axon outgrowth via

Cdh1/APC interaction *in vitro* (Konishi et al., 2004; Stegmuller et al., 2006) and SnoN deficiency leads to defects in CGN development *in vivo*. SnoN knockdown reduces axon regeneration of hippocampal and cortical cultures *in vitro* (Stegmuller et al., 2006) and of DRG sensory axons *in vitro* and in the spinal cord *in vivo* (Do et al., 2013). SnoN activates JNK signaling and promotes the expression of the actin binding protein ccd1, which has been proposed to mitigate SnoN's effect on neurite outgrowth in CGNs (Ikeuchi et al., 2009)

1.6.1.10 KLF

KLF1 - 17 are part of the Krueppel like factor family of zinc-finger transcription factors that bind DNA at CACCC/GC/GT boxes that are highly represented throughout the genome (Suske et al., 2005). KLFs are most well studied in cancer biology where they regulate events like cell cycle progression and cell death (Black et al., 2001). KLF activity is regulated by a variety of co-factors like Sin3A, CtBP1and corticosterone (Imataka et al., 1992; Zhang et al., 2001; Liu et al., 2009; Denver and Williamson, 2009; Bonett et al., 2009). Many of the KLFs are expressed in RGCs during development and upon regeneration (Moore et al., 2009). Certain forms of KLF are expressed throughout the brain during development and adulthood (Denver et al., 1999; Martel et al., 2002; Morita et al., 2003; Hwang et al., 2001; D'Souza et al., 2002; Otteson et al., 2004; Imhof et al., 1999; Yanagi et al., 2008). Overexpression of embryonically active KLFs (like KLF6 and 7) increases neurite outgrowth, and overexpression of KLFs active in adulthood (like KLF4 and 9) decreases neurite outgrowth in culture (Laub et al., 2001; Lei et al., 2001; Jeong et al., 2009; Laub et al., 2005;

Laub et al., 2006; Veldman et al., 2007). KLF7 regulates the expression of L1, GAP43 and TrkA as well as genes involved in cytoskeletal dynamics and synaptogenesis (Laub et al., 2001; Laub et al., 2005; (Lei et al., 2006; Kajimura et al., 2007).

1.6.2 Regulation of Gene Expression: DNA Methylation and Histone Acetylation

All cells in the body are genetically identical; nevertheless we encounter a huge variety of different cell types, especially in the nervous system. This variety is in part established by epigenetic regulation via chromatin modifications such as DNA methylation, which occurs predominately at CpG nucleotides such as 5-methylcytosine (5mC). Differential DNA methylation within promoter regions leads to repression of gene transcription (Herman and Baylin, 2003). DNA demethylation occurs in cell reprogramming events that induce pluripotency (Simonsson and Gurdon, 2004; Jullien et al., 2011; Mikkelsen et al., 2008).

Retinal regeneration in teleosts such as zebrafish is characterized by dedifferentiation and proliferation of Mueller glia to produce new neurons and glial cells (Fausett and Goldman, 2006; Raymond et al., 2006; Bernardos et al., 2007; Fimbel et al., 2007; Fausett et al., 2008; Ramachandran et al., 2010) possibly involving demethylation of DNA (Powell et al. 2012).

Apobec proteins have been shown to be involved in the demethylation of DNA during zebra fish development (Rai et al., 2008; Guo et al., 2011; He et al., 2011). The expression of apobec2a and apobec2b has been shown to be induced during the regeneration of Mueller glia cells in zebra fish following injury

(Powell et al. 2012). Apobec2b knockdown led to a reduction in reprogramming of Mueller glia after injury and Apobec2a knockdown reduced retina regeneration (Powell et al. 2012). These findings indicate an involvement of DNA demethylation during axon regeneration; however direct evidence has yet to be provided.

Histone modification is another way to regulate transcription. Histone and transcription factor acetylation is regulated by histone acetyltransferases and deacetyltransferases that modulate chromatin structure (Yang and Seto, 2007). Developmentally active genes are often times silenced in adulthood by specific histone modifications, which lead to repression of mRNA transcription for certain developmentally active transcription factors. Overexpression of histone acetyltransferase p300 in mature RGCs was able to enhance optic nerve regeneration by reactivating the expression of developmentally regulated RAGs *in vitro* (Gaub et al., 2010) and *in vivo* (Gaub et al., 2011).

1.6.3 Regulation of Gene Expression: Posttranscriptional Regulation:

1.6.3.1 microRNAs

mRNAs have been shown to localize in axons and dendrites to enable local protein synthesis in response to axon guidance molecules, neurotransmitters, growth factors and injury signals (Piper and Holt, 2004; Sutton and Schuman, 2006; Bramham and Wells, 2007; Lin and Holt, 2007). The transport and translation of mRNA within neurites is mediated by mRNA-binding proteins (e.g. zipcode binding protein 1 (ZBP1) and cytoplasmic polyadenylation

element-binding protein (CPEB)) (Bassell and Kelic, 2004; Richter, 2007; Bassell and Warren, 2008).

microRNAs (miRNA) are 17-25 nucleotide long non-coding RNAs that regulate mRNA translation and metabolism (Eulalio et al., 2008; Fabian et al., 2010). miRNAs have been found in axons and dendrites, with recent findings that implicate them in the modulation of local gene translation during development and axon regeneration (Natera-Naranjo et al., 2010; Dajas-Bailador et al., 2012; Zhang et al., 2013; Sasaki et al., 2013). miRNAs play important roles in neuronal precursors, controlling neurogenesis (Fineberg et al. 2009; Liu and Zhao 2009; Li and Jin 2010), regulating dendritic branching, axon path finding and dendritic spine morphology (Davis et al., 2008) as well as synaptic function in mature neurons (Vo et al. 2010; Siegel et al. 2011). *In vitro* miRNAs have been shown to control axon outgrowth from embryonic neurons (Dajas-Bailador et al. 2012; Franke et al. 2012). miR-134 for example controls expression of LIMK, which controls growth cone collapse and dendritic spine formation (Schratt et al., 2006). miR-138 has been reported to suppress axon regeneration via its downstream effector SiRT1 (NAD-dependent histone deacetylase) in cortical neurons *in vitro*. SiRT1 on the other hand inhibits miR-138 expression in a feedback loop in response to peripheral nerve injury (Liu et al., 2013)

1.6.3.2 Methylation and Degradation

Over 100 hundred RNA modifications have been identified to date of which two thirds are methylations (Cantara et al., 2011; Czerwoniec et al., 2009). Methylation of all different kinds of RNAs occurs on either nitrogen or carbon

atoms of the bases or on the 2'hydroxyl group in the sugar moiety (Dunin-Horkawicz et al., 2006; Rozenski et al., 1999). It is estimated that 1-2% of the genetic information for all coding sequences is devoted to RNA modifications (Bjork et al., 1995). The methylation of RNA is mitigated by RNA-MTases, a group of enzymes that include >60 members categorized into 4 super families (Czerwoniec et al., 2009). The phenomena of methylation of the RNA cap-structure, which is necessary for transcriptional initiation, is widely studied. The function of other methylation sites in the mRNA is a topic of current investigations. So far no link between RNA methylation and axon regeneration has been reported. It is however more than likely that RNA methylation influences axon regeneration since they can control axonal mRNA and miRNA transport and activity.

Beside posttranscriptional modification, mRNA stability is regulated by sequences in the 3'UTR of the mRNA. In the case of neurofilaments, 3'UTRs are well conserved, even more so that the promoter regions, indicating that neurofilament transcriptional regulation is preferentially mediated by RNA stability rather than transcription (Thyagarajan and Szaro, 2004). In PC12 cells, NGF stimulated neurite outgrowth stabilized the 3.5kb isoform of neurofilament light chain mRNA but not the 2.3kb isoform, which led to a faster degradation of the 2.3kb mRNA isoform and increased translation of the 3.5kb mRNA isoform (Ikenaka et al., 1990)

Robo mRNA is expressed equally in all neurons of the nerve cord in drosophila. However Robo protein is highly expressed in the longitudinal axon

tracts but almost absent from the commissural neurons. The local presence of Robo receptors is a key element of the interaction with slit and directly influences axon midline crossing (Kidd et al., 1998). In precerebellar neurons in mice, posttranscriptional regulation of Robo3 is regulated by the RNA-binding protein Musashi1 (Kuwako et al., 2010).

Epidermal growth factor (EGF) stimulation of axonal outgrowth during development has been linked to the posttranscriptional regulation of the kappa opioid receptor (KOR) via the KOR 5′-UTR-binding protein, Grb7 (Tsai et al., 2010). GAP43 protein expression has been shown to be influenced by GAP43 mRNA stabilization via interaction with PKC and other 3'UTR-mRNA binding proteins (Perrone-Bizzozero et al., 1993; Kohn et al., 1996; Chung et al., 1997; Irwin et al., 1997; Tsai et al., 1997).

Several proteins have been shown to be involved in β-actin mRNA transport and regulation, such as the survival of motor neuron (SMN) protein (Rossoll et al., 2003). Actin mRNA is transported down the axon for local protein synthesis at the growth cone or sites of synaptic contacts. The spatial and temporal regulation of actin mRNA and its influence of gene transcription, cell motility and axon regeneration have been widely studied (Sotelo-Silveira et al., 2006; Louvet and Percipalle, 2009).

1.6.4 Regulation of Gene Function: Posttranslational Regulation.

A variety of posttranslational modifications have been identified such as the addition of functional groups (e.g. acetate, phosphate, carbohydrates and lipids), chemical alteration of amino acids (e.g. citrullination), 3-dimensional

structural changes (disulfide bridges) and removal of amino acids or peptidolysis. These modifications influence the function and interaction of the modified protein. Others determine the lifetime of proteins and thereby their temporal activity with a cell. Protein degradation can be achieved in different ways such as proteasome degradation, lysosome degradation and macroautophagy (Wang et al., 2013).

Most proteins are influenced by posttranslational modifications. Consequently proteins that influence axon regeneration are likely to be functionally affected by these modifications. Transcription factor activity can be regulated by phosphorylation, acetylation and ubiquitination, as in the case of TP53 (Tedeschi and Di Giovanni, 2009; Lavin and Gueven, 2006) or STAT3 (McDonald and Reich, 1999; Paulson et al., 1999; Shankaranarayanan et al., 2001; Yuan et al., 2005; Lim and Cao, 2006). The phosphorylation state of many others directly regulates their axon regeneration capacity, such as NfκB (Gutierrez et al., 2008), CREB (Gao et al., 2004) and c-Jun (Dragunow et al., 2000). The importance on posttranslational modifications on transcription factor activity is highlighted by the overexpression of two TP53 mutant forms. An acetylation mimic at amino acid 320 leads to increased neurite outgrowth and a non-acetylateable form to decreased neurite outgrowth, possibly controlled by effects on GAP43 expression (Tedeschi et al., 2009)

Tubulins are important structural molecules whose regulation of dynamic instability is critical for axon regeneration. Tubulins are acetylated and tyrosinated. In neurons, acetylated microtubules are mostly found in the axon hillock and represent stable, long lived microtubules that are resistant to

nocodazole depolymerization (Baas and Black 1990; Ahmad et al. 1993; Brown et al. 1993). Almost all α-tubulin subunits get tyrosinated directly after translation, a process where one tyrosine residue is added to the C-terminus. These tyrosinated subunits are incorporated in microtubules and over time become detyrosinated, which leaves them with a glutamine residue at the C-terminus (glutamylated tubulin) (Gundersen et al. 1987). Further modifications are deglutamylation after detyrosination by removing the C-terminal glutamine residue (Paturle-Lafanechere et al. 1991; Lafanechere and Job 2000). Deglutamylated tubulins are mostly found in the brain in differentiated neurons and represent stable, long-lived microtubules (Paturle-Lafanechere et al. 1994). The modification itself does not influence microtubule stability but rather the binding affinity of microtubule associated proteins (e.g. Tau, MAP2) that modify microtubule dynamics and stability.

1.6.5 Expressed Genes and Signaling Pathways Involved in Regeneration

Similar to axon injury in the PNS, in the CNS retrograde injury signaling occurs in waves: (1) Increase in intracellular calcium as a response to injury induces axonal action potentials that trigger various responses in the soma (Mandolesi et al., 2004), and (2) decreased trafficking of trophic factors and increased trafficking of injury-induced molecular signals to the soma created by changes in local protein translation at the injury site (Ambron and Walters, 1996; Rishal et al., 2010; Newbern et al. 2009)

1.6.5.1 Cell Body Response: Chromatolysis

Chromatolysis is an acute response within the neuronal cell bodies in response to axon injury of regenerative PNS and non-regenerative CNS neurons (Brodal 1981, Kreutzberg 1982, Lieberman 1971). Chromatolysis is characterized by a displacement of the nucleus to the periphery of the cell, dispersal of the Nissl substance, loss and retraction of synaptic terminals and a swelling of the cell body. After the initial phase of chromatolysis that is similar in regeneration competent and incompetent neurons, structural changes vary drastically. Cell bodies of regeneration competent neurons show signs of increased metabolism and protein synthesis characterized by increased free ribosomes and other organelles as well as maintained hypertrophy (Kreutzberg 1982, Lieberman 1971). Regeneration incompetent neuronal cell bodies on the other hand show reduced cell body size and dendritic arborization as well as signs of atrophy (Rossi et al. 1995).

1.6.5.2 PI3K-Akt Pathway

The serine/threonine kinase Akt has been shown to be involved in survival, metabolism, proliferation and differentiation as well as several aspects of neurite outgrowth, such as elongation, branching and caliber (Manning et al., 2007; Huang et al., 2003). There are 3 isoforms of Akt (Akt1, Akt2, Akt3), which consist of an N-terminal pleckstrin (PH) homology domain, a kinase domain and a C-terminal regulatory domain (Jones et al., 1991; Nakatani et al., 1999). Akt is located in an inactive state in the cytosol and can be activated by phosphorylation via transmembrane receptors. Akt is mostly regulated by

phosphatidylinositol 3-kinase (PI3K). PI3K gets recruited to the plasma membrane where it facilitates the phosphorylation of membrane phospholipid phosphatidylinositol (4,5)-diphosphate (PIP2), converting it to phosphatidylinositol (3,4,5)-triphosphate (PIP3). Akt translocates to the plasma membrane and binds PIP3, leading to phosphorylation and activation of Akt by the phosphoinositide-dependent kinase 1 (PDK1) (Alessi at al., 1996; Anderson et al. 1998). PI3K also regulates the small GTPases Rac and Cdc42 (Raftopoulou & Hall 2004), key regulators of cytoskeletal reorganization. Additional phosphorylation by mTOR leads to further activation of Akt (Sarbassov et al., 2005; Jacinto et al., 2006). Targets of Akt are CREB (Du et al. 1998), peripherin (Konishi et al., 2007), β-catenin (Kim et al., 2008), GSK3β (glycogen synthase kinase 3β) (Cross et al., 1995; Salas et al., 2003; Gong et al., 2005) and mTOR (Asnaghi et al., 2004; Nave et al., 1999). PI3K-Akt signaling is activated by NGF, BDNF and NT3 (Huang et al., 2003). Akt activity has been linked to enhanced neurite outgrowth in a variety of neuronal cells (Read and Gorman, 2009). GSK3β inactivation by AKT phosphorylation e.g. has been shown to stimulate neurite outgrowth (Kim et al. 2004; Ooms et al., 2006) via regulation of MAP2 phosphorylation influencing microtubule stability (Lim et al., 2008). PTEN phosphorylates GSK3β counteracting the activity of Akt (Zhou et al., 2004). Akt also acts directly on microtubule dynamics via the phosphorylation of Tau (Ksiezak-Reding et al.2003) and actin dynamics via Girdin (Enomoto et al., 2005) and Ezrin (Shiue et al., 2005).

1.6.5.3 Raf-MEK-Erk Pathway

The Raf-MEK-Erk pathway lies downstream of neurotrophic factor signaling via receptor tyrosine kinases (RTKs) and is involved in axon assembly (Atwal et al. 2000). During development, Erk is phosphorylated in response to netrin (Forcet et al. 2002), the neuronal cell adhesion molecule NCAM (Schmid et al. 2000) and semaphorin 7A (Pasterkamp et al. 2003). Erk has been shown to influence axon microtubule dynamics by phosphorylating MAP2 (Ray & Sturgill 1987) and also being involved in the regulation of actin dynamics (Atwal et al. 2003). Erk has been shown to be involved in local protein synthesis in the growth cone, mediating growth cone collapse in response to Semaphorin 3A (Campbell & Holt 2003). It could also be involved in the local translation of growth promoting molecules in the growth cone such as GAP43 (Smith et al. 2004); the actin binding protein cofilin (Willis et al. 2005) and β-actin (Bassell et al. 1998). Erk has been shown to regulate transcriptional regulators involved in axon regeneration, such as CREB and nFATc (Xing et al. 1998; Hogan et al. 2003). Increased neurite outgrowth of adult DRG neurons in response to LRP1 signaling has been shown to be mediated by Erk phosphorylation *in vitro* (Yoon et al., 2013). Erk inhibition resulted in annulation of BDNF stimulated neurite outgrowth in DRG neurons overexpressing the BDNF receptor trkB (Hollis et al., 2012). However most of these findings are from *in vitro* experiments and thus the involvement of Erk in axon regeneration *in vivo* remains to be investigated.

1.6.5.4 JAK-STAT Pathway (see also Chapter 1.6.1.4)

The glycoprotein 130 (gp130) family of cytokines acts via heterodimerization of signaling receptors. The gp130-receptor is always a component of the receptor complexes. In peripheral axotomized neurons, the gp130 signaling functions as an injury signal. Many structurally related ligands of the gp130 receptor complex have been shown to influence axon regeneration such as, LIF, IL-6, CNTF and oncostatin M (Taga and Kishimoto, 1997). Gp130 signaling after injury is necessary for regeneration associated neuropetide expression such as VIP and PACAP in superior cervical ganglion (SCG) neurons (Habecker et al., 2009). Upregulation of other RAGs after peripheral injury rely on gp130 signaling such as damage-induced neuronal endopeptidase (DINE) induced by LIF (Kiryu-Seo et al., 2000; Boeshore et al., 2004; Kato et al., 2002) and peripherin induced by IL-6 and LIF (Oblinger et al., 1989; Troy et al., 1990; Wong and Oblinger, 1990; Lecomte et al., 1998; Sterneck et al., 1996; Lecomte et al., 1998), as well as GAP43 induced by IL-6 (Cafferty et al., 2004). STAT3 phosphorylation is delayed in CNTF deficient mice after peripheral nerve injury. Some evidence indicates that the STAT3 activation via the gp130/JAK pathway is only present in peripheral but not central neurons (Schwaiger et al., 2000; Qiu et al., 2005)

Gp130 cytokines signal primarily through the activation of the Janus kinase (JAK) and the transcription factors of the STAT family but have also been reported to activate the Ras/MAPK/ERK pathway to regulated gene expression (Taga and Kishimoto, 1997). IL-6-induced allodynia in mouse hind paws has

been shown to signal via Erk phosphorylation leading to activation of eukaryotic initiation factor 4E (eIF4E) mediating enhanced protein translation (Melemedjian et al., 2010).

Pharmacological inhibition of Jak but not Erk has been shown to abolish the conditioning effect of adult DRG neurons *in vitro* (Liu and Snider, 2001) and vivo (Qiu et al., 2005). cAMP injection into DRG neurons that mimics the conditioning effect raises mRNA levels of IL6 and LIF (Wu et al., 2007), however functional blocking antibodies against gp130 could not interfere with the effect on enhanced neurite outgrowth (Cao et al., 2006).

SOCS (suppressors of cytokine signaling) family members are rapidly upregulated in response to cytokine signaling and inhibit the Jak/Stat pathway (Wang and Campbell, 2002; Heinrich et al., 2003). Deletion of SOCS3 in RGC led to gp130-dependent axon regeneration after crush injury (Smith et al., 2009). The application of CNTF further improved axon regeneration. This effect was not seen in double mutant mice for gp130 and SOCS3.

1.6.5.5 Cytoskeletal Proteins

Cytoskeletal proteins play important roles in all aspects of neuritogenesis, such as neurite initiation, growth cone formation and neurite elongation and branching. There are 3 major classes of cytoskeletal proteins in the nervous system. Microtubules, neurofilaments and actin (microfilaments), all of which have distinct roles during axon elongation, maturation and regeneration after injury (Coulombe et al., 2000; Perrot et al., 2008; Walker et al., 2001). After peripheral nerve injury, regeneration associated cytoskeletal proteins, such as

tubulin and actin isoforms are upregulated, where neurofilament isoforms are downregulated in DRG neurons (Bomze et al., 2001; Bulsara et al., 2002). During the development of the nervous system the expression pattern is similar: when NF expression is upregulated, actin and regeneration associated tubulin isoforms are downregulated (Hoffman et al., 1989)

Neurofilaments (NF) consists of peripherins (a type III IF), α-internexins and the NF triplet proteins (light, medium and heavy chain) (Chinnakkaruppan et al., 2009; Asch et al., 2000; Carter at al.,1998; Lee at al., 1993; Ching et al. 1993; Leterrier et al., 1987; Scott et al., 1985). The spatiotemporal expression and regulation via phosphorylation of neurofilaments subunits is critical since transgenic mice with altered stoichiometry of NFs subunits show the formation of NF aggregates resembling human diseases such as amyotrophic lateral sclerosis (ALS) (Xiao at al., 2006; Bruijn et al., 2004; Lariviere et al., 2004). NF light and medium chains are expressed earlier in axonal development and NF heavy chain is only expressed in large diameter axons, correlating with increases in the velocity of axonal transport. NF light, heavy and intermediate chain isoforms are downregulated after sciatic nerve injury (Wong and Oblinger, 1990), whereas peripherin is upregulated in DRG neurons upon regeneration, resembling developmental expression patterns (Oblinger et al., 1989; Troy et al., 1990; McGraw et al., 2002). Regenerating xenopus RGCs on the other hand show increased NF expression (Gervasi et al., 2003)

Microtubules are hollow tubes that consist of the assembly of mostly α/β-tubulin dimers. They display polarity with a fast growing (+) end pointing towards

the synapse and a slow growing (-) end pointing towards the soma (Desai et al., 1997). Tubulins are categorized in 5 distinct families (α, β, γ, δ, ε- tubulins); however, α and β subunits are most common. βIII-tubulin is neuron specific and upregulated after peripheral nerve injury. α1-tubulin and β2-tubulin have also been found to be upregulated in adult DRG neurons after peripheral nerve lesion (Hoffmann, 1989; Miller et al., 1989; Costigan et al., 2002; Hoffman and Cleveland, 1988). β2-tubulin has been shown to interact with the E3 ubiquitin-protein ligase ZNRF1 to potentially induce neurite formation (Yoshida et al., 2009). When cAMP concentration is increased in DRG neurons via db-cAMP injection, βIII-tubulin expression is upregulated similar to a conditioning lesion (Han et al., 2004). α1-tubulin is also upregulated in injured rubrospinal and facial nerves (Tetzlaff et al., 1991) and β-tubulin mRNA is upregulated during regeneration of RGC in mammals (McKerracher et al., 1993).

Actin is an essential component of growth cone initiation, axon elongation and branching as well as synapse formation. Actin expression is upregulated after sciatic nerve injury in adult DRG neurons and associated with axon regeneration. Expression of axonally targeted β-actin mRNA has been shown to increase neurite branching in sensory axons within the chick spinal cord *in vivo* (Donelly et al., 2013). Actin has been shown to interact with other neurite outgrowth promoting molecules after sciatic nerve crush injury, such as SPRR1A (Bonilla et al., 2002) and HSP27 (Williams et al., 2006)

1.6.5.6 GAP43

Growth associated protein (GAP43/B50) expression in neurons correlates with axon elongation, synaptogenesis and sprouting during development and adulthood (Skene 1989; Bisby and Tetzlaff 1992). After peripheral nerve injury, GAP43 expression is upregulated and remains elevated until the axons reinnervate the sensory receptors. GAP43 has been shown to modulate growth cone formation and stability (Aloyo et al., 1982, Tetzlaff et al., 1989, Coggins and Zwiers, 1991 and Strittmatter et al., 1994). The upregulation of GAP43 occurs in regenerating DRG neurons in mice and rats as well as in regenerating RGC neurons in zebrafish (Kaneda at al., 2010). GAP43 deficient mice show path finding errors of RGC axons during development but DRG cultures from those mice show no alterations in axonal outgrowth (Stritmatter et al., 1995). However GAP43 knockdown in chick DRG displayed reduced neurite outgrowth *in vitro* (Donelly et al., 2013). Transgenic mice overexpressing GAP43 show increased sprouting of axons at the neuromuscular junction (Aigner et al., 1995). GAP43 overexpression in non-neuronal cells has been shown to induce neurite formation. Expression of axonally targeted GAP43 mRNA has been shown to increase neurite outgrowth in sensory axons within the chick spinal cord *in vivo* (Donelly et al., 2013).

1.6.5.7 CAP23

CAP23 is a cytoskeleton-associated and calmodulin binding protein that is widely expressed during development but mostly restricted to neurons in adulthood (Widmer and Caroni 1990). Cap23, similar to GAP43, is upregulated

after peripheral nerve injury and overexpression of CAP23 results in increases sprouting similar to that seen with GAP43 overexpression (Caroni et al. 1997). CAP23 deficient mice show deficits in stimulus induced sprouting at the neuromuscular junction in adulthood. Replacement of CAP23 with GAP43 resolved this phenotype indicating that both proteins share similar functions and can substitute for one another (Frey at al., 2000). CAP23 also modulates growth cone morphology. Transgenic mice overexpressing both GAP43 and CAP23 exhibit vastly increased DRG sensory axon regeneration (Bomze et al., 2001).

1.6.5.8 cAMP

3'-5'-cyclic adenosine monophosphate (cAMP) is developmentally regulated and its expression declines in later stages of vertebrate embryogenesis in Xenopus RGC (Shewan et al., 2002) and rat DRG neurons (Caiet al., 2001). After sciatic nerve injury, cAMP levels are increased by 3-fold up to one week. If dibutyryl-cAMP, a non-hydrolysable form of cAMP is injected into DRG neurons with a central only lesion, regeneration of the central branch is comparable to the regeneration induced by the conditioning effect (Neumann and Woolf, 1999). The elevated cAMP levels overcome neurite outgrowth inhibition by MAG or myelin (Qui et al., 2002; Neuman et al., 2002), and they trigger a switch from growth cone repulsion to attraction when encountering these substrates (Song et al., 1998). Combinatorial approaches elevating cAMP in DRG neurons and providing a NT3 gradient in the spinal cord lead to long distance sensory axon regeneration through a cervical injury site that was dependent on both manipulations (Lu et al., 2004). The effects of elevated cAMP on axon outgrowth

have been associated with an activation of protein kinase A (PKA) leading to CREB activation and increased expression or Arginase 1 (Cai et al., 2002). Arginase I promotes the synthesis of polyamines such as putrescine. Arginase I expression and thereby polyamine synthesis is upregulated after sciatic nerve injury in adult DRG neurons. If the polyamine synthesis is blocked the conditioning effect is partly inhibited. The conversion of putrescine to spermidine seems to be a key event in this mechanism. Further, spermidine promotes optic nerve regeneration *in vivo* and aids to overcome MAG inhibition *in vitro* (Deng at al., 2009).

1.6.5.9 DLK1

DLK1 (dual leucine zipper kinase 1) MAP kinase pathway was identified as an important regulator of axon regeneration in C. elegans via its effect on the formation of growth cones (Yan et al. 2009). Other members in the MAPK pathway are the MAPKK MKK-4 and the p38 kinase PMK-3 (Nakata et al., 2005). It has also been shown that DLK1 is important for axon regeneration but not for axon development (Hammarlund et al. 2009). DLK1 induces stabilization of CEBP-1 mRNA in axons following injury (Yan et al. 2009). In mice, C/EBPβ is also induced after injury activating the transcription of α-tubulin and GAP-43 (Korneev et al. 1997, Nadeau et al. 2005). This indicates that the local translation of regeneration associated tubulins and growth associated proteins could be responsible for growth cone formation after axon lesion in a DLK1 dependent pathway.

1.6.5.10 Neuropeptides

A variety of neuropeptides have been reported to be upregulated after injury in several neuron types. Sciatic nerve injury causes upregulation of galanin, Neuropeptide Y (NPY), vasculointestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) (Xu et al. 1990; Noguchi et al. 1993; zhang et al., 1997). In sympathetic neurons galanin, VIP and substance P are upregulated after injury (Dumoulin et al. 1991; Habecker et al. 2009). Some of the neuropeptides have been identifies as downstream effectors of the regeneration activated transcriptional regulators. The deletion of STAT3 and c-Jun inhibits upregulation of galanin and CGRP in facial motor neurons (Raivich et al.2004; Ruff et al. 2012). Deletion of galanin led to reduced axon regeneration after sciatic nerve crush (Holmes et al. 2000) and interference with CGRP expression led to reduction of axon growth between the distal and proximal nerve stump (Toth et al. 2009). VIP and galanin have been shown to stimulate neurite outgrowth of PC12 cells *in vitro* (Deutsch et al., 1993; Okumura et al., 1994; Klimaschewski et al., 1995). Galanin also stimulates neurite outgrowth of adult and PACAP of embryonic DRG neurons in culture (Mahoney et al., 2003; Suarez et al., 2006; Lioudyno et al., 1998).

1.6.5.11 mTOR Pathway

PTEN (tumor suppressor phosphatase and tensin homolog) deletion has been shown to promote axon regeneration of RGC (Park et al. 2008) and regeneration and sprouting of CST axons (Liu et al., 2010) in mice *in vivo*. Application of the mTOR (mammalian target of rapamycin) inhibitor rapamycin

abolished the effect of PTEN deletion on axon regeneration in RGCs (Park et al. 2008), indicating that mTOR signaling acts downstream of PTEN to regulate axonal growth. siRNA mediated PTEN knockdown increases axon outgrowth of motor neurons *in vitro* (Ning et al., 2010). Overexpression of the mTOR activator Rheb promotes axon regeneration of dopaminergic neurons (Kim and Chen, 2011). Embryonic spinal cord derived NSCs grafted into an adult spinal cord lesion showed a 50% reduction of graft derived axons extending into the host matter when treated with rapamycin (Lu et al., 2012)

In the PNS, the deletion of PTEN or the mTOR inhibitor TSC2 (tuberous sclerosis complex 2) increases sensory axon regeneration of adult DRG neurons via activation of the mTOR pathway *in vitro* and *in vivo* (Abe et al. 2010, Christie et al. 2010). Similarly, inhibition of the mTOR pathway by rapamycin reduces axon regeneration of adult DRG axons *in vitro* in response to a conditioning injury.

mTOR has two direct downstream targets S6K1 (p70-S6 Kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E (eIF4E) binding protein 1). Phosphorylation of S6K1 and 4E-BP1 leads to an activation of transcription (Dobashi, et al., 2011; Watanabe et al., 2007; Miwa et al., 2011; Proud et al., 2007). Inhibition of transcription via cyclohexamide has been shown to abolish axon regeneration in response to a condition injury.

The results on axon regeneration of the CST upon PTEN deletion are promising; however they have to be critically interpreted. PTEN was deleted at the age of 4 weeks and not during adulthood. This could lead to an extension of developmental growth programs rather than promoting real adult CST

regeneration. This extension of embryonic growth programs could partly account for the observed increase in CST axon regeneration at adulthood. Dr. Samara from our lab who is a co-author on the study from Liu et al., 2010 failed to replicate a positive regenerative effect of PTEN deletion in a dorsal column lesion model when cre-mediated PTEN deletion was performed at adulthood (Samara et al., manuscript in preparation).

1.6.5.12 Notch Signaling Pathway

Notch signaling not only controls the development of a variety of cells in almost all tissues in the body of all metazoans, including mammals, during development via cell to cell signaling (Artavanis-Tsakonas et al., 1999) but also has important functions in survival, maintenance and differentiation of stem cells (Androutsellis-Theotokis et al., 2006), as well as axon growth and guidance and synaptic function (Ables et al., 2011; Giniger et al., 1993). Furthermore, notch signaling has been shown to influence axon regeneration (El Bejjani and Hammarlund, 2012). So far 4 different single pass transmembrane notch receptors are known (notch1-4) that interact with a variety of ligands including the notch receptors themselves and delta-like proteins (Ables et al., 2011; Kopan et al., 2009; D'Souza at al., 2010) as heterodimers. In the canonical notch pathway, ligand binding to the notch rector leads to proteolytic cleavage of the notch receptor and the intracellular domain is transported to the nucleus to alter gene transcription (Ables et al., 2011).

Notch1 expression is increased after rat spinal cord injury and expression of CA-notch1 leads to inhibition of the differentiation of neuronal precursors into

neurons and glial cells *in vitro*, indicating that after spinal cord injury the differentiation of adult stem cells is inhibited via a notch dependent pathway (Yamamoto et al., 2001).

Increased notch activity in cortical neurons has been shown to reduce axon growth and axon retraction *in vitro* (Sestan et al., 1999), possibly via the modulation of microtubule dynamics (Ferrari-Toninelli et al., 2008). Similarly in c.elegans notch signaling has been shown to inhibit axon regeneration (El Bejjani and Hammarlund, 2012).

Over expression of notch1 which is expressed at high levels during development and low levels in adulthood, lead to reduced neurite outgrowth in hippocampal neurons *in vitro* (Berezovska et al., 1999). Notch can also signal via the non-canonical notch pathway which has been shown to regulate axon guidance in drosophila (Le Gall et al., 2008). In addition, notch signaling has also been involved in the dedifferentiation of Schwann cells after peripheral nerve injury highlighting its significance in several aspects of axon regeneration (Mirsky et al., 2008)

1.6.6 Conditioning Lesion

One of the most impressive degrees of central axon regeneration can be observed specifically in sensory axons emerging from dorsal root ganglion (DRG) neurons within the conditioning paradigm (Richardson & Issa, 1984, Neumann and Woolf, 1999).

When sensory axons of the sciatic nerve are injured, they create a retrograde injury signal to the cell body, which leads to the activation of

regeneration associated genes (RAGs). These RAGs are necessary for peripheral axon regeneration and are expressed as early as a few hours after injury up to several weeks until the regenerating axons re-innervate the sensory receptors. Regulation of these RAGs include upregulation of neuropeptides, transcription factors like ATF3, Sox11, C-Jun, JunD, STAT3 and growth associated proteins like GAP43 and tubulins as well as down regulation of transcription factors like ATF2 and other proteins like neurofilaments and neurotransmitters (Schreyer & Skene, 1993, Costigan et al., 2002; Jenkins and Hunt, 1991; Leah et al., 1991; Herdegen et al., 1992; Martin-Villalba et al., 1998; Schwaiger et al., 2000; Tsujino et al., 2000; Tanabe et al., 2003; Lee et al., 2004; Jankowski et al., 2006; Seijffers et al., 2007).

If this peripheral nerve injury occurs one to two weeks prior to a central lesion within the spinal cord of the same DRG neuron, then enhanced regeneration of the central branch is visible. This phenomenon is called the “conditioning effect”, since the adult DRG neurons are conditioned for central branch regeneration by a preceding peripheral branch injury. This enhanced regeneration is a result of altered gene expression and abolished upon inhibition of DNA transcription (Smith & Skene, 1997; Qiu et al., 2002)

The condition effect shows enhanced central branch regeneration *in vivo* and increased neurite outgrowth of DRG explant or dissociated cultures *in vitro*. Elevation of cAMP has been shown to be key aspect of the conditioning effect and can be mimicked by the injection of a non-hydrolysable form of cAMP into DRG neurons with a central only lesion (Neumann and Woolf, 1999). Gp130

cytokines also play a crucial role in the conditioning effect which is abolished in noradrenergic autonomic neurons that are deficient in gp130 (Hyatt et al., 2010)

A similar conditioning effect has been described in RGCs of the CNS. When a lens injury precedes the lesion of RGC axons, their regeneration is increased (Yin et al., 2003). One identified factor is the release of oncomodulin by macrophages that has been shown to be sufficient to promote RGC axon regeneration via a Ca2+/calmodulin-dependent pathway (Yin et al., 2003). Lens injury further leads to secretion of CNTF by retinal astrocytes resulting in increased expression of STAT3 in the RGCs (Muller at al., 2000; Park et al., 2004). The regeneration-associated transcriptome of lens injury induced axon regeneration of RGCs is similar to that of conditioned DRG neurons (Leon *et al.*, 2000; Fischer *et al.*, 2001; Fischer *et al.,* 2004).

1.6.7 Retrograde Injury Signal: β-importins

Regeneration of axons has been shown to be dependent on transcription and translation in the neuronal cell body and the axon (Caroni, 1998; Goldberg, 2003; Snider et al., 2002). This change in transcriptional regulation has to be triggered by a signaling cascade stemming from the injured axon being transmitted to the nucleus. Three retrograde signaling methods have been reported: (1) rapid response to the injury by injury-induced action potentials (2) interruption of retrograde transport of molecules from the distal end (3) retrograde transport of signaling molecules from the proximal axon stem (Enes et al., 2010; Udina et al., 2008; Perlson et al., 2004).

The third mechanism of retrograde signaling has been shown to be mediated in part by members of the importin/karyopherin family within hours of the injury. These soluble transport factors mediate translocation of substrates through the nuclear pore complex (Chook and Blobel, 2001; Gorlich and Kutay, 1999). The interaction of these factors with their substrates is mediated by the binding of nuclear localization signals (NLS). Importin-α as a monomer has been shown to bind to NLS with low affinity, but with high affinity as a heterodimer with importin-β (Jans et al., 2000; Kohler et al., 1999).

Importin-β protein levels increase via local mRNA translation in the axon at the sites of injury, thereby leading to the local formation of the importin-α/β-heterodimer (Giuditta et al., 2002; Steward, 2002). This heterodimer uses the motor protein dynein for retrograde axonal transport to the cell body and then translocates into the nucleus to initiate changes in gene transcription (Hanz et al., 2003). Application of a NLS-peptide that competes with NLS-protein binding has been shown to nearly completely abolish the conditioning effect, supporting the role of NLS transport based retrograde injury signaling as a key part of the conditioning effect (Hanz et al., 2003).

1.6.8 Inflammation and Regeneration

Inflammation in the nervous system and axon regeneration are tightly coupled since injury ultimately leads to inflammation. Within hours after injury, innate immune cells such as neutrophils, dendritic cells and monocytes are recruited to the injury site leading to further recruitment of adaptive immune cells such as T and B lymphocytes after a few days. Monocytes differentiate into

macrophages that recruit further leukocytes and promote the remodeling of the ECM and revascularization. Macrophages also secrete cytokines that are important for the recruitment and survival of lymphocytes. Neutrophils are bacterial recognition cells that recruit monocytes. T and B lymphocytes have been shown to be essential for neuron survival and axon regeneration in the PNS (Serpe et al., 2002; Vargas et al., 2010).

Inflammatory cells have been shown to have a key function in removing myelin debris after sciatic nerve injury (Perry and Brown, 1992). Deletion of microglia, neutrophils and macrophages lead to reduced growth factor (NGF, BDNF, NT3, NT4/5) secretion and reduced axon regeneration of PNS axons or CNS axons in peripheral nerve grafts, despite the activation of GAP43, CAP23 and tubulin expression (Barrette et al., 2008). Injecting pro-inflammatory agents into DRG neurons can mimic the conditioning effect (Steinmetz et al., 2005; Lu et al., 1991).

Activated macrophage transplants from the peripheral nerve to an RGC promote axon regeneration after injury (Lazarov-Spiegler et al., 1996). The macrophage secreted factor oncomodulin has been shown to promote RGC axon regeneration *in vitro* (Cen at al., 2007; Yin et al., 2003; Yin et al., 2006) and *in vivo* (Kurimoto et al., 2010; Yin et al., 2009). Oncomodulin is upregulated by neutrophils and macrophages infiltrating the eye after lens injury (Luo et al., 2007). The positive effect on regeneration of the lens injury is completely blocked in mice that are deficient in both CNTF and LIF suggesting an essential role for

injury-induced cytokines during inflammation mediated RGC axon regeneration (Leibinger et al., 2009)

Macrophages that were pre-exposed to the injured sciatic nerve promoted spinal cord axon regeneration after transplantation (Rapalino et al., 1998). This treatment went into clinical phase II trials but was discontinued in 2006 due to the lack of funds and, likely marginal clinical effects (Jones et al., 2010). The inhibition of neutrophil and monocyte accumulation via transient antibody-mediated blockade of CD11d integrin improved functional recovery after SCI in rats (Saiwai et al., 2010) and interference with LTB4/BLT1 signaling, reducing neutrophil influx promoted motor recovery (Gris et al., 2004).

CD4+ cells have been shown to be necessary to promote CST axon regenerative sprouting through uninjured tissue following an NT3 gradient (Chen et al. 2008). We found that lesion of CST axons themselves is necessary for regenerative sprouting into a growth permissive NSC graft in rats (Kadoya et al., manuscript in preparation).

The immune response is critically linked to axon injury and regeneration beside positive effects on regeneration, inflammation also leads to the secretion of toxins and reactive oxygen and nitrogen species. There are clear differences between the immune response in the PNS and CNS that most likely influence the regeneration such as the removal of myelin debris after injury that is favored in the PNS.

1.7 CONCLUSIONS

Despite decades of research in spinal cord injury and over 50,000 publications in the field, there are no useful neuroprotective or pro-regenerative therapies. Several clinical trials in the last few decades have either failed or were discontinued for lack of perceived efficacy. Given the number of publications (~ 5000) claiming some functional recovery after injury in animal models, one might expect a different outcome.

The reason for the lack of therapies is likely the numerous mechanisms that limit axonal regeneration in the CNS. Several aspects have to be addressed, such as providing a permissive cell graft for axon growth, reestablishing neurotrophic gradients to guide regenerating axons, reactivating intrinsic growth profiles in the injured adult neurons combined with appropriate rehabilitation. But even after axons are guided back to their appropriate targets and form synapses, functional gain might not occur and spasticity of the muscles and increased pain perception might be the result.

Promising approaches using stem cell therapy have been emerging in the field over the last decade (~1500 publications). Stem cells bring several advantages, such as intrinsic growth ability, repopulation of the injury site and differentiation into glia and neurons to recreate the spinal cord environment. But they also harbor problematic aspects such as ethical concerns, the risk of tumor formation and stimulation of immune responses. Patient derived Induced pluripotent stem cells (IPSC) are a promising alternative to classical stem cell therapies since they are not subject to the same ethical debate as embryonic

stem cells and have the potential to be tolerated immunologically. It will probably take another decade or two to address the tumor formation issue of IPSCs and until we can apply IPSC stem cell therapies risk free in human clinical trials.

Nevertheless it is clear that moving forward we will need to apply combinatorial therapies that combine the power of stem cell grafts with gene and drug therapy to promote intrinsic axon regeneration into stem cells grafts that can than act as functional relays connecting injured axons with their appropriate targets.

Several intrinsic mechanisms of axon regeneration have been identified to date in phylogenetically diverse animal models such as c. elegans, drosophila, zebrafish and mammals. Many of the identified molecules and pathways promoting axon regeneration interact with one another and the more we learn about each individual pathway, the more we realize that most pathways are interconnected in several aspects. One of the challenges is to identify key mechanisms that are inherent to several axon regenerative pathways. Once the key molecules are identified, targeted gene therapy or specifically designed drugs can be explored as therapeutically relevant interventions.

Several intrinsic mechanisms have been reviewed in this introduction and can be summarized in a general concept of axon regeneration. Axon injury leads to retrograde injury signal transport to the cell body to alter gene transcription. Several mechanisms include: Fast electrical signals immediately at the time of injury, interruption of axonal transport from the synapse and active mRNA translation at the injury site and retrograde transport of synthesized molecules.

The incoming injury signal to the nucleus activates transcription factors (e.g. c-Jun, TP53, ATF3, STAT3, SOX11, NFATc, SnoN, KLF4 and NFκB) that regulate the expression of regeneration associated genes that in most cases act on cytoskeletal rearrangements, dynamics and stability, such as GAP43, CAP23, microtubule and actin binding proteins (e.g. Tau, MAP2, Arpc3) and cytoskeletal proteins (e.g. tubulin, actin and neurofilament isoforms). They further regulate the expression of cell adhesion molecules involved in axon guidance, such as NCAM, L1CAM, integrins and tenascins, as well as enzymes regulating polyamine synthesis necessary for axon growth such as arginase1. Beside the retrograde injury signal within the axon, the secretion of neurotrophic factors (e.g. BDNF, NGF, NT3, NT4/5, FGF, GDNF) and cytokines (e.g. CNTF, interleukines, LIF oncostatin) by glia and immune cells trigger signaling cascades that enable transcription factor activation and the expression of RAGS and neuropeptides (e.g. VIP, Galanin, NPY, PACAP and CGRP). These signaling cascades include the Pi3K-Akt-GSK3β, Raf-MEK-Erk, gp130-Jak-STAT and PTEN-mTOR pathways that have been shown to be involved in axon regeneration.

All the discovered signaling pathways to date rely heavily on the interaction of extrinsic factors with intrinsic signaling pathways, leading to intrinsic changes in gene expression that then promote axon regeneration. The regenerating axon itself is, throughout its regenerative growth phase, further interacting with extrinsic cues that modulate their behavior.

1.8 Corticospinal axon regeneration

1.8.1 Importance of cortical spinal tract (CST) in humans

In humans, the corticospinal tract (CST) is the most important projection controlling all voluntary motor functions. Uncountable attempts to regenerate the corticospinal tract have largely failed in the past decades, displaying a limiting factor in advancing potential regenerative therapies to human trials. Unilateral pyramidotomy, lesioning the corticospinal tract in humans leads to complete hemiparalysis, however in rat or mice it leads to impairment of fine finger grasping motions in the forelimb on the affected site (Ropper et al., 1979). The anatomical and functional difference of the corticospinal tract between mammals allow for (1) rigorous investigation of complete corticospinal tract lesions in rodent models due to the minor impact on functional deficits, but (2) has then to be further confirmed in non-human primate models before clinical translation is possible (Lemmon and Griffiths, 2005).

1.8.2 Refractory nature of the CST

Even though regeneration of supraspinal inputs from the cortex or brain stem have been shown to have a greatly reduced regenerative potential compared to axons of the peripheral nervous system, there are certain incidences where the regeneration of those CNS axons is stimulated. Axons emerging from the brain stem, such as the reticulospinal, rubrospinal and raphespinal tract display the intrinsic ability to regenerate after injury when provided with a permissive peripheral nerve graft (David & Aguayo 1981, Richardson et al. 1984). The corticospinal tract, however, failed to regenerate in

this model underlining the refractory nature of specifically the corticospinal compared to other supraspinal inputs.

However, if the spinal cord is injured during the developmental phase, before corticospinal axons have grown into the injury site, then corticospinal axons circumvent the injury site and innervate appropriate targets caudal to the injury (Bregman et al. 1989). This time window can be extended if fetal spinal cord grafts are presented to injured corticospinal axons (Bregman et al. 1989). This indicates that intrinsic embryonic growth mechanisms within the corticospinal neurons are active during this developmental window and allow for corticospinal axon regeneration, but are then lost in adulthood.

1.8.3 Sprouting of spared corticospinal axons

Spared corticospinal axons sprout at the lumbar level into NT3-expresssing grey matter after an acute injury of the corticospinal tract at the level of the medulla (Chen at al., 2008).

In the adult rat (Weidner et al., 2001) as well as in the adult rhesus monkey (Rosenzweig et al. 2010), partial lesion of the corticospinal tract leads to sprouting and midline crossing of spared corticospinal axons within the spinal cord. Weidner and colleagues showed further that dorsal corticospinal tract lesion leads to excessive spontaneous sprouting of the uninjured ventral corticospinal tract in rats, which correlates with recovery of skilled motor function and can be abolished by subsequent lesion of the ventral corticospinal tract (Weidner et al., 2001). Corticospinal axons have further been shown to spontaneously sprout and form functional synapses onto propriospinal

interneurons neurons within the lumbar cord after mid thoracic dorsal hemi section that are then substrate to function related axonal pruning (Bareyre at el., 2004). In the rhesus monkey, a C7 lateral hemi section leads to spontaneous sprouting of the contralateral corticospinal tract restoring axon density to 60% of normal levels, which correlates with substantial improvements in both hand function and locomotion (Rosenzweig et al. 2010).

1.8.4 Manipulation of the injury site to promote corticospinal regeneration

1.8.4.1 Myelin associated inhibitors

Myelin associated inhibitors, such as Nogo, the myelin associated glycoprotein (MAG) and the oligodendrocytes myelin glycoprotein (OMgp) have been shown to inhibit axonal regeneration in vitro and in vivo (Yiu and He et al., 2006). Several studies have investigated the effects on corticospinal axon regeneration in vivo.

Nogo-A deficient mice fail to show increased corticospinal axon regeneration (Zheng et al. 2003; Lee et al. 2009). Although controversial, inhibition of Nogo-A by functional blocking antibodies has been reported to increase regeneration and sprouting of corticospinal axons (Schnell and Schwab, 1990; Bregman et al. 1995; Thallmair et al. 1998).

In an OMpg knockout mouse sprouting of serotonergic raphespinal axons was increased after injury, however corticospinal sprouting or regeneration was not affected (Ji et al., 2008).

MAG knockout did not show any increased optic nerve or corticospinal axon regeneration (Bartsch et al., 1995).

Triple knockout mouse of MAG, Nogo and OMpg lead to increased compensatory sprouting of uninjured serotonergic raphespinal axons after T8 lateral hemisection, but no increase sprouting of spared corticospinal axons after unilateral pyramidotomy. Both raphespinal and corticospinal axons failed to regenerate after lesion (Lee et al., 2010). Another study investigated sprouting of corticospinal axons after a T8 dorsal hemisection in the triple KO-mouse (NogoA/B, MAG, OmpG) and reported increased number of corticospinal axons caudal to the lesion site compared to wildtype littermates (Cafferty et al., 2010).

1.8.4.2 Chondroitinase ABC (ChABC)

ChABC cleaves the glucosaminoglycan (GAG) side chains of proteoglycans and can thereby substantially revoke the inhibitory potential of proteoglycans that are secreted by reactive astrocytes forming the glia scar after spinal cord injury (McKeon et al., 1995). The expression of ChABC in reactive astrocytes under the GFAP promoter has been shown to improve corticospinal regeneration within the lesion site but not caudal to the lesion site in transgenic mice. No functional motor, but sensory recovery was observed after dorsal hemisection or dorsal rhizotomy respectively (Cafferty et al., 2007).

1.8.4.3 Brain derived neurotrophic factor (BDNF)

BDNF delivery promotes the survival of corticospinal neurons (neuroprotection) (Giehl & Tetzlaff, 1996; Giehl et al. 2001; Lu et al. 2001), but fails to promote corticospinal axon regeneration (Lu et al. 2001).

Combinatorial strategies providing the neurotrophic guidance molecule and the corresponding receptor have led to successful corticospinal regeneration

via trkB/BDNF expression (Hollis et al., 2009). This corticospinal regeneration was limited to a model where the BDNF expressing cells were grafted into a subcortical lesion. When the BDNF-expressing cell graft was provided within the spinal cord, no corticospinal axon regeneration was detectable.

1.8.5 Gene therapy approaches

1.8.5.1 Pten (tumor suppressor phosphatase and tensin homolog)

Pten deletion via cre mediated gene exertion has been shown to promote regeneration and sprouting of corticospinal axons after T8 complete crush in mice. In this study a cohort of corticospinal axons has been shown to cross the injury site and innervate the caudal host spinal cord (Liu et al., 2010). Pten mediated corticospinal axon regeneration across a T8 complete spinal cord crush has further been shown via shRNA mediated Pten knock down, with postnatal induction of Pten shRNA expression (Zukor et al. 2013). The results on axon regeneration of the corticospinal upon Pten deletion are promising; however they have to be critically interpreted. Pten was deleted at the age of 4 weeks or postnatally and not during adulthood. This could lead to an extension of developmental growth programs rather than promoting real adult corticospinal regeneration. This extension of embryonic growth programs could partly account for the observed increase in corticospinal axon regeneration at adulthood.

1.8.5.2 KLF7 (Krüppel-like Factor 7)

KLF7 has been shown to be important for cortical axon development (Laub et al., 2005) and to be involved in axon regeneration in zebrafish (Veldman et al., 2007). It is developmentally regulated in the motorcortex and suppressed

after neurons mature (Laub et al., 2001). Blackmore and colleagues have shown in 2012 that over expression of KLF7 in the adult mouse leads to regenerative sprouting of corticospinal axons after a dorsal hemisection. The authors postulate that the regrowing axons do not emerge from the severed axons tips but rather from collateral sprouting of the injured corticospinal fibers. They have also shown an increase in sprouting and midline crossing of intact corticospinal fibers after unilateral pyramidotomy in the case of KLF7 overexpression (Blackmore et al., 2012)

1.8.6 Embryonic Spinal cord graft

The Laboratory of Barbara Bregman reported in 2001 that embryonic spinal cord tissue grafted into an adult rat with a full transection was able to stimulate the regeneration of adult corticospinal axons. This study did not get a lot of attention, as the amount of regenerating corticospinal axons was sparse and the grafting paradigm included the osmotic delivery of NT3 or BDNF making the interpretation of the findings more complex (Coumans et al. 2001). In 2006 the Bregman lab published a study where a 2-4 week delayed graft of embryonic spinal cord tissue into a C5/C6 lateral over-hemisection shows enhanced corticospinal regeneration and functional recovery in the presence or absence of BNDF or NT3. The functional recovery was visible in skilled and unskilled forelimb tasks. The improvement is most likely not mediated by corticospinal axon regeneration alone but rather by a combination of regeneration of descending rubrospinal and serotonergic fibers, and regenerative sprouting of

spared rubrospinal and corticospinal axons from additional distal anatomical locations.

We adapted the improved paradigm of grafting embryonic spinal cord derived neural stem cells (NSC) from Lu et al., 2012 and achieved significant and extensive corticospinal axon regeneration into the NSC grafts (Kadoya et al., manuscript submitted). The optimized grafting paradigm utilizes single cell suspensions of NSC embedded in a fibrin matrix instead of grafting whole tissue pieces of the embryonic spinal cord as in Coumans et al. 2001. This new method results in an increased area of contact between grafted cells and injured corticospinal axons. The number of corticospinal axons responding to the graft substantially exceeds reports of Coumans and colleagues. Chapter 2 of this thesis investigates the changes in intrinsic gene expression of regenerating adult corticospinal neurons.

1.8.7 Summary

The evidence for successful approaches to archive corticospinal axon regeneration is rather sparse. In most studies, corticospinal axon sprouting rather than true regeneration of uninjured fibers was observed or cannot be excludes. There is an enormous need for therapies that promote corticospinal axon regeneration after injury in humans. However the number of scientific reports that how robust corticospinal axon regeneration is negligible. Stem cell therapies that trigger adult axon regeneration seem promising but have a long way to go to be successfully and safely adaptable to the clinic, hence it is essential to identify a regeneration transcriptome of corticospinal neurons to design alternative gene

therapy approaches. Gene candidates that prove to promote axon regeneration in rodents have then sub sequentially to be tested in a non-human primate model before clinical translation, due to the diverse nature of the corticospinal tract observed in different mammals.

Through adaptation of the embryonic spinal cord grafting paradigm (Lu et al., 2012) combined with the utilization of BAC-TRAP-CST mouse lines (Dyole et al., 2008), allows us for the first time to identify a regeneration transcriptome of actively regenerating adult corticospinal neurons.

Candidate genes that are activated upon corticospinal regeneration can now be tested in an in vivo model of spinal cord injury in mice, be confirmed in a non-human primate model and upon success be submitted to clinical trials.

This thesis investigates the regenerative transcriptome of adult mouse corticospinal neurons and hence has the potential to identify gene candidates that could trigger cortical spinal axon regeneration after injury and promote functional recovery in mice, non-human primates and human patients.

1.9 EXPERIMENTAL PROPOSAL AND HYPOTHESIS

In order to manipulate axon regeneration via gene or drug therapies, key molecules that are differentially expressed or activated during regeneration have to be identified. Most molecules and mechanisms mentioned in this introduction have been found to be involved in the regeneration of DRG neurons of the peripheral nervous system or from CNS axonal tracts that retain some reduced ability for axon growth after injury in adulthood (e.g. RGC, sensory, reticulospinal, rubrospinal and raphespinal tract). Unfortunately little data is known about

regenerative mechanisms in the most refractive system – the corticospinal tract (CST). As the CST in humans controls functionally critical voluntary motor functions, and since each axonal tract reacts different to manipulations, **we proposed to identify the regeneration associated transcriptome of the CST neurons (Chapter 2)**.

This proposal is based on the hypothesis that: **Intrinsic mechanisms determine the regenerative capacity of CST neurons after injury in adulthood**

Not only are changes in intrinsic gene expression a key component of manipulations to achieve axon regeneration, the role of the extrinsic environment must also being considered. To investigate the influence of Schwann cells on central axon regeneration of DRG neurons, **we proposed to investigate if proper Schwan cell function is an essential part of the conditioning effect of adult DRG neurons (Chapter 3).**

This proposal is based on the hypothesis that:**Schwann cells contribute an essential part to the regeneration of the central branch of adult DRG neurons in response to a sciatic nerve crush**

This thesis will investigate glial mechanisms contributing to the conditioning effect, and intrinsic neuronal mechanisms associated with corticospinal tract growth.

2 THE REGENERATION TRANSCRIPTOME OF INJURED CORTICOSPINAL NEURONS

2.1 Abstract

In humans, the corticospinal tract (CST) is the most important motor system. Despite its crucial role, it has emerged as perhaps the most refractory axonal system in the adult spinal cord from which to experimentally elicit axonal regeneration. We were able to promote regeneration of adult CST axons by grafting spinal cord-derived multipotent neural stem cells (NSCs) to sites of adult spinal cord injury. The presence of this neural stem cell graft triggers the expression of regeneration-associated genes in the lesioned CST. We utilized translational ribosome affinity purification (TRAP) combined with RNA-sequencing to specifically identify and then interrogate the pool of actively transcribed regeneration-associated genes in the injured adult CST. Among the key molecular mechanisms that we identified were the Huntingtin (HTT) gene, the transcriptional regulator NFE2L2 and several ephrins and semaphorins. The overall pattern of differentially expressed genes suggests that injured corticospinal neurons particularly recruit redifferentiation, neurite outgrowth and axon guidance mechanisms to achieve regeneration, and do so using mechanisms that are distinct and common to other populations of regenerating CNS axons, including sensory axons. Genes identified in this experiment constitute a database that can be translated to high throughput drug screening tools and *in vivo* models of spinal cord injury.

2.2 INTRODUCTION

Adult corticospinal tract (CST) neurons lack the ability to regenerate, causing a loss of voluntary motor control in humans after spinal cord injury. The failure of adult axons of the CNS to regenerate arises from mechanisms both intrinsic (Kobayashi et al., 1997; Neumann et al. 2002; Qiu et al. 2002; Lu et al. 2004; Pearse et al. 2004; Alto et al. 2009; Kadoya et al. 2009) and extrinsic (Chen et al. 2000; Fournier et al. 2001; DeBellard et al. 1996; Galtrey and Fawcett, 2007) to the injured neuron. To date, there is little convincing evidence that injured adult CST neurons have sufficient intrinsic capacity to extend axons into a lesion site in the injured spinal cord (Sivasankaran et al., 2004; Liu et al., 2008). Even in the presence of growth factors such as neurotrophin expressing cellular grafts, the number of CST axons penetrating a cellular matrix in a lesion site is negligible.

In contrast to adult neurons, embryonic CNS neurons exhibit an extensive axonal growth capacity and readily extend axons over long distances, even in the adult host environment (Gaillard et al., 2007, Lu et al., 2012). These studies indicate that mechanisms intrinsic to the neuron and active during development are sufficient to overcome limitations in regeneration exhibited by adult CST axons.

Despite these obstacles to regeneration, there is emerging evidence that adult CNS neurons retain the capacity for structural plasticity, particularly sprouting of spared projections, under certain circumstances. Following partial spinal cord transections, spared CST axons spontaneously sprout into

denervated regions of the spinal cord (Weidner et al., 2001; Freund et al., 2007; Rosenzweig et al., 2010). Moreover, we recently discovered that adult CST neurons can regenerate fully transected axons when provided with a permissive environment for growth: multipotent neural stem cells grafted to sites of adult spinal cord injury are sufficient to support regeneration of adult CST axons (Kadoya et al., manuscript in preparation). Indeed, this regeneration is extensive, demonstrating the potency of *environmental factors* in allowing regeneration of even this most refractory of adult axonal systems to regeneration (Blesch & Tuszynski, 2009). Thus, interplay of environment and intrinsic neuronal mechanisms appears to optimize the potential for injured adult axons to regenerate.

The observation that injured adult axons can regenerate into a spinal graft of neural stem cells presents a remarkable opportunity to identify molecular mechanisms that are activated when adult corticospinal axons regenerate. Another opportunity to identify corticospinal neuronal mechanisms supporting axonal growth after injury in the adult is provided by models that permit sprouting of these axons after partial injuries. We used these observations to design a set of experiments to identify modifications to the corticospinal neuronal transcriptome during active axon regeneration in the adult.

Our model system took advantage of the unique opportunities provided by mouse genetics. We utilized a transgenic BAC-TRAP mouse line created by the Heintz laboratory, in which a GFP-tagged ribosomal protein under the Glt25D2-promoter is specifically restricted to layer5b cortical neurons (Doyle et al., 2008).

The ribosomal GFP-tag allows for immunoprecipitation of mRNA which is bound to the poly-ribosomes and hence actively transcribed ***solely in CST neurons***. Adopting this model, we subjected adult Glt25d2-EGFPL10a mice to lesion models in which corticospinal motor axons: 1) ***regenerate*** into neural stem cell grafts placed in sites of adult corticospinal tract injury, and 2) extensively ***sprout*** into sites of partial spinal cord transection. At time points when we predicted CST axons would be in an active growth state in these models, we performed mRNA sequencing to identify the “regeneration transcriptome”. Sampling of early growth time points would provide information on early transcriptional activators of the CST growth response; later time points would identify the direct cellular mechanisms employed by axons that are actively growing.

We now report that the growing adult corticospinal neuron expresses specific sets of genes that allow its growth in adulthood. These mechanisms include the activation of transcription factors such as NFATc2 and TP53, and the repression of Jun. Notably, these mechanisms are common to mechanisms contributing to the regeneration of other neuronal systems in the adult CNS (e.g., DRG axons regenerating centrally). Notch3 was found to be activated upon CST axon regeneration which is contradictory to previously described functions of notch signaling in interfering with axon regeneration. We identified Huntingtin and NFE2L2 as new possible molecular mechanisms supporting axonal regeneration that are unique to growing adult CST neurons. Thus, unique and common mechanisms define the ability of the most important functional motor system in the adult CNS to grow after injury. These findings establish a set of therapeutic

candidates that can be exploited in high throughput drug screens, *in vitro* neurite outgrowth assays and *in vivo* injury models to identify means for potentially improving outcomes after spinal cord injury.

2.3 RESULTS

2.3.1 Experimental paradigm

Model 1: CST Axonal Regeneration in Grafts of Multipotent Neural Stem Cells We performed a T1 dorsal column wire knife lesion and grafted neural stem cells (NSCs) into the lesion site (Fig. 2.1) in adult mice of age 10 - 20 weeks. The NSCs were derived from GFP-expressing mouse embryos under the CAG promoter. The grafted cells proliferated and adopted fates in the adult spinal cord of both neurons and glia (Lu et al., 2012; supplemental Figure S2.2). Host axons regenerated into these grafts (Coumans et al. 2001; Lu et al., 2012), thereby triggering a “regeneration transcriptome”. The mice that received these grafts express the GFP-tagged ribosomal protein L10A (Rpl10a) under the Glt25d2 promoter. Use of this BAC-TRAP (Bacterial Artificial Chromosome – Translational Ribosome Affinity Purification) mouse line allows highly specific mRNA isolation from layer 5b motor cortex neurons that largely consist of CST neurons. Donor NSCs grafts were placed in the lesion site, and additionally into C4 and C6 dorsal column sites to increase the proportion of cortical CST neurons from which a regeneration response was elicited. All cells were grafted one week post lesion (a clinically relevant time point that also appears to optimize cell survival). At time points of 3, 7 and 14 days, we dissected the motor cortex and extracted mRNA from cortical layer 5b, using immunoprecipitation with GFP-

antibody coated magnetic beads, a process that isolates the actively transcribed mRNAs specifically from layer 5b neurons. The samples were then purified, amplified and subjected to RNA sequencing.

Several additional steps were carried out prior to gene expression analysis, to optimize data collection. These steps include identification of the embryonic stage of development that elicited the greatest CST regenerative response, and identifying time points at which CST axons first regenerated into grafts.

Model 2: CST Axonal Sprouting After Partial Spinal Cord Lesions

In the second paradigm we performed a unilateral pyramidotomy of the CST tract before the pyramidal decussation in adult Glt25d2 BAC-TRAP mice. This lesion resulted in complete denervation of the ipsilateral motor neurons within the spinal cord grey matter. The contralateral, uninjured cortical hemisphere of the CST tract responds with sprouting of CST axons, which cross the spinal cord midline and grow into the spinal cord caudal to the lesion. We extracted the motor cortex and isolated the mRNA as described above from the ipsilateral motor cortex, whose axons undergo extensive sprouting. The mRNA underwent purification, amplification and was subjected to microarray gene chip analysis.

Neural Stem Cell Grafts from E12 Spinal Cord Promote Extensive Adult CST Regeneration

To maximize the number regenerating CST axons we compared NSC grafts derived from different developmental stages (Fig. 2.2). Mouse spinal cords

from E10 – E15 were extracted and grafted to sites of a C5 dorsal column lesion. NSCs from E12 - E13 elicited the strongest regenerative response. We therefore performed all subsequent experiments using NSCs derived from E12 donor mice.

2.3.2 Characterization of distinct chronological stages of CST axon regeneration identify 3 days, 1 week and 2 weeks as optimal time points for RNAseq

We investigated several time points after cell grafting and qualitatively assessed CST axon regeneration accordingly. Figure 2.3 shows the time course of 7, 10, 14 and 21 days after cell grafting. We observed the first sign of CST axon regeneration at 10 days post graft (10 DPG). At 7 DPG there was no axon regeneration detectable. We therefore assume that axon regeneration initiates shortly after 7 DPG. We found prolonged axon growth at 14 DPG and extended axon growth at 21 DPG. To achieve a translational representation of distinct stages of axon regeneration, we decided to investigate 3 time points with RNAseq that are representative of unique developmental stages of axon regeneration. Thus, we analyzed mRNA expression of 3 DPG, where no axon regeneration is occurring, but we would expect the activation of immediate early genes that prime the CST neurons to switch expression profiles from an adult stage to a more immature state where they are capable of extending axons. We further selected 7 DPG, a stage immediately before axons are extending, to investigate genes that are actively transcribed to initiate the growth of axons. Finally we performed RNAseq on 14 DPG, where we observed prolonged axon

extension and expected to identify genes involved in axon guidance supporting specifically the growth of CST axons.

2.3.3 Summary of gene expression analysis

We performed RNAseq on the immunoprecipitated (IP) and the unbound (UB) fraction. The IP fraction entails all mRNA derived specifically from layer5b neurons of the motor cortex and the UB fraction encompasses mRNA from all other cells in the dissected tissue of the motor cortex, including neurons, glia, blood and other cell types. For the IP and UB fraction, we compared the E12 graft to a lesion-only condition for the three time points. Genes detected in the IP comparison reflect genes specifically active upon CST regeneration, whereas genes detected in the UB fraction reflect non-specific gene changes in the motor cortex. For that reason, we subtracted the genes that are differentially regulated in the UB fraction from those of the IP fraction. It is important to note that significant candidate genes may be neglected with this subtraction strategy as the UB fraction does not get completely depleted of genes bound to GFP-tagged ribosomes. Thus there is the chance that genes specific to layer5b neurons are also detected in the UB fraction. For this reason, we performed a more detailed analysis of differentially regulated genes present in both fractions. We also grafted mouse bone marrow stromal cells (MSCs) in a third group as control: these cells do not support corticospinal tract regeneration. This additional control was performed at the 2 week time point. When we analyzed the genes that are differentially regulated in the 2 week time point, we subtracted the UB fraction of the “E12 graft versus lesion only”, and the IP and UB fraction of the ”MSC graft

versus lesion only" from the IP fraction of "E12 graft versus lesion only". This resulted in a list of genes for the 2 week time point more stringently filtered to only include genes involved in the regeneration of CST axons

Figure 2.4 shows a summary of the up and downregulated genes in response to the NSC graft at 3 days, 1 week, 2 weeks and the MSC graft for two weeks compared to the lesion only controls. The overall trend shows more differentially regulated genes in the IP fractions compared to the UB fractions within a given time point. This gives validity to the specificity of the treatment, since we mostly expect to observe differentially regulated genes within the CST neurons responding to the E12 graft (IP) and not in the other cell types of the motor cortex (UB). Additionally we observe very little differentially regulated genes within the MSC grafted group at the 2 week time point compared to the NSC graft at 2 weeks, indicating that the observed genes changes in response to the NSC are evoked by CST axon regeneration and not by the cell grating procedure. This further confirms the specificity of the NSC grafts triggering a regenerative gene expression response in the CST neuron cell bodies, which is not evoked by the MSC graft. A bit of a concern is the low number of significantly differentially expressed genes at one week time point. This might either be due to a real biological effect or due to sample quality; however, quality control tests showed no deficiencies in the one week samples. Moreover, RNAseq was comparable at all time points, since the number of reads was comparable at all time points (~40 million per sample).

2.3.4 Overview over methods of data analysis

We applied a cutoff threshold for selecting significantly differentially regulated genes with a false detection rate below 10% (FDR ≤ 0.1). Only in the case of the one week time point we lowered the cutoff threshold to a p-Value ≤ 0.05 to identify affected signaling pathways and downstream bio-functions. We applied different methods of analyzing the data. We utilized a web based interface called REPAIR (REPository of APNRR Integrated Research) that is designed and maintained by the Geschwind lab at UCLA. This interface allows us to perform comparative analysis between the different RNAseq datasets. We used this manipulation to subtract differentially regulated genes in the UB from the IP fraction. We also subtracted the differentially expressed genes found in the MSC transplant group from the 2 week IP fraction. We further identified the top up- and down-regulated genes within each group (Table 2.1 – 2.3)

We then exported the dataset from the REPAIR interface and imported them into the Ingenuity pathway analysis software (IPA). IPA integrates published literature to put the differential expression data into functional and regulatory context.

The data is organized in these categories:

Top differentially regulated genes: Top 10 down- and up-regulated genes per time point (Table 2.1 – 2.3)

Canonical pathway analysis: Determines the most significantly affected pathways

Upstream regulator analysis: Predict upstream molecules, including microRNA and transcription factors, which may be causing the observed gene expression changes

Downstream Effects Analysis: Identify whether significant downstream biological processes are increased or decreased based on gene expression results.

Network Analysis: Transcriptional networks, microRNA-mRNA target networks, phosphorylation cascades and Protein-Protein or Protein-DNA interaction networks.

2.3.5 Top differentially regulated CST axon regeneration associated genes

Table 2.1 - 2.3 lists the strongest up (red) and down (green) regulated genes identified at 3, 7 and 21 days. Interestingly, there is a trend from higher down-regulation at 3 days towards higher gene upregulation at 2 weeks. This indicates that early processes involved in regeneration and/or neuroprotection are more likely to suppress gene expression, and later processes are more likely to stimulate gene expression or that regenerating neurons actively metabolize mRNAs that were present from before the lesion. It further suggests that at early times points of regeneration, functions of mature conducting CST neurons like synaptic vesicle trafficking and maintenance of synapses have to be silenced. Early processes would include downregulation of maintenance genes, followed by neuronal reprogramming to then enter a phase of neurite elongation followed by reestablishing of synaptic contact. For this regenerative phase more genes would be upregulated to drive active axonal growth, guidance, maturation and

synaptogenesis. Table 2.1 – 2.3 indicates that the expression of transmembrane receptors are preferentially downregulated at 3 days (e.g. protein tyrosine phosphatase, receptor type, C; G protein-coupled receptor 156 and integrin, beta 2) and upregulated at 2 weeks (e.g. G protein-coupled receptor 101; immunoglobulin superfamily, member 1; frizzled family receptor 10). This finding indicates that the downregulated receptors are rather utilized in maintenance of mature axons and the later upregulated receptors are likely to be involved in axon outgrowth.

2.3.6 Canonical pathway analysis reveals distinct activation of pathways resembling the anatomical time points of regeneration

IPA has an actively curated database of 301 metabolic and 341 signaling pathways. These pathways include molecules that have been reported in the literature to engage in a particular canonical pathway. IPA uses the differential expression data for each gene within a given comparative dataset to rank identified canonical pathways according to the significance of activation of each pathway.

2.3.6.1 The top canonical pathways at 3 days indicate reprogramming of CST neurons to a more immature state

Figure 2.5 shows an interconnectivity map of the top canonical pathways that are activated at 3 days post grafting. The blue lines represent at least two molecules that are consistent in 2 connected pathways. For more detail on the number of overlapping molecules see also supplemental material (Fig. S2.9). The intensity of the red color represents the significance for activation of a certain

pathway. (darker red = lower p-Value). Several pathways are involved in metabolic signaling that counteract apoptotic signals and reactivate the metabolic machinery to induce regenerative programs. Of greater interest is the activation of the “human embryonic stem cell pluripotency” and cancer signaling pathways that indicate dedifferentiation of the adult CST neurons to a more immature growth phenotype. This pattern correlates with the anatomical findings that at 3 days post graft, no axons are yet regenerating, but the neurons have to reactivate gene expression necessary for initiation and elongation of axonal growth. Already at 3 days we found an activation of axon growth and guidance signaling pathways, indicating that the axons of the injured CST neurons are already responding to the environment and preparing for axon growth. A detailed view of the “human embryonic stem cell pluripotency” pathway and the differentially regulated molecules involved can be viewed in the supplemental materials (Fig. S2.10)

2.3.6.2 The top canonical pathways at 1 week indicate activation of axon growth mechanisms and guidance

Since at one week the amount of differentially regulated molecules was below 200, we applied less stringent cutoff credentials to investigate the canonical pathways for one week compared to 3 days and 2 weeks. Instead of the FDR ≤ 0.1 we applied the cutoff of p-Value ≤ 0.05. We only did this for the analysis of canonical pathways. Individual genes within these pathways have to be investigated with great care before selecting them as individual candidates for follow up studies. According to the anatomical studies, axon outgrowth can first

be detected at 7 days post graft. This is reflected by the activation of distinct canonical pathways, like the mTOR signaling pathway, which has previously been reported to be involved in axon outgrowth (Park et al. 2008; Liu et al., 2010; Ning et al., 2010; Lu et al., 2012). PKA and Ephrin receptor signaling are also activated and are known to be involved in axon outgrowth (Spencer et al. 2008; Cai et al., 2002; Hannila and Filbin, 2008) and guidance (Xu et al., 2012; Kao et al., 2012) (yellow box). In addition there is activation of glucose and ATP production, which are necessary for axon growth. Also we see activation of mitochondrial function, including transport of mitochondria down the axon (Fig. 2.6, red box). We observed a down regulation of the transcriptional regulator Jun which has been implicated to induce apoptosis in neurons (Shi et al., 2005; Connor et al., 1998; Earnshaw et al., 1999), so a down regulation can be viewed as a pro-survival signaling.

2.3.6.3 The top canonical pathways at 2 weeks show activation of axon guidance and synaptogenesis signaling

At 2 weeks, we observed prolonged axon extension that is reflected by the activation of several axon guidance signaling pathways (Fig, 2.7). In addition, we observed activation of synaptic long term potentiation and depression pathways as well as PKA signaling and cAMP signaling. G-protein coupled receptor signaling was also activated indicated by differential expression of G-protein coupled receptors such as ADORA2A, Htr5b, VIPR2 and CHRM3, down regulation of downstream molecules such as PKA and upregulation of and Erk and CREB, which all have been implicated in axon regeneration (see Chapter

1.6.1.6 and 1.6.5.3). At this stage of regeneration, axons are being guided by known classical axon guidance signaling mechanisms. We observed an activation of Netrin and Slit signaling, Semaphorin and Wnt signaling (supplemental Figure S2.14) beside many others. As expected, besides axon extension we also observed the activation of pathways involved in synapse formation and maintenance. Grafted neurons are in close proximity to the regenerating CST axons, so synaptic contacts can be established shortly after axonal outgrowth (supplemental Figure S2.15).

2.3.7 Upstream regulator analysis revealed potential candidate gene for CST regeneration

This analysis was used to identify transcription factors that may be responsible for the gene expression changes observed at different time points during CST regeneration. Based on known and predicted transcription factor binding sites, downstream signaling pathways and protein-protein interactions, IPA predicts which transcription factors are activated or inhibited to explain the upregulated and downregulated genes observed. Transcription factors include: transcription regulators, sequence-specific DNA-binding transcription factors, and ligand-dependent nuclear receptors. In the dataset, a total of 192 (3 days), 34 (1 week) and 188 (2 weeks) transcription factors were found to be differentially regulated in a significant manner (FDR ≤ 0.1). The activity of transcription factors is often regulated via phosphorylation or other mechanisms, the importance for a transcriptional regulator to have an impact on the observed phenotype cannot solely be proposed upon differential expression. Table 2.4 lists transcription

factors with predicted functions that are themselves not differentially expressed. IPA computes a z-score that reflects the validity of a certain transcription factor to be activated or inhibited. The computation of the z-score is based on the regulation of known or predicted downstream molecules. The more downstream molecules that are regulated in a certain direction (e.g. activation of a certain transcription factor) the higher the z-score. IPA thus only assigns a prediction of activation or inhibition for a certain transcription factor if the z-score is above/below ±2. Table 2.4 shows an overview of all transcription factors that have been classified as activated or as inhibited at 3 days and 2 weeks that pass the prediction threshold (2≤ z-score ≤-2) and their corresponding z-scores. Unfortunately no prediction could be made with confidence for the 1 week time point, since the number of differentially regulated genes was relatively low compared to 3 days and 2 weeks.

2.3.8 Known regeneration associated transcription factors

We identified the transcriptional regulators TP53 and Nfatc2 to be activated upon CST regeneration. TP53 has been shown to have a positive benefit on axon regeneration *in vitro* and *in vivo* (Di Giovanni et al., 2005; Di Giovanni et al., 2006).NFATc2 has been shown to be involved in the development of sensory axonal projections *in vivo* and in the axon outgrowth response towards neurotrophic factors *in vitro* (Greaf et al., 2003). The expression transcriptional regulator c-Jun and JunD was down-regulated in regenerating CST neurons. Contradictory to our findings both c-Jun and JunD upregulation has been associated with axon regeneration (Kenney and Kocsis,

1998; Herdegen and Zimmerman, 1994, Herdegen et al., 1991; Jenkins and Hunt, 1991; Leah et al., 1991; Herdegen et al., 1997; Lindwall and Kanje, 2005).

2.3.9 Novel Regeneration Associated Transcription Factor: Huntingtin (HTT)

We identified an up-regulation of the Huntingtin (HTT) gene using microarray gene expression analysis of sprouting CST axons and a predicted activation of HTT upon CST axon regeneration (Fig 2.8 and Supplemental Figure S2.16). HTT positively regulates the production of the brain derived neurotropic factor (BDNF) that has important roles in neuronal survival and neurite outgrowth. In addition to regulating BDNF levels transcriptionally, HTT protein is transported down the axon, where it interacts with the motor protein dynein, beta-tubulins and BDNF containing secretory vesicles. Mutation of HTT and its loss of function leads to a detrimental motor neuron disease called Huntington disease. These findings make HTT an interesting candidate that could be involved in CST axon regeneration.

Fig 2.8 shows the HTT gene regulation network and its downstram effectors at 2 weeks respectively. HTT was only identifed for the 1 week time point with less stringent condtition of a p-Value of ≤ 0.5 (Suplemenal Figure S2.16). Orange and blue arrows identify interactions that either increase or deacrease the expression of downstream molecules, respectively. Grey arrows indicate that differential regulation of downstream molecules has been described, but the literature findings are not conclusive. Yellow arrows reflect an inconsistent finding compared to the literature. The overall prediction of activation

or inhibition of the transcription factor is a summation of all these connections, reflected by the z-score.

2.3.10 Network Analysis revealed NFE2L2 as potential candidate gene for CST regeneration

A network consists of up to 35 molecules that have been reported to be connected within the literature. These interactions can be regulatory in nature or exert their effects by direct protein-protein interactions. The network analysis does not take directionality (up or down regulation of a specific member) into account when creating a functional network. Networks are ranked based on the number of molecules that are found to be differentially regulated within a dataset as well as on statistical criteria. The power of network analysis is the ability to identify genetic hubs from differential gene expression datasets, which might be themselves not differentially regulated, but which are of central importance to all other significantly differentially regulated molecules within its functional network. An overview of the 20 highest ranked networks, as well as a more detailed view on the highest ranked networks for the different time points can be found in the supplemental material (Tables S2.1 – S2.3 and Figures S2.17 – S2.19)

We identified the transcription factor NFE2L2 as a potential candidate for CST regeneration. NFE2L2 is upregulated 1.8 fold by 1 week post graft and rises to a peak of 3.4 fold after 2 weeks. In addition NFE2L2 was identified as a genetic hub by the IPA network analysis for the 1 and 2 week time point. If we compare the network to 1 week (Fig 2.9) and 2 weeks (Fig 2.10), we observe an increase in the number of connected partners within the network around

NFE2L2. The IPA analysis demonstrates that, not only is the magnitude of NFE2L2 gene expression increasing over time, but also the amount of molecules that are linked to its expression and function. In addition, NFE2L2 was classified as an activated upstream regulator and part of a functional network in the CST sprouting dataset at one week after pyramidotomy.

NFE2L2 is a member of a small family of basic leucine zipper (bZIP) proteins that shuttles between the plasma membrane and the nucleus. Its activation is linked to oxidative stress. A NFE2L2 deficient mouse has displayed increased functional motor deficits upon treatment of 3-nitropropionic acid (3NP) and malonate, which are known to cause striatal damage reminiscent of Huntington's disease (Calkins et al., 2005)

2.4 Criteria for candidate selection and confirmation

Most transcription factors are regulated in their activity by post transcriptional modifications, such as phosphorylation or acetylation. Thus changes in the differential expression of a transcriptional regulator are not absolutely necessary for its role in promoting axon regeneration. The power of the Ingenuity pathway analysis (IPA) software is that it can predict the activation state of a specific transcription factor via the investigation of the regulation of its downstream effectors. IPA maintains an actively curated database of literature findings that incorporates the relationships of expression level, activity and direct protein interaction between specific genes. This analysis can integrate the regeneration associated transcriptome into known signaling pathways as well as identify upstream and downstream effectors that are suggested to be activated or

inhibited to maintain the observed expression data. In order to identify individual candidates for verification of the quantitative RNAseq data, we categorized candidate selection upon the goal of data verification into (1) verification of expression levels of gene candidates to confirm changes in gene expression measured by RNAseq, (2) verification of predicted activity of transcriptional regulators (3) functional verification of selected candidate genes to determine their influence on corticospinal axon regeneration

2.4.1 Verification of expression levels of gene candidates

Despite the fact that RNAseq data is quantitative and as reliable as quantitative PCR, there is certain noise introduced by the method of mRNA purification and amplification due to low yields of mRNA. The extraction of mRNA specifically from cortical layer 5b neurons of the motor cortex via immunoprecipitation and thus additional overnight incubation steps increases the potential for mRNA degradation. We did however not see a reduced mRNA quality of the CST-specific mRNA that underwent immunoprecipitation compared to the unbound fraction contain all RNA species from several cell types. One has to keep in mind that the immunoprecipitated mRNA is enriched for ribosomal RNA and that this could also influence the RNA integration number (RIN) since it is a result of expression levels the 28s and 18s rRNAs. Due to the nature of the CST mRNA specific extraction, the amount of mRNA is below 100ng per sample and has to be amplified for preparation of the cDNA library. Amplification of low amounts of mRNA can lead to biases that can influence the outcome the RNAseq. It is such essential to confirm specific candidates in their expression

levels. Since during the process of RNAseq the mRNA is heavily amplified, a direct correlation of the fold change of mRNA levels and protein level cannot be drawn. Thus we had to pick several candidates for expression level verification, assuming that some of those might not show the expected change in protein expression. We selected candidates that are either strongly changed upon regeneration or that are highly abundant (high number of reads) in both the regenerating and non-regenerating CST neurons as well as based on antibodies that have been optimized for immunohistochemistry in our lab.

2.4.2 Verification of predicted activity of transcriptional regulators

Upstream analysis, such as prediction of transcription factor activity are essential to identify candidate genes that are themselves not differentially regulated but could potentially be critically involved in regulating the observed changes in gene expression that lead to cortical spinal axon regeneration. Before functional candidate validation of predicted candidate genes, it is essential to verify their activity during the regeneration of corticospinal neurons. Verification of activity of transcription factors can be assed in several ways. Immunohistilogical labeling with phosphospecific antibodies can be used to quantify an increase in phosphorylation of specific transcription factor and hence its state of activity in response to treatments that induce corticospinal axon regeneration. All transcription factors are translated in the cell body and contain a nuclear localization sequence that targets them to the nucleus. The translocation of many transcription factors to the nucleus is reflecting their state of activity. Hence, localization studies via immunohistochemistry can reveal information

about the activity of a specific transcription factor. Huntingtin (HTT) e.g. is present in neuronal cell bodies and axons and translocates to the nucleus when its transcriptional regulatory function is activated.

2.4.3 Functional verification

Changes in expression level or activity of a specific gene confirm the validity of the RNAseq data but do not necessarily indicate that this gene is involved in corticospinal axon regeneration per se. The observed changes could be of neuroprotective nature, could symbolize an immune response event or could result from secondary gene regulation that are not primarily involved in mitigating the axon regeneration event. Thus to identify candidate genes that are directly involved in axon regeneration it is essential to verify candidate genes on a functional level. We propose to investigate the implication of selected candidate genes in axon regeneration in a small scale in vitro and in vivo screen.

2.4.3.1 Small scale in vitro screen

We will take two approaches to investigate the functional implication of candidate genes in vitro to (1) determine their necessity for cortical axon growth and (2) their potential to promote axon regeneration of primary neurons in culture. To investigate their necessity we will reduce the expression of candidate genes with siRNA mediated gene silencing in embryonic cortical neurons and determine influences in neurite outgrowth. If a specific gene is necessary to mediate neurite outgrowth in the readily growing embryonic cortical neurons, we expect to observe a reduction of neurite extension compared to control siRNA treated neurons. We have to perform this essay with embryonic rather that adult

cortical neurons since adult cortical neurons are very difficult to culture and therefor make a rapid screening of candidates impossible. Secondly, adult cortical neurons extend axons not as readily as embryonic cortical neurons and are therefore not feasible for a “gene necessity screen”.

To investigate if the expression of a specific candidate is promoting neurite growth, we propose to overexpress the gene candidates in adult DRG neurons on permissive and inhibitory substrates and compare neurite extension to control plasmid expressing neurons. If neurite extension on either substrate is increased it indicates that the overexpressed gene is promoting neurite outgrowth and therefore could be involved in corticospinal axon regeneration after injury. The rationale for investigating overexpressed genes in adult DRG neurons is that these neurons are the only primary neurons that can be cultured in an adult state, thus resemble the developmental stage of the injured adult corticospinal neurons more closely. The genetic characteristics of adult corticospinal neurons and DRG neurons differ significantly hence candidates identified in regenerating corticospinal neurons might not be involved in DRG axon regeneration. Therefor we are performing the gene silencing of candidates in cortical neurons since it is more likely that similar genes are involved in regeneration of immature and mature cells of the same linage. If the function of a candidate gene mechanistically promotes axon regeneration, than overexpression of that gene could lead to increased regeneration in several neuronal cell types. In this case adult DRG neurons have the benefit of resembling the developmental stage of adult corticospinal neurons and do not as

readily extend axons as embryonic cortical neurons, which is an important factor to identify and increase in axon elongation, since the candidate gene ideally has to be overexpressed before axon elongation occurs to elicit it full effects on axon growth.

2.4.3.2 In vivo verification of candidate genes

We propose to investigate a few selected candidate genes in mouse models in vivo. These candidates have to fulfill several criteria to be selected, such as regulation of gene expression, being identified as upstream modulator in the RNAseq dataset, are differentially regulated upon corticospinal regeneration and have been reported in the literature to be involved in neuronal function.

NFE2L2 is a transcription factor that is upregulated by 1.8 fold at 1 week and by 3.4 fold at 2 weeks after grafting of NSCs. NFE2L2 has also been identified by IPA as an important genetic hub at both time points. NFE2L2 was additionally identified as upstream regulator in the ”corticospinal sprouting after unilateral pyramidotomy” dataset. NFE2L2 deficiency in mice has been associated with increased functional motor deficits upon treatment of 3-nitropropionic acid (3NP) and malonate, which are known to cause striatal damage reminiscent of Huntington's disease (Calkins et al., 2005). The NFE2L2 deficient mouse is available as life stock at Jackson Laboratories for purchase. All of these criteria make NFE2L2 an interesting candidate that could potentially be involved in corticospinal axon regeneration and can easily be tested in an in vivo model of corticospinal axon regeneration into a NSC graft.

Huntingtin is a protein that is mutated in motor neurons of Huntington diseased patients. The mutated protein displays a CAG repeat with varying copy numbers (up to 250 copies) and leads to loss of function. Huntingtin was itself not differentially regulated upon corticospinal regeneration but was identified us upstream regulator at one and two weeks after NSC grafting. We additionally found an up regulation of Huntingtin in the ”corticospinal sprouting after unilateral pyramidotomy” dataset. Huntingtin can function as transcriptional regulator via translocation into the nucleus. It regulates the expression and axonal transport of the brain derived neurotrophic factor (BDNF), which has been shown to promote corticospinal neuron survival. Hunting mutant mice are available as life stock and show an onset of the Huntington disease phenotype at around 10 weeks of age. These mice could be utilized to investigate the involvement of Huntingtin in corticospinal axon regeneration after NSC graft.

2.5 DISCUSSION

A central issue of regenerative biology is the identification of transcriptional regulators that govern critical phases during the regenerative process, often closely resembling developmental expression profiles. The corticospinal tract is the most important motor system in humans. Its regeneration has been extraordinarily difficult to elicit, and the present findings provide a list of candidate mechanisms for the first time that might be studied and augmented to enhance regeneration or sprouting after injury in the adult.

We have shown that adult mouse CST axons regenerate after lesion. Through mRNA sequence analysis we generated a regeneration associated transcriptome that identifies distinct aspects of regeneration.

Initially during the regenerative process, CST neurons express embryonic pluripotency pathways at 3 days post cell grafting. We observed an activation of the Wnt/β-catenin signaling pathway that is known to be involved in development, axon pathfinding and adult neurogenesis (Zhang et al., 2013; Varela-Nallar et al., 2013, Carter et al., 2007). Furthermore, we see a down regulation of BMP6 that function in neurogenesis and neuronal differentiation (Choi et al., 2011; Segklia et al. 2012; Dreau et al., 2012; Sánchez-Camacho et al., 2011; Ortega et al., 2010)

Interestingly, we also found activation of the reelin signaling pathway. Reelin is an extracellular protein essential for neuronal migration and brain development (D'Arcangelo et al., 1995; Alcantara et al., 1998; Rice and Curran, 2001; Cooper, 2008). Reelin signaling is involved in axon outgrowth and guidance (Borell at al., 1999; Li et al., 2005), arborization of cortical processes (Hoe et al., 2009) and synapse formation in the hippocampus (Borell at al., 1999). In addition, Reelin can increase neurogenesis in the dentate gyrus (Pujadas et al., 2010). Reelin signals via ApoER2, VLDLR and α3β1-Integrin. These pathways converge on the reelin signal transducer DAB1 (Delahaye et al., 2007; Bock et al., 2003; Pylayeva et al., 2006), All these downstream targets of reelin were found in this study to be significantly differentially regulated at 3 days in the regenerating CST (supplemental Figure S2.21). DAB1 activation can

increase neuronal differentiation in neuronal stem cells (Kwon et al., 2008) and was also significantly upregulated. In summary, the reelin signaling pathway is active during development of the cortex, involved in neuronal differentiation, migration, axon outgrowth, guidance and synapse formation. An activation of this pathway as early as 3 days after cell graft suggests that these transcriptional steps are important to prime the injured neurons for axonal regeneration.

At later stages of maturation these expression profiles shift drastically to axon guidance singling and preparation for synaptogenesis and maintenance.

At one week post grafting we identified activation of the mTOR and Ephrin signaling pathways, which have been implicated in neurite outgrowth and axon guidance. (These analyses were based on the statistical criterion of p-Value ≤ 0.05 rather than FDR ≤ 0.1, given the smaller number of genes differentially expressed at this time point.) Inhibition of the mTOR signaling pathway via Rapamycin has been shown to inhibit axonal outgrowth from grafted embryonic stem cells into the adult cord (Lu et al., 2012). Ephrins are tyrosin kinase receptors that are widely described as axon guidance molecules (Helmbacher et al., 2000; Luria at al., 2008; Wang et al., 2001) and as molecules involved in synaptogenesis (Grunwald et al., 2001; Nolt et al., 2011). We also found important regulators of the actin skeleton to be differentially regulated. Proper remodeling of the actin cytoskeleton is an essential part of axon outgrowth and regeneration. We found a 3.4 fold upregulation of NWASP, which is part of the Arp/23-potein complex that mediates actin filament branching (Uruno et al., 2003). We further found an upregulation of ROCK and a downregulation of SHH

(supplemental Figure S2.22). Both are involved in actin filament turnover and stability via regulation of the actin severing protein cofilin (Govek et al., 2005; Nashita et al., 2005). The transcription machinery is in general specifically activated at the 1 week time point underlining the effort of the CST neurons to increase their regenerative capacity.

At two weeks post graft, we saw anatomically that axons are readily regenerating and elongating. The corresponding transcriptional profile shows an activation of multiple axon guidance, synaptogenesis and synapse maintenance pathways. We found reduced expression of the Ephrin A and Ephin B receptors as well as Slit and Robo. Additionally, there was an increase in expression of integrins as well as netrin and its receptor DCC. All of which are critical axon guidance molecules during development of the nervous system (Andrews et al., 2007; Price et al., 2006; Nikolopoulos and Giancotti, 2005). As expected, we saw an increase in growth factor expression with upregulation of NT-3, NGF and noggin as well as FGF1 and IGF1 and IGF2. Growth factors have been shown to play essential roles in axon regeneration and neural survival in the peripheral and central nervous system (Patapoutian and Reichardt, 2001; Blesch et al., 2012). We identified differential expression of a class of axon guidance molecules called semaphorins. Semaphorins are key regulators of axon outgrowth in the cortex (Bagnard et al. 1998, 2000, 2001; Polleux et al. 1998, 2000). We found differential expression of semaphorins that either support axon growth or inhibit axon growth and cause growth cone collapse. Regulation of growth inhibiting semaphorins includes: Increased expression of Sema4D and Sema5A that inhibit

axonal outgrowth and cause growth cone collapse *in vitro* (Goldber et al., 2004; Govek et al., 2005).

Other axonal outgrowth inhibiting semaphorins were found to be downregulated, suggesting a supportive role in axon outgrowth of CST neurons at 2 weeks post grafting. These include Sema3C/D/E and their receptor Neurophilin1. Overexpression of Sema3s causes growth cone collapse upon binding to Neurophilin (He et al., 1997; Takahashi et al., 1998). We also found that Sem6B was downregulated. Sem6B is a newly identified Semaphorin that promotes growth cone collapse in hippocampal mossy fibers (Tawarayama et al., 2010). Other semaphorins that were differentially regulated have not yet been found to conclusively promote or inhibit axon outgrowth. For example; Sema3A was upregulated at 2 weeks. It has been reported to act as a chemo-repellant but also as a chemo-attractant for cortical apical dendrites (Polleux et al., 2000).

The complex spatial and temporal expression patterns and interactions of these diverse axon guidance molecules highlight the difficulty in manipulating one specific effector gene when designing a gene therapy approach. For this reason, we are focusing mostly on transcriptional regulators that act as upstream signaling hubs for future investigations. From the combination of transcriptional profiling and network analysis presented in this study, it is apparent that differential expression of several genes will be necessary for successful CTS axon regeneration.

The regeneration specific transcriptome described in detail in this study not only identifies the expected distinct steps of maturation, but provides deeper

insight into the transcriptional networks responsible for of each step. We can now start to manipulate the pathways that are activated during each step of regeneration to test its contribution to the regenerative process.

By interfering with each aspect of neuronal maturation we can identify key components of each step. We propose to investigate candidate genes that were identified in this project as transcriptional hubs using an *in vitro* neurite outgrowth model. Candidate factors whose activity is predicted to be important for promoting neural regeneration will be silenced using siRNAs in developing embryonic cortical neurons or retinal ganglion cells in culture to test the hypothesis that axonal outgrowth will be reduced. Based on the hypothesis that reprogramming of adult CST neurons to a more immature phenotype is necessary for enhanced axonal outgrowth, silencing of these factors in a developmentally immature cortical neuron will assess if they are necessary or sufficient to promote differentiation and axonal regeneration independently of reprogramming.

Loss of function of these candidate factors will also be assessed in an *in vivo* CST axon injury model in the presence of the growth triggering embryonic spinal cord graft. We will quantify the number of regenerating CST axons in a mouse line deficient for the transcription factor NFE2L2. If NFE2l2 has an essential role in any of the distinct stages of regeneration, we expect to observe a reduction in regenerating CST axons.

The same surgical paradigm will be applied to a Huntington mutant mouse line. Wild type Huntingtin (HTT) gene contains 6-36 glutamine repeats in exon 1.

In individuals suffering from Huntington disease, this number ranges up to 250. Patients with higher copy number of trinucleotide repeats typically have earlier onset of the disease. If HTT is an essential player in the regenerative process and its activation is necessary, then we expect to observe a reduction in the number of regenerating CST axons due to activation of mutant HTT. Mutant HTT has been shown to result in failure of regenerative sprouting of second order motor neuron axons in the process of reinnervating the neuromuscular junction (Ribchester at al., 2004). However, we expect from our data that overexpression of wt HTT might increase axon growth.

2.6 CONCLUSION

Regenerating CST axons show anatomically distinct chronological phases from axon regeneration to synapse formation. These steps are supported by activation of distinct signaling pathways ranging from neurogenesis and neuronal differentiation, over activation of axonal growth programs, mitochondrial organization and ATPs synthesis, to activation of axon guidance and synaptogenesis related mechanisms. We identified unique molecules and pathways activated upon CST regeneration and others that resemble known mechanism of axon regeneration in other CNS or PNS systems. In order to therapeutically apply this knowledge we will screen selected hit candidates using in *vitro* and *in vivo* assays of axonal regeneration to identify targets for gene therapy treatment of spinal cord injury and other neurological diseases.

2.7 EXPERIMENTAL PROCEDURES

2.7.1 Animals

All procedures involving animals were carried out in strict adherence to guidelines provided by The Guide for the Care and Use of Laboratory Animals (The Institute of Laboratory Animal Resources, 2011), The Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH, 1986), The Animal Welfare Act/Regulations and subsequent amendments (PL 89-544), and The Veterans Health Administration Handbook 1200.07 "Use of Animals in Research" (2011); VA San Diego Healthcare System (VASDHS) Research Services Policy 01 section 151-04 (Institutional Animal Care and Use Committee, IACUC) and VASDHS IACUC Policy 03 (Pre and Post-procedural Care of Laboratory Rodents). The animal use protocol was approved by the VASDHS IACUC (Protocol number 11-010). The Glt25d2 bacteria artificial chromosome (BAC) with EGFP-tagged ribosomal protein for translational ribosome affinity purification (TRAP) (Glt25d2-EGFPL10a) mice were obtained from the GENESAT project and maintained at the VA hospital in San Diego as described previously (Doyle et al., 2008). C57BL/6-Tg(CAG-EGFP)1Osb/J (GFP-mice) expressing "enhanced" GFP (EGFP) cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer were bred with C57BL/6 (wt-mice) females to produce GFP-positive embryos. GFP-mice and wt-mice were obtained from Jackson Laboratory. All mice were maintained on a 12-hr light/dark cycle and given ad libitum access to food and water.

2.7.2 Mouse Surgery

All surgery was done under deep anesthesia by using a combination of Ketamine (80-100 mg/kg) and Xylazine (5-10 mg/kg). Euthanasia for tissue harvesting as performed by injection of an overdose amount of anesthesia cocktail in accordance with AVMA Guidelines for Euthanasia of Laboratory Animals (2013).

2.7.3 Dorsal column lesion

To assess the regeneration of corticospinal tract axons subjects underwent C5 or T1 dorsal column lesions, as previously described (Weidner et al., 2001). Briefly, a Kopf wire knife (David Kopf Instruments, Tujunga, CA) was inserted 0.3 mm lateral to midline and 1.1 mm under the dorsal surface of the spinal cord; the knife was extruded 1.5 mm and lifted to transect the dorsal columns, with coincident compression with a 28 gage blunt tip from above to ensure lesion completeness.

2.7.4 Right Unilateral Pyramidotomy

Adult transgenic bacTRAP CST mice (DU9 line, Heintz Laboratory, Rockefeller Univ. NY) were anesthetized parentrally. The anterior neck of the mice was then shaved and sterilized. A midline incision was made and the trachea and esophagus retracted. Blunt dissection was performed to expose the anterior surface of the occipital bone. Micro-scissors and blunt forceps (FST, Foster City CA) were used to remove the caudal occipital bone. The Dura mater was punctured with a 6 mm insulin syringe (Becton Dickinson & Co., Franklin Lakes, NJ) and the right pyramidal tract was lesioned with a microknife (Fine

Science Tools, Foster City CA), above the level of the decussation, similar to the methods outlined by Starkey et al. 2008. The trachea and esophagus were then replaced and the surgical opening closed with sutures. Lesioned mice were then stratified into three different experimental groups based on survival time point: 1day, 1 week, and 2 weeks.

2.7.5 Transplantation surgeries

For mouse-donor studies, grafting was performed either 1 week after T1 dorsal column wire-knife lesion or at the time of lesion. Embryonic day 10-15 (E10 - E15) spinal cords from transgenic GFP-mice were dissected in ice cold HBSS, digested in 0.25% Trypsin for 20 min at 37C, washed 3 times with 10% FBS in Neurobasal medium, triturated in 5 ml Neurobasal medium + B27, filtered with 70 um mesh, spun at 200g for 5 min at room temperature, resuspended in 2 ml Neurobasal + B27 and kept on ice. Before grafting cells were spun at 200g for 3 min at room temperature, supernatant was aspirated and cell pellets were resuspended at a concentration of 200,000 cells/µl in a fibrin matrix (25 mg/ml fibrinogen and 25 U/ml thrombin, Sigma F6755 and T5772) containing growth factors to support graft survival (Willerth et al., 2007;Grumbles et al., 2009; Kadoya et al., 2009): BDNF (50 µg/ml, Peprotech, 452-02), neurotrophin-3 (NT-3; 50 µg/ml, Peprotech, 450-03), platelet-derived growth factor (PDGF-AA; 10 µg/ml, Sigma, P3076), insulin-like growth factor 1 (IGF-1; 10 µg/ml, Sigma, I8779), epidermal growth factor (EGF; 10 µg/ml, Sigma, E1257), basic fibroblast growth factor (bFGF; 10 µg/ml, Sigma, F0291), acidic fibroblast growth factor (aFGF; 10 µg/ml, Sigma, F5542), glial-cell-line-derived neurotrophic factor

(GDNF; 10 µg/ml, Sigma, G1401), hepatocyte growth factor (HGF; 10 µg/ml, Sigma, H9661), and calpain inhibitor (MDL28170, 50 µM, Sigma, M6690). Thrombin and Fibrinogen cell suspensions were injected separately to allow for fibrin matrix formation within the spinal cord. Fibrinogen suspension was injected in all sites prior to thrombin suspension. The graft mixture with either embryonic spinal cord cells or MSC was microinjected into the lesion cavity at T1 and into the intact spinal cord at C4 and C6 in 2 locations each. Each injection site received 0.83 µl total cell suspension, and subjects survived another 3 days till 6 weeks. GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells (MSC) were purchased from Invitrogen and cultured according to the manufactures manual till 80-90% confluency, trypsinated for 5 min with 0.25%, spun down for 5 min at 300g, resuspended at 75k cells/µl growth factor cocktail and kept on ice till injection.

2.7.6 CST-axon tracing

Nine days before perfusion, corticospinal tract axons were anterogradely labeled by injection of 0.3 µl of 10% biotinylated dextran amine (BDA; MW 10,000, Molecular Probes) into each of 10 sites per hemisphere spanning the motor cortex (from bregma, rostral-caudal: +/- 0.0, 0.5, 1 mm; lateral: +/- 1.2, 2.2 mm; depth: 0.7 mm).

2.7.7 Fluorescent staining

Spinal cords were removed from the vertebrae and serially sectioned in the sagittal plane at 35 µm intervals. Every sixth section was used for label detection. All steps were performed at room temperature if not noted otherwise.

Sections were washed three times in TBST (0.025% triton-X100 in TBS) for 10 min, washed with 50 % methanol in TBS for 5 min, dehydrated in 100 % methanol for 15 min, washed with 50 % methanol in TBS for 5 min and blocked in TBST + 5% donkey serum for 1 hour. Incubated overnight in TBST + 3% donkey serum with primary antibodies at 4℃. Sectio ns were washed 3 times in TBST + 3% horse serum and incubated 2.5 h with secondary antibodies. Washed 3 times with TBS for 10 min, mounted on glass slides and coverslipped Fluoromount G (Southern Biotechnology, Birmingham, AL).

2.7.8 Primary antibodies

Alexa 594-conjugated Streptavidin (1:300), mouse anti-NeuN (1:250, Millipore), rabbit anti-GFP (1:1000, Invitrogen), rabbit anti-GFAP (1:1500; Chemicon, Temecula, CA), mouse anti-APC (1:200, Millipore), rabbit anti-IBA1 (1:1500, Wako Chemicals, Richmond, VA)

2.7.9 Secondary antibodies

Alexa 488-conjµgated donkey anti-rabbit and Alexa 647-conjµgated donkey anti-mouse (1:200; Jackson ImmunoResearch, West Grove, PA). Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; [1 µg/mL]; Sigma-Aldrich, St. Louis, MO).

2.7.10 Translating Ribosome Affinity Purification

Three to four adult mice were pooled for each sample and three biological replicates were collected for each condition. All polysome purifications and mRNA extractions were carried out as described previously (Heiman et al.,

2008). RNA quantity was measured with a Nanodrop 1000 spectrophotometer and quality was assayed on an Agilent 2100 Bioanalyzer.

2.7.11 Gene Expression Analysis

2.7.11.1 RNASeq Library Preparation and Sequencing

Total RNA integrity was examined using the Agilent Bioanalyzer 2000 (Agilent) and quantified with NanoDrop®. Twenty nanograms of total RNA were used to generate cDNA using Ovation® RNA-Seq System V2 (NuGEN) following the manufacturer's instruction. One hundred nanograms cDNA were used in the library preparation using Ovation® Ultralow Library Systems (NuGEN). The cDNA was fragmented to 300 bp using the Covaris M220 (Covaris) then followed the manufacturer's instruction for end repair, adaptor ligation and library amplification. The libraries were pooled and sequenced using HiSeq™2500 (Illumina) for PE 50-bp reads following the manufacturer's instruction.

2.7.11.2 Microarray Gene Expression Analysis

The total RNA integrity was examined using the Agilent 2200 Tapestation (Agilent) and quantified with Ribogreen (Life Technologies). One hundred nanograms of total RNA were used in cRNA generation using Illumina® TotalPrepTM-96 RNA Amplification Kit following the manufacture's instruction. The cRNA was hybridized to the MouseRef-8 V2 Expression BeadChips (Illumina), and the signal was scanned with iScan™ (Illumina) following the manufacturer's protocol.

2.7.11.3 Sequence data generation

The 48 mouse RNA-seq samples for this project were sequenced by the UNGC project 2013 092. RNA- sequencing was carried out using the TruSeq RNA library prep. Preparation included 50 bp paired end reads.

2.7.11.4 Read Trimming

If the overall base quality is low, or the nucleotide composition QC show contamination (such as with adaptor sequences) then reads can be trimmed and/or filtered to remove problematic bases. No read trimming or filtering was done with this data set because the quality distribution and variance appear normal. Had quality been low and or nucleotide distribution highly inconsistent, read trimming could have been applied at this stage.

2.7.11.5 Alignment

Short reads were aligned using STAR to the mouse reference genome (mm9), with default parameters. STAR is a short-read aligner using a memory efficient compressed suffix array, which is fast, sensitive, accurate and integrated well with standard sequence analysis tools (Dobin et al., 2012). Additional QC is performed after the alignment to examine: the level of mismatch rate, mapping rate to the whole genome, repeats, chromosomes, and key transcriptomic regions (exons, introns, UTRs, genes).

2.7.11.6 Coverage Effective for Gene Expression Analysis

Gene expression can be measured in terms of total read counts per gene. For paired end experiments, total fragments are considered. Counts of fragments aligned to known exonic regions based on the RefSeq (refFlat) annotation are

considered for expression using HTSeq package (Anders, 2012). If both pairs are aligned to a gene then they are counted once as a fragment. If only one read from a pair is aligned to a known region then it is also counted once. Reads that align to exons shared by more than one gene are considered ambiguous and are not counted. Reads aligned to multiple locations are also not considered for expression quantification. Averagely across the samples 50% of the annotated genes have been detected by at least 50 reads.

2.7.11.7 Clustering of Samples

We use multiple clustering methods to examine the quality of replicates and to highlight possible outlier samples which can eventually be excluded if necessary. Samples are analyzed in this stage of the pipeline. Clustering techniques are applied to raw count expression levels, normalized raw counts via variance stabilized normalized count expression values (which will be explained in more detail the coming sections where it is applied).

2.7.11.8 Gene Ontology of Differentially expressed genes

We also test for gene ontology (GO) category enrichment amongst differentially expressed (DE) genes. Complete gene annotations are output in a separate excel file. The R package GeneAnswers (Feng et al. 2010) is used to test for enrichment. The Bioconductor annotation data package ’org.Mm.eg.db’ is used as the ontology library for the Mouse organism (Carlson et al). False discovery rate correction of the enrichment test p-value is set at ≤ 0.1. Below are figures for visualization of categorical enrichment. In figure 20 the three following gene ontology terms are shown; Cellular Component (CC), Biological Process

(BP) and Molecular Function (MF) ranked by the top (maximum 25) terms by p-value and the bars are the -log of the p-values. In the bar you have the term and to its right the number of DEG in that term.

Chapter 2, in its entirety, is currently being prepared for submission for publication of the material. Gunnar H.D. Poplawski, Audris Fan, Richard Lie, Paul Lu, Qing Wang, Giovanni Coppola and Mark Tuszynski. The dissertation author was the primary investigator and first author of this paper.

2.8 FIGURES

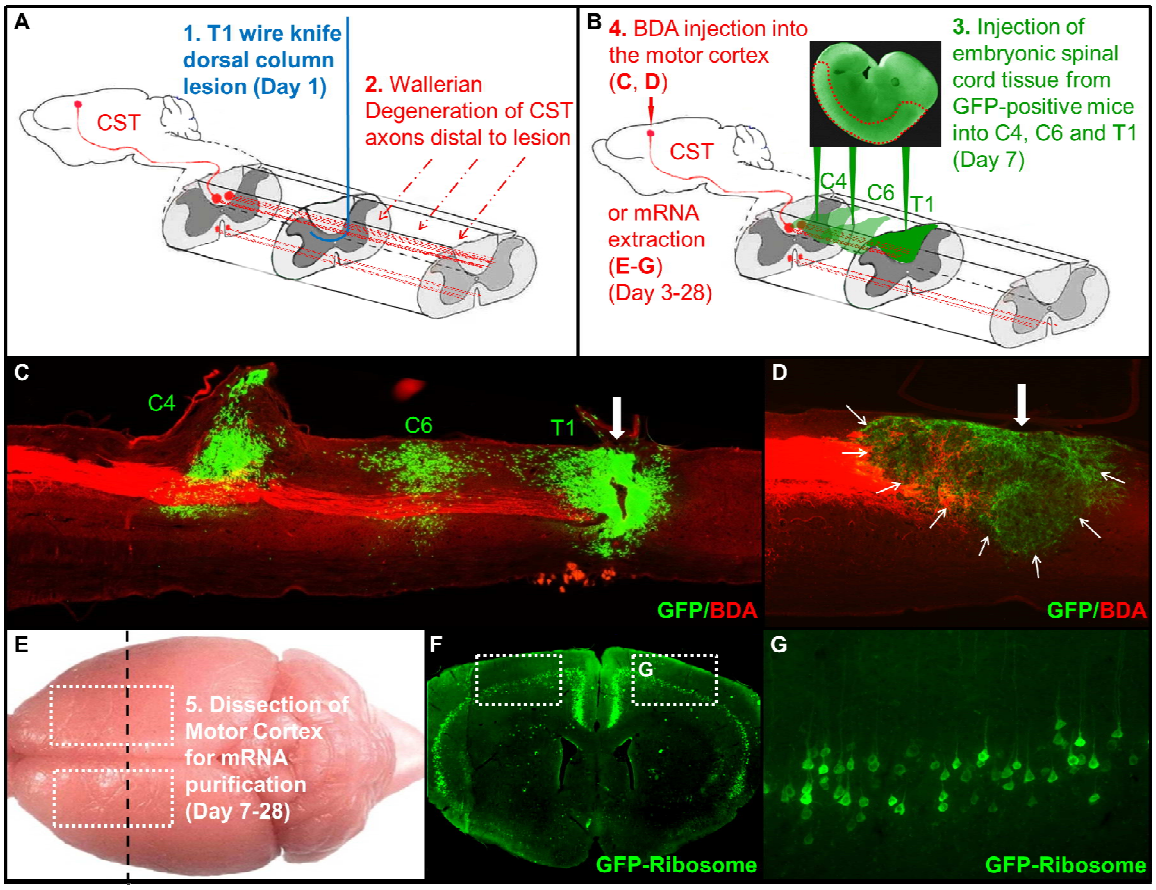

Figure 2.1: Experimental paradigm. Schematic view of mouse brain and spinal cord. CST-tract is labeled in red. Wireknife lesion was performed at thoracic level 1 (T1). (**B**) Isolation of GFP expressing embryonic mouse spinal cord, followed by grafting of triturated cells into lesion site at T1 and into intact cord at cervical level 4 (C4) and C6. (**C, D**) BDA injection into the motor cortex for anatomical studies or (**E-F**) tissue isolation for mRNA purification. (**C, D**) Sagittal section of mouse spinal cord showing GFP-expressing grafted cells (**green**) and BDA labeled CST-axons (**red**) (**C**) 1 week and (**D**) 6 weeks post graft. Bold white arrow indication lesion site. Thin white arrows indicate graft site. Regenerating CST axons that penetrate grafts are shown at higher magnification in Figure 2. (**E**) Schematic view of mouse brain for tissue collection. Dotted areas of motor cortex were dissected for mRNA harvesting (white). (**F**) Coronal section of BAC-TRAP mouse brain showing GFP-labeled layer 5B neurons (**green**). Dotted areas represent dissected tissue for mRNA harvesting (**white**). (**G**) Magnified image of dissected motor cortex, showing neurons expressing GFP-tagged ribosomal protein L10A (Rpl10a).

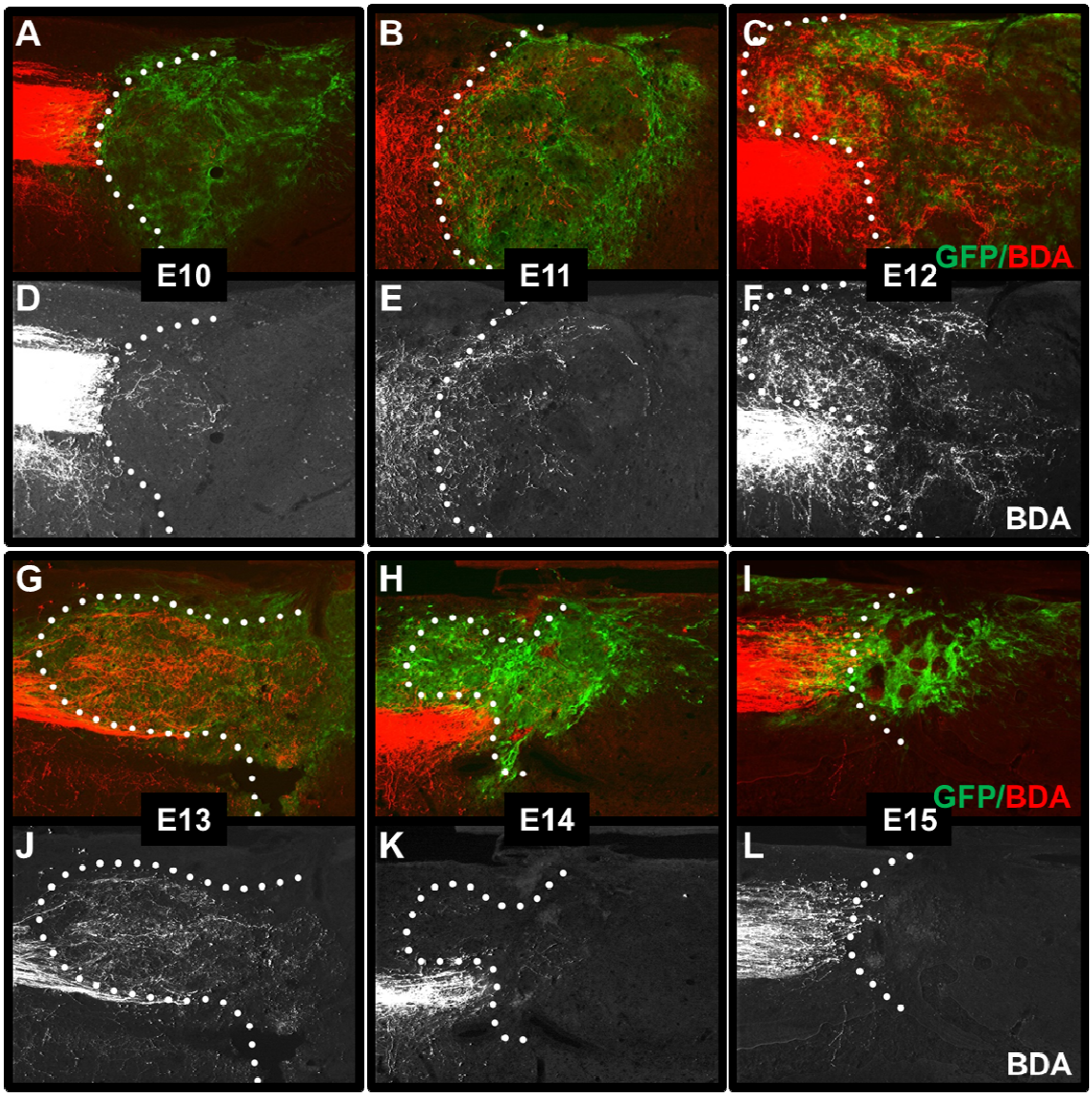

Figure 2.2: CST axon regeneration into NSC grafts derived from E10, E11, E12, E13, E14 and E15. (**A – C, G – I**) Sagittal sections of mouse spinal cord showing GFP-expressing NSC grafts in lesion sites (**green**) and BDA labeled CST axons (**red**). Grafts were derived from embryonic day E10 - E15. (**D –F, J – L**) Same section as in (A – C, G – I) showing greyscale image of the BDA-labeled CST axons (**white**) for better visualization of regenerating axons. White dotted lines outline the lesion graft interface.

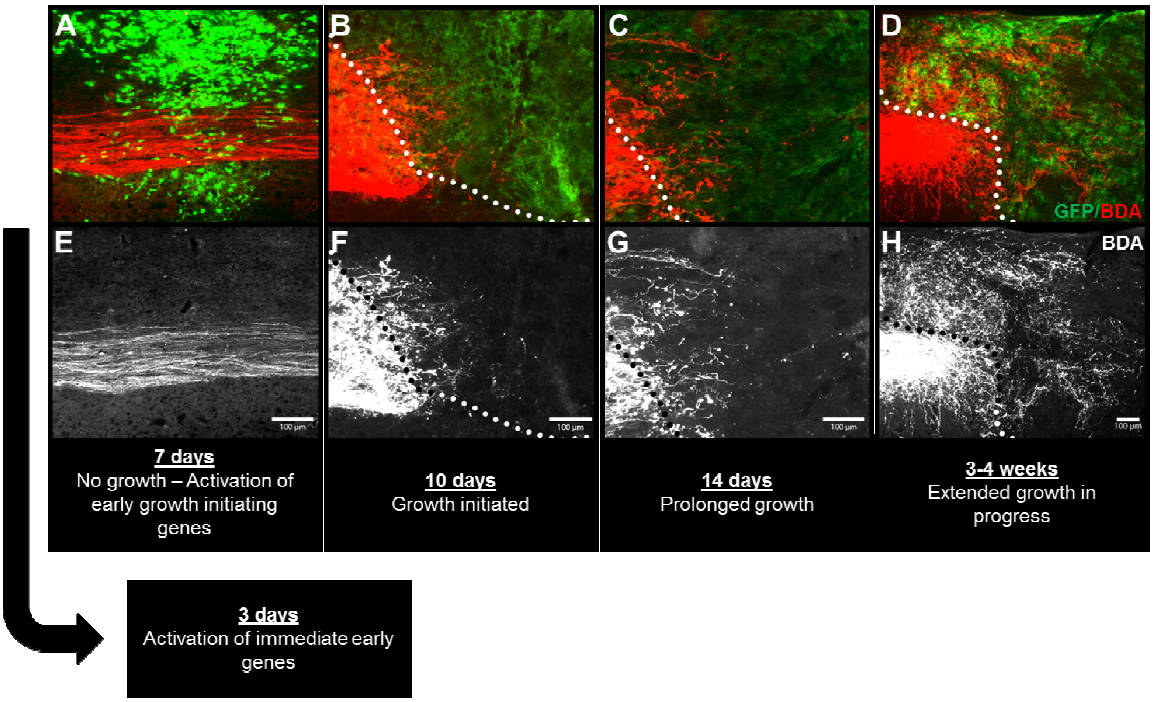

Figure 2.3: Chronological assessment of CST-axon regeneration into E12 spinal cord derived cell grafts reveals distinct stages of regeneration. (**A – D**) Sagittal section of mouse spinal cord showing GFP positive grafted cells (**green**) and BDA labeled CST-axons (**red**). (**E – H**) Same section as in (A – D) showing image of the BDA positive CST axons (**white**) for better visualization of regenerating CST axons. (**A, E**) 7 days after cell graft. Grafted cells migrate into host white matter and proliferate and differentiate. No CST axon growth detectable. (**B, F**) 10 days after cell graft. Grafted cells expanded and differentiated. CST axon growth is initiated and axons have grown over short distances. (**C, G**) 2 weeks after cell graft. CST axons are in prolonged growth phase. (**D, H**) 3 weeks after cell graft. Extended CST axon growth is in progress. (**A- C, E – G**) 20x images and (**D, H**) 10x images. Scale bar is 100 µm.

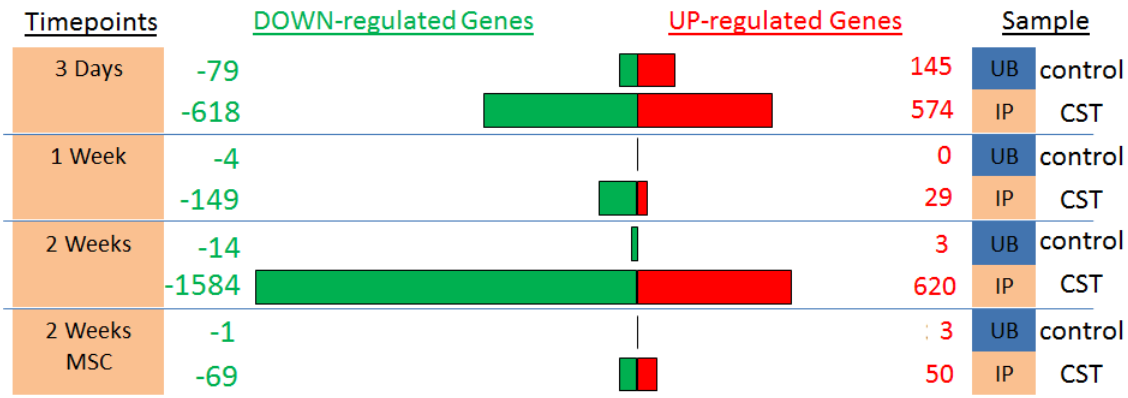

Figure 2.4: Differentially expressed genes across comparisons at FDR ≤ 0.1. Listed are from top to bottom, regeneration associated genes ratio comparisons for 3 days, 1 week, 2 weeks and 2 weeks with bone marrow stromal cell (MSC) graft. The 2 week MSC condition reflects gene ratios that are not related to CST regeneration. For each time point the UB and the IP fraction is listed. Green indicates down-regulation and red indicates up-regulation for each corresponding condition. The number of down and upregulated genes for each comparison are on the left and right side respectively. Please note that the UB fraction (unspecific) contains significantly less differentially regulated than the IP fraction (CST specific).

Table 2.1: Highest up- and down-regulated molecules 3 days after cell graft. Listed are the top 10 up and down-regulated genes at the 3 day time point. From left to right: Genesymbol, Entrez Gene name and the fold change of the E12 spinal cord graft at 3 days compared to lesion only control at 3 days. Up-regulated genes in red and down-regulated genes are listed in green.

Gene Symbol	Entrez Gene Name	Fold Change
HP	haptoglobin	26.4
IGFLR1	IGF-like family receptor 1	8.1
FXYD4	FXYD domain containing ion transport regulator 4	5.3
C230035I16Rik	RIKEN cDNA C230035I16 gene	4.9
GYPC	glycophorin C (Gerbich blood group)	4.7
EVC	Ellis van Creveld syndrome	3.1
CD93	CD93 molecule	3.0
SIX5	SIX homeobox 5	2.9
GIMAP1	GTPase, IMAP family member 1	2.9
MARVELD2	MARVEL domain containing 2	2.8
AURKB	aurora kinase B	-26.5
SLPI	secretory leukocyte peptidase inhibitor	-26.1
HSPB2	heat shock 27kDa protein 2	-21.5
SALL4	sal-like 4 (Drosophila)	-20.5
PTPRC	protein tyrosine phosphatase, receptor type, C	-18.2
GPR156	G protein-coupled receptor 156	-12.8
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	-7.7
Gucy1b2	guanylate cyclase 1, soluble, beta 2	-6.9
IRF4	interferon regulatory factor 4	-5.6
WNT3	wingless-type MMTV integration site family, member 3	-5.5

Table 2.2: Highest up- and down-regulated molecules 1 week after cell graft.

Listed are the top 10 up and down-regulated genes at the 1week time point. From left to right: Gene symbol, Entrez Gene name and the fold change of the E12 spinal cord graft at 1 week compared to lesion only control at 1 week. Up-regulated genes in red and down-regulated genes are listed in green.

Gene Symbol	Entrez Gene Name	Fold Change
ANXA9	annexin A9	14.3
IFI30	interferon, gamma-inducible protein 30	7.4
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	6.4
CCDC114	coiled-coil domain containing 114	6.0
TSHB	thyroid stimulating hormone, beta	5.5
TMIE	transmembrane inner ear	5.2
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	4.9
EVC	Ellis van Creveld syndrome	4.2
LRRN4	leucine rich repeat neuronal 4	4.1
HIF3A	hypoxia inducible factor 3, alpha subunit	3.7
COLQ	collagen-like tail subunit of asymmetric acetylcholinesterase	-26.4
CD82	CD82 molecule	-2.9
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	-2.4
ATG7	autophagy related 7	-2.3
IRF6	interferon regulatory factor 6	-2.3
IGF2BP2	insulin-like growth factor 2 mRNA binding protein 2	-2.2
KLHL1	kelch-like family member 1	-2.1
KDM8	lysine (K)-specific demethylase 8	-2.1
ZBTB3	zinc finger and BTB domain containing 3	-1.9
DPM3	dolichyl-phosphate mannosyltransferase polypeptide 3	-1.9

Table 2.3: Highest up- and down-regulated molecules 2 weeks after cell graft.

Listed are the top 10 up and down-regulated genes at the 2 week time point. From left to right: Gene symbol, Entrez Gene name and the fold change of the E12 spinal cord graft at 2 weeks compared to lesion only control at 2 weeks. Up-regulated genes in red and down-regulated genes are listed in green.

Gene Symbol	Entrez Gene Name	Fold Change
GPR101	G protein-coupled receptor 101	47.9
RBM47	RNA binding motif protein 47	31.2
IGSF1	immunoglobulin superfamily, member 1	29.1
RPE65	retinal pigment epithelium-specific protein 65kDa	27.3
PPAPDC1A	phosphatidic acid phosphatase type 2 domain containing 1A	23.5
ONECUT1	one cut homeobox 1	18.5
FZD10	frizzled family receptor 10	17.2
PIK3AP1	phosphoinositide-3-kinase adaptor protein 1	17.1
FOSL1	FOS-like antigen 1	16.4
NDST4	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 4	14.7
CYR61	cysteine-rich, angiogenic inducer, 61	-3.8
HIST1H2AD	histone cluster 1, H2ad	-2.8
CKAP2L	cytoskeleton associated protein 2-like	-2.4
KLHL1	kelch-like family member 1	-2.4
CUZD1	CUB and zona pellucida-like domains 1	-2.2
HES1	hairy and enhancer of split 1, (Drosophila)	-2.2
NEURL2	neuralized homolog 2 (Drosophila)	-2.1
SPC24	SPC24, NDC80 kinetochore complex component, homolog	-2.1
JUNB	jun B proto-oncogene	-2.1
C11orf82	chromosome 11 open reading frame 82	-2.0

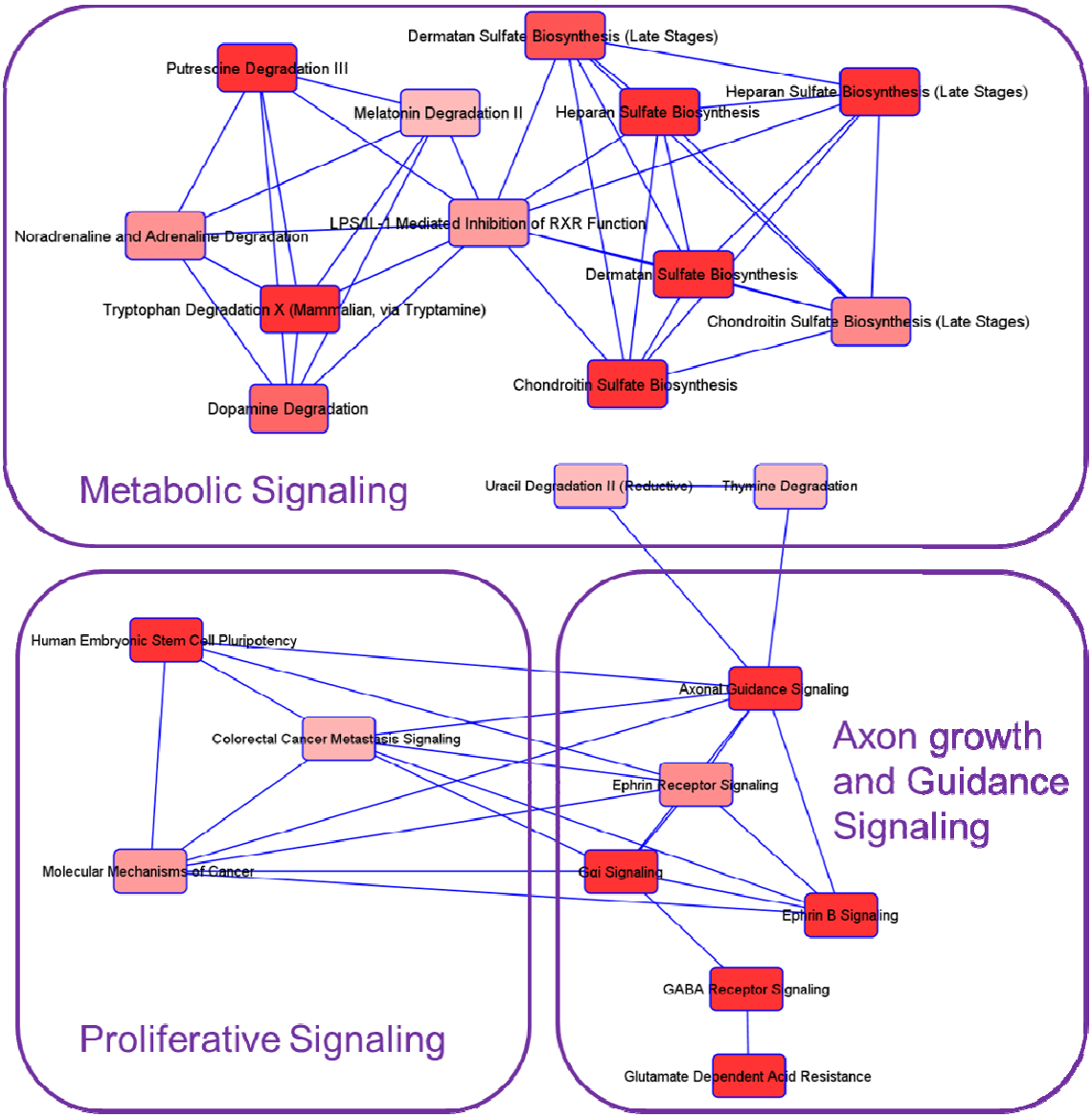

Figure 2.5: Interconnectivity Map of Activated Canonical Pathways 3 days post cell grafting. Each red box represents a canonical pathway that is activated in the dataset. The intensity of the red-color represents the pValue (higher intensity = lower p-Value for a certain canonical pathway). The blue lines indicate overlaps between two or more molecules between pathways. Each blue line represents at least one overlapping molecule. For more detail see also supplemental Figure S2.9 and S2.10

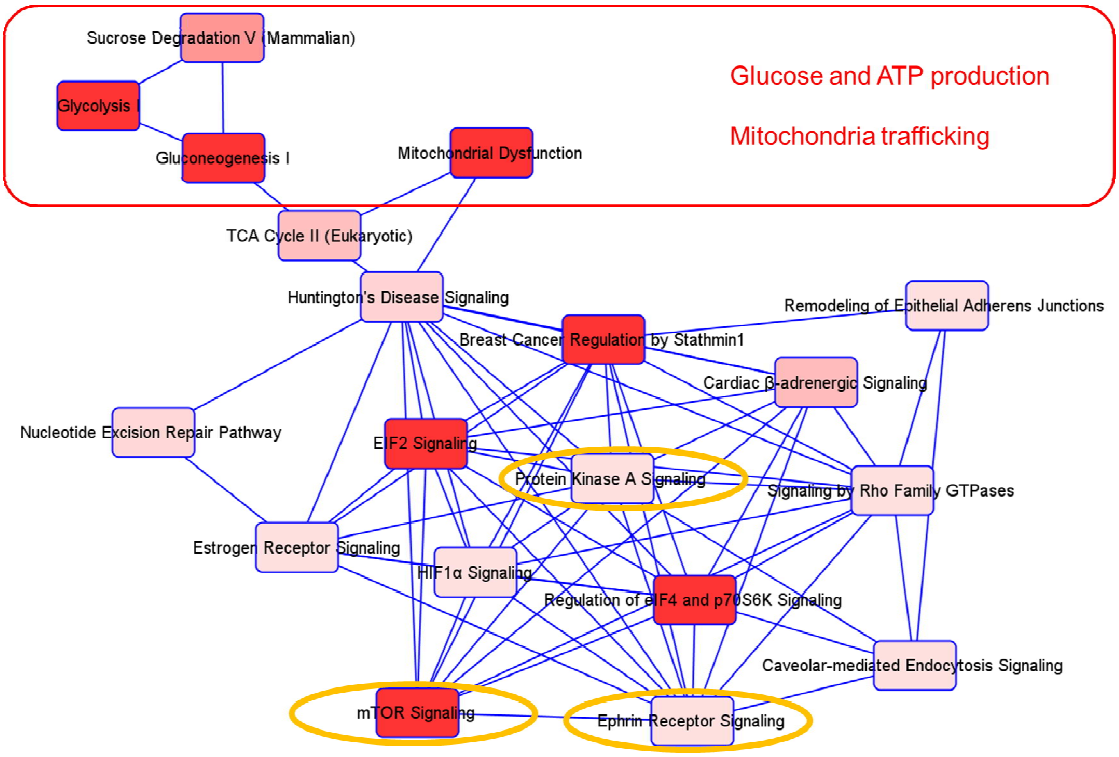

Figure 2.6: Interconnectivity Map of Activated Canonical Pathways 1 week post cell grafting.

Each red box represents a canonical pathway that is activated in the dataset. The intensity of the red-color represents the pValue (higher intensity = lower pValue for a certain canonical pathway). The blue lines indicate overlaps between two or more molecules between pathways. Each blue line represents at least one overlapping molecule. For more detail see also supplemental Figure S2.11 and S2.12

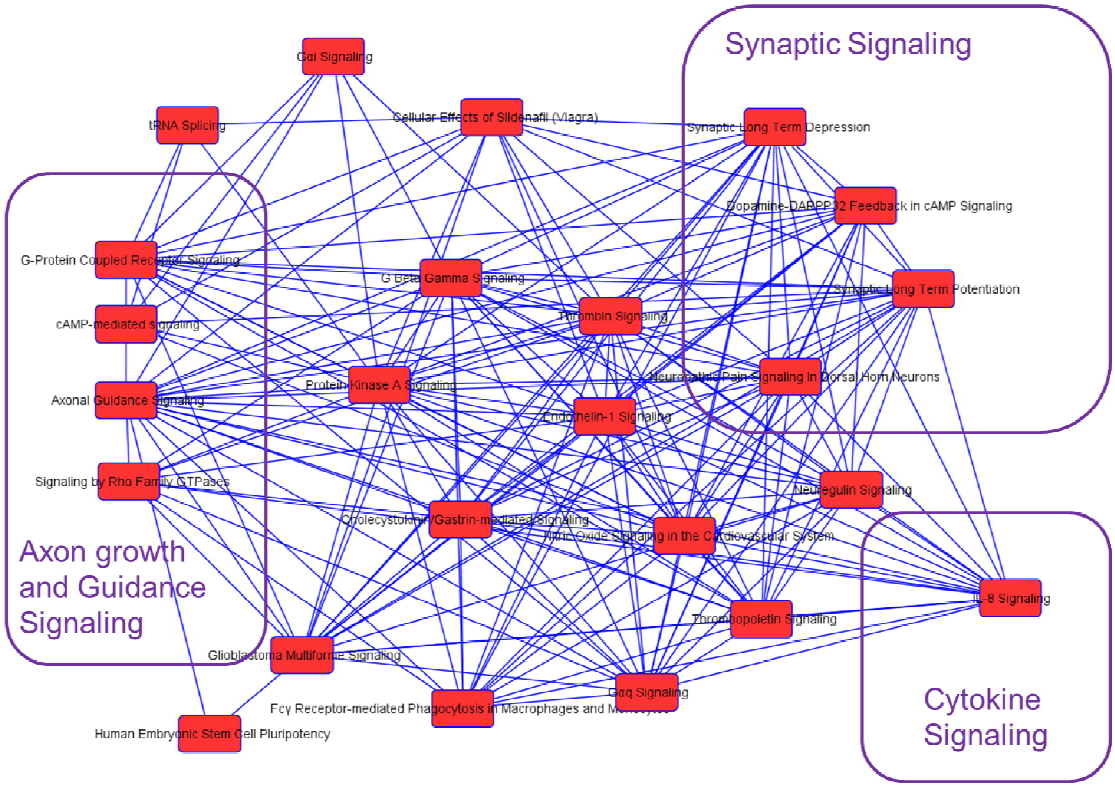

Figure 2.7: Interconnectivity Map of Activated Canonical Pathways 2 week post cell grafting.

Each red box represents a canonical pathway that is activated in the dataset. The intensity of the red-color represents the pValue (higher intensity = lower pValue for a certain canonical pathway). The blue lines indicate overlaps between five or more molecules between pathways. Each blue line represents at least one overlapping molecule. For more detail see also supplemental Figure S2.13 - S2.15.

Table 2.4 Upstream Regulator Analysis: Transcription Factors with Predicted Function.

The table lists the transcription factors that show a prediction of activation or inhibition based on the regulation of differentially regulated downstream molecules in the dataset (time point). From left to right: Upstream regulator, predicted activation state (red = activated, green = inhibited) and corresponding –score for 3 days, 1 week and 2 weeks. Upregulation is marked in red and down-regulation in green. (2≤ z-score ≤-2). Since the low amount of differentially regulated genes at 1 week, no prediction for transcriptional regulation could be made with confidence.

Upstream regulators at 3 days		
Upstream Regulator	**Predicted Activation State**	**Activation z-score**
CEBPB	Inhibited	-2.969
CEBPA	Inhibited	-2.534
KLF15		-1.987
SREBF1		-1.98

Upstream regulators at 2 weeks		
Upstream Regulator	**Predicted Activation State**	**Activation z-Score**
CTNNB1	Activated	3.014
FOS	Activated	2.377
TP53	Activated	4.368
CEBPB	Activated	2.031
SIX5	Activated	2.813
SMARCD3	Activated	2.449
ERG	Activated	2.683
T	Activated	2.449
NOTCH3	Activated	2
EGR1	Activated	2.779
MITF	Activated	2.804
NFATC2	Activated	2.673
FOXC1	Activated	2
LHX1	Activated	2.168
SMARCB1	Activated	2.63
HNF1A	Activated	2.324
TOB1	Activated	2.236
HTT	Activated	2.183
RBPJ	Inhibited	-2.533
POU4F2	Inhibited	-2.789
TAF4	Inhibited	-2.375
GFI1	Inhibited	-2.508
CREB1	Inhibited	-2.595
CREM	Inhibited	-2.902
TRIM24	Inhibited	-2.496

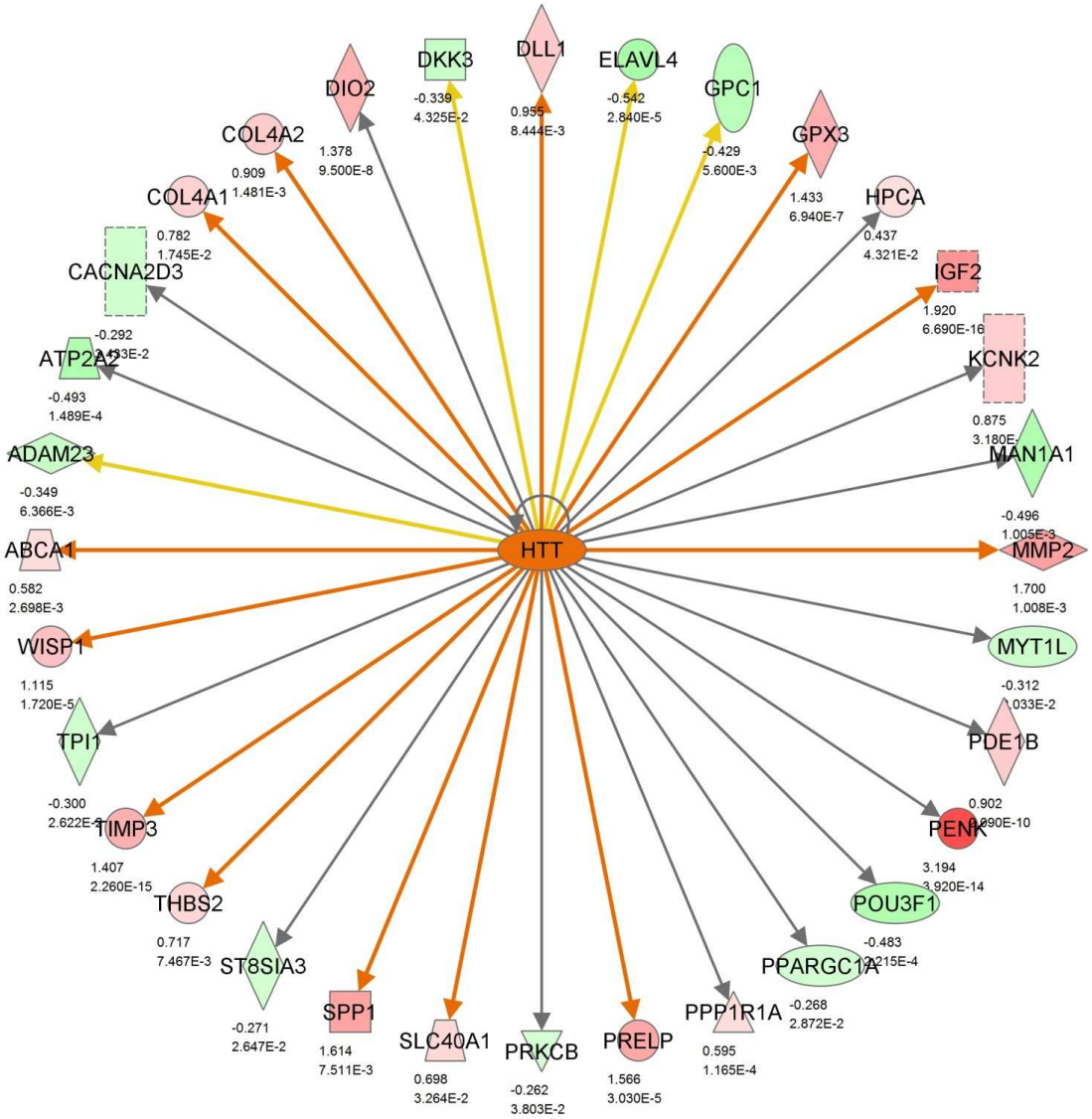

Figure 2.8: Downstream Effector Network of the Transcriptional Regulator Huntingtin (HTT) at 2 weeks after cell transplant.

Htt is predicted to be activated in this dataset. Thirteen downstream molecules (red) have bed shown to be positively regulated by HTT. Orange arrows show a prediction of activation. Grey arrows represent an influence on expression of downstream effector, but no clear trend of regulation can be extracted from the literature. Yellow arrows represent a controversial finding to the literature. Here are 4 molecules that are downregulated in this dataset, but are predicted to be positively regulated by HTT. Downregulated effector genes are in green. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation. (See also figure legend of Fig. S2.8)

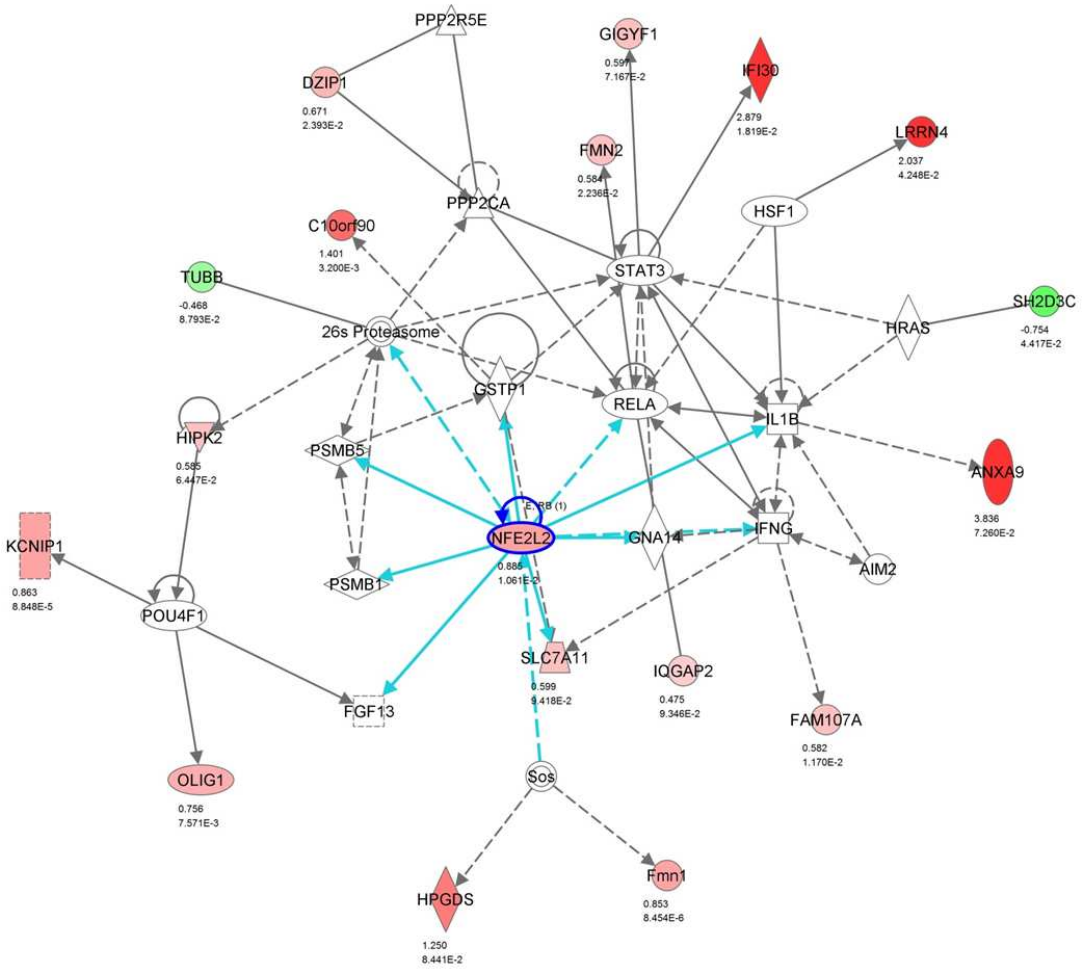

Figure 2.9: Functional Network around NFE2L2 identified at 1 week post grafting. The expression of NFE2L2 is increased by 1.8 fold at this intermediate stage of regeneration. Dashed lines indicate indirect interactions; solid lines indicate direct interactions. The arrow indicates the directionality of the relationship. Downregulated genes are shown in **green** and upregulated genes in **red**. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation. For detailed information on individual genes within the network and on their interaction see the legend in the supplemental material (Fig. S2.20)

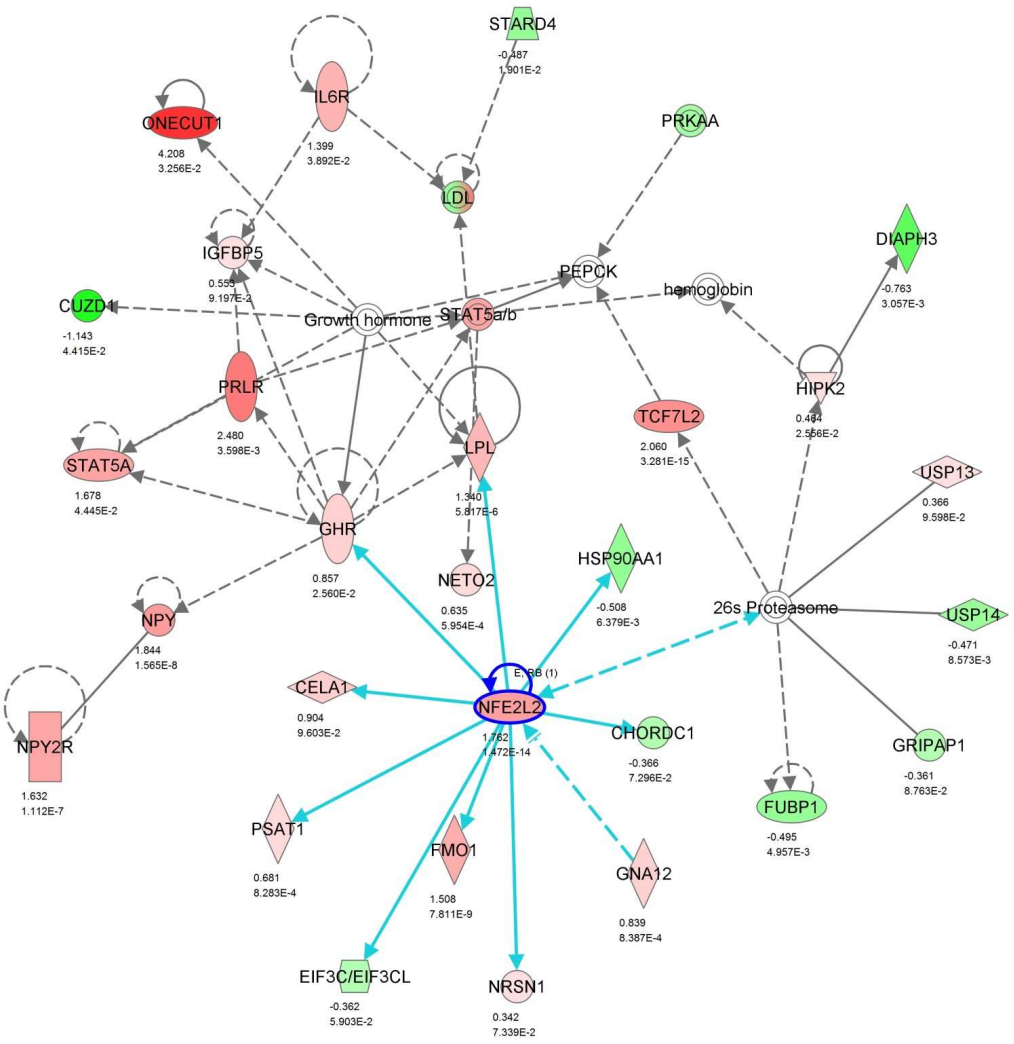

Figure 2.10: Functional Network around NFE2L2 identified at 2 weeks post grafting. The expression of NFE2L2 is increased by 3.4 fold at this mature stage of regeneration. Dashed lines indicate indirect interactions; solid lines indicate direct interactions. The arrow indicates the directionality of the relationship. Downregulated genes are shown in **green** and upregulated genes in **red**. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation. For detailed information on individual genes within the network and on their interaction see the legend in the supplemental material (Fig. S2.20)

2.9 SUPPLEMENTAL MATERIAL

2.9.1 Characterization of differences in grafts derived from E10, E12 and E15 spinal cord cells

To gain deeper insight, why lesioned adult CST axons preferentially regenerate into grafts derived from E12-E13 spinal cord cells, we analyzed the amount of axons emerging from the graft into the host matter at E10, E12 and E15. Additionally, we investigated the cell identity within those grafted cells 5-6 weeks post transplantation. We found that significantly more axons are emerging from E12 derived grafts than compared to E10 and E15 (Fig. S2.1). This finding correlates positively with the percentage of mature neurons within the graft. No correlation was found between the number of regenerating CST axons and the number of astrocytes (GFAP-positive), oligodendrocytes (ACP-positive) or macrophages (IBA-positive) within the graft (Fig. S2.2)

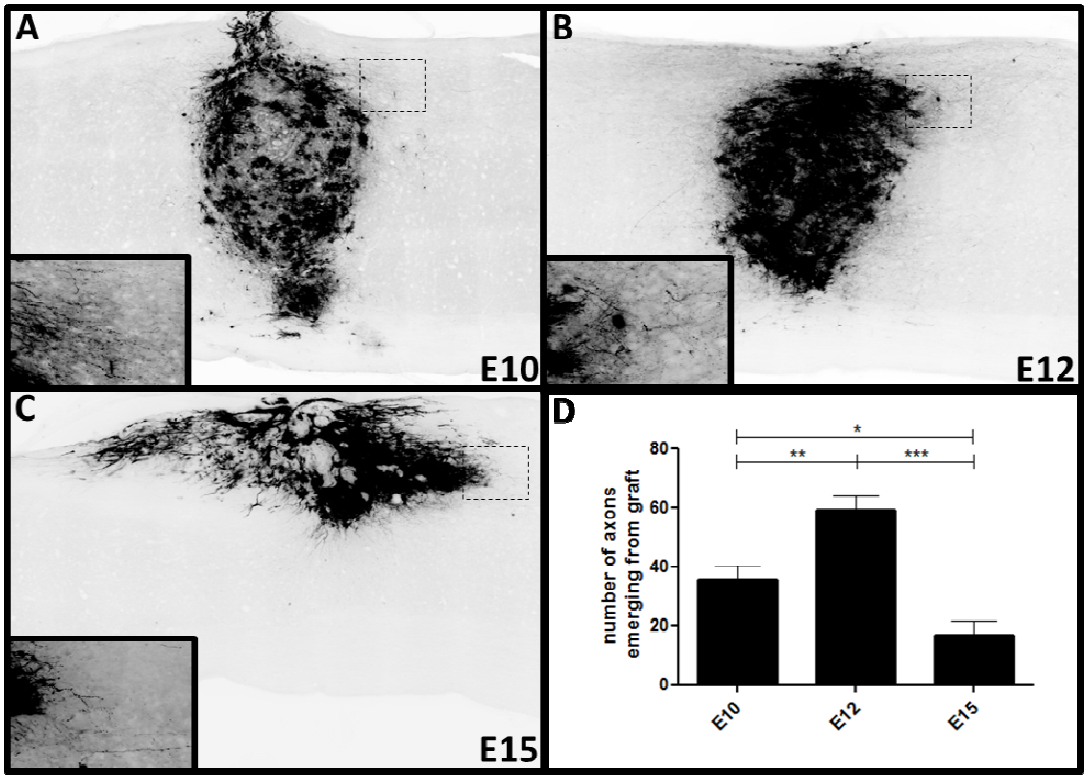

Figure S2.1: Quantification of axons emerging from embryonic spinal cord grafts derived from E10, E12 and E15. (**A-C**) Sagittal view of GFP-positive spinal cord graft into C5 dorsal column lesion 5-6 weeks post transplantation. GFP is labeled in black, inset shows close up of GFP-positive axons emerging caudally from graft and entering adult host white matter. (**A**) Graft derived from E10 spinal cords. (**B**) Graft derived from E12 spinal cords. (**C**) Graft derived from E15 spinal cords. (**D**) Quantification of axons emerging from grafted cells rostral and caudal. (one-way ANOVA with Tukey's multiple comparison test, P value *<0.05, **<0.01, ***<0.001; 3 animals with at least 2 sections analyzed per group)

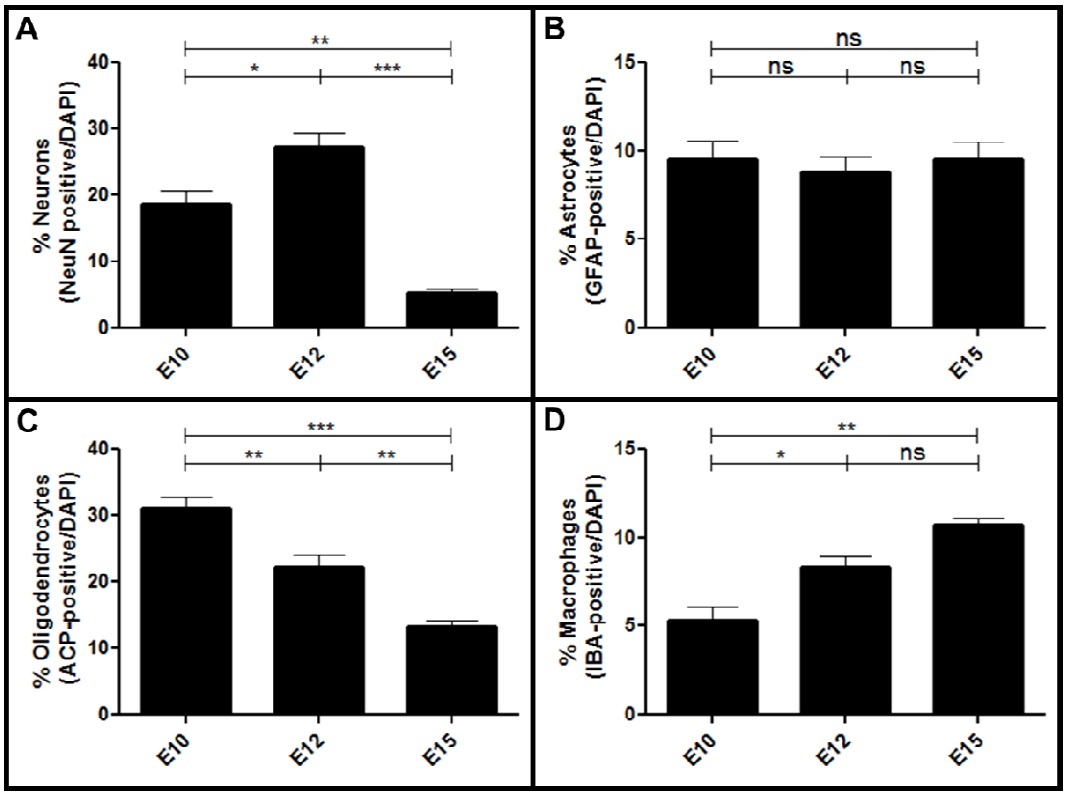

Figure S2.2: Quantification of cell differentiation from embryonic spinal cord grafts derived from E10, E12 and E15. (A-D) Percentage of investigated cells in graft in comparison to Nuclei stain. (**A**) Neurons based upon NeuN staining. (**B**) Astrocytes based upon GFAP-staining. (**C**) Oligodendrocytes based upon ACP-staining. (**D**) Macrophages based upon IBA-staining. (one-way ANOVA with Tukey's multiple comparison test, P value *<0.05, **<0.01, ***<0.001; 3 animals with at least 2 sections analyzed per group)

2.9.2 Identification of lesion boundaries and caveats of host graft interface

We want to first address an important caveat of this study: the definition of the lesion boundary. In general we would use the GFAP staining to outline the graft site, unfortunately the embryonic spinal cord derived cell grafts also differentiates into astrocytes that express GFAP. Thus the GFAP staining cannot be utilized to identify the host/graft interface. Another common approach is to define the host/graft interface by GFP expression in the grafted cells. However, early after grafting GFP positive cells migrate into the host cord rostral and caudal to the injection site (Fig. S2.3A). After maturation of the graft it is impossible to distinguish between grafted cells filling the lesion site within the original lesion boundaries and expanded areas of the graft resulting from proliferation of migratory cells (Fig. S2.3B).

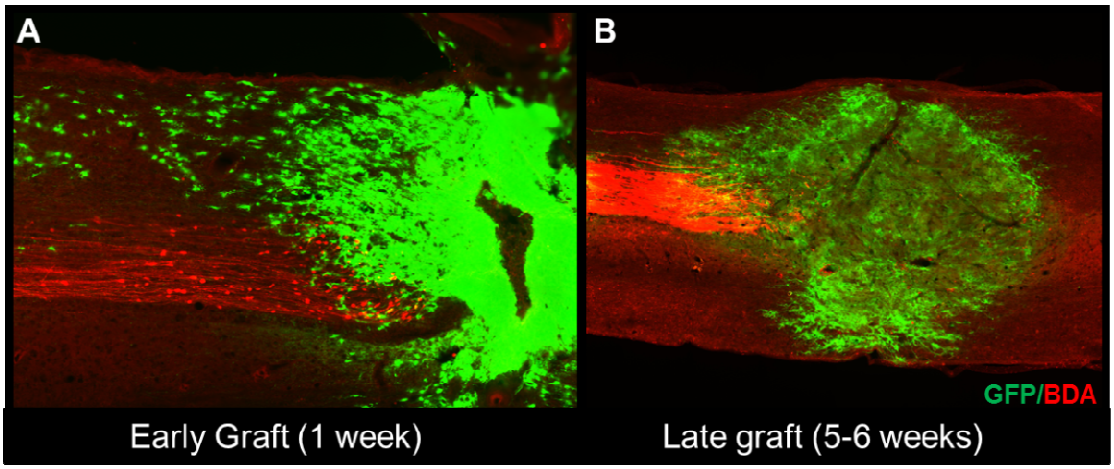

Figure S2.3: Grafted embryonic spinal cord cells migrate into host matter and expand within 5-6 weeks. (**A**) Sagittal view of GFP-positive embryonic spinal cord derived cells (green) one week after transplant. Cells have migrated into host cord rostral to lesion site and also infiltrated BDA-positive CST-axons (**red**). (**B**) Sagittal view of GFP-positive embryonic spinal cord derived cells (**green**) 5-6 weeks after transplant. Grafted and migrated cells have differentiated and proliferated in host cord matter.

2.9.3 Classification of regenerating and non-regenerating CST axons

Since GFP-signal and GFAP-staining could not be used to identify the host graft interface, we had to find a way to identify non-regenerative and regenerative CST host axons. In figure S2.4 we compare the morphology of non-regenerating versus regenerating CST axons. Non-regenerative CST axons are thicker in diameter, organized in a parallel manner and display retraction bulbs at the axon tip. Regenerative CST axons on the other hand are characterized by a smaller diameter (which is common for newly generated axons), non-directed organization and the absence of retraction bulbs. These characteristics recapitulate the morphology of uninjured CST axons in the white matter and grey matter respectively.

White matter tracts are organized in parallel manner and thicker in diameter. When the CST axons branch of the white matter tract to enter the grey matter, they display a thinner axon diameter and branchy morphology, necessary to find their way through grey matter neurons and to establish synaptic connections. Since the grafted cells mature to 30% into NeuN-positive neurons, this matrix provided for axon growth resembles structurally more grey than white matter. Thus it is not surprising that the regenerating CST axons resemble the morphology of non-injured, grey matter innervating axons.

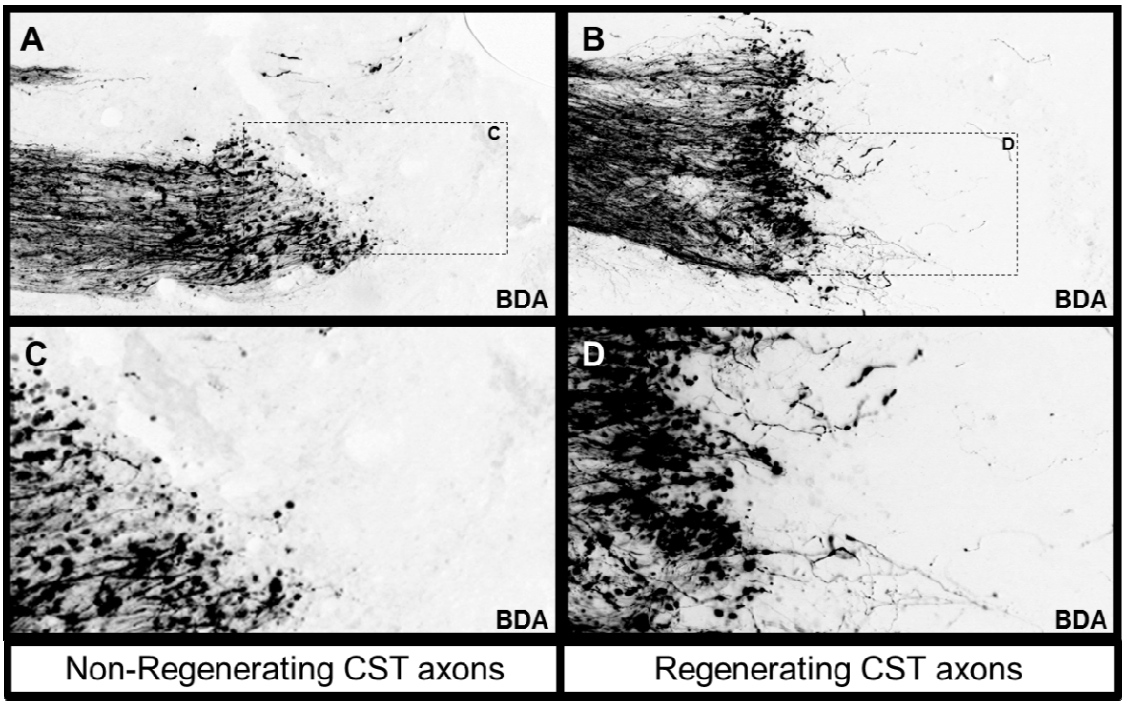

Figure S2.4: Morphology based classification of regenerating versus non-regenerating CST axons. Sagittal view of BDA-labeled (**black**) CST axons after lesion in adult mouse spinal cord. (**A, C**) Low and high magnification of non-regenerating CST axons. (**B, D**) Low and high magnification of regenerating CST axons.

2.9.4 Grafted embryonic spinal cord cells resemble “grey matter like areas” after differentiation

Grafted embryonic spinal cord derived cells expand to the point where they displace CST white matter tracts and compress the host grey matter (Fig S2.5A). It is important again to make a distinction on one hand between uninjured CST axons within the host grey matter that has been infiltrated by grafted cells and on the other hand regenerating CST axons innervating newly created NeuN-positive “grey matter-like areas”. Figure S2.5 (B-E) show distinct “grey matter-like areas” that contain numerous BDA-labeled CST axons, which also show NeuN labeling (mature neurons).

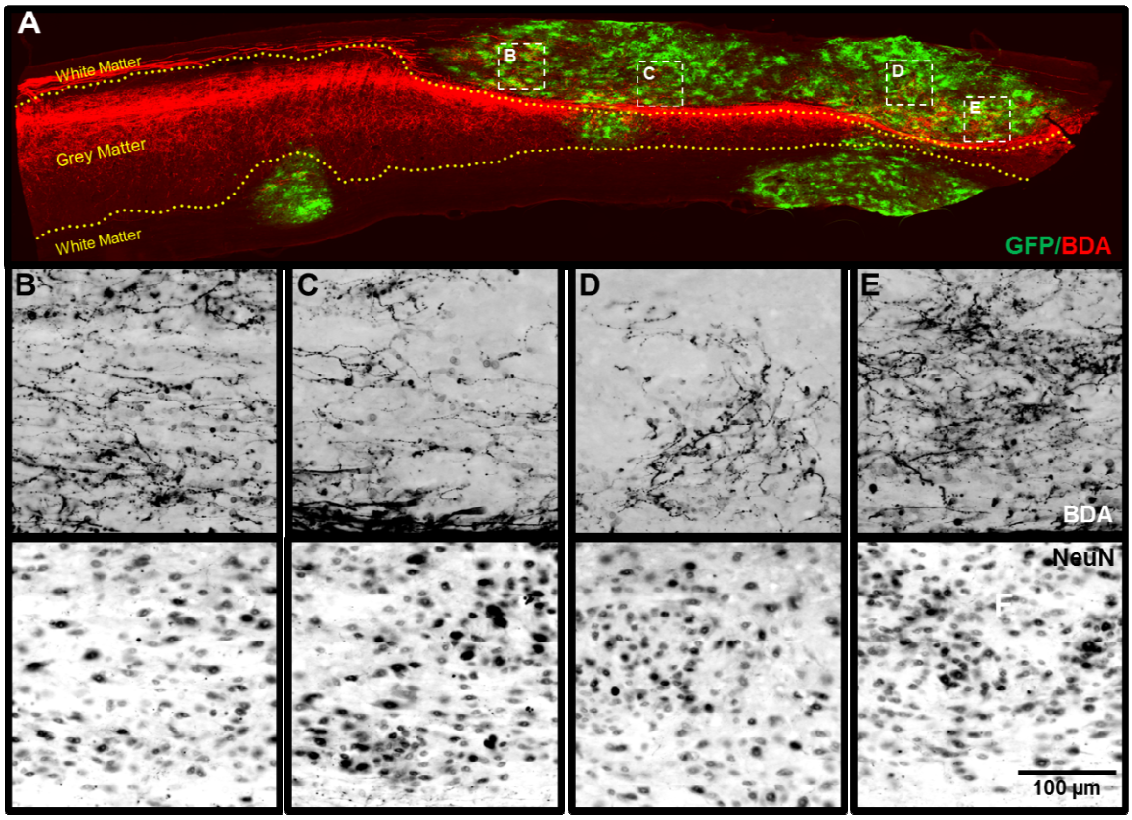

Figure S2.5 Regenerating CST axons colocalize with NeuN positive areas of the grafted embryonic spinal cord cells. (**A**) Sagittal section of adult mouse spinal cord, showing several areas of grafted GFP-positive embryonic spinal cord derived cells (**green**) and BDA-labeled CST axons (**red**). Compressed grey matter is outlined by yellow dots. (**B-E**) magnified areas for (A) showing in the upper panel: BDA labeled regenerating CST axons (**black**) and lower panel: NeuN labeled mature neurons (black)

2.9.5 Host grey matter and “grey matter like areas” show a clear morphological distinction

Figure S2.6 shows are more detailed display of NeuN positive graft areas. There is a clear distinction between host grey matter and “grey matter-like areas”. The neurons in the host grey matter are distributed more sparsely and appear larger in diameter while neurons of the “grey matter-like areas” appear smaller and show more dense distribution. This NeuN staining pattern gives more validity to the hypothesis that CST axons regenerate or sprout into the grafted areas. Especially in Figure S2.6B, there is a clear distinction between the higher density “grey matter-like area” and the lower density host grey matter (NeuN staining). The expanded “grey matter-like area” pushes the white and grey matter dorsal and displays a clear interface of graft, white matter and grey matter. We classify BDA-labeled CST axons within those distinct GMLAs as regenerating CST axons.

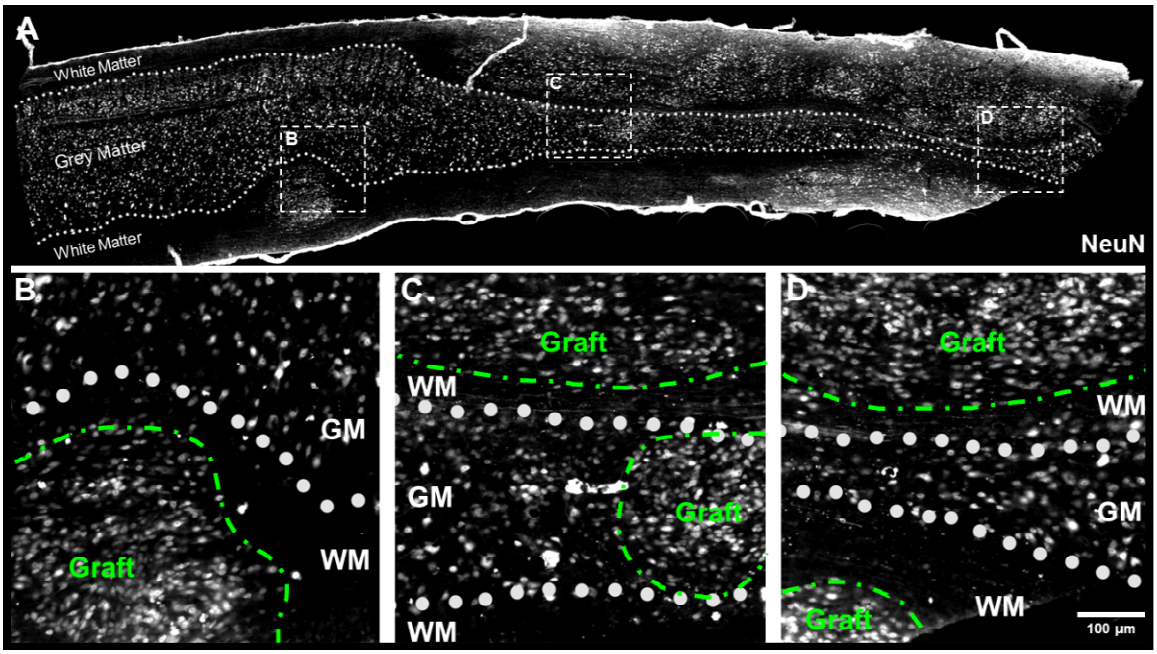

Figure S2.6: Distinction between host grey matter and "grey matter like areas". (**A**) NeuN staining of sagittal section from Fig S2.5A (**white**). Compressed grey matter is outlined by white dots. (**B-D**) magnified areas for (A) showing NeuN labeled mature neurons (**white**), Grafted areas are outlined in **green** and host grey (GM) and white matter (WM) are outlined by white dots.

2.9.6 Signal to noise ratio

The larger the signal to noise ratio, the more meaningful the results of the RNAseq and the more likely it is to identify candidate genes that influence CST regeneration. In this study the signal corresponds to CST neurons responding to graft leading to altered gene expression resulting in CST axon regeneration. The noise encompasses all CST neurons, that are not responding to the graft, hence do not express regeneration associated genes (RAGs) resulting in no CST axon regeneration. We found that (Fig. S2.7) when the area of CST axon interaction with the grafted cells is increased, we observe more CST axon growth.

According to these findings we adapted our transplantation model from single graft injection into the lesion site to multiple graft injection sites as described in Figure 2.1. This allows for CST axons being in direct contact with “grey matter-like areas” over several mm, resulting in an increased overall number of regenerating CST axons per animal. Thus increasing the number of CST neurons in the motor cortex that is activating RAGs, thereby reducing the number of CST neurons in the motor cortex that do not activate RAGs, leading to an increase in the signal to noise ratio.

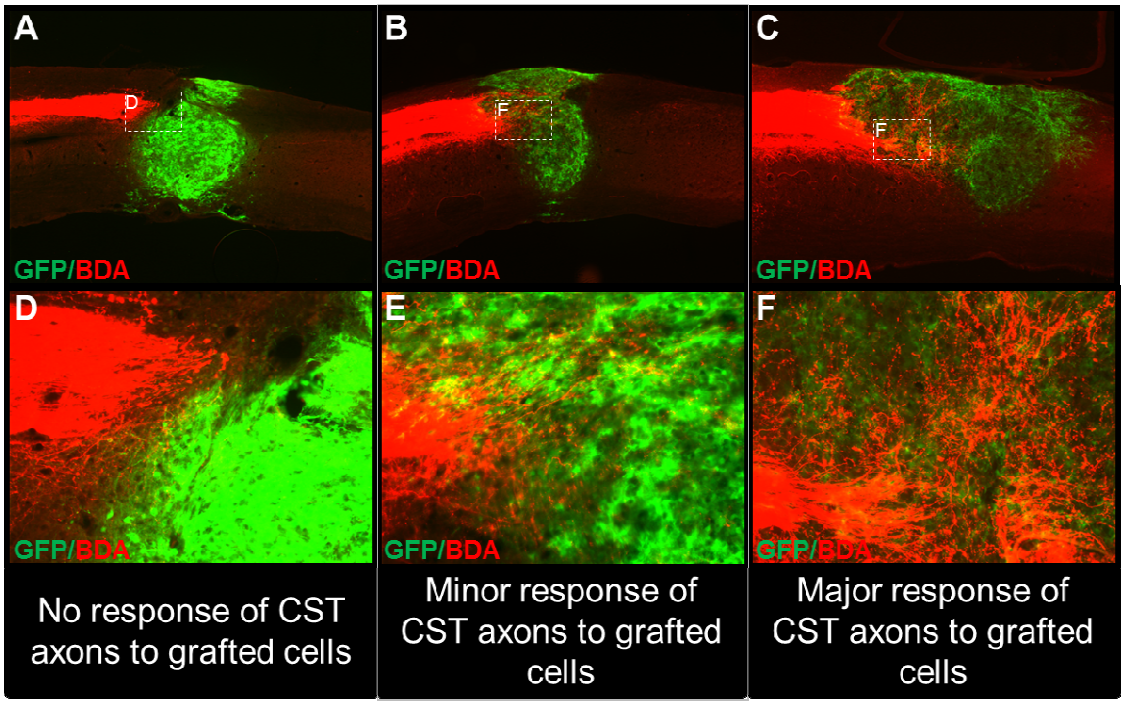

Figure S2.7: Extent of Graf/CST axon interaction determines the extent of the regenerative response. Sagittal section of adult mouse spinal cord, grafted GFP-positive embryonic spinal cord derived cells (**green**) and BDA-labeled CST axons (**red**). (**A, D**) NO graft/CST axon interaction leads to negligible regenerative response. (**B, E**) Medium graft/CST axon interaction leads to minor regenerative response. (**C, F**) Extended graft/CST axon interaction leads to major regenerative response.

2.9.7 Overview of differential gene expression: Heatmaps

Figure S2.8 shows heat maps of the 3 individual samples per time point. The heat maps show the top ranked differentially expressed genes within the 3 time points. The ranking is based on general abundance of a specific gene mRNA within the pool of all investigated mRNA. They more abundant a gene is, the more reads it will create in the sequencing process and the higher the rank will be. The genes in Fig S2.8 are clustered based upon functional groups. The heat map provides an overview of the consistency of the individual samples (3) per time point.

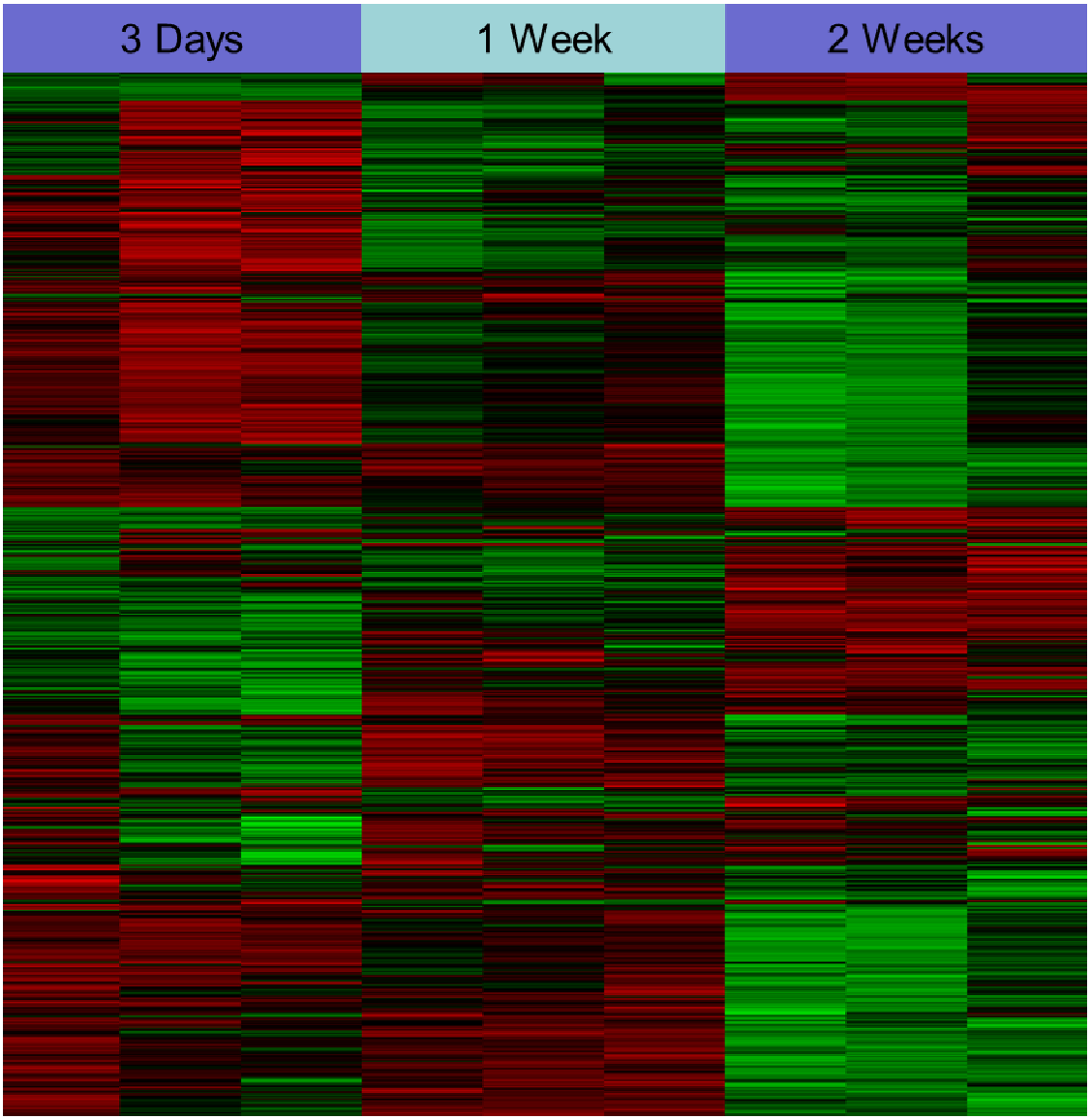

Figure S2.8: Sample log fold change of highest ranked differentially regulated genes (FDR≤0.1). Heatmaps of up (**red**) and down (**green**) regulated genes grouped by functionality show differential gene expression of IP-fraction from E12 graft versus IP-fraction of lesion only. From left to right: 3 day, 1 week and 2 week time point (3 independent samples per time point).

2.9.8 Criteria for assessing differential gene expression

After mRNA purification, we performed Bioanalyzer (Agilent Technologies, Inc.) mRNA quality control. The Bioanalyzer determines the amount of the abundant 28S and 18S ribosomal RNAs and calculates a ratio between those concentrations. The RNA Integrity Number (RIN) is based on that 18S to 28S ratio. RNA degrades slowly over time and the RNA degradation will alter the 18/28S ratio. It will also increase the baseline signal between the 18S and 28S peak, due to an increase in smaller RNA fragments. RIN is computed by taking all these variables into account to determine the quality of the analyzed RNA. The RIN software algorithm allows for classification of eukaryotic RNA ranging from 1 (completely degraded RNA) to 10 (completely intact RNA). All samples used for RNAseq where in between a RIN of 7 to 9.5. The average RIN was 8.1.

We applied a false detection rate (FDR) of 10% and a pValue below 0.05 (1 week time point) as a cutoff for the detection of differentially expressed genes.

2.9.9 The top canonical pathways at 3 days indicate reprogramming of CST neurons to a more immature state

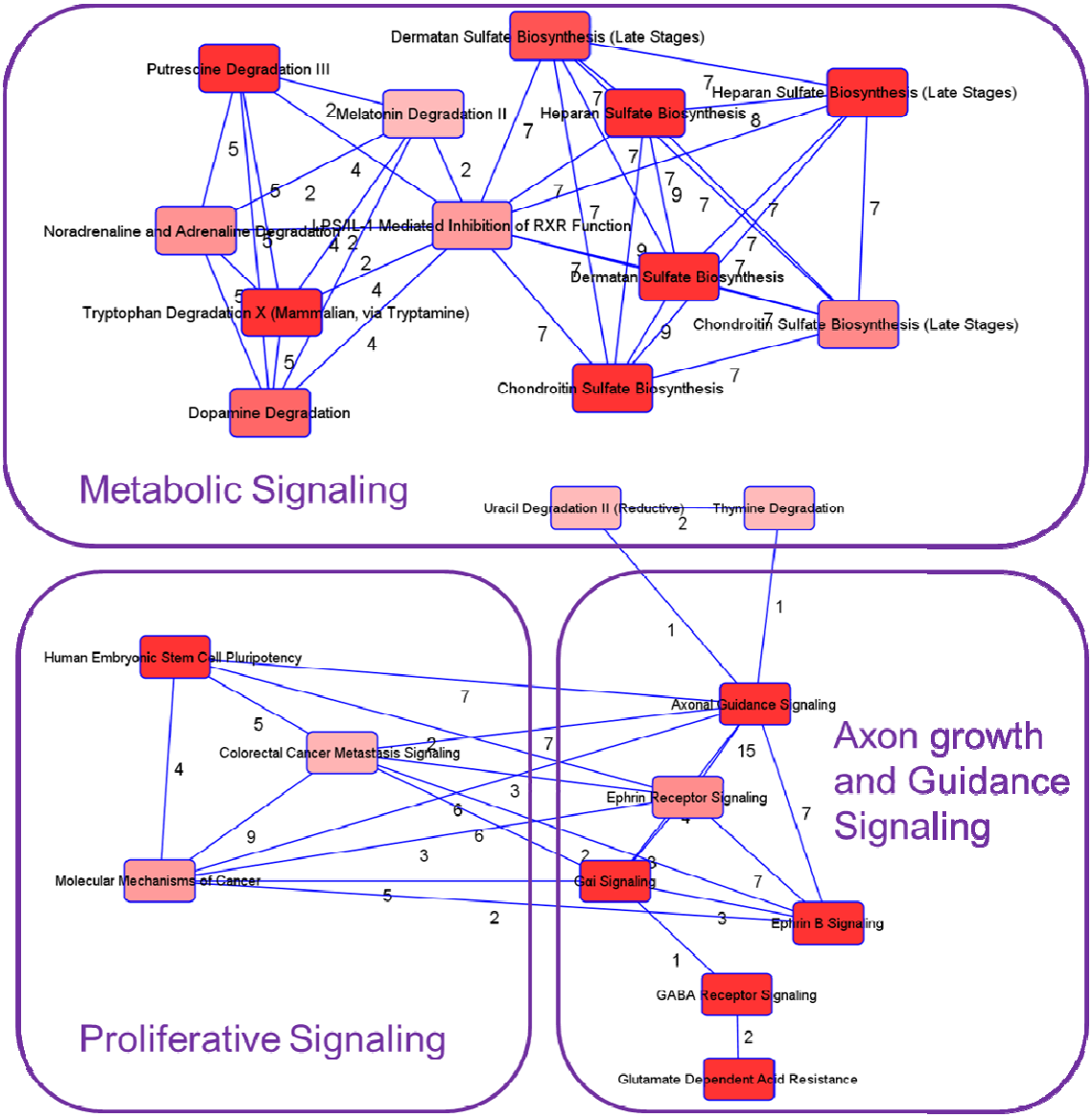

Figure S2.9: Interconnectivity Map of Activated Canonical Pathways 3 days post cell grafting including number of overlapping molecules
Each red box represents a canonical pathway that is activated in the dataset. The intensity of the red-color represents the pValue (higher intensity = lower pValue for a certain canonical pathway). The blue lines indicate overlaps between two or more pathways, the number represents the amount of overlapping molecules.

2.9.10 Human embryonic Pluripotency Pathway (3 days)

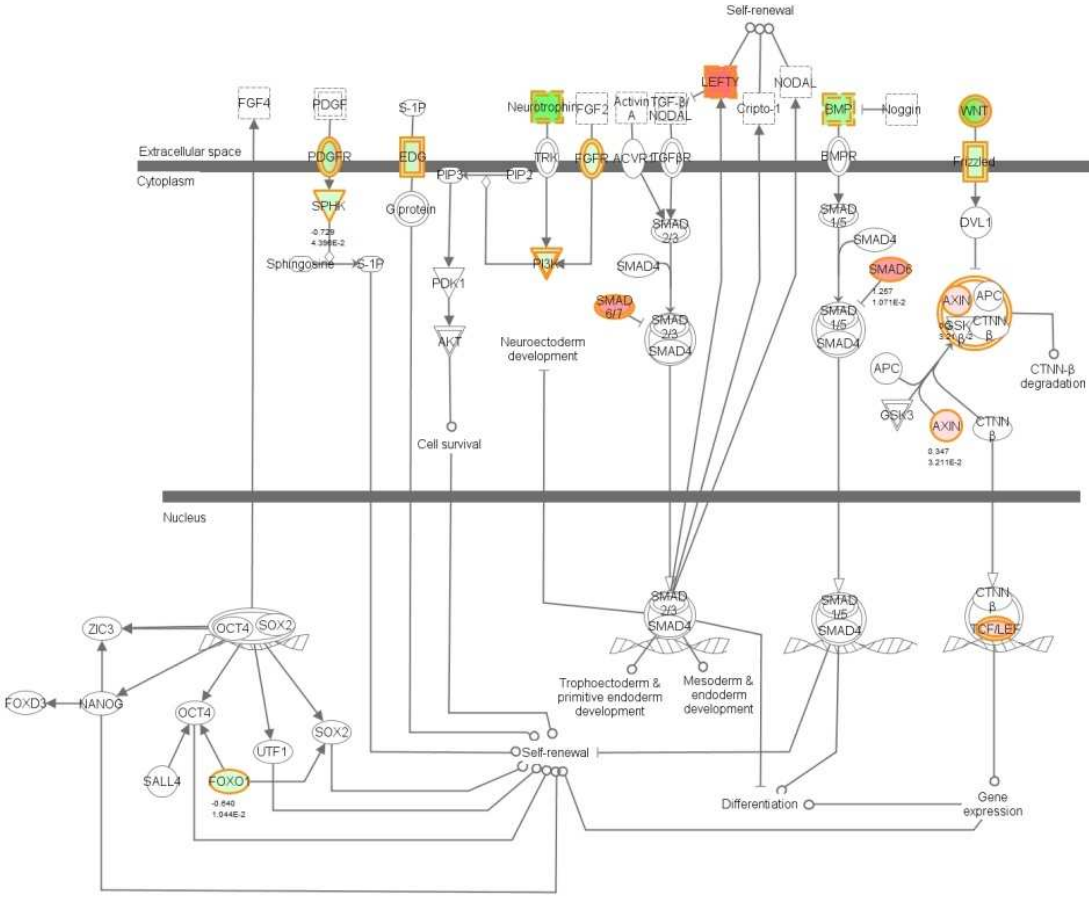

Figure S2.10: Detailed canonical Pathways (3 days):Human Embryonic Pluripotency Pathway. Molecules in **green** are down and in **red** are upregulated at 3 days post graft.

2.9.11 The top canonical pathways at 1 week indicate activation of axon growth mechanisms and guidance

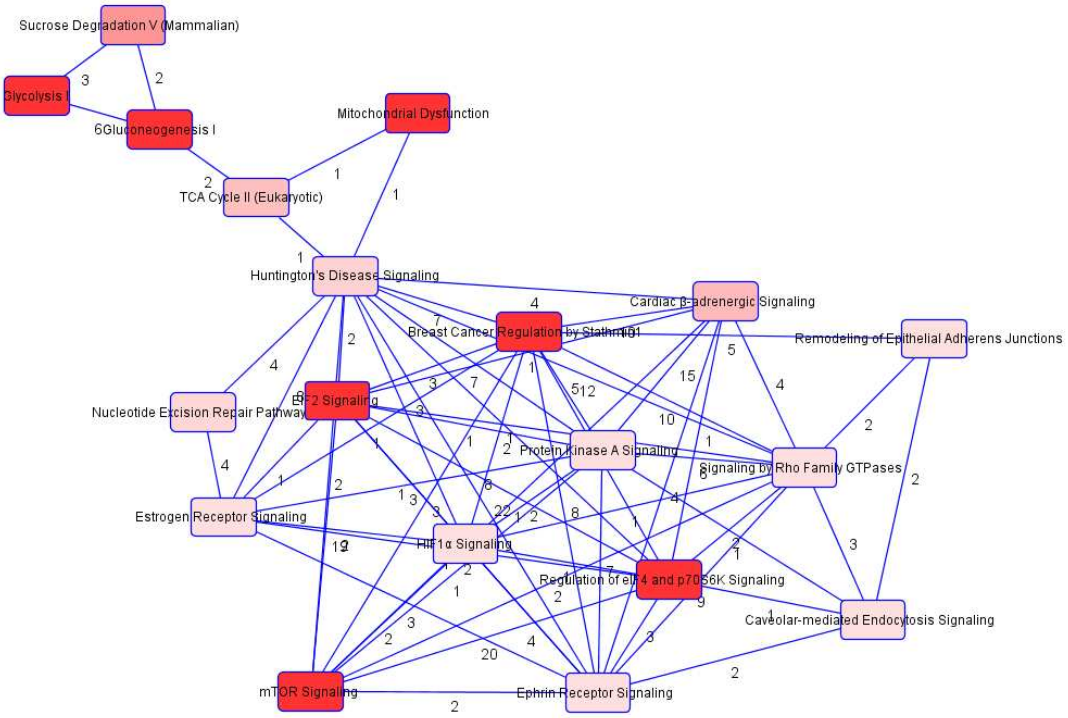

Figure S2.11 Interconnectivity Map of Activated Canonical Pathways 1 week post cell grafting including number of overlapping molecules
Each red box represents a canonical pathway that is activated in the dataset. The intensity of the red-color represents the pValue (higher intensity = lower pValue for a certain canonical pathway). The blue lines indicate overlaps between two or more pathways, the number represents the amount of overlapping molecules.

2.9.12 mTOR signaling Pathway (1 week)

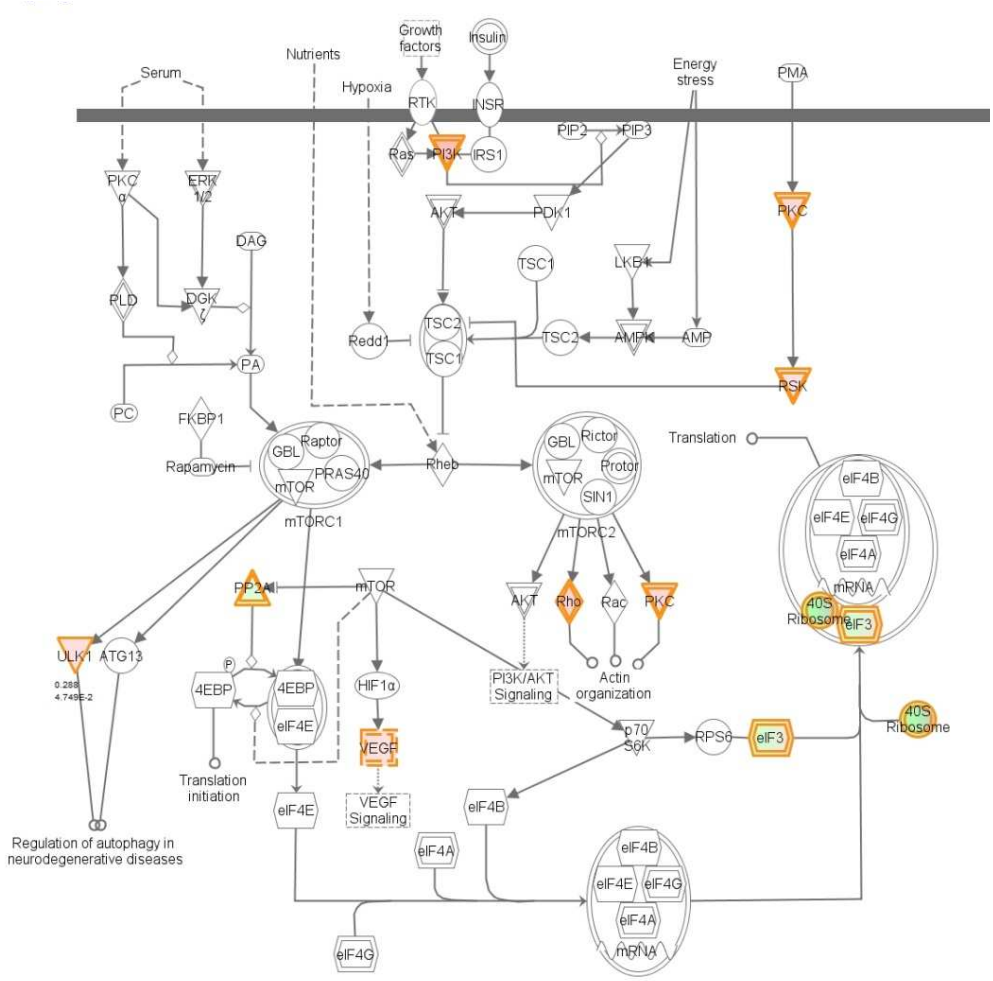

Figure S2.12: Detailed Canonical Pathways (1 week): mTOR signaling Pathway. Molecules in **green** are down and in **red** are upregulated at 1 week post graft.

2.9.13 The top canonical pathways at 2 weeks show activation of axon guidance and synaptogenesis signaling

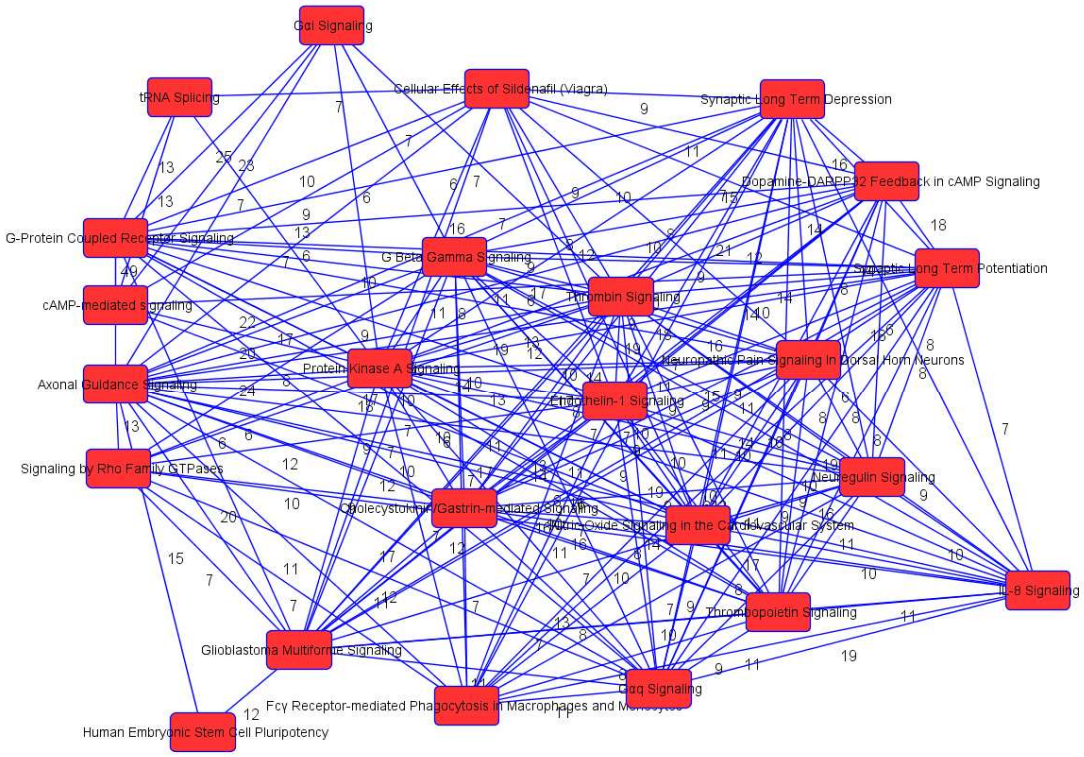

Figure S2.13: Interconnectivity Map of Activated Canonical Pathways 2 weeks post cell grafting including number of overlapping molecules
Each red box represents a canonical pathway that is activated in the dataset. The intensity of the red-color represents the pValue (higher intensity = lower pValue for a certain canonical pathway). The blue lines indicate overlaps between two or more pathways, the number represents the amount of overlapping molecules.

2.9.14 Axon guidance Signaling (2 weeks)

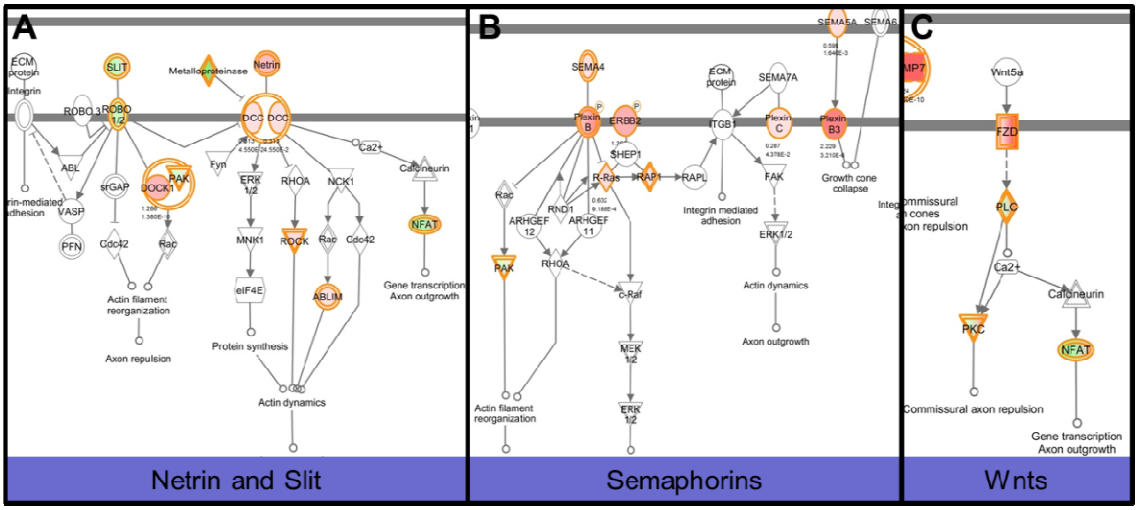

Figure S2.14: Detailed Canonical Pathways (2 weeks): Axon guidance Signaling. Molecules in **green** are down and in **red** are upregulated at 2 weeks post graft. (**A**) Netrin and slit pathway. (**B**) Semaphorins. (**C**) Wnts.

2.9.15 Synaptic Long Term Potentiation (2 weeks)

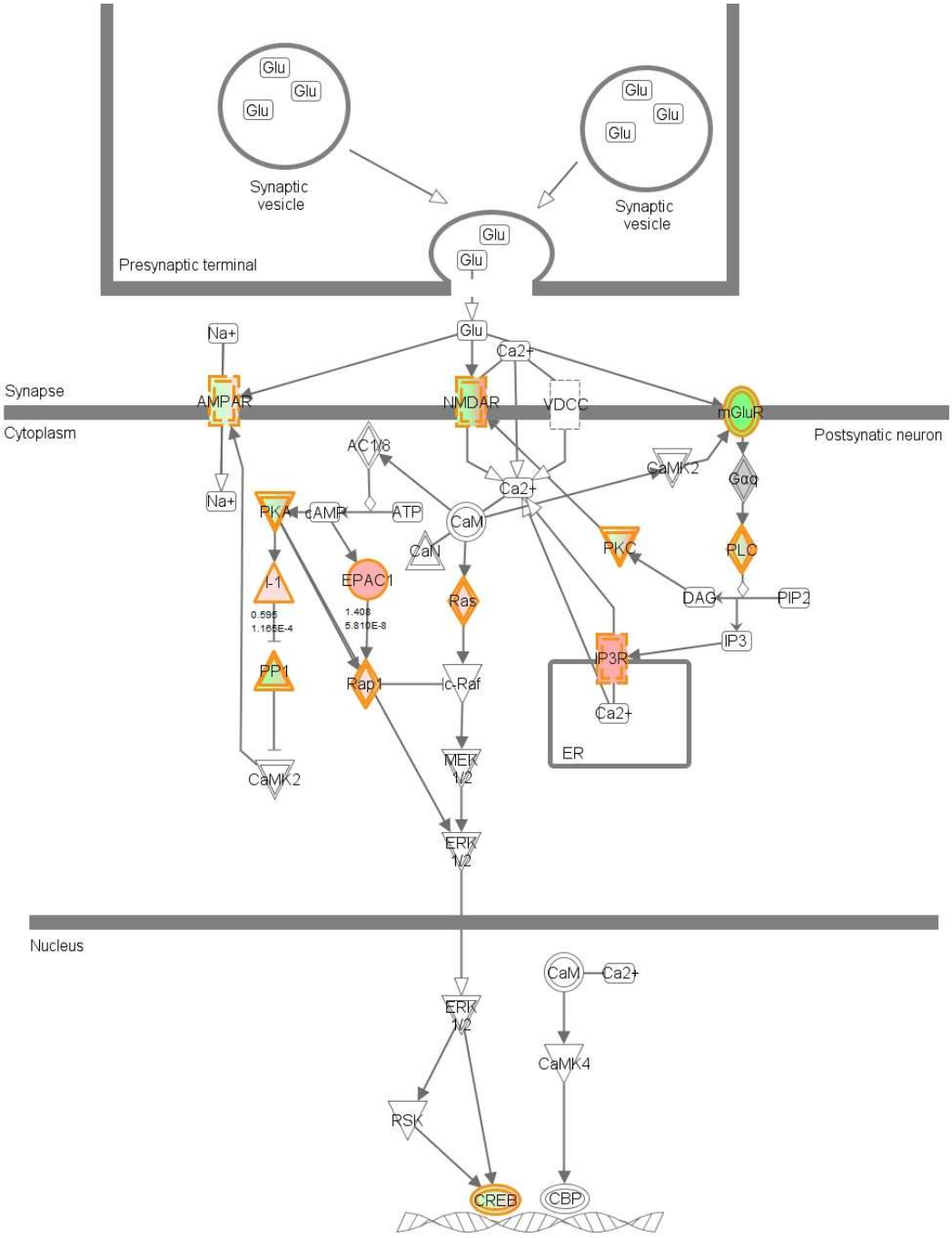

Figure S2.15: Detailed Canonical Pathways (2 weeks): Synaptic Long Term Potentiation. Molecules in **green** are down and in **red** are upregulated at 2 weeks post graft.

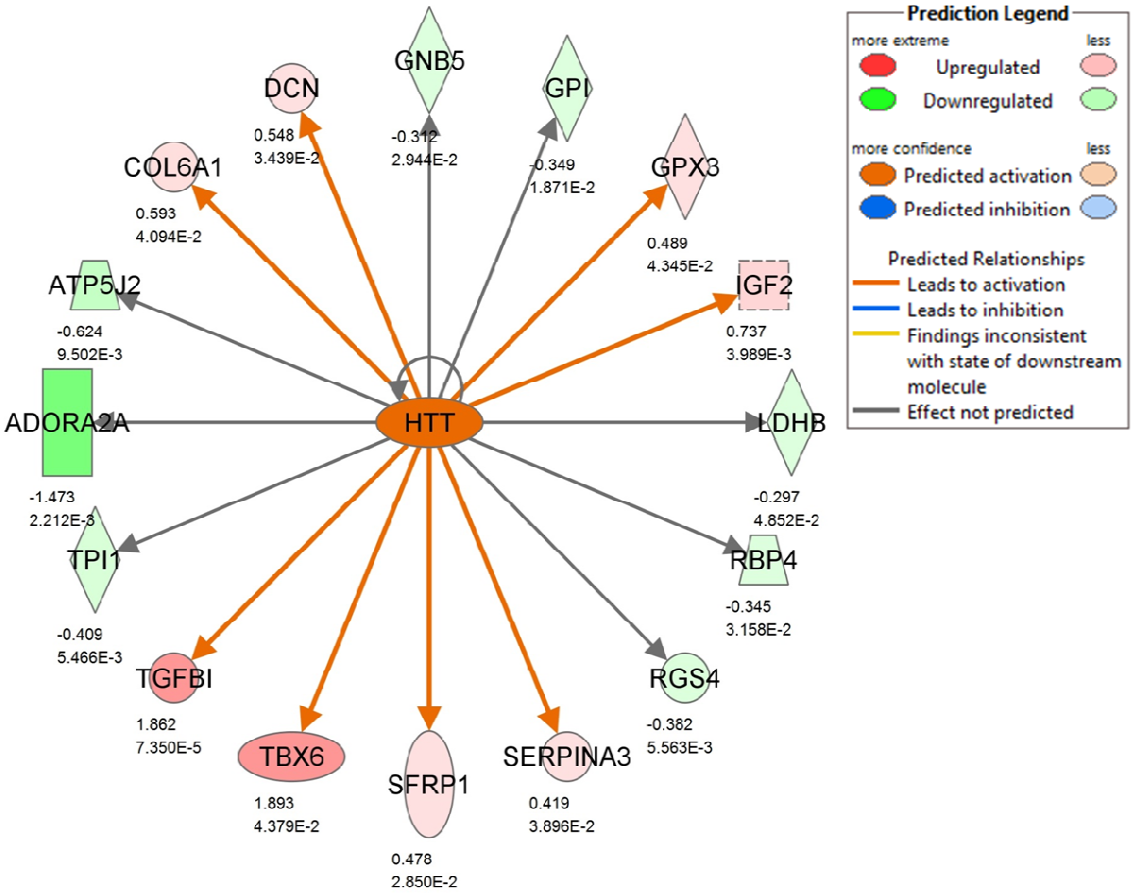

Figure S2.16: Downstream Effector Network of the Transcriptional Regulator Huntingtin (HTT) at 1 week after cell transplant (p-Value ≤ 0.05). Htt is predicted to be activated in this dataset. Eight downstream molecules (**red**) have bed shown to be positively regulated by HTT. **Orange** arrows show a prediction of activation. Grey arrows represent an influence on expression of downstream effector, but no clear trend of regulation can be extracted from the literature. Downregulated effector genes are in **green**. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation.

2.9.16 Network Analysis

Table S2.1: 20 highest ranked networks for 3 days

Top Network Functions at 3 days	Focus Molecules
Carbohydrate Metabolism, Small Molecule Biochemistry, Connective Tissue Disorders	31
Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry	31
Tissue Morphology, Behavior, Hereditary Disorder	30
Tissue Morphology, Cancer, Hematological Disease	30
Cellular Growth and Proliferation, Gene Expression, Cellular Development	30
Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cell Morphology	29
Cell Morphology, Cellular Assembly and Organization, Cellular Development	28
Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	27
Hereditary Disorder, Neurological Disease, Psychological Disorders	27
Cellular Function and Maintenance, Hematological System Development and Function, Cell Death and Survival	26
Cellular Assembly and Organization, Cellular Function and Maintenance, Embryonic Development	24
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	21
Inflammatory Response, Cellular Development, Cellular Growth and Proliferation	16
Digestive System Development and Function, Endocrine System Development and Function, Organ Morphology	20
Cell Cycle, Cell Death and Survival, Respiratory System Development and Function	18
Tissue Morphology, Visual System Development and Function, Cancer	18
Cell Signaling, Molecular Transport, Vitamin and Mineral Metabolism	18
Cell Cycle, Cardiovascular System Development and Function, Embryonic Development	17
Neurological Disease, Hereditary Disorder, Psychological Disorders	17
Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism	17
Cell-To-Cell Signaling and Interaction, Molecular Transport, Small Molecule Biochemistry	17
Inflammatory Response, Cellular Development, Cellular Growth and Proliferation	17
Hematological System Development and Function, Tissue Morphology, Cellular Movement	17
Free Radical Scavenging, Molecular Transport, Small Molecule Biochemistry	16
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	16

Table S2.2: 20 highest ranked networks for 1 week

Top Network Functions at 1 week	Focus Molecules
Organismal Development, Endocrine System Development and Function, Molecular Transport	26
Embryonic Development, Organismal Development, Tissue Development	24
Cell Death and Survival, Gene Expression, Cancer	18
Endocrine System Development and Function, Molecular Transport, Small Molecule Biochemistry	12
Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Molecular Transport	12
Cellular Development, Skeletal and Muscular System Development and Function, Tissue Development	11
Cellular Growth and Proliferation, Endocrine System Development and Function, Molecular Transport	10
Cell Cycle, Developmental Disorder, Hereditary Disorder	1
Cellular Function and Maintenance, Cellular Growth and Proliferation, Reproductive System Development and Function	1
Cancer, Carbohydrate Metabolism, Cellular Function and Maintenance	1
DNA Replication, Recombination, and Repair, Developmental Disorder, Gastrointestinal Disease	1
Cellular Development, Cellular Growth and Proliferation, Molecular Transport	1
Cancer, Inflammatory Disease, Inflammatory Response	1
Cell Morphology, Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization	1
Cell Signaling, Cancer, Neurological Disease	1

Table S2.3: 20 highest ranked networks for 1 weeks

Top Network Functions at 2 weeks	Focus Molecules
Cell Morphology, Cell-To-Cell Signaling and Interaction, Cellular Function and Maintenance	34
Cell Signaling, Small Molecule Biochemistry, Connective Tissue Disorders	33
Cellular Development, Reproductive System Development and Function, Lymphoid Tissue Structure and Development	32
Cellular Development, Nervous System Development and Function, Hereditary Disorder	32
Cell Morphology, Cellular Assembly and Organization, Cellular Development	32
Embryonic Development, Organ Development, Organismal Development	32
Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism	32
Cellular Movement, Embryonic Development, Cell-To-Cell Signaling and Interaction	32
Cancer, Cell Morphology, Cellular Function and Maintenance	32
Tissue Development, Cell Morphology, Cellular Assembly and Organization	31
Nutritional Disease, Metabolic Disease, Cellular Development	31
Developmental Disorder, Skeletal and Muscular Disorders, Cell Signaling	31
Digestive System Development and Function, Organ Morphology, Cell Death and Survival	31
Cellular Development, Gene Expression, Organismal Development	30
Cardiovascular System Development and Function, Organismal Development, Tissue Development	30
Cell Death and Survival, Nervous System Development and Function, Cellular Movement	28
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	28
Cell Morphology, Nervous System Development and Function, Cell Death and Survival	28
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Cellular Function and Maintenance	28
Connective Tissue Development and Function, Tissue Morphology, Nutritional Disease	28
Neurological Disease, Hereditary Disorder, Psychological Disorders	27
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Cellular Function and Maintenance	27
Renal and Urological Disease, Neurological Disease, Ophthalmic Disease	27
Cell Morphology, Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization	27
Cellular Growth and Proliferation, Cardiovascular System Development and Function, Organ Morphology	26

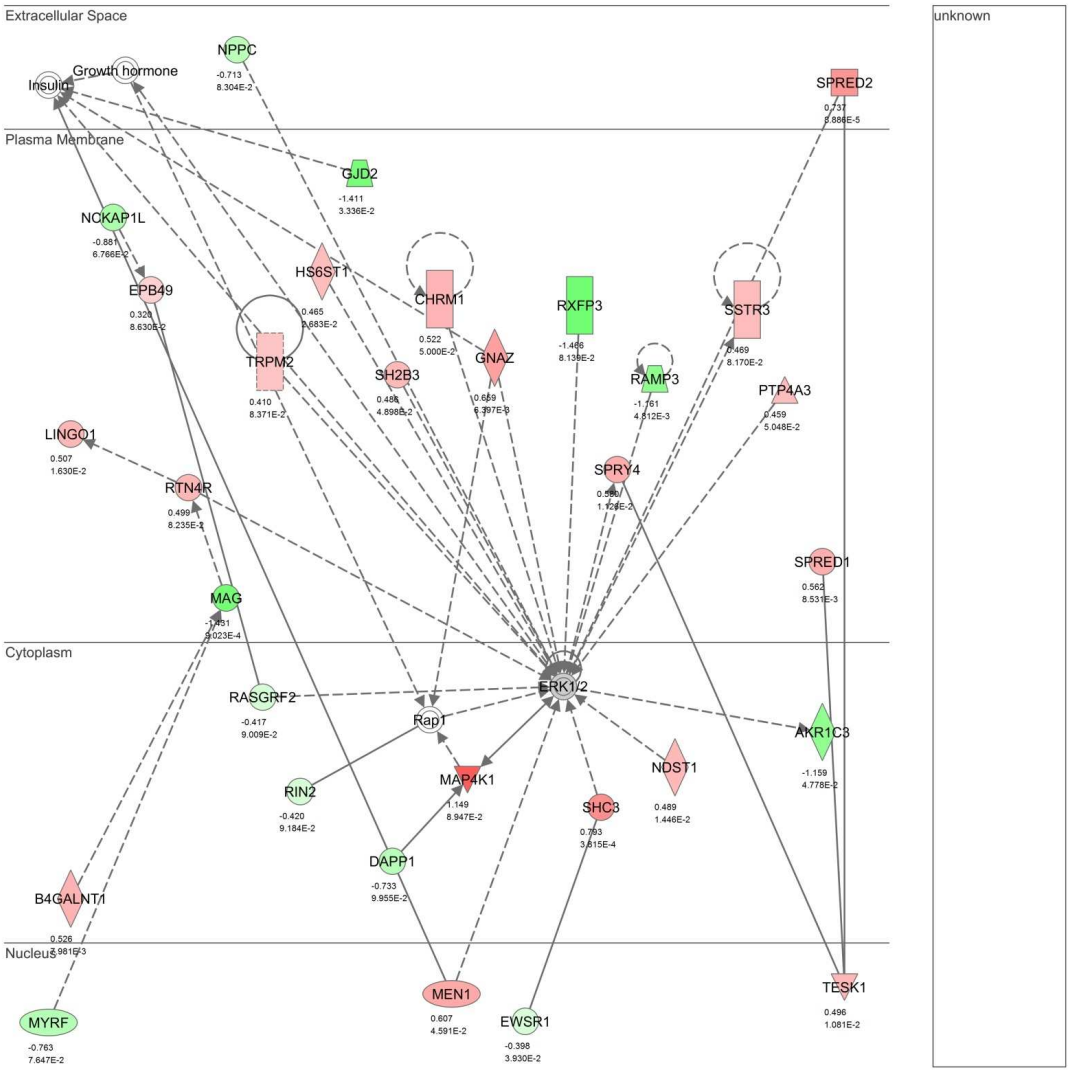

Figure S2.17: Highest ranked functional network at 3 days containing 31 molecules with reported interaction. Dashed lines indicate indirect interactions; solid lines indicate direct interactions. The arrow indicates the directionality of the relationship. Downregulated genes are shown in **green** and upregulated genes in **red**. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation. For detailed information on individual genes within the network and on their interaction see the legend in the supplemental material (Fig. S2.20)

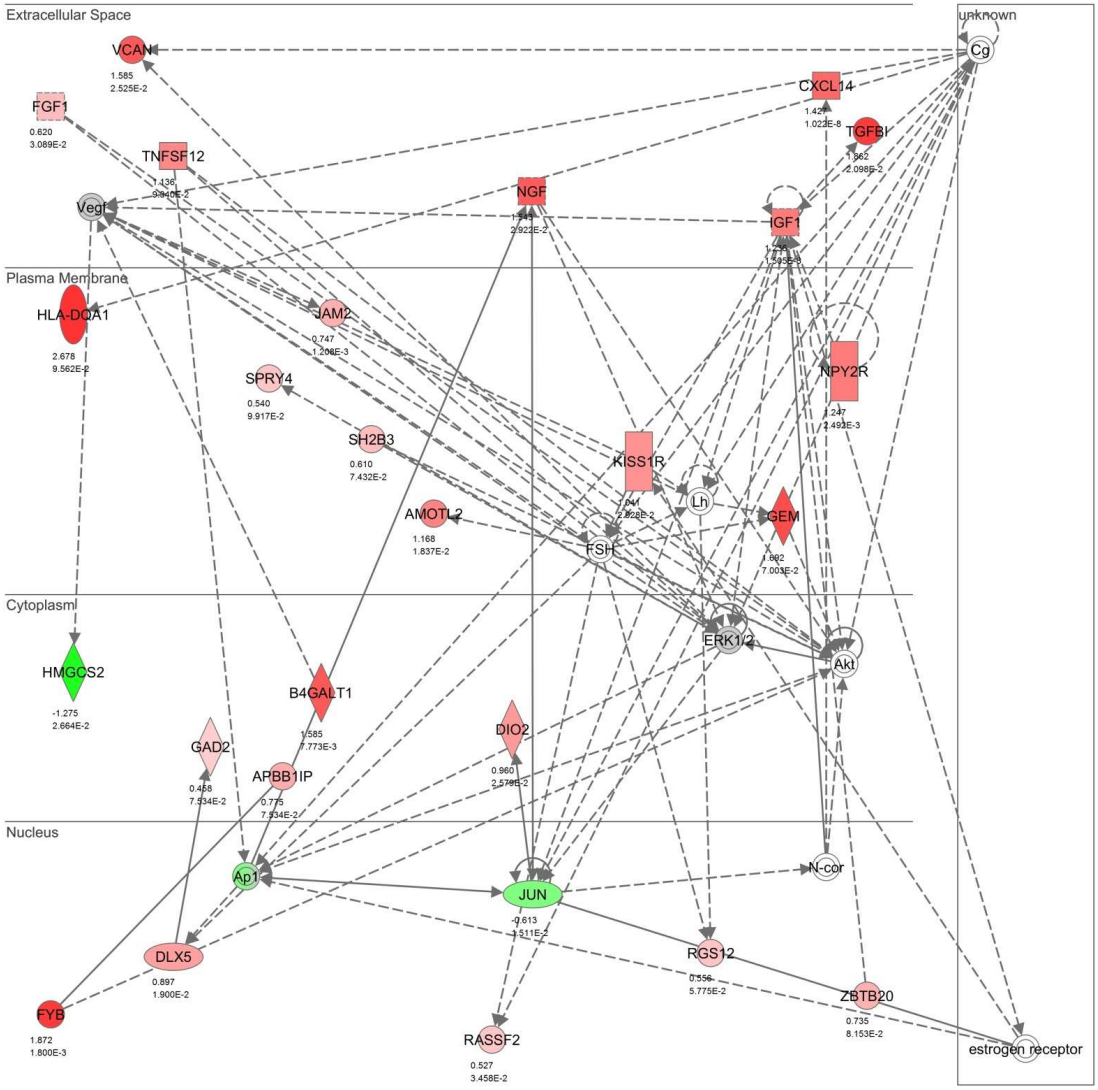

Figure S2.18: Highest ranked functional network at 1 week containing 26 molecules with reported interaction. Dashed lines indicate indirect interactions; solid lines indicate direct interactions. The arrow indicates the directionality of the relationship. Downregulated genes are shown in **green** and upregulated genes in **red**. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation. For detailed information on individual genes within the network and on their interaction see the legend in the supplemental material (Fig. S2.20)

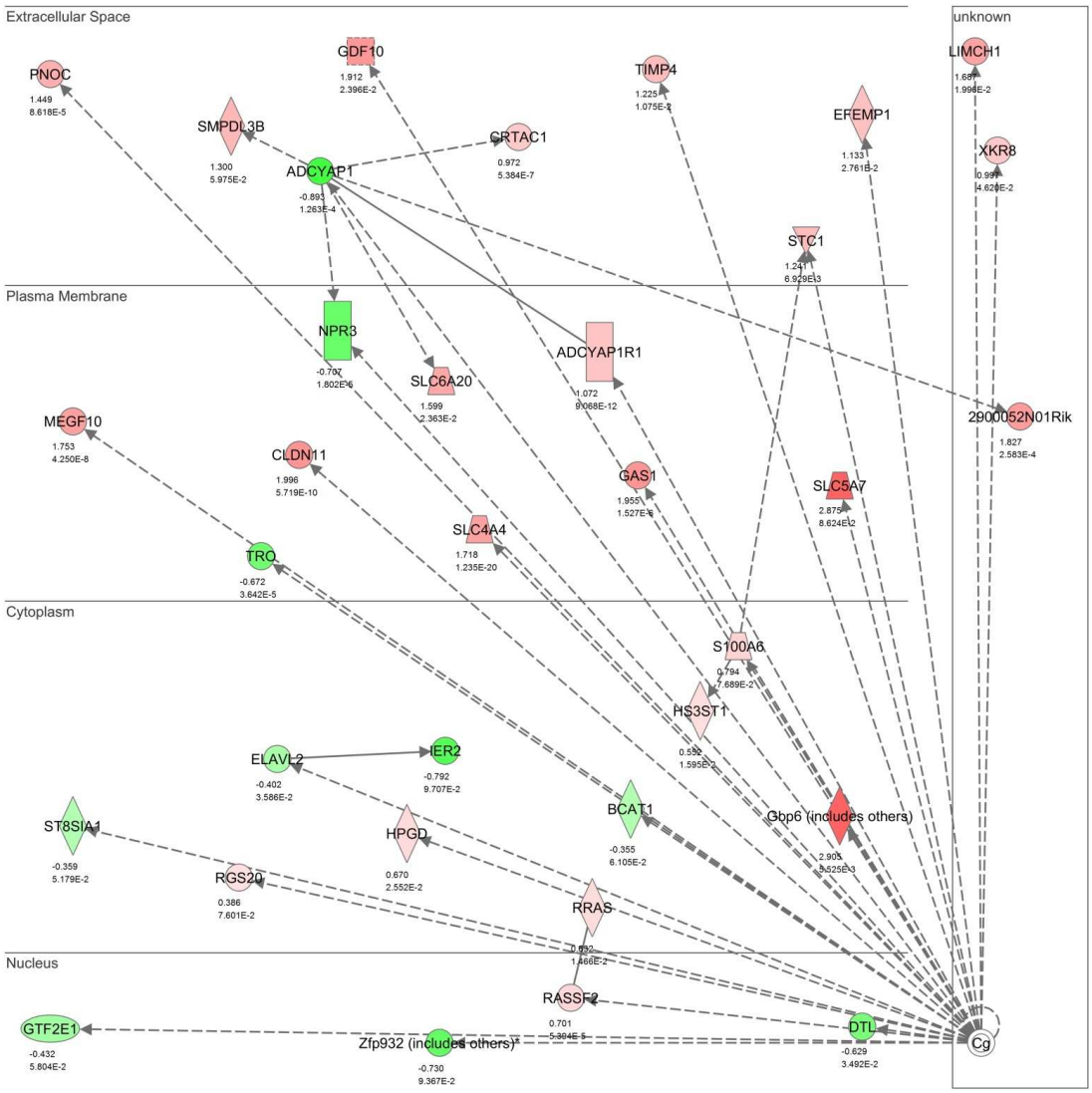

Figure S2.19: Highest ranked functional network at 2 weeks containing 34 molecules with reported interaction. Dashed lines indicate indirect interactions; solid lines indicate direct interactions. The arrow indicates the directionality of the relationship. Downregulated genes are shown in **green** and upregulated genes in **red**. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation. For detailed information on individual genes within the network and on their interaction see the legend in the supplemental material (Fig. S2.20)

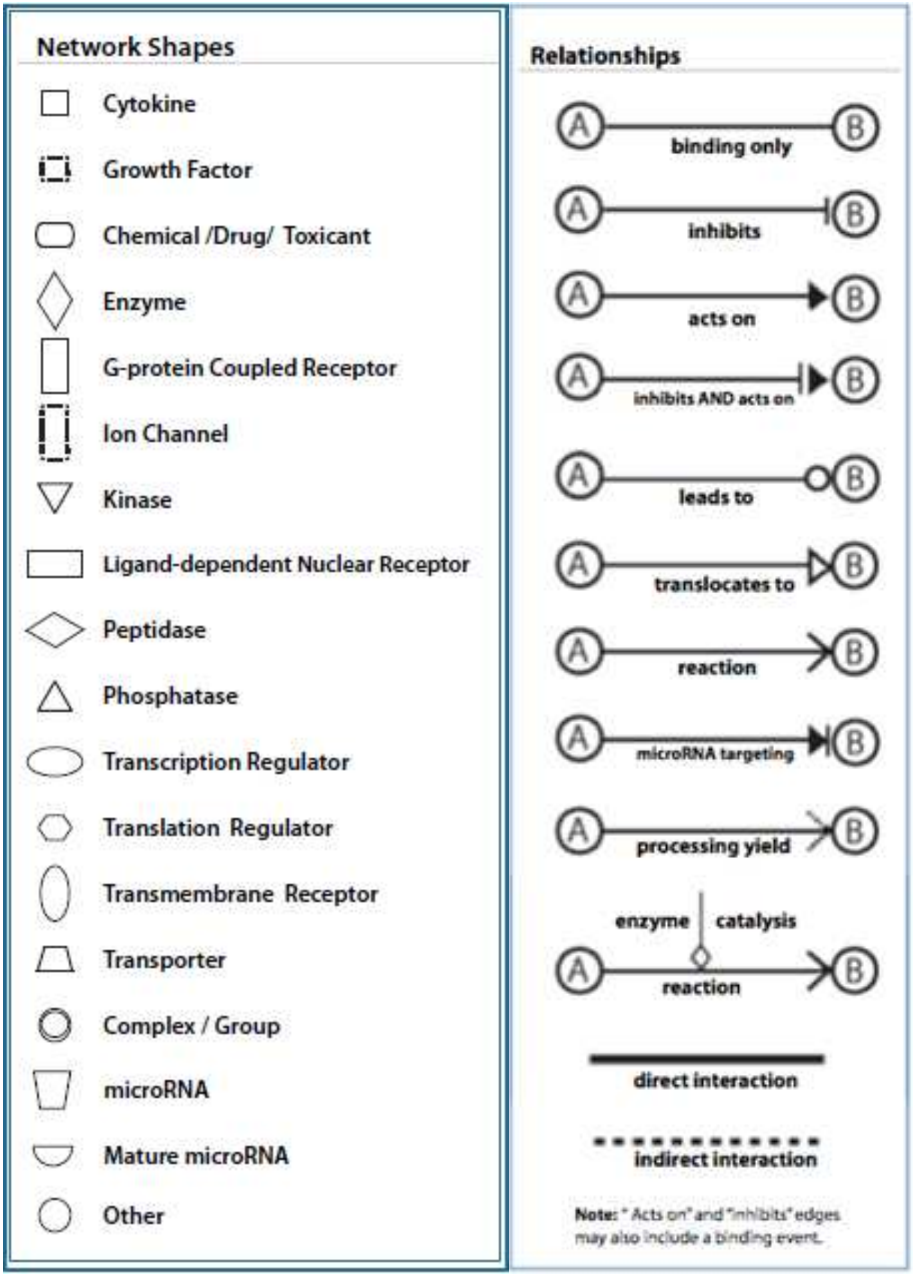

Figure S2.20: Legend for Molecule type and relationships status in IPA network analysis

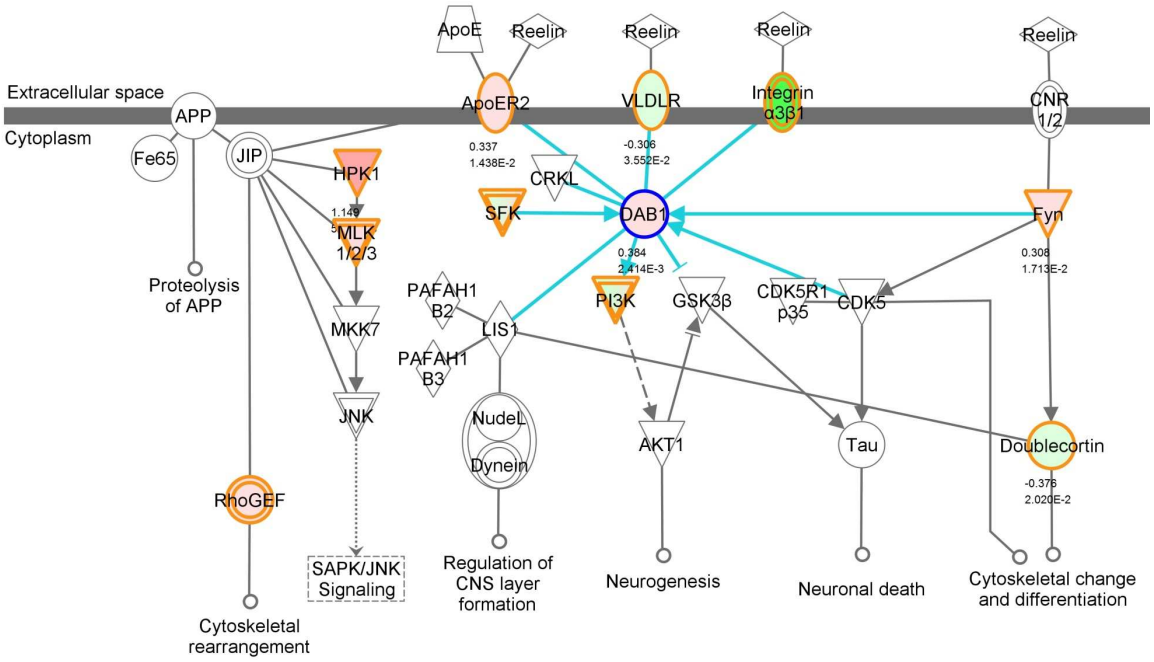

Figure S2.21 Functional Network around DAB1 in reelin signaling pathway identified at 3 days post grafting. Dashed lines indicate indirect interactions; solid lines indicate direct interactions. The arrow indicates the directionality of the relationship. Downregulated genes are shown in **green** and upregulated genes in **red**. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation. For detailed information on individual genes within the network and on their interaction see the legend in the supplemental material (Fig. S2.20)

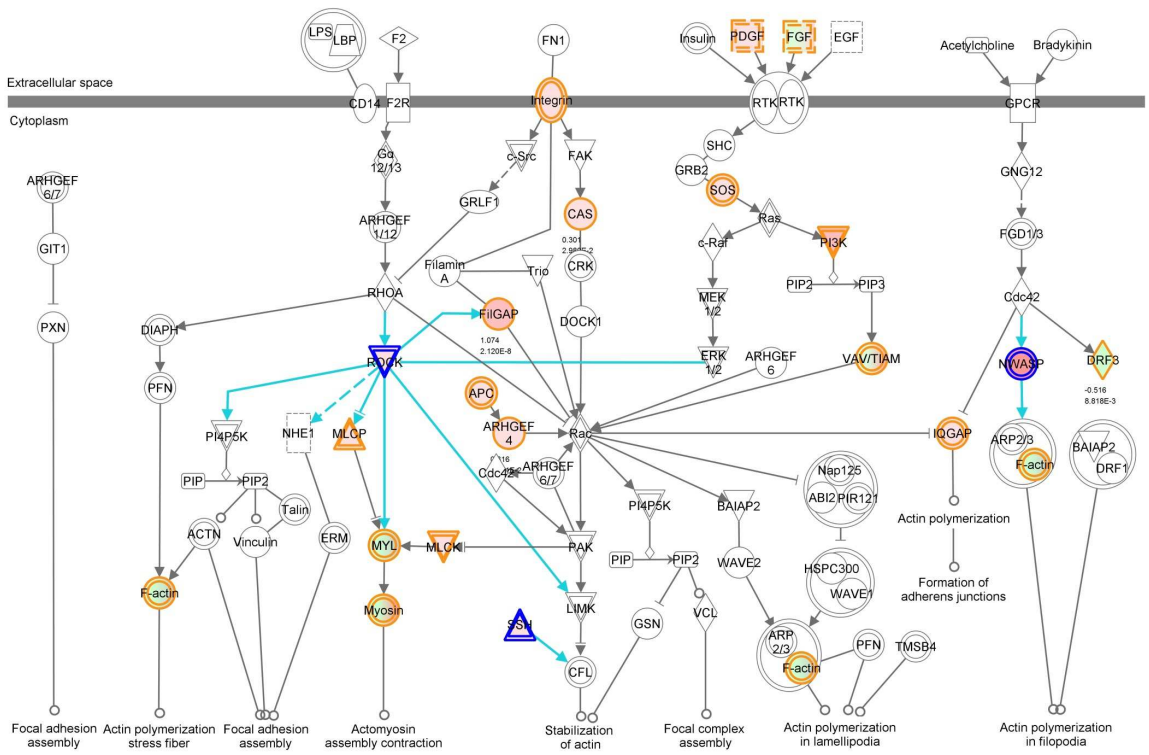

Figure S2.22 Functional Network around ROCK, SSH and NWASP in actin cytoskeleton signaling pathway identified at 3 days post grafting. Dashed lines indicate indirect interactions; solid lines indicate direct interactions. The arrow indicates the directionality of the relationship. Downregulated genes are shown in **green** and upregulated genes in **red**. The log ratio and the pValue are shown for each downstream molecule. An increase of the intensity in color (red and green) resembles stronger gene regulation. For detailed information on individual genes within the network and on their interaction see the legend in the supplemental material (Fig. S2.20)

3 SCHWANN CELLS ARE ESSENTIAL FOR THE CONDITIONING RESPONSE IN DRG NEURONS

3.1 Abstract

Axons in the peripheral nervous system (PNS) undergo long distance regeneration after injury. The injury signal is transported retrogradely to the cell body and initiates the expression of regeneration associated genes (RAGs). An injury of the central branch of the same neurons does not activate the expression of those RAGs. If however, the central branch lesion is preceded by an injury of the peripheral branch, activated RAGs are now able to also promote central branch regeneration. This phenomenon is known as the conditioning effect. Schwann cells are essential to regeneration of the peripheral axon after injury. We investigated the hypothesis that Schwann cells constitute an essential component of the conditioning effect. Schwann cells can be rendered deficient to the injury response by conditional deletion of the Low-density lipoprotein Receptor-related Protein-1 (LRP-1) gene; as a result, Schwann cells exhibit deficits in survival, proliferation, migration, and guidance/myelination of regenerating axons. We found that conditional LRP1-deletion in Schwann cells leads to a reduction of the conditioning effect *in vitro* and a trend towards reduction of central axon regeneration following conditioning lesions *in vivo*. Moreover, LRP1 deletion in Schwann cells results in deficient expression of neuronal growth factors, tubulins, neurofilaments and matrix metalloproteases in the injured DRG neuron, likely accounting for reduction of the conditioning effect. Thus, Schwann cells constitute an essential mechanism underlying the conditioning effect on central axon regeneration.

3.2 INTRODUCTION

Central nervous system (CNS) injuries are devastating and leave patients with lifelong deficiencies in motor and sensory function. The regeneration of CNS axons after injury still remains a major challenge compared to axons of the PNS, which regenerate over long distances after crush injury. DRG neurons extend axons into both the PNS and the CNS, which regenerate after PNS lesions but not after CNS lesions.

However, several studies have demonstrated that the central component of a DRG neuron can regenerate if a “conditioning lesion” is performed, wherein the peripheral branch of a DRG neuron is crushed several days preceding the central lesion (Richardson & Issa, 1984; Schreyer & Skene, 1993; Neumann and Woolf, 1999). Several regeneration associated genes (RAGs) and transcriptional regulators that promote regeneration of DRG neurons *in vitro* and *in vivo* have been identified following conditioning lesions (Costigan et al., 2002; Jenkins and Hunt, 1991; Leah et al., 1991; Herdegen et al., 1992; Martin-Villalba et al., 1998; Schwaiger et al., 2000; Tsujino et al., 2000; Tanabe et al., 2003; Lee et al., 2004; Jankowski et al., 2006; Seijffers et al., 2007). First, injury of the sciatic nerve induces a signal that is retrogradely transported to the nucleus to alter gene transcription, which is in part mediated by importin-β/importin-3 (Hanz et al., 2003). However it is not completely understood how this retrograde signal is specifically induced in the peripheral but not the central branch of the DRG neurons. One major difference is that in the PNS axons are associated with Schwann cells, whereas CNS axons are associated with oligodendrocytes and

astrocytes. Moreover, Schwann cells exert an essential role in peripheral axonal regeneration (Bradley and Asbury, 1970; Scherer et al., 1994; Jessen and Mirsky, 2008). Following peripheral nerve injury, Schwann cells secrete extracellular matrix proteins, growth factors and cytokines (Bunge and Pearse, 2003; Chen et al., 2007) which contribute to axonal regeneration. Accordingly, we hypothesized that signaling between Schwann cells and axons is essential not only for the regeneration of peripheral axons, but also in generating the conditioning effect that leads to central axon regeneration. Thus, we tested the hypothesis that Schwann cell deficiency leads to a reduction in the conditioning effect both *in vitro* and *in vivo*.

We utilized a strain of mouse in which the Schwann cell receptor LRP1 is conditionally deleted. LRP1 exerts an essential role in the function of mature Schwann cells, promoting Schwann cell migration via Erk (Mantuano et al., 2010) and survival by antagonizing the unfolded-protein response (Mantuano et al., 2011, Campana et al., 2006; Mantuano et al., 2008). In the conditionally deleted LRP1 mouse, the LRP1 gene is flanked by loxP sites. When this mouse is crossed with mice expressing the cre recombinase under the P0 promoter (responsible for the expression of the Schwann cell specific protein myelin protein zero), offspring becomes deficient for LRP1 specifically in Schwann cells at embryonic day 13.5 - 14.5 (Feltri et al., 1999, 2002). Thus, crossed P0-cre / LRP1 flox mice develop normally, and as intact adults exhibit a modest phenotype displaying impaired formation of Remark bundles, including increased axon diameter and reduced SC cytoplasm between axons as well as a reduced

thickness of the myelin sheets with no effect on axon diameters leading to increased G-ratios (Orita et al., 2013). However, after peripheral nerve injury, Schwann cells fail to function normally, evidenced by defects in Schwann cell migration, proliferation and re-myelination as well as increased mechanical allodynia and delayed recovery of motor function (Campana et al., 2006; Orita et al., 2013). We used this mouse model of impaired Schwann cell responses to injury to test the hypothesis that Schwann cells are essential for the conditioning effect, and explored candidate molecular mechanisms that might underlie Schwann cell dependency for conditioning.

We now report a reduction of the conditioning effect in SC-LRP1-KO mice *in vitro* and a trend towards reduced central branch regeneration following a SNC *in vivo*. Further, Schwann cell dysfunction results in molecular deficiencies in the conditioned DRG neurons, including altered expression of regeneration associated cytoskeletal proteins and growth factors, that likely account for the impaired conditioning effect on central axon regeneration.

3.3 RESULTS

3.3.1 SC-LRP1-KO mice show significantly reduced axonal outgrowth of conditioned DRGs in vitro.

To test whether Schwann cell dysfunction is associated with a reduction in the conditioning effect *in vitro*, we performed a sciatic nerve crush one week prior to the extractions of L4, L5 and L6 DRGs in SC-LRP1-KO and wildtype (WT) mice. We combined the L4, L5 and L6 DRGs followed by dissociation and culturing on either the permissive substrate poly-D-lysine (PDL) or the stimulatory substrate laminin. We fixed the cells after 2 days *in vitro* (DIV) and assed neurite morphology via immunolabeling and imaging of the neurons specific microtubule subunit βIII-tubulin via fully automated imaging and image analysis (ImageXpress, Molecular Devices). Sciatic nerve crush of adult mouse DRGs one week prior to DRG neuron culturing leads to a 3-fold increase in axon outgrowth *in vitro* (Neumann et al., 1999). We replicated this finding in wild type mice. Moreover, we identified a significant reduction of the DRG conditioning response in SC-LRP1-KO mice (Two tailed T-test. p-Value *<0.05 show significant reduction in SC-LRP1-KO mice compared to WT mice; Fig. 3.1, 3.2). Total axon outgrowth, axon branching and longest axon per neuron were significantly reduced one week after conditioning SNC. Similar results occurred on two different substrates, poly-D-lysine (Fig. 3.1) and laminin (Fig. 3.2).

3.3.2 SC-LRP1-KO mice show a trend towards reduced central axonal regeneration one week after sciatic nerve crush in vivo.

To test whether Schwann cell dysfunction is associated with a reduction in the conditioning effect *in vivo*, we performed a sciatic nerve crush one week prior to a C4 dorsal column wire-knife lesion. Sensory axons were labeled with a retrograde and anterograde tracer (cholera toxin subunit β, CTB) prior to perfusion 5 weeks after the dorsal column lesion. There was a trend towards a reduction of the conditioning effect *in vivo* in adult SC-LRP1-KO mice after C4 spinal cord dorsal column lesions. We quantified the number of axons crossing the lesion center (Fig. 3.4B) and determined the integrated pixel density of CTB-labeled dorsal column axons within the lesion boundaries (Fig. 3.4A). Both methods indicated a trend toward reduction in regenerating central sensory axons in SC-LRP1-KO mice (P-Value = 0.08; N=3 per group; Fig. 3.4A). These experiments are undergoing repetition and expansion in animal subject number. If findings confirm the strong trend toward a reduction in central axonal regeneration after conditioning lesions in Schwann cell-deficit mice, then we will conclude that Schwann cells constitute an essential component of the conditioning effect. (More detail on the quantification methods of the integrated density and axon count are presented supplemental materials, Figure S3.1).

3.3.3 Gene expression analysis

To identify candidate mechanisms underlying Schwann cell effects on the conditioning response, we performed RNAseq of DRGs in their intact state and one week after sciatic nerve crush in WT and in SC-LRP1-KO mice (Figure 3.3).

The greatest number of genes were differentially regulated when comparing conditioned SC-LRP1-KO (4432 genes) and conditioned wildtype (3468 genes) groups to intact animals (Fig. 3.5A,B). Surprisingly, we found little difference between the SC-LRP1-KO and the wildtype mice in the conditioned state (66 genes; Fig. 3.5D) and even fewer genes differentially regulated between SC-LRP1-KO and WT mice in the naïve state (46 genes; Fig. 3.5B) using a significance criteria of FDR ≤ 0.1. We therefore focused our analysis on the comparison of differentially regulated genes upon conditioning within the SC-LRP1-KO to differentially regulated genes upon conditioning within the WT group. 1870 of these differentially regulated genes overlapped between both SC-LRP1-KO and wildtype mice and contained genes have been previously described in the literature, including GAP43, SOX11 and ATF3 (Table 3.1). 1598 genes were differentially regulated specifically in the conditioned wildtype DRGs, including neurofilament and tubulin isoforms. 2362 genes were differently regulated in conditioned SC-LRP1-KO DRGs such as the transcriptional regulator JunD and ATF2 (Figure 3.5 and Table 3.1)

3.3.4 Differentially regulated genes with potential function in neurons

3.3.4.1 Cytoskeletal proteins involved in axon regeneration

We found β1-tubulin and α1b-tubulin uniquely upregulated in conditioned wildtype DRGs and not changed in the conditioned SC-LRP1-KO DRGs (Table 3.2). The upregulation of β2-tubulin gene expression was 41% greater in wildtype (3.1-fold) than in the SC-LRP1-KO (2.2-fold) animals (Table 3.4). Downregulation of neurofilaments is another indicator of axon regeneration: we found a

downregulation of light, medium and heavy chain neurofilaments in conditioned WT DRGs (Table 3.2), but no change in conditioned SC-LRP1-KO DRGs. Upregulation of these tubulins and down regulation of neurofilaments has been a reported characteristic for regenerating DRG axons (Hoffman et al., 1989; Miller at al., 1989; Gold et al., 1991; Wong et al., 1990; Tetzlaff at al., 1988; Troy et al., 1990)

3.3.4.2 Neuronal growth factors involved in axon regeneration (Supplemental table S3.3)

The expression of neuronal growth factors has been associated with enhanced axon regeneration (Lee et al., 2001; Bibel & Barde, 2000). Schwann cells secrete neurotrophic factors such as NGF and BDNF to promote their own survival and peripheral axon regeneration after injury (Meyer et al., 1992; Funakoshi et al., 1993). Brain derived neurotropic factor (BDNF) was 2-fold upregulated in both conditioned wildtype and SC-LRP1-KO DRGs. Nerve growth factor (NGF) was only upregulated (2-fold) in the conditioned wildtype and unchanged in the conditioned SC-LRP1-KO; and neurotropic factor 3 (NT-3) was actually *downregulated* in the SC-LRP1-KO DRGs (-4.2-fold; Supplemental table S3.3). The neurotrophic support from Schwann cells to injured DRG neurons is an important mechanism for central axon regeneration after conditioning, thus the lack of NT3 and NGF in the inured SC-LRP1-KO mice might be responsible for reduced central axon regeneration. In addition we observed a 20-fold reduction of fibroblast growth factor 17 (FGF17) specifically in the SC-LRP1-KO mice upon conditioning and no change in the conditioned WT mice. FGF17 has

been shown to regulate frontal cortex subdivision patterning in mice (Cholfin and Rubenstein, 2008) and to control brain size during neurogenesis (Jen et al., 2009). The overall growth factor expression is reduced in the SC-LRP1-KO DRGs most likely due to reduces growth factor expression from the LRP1-defitient Schwann cells.

3.3.4.3 Retrograde injury signaling to initiate RAGs

Hanz and colleagues (2003) reported that a retrogradely transported injury signal is necessary to initiate the expression of RAGs to support peripheral axonal regeneration, acting via local synthesis in the injured axon of importin-3. Notably, we found an upregulation of importin-3 mRNA within DRGs only in conditioned wildtype DRGs (Table 3.2) and not in SC-LRP1-KO animals. Since the mRNA is transported down the axon for local protein translation, it is feasible, that in the SC-LRP1-KO mice, less importin-3 mRNA is produced and thereby leads to reduced local importin-3 translations resulting in an altered regenerative response. These alterations might cause differential gene expression such as seen for ATF2 and JunD specifically in SC-LRP1-KO DRGs.

3.3.4.4 Transcriptional Regulators Involved in Regeneration (supplemental table S3.4)

We found activation of several transcriptional regulators in DRGs upon conditioning of WT and SC-LRP1-KO mice (e.g. c-Jun, ATF3, CREB, SOX11). However, KO mice exhibited a downregulation of the transcriptional regulator JunD and an upregulation of ATF2, which is contrary to predicted patterns of regulation in WT mice (Herdegen at a., 1992; Martin-Villalba et al., 1998). We

also found a unique upregulation of the Jun family member JunB upon conditioning of WT DRGs. Since JunB is the only member that has not yet been associated with axon regeneration, it is possible that JunB is specifically activated in neurons in response to normal Schwann cell signaling. Triiodo-thyronine (T3) plays an important role in development and regeneration, and has been shown to upregulate JunB expression in Schwann cells (Mercier et al., 2001). It is likely that this mechanism is dependent on JunB upregulation and therefor altered in Schwann cells of SC-LRP1-KO mice. Additional differentially regulated transcription factors involved in axon regeneration are listed in supplemental table S3.4.

3.3.5 Differentially regulated genes with potential function in Schwann cells

3.3.5.1 Unique activation of bio functions mediating cell migration and the immune response specifically in conditioned wildtype DRGs

When we compared the gene expression profile between conditioned SC-LRP1-KO and conditioned wildtype DRGs, we found that genes involved in cell migration and immune response are most prominently differentially regulated in the conditioned wildtype DRGs. Supplemental Table S3.2 lists the activated bio functions determined based on genes that are differentially expressed in conditioned WT DRGs and not changed in conditioned SC-LRP1-KO DRGs.

3.3.5.2 Unique expression of Calcium interacting proteins of the S100 family specifically in conditioned wildtype DRGs

A prominent differentially regulated class of molecules are the S100A proteins that are involved in the regulation of proliferation, differentiation,

apoptosis, Ca2+ homeostasis, energy metabolism, inflammation and migration/invasion (Donato et al., 2013). Several isoforms (S100A1,4,6,8,9,13) are uniquely upregulated in the wildtype mouse upon conditioning (S100A6,13 are even down in SC-LRP1-KO). S1009 e.g. activates the expression of β2-integrin (Newton at al., 1998). β2-integrin was also uniquely upregulated in the conditioned wildtype DRGs promoting cell migration (Ding at al., 1999), synapse formation (Carrasco et al. 2004) and polymerization of the actin cytoskeleton (Van Buul et al., 2004), and not in KO mice.

3.3.5.3 Metalloproteases

The LRP1 ligand metalloproteinase-9 (MMP9) has been shown to be upregulated in Schwann cells after nerve damage to allow for Schwann cell motility and infiltration of the injured nerve, to phagocytose debris and promote axon regeneration (Chattopadhyay at al., 2009). MMP9 further serves as chemo attractant for macrophages leading to infiltration of macrophages into the injured nerve (Shubayev et al., 2006). We found a 3.4-fold upregulation of MMP9 only in the conditioned wildtype DRGs (Table 3.2) and not in KO mice. This could lead to fundamental deficiencies in Schwann cell signaling to the injured neuronal soma.

3.3.6 Nitric Oxide signaling with potential function in Neurons and Schwann cells

Neuronal nitric oxide synthase (nNOS) is present in axons and Schwann cells of the sciatic nerve (Qui et al., 2001). Sciatic nerve injury leads to upregulation of nNOS in DRG neurons, which is centrifugally transported and accumulated in growing axons. One week after SNC, nNOS and its upstream

mediator PSD95 are mostly upregulated in Schwann cells (Gao et al., 2008). Downregulation of nNOS has been shown to increase avulsion-induced motor neuron death (Zhou and Wu, 2006). nNOS synthesizes NO from L-arginine, which has been shown to be neuroprotective for DRG neurons *in vitro* (Thippeswamy et al., 1997) and *in vivo* (Thippeswamy et al.,2001). We found a downregulation of PSD95 and of nNOS specifically in the SC-LRP1-KO DRGs one week after SNC (supplemental Figure S3.3). Since reduced expression of nNOS leads to a decreased production of NO, neuroprotection and possibly Schwann cell survival itself might be inhibited in the SC-LRP1-KO DRGs. This reduced survival of Schwann cells and DRG neurons might result in reduced central branch regeneration *in vivo* and reduced axon outgrowth *in vitro*.

3.4 DISCUSSION

It is understood in the field of spinal cord injury research that fundamental mechanisms supporting successful peripheral nerve regeneration can lead to means of enhancing the regeneration of more refractory CNS axons. The conditioning effect of adult DRG neurons is an ideal system to apply this principle, since we know that a peripheral regenerative response can trigger a central regenerative response via the activation of common RAGs. In this study we investigated the Schwann cell component of the conditioning effect on adult DRG neurons *in vitro* and on central axon regeneration *in vivo*, and now find significantly reduced conditioning effects *in vitro* and strong trends toward impaired central regeneration *in vivo* in mice with dysfunctional Schwann cells.

These findings support the hypothesis that an essential component of the conditioning effect is the Schwann cell.

After sciatic nerve crush activated wild-type Schwann cells de-differentiate at the crush site, proliferate, migrate and remyelinate regenerating axons (Chen et al., 2007). They further phagocytose debris and guide axons by secreting extracellular matrix proteins and growth factors (Bunge and Pearse, 2003; Chen et al., 2007). During this process the LRP1-receptor is substantially upregulated in schwann cells, activating ERK/MAP kinase and Akt, promoting schwann cell survival and migration (Campana et al., 2006; Mantuano et al., 2008a,b). The group of professor Campana has developed a conditional knockout of the LRP1-receptor specifically in Schwann cells (SC-LRP1-KO) driven by a P0-promoter activated cre-recombinase. After SNC these mice show defects in Schwann cell migration, proliferation and re-myelination as well as delayed recovery of sensory and motor function. In the uninjured state defects are visible in the non-myelinating/ensheathing Schwann cells that show impaired formation of Remark bundles, including increased axon diameter and reduced SC cytoplasm between axons. The LRP1-deficient myelinating Schwann cells show a reduced thickness of the myelin sheets with no effect on axon diameters leading to increased G-ratios (Orita et al., 2013).

3.4.1 Differential expression of known RAGS

We found a significant reduction in the axonal outgrowth *in vitro* on permissive and stimulatory substrates. This result indicates that fully functional Schwann cells are critical leading up to the time of SNC and up to one week

afterwards. Initially local mRNA translation at the injury site of importin-3 establishes the retrograde injury signal that leads to activation of RAGs. We found an increase of the importin-3 mRNA in the DRGs in the wildtype but no change in the SC-LRP1-KO mice. This altered expression of importin-3 could mean that the retrograde signaling is reduced or altered in the SC-LRP1-KO mice and the regenerative response is delayed or altered. Contradictory to this idea is that we found several known RAGs to be similarly regulated in the wildtype and SC-LRP1-KO DRGs one week post crush. This could also mean that the mRNA trafficking is disrupted in the SC-LRP1-KO mice reducing the pool of importin-3 mRNA within the cell body. We did identify the upregulation of well know RAGS in similar quantities in conditioned wildtype and SC-LRP1-KO mice respectively (Neuropeptide Y (7.59-fold; 8.76-fold), ATF3 (5.6-fold, 6.11-fold), Galanin (4.14-fold, 3.77-fold), Sox11 (3.04-fold, 3.48-fold), GAP43 (1.5-fold, 1.68-fold)). This indicates that the initial signal to activate RAGs must be comparable between both mice.

We did however identify the lack of reduction in neurofilament expression in the SC-LRP1-KO DRGs. This reduction of neurofilaments has been widely described as critical factor in peripheral and central axon regeneration. In addition we found a specific upregulation of several tubulin isoforms in the wildtype DRGs. The upregulation of tubulins in regenerating axons is also a well descried phenomenon in the literature. During axonogenesis tubulins are solely expressed as cytoskeletal proteins and neurofilaments are only expressed during later phases of axon maturation and thickening of large diameter axons. During

the regenerative process of lesioned axons, these developmental patterns of gene expression of cytoskeletal components have to be reactivated. This leads to a reduced expression of neurofilaments and an increased expression of tubulins within the neurons to facilitate rapid axon elongation.

3.4.2 Reduced trophic support in LRP1-deficient Schwann cells might reduce axon regeneration

We found an increased expression of NGF in the wildtype and a decreased expression of NT3 and FGF17 in the SC-LRP1-KO DRGs. Both NGF and NT3 have been shown to support the (Alberts et al., 1994) survival of DRG neurons and the elongation of their axons. NT3 seems to support axon outgrowth in earlier stages of neurite initiation and elongation, and NGF at later stages of elongation and target innervation. Schwann cells secrete both NGF and NT3 upon sciatic nerve injury to facilitate neuronal survival and axon regeneration. NGF also promotes myelination of axons by Schwann cells (Chan et al., 2004). Schwann cells secrete NT3 in an autocrine loop to secure their own survival in the absence of an axon after injury (Meier et al., 1999). The observed reduction in NT3 expression in the SC-LRP1-KO is potentially due the function deficit in the Schwann cells. This might also be part of the signaling cascade that leads to increased apoptosis of Schwann cells in the LRP1-defitient mice after SNC. Reduced NT3 secretion would also lead to increased Schwann cell death, potentially explaining the reduced secretion of NGF in the SC-LRP1-KO DRGs. We will further investigate NT3, NGF and FGF17 expression within the sciatic nerve after sciatic nerve crush to determine if the differential expression of those

growth factors is mediated by altered Schwann cell function or by the DRG neurons themselves.

3.4.3 Pro and anti-apoptotic signaling via nNOS

The reduced expression of PSD95 and nNOS specifically in SC-LRP1-KO mice indicates that anti-apoptotic signaling is decreased in the conditioned DRG neurons. This decrease could be part of the increased Schwann cell death that has been observed after SNC in LRP1-deficient Schwann cells, or it could be involved in pro-apoptotic signaling towards the DRG neurons. Increased apoptosis of DRG neurons in response to SNC would lead to reduced number of axons being able to regenerate ultimately leading to the observed trend that suggest a reduction of central branch regeneration.

3.4.4 The unique transcriptome of conditioned WT DRGs is heavily involved in mitigating immune responses

We found 1598 genes that were differentially regulated specifically in DRGs of WT mice upon conditioning. Hundreds of the upregulated genes were identified by IPA to be part distinct functional categories, such as immune cell trafficking (134 molecules), inflammatory response (220), cellular movement (183 molecules), cell to cell signaling and interaction (145 molecules) and hematological system development and function (225 molecules). Supplemental Figure S3.4 shows a heatmap displaying these functions in a graphical representation. It is visually striking that most of these pathways are highly activated in the unique genome of the WT DRGs upon conditioning. When compared to SC-LRP1-KO DRGs upon conditioning (Supplemental Figure S3.5)

none of the immune response functions is clearly activated as seen in the WT transcriptome. The only functional group where a trend is visible is the cell death and survival category, where a lot of functions are decreased. LRP1 has been show to act as a pro-survival receptor in Schwann cells and SC-LRP1-KO mice show increased Schwann cell death after SNC (Campana et al., 2006). Thus the deletion of LRP1 in Schwann cells not only reduces Schwann cell survival but also leads to decreased activity of immune cells that are necessary to promote events such as removal of myelin and axon debris, secretion of cytokines and growth factors and possibly extracellular matrix molecules, necessary for peripheral axon regeneration. Since central branch regeneration shows a clear trend to be reduced as well in SC-LRP1-KO mice, it seems plausible that the reduced growth factor expression from Schwann and immune cells is the reason for a general decrease in the ability of DRG neurons to regenerate axons after injury.

3.4.5 Future Directions

To confirm the RNAseq analysis we will validate gene candidates on the protein level via western blot. We will investigate the protein expression of neurofilaments, tubulins, MMP9, NGF and NT3. We will add more animals to the *in vivo* study of central branch regeneration to investigate if the trend of reduced central branch regeneration in the SC-LRP1-KO mice is confirmed.

We will further analyze gene expression in the sciatic nerve one day post crush. Growth factor secretion by Schwann cells post crush is a critical stimulus to neuronal survival and axon regeneration within the sciatic nerve. We will

investigate the hypothesis that LRP1-deficient Schwann cells produce less growth factors that are critical to support peripheral and central axon branch regeneration.

3.5 CONCLUSION

We provided evidence that mice with a deletion of the LRP1 receptor in Schwann cells show a reduced conditioning effect *in vitro* and a trend towards a reduction of central axon regeneration *in vivo*. DRGs of both transgenic and wildtype mice express well characterized RAGs one week after sciatic nerve crush. However distinct known molecules associated with axon regeneration are expressed differentially in both groups. These differences are most likely due to reduced growth factor and cytokine expression in the transgenic DRGs possibly as a result of greater Schwann cell apoptosis and impaired immune responses. These findings indicate an essential role for Schwann cells in the conditioning effect in adult DRG neurons.

3.6 EXPERIMENTAL PROCEDURES

3.6.1 Animals.

Breeding and genotyping of LRP1-deficient mice was performed as described earlier (Orita et al., 2013). Mice that are deficient in Schwann cell LRP1 are called scLRP1-/- mice and littermate controls containing Schwann cell LRP1 are called scLRP1+/+ mice. NIH guidelines for laboratory animal care and safety were strictly followed. All mice were housed with a 12/12 h light/dark cycle with ad libitum access to food and water. All the animals used for surgeries were adult female LRP1-/- and LRP1+/+ mice (20–40 g).

3.6.2 Mouse Surgery.

All surgery was done under deep anesthesia by using a combination of Ketamine (80-100 mg/kg) and Xylazine (5-10 mg/kg). Euthanasia for tissue harvesting as performed by injection of an overdose amount of anesthesia cocktail in accordance with AVMA Guidelines for Euthanasia of Laboratory Animals (2013).

3.6.3 Sciatic nerve crush

An incision was made along the long axis of the femur. The sciatic nerve was exposed at mid-thigh level by separating the biceps femoris and the gluteus superficialis and then carefully cleared of surrounding connective tissue. The nerve was followed towards the spinal cord and exposed till the spinal cord entry point. The sciatic nerve was crushed once for 15 s with flat forceps 5 mm distal to the spinal cord entry point. The muscle was closed using 5.0 silk sutures and skin layers were closed using staples..

3.6.4 Dorsal column lesion

To assess the regeneration of the central branch of the L4-6 DRG neurons subjects underwent C5 dorsal column lesions, as previously described (Weidner et al., 2001). Briefly, a Kopf wire knife (David Kopf Instruments, Tujunga, CA) was inserted 0.3 mm lateral to midline and 0.9 mm under the dorsal surface of the spinal cord; the knife was extruded 1.5 mm and lifted to transect the dorsal columns, with coincident compression with a 28 gage blunt tip from above to ensure lesion completeness. Some of the groups had received a bilateral SNC 8 days prior to the DCL and other groups only received the DCL.

3.6.5 Axon tracing

Two to six weeks after initial surgery, dorsal-column sensory axons were labeled transganglionically using cholera toxin-B (CTB) injections (3 µl of 1% solution injected in each sciatic nerve) (Bradbury et al., 1999; Lu et al., 2003). Animals were perfused 2 days after tracer injection with 4% paraformaldehyde. Spinal columns were removed and post-fixed in 4% PFA at 4 ℃ overnight, then cryoprotected in 30% sucrose.

3.6.6 Light level and fluorescent staining

Spinal cords were removed from the vertebrae and serially sectioned in the sagittal plane at 35 µm intervals. Every sixth section was used for label detection. All steps were performed at room temperature if not noted otherwise. Sections were washed three times in TBS for 10 min, quenched in 0.6% H_2O_2 for 15 min, washed two more times in TBST (0.025% triton-X100 in TBS) for 5 min and blocked in TBST + 5% horse serum for 1 hour. Incubated for 3 days in TBST + 3% horse serum with primary antibodies at 4°C. Sections were washed 3 times in TBST + 3% horse serum and incubated 2.5 h with secondary antibodies. Washed 3 times with TBS for 10 min, incubated with ABC-solution for 1 hour, washed 3 times with TBS for 10 min, developed in 3,3′-diaminobenzidine (DAB) for 10 min and washed 3 times in TBS for 10 min. Sections were coverslipped with Cytoseal 60 (Thermo Scientific) for light-level and fluorescence and with Fluoromount G (Southern Biotechnology, Birmingham, AL) for fluorescence only

.

3.6.7 Primary antibodies

Light-level CTB immunolabeling was used to examine growth of dorsal-column sensory axons (polyclonal goat antibody, 1:80,000; List Biological Laboratories, Campbell, CA) (Lu et al., 2003). CTB-labeled sensory axons, host astrocytes and host schwann cells were detected by triple fluorescent labeling using antibodies against CTB (sensory axons, 1:5000), rabbit anti-GFAP (1:1500; Chemicon, Temecula, CA), S100 (monoclonal antibody, clone SH-A1, 1:500; Sigma-Aldrich, St. Louis, MO).

3.6.8 Secondary antibodies

Sections were incubated with Biotinylated Horse anti-Goat (6 µl per 1ml) for light-level and for fluorescence with cyanine 5-conjµgated donkey anti-goat, Alexa 488-conjµgated donkey anti-mouse (1:400), and Alexa 594-conjµgated donkey anti-rabbit (1:200; Jackson ImmunoResearch, West Grove, PA). Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; [1 µg/mL]; Sigma-Aldrich, St. Louis, MO)

3.6.9 Axon quantification

Sensory axon penetration into and beyond injury site was quantified in every subject in a series of three-in-seven CTB-labeled 30-µm-thick sagittal sections. Quantification was performed separately within grafts and in host spinal cord blocks located rostral to the lesion site at the following distances: 0-250, 250-500, 500-1000, and 1000-2000 µm. Within each block, the total number of CTB-labeled axons was counted in a microscopic field consisting of 100 boxes in a 10 × 10 matrix using 20× high-numerical aperture optics. Branched axons were

counted only once. Data are presented as mean ± SEM axon number. The host-lesion boundary was evident under phase-contrast optics as a distinct change in the appearance of neurons or white matter to the lesion cavity that contained uniform sheets of smaller, rounded cells. In addition, double fluorescent labeling for CTB-labeled axons and GFAP (astrocytes) was used as a reference to confirm the location of the lesion boundary in each animal. Observers were blinded to group identity.

3.6.10 DRG culture

Adult LRP1+/+ and LRP1-/- mice were euthanized and DRGs were removed, stripped of their roots and collected in HBSS on ice. DRGs were washed once with HBSS, digested with HBSS + 0.25% collagenase XI (Sigma-Aldrich) + 5 mg/ml Dispase (Worthington, Lakewood, NJ) for 30 min at 37℃, washed once with media (DMEM/F12 w/Glutamax, 1% penicillin and streptomycin (1000 U per 10 mg/ml final concentration), 10% FBS, 1x B27 (Invitrogen, Grand Island, NY)), triturated in media, counted and plated onto 24 well tissue culture treated plates (Thermo Scientific, Logan, UT) at 1000 DRG neurons in 1 ml media per well. Plates were precoated with Poly-D-lysine hydrobromide (PDL, 20 µg/ml in water, Sigma-Aldrich) overnight at room temperature, washed 3 times with sterile water and air dried. Some wells were coated in addition with laminin (0.5 µg/ml in PBS, Sigma-Aldrich) for 4 hours at room temperature, washed 3 times with media and not dried before plating. All DRG neurons were cultured at 37℃ in 5% CO_2 for 24 or 48 hours. Neurons were fixed with 4% Formaldehyde in TBS for 15 min at 37°C, washed 3 times with

TBS, permeabilized with TBST for 15 min at 37℃, wa shed 3 times with TBS, blocked in TBS + 5 % horse serum for one hour at 37℃, incubated with primary antibody mouse anti-ßIII-tubulin (1:1000, Promega, Fitchburg, WI) in TBS + 3 % horse serum overnight at 4℃, washed 3 times with T BS + 3 % horse serum, incubated with secondary antibody Alexa488 anti-mouse (1:1000) and DAPI (1 µg/ml) in TBS + 3 % horse serum for one hour at 37℃ , washed 3 times with TBS and imaged with ImageXpress (Molecular Devices, Sunnyvale, CA). Data was analysed with MetaXpress (Molecular Devices), graphs were created and statistical analysis were performed with Prism (GraphPad, La Jolla, CA).

3.6.11 RNA sequencing

Please refer to Experimental Procedures in chapter 2.6.10

Chapter 3, in its entirety, is currently being prepared for submission for publication of the material. Gunnar H.D. Poplawski, Tetsuhiro Ishikawa, Edward Wang, Qing Wang, Giovanni Coppola, W. Marie Campana and Mark Tuszynski. The dissertation author was the primary investigator and first author of this paper.

3.7 FIGURES

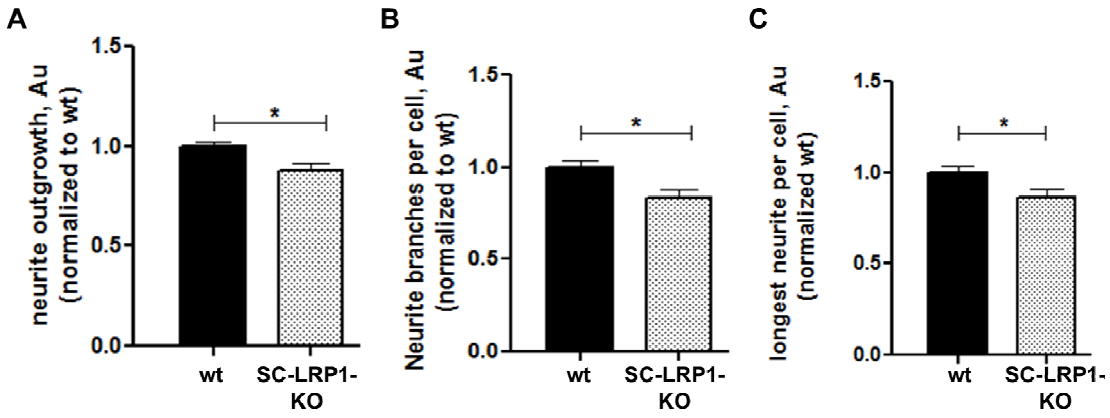

Figure 3.1: Axonal outgrowth is reduced in conditioned DRG neurons from SC-LRP1-KO compared to wildtype mice. (**A – C**) Black bar shows normalized data from wildtype and grey bars from SC-LRP1-KO DRG neurons after 2 days in culture grown on Poly-L-Lysine. Data is normalized to wildtype. (**A**) Total neurite outgrowth per cell based on βIII-tubulin staining. (**B**) Neurite branching. (**C**) Average length of longest axon per neuron. All graphs combine data from 4 independent experiments with 2-3 wells analyzed per condition (Two tailed T-test. p-Value *<0.05).

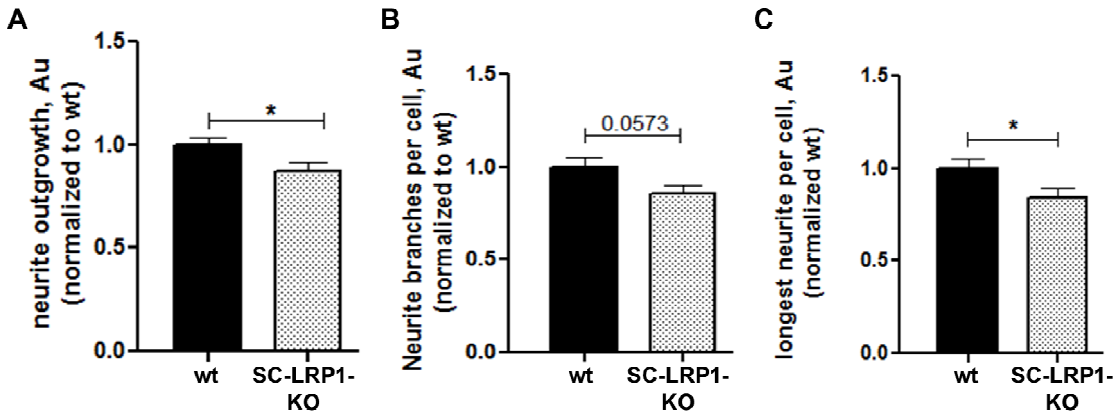

Figure 3.2: Axonal outgrowth is reduced in conditioned DRG neurons from SC-LRP1-KO compared to wildtype mice. (**A – C**) Black bar shows normalized data from wildtype and grey bars from SC-LRP1-KO DRG neurons after 2 days in culture grown on laminin. Data is normalized to wildtype. (**A**) Total neurite outgrowth per cell based on βIII-tubulin staining. (**B**) Neurite branching. (**C**) Average length of longest axon per neuron. All graphs combine data from 4 independent experiments with 2-3 wells analyzed per condition (Two tailed T-test. p-Value *<0.05).

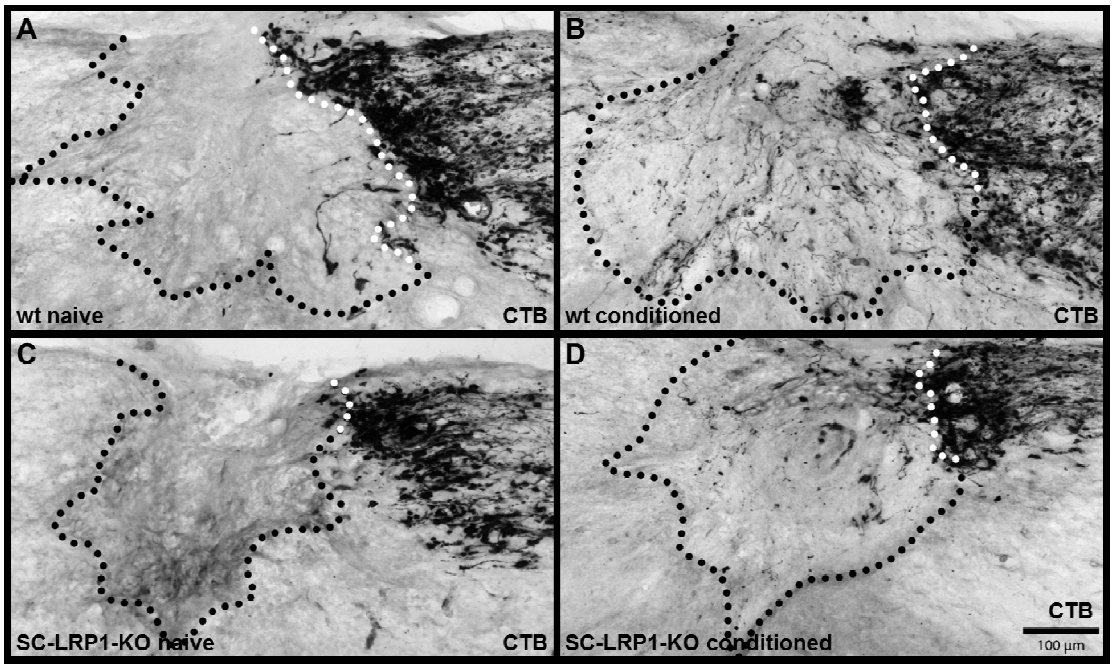

Figure 3.3: Central axon regeneration shows a trend of reduction in conditioned DRG neurons from SC-LRP1-KO compared to wildtype mice. Representative images of sagittal sections of mouse spinal cord showing CTB labeled sensory axons (**black**) 6 weeks post sciatic nerve crush. Lesion boundaries are outlines with dotted lines based upon GFAP-staining and DIC-light level microscopy. (**A**) no SNC in wiltype mouse. (**B**) SNC in wildtype mouse. (**C**) No SNC in SC-LRP1-KO mouse. (**D**) SNC in SC-LRP1-KO mouse.

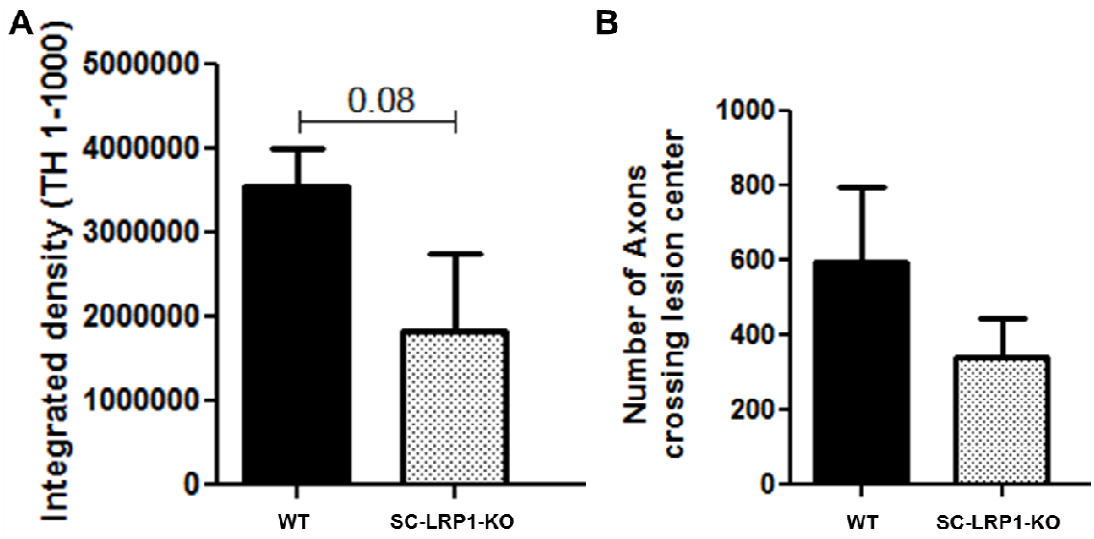

Figure 3.4: Central axon regeneration shows a trend of reduction in conditioned DRG neurons from SC-LRP1-KO compared to wildtype mice. Quantification of central axon regeneration based on CTB staining (every sixth spinal cord section section was quantified). Black bar shows raw data from wildtype and grey bars from SC-LRP1-KO mice 6 weeks after SNC. (**A**) Quantification of integrated pixel density of CTB signal in lesion site. (**B**) Manual axon count based on CTB labeling of axon rostral to lesion center (One-tailed T-test. pValue = 0.08, n = 3 mice per group).

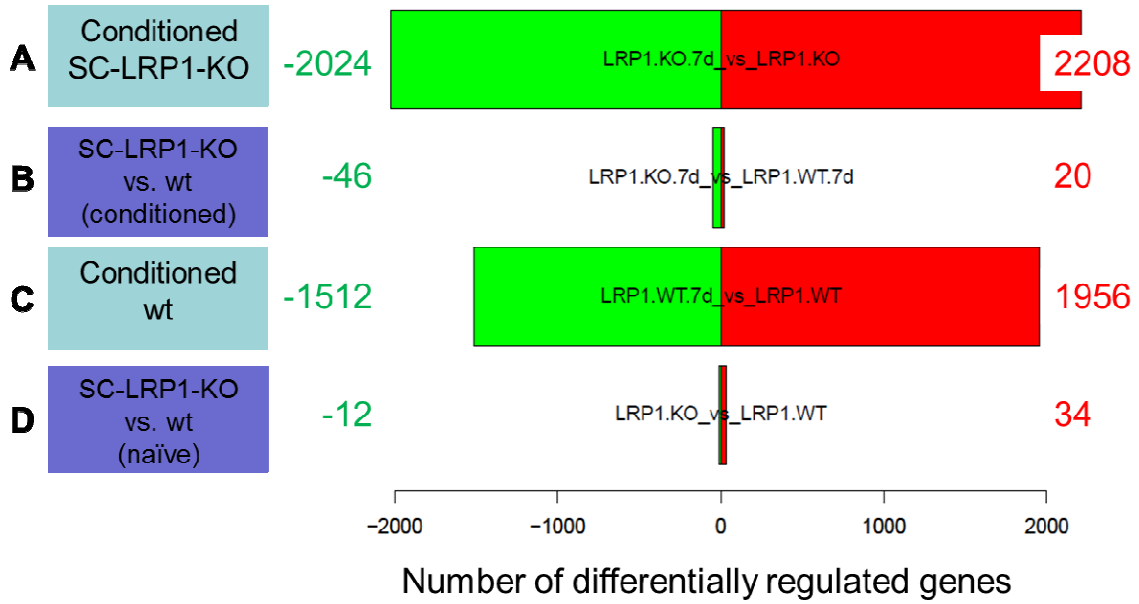

Figure 3.5: Differentially expressed genes across comparisons at ≤ 0.1 FDR (**A**) Differentially regulated gene ratio comparisons for conditioned SC-LRP1-KO mice. (**B**) Comparison of conditioned SC-LRP1-KO versus conditioned wildtype mice. (**C**) Conditioned wildtype mice. (**D**) Comparison of naive SC-LRP1-KO versus naive wildtype mice. Green indicates down and red up-regulation. The scale bar at the bottom represents number of up- and downregulated genes. Down regulated genes are listed in (green) and upregulated genes in (red).

Table 3.1: Differential regulation of known RAGs upon conditioning of SC-LRP1-KO and wildtype mice. Table is categorized in transcription factors, neuropeptides and others. Genes that are expected to be up and downregulated are shown in red and green respectively.

Transcription Factors	Neuropeptides	Other
c-Jun	Neuropeptide Y	GAP-43
JunD (Down in SC-LRP1-KO)	Galanin	tubulins (alpha, beta)
ATF3	vasoactive intestinal peptide	IL6
CREB		SPRR1A
Sox-11		arginase
CAP-23		Hsp27
STAT3		Neurofilaments
ATF2 (Up in SC-LRP1-KO)		

Exclusively differentially expressed in SC-LRP1-KO	Expected to be Upregulated
Exclusively differentially expressed in wt	Expected to be Downregulated
Differentially expressed in SC-LRP1-KO ad wt	

Table 3.2: Selected differentially-regulated genes unique to conditioned wildtype DRGs. From left to right: Genesymbol, Entrez Gene name and the fold change. Up and down-regulated genes are listed in red and green respectively.

Gene Symbol	Description	Fold change
XIRP2	xin actin-binding repeat containing 2	28.6
S100A8	S100 calcium binding protein A8 (calgranulin A)	6.1
S100A9	S100 calcium binding protein A9 (calgranulin B)	5.2
NGP	neutrophilic granule protein	4.8
ITGB2L	integrin beta 2-like	3.5
MMP9	matrix metallopeptidase 9	3.4
FGFBP1	fibroblast growth factor binding protein 1	3.3
PTGDS	prostaglandin D2 synthase (brain)	3.2
JUNB	Jun-B oncogene	2.5
NGF	Neuronal Growth Factor	2
TUBA1B	tubulin, alpha 1B	1.5
TNPO2	transportin 2 (importin 3, karyopherin beta 2b)	1.3
NEFM	neurofilament, medium polypeptide	-1.8
NEFH	neurofilament, heavy polypeptide	-1.7
SNAP25	synaptosomal-associated protein 25	-1.7
NEFL	neurofilament, light polypeptide	-1.7
SYP	synaptophysin	-1.4
SV2B	synaptic vesicle glycoprotein 2 b	-1.4
NCAN	neurocan	-1.2

Table 3.3: Selected differentially-regulated genes unique to conditioned SC-LRP1-KO DRGs. From left to right: Genesymbol, Entrez Gene name and the fold change. Up and down-regulated genes are listed in red and green respectively.

Gene Symbol	Description	Fold change
ADRA1A	adrenergic receptor, alpha 1a	3.3
NEUROG1	neurogenin 1	2.8
RAB31	RAB31, member RAS oncogene family	2.1
CHRNA1	cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	2.1
IER5	immediate early response 5	2
ATF2	activating transcription factor 2	1.3
FGF17	fibroblast growth factor 17	-20.8
MYO3A	myosin IIIA	-7.2
TNNT1	troponin T1, skeletal, slow	-5.7
AVPR1B	arginine vasopressin receptor 1B	-5.3
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	-4.3
NTF3	neurotrophin 3	-4.2
SP9	trans-acting transcription factor 9	-3.8
JunD	Jun proto-oncogene related gene d	-1.4

Table 3.4: Selected differentially-regulated genes found in both conditioned SC-LRP1-KO and wildtype DRGs. From left to right: Genesymbol, Entrez Gene name and the fold change for conditioned wildtype and conditioned SC-LRP1-KO. Up and down-regulated genes are listed in red and green respectively.

Gene Symbol	Description	Fold change wt	Fold change SC-LRP1-KO
TUBB2B	tubulin, beta 2B	3.1	2.2
VGF	VGF nerve growth factor inducible	3.2	2.4
NNAT	neuronatin	2.4	1.4
KIF22	kinesin family member 22	2.6	2
ACTA1	actin, alpha 1, skeletal muscle	20.8	-4
TGM7	transglutaminase 7	9.4	-4.5
CACNA1S	calcium channel, voltage-dependent, L type, alpha 1S subunit	4.6	-2.5
KRTDAP	keratinocyte differentiation associated protein	3.7	-2.9
DLK1	delta-like 1 homolog (Drosophila)	2.8	-2.6
ALPK3	alpha-kinase 3	2.3	-3.5
LAMB2	laminin, beta 2	1.2	-1.2

3.8 SUPPLEMENTAL MATERIAL

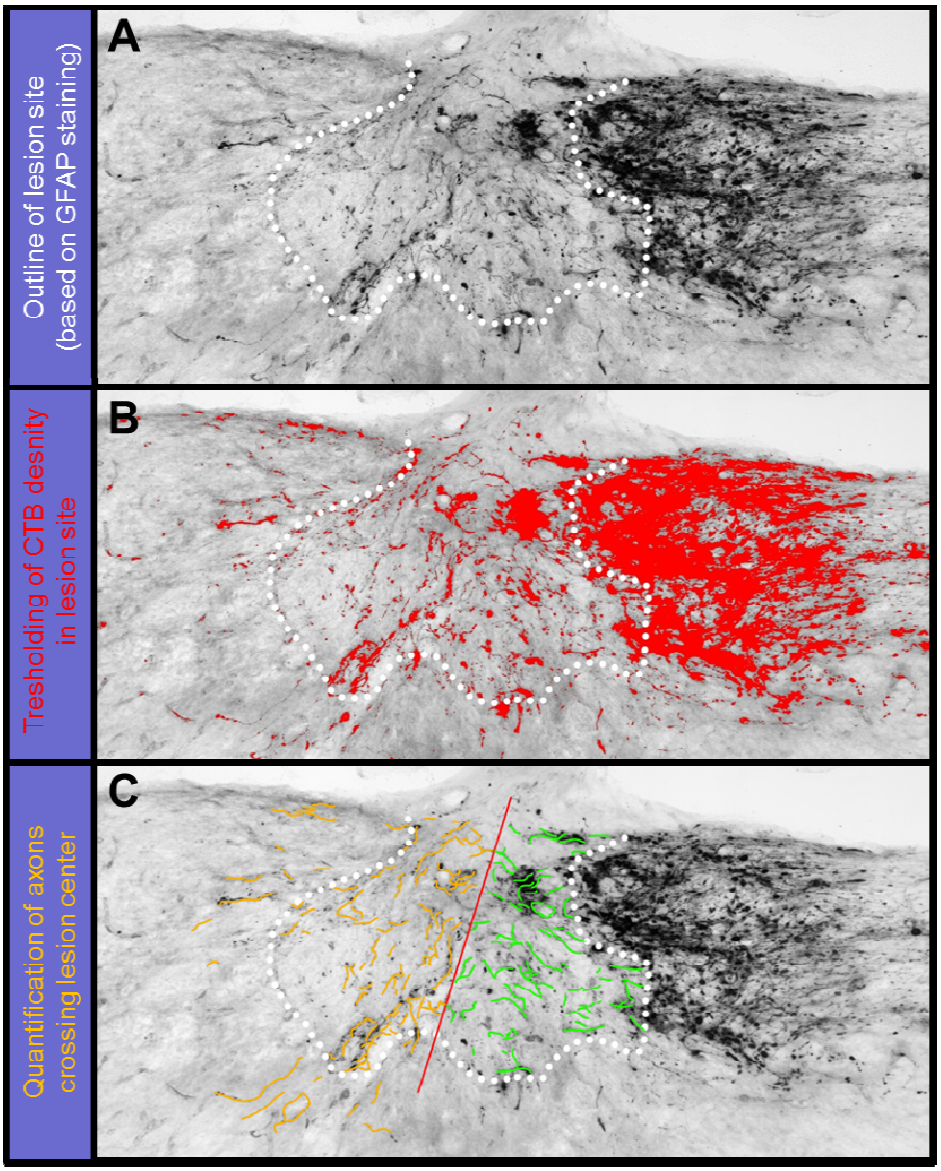

Figure S3.1: Methods of quantification. Sagittal sections of mouse spinal cord showing CTB labeled sensory axons (black) 6 weeks post sciatic nerve crush. (**A**) Lesion boundaries are outlines with dotted lines based upon GFAP-staining and DIC-light level microscopy. (**B**) Tresholding of CTB signal intensity. All positive recognized pixels are overlayed in **red**. Only pixels within lesion boundaries were quantified. All sections were treated together with the same solutions , imaged with the same exposure settings and the same threshold was applied. (**C**) **Red** line defines lesion center based on lesion boundary and background tissue. All axons rostral to center line were traced in **orange** and counted.

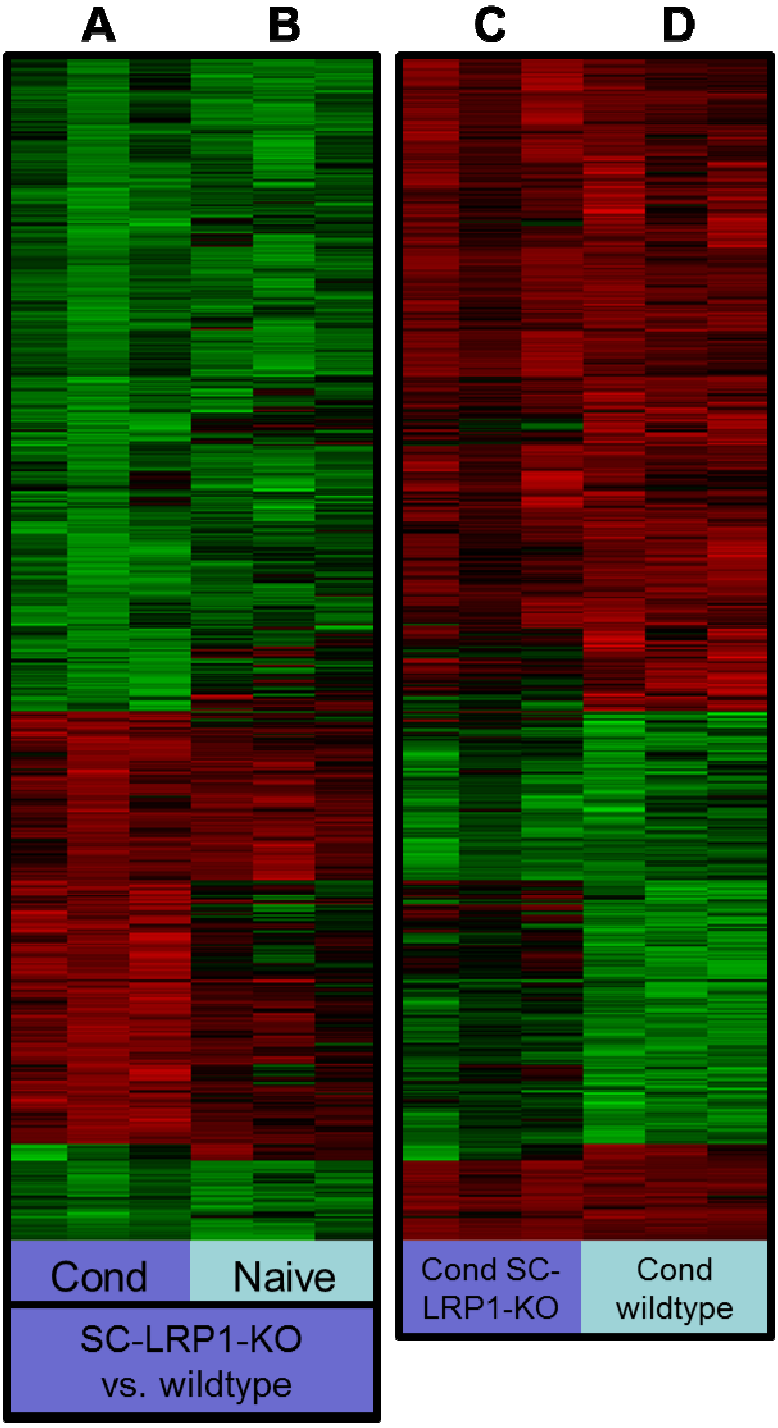

Figure S3.2: Sample log fold change of highest ranked differentially regulated genes (FDR<0.1). Heatmaps of up (red) and down (green) regulated genes grouped by functionality. (**A**) Conditioned SC-LRP1-KO versus wildtype DRGs. (**B**) Naïve SC-LRP1-KO versus wildtype DRGs. (**C**) Conditioned SC-LRP1-KO DRGs. (**D**) Conditioned wildtype DRGs.

Table S3.1: Selected differentially-regulated genes unique to conditioned wildtype DRGs (muscle related). From left to right: Genesymbol, Entrez Gene name and the fold change. Up and down-regulated genes are listed in red and green respectively.

Gene Name	Description	Fold change
MYH4	myosin, heavy polypeptide 4, skeletal muscle	48.8
MYL1	myosin, light polypeptide 1	48.8
TNNT3	troponin T3, skeletal, fast	31.3
TNNC2	troponin C2, fast	25.6
TTN	titin	19.4
MYBPC1	myosin binding protein C, slow-type	14.8
MYH1	myosin, heavy polypeptide 1, skeletal muscle, adult	14.2
MYLPF	myosin light chain, phosphorylatable, fast skeletal muscle	6.2
ACTN2	actinin alpha 2	5.4
TNNC1	troponin C, cardiac/slow skeletal	5.2
ACTN3	actinin alpha 3	4.7
NRAP	nebulin-related anchoring protein	4
NEB	nebulin	3.7
MSLN	mesothelin	3.7
PYGM	muscle glycogen phosphorylase	3.5
DSP	desmoplakin	3.2

Table S3.2: Top significantly increased bio functions according to gene expression changes specifically in conditioned WT DRGs. From left to right: Bio function, differentially regulate molecules that lead to the prediction.

Functions Annotation	# Molecules	Functions Annotation	# Molecules
accumulation of cells	41	engulfment of phagocytes	19
accumulation of granulocytes	18	extravasation	11
accumulation of leukocytes	34	fatty acid metabolism	60
accumulation of myeloid cells	23	function of blood cells	85
accumulation of neutrophils	11	function of leukocytes	81
accumulation of phagocytes	21	homing	61
activation of blood cells	67	homing of cells	58
activation of cells	82	homing of granulocytes	27
activation of leukocytes	59	homing of leukocytes	48
activation of lymphocytes	43	homing of neutrophils	26
activation of macrophages	13	hyperactive behavior	19
activation of mononuclear leukocytes	45	hypersensitive reaction	69
activation of myeloid cells	21	immune response of antigen presenting cells	19
activation of neutrophils	9	immune response of neutrophils	13
activation of phagocytes	25	immune response of phagocytes	22
activation of T lymphocytes	34	infiltration	48
adhesion of blood cells	43	infiltration of cells	47
adhesion of granulocytes	13	infiltration of granulocytes	28
adhesion of immune cells	38	infiltration of leukocytes	43
adhesion of red blood cells	6	infiltration of leukocytes	43
antimicrobial response	31	inflammatory demyelinating disease	23
arthritis	135	metabolism of reactive oxygen species	44
arthropathy	138	migration of antigen presenting cells	18
binding of cells	45	migration of dendritic cells	13
binding of leukocytes	22	migration of monocytes	11
binding of phagocytes	14	migration of phagocytes	33
cell movement of antigen presenting cells	37	morphology of cardiovascular system	78
cell movement of eosinophils	16	Movement Disorders	136
cell movement of macrophages	28	phagocytosis by macrophages	13
cell movement of monocytes	20	phagocytosis of antigen presenting cells	14
cell movement of neutrophils	43	phagocytosis of bacteria	6
cell viability of phagocytes	14	phagocytosis of blood cells	18
chemotaxis	60	phagocytosis of myeloid cells	17
chemotaxis of cells	57	phagocytosis of neutrophils	9
chemotaxis of granulocytes	26	phagocytosis of phagocytes	17
chemotaxis of leukocytes	47	priming of T lymphocytes	9
chemotaxis of myeloid cells	38	production of reactive oxygen species	34
chemotaxis of neutrophils	25	quantity of helper T lymphocytes	28
chemotaxis of phagocytes	39	quantity of lymphocytes	95
contractility of skeletal muscle	6	quantity of mononuclear leukocytes	97
cytotoxicity of cells	24	quantity of T lymphocytes	69
cytotoxicity of leukocytes	21	recruitment of cells	33
cytotoxicity of lymphocytes	20	recruitment of cells	33
degradation of protein	53	recruitment of leukocytes	32

Table S3.3: Differentially-regulated growth factors in conditioned wildtype and SC-LRP1-KO DRGs. From left to right: Genesymbol, Entrez Gene Name, fold change WT and fold change SC-LRP1-KO. Up and down-regulated genes are listed in **red** and **green** respectively. (-) demarks no significant change in gene expression.

Symbol	Entrez Gene Name	Fold Change wt	Fold Change SC-LRP1-KO
REG1A	regenerating islet-derived 1 alpha	246.9	229.9
GRP	gastrin-releasing peptide	59.5	7.2
FGF3	fibroblast growth factor 3	50.6	33.8
AMELX	amelogenin, X-linked	4.6	-
INHBB	inhibin, beta B	4.5	8.2
MSTN	myostatin	3.7	7.3
VGF	VGF nerve growth factor inducible	3.2	2.4
FGF11	fibroblast growth factor 11	2.7	2.9
NGF	nerve growth factor (beta polypeptide)	2	-
NOV	nephroblastoma overexpressed	2	-
BDNF	brain-derived neurotrophic factor	1.9	1.9
OGN	osteoglycin	1.9	1.9
BMP3	bone morphogenetic protein 3	1.7	-
BMP5	bone morphogenetic protein 5	1.6	1.5
IGF1	insulin-like growth factor 1 (somatomedin C)	1.5	2
NDP	Norrie disease (pseudoglioma)	1.4	1.4
KITLG	KIT ligand	1.3	1.6
PDGFC	platelet derived growth factor C	-	1.7
TGFA	transforming growth factor, alpha	-	1.4
JAG2	jagged 2	-1.4	-1.4
TGFB2	transforming growth factor, beta 2	-1.4	-
FGF1	fibroblast growth factor 1 (acidic)	-1.5	-
Nrg1	neuregulin 1	-1.5	-
NRG2	neuregulin 2	-1.6	-1.8
PDGFA	platelet-derived growth factor alpha polypeptide	-1.6	-
FGF5	fibroblast growth factor 5	-1.7	-
FGF18	fibroblast growth factor 18	-1.9	-
EGF	epidermal growth factor	-2.1	-
NOG	noggin	-2.2	-3.5
FGF9	fibroblast growth factor 9 (glia-activating factor)	-2.2	-
GAS6	growth arrest-specific 6	-	-1.4
BMP15	bone morphogenetic protein 15	-	-1.8
NELL1	NEL-like 1 (chicken)	-	-1.8
AGT	angiotensinogen	-	-1.9
NTF3	neurotrophin 3	-	-4.2
FGF17	fibroblast growth factor 17	-	-20.8

Table S3.4: Differentially-regulated transcription factors with known function in neurite outgrowth in conditioned wildtype and SC-LRP1-KO DRGs. From left to right: Genesymbol, Entrez Gene Name, fold change WT and fold change SC-LRP1-KO. Up and down-regulated genes are listed in **red** and **green** respectively.

Gene Symbol	Entrez Gene Name	Fold Change wt	Fold Change SC-LRP1-KO
ATF2	**activating transcription factor 2**	-	1.3
ATF3	**activating transcription factor 3**	48.4	69.2
ATF4	activating transcription factor 4 (tax-responsive enhancer element B67)	1.6	1.3
CREB1	**cAMP responsive element binding protein 1**	1.3	1.4
CREB3	**cAMP responsive element binding protein 3**	1.6	-
CREB3L1	cAMP responsive element binding protein 3-like 1	-	-1.5
CREBBP	CREB binding protein	-1.5	-
CREBL2	cAMP responsive element binding protein-like 2	-	-1.4
CREBZF	CREB/ATF bZIP transcription factor	-1.3	-
CREM	cAMP responsive element modulator	1.8	2.5
JDP2	Jun dimerization protein 2	-	-1.8
JUN	**jun proto-oncogene**	2.9	3.1
JUNB	**jun B proto-oncogene**	2.5	-
JUND	**jun D proto-oncogene**	-	-1.4
KLF10	Kruppel-like factor 10	1.6	1.7
KLF15	Kruppel-like factor 15	-	-2.0
KLF2	**Kruppel-like factor 2 (lung)**	-	-1.6
KLF6	**Kruppel-like factor 6**	1.9	2.2
KLF7	**Kruppel-like factor 7 (ubiquitous)**	-	1.4
KLF9	**Kruppel-like factor 9**	-1.4	-
NFKBIA	nuclear factor of kappa light polypeptide inhibitor, alpha	-	-1.5
NFKBIZ	nuclear factor of kappa light polypeptide inhibitor, zeta	2.0	1.5
SOX10	SRY (sex determining region Y)-box 10	-1.7	-
SOX11	**SRY (sex determining region Y)-box 11**	8.2	11.2
SOX17	**SRY (sex determining region Y)-box 17**	-2.2	-
SOX5	SRY (sex determining region Y)-box 5	-	1.6
SOX7	**SRY (sex determining region Y)-box 7**	3.7	3.6
SOX9	SRY (sex determining region Y)-box 9	2.9	2.4
STAT3	**signal transducer and activator of transcription 3 (acute-phase response factor)**	-	-
STAT5A	signal transducer and activator of transcription 5A	2.3	2.0
STAT5B	signal transducer and activator of transcription 5B	1.5	-

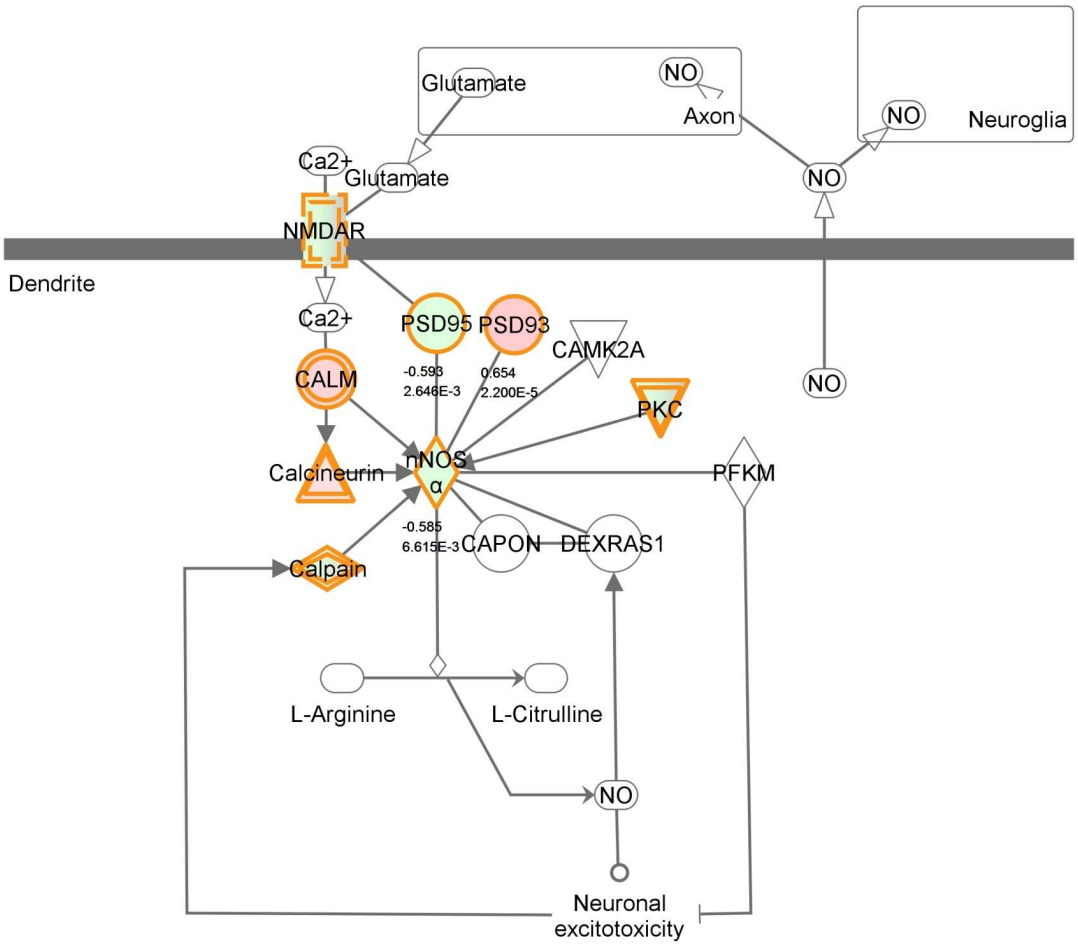

Figure S3.3: nNOS signaling pathway is specifically activated in conditioned SC-LRP1-KO DRGs: Dashed lines indicate indirect interactions; solid lines indicate direct interactions. The arrow indicates the directionality of the relationship. Downregulated genes are shown in green and upregulated genes in red. An increase of the intensity in color (red and green) resembles stronger gene regulation. For detailed information on individual genes within the network and on their interaction see the legend in the supplemental material (Fig. S2.19)

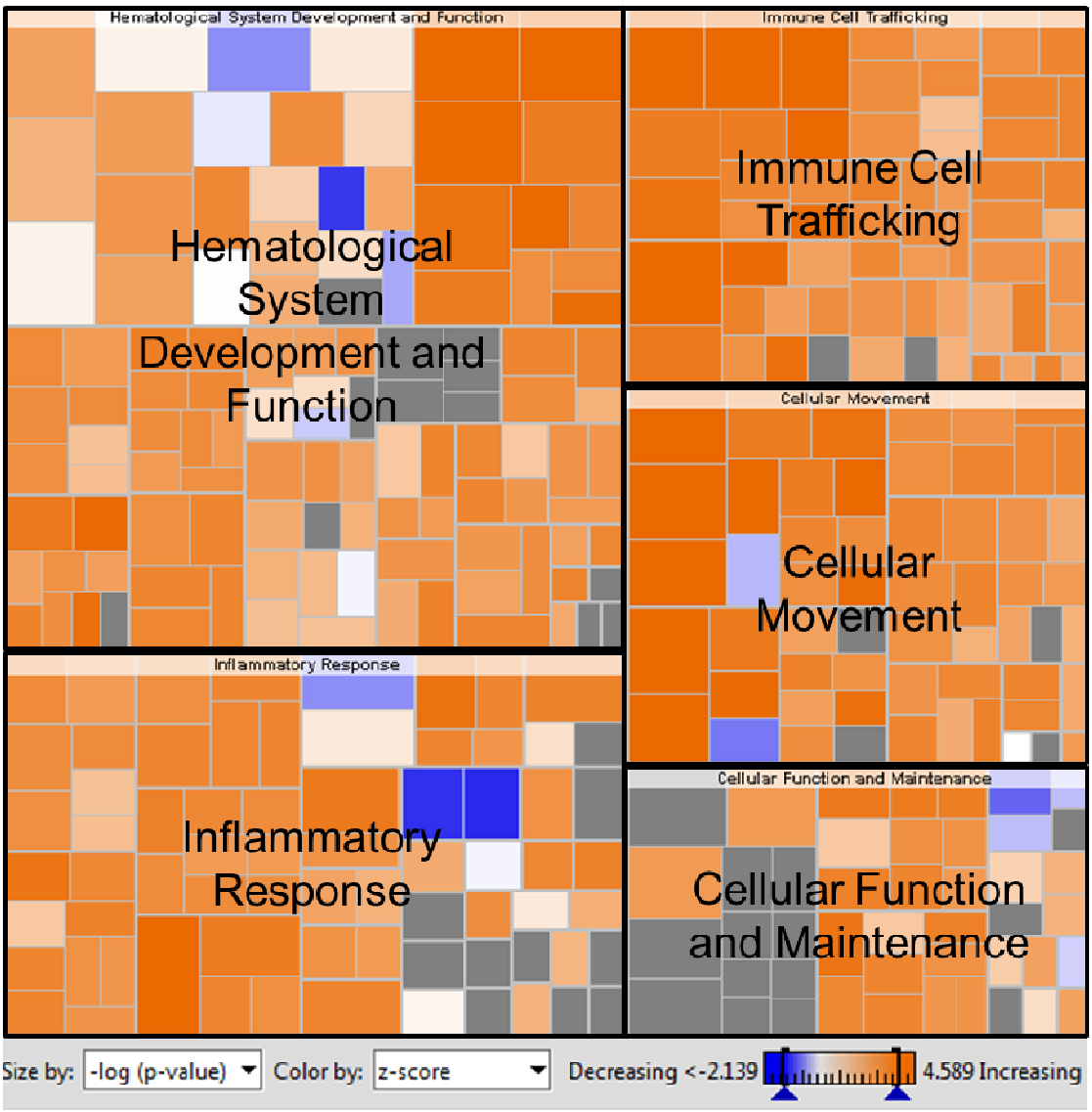

Figure S3.4: Heatmap of downstream analysis of biological functions resulting from gene expression changes upon conditioning of WT DRGs: Each square represents a functional group that can represent hundreds of differentially regulated molecules that either activate or inhibit a specific function. The color represents the z-score of activation (**orange**), inhibition (**blue**) or no direction (**grey**) of a certain function. Individual bio functions (squares) are clustered together and form a functional groups (**black boxes**). The size of an individual square represents the averaged significance (pValue) of the genes within a specific function with (bigger squares = lower pValue).

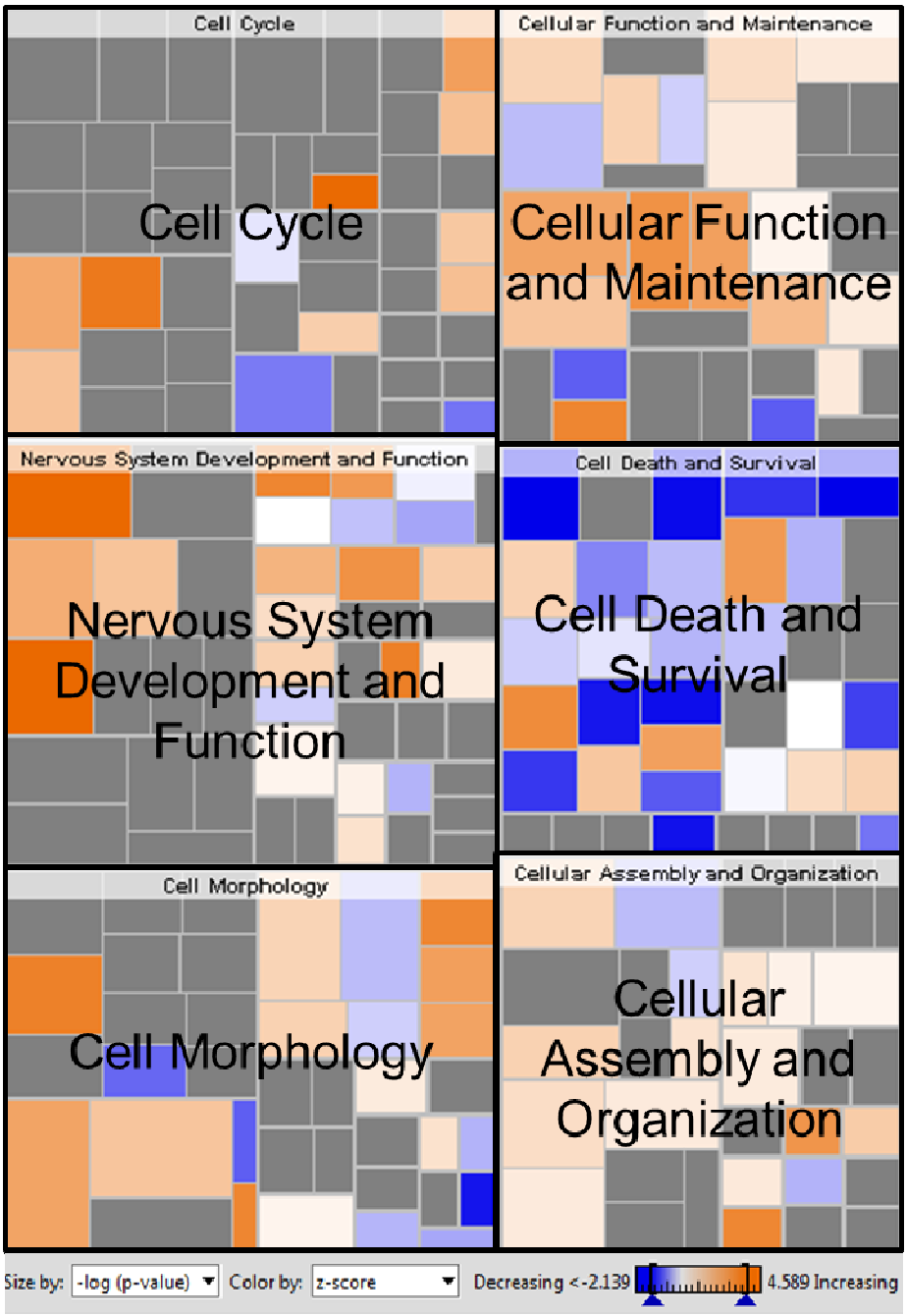

Figure S3.5: Heatmap of downstream analysis of biological functions resulting from gene expression changes upon conditioning of SC-LRP1-KO DRGs: Each square represents a functional group that can represent hundreds of differentially regulated molecules that either activate or inhibit a specific function. The color represents the z-score of activation (**orange**), inhibition (**blue**) or no direction (**grey**) of a certain function. Individual bio functions (squares) are clustered together and form a functional groups (**black boxes**). The size of an individual square represents the averaged significance (pValue) of the genes within a specific function with (bigger squares = lower pValue).

4 CONCLUSIONS

The primary hypothesis of this thesis was that axon regeneration is mediated by intrinsic gene expression, which is directly influenced by the immediate environment interacting with the injured axons. We further hypothesize that these changes in gene expression will in return influence the regenerative capacity of central nervous system axons after injury. We investigated two model systems where extrinsic (environmental) mechanisms influence the intrinsic gene expression in either adult cortical spinal neurons or adult dorsal root ganglion neurons. Both of these systems can be primed for regeneration.

The regeneration of CST neurons is triggered by the application of neural stem cell grafts placed into the injury site. These grafts have unique features, including derivation from embryonic spinal cord cells from a specific developmental age (embryonic day 12). Other embryonic derived neural stem cells from areas such as the cortex or hippocampus do not support CST axon regeneration. The unique nature of these grafts indicates that the grafted cells display specific signaling molecules, or a stoichiometric combination of general signaling molecules, to the injured CST axons that are able to trigger retrograde signaling responses to the cell body to alter gene transcription and establish the intrinsic basis for axonal regeneration. It also indicates that the injured CST axons display a combination of transmembrane receptors, that when stimulated in the right pattern, can elicit retrograde signaling to the cell body to initiate axon

regeneration. This indicates that one could study the critical steps involved in achieving CST axon regeneration with different approaches.

One can investigate the transcriptional response to injury and regeneration to identify master regulators of transcription that control the differential expression of additional downstream transcription factors. These in turn mediate multiple signaling pathways that activate CST axon growth in response to a permissive graft environment following injury. The investigation of those master switches of regeneration can be further enhanced by the search for common promoter sequences that regulate whole sets of immediate early transcription factors. Or, one can identify the transmembrane signaling molecules that are present on both the grafted neural stem cells and the injured CST axons, whose specific interaction most likely leads to local protein translation which triggers the retrograde injury response to induces differential gene transcription ultimately leading to regeneration. Both scenarios however merge at some point in the nucleus in the control of gene transcription, which can be identified and described using quantitative analysis of the CST specific regeneration transcriptome. The ultimate goal of this study is to identify those master switches of regeneration that are activated in response to cell membrane receptor signaling, and manipulate their expression to increase the regenerative potential of CST neurons.

In the second aim we investigated the hypothesis that the Schwann cell is an essential component of the conditioning effect in adult dorsal root ganglion

neurons. When a sciatic nerve crush is performed, cell intrinsic mechanisms within the peripheral axon are activated that lead to a retrograde injury signaling response to the cell body. This alters gene transcription and activates the expression of regeneration-associated genes. These retrograde signaling mechanisms have been shown to involve local protein translation in the axon and active retrograde protein transport by the microtubule dependent motor protein dynein. This indicates that the regenerative response is mediated by intrinsic factors alone and not like in the CNS triggered by a specific cell graft. However it has been shown that Schwann cells are essential for the regeneration of peripheral axons. This indicates again that the extrinsic signaling stemming from the environment is necessary to promote axon regeneration.

4.1 Commonalities in the regenerative signaling of CST and dorsal root ganglion neurons

In both systems, several components are necessary to elicit axon regeneration. (1) An injury signal has to be present to initiate changes in gene transcription (unpublished data from our lab has shown that for CST axons to regenerate into a neural stem cell graft, an injury has to performed to CST axons at some point); (2) the environment with which the injured axon interacts has to be supportive for axon regeneration (NSCs or Schwann cells); (3) axon regeneration is accompanied by changes in gene transcription in the neuronal cell body. The environmentally triggered signaling mechanisms could either be activated by soluble factors, by cell-to-cell interactions or a combination of both. It

is known that in the peripheral nerve, the secretion of neurotrophic factors is essential in the regeneration of peripheral axons; this does however not exclude the possibility that Schwann cells also promote peripheral axon regeneration via direct cell to cell interactions. In the CNS the interactions seems to be mainly based on direct cell to cell interactions, since the proximity of the grafted neural stem cells influences the amount of CST axons responding to the grafted cells. This does not however rule out localized effects mediated by soluble secreted factors, as the dependence on proximity could in part be due to spatially limited diffusion of neurotrophic factors in the adult CNS host tissue.

We found that Schwann cells constitute an essential part of the conditioning effect in vitro and show a trend to influence central branch regeneration in vivo. We utilized a transgenic mouse wherein the LRP1-receptor was conditionally deleted in Schwann cells. Since LRP1 functions as a pro-survival receptor after injury, Schwann cell LRP1-deficient mice show increased Schwann cell death after peripheral nerve crush. Since Schwann cells are essential for the regeneration of peripheral axons, it is not surprising that the regeneration of peripheral axons is impaired in these mice (unpublished data from the Campana laboratory). Out results indicate a further role for the Schwann cells besides supporting the regeneration of the peripheral nerve. We found significant changes in gene expression within the injured DRGs in the LRP1-deficient mice compared to wildtype mice. This indicated that Schwann cells not only have a supporting role in peripheral branch regeneration but also influences

central branch axon regeneration. In the conditioning effect, gene expression changes induced by peripheral nerve crush and hence induction of regeneration is utilized by the DRG neurons to enhance central branch regeneration. Since we found a significant alteration in gene expression in the LRP1 deficient mice, it is very plausible that some of these gene expression changed will influence central branch regeneration, and we have shown that they influence neurite outgrowth in vitro.

In both the CST and DRG system, changes in gene expression following injury combined with the presence of a permissive cell graft lead to regeneration. The difference between both systems lies within the signaling mechanism between the environment (NSC or glia cell) and neuron intrinsic transmembrane cell signaling receptors and ligands. These are most likely differentially expressed cell signaling receptors that are active during development to guide axons to their appropriate targets. Therefore Schwann cells must likely express a different combination of cell guidance cues than are needed for CST axon growth. It would therefore seem likely that, whilst basic intrinsic regenerative pathways may be conserved, the regenerative transcriptome will differ significantly between regenerating CST and DRG neurons. We found the potential activation of common transcription factors such as TP53, STAT3 and NFATc in regenerating CST neurons. An activation of those transcription factors has also been described in the regeneration of the peripheral nervous system. Other transcriptional regulators however have been uniquely identified upon

corticospinal regeneration, such as HTT and NFE2L2 and might therefor present transcriptional regulators that are unique to central nervous system axon regeneration.

4.2 Caveats of the Schwann cell component of the conditioning effect:

To completely investigate the contribution of Schwann cells towards the conditioning effect, one would need a mouse model that is completely deficient of Schwann cells, or a technique to eliminate all the Schwann cells in the sciatic nerve. No such mouse models currently exist and would probably be embryonically lethal if produced, since Schwann cells are essential for the development of the peripheral nervous system. One could inject ethidium bromide into the sciatic nerve to kill off Schwann cells, but this treatment is very harsh and might cause changes to the peripheral axons that are hard to distinguish from the effects of Schwann cell deletion. In the SC-LRP1 deficient mouse, the reduction of axonal outgrowth of conditioned DRG neurons in vitro was about 15% and we are still confirming the reduction in vivo. This partial but significant effect might be increased with the complete lack of Schwann cells. This might be expected, as we show that the Schwann cell has a significant effect on the condition effect. Therefore, if Schwann cells are still present after sciatic nerve crush, they are still promoting the condition of DRG neurons. Another caveat is that the LRP1-deficient Schwann cell has shown several functional deficiencies, such as ensheathing and myelination of axons, migration,

re-myelination and survival after injury. All of these components could be part of the mechanisms that mediate how Schwann cells influence the condition effect. Altered LRP1 mediated signaling between the Schwann cell and the neuron itself might be the trigger for reduced axon regeneration. It would be interesting to perform a rescue experiment where LRP1 is deleted in Schwann cells but the pro-survival signaling properties of LRP1 are conserved. In this regard it would also be interesting to investigate if the infiltration of immune cells into the DRGs is dependent on altered LRP1 signaling or on increased Schwann cell death or both.

4.3 Caveats of sample reproducibility and Ingenuity Pathways Analysis Software

We performed 3 biological replicates per time point and treatment. To assure that each replicate would show the expected biological effect, we adapted the grafting paradigm so that there is sufficient contact between grafted neural stem cells and injured CST axons. This also resulted in the effect that injured CST axons are immediately in contact with neural stem cells at the time of grafting. If we would graft cells only into the injury site, the grafted cells would proliferate and migrate and some would be earlier in contact with the injured CST axons than others. This would lead to a temporal discrepancy of gene expression between different CST neurons from the same animal. By injecting cells in 9 positions along the spinal cord we improve simultaneous activation of regenerative programs in more CST neurons.

To further increase the signal to noise ratio, we pooled the motor cortexes from 3 – 4 mice per sample. This procedure further increases the chance that each biological replicate contains more reproducible amounts of CST neurons that are actively responding to the neural stem cell graft with differential gene expression.

The average mRNA quality was measured at RIN = 8.1 so no extra step to remove ribosomal RNA was necessary. Due to the nature of the sample preparation via immunoprecipitation of mRNA solely from CST neurons of layer5b of the motor cortex, the amount of mRNA per sample was around 50ug. We needed to amplify the mRNA to create the cDNA library. RNA samples above 100ug do not need amplification for cDNA library construction. We have to be aware that amplification of RNA can create amplification bias, thus follow up verifications of candidates is critical, especially for candidates that have low reads in the RNA sequencing process.

The Ingenuity pathway analysis software (IPA) predicts activation of up- and downstream regulators according to the expression profiles of affected genes based on literature findings. This analysis tool is instrumental in identifying master switches of gene regulation such as transcription factors or genetic hubs. One has to be aware of the short comings that the activation of a certain transcription factor is solely a prediction and can of course be misled. It is essential to further investigate the regulation of those predicted genes. In many cases the expression itself of the predict genes is not differentially regulated but

its activity is altered by post translational modifications such as phosphorylation or acetylation. These modifications can influence the activity or longevity as well as subcellular location a protein. In the case of transcription factors, activation via phosphorylation is often times correlated with translocation to the nucleus. Verification of these candidate genes cannot achieved by verification of their expression levels but rather by determining attributes like phosphorylation status with phospho-specific antibodies or determination of subcellular locations via immunohistochemistry.

4.4 Power of the investigation of the Regeneration transcriptome of injured corticospinal tract neurons

The BAC-TRAP mouse line Glt25d2-EGFPL10a allows for the selective enrichment of mRNAs that are bound to polyribosomes, the majority of which would be expected to be undergoing active translation. The advantage over common mRNA sequence analysis is that mRNAs undergo posttranscriptional modifications, and the presence of a specific mRNA molecule does not necessarily mean that it is actively transcribed into a protein. Thus the overall transcriptome at a certain time point contains mRNAs that potentially have been transcribed earlier and are marked for degradation, are actively silenced or have been freshly transcribed and not yet utilized for protein translation. Investigating the actively translating pool of polyribosomal bound mRNAs provides a “snapshot” of the transcriptome exactly at time of tissue harvesting and is more likely to contain mRNA that code for proteins that are essential for the state of

regeneration at a given time point, enhancing the chances of identifying candidate genes relevant to CST axon regeneration.

4.5 Caveats of the investigation of the Regeneration transcriptome of injured corticospinal tract neurons

The BAC-TRAP mouse line Glt25d2-EGFPL10a expressed the ribosomal GFP-fusion protein L10a-GFP under the Glt25D2 promoter. This promoter is not solely expressed in CST neurons but also in cortico-pontine and cortical-striatal neurons of the layer 5b in the motor cortex. The labeled neurons in the layer5b however are mostly corticospinal neurons. However there is a certain error included that could result from the other projecting neurons. This error is expected to be negligible since the lesion is only performed on the corticospinal tract and the treatment is localized within the dorsal column of the spinal cord with C4 as most rostral point of intervention. Thus the distance of the surgical manipulations and cell grafts should not reach past C3 and is a sufficient distance from the pons or the striatum that they should not influence gene expression of those projecting neurons. It is however possible that inputs to the cortico-pontine or cortical-striatal neurons are influenced by the regeneration or the lesion of the corticospinal neurons, and thereby affect gene transcription in those neurons that can then influence the expression analysis. It is unknown if corticospinal neurons innervate cortico-pontine or cortico-striatal neurons. Further this is an issue that cannot easily be resolved and one has to keep that in mind when interpreting the data.

4.6 Immune response promotes axon regeneration

The transcriptional analysis of conditioned DRGs showed hundreds of significantly regulated genes that were uniquely altered in wildtype and unchanged in the DRG of SC-LRP1-defitient mice that promote the infiltration of immune cells into the DRGs. It has recently been shown that macrophage infiltration after sciatic nerve crush constitute an essential part of the conditioning effect in vitro and in vivo (Kwon et al., 2013). The potential reduction of macrophage and other immune cell infiltration into the DRGs of SC-LRP1-defitient mice, caused by altered LRP1 signaling might be part of the reduction that we observed in the conditioning effect. Schwann cells secrete growth factors and cytokines that attract macrophages and other immune cells. Thus it is plausible that part of the effect we are observing is mediated by insufficient immune cell recruitment as a result of LRP1-deletion in Schwann cells. Macrophage infiltration and the secretion of inflammatory cytokines, such as oncomodulin, have been reported to enhance retinal ganglion regeneration after a conditioning lens injury (Leon et al., 2000; Yin et al., 2006).

If the infiltration of immune cells into the injured nerve is a critical component of not just peripheral but also central axon regeneration, then it is possible that the neural stem cell graft also promotes CST axon regeneration by recruitment of immune cells or activation of microglia within the spinal cord and that this activation or recruitment demarks an essential part of the regeneration triggering ability of the neural stem cell graft. It might even be that grafted

microglia cells or precursors of those stemming from the grafted embryonic spinal cord contribute to the observed CST regeneration. This hypothesis could be investigated by administration of microglial-inhibiting drugs such as minocycline and quantification of regenerating CST axons into the neural stem cell graft.

4.7 Extrinsic Factors that promote intrinsic gene expression vs. facilitation of axon growth

We found that Schwann cells constitute an essential component to the conditioning effect, which is mediated by differential gene expression within the DRG neurons. If we can confirm (with the addition of more animals) the trend of reduced central axon regeneration in vivo, then this change in gene expression is sufficient to reduce axon regeneration. This also means that there are two components stemming from the extrinsic environment: (1) signaling that leads to altered gene expression that eventually leads to central axon regeneration (2) secretion of e.g. extracellular matrix molecules that facilitate axon regeneration of the peripheral branch. An event of group (1) would be the infiltration of immune cells in response to cytokine release by Schwann cells leading to altered gene expression in the DRG neurons. Whereas Schwann cells secreting extracellular molecules, e.g. laminin, to promote axon regeneration would fall in category (2).

These two distinct mechanisms that are performed by Schwann cells in the peripheral nerve could be fulfilled by different cell types in the neural stem cell graft. The neural stem cell graft consists of neuronal and glial precursors that

differentiate into astrocytes, oligodendrocytes, neurons and potentially other cells types. It is possible that some component of this graft would trigger changes in gene expression in the injured CST neurons and that other components would facilitate the growth of axons. If we could differentiate between those two pathways, it would be possible to manipulate the graft in either way to (1) increase gene expression triggering CST growth or (2) CST axon extension from regenerating neurons.

4.8 Expression of genes involved in post synaptic signaling

Ingenuity pathway analysis software identified the activation of long term depression and long term potentiation pathways at 2 weeks after grafting of neural stem cells into the injured spinal cord. This classification was based on differential expression of post –synaptic AMPAR, NMDAR, mGluR and GRID receptors as well as several downstream molecules involved in these pathways. This could either mean that these post-synaptic receptors either have functions in axon regeneration in the axon tip, or more likely that the CST neurons at 2 weeks are integrating signals from other neurons. If the latter is the case, it would mean that either the regeneration of the axon and the associated signaling stimulates inputs onto CST neurons, or that some of the CST axons already formed synapses with graft derived neurons that now function as relays. It would be interesting to investigate if these post-synaptic receptors have dual functions and are also involved in axon regeneration. One would expect acute cortical reorganization following the traumatic injury but these events are expected to

occur within days after injury. Thus it is intriguing that we observed the activation of those signaling pathways at the latest time point that we investigated, with is two weeks after grafting of neural stem cells and 3 weeks after CST lesion. At this time point cortical reorganization and lingering plasticity should be over.

4.9 Possible Future Directions

It is possible that inhibiting synapse formation by regenerating CST axons in the graft could prevent premature termination of axon growth, resulting in longer distance regeneration in the absence of “stop” signals (synapse formation). These axons could be guided by neurotrophic gradients (e.g. NT3) possibly out of the lesion site and into the host cord to achieve long distance CST axon regeneration that bridges across an injury site. If one would investigate the transcriptome of later time points of CST axon regeneration, when regeneration has ended and synapses are formed (e.g. several weeks after grafting) one could identify those presynaptic molecules and genetically delete them in regenerating CST neurons or pharmacologically inhibit synapse formation. Alternatively, one could genetically delete the post-synaptic molecules in the grafted neural stem cells and use the grafts just to initiate CST growth.

4.10 Conclusions

It is well understood that central nervous system (CNS) axon regeneration is mediated by both extrinsic mechanisms of the environment and intrinsic mechanisms within the injured axon. This was demonstrated initially in the early

1980s when Aguayo and colleagues showed that a subset of CNS axons maintain the ability to regenerate throughout adulthood when presented with a growth permissive substrate of the peripheral nerve environment.

The work presented here highlights the importance of the interaction of environmental extrinsic mechanisms with intrinsic gene regulation as mediators of central axon regeneration in two distinct model systems.

(1) Adult corticospinal motor neurons can be triggered to regenerate when presented with a growth permissive NSC graft. We identified the regeneration transcriptome of injured adult corticospinal neurons, which shows differential regulation of known growth promoting pathways and axon guidance molecules as well new candidate regulators specific to corticospinal axon regeneration. Validation and further characterization of individual candidate genes emerging from this transcriptional analysis may be a good foundation for the rational development of future clinical therapies to promote axon regeneration after spinal cord injury in humans.

(2) Schwann cells which are essential for peripheral axon regeneration also elicit their signaling mechanism to promote central axon regeneration after a conditioning injury. We tested this hypothesis by utilizing a mouse line with conditional LRP1-deletion in Schwann cells, leading to increased Schwann cell death after sciatic nerve crush. Reduced Schwann cell viability after injury influenced the intrinsic gene expression of dorsal root ganglion neurons. Thus the intrinsic gene expression profiles of the conditioned dorsal root ganglion

neurons from SC-LRP1-KO mice differ from wildtype littermates reflecting the reduction in axon regeneration and implying a role for Schwann cells in supplying neurotrophic support and mitigating the appropriate immune response to promote central axon regeneration.

The manipulations of extrinsic environmental mechanisms directly influence the regeneration of central nervous system axons and their intrinsic gene expression profiles in the investigated sensory and motor system. To further identify the master switches of central axon regeneration one could mine the datasets for regenerating CST or DRG neurons for promoter sequences that are common to immediate early transcriptional regulators. Activation of those promoter sequences could be utilized to trigger or enhance central axon regeneration. We will undertake further studies to potentially identify those common promoter sequences in the transcriptome of regenerating CST neurons.

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