# REGENERATION AND PLASTICITY OF DESCENDING PROPRIOSPINAL NEURONS AFTER TRANSPLANTATION OF SCHWANN CELLS OVEREXPRESSING GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR FOLLOWING THORACIC SPINAL CORD INJURY IN ADULT RATS

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# REGENERATION AND PLASTICITY OF DESCENDING PROPRIOSPINAL NEURONS AFTER TRANSPLANTATION OF SCHWANN CELLS OVEREXPRESSING GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR FOLLOWING THORACIC SPINAL CORD INJURY IN ADULT RATS

After spinal cord injury (SCI), poor axonal regeneration of the central nervous system, which mainly attributed to glial scar and low intrinsic regenerating capacity of severely injured neurons, causes limited functional recovery. Combinatory strategy has been applied to target multiple mechanisms. Schwann cells (SCs) have been explored as promising donors for transplantation to promote axonal regeneration. Among the central neurons, descending propriospinal neurons (DPSN) displayed the impressive regeneration response to SCs graft. Glial cell line-derived neurotrophic factor (GDNF), which receptor is widely expressed in nervous system, possesses the ability to promote neuronal survival, axonal regeneration/sprouting, remyelination, synaptic formation and modulate the glial response.

We constructed a novel axonal permissive pathway in rat model of thoracic complete transection injury by grafting SCs over-expressing GDNF (SCs-GDNF) both inside and caudal to the lesion gap. Behavior evaluation and histological analyses have been applied to this study. Our results indicated that tremendous DPSN axons as well as brain stem axons regenerated across the lesion gap back to the caudal spinal cord. In addition to direct promotion on axonal regeneration, GDNF also significantly improved the astroglial environment around the lesion. These regenerations caused motor functional recovery. The dendritic plasticity of axotomized DPSN also contributed to the

functional recovery. We applied a G-mutated rabies virus (G-Rabies) co-expressing green fluorescence protein (GFP) to reveal Golgi-like dendritic morphology of DPSNs and its response to axotomy injury and GDNF treatment. We also investigated the neurotransmitters phenotype of FluoroGold (FG) labeled DPSNs. Our results indicated that over 90 percent of FG-labeled DPSNs were glutamatergic neurons. DPSNs in sham animals had a predominantly dorsal-ventral distribution of dendrites. Transection injury resulted in alterations in the dendritic distribution, with dorsal-ventral retraction and lateral-medial extension of dendrites. Treatment with GDNF significantly increased the terminal dendritic length of DPSNs. The density of spine-like structures was increased after injury and treatment with GDNF enhanced this effect.

In conclusion, this work provides a thorough overview of the morphologic mechanism of descending propriospinal tract responding to the axotomy injury and combinatory treatment of Schwann cell and GDNF.

Xiao-Ming Xu, Ph.D., Chair

## TABLE OF CONTENTS

## LIST OF TABLES

**LIST OF FIGURES**

# CHAPTER 1

## INTRODUCTION

### Background:

After spinal cord injury (SCI), rostrocaudal axonal regeneration is crucial for significant functional recovery; however, neurons of the mature central nervous system (CNS) are believed to have low regenerative ability. In some central axons, an early growth response may be seen, but this response is abortive and generally does not create meaningful connections (Hall and Berry, 1989; Steward et al., 2008; Zeng et al., 1994). The abortive regeneration of CNS axons contributes heavily to the poor recovery observed after SCI. In the early 1980s, the elegant experiments done by Aguayo and colleagues demonstrated that the peripheral nerve (PN) milieu, mainly composed of Schwann cells (SCs), was more favorable for regeneration of injured CNS axons than the CNS environment (Bray et al., 1987; David and Aguayo, 1981; Richardson et al., 1980; Richardson et al., 1982; Richardson et al., 1984). Since then, many therapeutic strategies have been established through transplantation of either a PN segment containing SCs or SCs isolated from the PN (Decherchi and Gauthier, 2000; Houle, 1991; Houle et al., 2006; Iannotti et al., 2003; Levi et al., 2002; Oudega et al., 2001; Xu et al., 1995b; Xu et al., 1997). These experiments strongly support the premise of using SCs for repair after SCI (Wiliams and Bunge, 2012). To date, most regeneration studies after SCI have focused on long supraspinal pathways that project from the brain or brainstem to the spinal cord such as the corticospinal (CST) and rubrospinal (RST) tracts. However, regeneration of these long supraspinal tracts is difficult to achieve (Deng et al., 2013; Guest et al., 1997a; Kanno et al., 2014; Lee et al., 2013; Papastefanaki et al., 2007; Tuszynski et al., 1998; Xu et al., 1995a). On the contrary,

although greatly understudied, propriospinal tracts (PSTs) possess greater innate regenerative capacity, and have been shown to strongly respond to PN/SC grafts (Deng et al., 2013; Iannotti et al., 2003; Xu et al., 1995b; Xu et al., 1997). The regenerative response of the PSTs could even result in significant functional recovery after SCI (Deng et al., 2013).

**The Propriospinal tract (PST)**

The propriospinal tract (PST) is important in mediating and maintaining a variety of normal spinal functions including reflexes, posture, and locomotion (Cowley et al., 2008; Jankowska, 1992; Kostyuk and Vasilenko, 1979). The neurons within the PST constitute an uninterrupted cell column and their axons project either unilaterally or bilaterally in the rostrocaudal plane and directly affect motoneurons and interneurons in multiple cord segments (Szentagothai, 1964). Anatomically, PSTs are classified as either “short” or “long” PST based on the distances of their axon projections (Cowley et al., 2010). Although argument still exists as to what defines a short or long PN, we consider short PSTs (sPSTs) as those spanning less than six spinal segments, whereas long PSTs (lPSTs) project further than six spinal segments (Flynn et al., 2011). Short propriospinal pathways interconnecting several neighboring segments are located both in the lateral and ventral funiculus (Sterling and Kuypers, 1968), The sPSTs with cell bodies medially located in the grey matter often project contralaterally, while the sPSTs with cell bodies laterally located project ipsilaterally. The sPSTs can project bidirectionally (Burton and Loewy, 1976; Matsushita, 1970; Menetrey et al., 1985; Petko and Antal, 2000). In line with classical studies by Romanes and Sprague who demonstrated a medio-lateral division between motoneurons and their target muscle groups, within the limb enlargements (Romanes, 1951; Sprague, 1948), Kuypers and colleagues proposed a somatotopic organization of sPSTs. The sPSTs originating in neurons within the

ventromedial grey matter (lamina VIII and the medial portion of lamina VII), innervate and influence motoneurons supplying axial muscles as their axons terminate within and around medial motoneurons pools. Correspondingly, the soma of sPSTs located in lateral regions (lateral parts of laminae VII) innervate motoneurons supplying more distal limb muscles, as their axons terminate in the vicinity of the lateral motoneuron pools (Molenaar and Kuypers, 1978; Sterling and Kuypers, 1968). lPSTs that are involved in locomotor activity reciprocally connect cervical and lumbar enlargements and are concentrated in the ventral quadrants (Giovanelli Barilari and Kuypers, 1969). The anatomical distinction that can be made with respect to lPST is whether their cell bodies are located rostrally (within the cervical enlargement) and project caudally, or vice-versa. These two populations are termed long descending PST (ldPST) and long ascending PST (laPST), respectively (Giovanelli Barilari and Kuypers, 1969; Matsushita and Ueyama, 1973; Molenaar and Kuypers, 1978). The function of ldPSTs is involved in feed-forward inhibition of supraspinal command and reciprocal connection of cervical and lumbar motor circuits (Alstermark et al., 1991a; Alstermark et al., 1999; Isa et al., 2006). The laPST system was found to play an important role in locomotion by coupling neural activity in cervical and lumbar enlargements (Cote et al., 2012; Miller et al., 1973). Propriospinal neurons receive strong and convergent supraspinal innervations including those from the corticospinal (CST), rubrospinal (RST), reticulospinal (ReST) and vestibulospinal (VST) tracts (Alstermark et al., 1987; Alstermark et al., 1991b; Illert et al., 1977; Kostyuk and Vasilenko, 1978; Nishimura et al., 2009; Robbins et al., 1992; Skinner et al., 1979). Such signal relay has significance in transporting supraspinal command down to the spinal cord not only in normal physiological but also in pathological conditions (Figure 1).

Several critical studies concluded that supraspinal axons, which usually fail to regenerate through and beyond the lesion site, form 'new' contacts with spared intraspinal or propriospinal circuits projecting past a SCI lesion to lumbar segments. Such supraspinal-propriospinal reorganization formed an anatomical 'bridge' allowing transmission of descending signals below the lesion to activate the lumbar locomotor central pattern generator (CPG) (Bareyre et al., 2004; Courtine et al., 2008; Cowley et al., 2008; Vavrek et al., 2006). Such plasticity occurred based on the intact propriospinal system spared following an incomplete SCI. However, with a severe injury such as a complete SCI, axonal regeneration through and beyond the injury is required to achieve meaningful functional recovery. Descending propriospinal axons (dPSTs) are uniquely suited for reestablishing connections across the lesion since they show greater growth responses after SCI than long-tract axons (Deng et al., 2013; Iannotti et al., 2003; Xu et al., 1995b; Zhang et al., 2009). Therefore, the plasticity of CST axons that innervate dPST neurons and subsequent regeneration of dPST axons beyond the lesion site may provide an alternative pathway or "functional relay" for transmission of supraspinal motor commands down to the spinal cord to promote motor recovery.

**Schwann cells mediate endogenous repair of PNS and CNS injuries**

The peripheral environment has long been shown to be permissive for CNS axonal regeneration (David and Aguayo, 1985; Horvat et al., 1989; Salame and Dum, 1985). SCs are the major component of the grafted nerve that promotes such regeneration. Developmentally, SCs derive from the neural crest (Bhatheja and Field, 2006). Neural crest cells give rise to SC precursors from which immature SCs are generated. The immature SCs then differentiate into either myelin-forming or non-myelin-forming SCs (Corfas et al., 2004; Jessen and Mirsky, 2005). SCs are very important for guiding axonal growth and producing myelin sheath for peripheral axons. SCs are also essential

for endogenous repair of peripheral and central axons after injury (Arthur-Farraj et al., 2012; Oudega et al., 2005). SCs are involved very early in segmenting and incorporating degraded damaged myelin and recruiting macrophages for removal of myelin debris. Early after axonal injury, SCs in the distal nerve dedifferentiate into non-myelinating SCs and proliferate extensively. These newly formed non-myelinating SCs migrate within reorganized connective tissue to form column-like structures known as bands of Bungner (Arthur-Farraj et al., 2012; Jessen and Mirsky, 2005). This proliferative response is accompanied by downregulation of myelin-associated molecules that inhibit axonal regeneration, and upregulated cell adhesion molecules including neural cell adhesion molecule (NCAM), N-cadherin, L1, and several other trophic factors, creating a permissive environment for the repair of injured axons (Arthur-Farraj et al., 2012; Jessen and Mirsky, 2008; Svaren and Meijer, 2008; Taveggia et al., 2010). For different types of SCI such as contusion, laceration, and photochemical lesions, SCs can migrate into the injured spinal cord and myelinate or ensheathe a large number of regenerating axons, and more importantly, establish nodes of Ranvier with normal ion channel patterns on central axons and can maintain action potential conduction for over a year (Black et al., 2006). In experimental SCI, transplantation of either peripheral nerve or isolated and purified SCs establishes conduits for axon regeneration and remyelination, replaces lost glial cells, and improves neurological function (Honmou et al., 1996). Next, I define both PN and purified SC transplantation as SC transplantation for repair after SCI.

**Advantage of using Schwann cells/PN**

The PN auto-graft is one of the earliest experimental treatments to promote CNS axonal regeneration after SCI. A PN graft not only provides supportive SCs but also promotes the survival of axotomized spinal cord neurons by upregulating the expression of nitrous oxide (NO) and further activation of the NO-dependent cyclic-GMP pathway, a survival

effector, in these neurons (Yick et al., 1999). Moreover, nerve grafts induced expression of growth factors such as NGF and BDNF in the host spinal cord and attenuated delayed glial scar formation at the interface of the caudal spinal cord, which is crucial for successful regeneration (Kuo et al., 2011). Dissociated SCs were later utilized as a transplantation strategy to promote axon regeneration. After transplantation, the initially dissociated SCs align predominantly parallel to the length of the implant. Thus, dissociated SCs alone could both elicit axonal ingrowth and serve as an effective substrate or bridge for growing axons (Murray and Fischer, 2001). Compared to peripheral nerve grafts, one unique advantage of using purified SCs is the potential to engineer them to overexpress growth-promoting factors and/or adhesion molecules to enhance axon growth (Wiliams and Bunge, 2012).

**Schwann cells/PN transplantation promotes propriospinal regeneration**

The majority of regenerated axons in the grafted PN/SCs have been shown to be of spinal cord origin. Propriospinal neurons whose axons regenerated into the SC/PN grafts originated within Rexed lamina III-VII, the medial portion of lamina VIII, and lamina X and were distributed throughout the spinal grey matter as far rostral and caudal as C3 and S3 (Kao et al., 1977; Paino et al., 1994; Richardson et al., 1980; Richardson et al., 1982; Richardson et al., 1984; Xu et al., 1995b; Xu et al., 1997). The density of labeled neurons was the greatest in sections closest to the graft and diminished progressively at increasing rostral and caudal distances. Approximately half of the labeled cells were located in a 4 mm region surrounding the graft (Paino et al., 1994; Xu et al., 1995b). A bilateral distribution of retrograde-labeled propriospinal neurons within the spinal cord was observed, with a significantly higher occurrence of labeled cells on the ipsilateral side were reported in both PN nerve graft and isolated SCs graft models (Decherchi and Gauthier, 2000; Iannotti et al., 2003). However, due to the fact that intrinsic spinal cord

neurons, especially long projecting propriospinal neurons, have various projection patterns including projection distance, laterality, or branching within the graft (Saywell et al., 2011; Siebert et al., 2010b; Tuszynski and Steward, 2012; Verburgh and Kuypers, 1987), analysis of the size, distribution, and cytological features failed to yield specific identification of usual destinations of these labeled neurons. In addition to the anatomical differences, Siebert et al. (2010) observed phenotypic variation in the post-injury response to axotomy between ldPST neurons and sdPST neurons. The ldPST neurons lacked both cell death and regenerative responses, and down-regulated many genes important for regrowth. Instead of mounting a robust early response exhibited by short thoracic propriospinal neurons, ldPST neurons become relatively dormant or quiescent (Siebert et al., 2010b). This study demonstrated that ldPST neurons respond more like supraspinal neurons than sdPST neurons following low thoracic axotomy. This study did not combine propriospinal neuronal response with any other treatment such as the use of SC transplantation, and no information is available concerning how SC transplantation might affect the posttraumatic response of propriospinal neurons.

**Optimal transplantation time**

Some inhibitory influences associated with the mature CNS are downregulated over time after injury. The injury site environment may become more favorable for axonal growth at later time points following injury. For example, in the acute phase of SCI, regeneration of propriospinal axons into grafted PN is often hindered by a lesion cavity between the cord stumps and the grafts, which is caused by coalescence of several small cysts. Delayed spinal cord grafting after the trauma appeared to prevent such cyst formation (Kao et al., 1977). In addition, Wardrope and Wilson found that the lytic enzymes released by the damaged axons were no longer found in the extracellular space one week following injury (Wardrope and Wilson, 1986). Moreover, it is likely that the differences between

acute and delayed transplant injury conditions are not restricted to the environment alone. Rather, there may be differences in the ability of neurons themselves to mount a regenerative response. The process of re-exposing the lesion site 2 weeks after injury and clearing away the glial scar at the injury site prior to transplantation may actually elicit a “conditioning lesion” (Neumann and Woolf, 1999; Richardson et al., 1984). That is, neurons that have been injured previously may be primed to upregulate cellular and molecular programs associated with axonal growth. After axotomy at a distance from the cell body, a strong initial inflammatory as well as early regeneration and cell death responses occur. An early up-regulation of several growth factor receptors, as well as a down-regulation of receptors to several factors that inhibit axonal growth may indicate that potential therapies to protect PST neurons from early cell death post-axotomy and to maximize and sustain the early regenerative response should be applied during an acute phase. On the other hand, when acutely injured propriospinal axons displayed a strong growth capacity, chronically injured axons are more impaired in their propensity to regenerate. Decherchei and Gauthier found that a time window of three weeks post-lesion is a critical period for axonal regeneration after which injured neurons may attenuate regeneration potential (Decherchi and Gauthier, 2000). Sandrow et al. also observed a 50% reduction in the mean number of contributing propriospinal neurons after extended delay (Sandrow et al., 2008). Siebert and colleagues further confirmed that the thoracic propriospinal neurons mounted a very dynamic response following low thoracic injury. In the chronic phase, the transitory enhanced expression of regeneration-associated genes diminished. Contrarily, gene expressions of several inhibitory receptors for axonal growth were initially down regulated but recovered to control level in later periods post-injury (Siebert et al., 2010b).Therefore, acute or sub-acute phase (one

or two weeks after injury) is the optimal time window for the treatment of the propriospinal axonal regeneration.

**Combination with neurotrophic factors**

Another therapeutic approach for axonal regeneration involves the use of exogenous neurotrophic factors. The combination of neurotrophic factors with transplants heightens the regenerative effort of injured neurons. Exogenous application of neurotrophic factors increases the intrinsic capacity of mature neurons for regrowth, and prevents atrophy of axotomized neurons (Coumans et al., 2001). Several observations indicated that GDNF, GFRα1, and GFRα2 mRNAs were highly expressed in the ventral horn, and moderately expressed in the intermediate zone and dorsal horn of the spinal gray matter, and injury induced up-regulation of receptor genes for GDNF shortly after SCI (Satake et al., 2000; Widenfalk et al., 2001). These observations suggest that propriospinal neurons may respond to GDNF and regenerate axons into the GDNF-enriched graft. GDNF may exert a direct effect via receptors expressed on injured axons or via retrograde transport from the site of implantation to the cell body of injured axons. Intervention to rescue injury-induced death of propriospinal neurons should start quickly following injury, prior to onset of cell death to sustain their survival and regenerative responses (Paratcha et al., 2001; Trupp et al., 1999 Seibert et al., 2010b). Neurotrophins combined with SC transplantation act synergistically to maximize neuroprotective or regenerative responses. After transplantation of a SC-seeded guidance channel into an acutely transected spinal cord, approximately two-thirds of a total 2,500 myelinated and unmyelinated axons of propriospinal origin were present within the transplant cable (Xu et al., 1997). When combined with GDNF, a significant and synergistic increase in axonal regeneration and myelination occurred. Retrograde tracing revealed that GDNF-induced enhancement of axonal regeneration mainly originated from propriospinal

neurons (Iannotti et al., 2003; Zhang et al., 2009). In contrast, other factors like BDNF failed to increase the number of axons that regenerated back into the spinal cord which may be attributed to an insensitivity of propriospinal axons to BDNF (Tom et al., 2013). Application of neurotrophic factors is more important in chronic injuries when growth factor related gene expressions are greatly reduced (Dolbeare and Houle, 2003; Tom et al., 2009).

**Graft-host interface**

To achieve meaningful functional regeneration, it is necessary to promote axons to regenerate beyond the lesion site, reenter the host spinal cord and reconnect with target neurons. Following SCI, CNS axons do not regenerate spontaneously due to the presence of inhibitory molecules associated with glial scar and CNS myelin at the lesion site, as well as the shortage of neurotrophic support in the host spinal cord. PN or isolated SC transplantation could improve glial environment to certain extent, however, such transplantation did not elicit regeneration of propriospinal axons beyond the graft environment, despite robust ingrowth of axons into the graft (Deng et al., 2011; Wiliams and Bunge, 2012). Without additional treatment at the graft-host interface, regenerating axons within the SC graft are hindered by properties of the interface similar to the SC-CNS parenchymal interface seen at the dorsal root entry zone (DREZ), which is normally impenetrable by regenerating sensory axons in the adulthood (Fernandez et al., 1985; Guest et al., 1997b; Moissonnier et al., 1996; Xu et al., 1999). More recently, dorsal root axons have been found to regenerate into CNS territory in a dorsal root crush model; however, they rapidly stalled and then remained completely immobile or stable, even after conditioning lesions that enhanced growth along the root (Di Maio et al., 2011). The molecular environment encountered by regenerating axons including connective tissue and host astrocytes at the distal graft/host interface, appears to be more important for

progress of regrowth than quantity of fibers within the graft. To promote regenerated axons within the PN graft into the host spinal cord where they can establish functional connections, different therapeutic combinations aimed at modifying glial scar at the graft-host interface have been tested. In 2004, Chau et al. used chondroitinase ABC (ChABC) to directly digest the glial scar caudal to a SC-seeded transplant after acute SCI. By 1 month post ChABC treatment, significant propriospinal axons regenerated through the graft-host interface and extended into the host cord as far as 5 mm (Chau et al., 2004). However, no functional evaluation was performed in this study. Tom et al. (2008, 2009) also applied ChABC to the distal graft-host interface in an acute PN graft model allowing regenerating axons to extend beyond the graft spanning the cavity and promoted some functional recovery. However, the sources of axon outgrowth in these studies were not discerned (Tom and Houle, 2008; Tom et al., 2009). It is quite possible that these regenerated axons primarily originated from propriospinal neurons.

In addition to modifying the glial scar at the graft-host interface, activation of injured neurons by different growth factors will add another important dimension to axonal regeneration (Dolbeare and Houle, 2003; Storer et al., 2003). For example, Lee et al. combined the transplantation of multiple PN autografts (PNGs), embedded within acidic fibroblast growth factor (aFGF)-laden fibrin matrix, and ChABC delivered to both the graft and at the graft/host interfaces after a complete spinal transection (Lee et al., 2013). In this experiment, dPST axons were found to regenerate beyond the lesion site. Interestingly, another trophic factor GDNF was found to modify the SC graft-host interface, promote the migration of host astrocytes into the SC bridge, reduce reactive astrogliosis, macrophage infiltration, and cavitation (Deng et al., 2011; Iannotti et al., 2003). Within the SC graft, elongated processes of astrocytes extended parallel to the graft axis and in close alignment with regenerated axons (Figure 2). This morphological

change of reactive astrocytes at the graft-host interface proved permissive for regenerative axon growth. However, the origins of regenerated axons were not traced in these studies. Recently, William and Bunge reported that, compared to pre-gelled bridges, fluid bridges of SCs and Matrigel affected morphology and distribution of host astrocytes, induced immature astrocyte characteristics and elongated processes into the bridge forming a tight-meshwork that walled off the graft-host interface (Williams et al., 2013). This modification was closely associated with enhanced regeneration of brainstem axons across the rostral interface and improvement in hindlimb locomotion. Deng et al. expanded upon the previous work and constructed a continuous growth-promoting pathway in adult rats, formed by transplantation of SCs overexpressing GDNF both in the lesion gap and caudal host cord (Deng et al., 2013) (Figure 3). This pathway, extending from the cut axonal ends to the site of innervation in the distal spinal cord, promoted regeneration of dPST axons through and beyond the lesion gap of a spinal cord hemisection. Within the distal host spinal cord, regenerated dPST axons were myelinated and formed synapses with host neurons providing anatomical basis for its functional recovery.

**Functional recovery**

Anatomical and physiological investigations have confirmed that the intraspinal network of propriospinal neurons plays a critical role in motor reflexes, voluntary movement, and sensory processing. A population of propriospinal neurons located in the upper cervical segments is critical for certain CST-dependent forelimb motor tasks. Their role is to transmit CST input, as well as convergence input from the rubro-, tecto-, and reticulo-spinal tracts, to motoneurons in segments C6 to T1 that innervate the forelimb (Flynn et al., 2011; Isa et al., 2013). These connections may be important for preparatory movements of the hindlimbs prior to and during targeted reaching by the forelimbs

(Kostyuk and Vasilenko, 1978). Two special populations of propriospinal neurons were long projecting neurons, including laPST neurons projecting from the lumbosacral enlargement rostrally to the cervical enlargement, and ldPST neurons projecting caudally from the cervical enlargement to the lumbosacral enlargement. These two types of long distance PST neurons send reciprocal projections between the upper and lower limb segments and function in the regulation and fine-tuning of locomotion, limb coordination, and postural support (Flynn et al., 2011). In addition, the ability of propriospinal neurons to activate and coordinate spinal CPGs makes them ideally suited to facilitate significant locomotor recovery. Even non-specific electrical stimulation of propriospinal networks (leading to lumbar CPG activation) following SCI results in significant locomotor performance improvements (Ballion et al., 2001; Cowley et al., 2008; Juvin et al., 2005). Short thoracic propriospinal neurons arise from the thoracic levels of the spinal cord and their axons project only a few segments in both the rostral and caudal directions. These neurons are primarily involved in regulating axial musculature and postural mechanisms, working in concert with ldPST and laPST (Flynn et al., 2011).

It has been suggested that the propriospinal system plays a key role in functional recovery after SCI (Jane et al., 1964; Selzer, 1978). Several recent studies have provided compelling evidence supporting this notion (Bareyre et al., 2004; Courtine et al., 2008; Vavrek et al., 2006). These publications reported motor improvement in animals following SCI via generation of de novo intraspinal circuits involving propriospinal neurons. These data suggest that descending supraspinal signaling may be reestablished through new connections with intact propriospinal neurons projecting past the lesion site and contact neurons and circuits that can shape motor function. A recent study by Fenrich and Rose (2009) further emphasizes the importance of commissural

propriospinal neurons in recovery from SCI. They demonstrated that severed axons of commissural propriospinal neurons can regenerate and make functional synaptic connections with spinal motoneurons (Fenrich and Rose, 2009).

A basic function of an axon is to conduct action potentials. There are some studies in which electrophysiological testing was applied after different grafting paradigms following SCI. Pinzon et al. showed that propriospinal neurons, primarily within laminae IV, V, and VII, of the adjacent cord segments regenerated axons through a PN graft and gave rise to evoked potentials characterized by low amplitude, long latency responses and undetectable late responses with low voltage stimulation, compared to evoked responses in normal animals (Pinzon et al., 2001). The approximate conduction velocities of the identified responses are in the range of myelinated axons indicating that the regenerated propriospinal axons were myelinated. Deng et al. demonstrated that provision of a SC-GDNF growth promoting pathway promoted dPST axons to regenerate through a hemisection lesion to enter the caudal host spinal cord. These regenerated axons conducted action potentials across the lesion gap (Deng et al., 2013). The major hurdle remains in that only a limited number of regenerating axons grew back into the host spinal cord despite the use of different kinds of combinatorial treatment strategies.

One important question is whether a small number of regenerated propriospinal neurons can influence functional recovery. The ability of propriospinal neurons to contribute to functional recovery following complete spinal cord transection has been demonstrated in the lamprey (Selzer, 1978). The role for propriospinal neurons to enhance recovery following SCI and cell transplantation in the mammal has been understudied. Kinematic analysis during treadmill walking in spinal cord-transected rodents with open-ended SC grafts has provided no clear evidence of fore- to hind-limb coordination although animals grafted with SC-filled channels exhibited more frequent

and longer episodes of alternating dorsal stepping (Guest et al., 1997b). Animals recovered some behavioral function when a continuous growth-promoting pathway of SCs-GDNF was provided (Deng et al., 2013). Interestingly, significant differences existed only in stride length but not other parameters between the SCs-GDNF-treated group and the other treatment groups in footprint analysis indicating that functional restoration occurs in more proximal than distal muscles, which could be innervated by different spinal pathways (Deng et al., 2013). Several reasons could account for these surprising results. First, after SCI, the original anatomical structure is interrupted. Axonal regeneration is disorderly along the rostral-caudal orientation under transplantation interference. Second, in addition to the heterogeneous types of propriospinal neurons, the target neurons of regenerated axons are basically randomly chosen which may complicate behavioral results and sometimes worsen the motor function recovery. Third, although axonal regeneration, remyelination, and synaptic formation in our studies appear to be functional, it is unclear how efficient these connections were in transducing information. It is possible that the regenerated axons remained in a pathological state with decreased conduction velocities even after regeneration, possibly due to chronic demyelination. Fourth, although we show evidence that regenerated axons are myelinated, we do not know the extent of myelination along the axons or whether the thickness of myelin surrounding the axons approaches normal. Last, but not least, despite the observation that regenerated axons formed new synapses on host neurons, the efficiency of these connections might not be suboptimal.

**New sources of Schwann cells for transplantation**

Due to the great potential to amplify and genetically manipulate SCs *in vitro*, transplantation of purified SCs has become an important strategy for possible experimental and clinical treatment for SCI. In addition to the expansion of isolated SCs

from PN, Schwann cell precursors (SCPs) with a SC phenotype derived from bone marrow stromal cells, subcutaneous fat tissue, skin, or even human umbilical cord have recently been created *in vitro* (Agudo et al., 2008; Ban et al., 2009; Biernaskie et al., 2007; Chi et al., 2010; Kamada et al., 2011; Park et al., 2010; Someya et al., 2008; Yan-Wu et al., 2011). These SC-like cells display impressive regenerative potential *in vivo* following SCI. These cells resemble PN-derived SCs in terms of expression of SC markers, as well as the ability to fill the lesion and myelinate central and peripheral axons (Biernaskie et al., 2007). However, the SCPs exhibit striking differences from mature SCs upon transplantation into the CNS. For example, integration ability into host tissue exceeds PN-derived SCs. They may be able to modify adjacent host tissues, specifically reducing reactive gliosis (Biernaskie et al., 2007). In addition, after PN injury, SCs located in the distal segment of the injury become activated. They increased proliferative ability and growth factor expression which enhances their regeneration-permissive capacity and make them an attractive cell type for promoting axonal regeneration (Dinh et al., 2007; Rasouli et al., 2006). Although several studies have taken advantage of these various sources of SCs for axonal regeneration, no further information is available concerning their effects on propriospinal axonal regeneration.

**Summary**

Since being described over one hundred years ago by Sir Charles Sherrington (Flynn et al., 2011), the propriospinal system has been shown to be important for normal spinal cord physiology as well as functional recovery after SCI in all mammals. However, the relative contribution of the propriospinal system to functional recovery in man can only be speculated at this stage. Studies in animal models of SCI provide compelling evidence that propriospinal neurons are the most promising targets for therapeutic interventions after SCI compared to neurons originated from other CNS regions such as

in the sensorimotor cortex and the red nucleus. Nevertheless, several important questions remain. For example, in severe SCI particularly in complete SCI, can regenerated propriospinal axons reestablish synaptic connections with distal spinal neurons? Can regenerated propriospinal neurons receive supraspinal innervation? Can propriospinal regeneration serve as a functional relay for motor and sensory recovery? What are the intrinsic mechanisms underlying regenerative response of propriospinal neurons to axotomy? Can propriospinal regeneration be further enhanced via different combinatorial strategies such as activity-dependent synaptic reorganization? Do different phenotypes of propriospinal neurons have different regenerative capacity? Do propriospinal neurons in different species have different capacity for regeneration? Since SC transplantation has been approved by FDA for clinical trials (Guest et al., 2013), addressing these questions would further facilitate the translation of SC transplantation to clinical treatment of SCI. To promote a complete functional regeneration, several critical steps have been proposed which may include (i) enhancing the intrinsic regenerative capacity of injured neurons, (ii) manipulating the interaction between the grafted SCs and host astrocytes making the graft-host interface more permissive for axon growth, and (iii) reducing or removing inhibitory molecules associated with the glial scar. These strategies may enhance the permissivity of the off-ramp as well as the on-ramp for axon growth. It will be very exciting to explore additional combinatory strategies to recruit the propriospinal system on the backbone of SC transplantation to target functional regeneration following SCI.

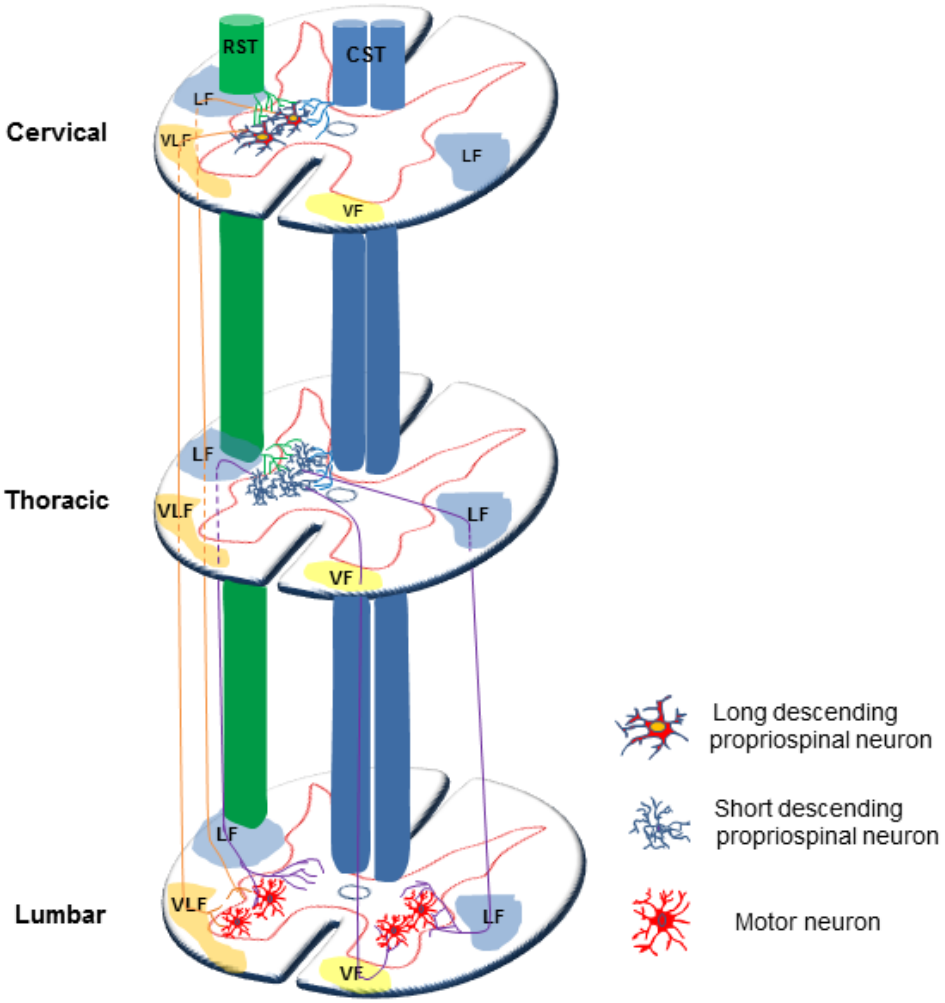



**Figure 1. Schematic diagram of descending propriospinal system.** Short propriospinal pathways interconnecting several neighboring segments are located both in the lateral and ventral funicula. The sPSTs with cell bodies medially located in the grey matter often project contralaterally, while the sPSTs with cell bodies laterally located project ipsilaterally. The sPSTs can project bidirectionally. The sPSTs originating in neurons within the ventromedial grey matter (lamina VIII and the medial portion of lamina VII), innervate medial motoneurons pools. Correspondingly, the soma of sPSTs located in lateral regions (lateral parts of laminae VII) innervate the lateral motoneuron pools. lPSTs that are involved in locomotor activity reciprocally connect cervical and lumbar enlargements and are concentrated in the ventral quadrants. Propriospinal neurons receive convergent supraspinal innervations including those from the corticospinal (CST), rubrospinal (RST). lPSTs: long propriospinal tracts; sPSTs: short propriospinal cord tracts; CST: corticospinal tract; RST: rubrospinal tract; LF: lateral funicula; VLF: ventral lateral funicula; VF: ventral funicula;.

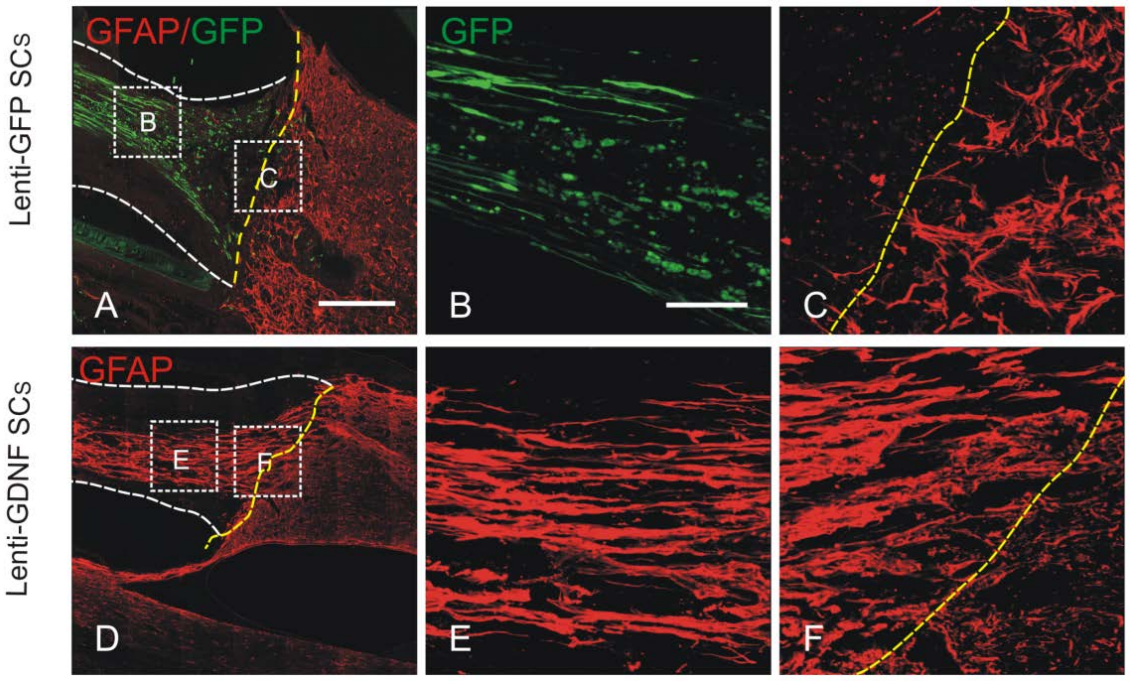



**Figure 2. GDNF induced migration of host astrocytes into the Schwann cell (SC) grafts.** Images shown in (A-F) are representative photomicrographs of the caudal graft-host interface from grafts that contained 1) lenti-GFP SCs, 2) lenti-GDNF SCs. (C-E) In the Lenti-GFP SC graft, a dense meshwork of hypertrophic astrocytes, labeled with GFAP, was seen at the host side of the caudal graft-host interface (yellow dashed line). Note that host astrocytes did not migrate into the SC graft in the absence of GDNF. The survival of grafted SCs, evidenced by GFP-staining, with elongated processes extending along the axis of the graft was clearly seen. (D-F) In the lenti-GDNF SC graft, remarkably more host astrocytes migrated into the graft environment for considerable distances. (B,C) and (E,F) are high magnifications of boxed areas of the graft proper and caudal graft-host interface in A and D, respectively. Yellow dashed lines indicate the graft-host interfaces. White dash lines in (A, C, D, F) depict the graft proper. Scale bars A, D: = 400μm; B, C, E, F, = 100μm. Modified from Deng et al. (2011).

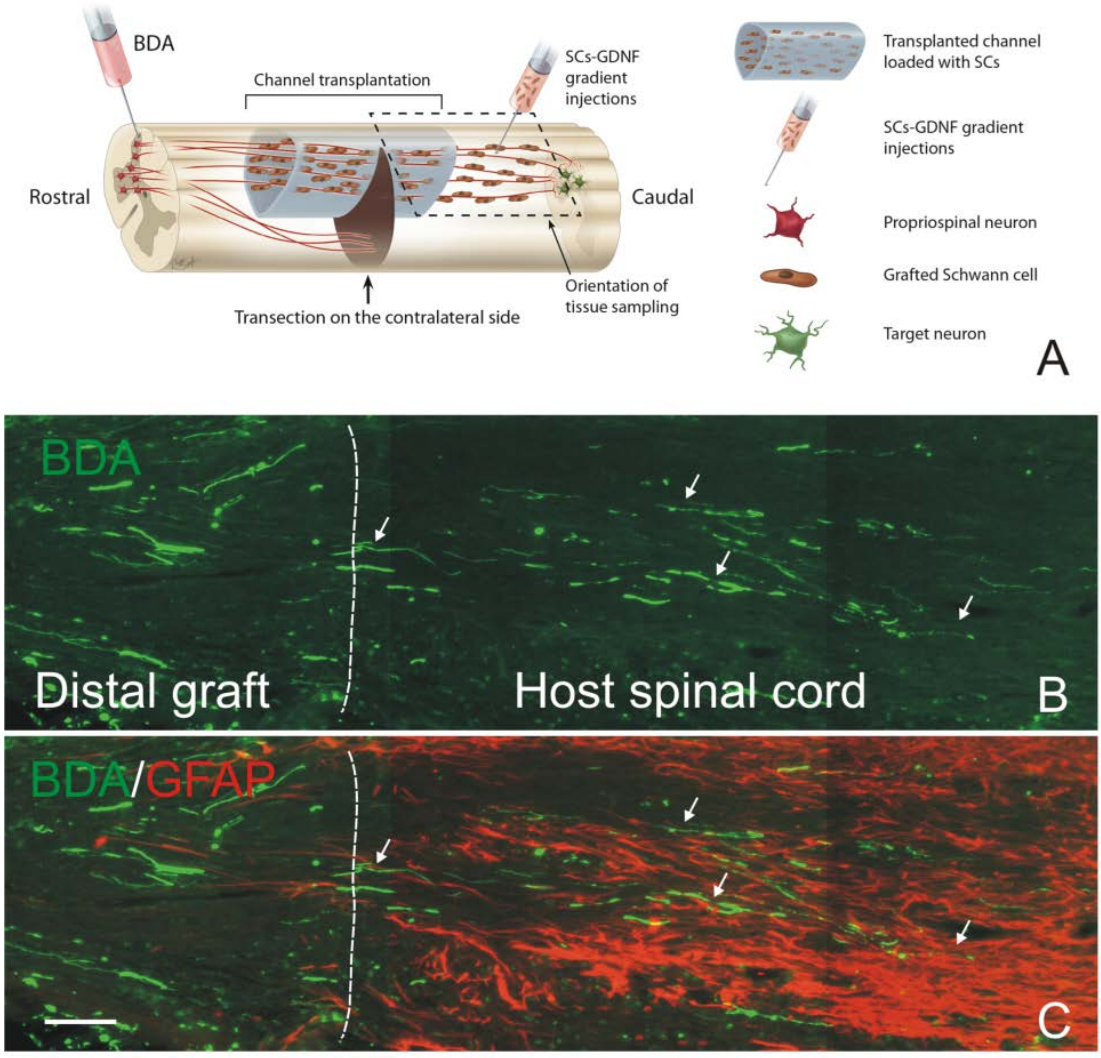



**Figure 3 Descending propriospinal axons regenerate across the caudal graft-host interface and grew back into the distal host spinal cord.** (A) Schematic drawing shows the experimental strategy and how tissue was sampled. Note that a continuous growth permissive pathway was established by graft of Schwann cell over-expressing GDNF (SCs-GDNF) both in the lesion and caudal spinal cord to promote the axonal regrowth beyond the graft-host interface. (B) BDA-anterogradely labeled propriospinal axons (green, arrows) were found to penetrate through the distal graft-host interface (white dashed line) and to elongate within the distal host spinal cord only in the group injected with SCs-GDNF into the caudal host tissue. (C) The distal graft-host interface was demarcated by GFAP-labeled astrocytes (red). B&C Scale Bar=100μm. Modified from Deng et al. (2013).

## CHAPTER 2

# FUNCTIONAL REGENERATION OF DESCENDING PROPRIOSPINAL AXONS THROUGH AND BEYOND A GROWTH-PROMOTING PATHWAY CONSTRUCTED BY TRANSPLANTATION OF SCHWANN CELLSS OVEREXPRESSING GDNF AFTER A THORACIC SPINAL CORD TRANSECTION IN ADULT RATS

**Hypotheses:** 1) Transplantation of SCs overexpressing GDNF into a transection lesion gap and caudal to it provides a continuous axonal growth pathway that leads to the regeneration of descending propriospinal axons across the lesion gap to reenter the caudal spinal cord and to form new synapses. 2) successfuldPSN axonal regeneration will result in the motor functional recovery.

### Introduction

After spinal cord injury, it is hard to achieve functional recovery. Mature CNS axons fail to regenerate spontaneously in adult mammals (Liu et al., 2008; Sivasankaran et al., 2004). Lesion cavity surrounded by glia scar and myelin debris in the extracellular matrix form physical and chemical barriers that prevent axonal growth (Silver and Miller, 2004; Yiu and He, 2006). Combinatorial strategies have been tested to target multiple inhibitory mechanisms following SCI (Bunge, 2008; Fortun et al., 2009; Olson, 2013). The most popular strategies tested so far include providing growth factors or guidance cues for axotomized axons; digesting the glial scar; and stimulating intrinsic regenerating capacity directly on neuronal soma with various degree of success.

Neurons in the CNS showed different capacity for regeneration after variety of treatments (Chau et al., 2004; Deng et al., 2013; Fouad et al., 2005; Kanno et al., 2014;

Lee et al., 2013; Lu et al., 2012; Pearse et al., 2004; Xu et al., 1995a). Schwann cell transplantation is one of the most promising cell-based strategies for SCI repair. Several CNS pathways can regenerate their axons into the SC transplant (Deng et al., 2013; Fouad et al., 2005; Williams et al., 2015; Xu et al., 1995b; Xu et al., 1997; Xu et al., 1999). However, critical limitations still exist which seriously retard the application of SC transplantation. One of the main challenges of SC-mediated strategy is to promote continuous growth of regenerated axons through the lesion gap into the host spinal cord so that these axons can make synaptic contacts with target neurons in the distal host spinal cord to reestablish neural circuitry between the damaged spinal cords. This is the key to enable the signals from the brain to the spinal cord caudal to the injury and subsequently enable the signals to get to their appropriate targets.

One of the reasons that axons find it difficult to cross the implant/spinal cord interfaces is the reaction of astrocytes and their production of some inhibitory molecules which usually surrounded the grafted SCs and impede their migration (Plant et al., 2001). The other reason is that graft environment is more favorable to the regenerated axons than surrounding host environment which also trapped the regenerated axons from further growth (Jones et al., 2001). Different efforts have been tried to either overcome the glia barrier in the graft-host interface (Chau et al., 2004; Fouad et al., 2005; Lee et al., 2013) or provide the extra growth promoting signal in host environment to facilitate the axonal growth back to the host cord (Blits et al., 2003; Lu et al., 2004; Taylor et al., 2006; Tom and Houle, 2008). Previously, we found that GDNF can not only directly promote axonal regeneration but also affect the interaction between grafted Schwann cells and host reactive astrocytes so that astrocytes formed less proteoglycans and become less inhibitory and more permissive to axon growth (Deng et al., 2011). Therefore, with GDNF, we can overcome different barriers which retard axonal regeneration.

Previously, in a model of partial SCI, we constructed an axonal growth-promoting pathway by grafting SCs-GDNF from lesion cavity extending to the caudal spinal cord. Axons from DPSN were found to grow through and beyond the implant and make synaptic connections with the distal host neuronal soma or dendrites (Deng et al., 2013). Although axonal regeneration of DPSNs and behaviorial improvement have been found in this model, it may face with much harder scenario in a complete spinal cord transection injury as other strategies do (Bunge, 2008; Levi et al., 2002; Steward et al., 2006). The model of spinal cord transection injury is not usually chosen in SCI research due to the extreme difficulty to overcome the large lesion gap caused by significant retraction of broken stumps, unstable graft/host interface, strong glial scar and severe neuronal damage. Spared axons are not available to contribute to the plasticity and recovery of function that can occur after partial injuries (Bareyre et al., 2004; Courtine et al., 2008). However, it is the gold standard to prove the efficacy of a strategy on true axonal regeneration across the lesion gap (Tuszynski and Steward, 2012). Furthermore, the majority of human injuries are severe in extent ("ASIA A") which corresponds to a complete and permanent loss of function below the lesion (Fawcett et al., 2007). Therefore, the model of complete transection spinal cord injury is highly clinical relevant. It is necessary to test any promising strategy in this most severe model. We therefore extended our successful SCs-GDNF transplantation strategy from a spinal cord hemisection model to a spinal complete transection model in adult rats.

## Materials and Methods

### Generation of purified population of Schwann cells (SCs)

SCs were purified as described previously (Deng et al., 2013). Purified SCs (purity >98%) at the third or fourth passage were collected for *in vitro* viral infection and transplantation.

**Transduction of SCs *in vitro***

SCs were pre-treated with 4-6 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) for 30-60 min, infected by lentiviruses expressing either green fluorescence protein (SCs-GFP) or GDNF (SCs-GDNF) At multiplicity of infection (MOI) of 4, resulting in about 50% infection of cells (Abdellatif et al., 2006; Deng et al., 2011).

**Animal groups and exclusion criteria**

An initial set of 51 adult female Sprague Dawley (SD) rats (180-200g, Harlan, Indianapolis, IN) were randomly divided into 3 groups (n=17). To ensure that the spinal cords were completely transected, the following highly conservative exclusion criteria were used: (1) incomplete interruption of GFAP-labeled astrocytes in the spinal cord examined in a set of sections spaced 100 μm apart; (2) too close a distance between the BDA tracer injection site and the lesion (<3 mm); (3) the animals did not survive all three major surgeries (spinal transection, cell transplantation, and tracer injection). Using these criteria, 5 rats in the control group that received caudal injection of vehicle were excluded (3 died after surgery and 2 were observed to have BDA leakage, final n=12); 3 rats in the group that received caudal injection of SCs-GFP were excluded (2 died after surgery and 1 observed BDA leakage, final n=14); 1 rat in the group that received caudal injection of SCs-GDNF was excluded (after surgery; final n=16). An additional group of 6 rats received transplantation of GFP transgenic SCs overexpressing GDNF (SCs/GFP-GDNF) into the lesion cavity and SCs-GDNF into the caudal host cord. The purpose of adding this group is to display the survival of grafted SCs and their interaction with the host astrocytes. Another group of 6 rats received graft of GFP transgenic SCs only (SCs/GFP) into the lesion cavity as a control for the SCs/GFP-GDNF group to determine whether SCs, combined with or without GDNF, had any effect on modifying host

astrocyte gliosis in this model. To keep all researchers blinded to the treatment groups during behavioral assessments and surgeries, we established a standard practice of coding all animals with numbers that are randomized and not reflective of treatment groups. Information about the type of treatment is separated from the coded numbers immediately following treatment and is not present during these procedures (Deng et al., 2013).

**Thoracic spinal cord transection and cell transplantation**

Female Sprague Dawley (SD) rats were anesthetized with an intraperitoneal injection of a ketamine (40-95 mg/kg)/xylazine (5-10 mg/kg) cocktail. All surgical procedures were performed under sterile conditions and all animal care was provided in accordance with the guidelines provided by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Lacrilube ophthalmic ointment (Allergan Pharmaceuticals, Irvine, CA) was applied to the eyes and the antibiotics Bicillin (C-R, 20,000 U, A. J. Buck, Inc., Owings Mills, MD) and triple antibiotic ointment Bacitracin (400 units/gram), Neomycin 3.5 mg/gram, Polymyxin B Sulfate (5000 units/g) were administered to surgical wound. The procedures for spinal cord transection and cell transplantation were described in previous publications (Deng et al., 2013). After a multilevel laminectomy was performed to expose T10-12 cord segments, the dura was incised longitudinally and the spinal cord was transected at T11. After transection, the rostral and caudal spinal stumps were retracted to leave a gap of 2-3 mm. The completeness of injury was judged by seeing the interior surface of the vertebral bodies. The dura and muscle were sutured. One week after injury, the spinal cord was re-exposed. Injections of 6 µl fibrinogen solution containing SCs-GDNF ($1x10^5$cell/µ) were made into the lesion cavity in all groups, then 6 µl thrombin solution was injected into the

same site to solidify the cellular bridge. For different groups, DMEM, SCs-GFP or SCs-GDNF was bilaterally injected into the caudal host spinal cord. The injections were made at 0.5 and 1.0mm caudal to the edge of lesion and at 0.5mm lateral to the midline and 1.0mm ventral to the dorsal surface of the spinal cord. At each injection point, 1.0 μl of DMEM, SCs-GFP, or SCs-GDNF (cell density: $1.0x10^5$ cells/μl) was injected.

**Behavior assessments**

Basso, Beattie and Bresnahan (BBB) locomotor rating scale was performed one week before the surgery and every week after SCI for up to nine weeks after transplantation (ten weeks after injury)(Deng et al., 2013).

**Anterograde tracing**

On the $10^{th}$ week after SC transplantation, bilateral and stereotaxical injections of biotinylated-dextran amine (BDA; 10%, 1μl/site; Molecular Probes) were made into the intermediate gray matter of the cord at distances from 3 to 6 mm rostral to the graft (for BDA, 1 injection/site/mm; total 3 injections/side) according to a previously published work (Deng et al., 2013).

**Immunohistochemistry**

Polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:100, Millipore Bioscience Research Re-agents), monoclonal mouse anti-synaptophysin antibody (1:1000; Millipore Bioscience Research Reagents), mouse monoclonal anti-serotonin antibody (1:200, abcam, Cambridge, MA, USA) and rabbit polyclonal anti-tyrosine hydroxylase antibody (1:200, abcam, Cambridge, MA, USA) were used to identify astrocytes, presynaptic components, regenerated serotonin and tyrosine hydroxylase

positive axons, respectively, were used according to an existing protocol (Deng et al., 2013).

**Electron microscopy**

Briefly, rats were perfused with 2% paraformaldehyde and 1% glutaraldehyde followed by 5% sucrose in phosphate buffer. Sections underwent a freeze–thaw procedure, incubated in avidinbiotin-peroxidase complex (1:50; Vector Laboratories) containing 2% normal goat serum in PBS, visualized with nickel-DAB/H2O2, and followed by conventional processes for transmission electron microscopy (Deng et al., 2013).

**Quantification of BDA-labeled axons**

For quantification of BDA-labeled axons, one set of serial BDA stained sagittal sections cut at a thickness of 20 μm spaced at 100 μm between adjacent sections were used for analysis. We defined the host–graft boundary by GFAP staining. We set the starting point “0” located inside the lesion which has an equal distance to the rostral and caudal boundary. The number of BDA-labeled axons on each section was counted and summed at the “0” point, caudal at graft/host interface, and at every 400 μm intervals to the caudal interface within the caudal host spinal cord. Percentage of axons which grow across the caudal graft-host boundary was calculated by equation: Percentage = the number of axons at a specific different distance to the caudal graft-host interface ÷ the number of axons appearing at the “0” point ×100%.

**Assessments of GFAP immunoreactivity in vivo**

Fluorescence intensity of GFAP immunoreactivity (IR) was measured to estimate the fold change in GFAP at the lesion border cross the groups. The GFAP-IR was used as a marker to outline the boundary between the grafted and host tissues. After outlining of

the astrogliotic region at the graft-host interfaces, the intensity of GFAP-IR in the territory 1mm caudal to interface were measured by using an Olympix digital camera attached to the Olympus BX60 microscope and analyzed using an NIH Image J software. For each animal, 20 μm serial sections at equal mediolateral distances were used for analysis. Sections from each group were processed with the same camera exposure time for fluorescence signal. The total intensity values were then averaged for each group.

**Statistical analysis**

Data are presented as mean ± standard deviation (SD). Two-way or one-way analysis of variance (ANOVA) was used to determine statistical significance followed with Tukey's post hoc analysis. All statistical values were calculated using GraphPad Prism 5.0 software (GraphPad), with a $p$ value < 0.05 considered statistically significant.

**Results**

**Axonal regeneration through and caudal to the lesion**

Strict criteria have been used to confirm true axonal regeneration in our study (Deng et al., 2013; Lu et al., 2012; Tuszynski and Steward, 2012). After spinal cord complete transection, a large lesion gap was creased with GFAP immunoreactivity (IR) clearly seen at the edge of the rostral and caudal host stumps (Fig. 5A). The regenerated axons exhibit irregular trajectories with abrupt turns (Fig. 5 G&I) rather than in tightly linear bundles typical of spared axons. Most of regenerated axons appearing in the lesion gap and caudal host cord were distributed within the territory of grafted Schwann cells overexpressing GDNF (Fig. 5). A small number of axons growing out of the graft preferred to grow into the gray matter that may be less inhibitory due to the presence of less myelin, a major contributor of myelin-inhibition mediated axonal growth-inhibition,

than the white matter. Many regenerated axons within the graft and caudal to it displayed unpredicted orientation (Fig. 5G) as compared to longitudinally oriented axons seen in the normal or sham-operated spinal cord.

**Descending propriospinal tract (dPST) axons contributed to axonal regeneration through and beyond the lesion**

Previously, we demonstrated that dPST axons regenerated through and beyond a pre-constructed SCs-GDNF growth-promoting pathway in a spinal cord hemisection model. In this model, most of the axons that regenerated into and beyond the injury were dPST neurons whose soma were located mainly in the lamina VII and VIII of the T8-10 spinal cords (Deng et al., 2013). To trace the course of regenerated propriospinal axons directly through and beyond the graft in a complete transection model, we injected an anterograde tracer biotinylated-dextran amine (BDA, 2%) into the intermediate gray matter of the rostral spinal cord at distances of 3-6 mm rostral to the graft (Fig. 5A). According to strict criteria that we established, 2 rats in the DMEM group, and 1 rat in the SCs-GFP group were excluded due to the BDA leaking into the graft. Histological evidence showed that all rats had complete transection injuries. In control groups in which either SCs-GFP or DMEM were injected into the distal host spinal cord, outgrowth was sparse and limited to within 800 μm of the graft-host interface, characterized by GFAP staining. In the DMEM group, only 12.4±2.3% of total regenerated axons (544.6±41.94) inside the graft midpoint passed across the caudal interface n = 12. In the SC-GFP group, 27.8±6.8% of the total regenerated axons inside the graft midpoint (583.6±61.4) passed across the caudal interface, n=14 (Fig. 5, 6). In contrast, in the SCs-GDNF group, significantly greater axons (61.4±6.2%) of the total axons in the graft, (611.4±54.4) penetrated though the distal graft-host interface (n=16). The longest dPST

axons that extended into the caudal cord were found to be at 3.2 mm from the distal interface (Fig. 5J, 6). Without GDNF treatment, the regenerated DPST axons usually stopped in the caudal graft-host interface although these axons can regenerate into the grafted SCs environment (Fig. 7).

**Supraspinal serotonergic axons also regenerated through and beyond a complete spinal transection**

supraspinal axonal regeneration can also benefit the motor functional recovery, we next examined whether our strategy can promote supraspinal axonal regeneration across the caudal graft/host interface. Descending brainstem tracts such as serotonergic axon, one of the major neuromodulators, normally maintains motoneuron excitability by regulating crucial persistent calcium currents (Murray et al., 2010). Restoration of 5-HT circuit can help motor neurons ready to respond to fast glutamate synaptic inputs and cause appropriate muscle contractions (Gerin et al., 2010; Hayashi et al., 2010; Hounsgaard et al., 1988). , In the group with grafts of SCs-GDNF administered both inside the lesion and caudal to it, the immunostaining demonstrated that supraspinal serotonergic (serotonin or 5-HT-IR) positive axons were found to enter the SCs-GDNF graft at the rostral host-graft interface defined by the GFAP-IR reactive astrocytes (Fig. 8 B-D). The regenerated 5-HT and TH axons also appeared in the lesion epicenter (Fig. 8 E-G). More importantly, the 5-HT supraspinal axons were also identified within the caudal host spinal cord (Fig. 8 H-M) although the number appears to be less than that of the propriospinal axons.

**Catecholaminergic axons regenerated through and beyond a complete spinal transection**

Supraspinal catecholaminergic (tyrosine hydroxylase, TH-IR) axons have been also shown to critically influence the output of the spinal central pattern generator for locomotion (Grillner and Jessell, 2009; Jordan et al., 2008; Kuscha et al., 2012). Immunostaining demonstrated that, in the group with grafts of SCs-GDNF administered both inside the lesion and caudal to it, TH axons were found to enter the SCs-GDNF graft at the rostral host-graft interface, across the lesion and grew back into the caudal host spinal cord (Fig. 9 B-M).

**Regenerated axons were myelinated and formed synapse in the caudal host spinal cord**

To determine whether regenerated axons form new synapses on target neurons in the distal host spinal cord after complete transection and construction of SCs-GDNF growth-promoting pathways, immunostaining for a presynaptic marker, synaptophysin, was performed. Colocalization of BDA-labeled axons with synaptophysin was observed (Fig. 10 A-C). To provide more solid evidence for synaptic formation, we performed immuno-electron microscopy (iEM) to identify BDA immunoreactivity at the EM level. We found that BDA-labeled regenerated axons formed presynaptic terminals with numerous synaptic vesicles, which were electron dense after immunolabeling for BDA reacted with streptavidin-horseradish (Fig. 10 D). Theses presynaptic terminals make contacts with postsynaptic components of other neurons/terminals (Fig. 10D). Importantly, BDA labeled axons were found to be remyelinated (Fig. 10 E, F) indicating that these regenerated axons might be functional in relaying transmission.

**SCs-GDNF remodeled astrocytic responses at the caudal graft-host interfaces**

GFAP is a hallmark of reactive astrogliosis. In our previous hemisection model, we found that GDNF can significantly reduce astroglial responses along the graft-host interface (Deng et al., 2011). We confirmed such a phenomenon in the transection model in the present study. The grafted SCs-GDNF significantly inhibited astrocytic glial responses as shown in the caudal graft-host interface where reduced immunofluorescence staining of GFAP was found (Fig. 11E-F, H). This attenuation of astrocytic glial response may facilitate the growth of dPST axons back into the caudal host spinal cord (Fig. 11F, I). Contrarily, in the control group where the caudal SC graft was either mixed with vehicle or expressing GFP, strong glial responses were found as compared to the SCs-GDNF group (Fig. 11B, C). Quantification data showed significant differences in the caudal GFAP intensity among the three groups (Fig. 11J; vehicle *vs* SCs-GDNF, $p < 0.001$; SCs-GFP *vs* SCs-GDNF, $p < 0.001$). In the control cases (vehicle or SCs-GFP groups) where strong astrocytic glial responses were found, axons within the graft failed to grow back into the caudal host spinal cord (Fig. 7B). The astrocytes in the caudal host side in the group received SCs-GDNF were characterized by a relatively smaller cellular soma and slim, long cellular processes and loose cellular distribution (Fig. 11E, H), different from the astrocytes in the same region of the control groups which displayed hypertrophic cellular morphology and dense cellular organization (Fig. 11B; 12B).

**SCs-GDNF promoted migration of host astrocytes into the SCs-GDNF-filled lesion gap where they are closely associated with regenerated dPST axon**

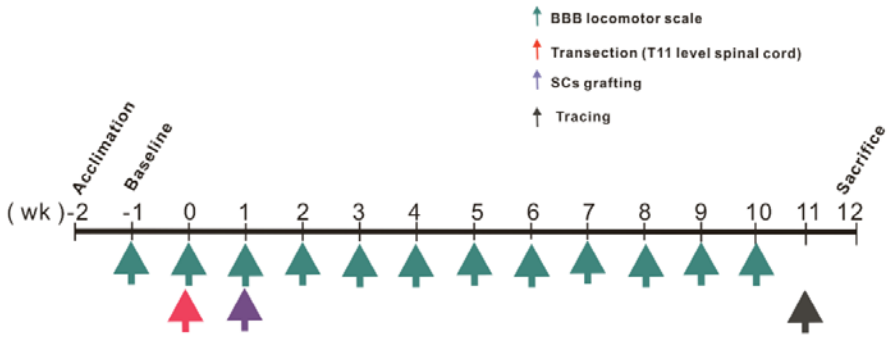
We also examined the effect of SCs-GDNF on the migratory ability of astrocyte, stained with GFAP, into the SCs-GDNF-filled lesion gap from the host side of the spinal cord. In our subgroups to determine the survival of grafted SCs in the lesion gap or caudal to it,

survival of GFP-positive SCs within the lesion gap was clearly seen and they were surrounded by a dense meshwork of reactive astrocytes (GFAP-IR) indicating that transplantation of SCs alone, without GDNF, may trigger astrocytic responses that sharply separate these two populations of glial cells at the lesion site (Fig. 13). In all groups that received SCs-GDNF, robust migration of astrocytes into the transplants was observed (Fig. 14). This migration caused the blurring graft-host interface particularly in the distal graft-host interface in cases where SCs-GDNF was grafted both within the lesion gap and caudal to it (Fig. 14). The migrated astrocytes into the SCs-GDNF transplant within the lesion cavity were characterized by elongated processes and rostral caudal orientation (Fig. 14D). Migratory astrocytes were closely associated with BDA-labeled regenerated dPST axons (Fig. 15E-G).

**The constructed SCs-GDNF growth-promoting pathway promoted improved hindlimb locomotor function**

To determine the functional efficacy of the SCs-GDNF transplantation within and caudal to the graft, Basso, Bettie, and Bresnahan (BBB) locomotor rating scale was assessed at 10 weeks post SCI or 9 weeks post-transplantation. No significant difference between the three groups that received caudal injection of the SC-GDNF, SC-GFP, or vehicle was found at or before 8 weeks post-injury (Fig. 16A). At 9 week post-SCI, the average score in the vehicle control group (n = 12) was 0.67 ± 0.93 (mean ± SD). In the group that received caudal injections of SC-GFP, the average score was 0.54 ± 1.2 (n = 14). In the group that received caudal injection of SC-GDNF, the average score was 1.91 ± 1.86 (n = 16); At the 10 week post-SCI, the average score in the vehicle control group (n = 12) was 0.33 ± 0.75 (mean ± SD). In the group that received caudal injections of SC-GFP, the average score was 0.17 ± 0.67 (n = 14). In the group that received caudal injection of SC-GDNF, the average score was 2.03 ± 1.92 (n = 16) (Fig. 16B, C). The average BBB

score of the caudal injection of the SCs-GDNF group was significantly higher than that of the other two groups ($p$<0.001). The group with SC-GFP transplants did not exhibit any significant improvements in the BBB score when compared with the vehicle control group. The results did not indicate that the recovery reached the plateau at 9 weeks after the transplantation, the time point at which testing was discontinued. There was correlation between individual functional recovery and number of regenerated BDA labeled descending propriospinal axons which grew across the caudal graft-host interface (r=0.81, $p$<0.001) (Fig. 16D).




Experimental timeline

| Group | T11 spinal cord transected lesion gap | Caudal host cord | Original rats number *(n)* | BDA leaking | Rat died | Final available number (n) | BBB data (n) | Histology data (n) |
|---|---|---|---|---|---|---|---|---|
| 1 | SC-GDNF | DMEM | 17 | 2 | 3 | 12 | 12 | 12 |
| 2 | SC-GDNF | SC-GFP | 17 | 1 | 2 | 14 | 14 | 14 |
| 3 | SC-GDNF | SC-GDNF | 17 | 0 | 1 | 16 | 16 | 16 |
| subgroup 1 | SC-GFP-GDNF | | 6 | | | | | 6 |
| subgroup 2 | SC-GFP | | 6 | | | | | 6 |

**Table 1 Summary of animal usage**

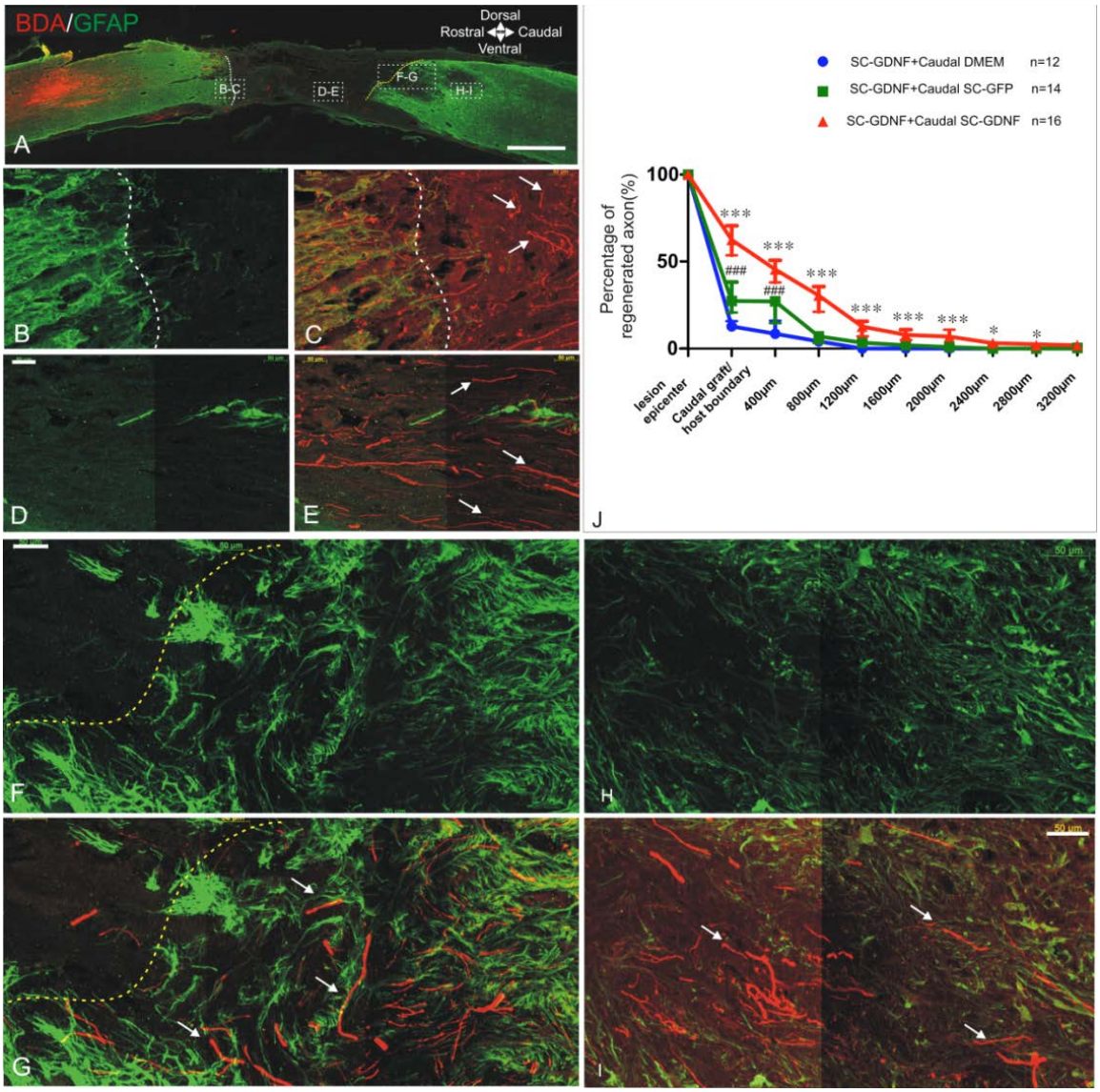

BDA/GFAP
Dorsal
Rostral
Caudal
Ventral
B-C
D-E
F-G
H-I
A
B
C
D
E
J
SC-GDNF+Caudal DMEM n=12
SC-GDNF+Caudal SC-GFP n=14
SC-GDNF+Caudal SC-GDNF n=16
Percentage of regenerated axon(%)
100
50
0
lesion epicenter
Caudal graft/ host boundary
400μm
800μm
1200μm
1600μm
2000μm
2400μm
2800μm
3200μm
F
H
G
I

**Figure 5 Descending propriospinal tract (dPST) axons regenerate into and beyond a T11 transection lesion gap after the construction of SCs-GDNF growth-promoting pathway.** (A) A montage image shows BDA-labeling (red), astrocytes (GFAP, green) in a sagittal section of a case received SCs-GDNF transplantation into the lesion gap at T11 and injection of the SCs-GDNF into the distal host spinal cord. The BDA injection site was located at 3 mm rostral to the lesion. Regions at different sites were boxed and examined at higher magnification at rostral graft-host interface (B, C), within the graft (D, E), caudal graft-host interface (F, G), and cord spinal cord proper (H, I), in the subsequent images. (B-C) At the rostral host-graft interface, regenerated DPSN axons (white arrows in C) crossed the host-graft boundary (white dash line) to grow into the graft. (D-E) Within the SCs-GDNF graft, continued growth of regenerated DPSN axons were found (white arrows in E). (F-G) At the distal grafted-host interface (yellow dash line), regenerated axons (white arrows in G) penetrated into the caudal host cord. (H-I) Within the distal host spinal cord, regenerated dPST axons (white arrows in E) were clearly seen. (J) Percentage of regenerated axons appearing in the caudal host spinal cord over those appearing within the graft mid-point at different distances from the caudal graft-host boundary among three groups. ***, $p < 0.001$, *, $p < 0.05$, SC-GDNF versus DMEM; ###, $p < 0.001$; #, $p < 0.05$, SC-GFP versus DMEM. Scale bars: A, 1000 μm; B–I, 50 μm. Animal number: Caudal injection of DMEM group, n=12; Caudal injection of the SC-GFP group, n=14; Caudal injection of the SC-GDNF group, n=16. Bar heights represent means ±SD.

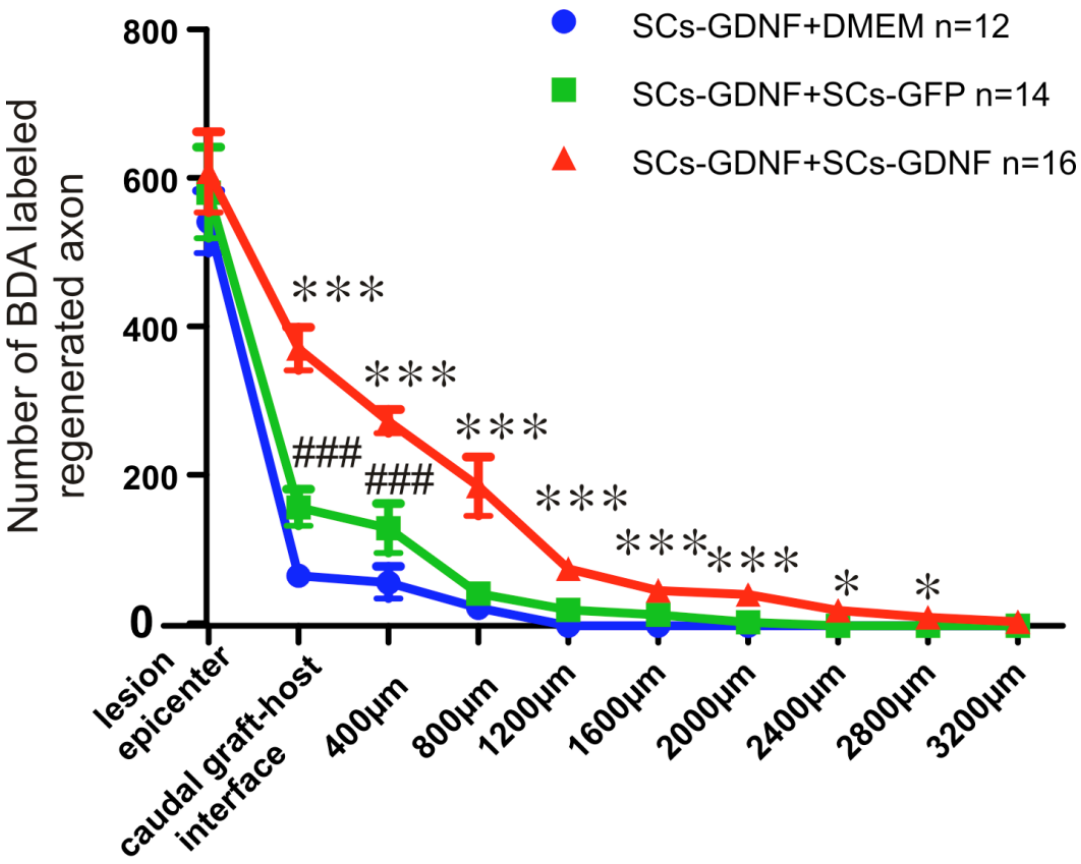



**Figure 6. The number of regenerated dPST axons that regenerated into the caudal host cord at different distances from the caudal graft-host interface.** ***, $p < 0.001$, *, $p < 0.05$, SC-GDNF (red) versus DMEM (blue); ###, $p < 0.001$; #, $p < 0.05$, SC-GFP (green) versus DMEM (blue). Bar heights represent means ±SD.

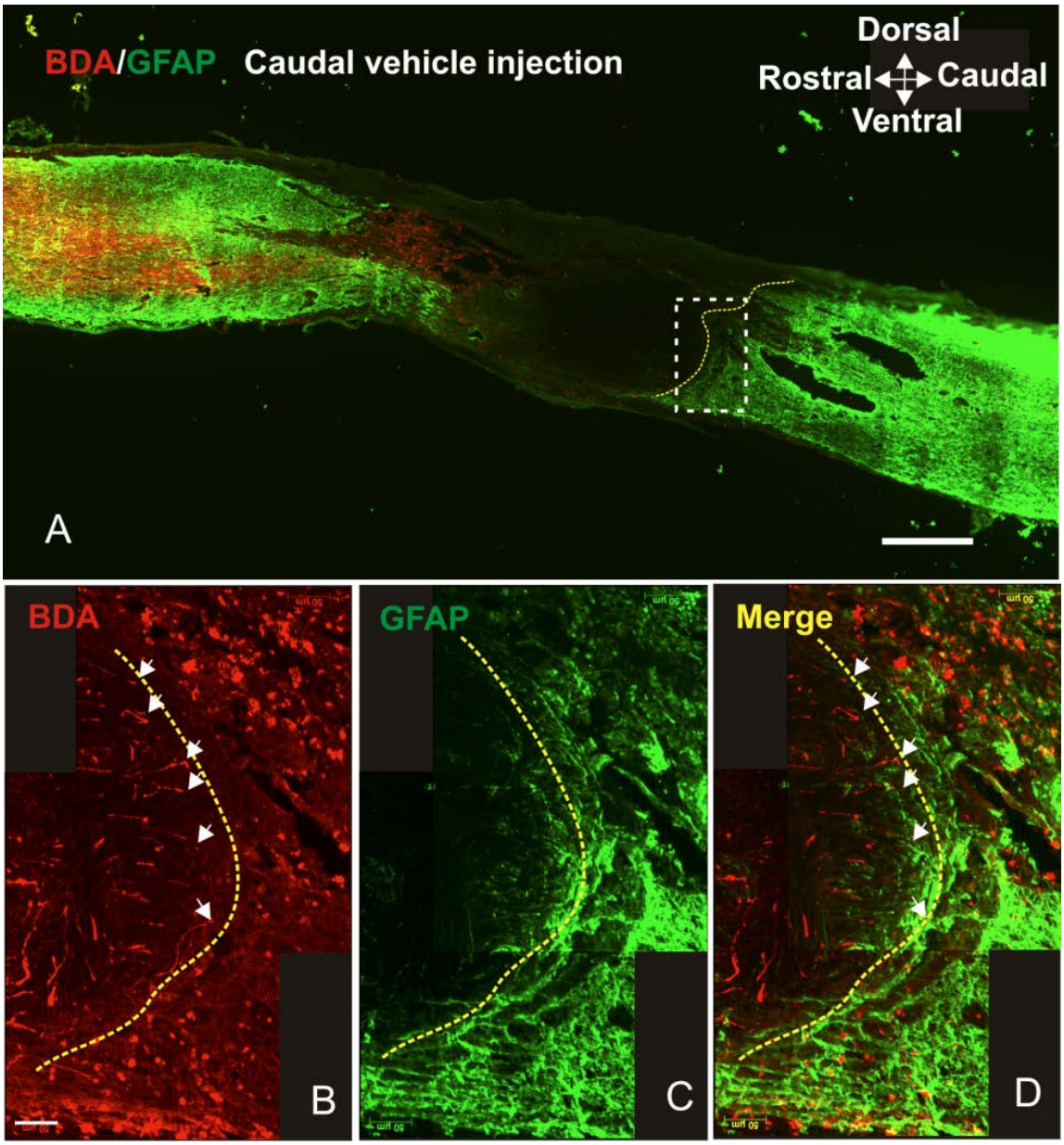



**Figure 7. Descending propriospinal tract (dPST) axons regenerated into the SCs-GDNF graft but stopped at the caudal graft-host interface in the absence of SCs-GDNF in the distal host spinal cord.** (A) A montage image of a sagittally-cut spinal cord showing the lesion site stained with BDA for regenerated dPST axons (red), and GFAP for astrocytes (green). (B-D) High magnification of the boxed area in A at the caudal graft-host interface shows that regenerated dPST axons (white arrows, B and D) failed to growth into the distal host spinal cord defined by a reactive astrocyte border (yellow dashed line, B-D).

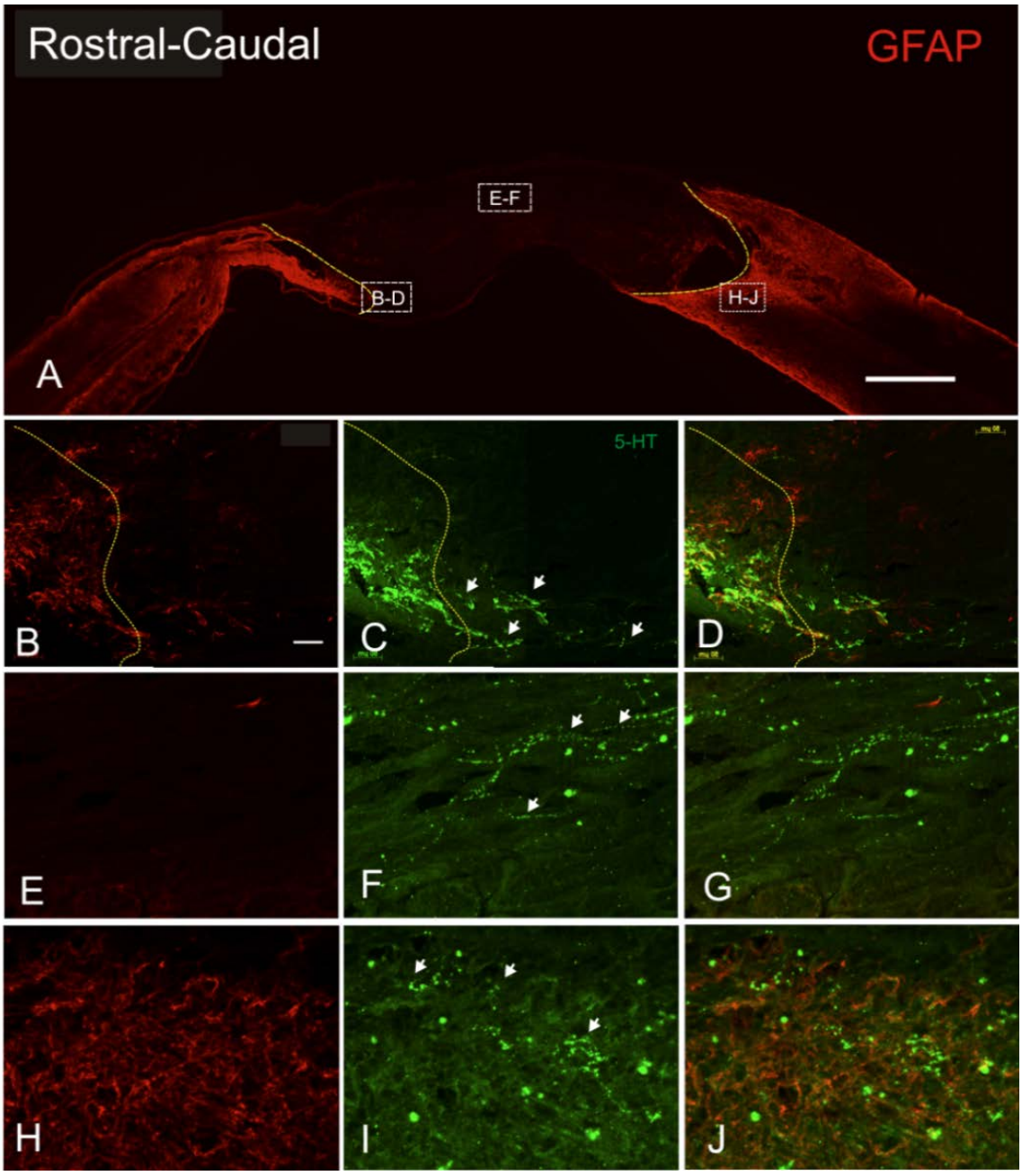

Rostral-Caudal
GFAP
E-F
B-D
H-J
A
5-HT
B
C
D
E
F
G
H
I
J

**Figure 8. Descending serotonergic axons regenerated into and beyond a spinal cord transection when a continuous SCs-GDNF growth-promoting pathway was provided within and caudal to a spinal cord transection.** (A) A montage image of a sagittally-cut section shows the lesion gap defined by GFAP-stained astrocytic glial border at both rostral and caudal ends of the host spinal cord (dashed lines). Boxed areas indicate regions of interests at the rostral graft-host interface (B-D), within the graft (E-G), and caudal graft-host interface (H-J), as shown in high magnification of the subsequent corresponding images. Regenerated serotonergic fibers ($5\text{-}HT^{+}$) were found to grow from the rostral spinal cord into the SCs-GDNF graft (B-D, arrows), within the graft (E-G, arrows) and into the distal host spinal cord (H-J, arrows). Scale bars: (A) 1000μm; (B–M) 50μm.

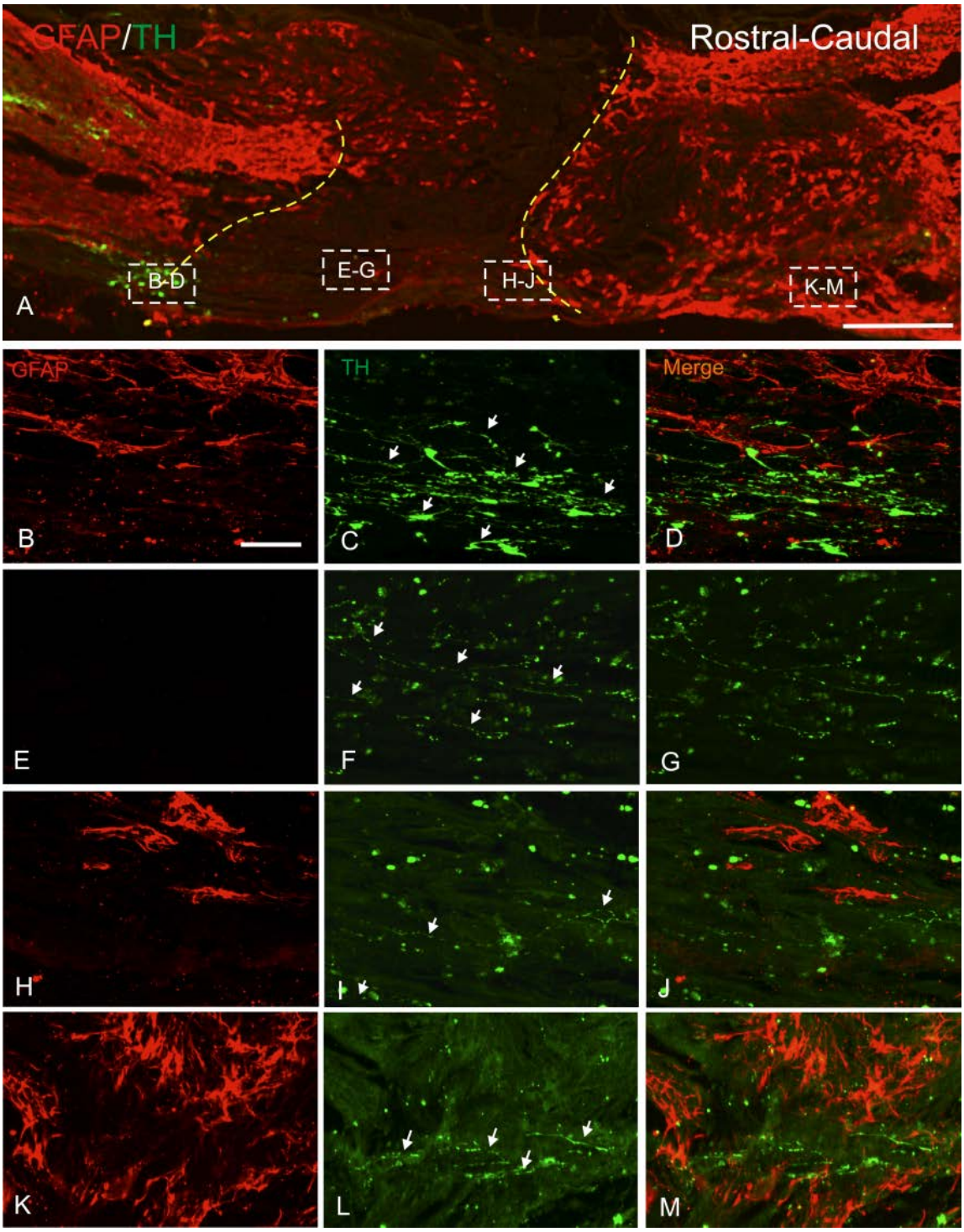

GFAP/TH
Rostral-Caudal
B-D
E-G
H-J
K-M
A
GFAP
TH
Merge
B
C
D
E
F
G
H
I
J
K
L
M

**Figure 9. Descending catecholaminergic axons regenerated into and beyond a spinal cord transection when a continuous SCs-GDNF growth-promoting pathway was provided within and caudal to a spinal cord transection.** (A) A montage image of a sagittally-cut spinal section shows the lesion gap defined by GFAP-stained astrocytic glial border at both rostral and caudal ends of the host spinal cord (dashed lines). Boxed areas indicate regions of interests at the rostral graft-host interface (B-D), within the graft (E-G), caudal graft-host interface (H-J) and the caudal spinal cord proper (K-M), as shown in high magnification of the subsequent corresponding images. Regenerated catecholaminergic fibers ($TH^+$) were found to grow from the rostral spinal cord into the SCs-GDNF graft (B-D, arrows), within the graft (E-G, arrows), and into the distal host spinal cord (H-M, arrows). Scale bars: (A) 1000µm; (B–M) 50µm.

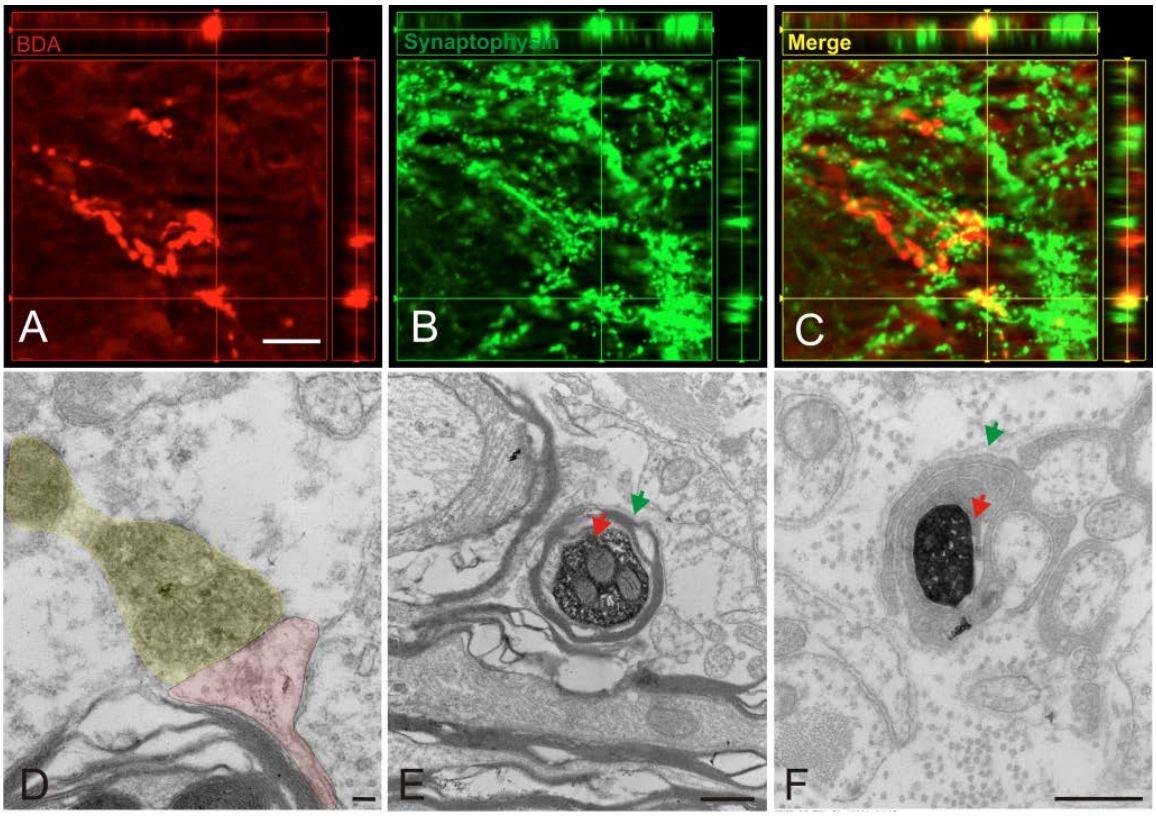
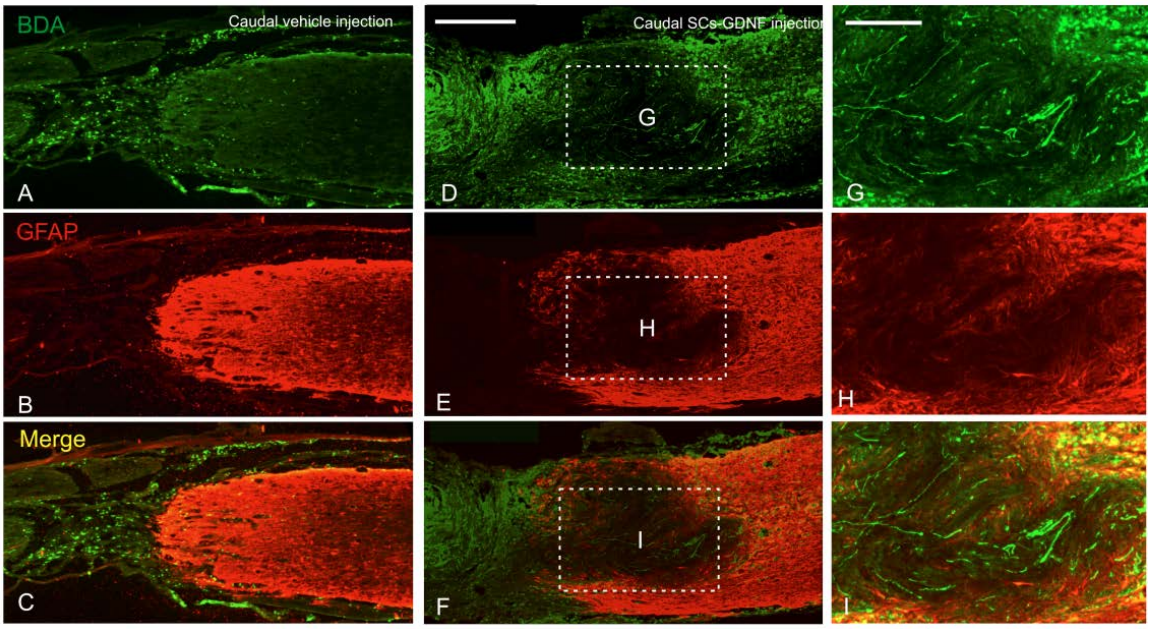



**Figure 10. Regenerated dPST axons formed synapse on host neurons and were myelinated in the distal host spinal cord when a continuous SCs-GDNF growth-promoting pathway was provided within and caudal to a spinal cord transection.** (A-C) BDA-labeled dPST axons (red) colocalize with a presynaptic marker synaptophysin (green), confirmed in xz and yz plane reconstructions. (D) Immuno-electron microscopy showed that a regenerated dPST presynaptic terminal, containing dark reaction product of BDA (yellow contour) formed a synapse with a host dendritic spine (pink contour). (E, F) Immuno-electron microscopy also showed that regenerated BDA-containing dPST axons (red arrow) were being myelinated (green arrows). Scale bars: (A-C) 20 µm; (D)100 nm; (E, F) 500 nm.


BDA
Caudal vehicle injection
A
Caudal SCs-GDNF injection
G
D
G
GFAP
B
H
E
H
Merge
C
I
F
I

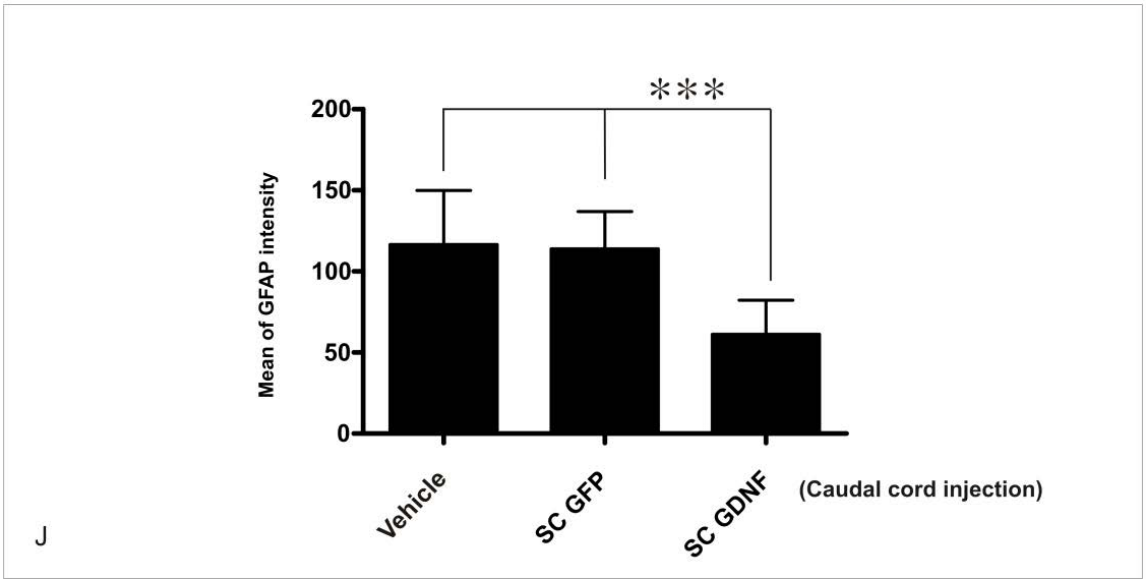

***
200
150
100
50
0
Mean of GFAP intensity
Vehicle
SC GFP
SC GDNF
(Caudal cord injection)
J

**Figure 11. SCs-GDNF grafted within and caudal to a spinal transection modified astroglial responses at the caudal graft-host interface.** Representative photomicrographs of GFAP expression in the caudal graft-host interface in a case that received DMEM (A-C) or a case that received SCs-GDNF (D-F) injections into the caudal host spinal cord. In the DMEM-injected case, increased expression of GFAP (B, C) was found at the distal graft-host interface. In contrast, in the SCs-GDNF-injected case, the expression of GFAP was considerably reduced (E, F). Such reduction was correlated with significant regeneration of dPST axons into the caudal host spinal cord (D, F). High magnification of boxed area in D-F were further appreciated in G-I colocalization of regenerated axons (BDA, red) (J) Quantitative analyses show that the differences in GFAP expression between the SCs-GDNF and other two groups are statistically significant (*: $p < 0.05$). Scale bar: A-F 500 μm; G-I 250 μm

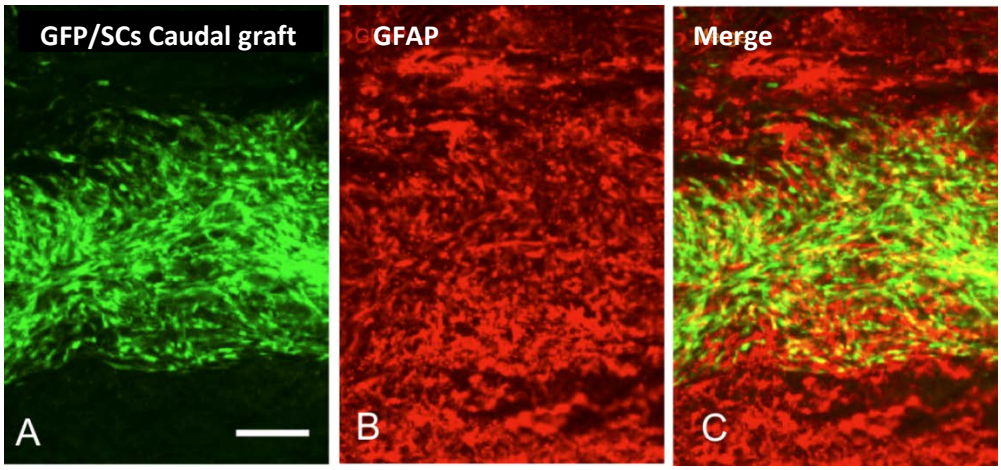



**Figure 12. Schwann cells expressing GFP (SCs-GFP) injected into the distal host spinal cord survived amongst host astrocytes.** (A) presence of SCs-GFP in the distal host spinal cord (green). (B) Host astrocytes (GFAP-IR, red), (C) Merge image indicated that SCs-GFP had no effect on the astrocytic expression of GFAP. Scale bar: 50μm

**Subgroup with SCs-GFP grafted in the lesion gap**

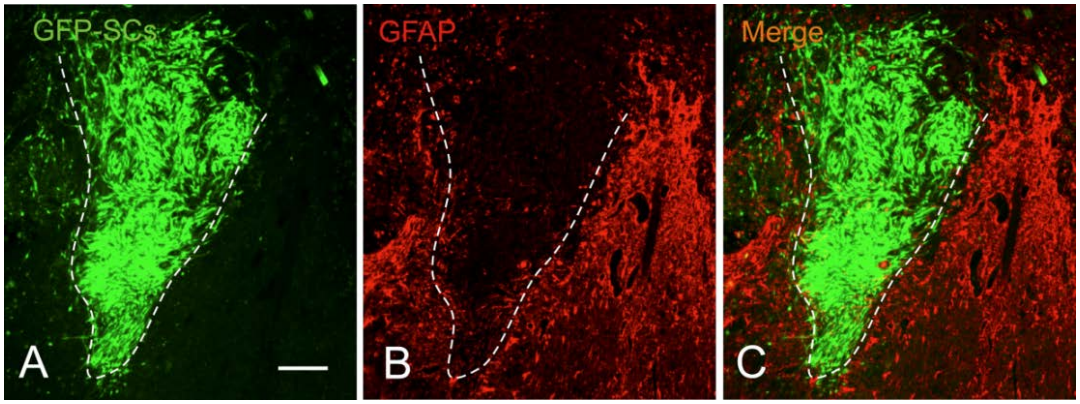



**Figure 13. In the absence of GDNF, grafted GFP expressing Schwann cells and host astrocytes were separated by a sharp boundary.** (A) Grafted SCs expressing GFP (SCs-GFP, green). (B) Host astrocytes (GFAP-IR, red), (C) Merged image. Scale bar: 50μm

**Subgroup with SCs-GFP-GDNF grafted in the lesion gap**

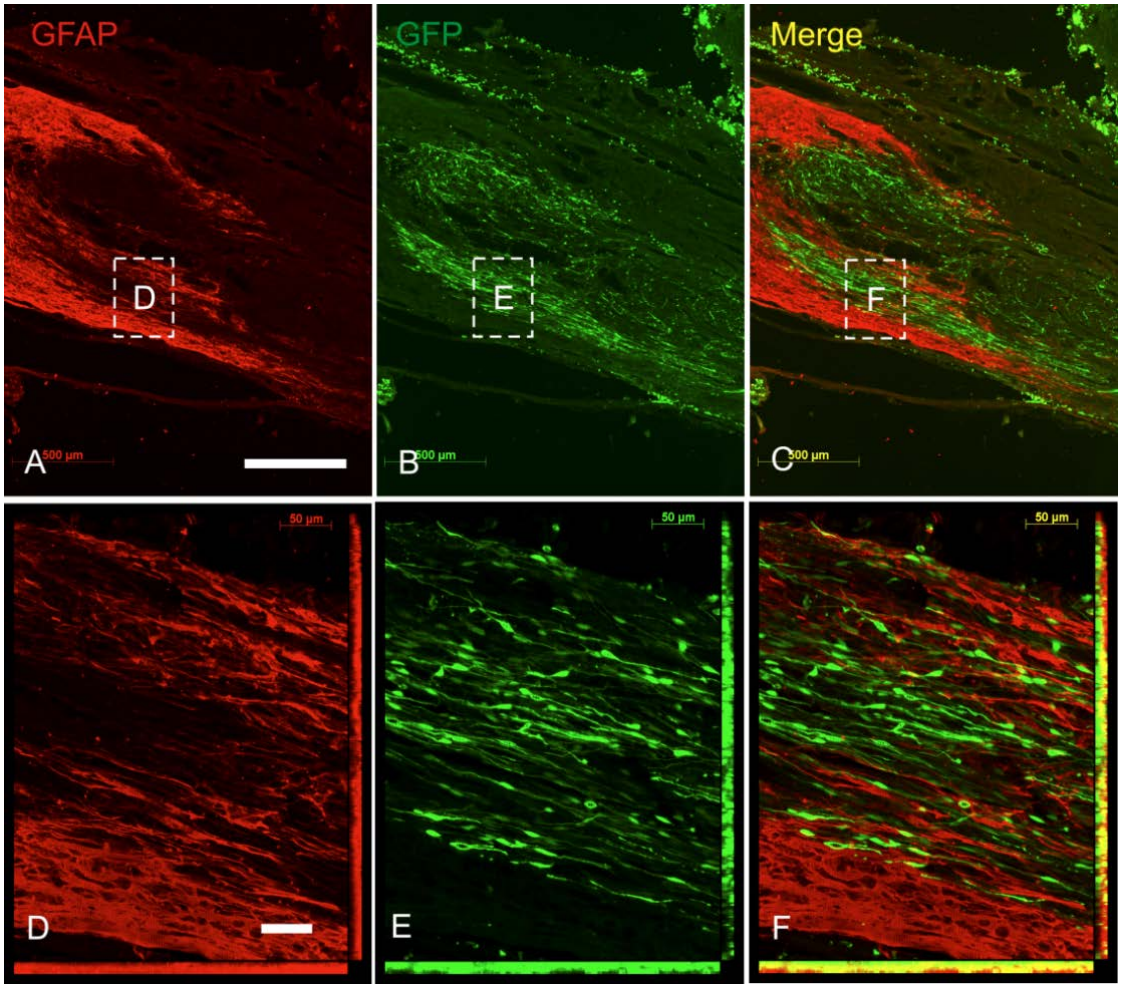



**Figure 14. GDNF, expressed by SCs-GFP, promoted bidirectional migration of astrocytes into the graft and SCs into the host.** (A-C) low magnification shows the bidirectional migration of astrocytes and SCs towards each other at the rostral graft-host interface. (D-F) High magnification of boxed area in A-C show the intermingling of astrocyte and SCs, creating a blurring boundary. Scale bar: (A-C) 500µm; (D-F) 50µm.

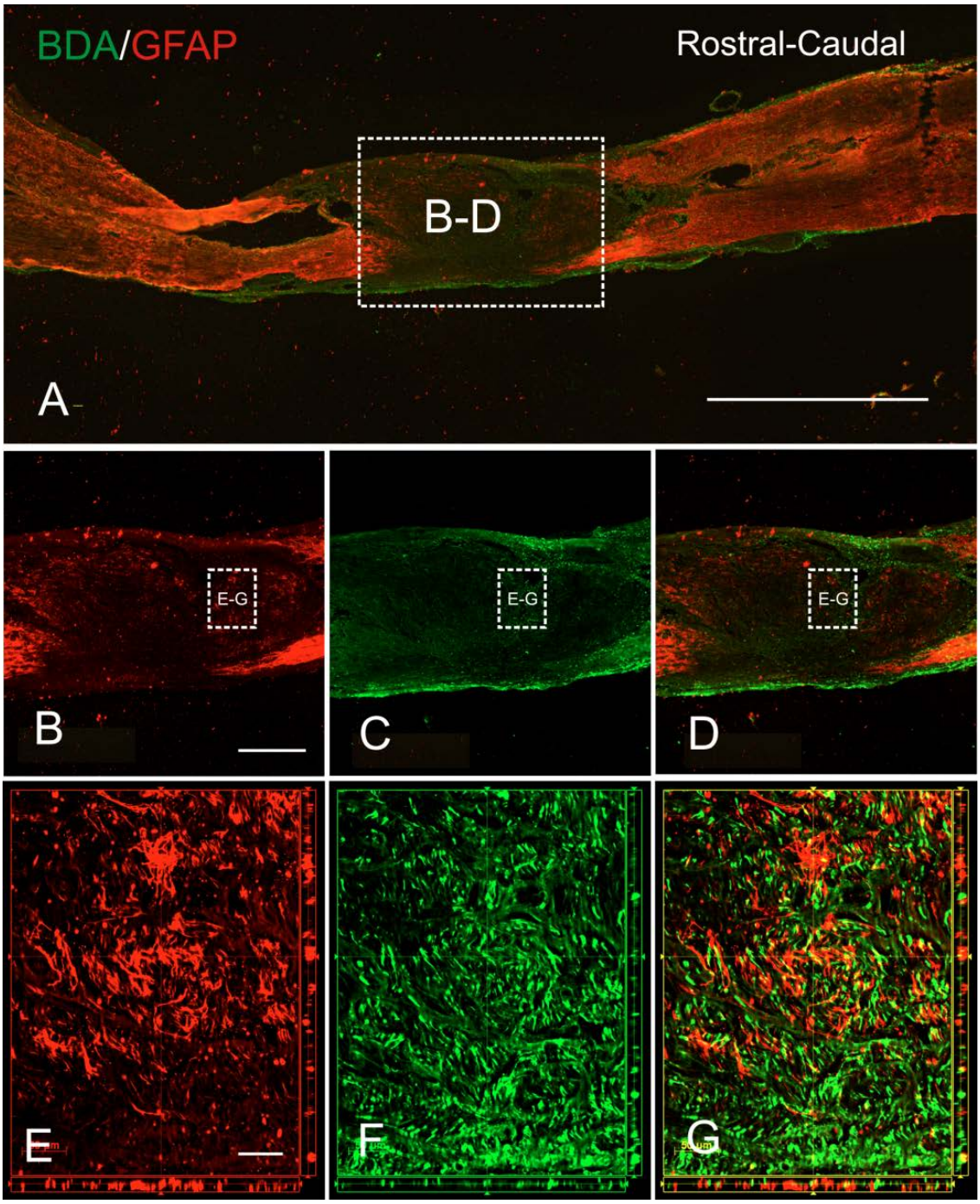



**Figure 15. SCs-GDNF induced parallel alignment between migratory astrocytes and regenerated axons.** (A-D) Low magnification images showed migration of host astrocytes (GFAP IR red) into the SCs-GDNF graft. (E-G) At high magnification, a significant amount of regenerated descending propriospinal tract (dPST) axons (F, BDA-labeled, green) were shown in close association with the host astrocytes migrated into the graft region (E, GFAP-IR, red) in XY, XZ and YZ planes. Scale bar: (A) 1000 μm; (B-D) 100 μm ; (E-G) 50 μm.

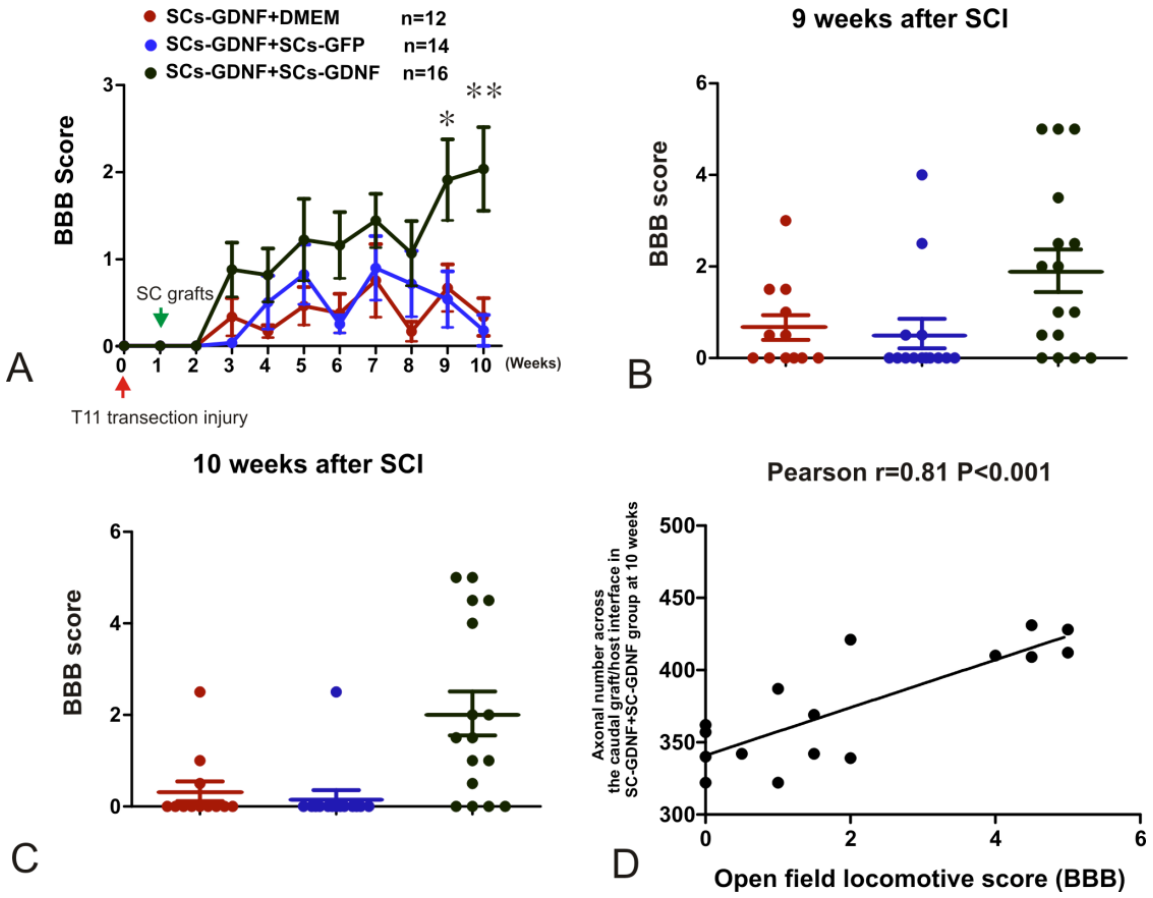



**Figure 16 Partial recovery of hindlimb motor function after axonal regeneration through and beyond a continuous SCs-GDNF growth-promoting pathway established after a complete spinal transetion.** (A) Improved BBB locomotor recovery was found in the group that received intraspinal transplantation and caudal injection of SCs-GDNF (green), compared with caudal injection of either SCs-GFP (blue) or DMEM (red) (**$p$ < 0.01, *$p$ < 0.05, SCs-GDNF vs SCs-GFP or vs DMEM). (B, C) Scatter plot showing the BBB score of different groups at the 9th (B) and 10th (C) week after spinal transection. (D) Correlation between the number of regenerating axons across the caudal boundary and the open field locomotor score. Data displayed as means ±SD.

## Discussion

### A continuous growth-promoting pathway of SCs-GDNF promoted regeneration of specific descending propriospinal and supraspinal axons through and beyond a spinal cord transection

At the level of the thoracic spinal cord, without any exogenous growth factors, only a small number of sensory and propriospinal axons regenerated into either SCs or PN grafts environment in transection injury (Cote et al., 2011; Xu et al., 1995b). SCs do not appear to provide the necessary (or sufficient) signals to promote regeneration of axons from the brainstem (Kamada et al., 2005; Kamada et al., 2011; Xu et al., 1997). Given that brainstem axons can enter the PN grafts placed adjacent to neuronal cell bodies, it seems unlikely that brainstem spinal axons are incapable of responding to SC cues. The distance between injured axonal end and neuronal soma matters here (Bunge and Pearse, 2003). To promote regeneration of long descending central nerve axons, stronger signal should be conveyed from the broken axonal stump to neuronal soma to awake the regenerative capacity (Bamber et al., 2001).

Neurotrophic factors are strong candidates for combination therapies, given their known ability to promote neuronal survival, axonal regeneration/sprouting and neuroprotection, and SC differentiation, and to enable growth of neurites on inhibitory substrates (Cai et al., 1999; Chan et al., 2001; Sharma, 2007). The response of different neuronal tracts to neurotrophic factors depends on the repertoire of receptors expressed as well as the signals elicited by these factors (Kalb, 2005). Among the most studied neurotrophic factors in the field of SCI repair are neurotrophin 3 (NT3), nerve growth factor (NGF), glia-derived neurotrophic factor (GDNF), α-Fibroblast growth factor (α-FGF) and brain-derived neurotrophic factor (BDNF), all of which exert specific (and sometimes

overlapping) effects on different populations of cells (Blesch and Tuszynski, 2001; Iannotti et al., 2003; Lee et al., 2013; Tuszynski et al., 2003; Vavrek et al., 2006; Xu et al., 1995a; Zhang et al., 2009). Regarding the motor functional recovery, corticospinal tract (CST), raphespinal tract (5-HT), rubrospinal tract and coeruleospinal tract (TH), are the chief organizers of motor function and have been widely studied (Lee et al., 2013; Pearse et al., 2004; Raineteau et al., 2002). The regeneration of these nerve fibers is important for hindlimb functional recovery (Shapovalov, 1975). dPST and their neurons (dPSN) have recently been implicated in mediating plasticity under incomplete SCI (Courtine et al., 2008). Previous studies indicate that different axonal tracts responded to different neurotrophic factors. GDNF protein promoted sensory and propriospinal axons regeneration in the model of partial injury (Deng et al., 2013; Iannotti et al., 2003). However, some supraspinal tracts such as coeruleospinal tracts indeed also express GDNF receptors which endow these tracts the potency to respond to the GDNF stimulation (Burazin and Gundlach, 1999; Trupp et al., 1997). In the current work, lentiviral-mediated GDNF expression, released from grafted SCs, may elicit much stronger signaling in these specific brain stem neurons for their regeneration. Long term caudal distribution of high concentration of GDNF provided stimulating signals in the caudal inhibitory milieu, which consequently significantly attracted specific propriospinal and supraspinal axons to regenerate into the distal host spinal cord. Since we used antibodies to identify regeneration of transmitter-specific supraspinal descending pathways to the graft and caudal spinal cord, there exists the possibility that other supraspinal descending pathways may also contribute to the overall regeneration stimulated by the SCs-GDNF growth-promoting pathway.

**GDNF modulated the interaction between grafted SCs and host astrocytes**

A big challenge in SC transplantation strategies is the poor integration between grafted SCs and host astrocytes. Astrocytes and SCs normally reside separately in the PNS and CNS. At the peripheral nerve entry zone, astrocytes contribute to the formation of glial limitans that prevents SCs from migrating into the CNS and generates barriers to axonal regeneration after injury (Fraher, 1997; Golding et al., 1997). This segregation also occurred after transplantation of SCs into the astroglial environment in spinal cord (Baron-Van Evercooren et al., 1992; Sims et al., 1999). Exogenous SCs can aggravate the astroglial response induced by the injury. The strong glial production such as proteoglycans built up along the graft/host interface at least partially accounted for the segregation between grafted SCs and host astrocytes (Santos-Silva et al., 2007). This glial scar not only limits the mutual migration between SCs and astrocytes but also retards the axonal regeneration into the SCs environment (Fitch and Silver, 2008). Furthermore, the poor integration of SCs to the surrounding host tissue has trapped regenerated axons within the graft causing presumably non-functional regeneration (Bunge, 2008). Interestingly, we previously found GDNF can modify the glial environment in a spinal cord hemisection and bridge transplantation of SCs-GDNF model. My current work confirmed that, with GDNF treatment, the abrupt boundary often seen in SC grafts has been replaced by a zigzagged or an even unidentified interface. The large number of migratory astrocytes was observed dispersed amongst and in close association with grafted SCs in the territory full of GDNF within both the lesion and caudal graft site, which indicated that SCs and astrocytes are less repellent to each other under the influence of GDNF. Such irregular borders are more permissive for axons to grow from host astrocyte environment to the grafted SC territory as well as

back to host cord (Deng et al., 2011; Iannotti et al., 2003; Kanno et al., 2014; Lee et al., 2013; White et al., 2011; Williams et al., 2015).

**Astrocytes within the GDNF territory displayed a permissive character for axonal regeneration**

There are many clues that the permissibility of astrocytes for axonal growth is related with developmental status, which is usually reflected by their morphological pattern (Tom et al., 2004; White et al., 2008). Radial glia, the immature cells that support axonal growth during development, have an elongated, bipolar phenotype,whereas mature astrocytes exhibit multiple processes and primarily express GFAP (Vaccarino et al., 2007). In vitro, the glial-precursor derived astrocytes cultured with bone-morphogenetic protein also displayed extended processes which promote axon regeneration. The astrocytes appearing in developmental phase can create several adhesive molecules permissive for axonal regeneration (Tom et al., 2004). After CNS injury, reactive astrocytes become hypertrophic and enhance expression of GFAP and other glial components inhibitory to axon regeneration while a subpopulation of reactive astrocytes express nestin, an embryonic marker, indicating an immature status although in a temporary and minor manner (Clarke et al., 1994). Several growth factors and cytokines can significantly keep astrocytes in a morphological state characterized by slim and thinner processes, reduced GFAP expression and marked transformation to a bipolar morphology similar to a relatively undifferentiated state permissive to axonal growth (Menet et al., 2001; Santos-Silva et al., 2007; White et al., 2008; Williams et al., 2015). Early studies of complete transection with SC transplantation reported that axons sprouting short distances into the lesion in association with sporadic migratory astrocytes (Guth et al., 1981; Matthews et al., 1979). These axonal sprouts were enclosed within a basal lamina continuous from these migratory astrocytes and grafted

SCs. These tunnel-like structures are distinct from the inhibitory sheets of basal lamina found in CNS scar tissue (Williams et al., 2015). These permissive post-traumatic astrocytes may be derived from de-differentiating and/or de novo astrocytes. My studies indicate that under GDNF in situ treatment, a significantly attenuated glial response was found and accompanied by transformed astrocyte morphology especially those migratory astrocytes with bipolar distribution of slim processes and reduced GFAP expression both in the lesion gap and in caudal area of the spinal cord. These migratory astrocytes associated with transplanted SCs escorted regenerating axons across the lesion. Thus, in agreement with the de-differentiation phenomenon discussed in previous studies, we found that GDNF switches the astrocyte from an inhibitory phenotype with a stellate morphology to a growth permissive phenotype with an elongated or radial-like morphology.

**Conclusion**

Following a spinal cord complete transection and construction of a SCs-GDNF growth-promoting pathway through and beyond a lesion gap of the thoracic cord, we observed that both descending dPST axons and brainstem-originated serotonergic and catecholaminergic axons regenerated through and beyond the lesion gap. In the distal host spinal cord, regenerated axons formed synapses and were myelinated. Such structural regeneration occurred in close association with an enhanced locomotor recovery. With GDNF treatment, regenerating axons appeared in close association with substrates expressing both inhibitory and stimulatory molecules, which suggested that axonal regeneration can take place in situations where both signals are present. For a therapeutic intervention to work, it needs to shift the balance between these two

opposing signals. As the major component in the glial scar, astrocytes are heterogeneous cells. Some populations display axonal growth-inhibitory characteristics but some are permissive. The high astrocytic responsiveness to local cues make them promising candidates as therapeutic targets to shift to the dominant beneficial pattern. GDNF combined with SCs transplantation can not only directly support axonal regeneration but also transform reactive astrocyte to be permissive. These dual effects endorse GDNF as a very powerful potential in repairing the spinal cord injury.

# CHAPTER 3

## INVESTIGATION OF DENDRITIC MORPHOLOGICAL PLASTICITY OF THORACIC DPSN RESPONDING TO THE AXOTOMY AND GDNF TREATMENT AND THEIR NEUROTRANSMITTER PHENOTYPE

**Hypotheses**: SCI induces morphological changes of dPST neurons at their somas and dendrites which may be reversed by treatments of GDNF. There are various neurotransmitter phenotypes in dPSN .

### Introduction

Over one hundred years ago it was recognized that a complicated intraspinal network of interneurons connecting segments was present in the spinal cord. Sir Charles Sherrington first described and named this unique population of spinal cord interneurons as propriospinal neurons (PNs)(Sherrington, 1885). Using electrophysiological techniques these neurons were later shown to play a critical role in motor reflexes, voluntary movement, and sensory processing (Alstermark et al., 2007; Cowley et al., 2010; Gerasimenko et al., 2009; Jankowska, 1992). PNs have been shown to play a critical role in the relay of signals from supraspinal afferents such as the cortical spinal tract (CST), rubro-, tecto-, and reticulo-spinal tracts to spinal motoneurons. PNs also receive input from muscle peripheral afferents, permitting feedback to muscle groups (Alstermark et al., 1984; Illert et al., 1977; Illert and Tanaka, 1978; Kostyuk and Vasilenko, 1978). This spinal circuitry allows for rapid correction of movement errors, while maintaining convergent supraspinal control and influence (Illert et al., 1977; Illert et al., 1978).

Descending propriospinal tract (dPST) and their neurons (dPSNs) have recently been implicated in mediating plasticity under pathological conditions. Low thoracic

axotomy invoked a strong regenerative response of descending thoracic propriospinal neurons to the injury (Siebert et al., 2010a). Severed axons of commissural PNs can regenerate and make functional synaptic connections with spinal motoneurons (Fenrich and Rose, 2009; Siebert et al., 2010a). dPSTs also respond to exogenous treatment with the neurotrophic factor GDNF and Schwann cells, resulting in successful regeneration through the lesion site (Deng et al., 2013; Iannotti et al., 2003; Xu et al., 1999; Zhang et al., 2009). Moreover, supraspinal axons, which usually fail to regenerate through and beyond the lesion site, form 'new' contacts with spared propriospinal neurons which project past a SCI lesion to lumbar segments (Bareyre et al., 2004; Courtine et al., 2008; Nishimura et al., 2009; Osborne et al., 2008; Vavrek et al., 2006). Therefore, reorganization of supraspinal-propriospinal bridges contribute to functional recovery after SCI.

Although the functional significance has been emphasized for many years, the anatomical structure of these descending propriospinal neurons remains unclear. Previous morphological studies of these propriospinal neurons have largely relied on conventional dye tracing after electrophysiological recording (Ritz and Greenspan, 1985). Due to their scattered distribution within the spinal cord and diffuse projections, previous studies have been limited by the lack of a reproducible and efficient labeling method to identify PNs (Sterling and Kuypers, 1968). Another fundamental limitation was that conventional retrograde tracers used were primarily restricted to labeling the cellular somata, making the morphological study of the dendritic details of neurons impossible (Naumann et al., 2000). More recently, the use of retrogradely infectious viruses expressing genetically encoded fluorophores has been developed through which details of neuronal morphology can be clearly labeled without immunohistochemical amplification. Recombinant rabies virus has been widely used to reveal detailed

neuronal morphology (Callaway, 2008; Ni et al., 2014; Ugolini, 2011; Weible et al., 2010; Wickersham et al., 2007). In this study, we used this recombinant rabies virus-based method to reveal the dendritic morphology of descending propriospinal neurons that projected their axons from low thoracic cord to the lumbar cord level in rats. Following a complete transection injury at the T11 spinal cord level, we also analyzed the effect of GDNF treatment on the dendritic plasticity of injured neurons.

**Materials and Methods:**

**Animals**

Twenty four Sprague Dawley adult female rats (180g-200g, Harlan) were used, twelve for the viral tracing study and the other twelve for the classification of neurotransmitter type. Animals were divided into three groups, receiving either sham injuries or complete spinal transections with or without GDNF treatment. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

**Viral and FluoroGold (FG) retrograde tracing**

Rats were anesthetized by an intraperitoneal (IP) injection of a ketamine(40-95 mg/kg)/xylazine (5-10 mg/kg) cocktail. A laminectomy was performed to expose the 2$^{nd}$ lumbar segment (L2) of the spinal cord. The dura was cut using a 26G needle to expose the spinal cord. Mutant monosynaptic rabies virus Rb-B19-ΔG-GFP (purchased from Salk Institute) was bilaterally injected into the intermediate gray matter (laminae V - VIII) of the L2 spinal cord (0.75µl/side, 10∧9TU/ml) using a stereotaxic apparatus. This G-

mutated virus had the envelope glycoprotein gene deleted from its genome and had been transformed into a first-order retrograde tracer (monosynaptic transfection).Compared to multiple synaptic retrograde transportation viruses such as pseudorabies virus, this G-mutated virus can specifically target the descending propriospinal neurons which directly project their axons to the infection area such as CPG area in the lumbar enlargement but not those other neurons indirectly affect the CPG through hierarchical contacts (Wickersham et al., 2007). In our study, the DPSNs labeled by this G-mutated rabies virus include only those directly projecting to the intermediate zone of the L2 lumbar cord. The injection procedure lasted for 5 minutes including leaving the needle in place for 1 minute prior to retraction to limit leakage. For FG labeling, 2% FG (Sigma 39286 ) was injected into the L2 level spinal cord (0.75µl/side) with the same stereotaxic parameters (Fig. 1).

**Spinal Transection**

Three days after viral or tracer injections, the animals in the experimental groups were anesthetized with ketamine/xylazine. A laminectomy was performed to expose the 11$^{th}$ thoracic segment (T11) of spinal cord. A pair of iridectomy scissors (Fine Scientific Tools; Foster City; CA) was used to transect the spinal cord. Successful transection was judged after exposure of the rostral and caudal stumps of the retracted spinal cords. Following transection, a probe was scraped along the inner wall of the vertebral canal through the lesion site to further ensure a complete spinal cord transection (Coulon et al., 2011). After bleeding in the lesion gap was stopped, gelfoam containing either 0.01M PBS or PBS containing GDNF (3 µg/µl) was placed into the lesion gap (Fig. 1). Sham animals received a laminectomy at the T11 level without spinal cord transection.

**Post-Operative Care**

Following each surgical procedure, the musculature was closed with 3.0 silk, and the skin was closed with 3.0 Nylon suture. External sutures were removed after the first week post-operatively. Buprenorphine hydrochloride (Buprenex injectable; Ben Venue Laboratories Inc.; Bedford; OH; 0.03 cc SQ) was given twice daily for the first 48 hrs post-surgery. They also underwent bladder expression until sacrifice to prevent urinary tract infection.

**Tissue processing, Imaging and 3D reconstruction of viral labeled dPSNs**

Four days after transection injury, animals were anesthetized using a ketamine/xylazine mixture and were perfused with cold PBS followed by 4% paraformaldehyde. The whole spinal cord was carefully removed from the vertebral spine. Continuous sections of T6-T10 spinal cord segment were transversely sectioned at 150 µm thickness with a vibratome.

Labeled cells from all sections of T6-T10 cord segments were examined under a two-photon laser scanning microscope (Prairie Technologies) with 910 nm excitation light. Sections with completely labeled neurons were selected for further imaging. Stacks of images were collected by using a 20 × water immersion lens and an increment of 1 µm. Initially, each stack of images was projected and saved as a .tif format; montage stack images of individual neurons were then created. The morphology of labeled cells in the stack images was reconstructed with Neurolucida (MBF Bioscience, Colchester, VT, USA).

**Criteria for classification of dPSNs**

Our analysis was restricted to DPSNs from T6 to T10 spinal cord segments with axonal projections to the L2 spinal cord segment. Only those neurons whose somata were located in the intermediate gray matter (Rexed Lamina VII) were included. Each neuron was analyzed with respect to a coordinate system. The orientation of dendrites was defined as medial (close to the central canal), lateral (far from the central canal), ventral (close to the anterior horn), and dorsal (close to the posterior horn). Accordingly, the XY-plane is the transverse plane of the spinal cord, the YZ-plane is the parasagittal plane, and the XZ-plane is the horizontal plane (Fig.2). Dendrites that could not be traced back to their soma were excluded. Neurons with incomplete dendritic trees were also excluded. Only neurons with labeled somata and more than 90% dendritic trees were considered complete cells and selected for final analysis.

**Quantitative morphometry**

Morphometric parameters were extracted with the Neurolucida analysis tool NeuroExplorer. To quantify the percentage of dendritic distribution in different orientations, we divided the spinal regions around each individual neuron into four quadrants and defined these quadrants as lateral (320°~50°), dorsal (50°~140°), medial (140°~230°) and ventral (230°~320°). The percentage of dendritic profiles appearing in these areas was calculated. To estimate the surface area of the three-dimensional cell bodies, the perimeter of each soma was first traced from its two-dimensional image. Then, by applying the formula for the surface area of an ellipsoid to the perimeter and two dimensional area measurements, the three-dimensional surface volume was calculated.

**Neurotransmitter immunohistochemistry**

To determine the transmitter phenotype of dPSNs, we performed experiments with FG retrograde labeling combined with immunohistochemistry. Four days after transection injury, animals received a lethal dose of ketamine/xylazine and were perfused transcardially with a fixation solution containing 4% paraformaldehyde and 0.3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. T7-T9 spinal cords were removed from the spines and cryo-cut into 20-µm thick transverse sections. We collected sections at every 100 µm interval as a set for each neurotransmitter staining. Immunohistochemistry was performed using antibodies raised against protein-conjugated amino acid neurotransmitters (anti-glutamate, G9282, 1:10,000, Sigma-Aldrich, USA; anti-GABA, A2052, 1:5,000, Sigma, USA; anti-glycine, 1:2,000, AB139, Chemicon, Temecula, CA, USA; Millipore anti-Choline anti-Choline acetyltransferase, 1:100, AB144P, Chemicon, Temecula, CA, USA) producing staining of distinct cellular populations. After incubating spinal cord sections with the primary antibody or antibodies for 48 h, secondary antibodies matching the species of the primary antibody and labeled either with Cy3 (ab6939 abcam MA, USA) or Alexa Fluor 488 (A-11001 Life Technologies, NY, USA) were employed to visualize their binding sites. To test the specificity of the primary antibody, they were omitted from control sections and no immunoreactivity was detected.

**Quantification of neurons**

Patterns of staining were visualized under a fluorescence microscope using appropriate fluorescence filter sets. Counting of neuronal cell bodies was performed by a blind observer using Image J software. All results were based on four different animals on one

set of sections per animal for each molecular marker and tracer. Counting fields were not allowed to approach the margins of the analyzed spinal cord regions to minimize inaccuracies due to edge effects that are likely to increase by difficult-to-discern cell types at their borders. Counting was done under high magnification objectives (20x). By focusing through the sections, marker and tracer co-localization was readily distinguished from stained elements at different depths. The numbers of cells stained for selected markers were counted and averaged for each combination of staining across all analyzed sections and experiments. Each set of section was stained for each different neurotransmitter. Neurons were selected only from intermediate grey matter. Quantitative analysis was based on the number of each section (n) through the caudal-rostral extent of the analyzed. In each set, percentage of DPSN neurons with specific neurotransmitter=number of neurons co-labeled by FG and neurotransmitter antibody in this set/ total number of neurons labeled by FG in this set.

**Statistical analysis**

All numerical data are presented as mean ± SEM. Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad, San Diego, CA). The Kolmogorov-Smirnov test was used to compare the patterns of cumulative or relative frequency plots in the histogram of individual values between sham and treatment groups (Kim et al., 2008). Due to the non-Gaussian distribution of morphology, the nonparametric Kruskal-Wallis test was used to determine the significance of group effect. One way ANOVA with Post hoc Tukey's test was performed to determine the effect of injury and GDNF treatment on other dendritic parameters. Two-way ANOVA was performed to determine the effect of either the factor of spinal cord level or the factor of injury/GDNF treatment (sham vs. injury vs. GDNF) independently or their possible interaction on neurotransmitter

distribution. Post hoc Bonferroni's test was performed to compare means between groups. Significance was set at the 95% confidence interval.

**Results**

A total of 48 dPSNs were labeled by GFP fluorescence. The enhanced green fluorescent protein (EGFP) expressed by the virus filled all of the neuronal processes. The fluorescence intensity was high enough to label fine processes of dendrites. Forty-two labelled cells in the intermediate laminar were reconstructed. After careful examination of dendritic morphology, 36 cells met the criteria and were fully analyzed (15 cells in the sham group; 10 cells in the injury group; 11 cells in the injury + GDNF group).

**Orientation of dendritic distribution**

Labeled dPSNs were present bilaterally, and to maintain consistency in orientation, cells on the left side of the spinal cord were mirror-reflected. DPSNs displayed a variety of dendritic morphologies (Fig. 18). dPSNs extended their dendritic branches in medial, lateral, ventral, and dorsal directions (Fig. 18A), with some having a predominant dendritic distribution along the medial-lateral axis (Fig. 18B) or ventral-dorsal axis (Fig. 18C). With NeuroExplorer, we analyzed the normal orientation of dendrites of DPSNs and their change after injury. dPSN neurons had relatively simple and sparse branches. To examine dendritic spatial orientation as a function of distance from the dendrite origin, polar histograms were constructed. The results from the polar histograms indicated a nonuniform distribution of dendritic profiles in sham animals (Fig. 19A) and this overall distribution pattern changed after transection injury and GDNF treatment (Fig. 19B, C; Sham & TX: $p < 0.05$, Sham & TX + GDNF, $p < 0.01$). To further analyze the change within specific orientations, we divided the spinal regions around each individual neuron into four quadrants and defined these quadrants as lateral: 320°~50°; dorsal: 50°~140°;

medial: 140°~230°; ventral: 230°~320°, and determined the percentage of the total dendritic length in each quadrant to reduce the variation. This result showed that as a group, the dendritic distribution of intact DPSNs displayed a dorsal-ventral predominate distribution (lateral 13.78±2.12%, dorsal 35.75±3.70%, medial 19.16±2.85%, ventral 31.3±3.77%; lateral versus dorsal $p < 0.001$; lateral versus ventral $p < 0.001$; dorsal versus medial $p < 0.01$; medial versus ventral $p < 0.05$) (Fig. 20C). After injury, the distribution changed to an unbiased pattern due to the retraction of dorsal and ventral dendritic profiles and extension of lateral and medial profiles (TX group: lateral 30.07±4.74%, dorsal 19.75±4.23%, medial 25.99±5.19%, ventral 24.18±3.49% $p > 0.05$; TX+GDNF group: lateral 26.12±3.21%, dorsal 20.97±2.19%, medial 29.82±4.21%, ventral 23.08±3.12% $p > 0.05$) (Fig. 20C). There was no significant difference in dendritic orientations between TX and TX+ GDNF groups.

**Changes in dendritic length and dendritic branches after injury and GDNF treatment**

With the data from NeuroExplorer, we analyzed parameters related to dendritic length and dendritic branches. Dendritic length included the total dendritic length (sum of all dendritic lengths), interval length (length between dendritic nodes) and terminal dendritic length, while parameters of dendritic branches included the number of total branches, interval nodes, and terminal branches. As shown in Fig. 21A, the total dendritic length in the sham group was 2946.66± 305.93 μm. It was increased to 3442.77± 413.35 μm after injury although it was not statistically significant ($p > 0.05$), and GDNF treatment enhanced this effect (3951.73± 340.53 μm, versus sham $p < 0.05$). The increase in total dendritic length may originate from the extension of individual dendrites, increased dendritic branches, or a combination of both. Increases in internal intervals and terminal length of dendrites would indicate increases in the extent of the dendritic arbor into the

surrounding neuropil. Injury with or without GDNF treatment did not affect the portion of the dendritic arbor proximal to the soma, and there was no significant difference in internal intervals among the three groups ($p > 0.05$, Fig. 21B). However, treatment with GDNF increased terminal length of dPSN dendrites of injured animals in a manner similar to that observed in total dendritic length, with terminal lengths of 2080.50 ± 176.29 µm in the sham group, 2563.84 ± 257.13 µm in the injury group (versus sham $p > 0.05$), and 3101.83 ± 269.67 µm in GDNF-treated animals (versus sham $p < 0.01$) (Fig. 21C). To examine the changes in dendritic number, we analyzed the number of dendritic branches, dendritic nodes (the points where dendrites bifurcate), and dendritic endings. Results showed that the number of total dendritic branches per cell was 16.39. ± 1.72 in the sham group, 18.7 ± 2.12 in the injury group, and 20.36 ± 1.47 in the GDNF treatment group (Fig. 21D), and these did not differ across groups ($p > 0.05$). The number of dendritic nodes (sham, 6.33 ± 0.81; injury, 7.4 ± 0.93; and injury + GDNF, 7.55±0.56) and dendritic endings (sham, 10.1±0.93, injury, 11.3±1.22, injury + GDNF, 12.3±0.89 ) also did not differ across groups ($p > 0.05$, Fig. 21E & F). The number of dendritic terminal branches in sham, injury and injury + GDNF animals were 2.62±0.27, 2.89±0.30, and 2.84±0.24 respectively ($p > 0.05$). The maximal order of a dendrite in sham, injury and injury + GDNF animals was 4.11±0.30, 4.4±0.27, and 4.45±031 respectively ($p > 0.05$) (Fig. 21D & H).

**Dendritic spine-like structures**

Because axotomy or GDNF treatment induced alterations of the dendritic arbors of neurons, this may have affected the distribution and density of spines as well. To investigate this, dendritic spine-like structures of neurons in the different groups were sorted and compared according to their occurrence on the dendrites. First, we found that dendritic spine-like structures of propriospinal neurons were distributed on all orders of

dendrites but not in abundance. Five types of spine-like structures were found on dendrites and were classified as stubby, mushroom, regular (between mushroom and thin spines in morphology), thin, and filopodia (Fig. 22A). In sham animals, the average number of spine-like structures of each neuron was 23.88 ± 2.15 (Fig. 22B); the average density of spines of each neuron was 0.008 / µm ± 0.001(Fig. 22C). In sham animals the majority of spine-like structures were classified as stubby (82.20% ±2.97), followed by regular (9.73% ± 2.39), mushroom (6.25% ± 2.34), thin (2.89% ± 1.35), and filopodia (0.37% ± 0.37%) (Fig. 22C & E). After transection of the spinal cord, the total number of spine-like structures in each neuron significantly increased to 48.47 ± 9.41 (Fig. 22, $p < 0.01$ versus sham group), and to 3 fold the number in sham animals (74.72± 12.38) after GDNF treatment ($p < 0.01$ versus sham group). The number of spine-like structures classified as stubby changed in the same pattern as that seen in the total number of spines, with 19.35± 1.87 in the sham group and increasing to 40.44.0 ± 8.46 after transection of spinal cord ($p < 0.01$ versus sham group), and to 64.09 ± 9.30 in the GDNF treatment group ($p < 0.01$ versus sham group, Fig. 22B & C). There were no significant changes in the numbers of the other types of spine-like structures among the sham, injury, and GDNF treatment groups (all $p > 0.05$ versus sham group, Fig. 22B & C). The densities (number of spines / µm) of the five types of spine-like structures changed in the same pattern as that seen in spine number in injury and GDNF treatment groups (Fig. 22C). The density of spine-like structures classified as stubby was 0.006 / µm ± 0.001 in the sham group, increasing to 0.011 / µm ± 0.002 after injury and to 0.015 / µm ± 0.002 with GDNF treatment ($p < 0.01$ versus sham group). The density of spine-like structures classified as filopodia was also significantly increased 8 fold between the sham and injury groups ($p < 0.05$ versus sham group), but the number was still small in

the injury group (0.0003 / μm ± 0.0001). Interestingly, the proportion of all five types of spine-like structures remained consistent in all three groups (Fig. 22E).

In order to determine if there were different responses of spine-like structures to injury or GDNF treatment in different order of dendrites, we investigated the number of spines in each order of dendrites. The number of spines in each order showed a similar pattern in which more spines were found in GDNF-treated group (Fig. 22D). The increases in spines were significant in the first three orders of branches. In the first order, the number of spines was 5.00 ± 1.24 in the sham group. It slightly increased to 8.89 ± 3.02 after injury, and to 10.00 ± 2.19 when treated with GDNF ($p < 0.01$ versus sham group). In the second order, the number of spines dramatically increased from 5.00 ± 1.24 (sham group) to 19.89 ± 3.30 (injury group), and to 31.63 ± 2.19 (GDNF group) (all $p < 0.01$ versus sham group). Changes in spine number in the third order were similar to that in the first order, with 5.14 ± 1.39 in the sham group and 9.11 ± 4.82 in injury group, but increased in the GDNF-treated group (20.09 ± 4.79, $p < 0.01$ versus sham group). The number of spines in the fourth and five orders were similar to that in the first order, and there were no significant differences across the groups (all $p > 0.05$ versus sham group).

**Somata**

The somata of dPSNs were randomly distributed lateral to medial in lamina VII. There was a significant difference in somal volume among three groups: sham, 22156.57±4046.75 μm³, injured, 31872.25±6854.58 μm³, and GDNF-treated, 46602.29 ± 8040.20 μm³ ($p < 0.05$ sham versus GDNF-treated). The surface areas of the somata averaged 5196.00±593.63 μm² in sham animals, 6401.38±1226.00 μm² in injured animals, and 7394.00±949.23 μm² in GDNF-treated animals ($p > 0.05$). To determine the relative contributions of the dendrites and somata as sites of afferent input, the surface

area of each soma was compared with the total dendritic area of each labeled dPSN. The somata accounted for a relatively small amount of the total dendrosomatic area of the cells, and there was no significant difference among the three groups (sham, 9.19±1.26, injured, 10.01±2.05, GDNF-treated, 7.45±0.98; $p$ >0.05).

**Neurotransmitter types**

In the thoracic segment, dPSNs labeled by FG were found mostly located in Rexed laminae VII. In order to characterize the neurotransmitter phenotypes of dPSNs, we employed glutamate and ChAT antibodies as markers for excitatory neurons and GABA and Glycine as markers for inhibitory neurons. Cells labeled by anti-glutamate, anti-GABA, anti-Glycine, and anti-ChAT were clearly distinguished from the fluorescent background by their intense labeling. FG (blue signal) labeled neurons quite consistently (Fig. 23). By merging the signals from the green, red, and blue channels, we could detect dPSNs with different neurotransmitters. We found that the majority of the DPSNs in sham animals were glutamatergic neurons (T7: 92.75±2.4%; T9: 90.49±1.5%). The other three types of neurons contributed less than 10 percent of the total dPSN population (ChAT: T7: 5.1±1.2%; T9: 5.51±0.63%); (GABA T7: 2.53±0.46%; T9: 2.38±0.75%); (Glycine T7: 1.99±0.32%; T9: 2.33±1.04%) ($p$ < 0.001, compared to Glutmate) (Fig. 24A). Although transection injury caused significant reductions in the number of FG-labeled neurons in rostral cord proximal to the injury [sham T7: 523.1±10.42, T9: 775.06±47.80; injury T7: 420.70±91.74, T9: 393±37.98; injury + GDNF: T7: 400.23±41.56, T9: 332.19±25.72, sham versus injury and sham versus injury + GDNF $p$ < 0.001) (Fig. 24B), GDNF treatment did not protect neurons from reductions in number likely through death after injury and did not change the distribution of the different types of dPSNs (Fig. 24C-F).

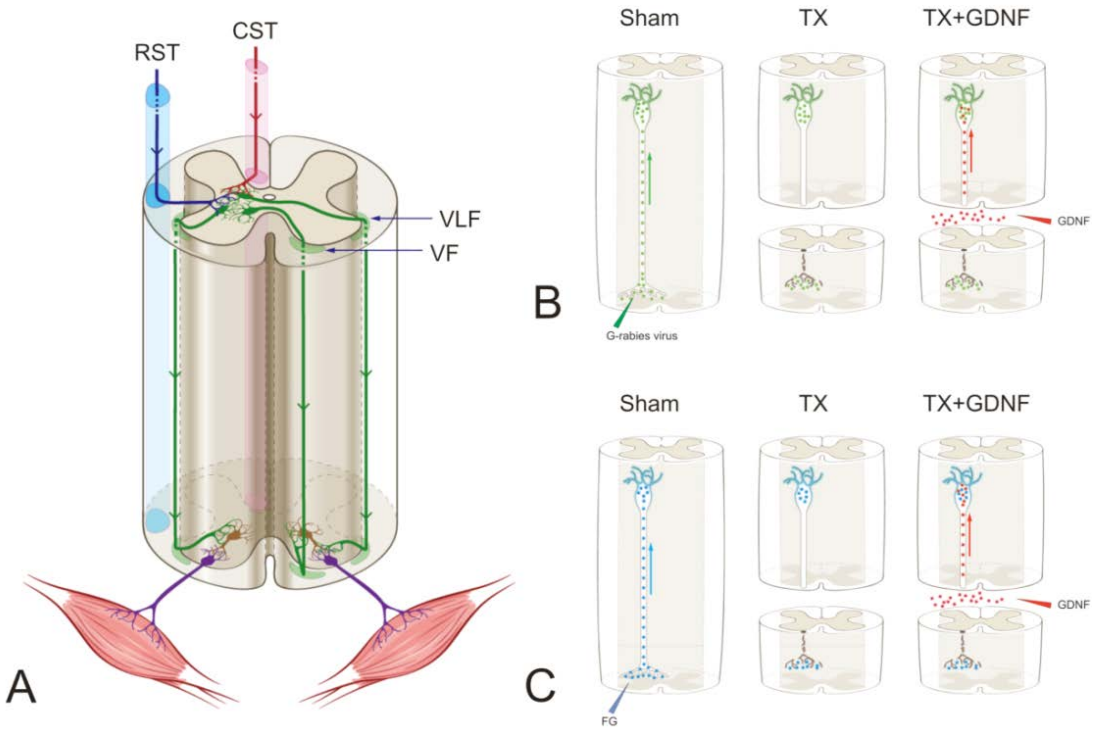



**Figure 17. (A) Schematic diagram of the descending propriospinal tract system (DPST).** dPST pathways descending several spinal segments are located in both the ventral and ventral lateral funiculae (VF and VLF, respectively). The dPST projects either contralaterally or ipsilaterally through the VF or VLF and innervates motoneuron pools directly or indirectly through interneurons. dPST neurons receive convergent supraspinal innervation, including those from the corticospinal (CST) and rubrospinal (RST) tracts. Descending propriospinal neurons are indicated in green, interneurons in brown and motoneurons in purple. (B, C) Schematic diagrams of the experimental designs for the morphological study (B) and the neurotransmitter study (C). The three figures in each panel, from left to right, show propriospinal neurons were first retrogradely infected by a G-mutated rabies virus (green particles) that expressed green fluorescence protein which filled the dendritic compartments of DPST neurons (B) or retrogradely labeled by FluoroGold (FG) (blue particles) (C). Spinal cords then received either a transection injury or a transection + glial derived growth factor (GDNF) (red dots) applied to the lesion site to be retrogradely transported to the soma.

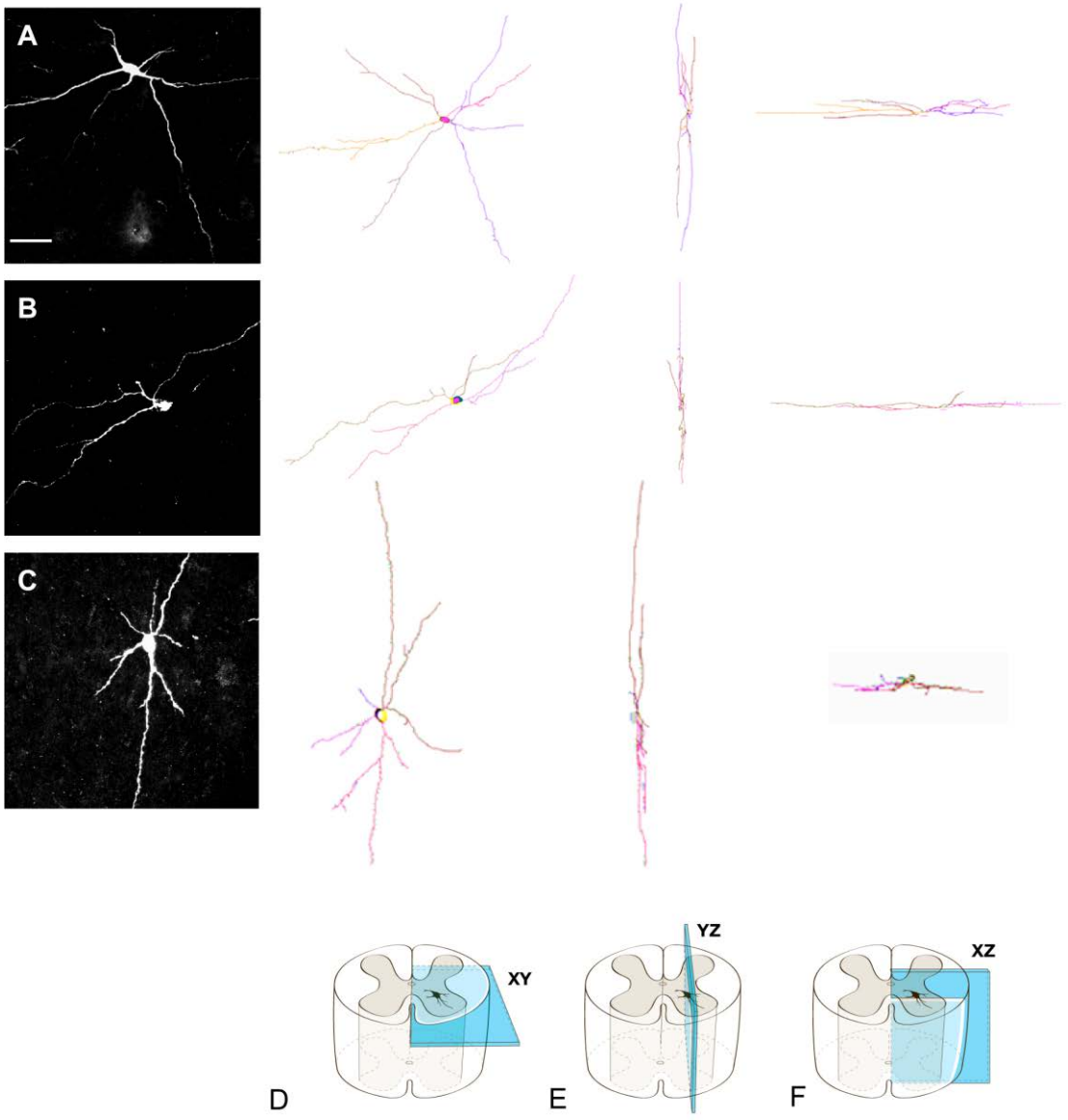



**Figure 18. Camera lucida reconstructions of three propriospinal neurons (rows A, B, C) with different dendritic patterns as if viewed in the transverse (D), parasagittal (E), and horizontal (F) planes.** Row A shows a dPSN that extended its dendritic branches in medial, lateral, ventral and dorsal directions. Row B shows a dPSN that extended its dendritic branches predominantly in medial and lateral directions. Row C shows a dPSN that has more dendritic branches extending in ventral and dorsal directions. Scale bar: 100µm.

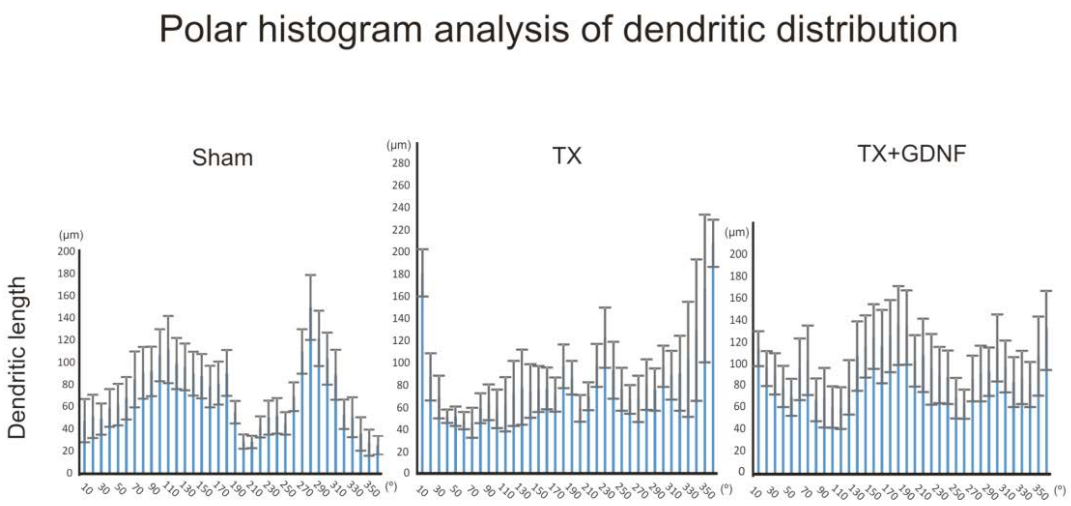



**Figure 19. Polar histogram analysis of dendritic distribution in different orientations in the sham , transection (TX), and transection + GDNF (TX + GDNF) groups.** The polar histograms indicate a nonuniform distribution of dendritic profiles in sham animals and non-biased distributions in the TX and TX + GDNF groups. Bar heights represent means ±SD.

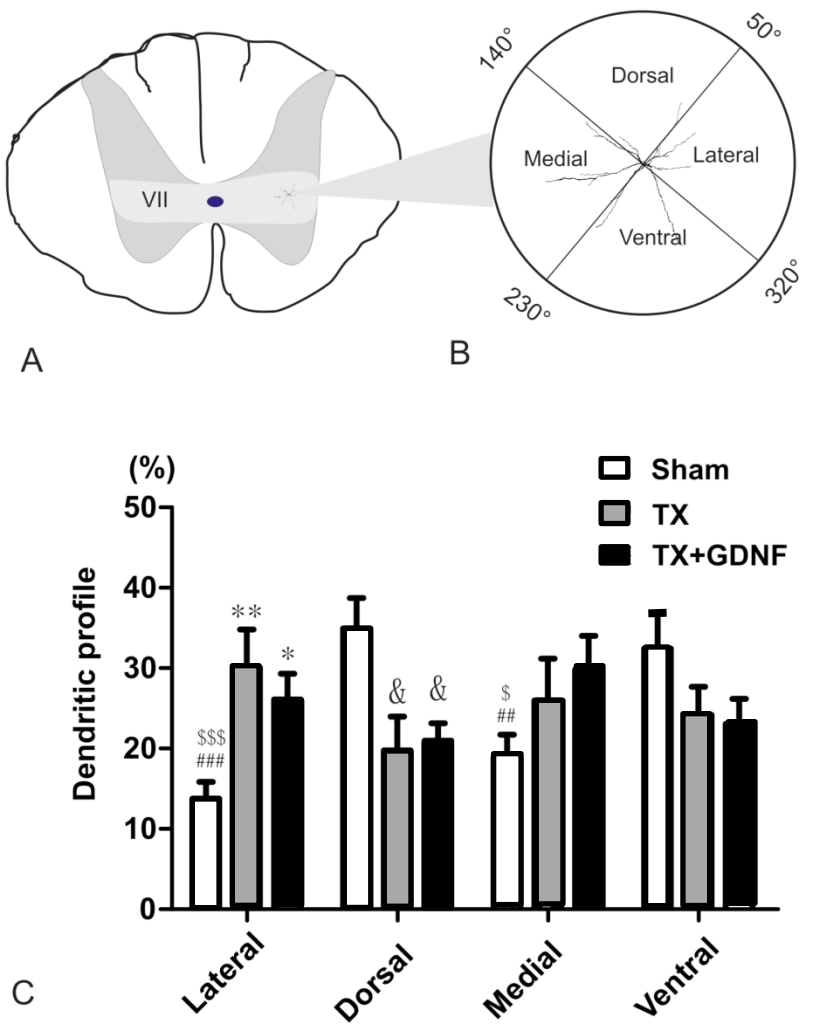



**Figure 20. Percentage of dendritic distribution in four quadrant areas.** (A) Schematic drawing of a spinal cord transverse section, light grey area corresponds to lamina VII in which all of the dPSNs in this study were located. A representative neuron is indicated at right in lamina VII. (B, C) The area surrounding the soma was separated into four quadrants. We defined these four quadrants as lateral: $320^0$~$50^0$; dorsal: $50^0$~$140^0$; medial: $140^0$~$230^0$; ventral: $230^0$~$320^0$. (D) Comparison of percentage distribution of dendrites among the sham, transected (TX) and TX + GDNF groups. * $p < 0.05$ TX + GDNF lateral versus sham lateral; ** $p < 0.01$ TX lateral versus sham lateral; & $p < 0.05$ TX dorsal or TX + GDNF dorsal versus sham dorsal; ## $p < 0.01$ sham medial versus sham dorsal; ### $p < 0.001$ sham lateral versus sham dorsal; $ $p < 0.05$ sham ventral versus sham medial; $$$ $p < 0.001$ sham ventral versus sham lateral..

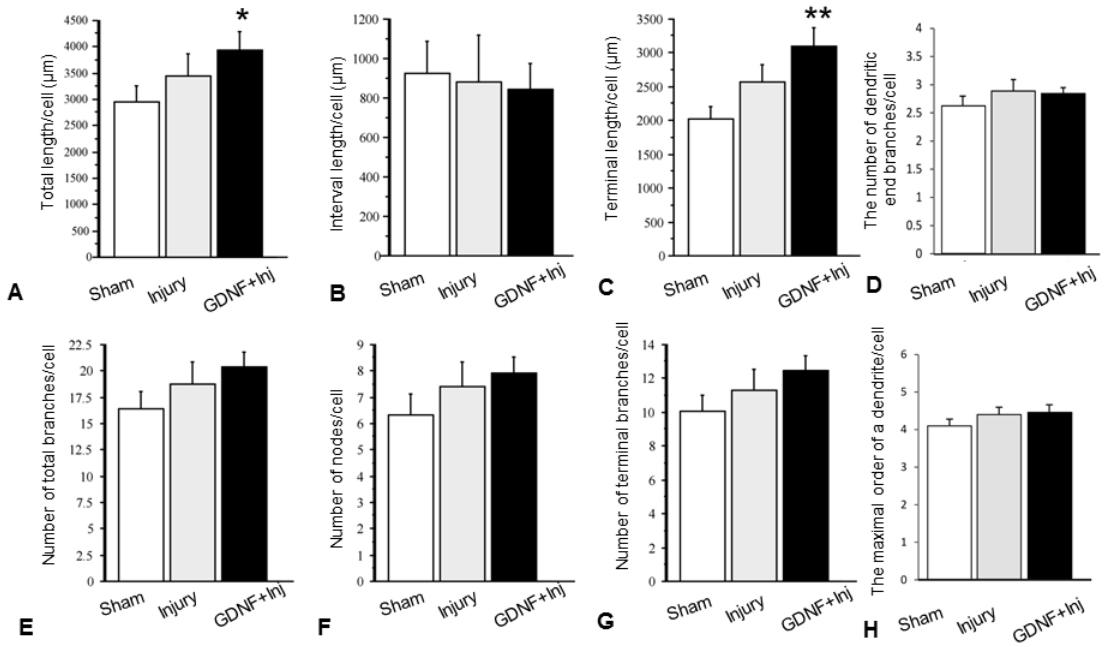



**Figure 21. Comparison of the dendritic features of the 36 GFP-labeled and fully reconstructed dPSN neurons from sham (n=15), transection injury (TX, n=10) and TX + GDNF treatment groups (n=11).** (A) total dendritic length; (B) dendritic interval length; (C) terminal dendritic length; (D) number of dendritic end branches/cell; (E) number of total dendritic branches; (F) number of dendritic nodes; (G) number of terminal dendritic branches; (H) maximal order of dendritic branches. *: $p < 0.05$; **: $p < 0.01$ (Compared to sham group). Bar heights represent means ± SEM.

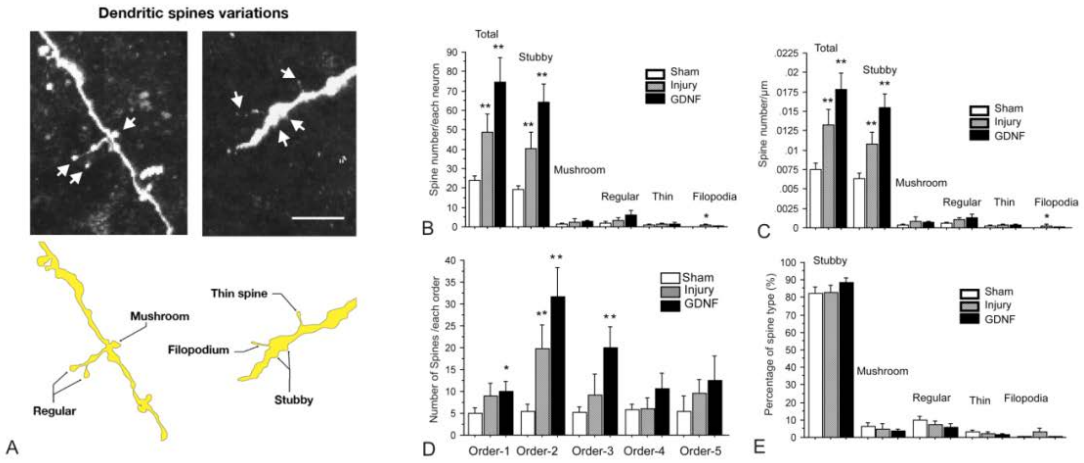



**Figure 22. Morphological plasticity of dendritic spine-like structures of dPSNs after axotomy injury treated with or without GDNF.** (A) Schematic drawings and representative images of the five different types of spine-like structures. Comparisons of the number (B), density (C), number by branch order, (D) percentage of types of spine-like structures overall and by the different types across the sham, TX, and TX + GDNF groups. * $p < 0.05$ compared with sham group. ** $p < 0.01$ compared with sham group. Bar heights represent means ± SEM.

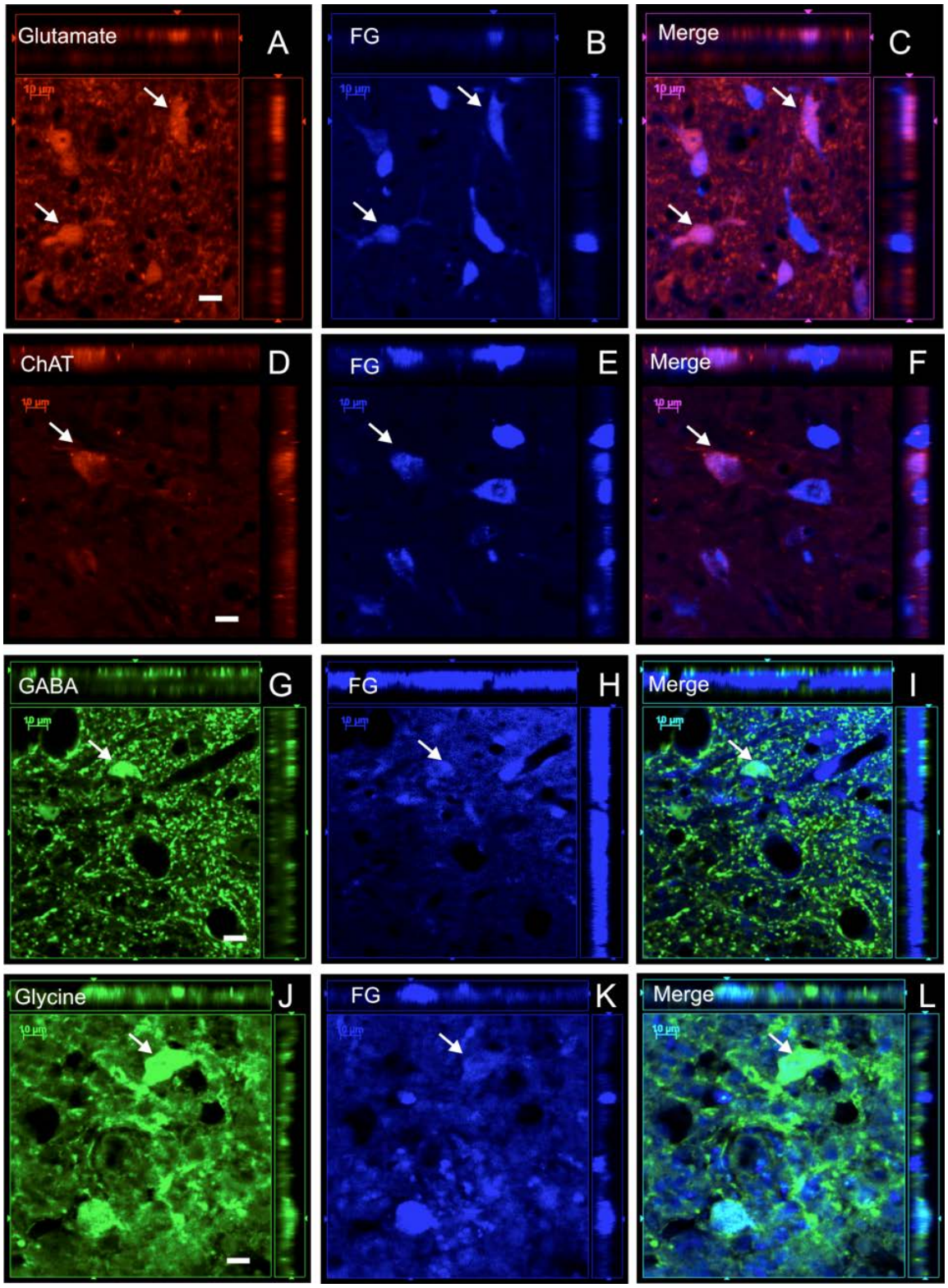

Glutamate
A
FG
B
Merge
C
ChAT
D
FG
E
Merge
F
GABA
G
FG
H
Merge
I
Glycine
J
FG
K
Merge
L

**Figure 23. Co-localizations of retrograde tracer FluoroGold (FG) with neurotransmitter markers in sub-populations of dPSNs.** (A-C) The majority of neurons labeled by FG were glutamatergic (white arrow, red glutamate, blue FG), (D-F) a small percentage of neurons labeled by FG were ChAT-positive neurons (white arrow, red ChAT, blue FG). (G-I) a small percentage of neurons labeled by FG were GABAergic neurons (white arrow, green GABA, blue FG). (J-L) a small percentage of neurons labeled by FG were glycinergic neurons (white arrow, green glycine, blue FG). Scale bar: 10μm.

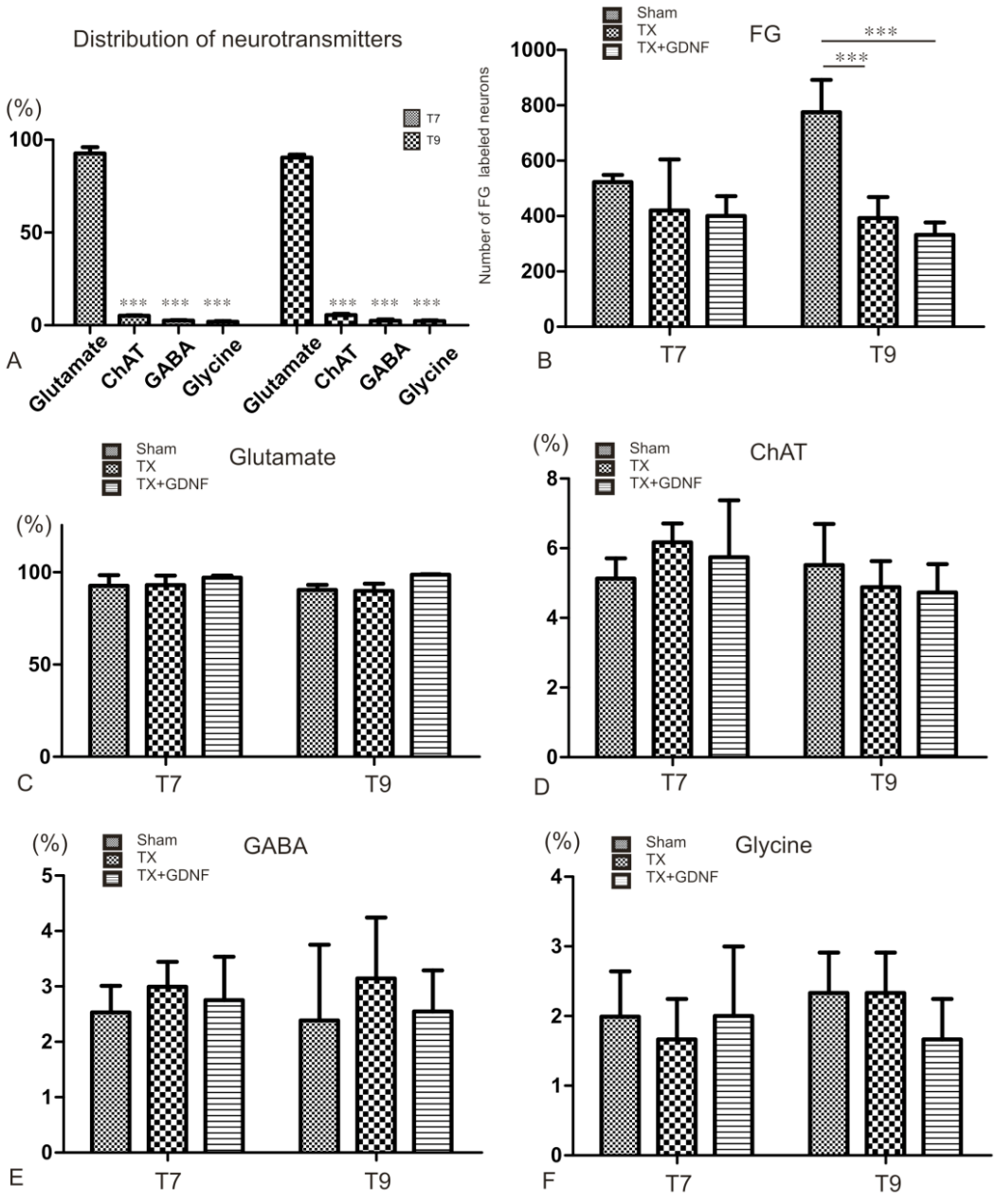

Distribution of neurotransmitters
(%)
T7
T9
100
50
0
***
Glutamate
ChAT
GABA
Glycine
A
Sham
TX
TX+GDNF
FG
Number of FG labeled neurons
1000
800
600
400
200
B
T7
T9
Glutamate
C
ChAT
8
6
4
2
D
GABA
5
4
3
2
1
E
Glycine
F

**Figure 24. Quantification of subpopulations of four different neurotransmitters in FG labeled dPSNs**.(A) Percentage distribution of subpopulations of four different neurotransmitters in the sham group. The majority of FG-labeled dPSNs are glutamatergic neurons. *** $p < 0.001$ compared with glutamatergic neurons. (B) Total number of FG-labeled neurons in T7 and T9 spinal cord in sham animals and after spinal transection (TX) with or without treatment with GDNF. The number of FG-labeled cells is decreased in T9 spinal cord after transection injury regardless of treatment with GDNF. *** $p < 0.001$ compared with sham group. (C-F) Percentage distribution of subpopulations of DPSNs with four different neurotransmitters post transection injury and GDNF treatment. Bar heights represent means ± SD

## Discussion

A neuron's morphology, especially the dendritic arbor, represents one of the most important distinguishing features of neuronal type and often accounts for a neuron's physiological role in a circuit (Markram et al., 2004). Neurons are driven by the code of synaptic inputs to conduct their physiological function (Kuhn et al., 2004). Several dendritic characteristics, including branching patterns, size, and orientation of dendritic trees determine how synaptic inputs are integrated (Cline, 2001; Henze et al., 1996). dPSNs have not previously been morphologically described. Identification of morphological properties of normal and injured dPSNs can provide fundamental knowledge of this group of neurons and elucidate the mechanisms of their contribution to the functional recovery after SCI. The complicated physiological roles of dPSNs system indicate their complex compositions which can be reflected by their diverse targeted neurons (Flynn et al., 2011). Thoracic intraspinal projections arising from the intermediate zone have been found to be directed to spinal motoneurons and to non-motoneuronal elements in the intermediate zone where central pattern generators (CPGs) are mainly located (Alstermark and Isa, 2002; Ballion et al., 2001; Gerasimenko et al., 2009). Therefore, dPSNs not only directly control the limb movement but also facilitate forelimb/hindlimb coordination through CPGs during natural stepping (Gerasimenko et al., 2009). Ni (2014) used a recombinant rabies virus-based method to trace a subset of the long descending propriospinal neurons that form monosynaptic connections directly with motor neurons of the hindlimb (Ni et al., 2014). Our study focused on another subgroup of dPSNs which project to the intermediate zone of the lumbar enlargement and make contact with either dendrites of motoneurons or non-

motoneuron elements including those that may contribute to CPG for locomotion (Guevremont et al., 2006; Sterling and Kuypers, 1968).

**Dendritic morphology of dPSNs**

Rodents lack direct connections from cortex to spinal motoneurons. Instead, interneuron circuits, especially the descending propriospinal system, relay supraspinal commands. Working in concert with supraspinal neurons, the descending propriospinal neurons residing in Lamina VII integrate both motor and sensory inputs and therefore finely tune locomotion, limb coordination, and postural support (Cowley et al., 2010; Delwaide et al., 1977; Miller et al., 1973; Skinner et al., 1980). Most of the dorsal CST has been found to terminate its axons in the intermediate zone (Rosenzweig et al., 2009). The “ventral intermediate zone” has also been found to be the chief area of termination of tectospinal fibers (Nyberg-Hansen, 1964; Nyberg-Hansen, 1965). In addition, spinal interneurons are connected with sensory afferents such as dorsal root fibers which penetrate the dorsal funiculus and traverse the dorsal horn (Ritz and Greenspan, 1985). This connection conveys feed forward information from sensory to motoneurons (Cote et al., 2012). Thus, major signals transduced through dPSNs come from afferent projections located both dorsally and ventrally relative to the dPSNs, which may account for the predominant dorsal-ventral dendritic distribution in the transverse plane of normal dPSNs found in our study. In cats and other mammals, Golgi stained neurons in the ventromedial, lateral, and central areas of lamina VII display a predominant dorso-ventral dendritic orientation. The dendritic distributions were extensive in the transverse plane but were restricted rostro-caudally. (Berkowitz et al., 2006; Brown, 1983; Scheibel and Scheibel, 1969). Therefore, we believe that this dendritic distribution pattern may be

conserved across the species (Fetcho, 1992; Kusuma and ten Donkelaar, 1979; Nieuwenhuys, 1964).

This orientation is in sharp contrast to the longitudinal dendritic orientation of islet interneurons in the dorsal horn as well as to the pronounced longitudinal dendritic bundles of the motoneurons (Fargo and Sengelaub, 2007; Maxwell et al., 2007; Sengelaub and Forger, 2008). Another common type of cells in lamina II, defined as stalked cells, also possess dendritic arbors with a dorsal-ventral orientation, with dendrites predominantly projecting ventrally from the cell body and extending in the rostro-caudal plane (Maxwell et al., 2007). The nociceptive-specific neurons and multi-receptive neurons located in the lateral portion of lamina V have dense dendritic arborizations and extensive spreading of their dendrites in all directions, including rostro-caudally. Dorsally directed dendrites either reach the nucleus proprius or the inner lamina I and ventrally directed dendrites extend to lamina VII. The medially directed dendrites extended into lamina V and lamina X over the central canal. Their dendrites can reach the white matter lateral to the dorsal horn (Grudt and Perl, 2002; Maxwell et al., 2007; Ritz and Greenspan, 1985). In contrast, dPSNs in lamina VII in our study had only a few dendrites, which for the most part did not have complicated branching. The extension of their dendrites, although not wide, still reach into lamina IX (the ventral horn motoneuron area) and lamina V but rarely white matter. The different dendritic patterns may reflect the functional variety of the neurons.

**Dendritic plasticity induced by axotomy**

The changes in dendritic morphology of neurons after ischemia/hypoxia have been reported by other investigators. For example, after neuronal damage there is an acute

reduction in apical and basal dendritic number in hippocampal neurons (Biernaskie and Corbett, 2001; Pokorny et al., 1982; Pokorny and Trojan, 1983). In contrast, Ruan (2006) reported that the dendrites of CA1 neurons undergo disorientation and an increase in apical dendritic length shortly after transient cerebral ischemia (Ruan et al., 2006). Interestingly, in our study, we also see an outgrowth in distally axotomized dPSN dendrites, especially in the terminal dendritic branches. More importantly, as a group, the dendritic distribution pattern changed from a predominantly dorsal-ventral distribution to an un-biased distribution due to the dorsal-ventral retraction and lateral-medial extension of the dendrites.

Several mechanisms might be involved in the axotomy-induced dendritic plasticity. Firstly, immediate post-injury dendritic outgrowth may be due to an excessive activation of glutamate receptors after injury. Activation of NMDA receptors stimulates dendritic outgrowth by increasing intracellular calcium (Cambray-Deakin and Burgoyne, 1992; Ciccolini et al., 2003; Hirai and Launey, 2000). Secondly, since the dPSN system is a point of convergence for various peripheral inputs, deafferentation caused by transection spinal cord injury can cause rapid dendritic atrophy of central neurons (Burke et al., 1992; Horch et al., 2011). Thirdly, the dendritic plasticity could reflect a compensatory response of the spinal cord to the functional deficits caused by the insult. Injured CST axons rostral to a spinal cord lesion are able to sprout into the gray matter and contact short and long propriospinal neurons. Many of these sprouts arborized in the intermediate laminae of the gray matter (Fouad et al., 2001). The increasing total dendritic length post-injury in our study may reflect this enhanced crosstalk between sprouting CST axons and dPSN dendrites.

The dendritic plasticity observed in the present study occurred within 7 days. This time point is very critical, as injured neurons can either suffer from neuronal degeneration

leading to possible death or they can initiate regenerative responses (Deng et al., 2014; Siebert et al., 2010a). In acute injury, a strong initial inflammatory response as well as early regeneration and cell death responses, occur in the cell body. There is an early up-regulation of several growth factor receptors, as well as a down-regulation of receptors to several factors that inhibit axonal growth which orchestrate the determination of cellular fate. However, different types of neurons may have different responses to injury (Siebert et al., 2010a; Siebert et al., 2010b). Lastly, intrinsic neurotrophic factors might play important roles in injury-induced dendritic outgrowth. Neurotrophic factors regulate the growth and maintenance of dendrites during development (Danzer et al., 2002; Gorski et al., 2003; Morfini et al., 1994; Snider, 1988). Spinal cord injury causes a rapid accumulation of GDNF in the rostral part of the spinal cord (Hara et al., 2012). Although such local increases of endogenous GDNF protein may not be sufficient for nerve regeneration and locomotor improvement, it may play a physiologic role in supporting spinal neurons including propriospinal neurons. Exogenous application of neurotrophic factors can further increase the intrinsic capacity of mature neurons for regrowth and prevent atrophy of axotomized neurons (Bregman et al., 1998; Coumans et al., 2001). The usage of GDNF in our study was based on observations that GDNF supports survival and regeneration of descending propriospinal neurons in adult and neonatal rats after SCI (Baumgartner and Shine, 1998; Deng et al., 2013; Dolbeare and Houle, 2003; Henderson et al., 1994; Li et al., 1995; Oppenheim et al., 1995; Watabe et al., 2000). The expression of GDNF receptors, such as the ligand-binding component (GFR-1) and the transmembrane Ret receptor tyrosine kinase (Trk) c-RET in descending propriospinal neurons, is upregulated after spinal cord injury (Airaksinen et al., 1999; Kokaia et al., 1999; Siebert et al., 2010a). The direct actions of GDNF on the pattern of dendritic arborization in developing neurons and injured neurons via a target-derived

retrograde transport mechanism has been reported, and axonal injury can enhance the transport rate (Kashiba and Senba, 1999; Neet and Campenot, 2001; Vrieseling and Arber, 2006). GDNF treatment is associated with up regulation of II-tubulin or GAP-43 mRNA (Storer et al., 2003; Vrieseling and Arber, 2006).

**Plasticity of dendritic spine-like structures induced by axotomy**

There is a large amount of evidence demonstrating that dendritic spines can change shape, size, and number following various injuries or disease insults (Fiala et al., 2002; Halpain et al., 2005; Tan and Waxman, 2012). Injured glia release their internal stores of glutamate into the environment, which can activate AMPA and NMDA receptors. The downstream signals such as CaMKII, PKC, PKA, MAPK, and Rac1 regulate dendritic spine morphology through actin cytoskeleton reorganization (Tan and Waxman, 2012). The function of these receptors can be enhanced by accumulation of endogenous neurotrophins, such as brain derived neurotrophic factor (BDNF), GDNF, and neurotrophin-3 (NT-3), in the environment after SCI. The number of spines may increase when inputs that contribute to the suppression of synapses are lesioned. The increased number of available axonal inputs may also lead to more spines. The remaining dendrites after injury may express more spines to compensate for the overall loss of afferents input (Ferrer et al., 1988; Sunanda et al., 1995). The dendritic spine remodeling induced by injury is similar to activity-dependent synaptic plasticity in models of learning and memory (Tan and Waxman, 2012). Increased dendritic spine density is observed in neurons that have entered into a more plastic state, indicating availability to form new or stronger synapses (Alvarez and Sabatini, 2007). Similar to stalked (Gobel, 1975), DPSNs in our study with similar dendritic patterns possessed only a few spine-like

structures. After injury and treatment with GDNF, we found increases in the density of spine-like structures which could indicate a more plastic state induced by injury and GDNF therapy. The plasticity in spine-like structures appeared in several orders of dendrites, and with spine-like structures classified as stubby and filopodia mainly contributing to this change. Compared to regular spines, stubby and filopodia spines are considered to be more dynamic, unstable, and immature (Yuste and Bonhoeffer, 2004). Following contusive SCI, increases in dendritic spine length and spine head diameters were observed 1-month post-injury. The change of synaptic density happened mainly in dendritic regions closer to the soma and occurred for all spine types, with a particularly marked redistribution occurring with mushroom spines (Zuo et al., 2005). The differences between our findings and those of Zuo et al. (2005) may be due to the different time periods during which the spines were assessed.

**Neurotransmitter phenotypes of dPSNs**

A major challenge for understanding how locomotor patterns are generated is in determining the intraspinal organization of chemically characterized groups of neurons that converge on different motoneuron pools. DPSNs play an essential role in coordinating rhythmic motor activity between the forelimb and hindlimb during locomotion. From physiological studies in mammals it is known that this coordination involves glycinergic, GABAergic, glutamatergic, and cholinergic spinal cord interneurons (Juvin et al., 2005; Mahmood et al., 2009; Restrepo et al., 2009). Other spinal interneurons, such as CPG neurons and premotor interneurons, have been characterized by neurotransmitter phenotype (Coulon et al., 2011; Ni et al., 2014). Little is known about neurotransmitter phenotype of dPSNs, and our study, for the first time,

describes the relative proportions of putative glycinergic, GABAergic, cholinergic, and glutamatergic dPSNs in the laminae VII of the mid-low thoracic spinal cord. Our results indicate that the majority of FG-labeled dPSN neurons are glutamatergic neurons. This proportion is much higher than that of other premotor neurons (Ni et al., 2014). Therefore, the dPSNs may represent a unique population of spinal interneurons. In the current study, after spinal transection, half of dPSNs died, although this mainly occurred in the segment proximal to the injury. However, the relative proportions of the different neurotransmitters present in dPSNs did not change. The response of dPSNs to the injury therefore mainly depended on the distance of soma from the injury but not the neurotransmitter phenotype (Siebert et al., 2010b). Interestingly, previous literature has provided some clues that link dendritic morphology to specific neurotransmitter phenotypes. Most GABAergic interneurons in the dorsal horn, defined as islet cells or central cells, also have a rostral-caudal distribution of their dendritic arbors (Gobel, 1975; Gobel et al., 1980; Maxwell et al., 2007). The dendrites of cholinergic interneurons in the dorsal horn of mouse spinal cord have been found to have a rostral-caudal preferential distribution and are more developed in spinal parasagittal sections in the rat (Mesnage et al., 2011). The stalk cell, with a dorsal-ventral dendritic distribution, has been proposed to be an exclusively excitatory component (Gobel, 1978; Gobel et al., 1980; Todd and McKenzie, 1989). A majority of glutamatergic cells in the laminae I and II are vertical/stalked cells (Gobel, 1978; Grudt and Perl, 2002; Maxwell et al., 2007). Although in our current study the morphology of dPSNs did not directly relate to the neurotransmitter phenotype, the predominant glutamatergic proportion, and the dorsal-ventral preferential dendritic distribution indicates that this subpopulation of dPSNs may be relatively homogenous. Their dendritic morphology suggests their functions as an integrated and relay unit of dorsal sensory afferent input and ventral brain stem-spinal

output. As in Maxwell et al. (2007), although the neurons in our study displayed a predominant morphology, there were other morphologies that may reflect some subtle functional difference in dPSNs.

**Limitations of the study**

There are some limitations in this study. The number of cells labeled by GFP was small which limited our classification of the neurons. Cells with extensively truncated dendritic trees were excluded from the sample but it is likely that some long dendritic branches lost as a consequence of the slice preparation. Further, it is impossible for us to directly connect our morphological results with specific neurotransmitter types, as the thick sections required for the morphometry would prevent adequate immunohistochemical staining. Future efforts may recruit transgenic animal model and apply tissue transparency technology (Chung et al., 2013; Ni et al., 2014) to address this question.

**Conclusion**

In summary, we characterized patterns of dendritic organization of DPSNs, the important component of intraspinal networks, and investigated the plasticity of the DPSNs dendritic arbor after injury through a monosynaptic transneuronal tracing technique. We demonstrated that dPSNs have a predominant neurotransmitter phenotype and morphological pattern which may be closely related to their function. However several critical questions remain: 1) How do these dPSNs relay the supraspinal commands or integrate sensory afferent signals? 2) How does the plasticity of dPSNs contribute to the functional recovery after spinal cord injury? 3) How can we optimize the potential

therapies that protect PST neurons from early cell death post-axotomy and maximize and sustain the early regenerative response?

# CHAPTER 4

# CONCLUSIONS

The propriospinal system has been shown to be important for normal spinal cord physiology in all mammals studied so far. Especially the dPST attracts more and more attention due to their great potency to contribute to the functional recovery after SCI. Although our work displayed some basic characters of dPST/dPSN in physiologic and pathological conditions, some of which have never been described before, the discoveries yet are still in the infant phase. To explore the greatest beneficial effects of dPST to the repair of the injured spinal cord, many important questions regarding the underlying mechanisms need to be answered.

## Anatomical mechanisms

The ability of regenerated dPST to promote functional recovery following SCI may be attributed to two broad, but parallel mechanisms. The first involves alterations in the strength of existing neural circuits through, for example, changes in neurotransmitter release or postsynaptic receptor density and reorganization of synaptic contact with supraspinal axonal terminal. The second involves sprouting, regeneration of axons and expansion of dendritic fields. The involvement of dPST in anatomical/circuit plasticity is now accepted and proved however indirectly. Our results provide the first direct evidence of post-injury reorganization of its dendritic pattern. However, the biological significance of this change is more important and yet unknown. Is this the random intrinsic reaction to the axotomy or determined by extrinsic signal including enhanced supraspinal motor command and lose of afferent stimulation? Which supraspinal tract

delivered the most influential signal? Although we have successfully promoted large amount of dPST axonal regeneration across the lesion, the functional recovery is still limited in a complete spinal cord transection model. So far most of regenerated axons originated from the neurons proximal to the injury in our model were mainly thoracic dPSNs. We believe that the promotion of more dPSNs regeneration, particularly from those far away from the injury, and the rewiring between supraspinal sprouting and regenerated dPSNs are both important for the functional outcome.

**Molecular mechanisms**

The molecular events that unfold following SCI have long been of research interest; however, the outcome emphasis and interpretation has many facets as intracellular dynamics of signal pathway networking is extremely complex. Critically, to take full advantage of this powerful neuronal subtype, we must further investigate their cellular/intrinsic properties. An important first step in this direction has been taken by Siebert et al.(2010a,b). Their genetic profiling of PNs after SCI has provided a greater understanding of the expression patterns of cell surface receptors for various neurotrophic factors and growth inhibitory molecules in PNs (Siebert et al., 2010a; Siebert et al., 2010b). One important observation was that the expression of GDNF were up-regulated after SCI. This means that the thoracic PNs may develop an increased sensitivity to GDNF (Markus et al., 2002). Our current and previous results confirmed this effect (Deng et al., 2013; Iannotti et al., 2003). However, this is not the end of the story. In Siebert's study, the experiment was not been designed to study the regenerating neurons. After SCI, without interference, the regeneration of propriospinal axons is limited, while in SC transplantation, the regeneration of dPST axons is very significant (Xu et al., 1995b). It is therefore important to uncover the downstream signal pathway which mediated this regeneration. In addition, we do not know yet whether the

different molecular reaction between long dPSN and short dPSN in Siebert's study is due to the different distance between soma and broken axonal stump or the different neuronal subtype. The molecular mechanism of GDNF in promoting axonal regeneration is not very clear. Several molecules of GDNF downstream signal pathway have been reported to be involved in neurite outgrowth(Sariola and Saarma, 2003; Yoong et al., 2009). To maximize the dPSN axonal regeneration, it is very necessary to explore the orchestration among intrinsic capacity of DPSN, SC and GDNF mediated signal pathway.

**Research tools**

The limited study related to dPSN is mainly due to their anatomical complexity, variety of genetic background and relay function. New tools in term of pathway-selective anatomical mapping and functional dissection should be developed for more accurate study. Kinoshita have developed a new technique to temporarily and selectively inactivate spinal interneurons and show that these neurons are required for reach and grasp movements of primate which open a door to study the function of intraspinal components (Kinoshita et al., 2012). A recombinant rabies virus-based method has been applied to label a specific type of long-projecting premotor descending propriospinal neurons in the mouse upper spinal cord that are monosynaptically connected to the motor neurons in the lumbar spinal cord(Ni et al., 2014). This order-controllable tran-synaptic marker can finely dissect the different orders of neurons along the neuronal circuit. Furthermore, the whole tissue transparent imaging methods provide an amazing opportunity to convincingly display the dPSN network under whole spinal scale (Zhang et al., 2014). Likewise, the field of developmental genetics, which has revolutionized our understanding of motor control, will also have a major influence in this field. As such, spinal interneurons have been classified based on transcription factor expression. Interneuron subtypes within these classifications usually express similar physiological

and morphological properties, including neurotransmitter phenotype and projection patterns (Grillner and Jessell, 2009). Clearly, determining the genetic background of PNs that contribute to recovery after SCI would infer a great deal of information regarding their physiology and subsequent role in motor control. Cellular electrophysiology techniques also have an important role to play in further elucidating the function of dPST axons in both physiological spinal circuits and regenerating pathway. The combination of these tools may lead to the identification of key dPSN types that mediate spontaneous functional recovery after incomplete SCI.

**Clinical translation**

Efficacy of promoting functional recovery in SCI through SC transplantation has been widely studied in rodent models with a small number of studies in larger animals, and even fewer in clinical phase I studies in humans (Guest et al., 2013). To translate rodent data into clinical practice, it is essential to perform a comparative analysis between the species. There may be many differences to be aware of, especially the organization of supra-propriospinal system and their species-specific responses to SC transplantation. For example, the distribution pattern of the CST in the cord is different between humans and rodents. In rodents, motor control from the CST is exerted largely upon interneurons in the dorsal horn of the spinal gray matter. However, in carnivores and primates, the number of corticospinal axons increases and corticospinal terminations shift progressively toward the interneurons of the intermediate zone and ventral horn, ultimately forming increasing numbers of synaptic terminations directly onto motoneurons. Humans have more direct cortico-motoneuron synapses than any other species (Schieber, 2007). It has been almost completely unknown so far how dPST

behavior after SCI in primate and human. Any translational studies including SC transplantation should take into account such differences.

**Summary**

Growing evidence suggests that dPST may be one of the most powerful targets for developing new or existing SCI therapies. For example, physical therapy-based approaches could benefit from understanding the types of physical activities that ‘engage’ or ‘recruit’ particular subsets of dPSNs (Harkema et al., 2012). This may lead to entrainment of these networks, increased plasticity, and even greater improvements in motor function as a result of such treatments.

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Xu, X.M., et al., 1999. Regrowth of axons into the distal spinal cord through a Schwann-cell-seeded mini-channel implanted into hemisected adult rat spinal cord. Eur J Neurosci. 11**,** 1723-40.
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Zuo, Y., et al., 2005. Development of long-term dendritic spine stability in diverse regions of cerebral cortex. Neuron. 46**,** 181-9.

**CURRICULUM VITAE**

LINGXIAO DENG

## Education

Ph.D. Anatomy & Cell Biology

2010-2015

Indiana University, Indiana University-Purdue University-Indianapolis

Advisor: Xiao-Ming Xu. M.D., Ph.D.

M.S. Orthopaedics

2003-2006

Fujian Medical University

Advisor: Jian-Hua Lin

M.D Medicine

1993-1998

Fujian Medical University

**Awards & Honors**

2008 “TOP 16 STUDENT POSTER COMPETITION FINALISTS & AWARDS” in The 26th Annual National Neurotrauma Symposium (United Stated).

2010 University Fellowship-Indiana University

2012 Honorable Mentioned (in 2011 Indiana University Student Research Competition) Sigma Xi Scientific Research Society.

2013 Indy Society of Neuroscience 1st place poster competition. Title of Poster: “Combinatory strategy among aligned filament, extracellular matrix molecule, Schwann cell and GDNF in adult rat thoracic spinal cord hemi-section injury”

2014 Travel Award winner in 2014 American Association of Anatomists Annual Meeting at Experimental Biology

2014 “Outstanding Abstract” at the joint symposium of 4th International Neural Regeneration Symposium (INRS2014) 6th International Spinal Cord Injury Treatment and Trials Symposium (ISCITT) 9th Asia Pacific Symposium on Neural Regeneration (APSNR). Abstract entitled "Characterization of dendritic morphology and neurotransmitter distribution of thoracic descending propriospinal neurons following complete spinal cord transection and GDNF treatment"

2015 “Student competition finalists & awards” the 33rd Annual Symposium of the National Neurotrauma Society in Santa Fe, New Mexico

**Professional Appointments**

Orthopaedist Surgeon

1998-2006

The Orthopaedics department,

The First Affiliated Hospital of Fujian Medical University

**Conferences Posters& Presentations**

2014. **Lingxiao Deng**, Yiwen Ruan, Chen Chen, Christian Corbin Frye, Wenhui Xiong, Xiaoming Jin, and Xiao-Ming Xu, Characterization of dendritic morphology and neurotransmitter distribution of thoracic descending propriospinal neurons in sham, axonatomy injury and GDNF treatment. Poster Presentation, the joint symposium of 4th International Neural Regeneration Symposium (INRS2014) 6th International Spinal Cord Injury Treatment and Trials Symposium (ISCITT) 9th Asia Pacific Symposium on Neural Regeneration (APSNR).

2014. **Ling-Xiao Deng**, Nai-Kui Liu, Shuang-Ni Yang, Xuejun Wen, Xiao-Ming Xu, Laminin-coated multifilament bridges, Schwann cell grafting, and GDNF administration promote directional axonal growth following a rat thoracic spinal cord hemisection, Poster Presentation, 2014 American Association of Anatomists Annual Meeting at Experimental Biology.

2013. **Ling-Xiao Deng**, Nai-Kui Liu, Shuang-Ni Yang, Xuejun Wen, Xiao-Ming Xu, Combinatory strategy among aligned filament, extracellular matrix molecule, Schwann cell and GDNF in adult rat thoracic spinal cord hemi-section injury, Poster Presentation, 2014 Indy Society of Neuroscience

2012. **Lingxiao Deng**, Ping Deng, Yiwen Ruan, Zao Cheng Xu, Naikui Liu, Geogre M, Smith and Xiao-Ming Xu, A novel growth-promoting pathway formed by GDNF-overexpressing Schwann cells promotes propriospinal axonal regeneration, synapse formation, and partial recovery of function after spinal cord injury. Poster presentation, Indiana University Student Research Competition, Sigma Xi Scientific Research Society

2008. **Lingxiao Deng**, Jianguo Hu, Naikui Liu, George Smith, Xiao-Ming Xu, GDNF modifies astrogliotic responses at graft-host interfaces allowing robust axonal regeneration into Schwann cell-seeded guidance channels grafted into hemisected adult rat spinal cords. The 26th Annual National Neurotrauma Symposium (United Stated).

**Publications**

Walker CL, Wang X, Bullis C, Liu NK, Lu Q, Fry C, **Deng L**, Xu XM. Biphasic bisperoxovanadium administration and Schwann cell transplantation for repair after cervical contusive spinal cord injury. *Experimental Neurology.* 2015 Feb;264:163-72. doi: 10.1016/j.expneurol.2014.12.002. Epub 2014 Dec 12. (PMID: 25510318)

**Ling-Xiao Deng**, Chandler Walker, Xiao-Ming Xu, Schwann Cell-Mediated Axonal Regeneration in the Central Nervous System, Book "Neuronal regeneration", Chapter , Science Press and Elsevier (in press)

Liu, Nai-Kui; **Deng, Ling-Xiao**; Zhang, Yi-Ping; Lu, Qing-Bo; Wang, Xiao-Fei; Hu, Jian-Guo; Oakes, Eddie; Bonventre:, Joseph; Shields, Christopher; Xu, Xiao-Ming, cPLA2 protein as a novel therapeutic target for spinal cord injury, Annals of Neurology 2014: an official journal of the American Neurological Association and the Child Neurology Society. DOI: 10.1002/ana.24134. (impact factor 11.6)

**Ling-Xiao Deng**, Chandler Walker, Xiao-Ming Xu, Schwann cell transplantation mediated propriospinal axonal regeneration. Brain Research 2014 http://dx.doi.org/10.1016/j.brainres.2014.09.038 (**Invited review**)

**Lingxiao Deng**, Ping Deng, Yiwen Ruan, Zao Cheng Xu, Naikui Liu, Geogre M, Smith and Xiao-Ming Xu, A novel growth-promoting pathway formed by GDNF-overexpressing Schwann cells promotes propriospinal axonal regeneration, synapse formation, and partial recovery of function after spinal cord injury, (2013), The Journal of Neuroscience, : the official journal of the Society for Neuroscience 33, 5655-5667. (impact factor 7.9)

Jianguo Hu, Xiaofei Wang, **Lingxiao Deng**, Naikui Liu, Xiang Gao, Jinhui Chen, Feng C Zhou, Xiao-Ming Xu, Co-transplantation of Glial restricted precursor cells and Schwann cells promotes functional recovery after spinal cord injury, Cell Transplant, 2013 Jan.

**Ling-Xiao Deng**, Chandler Walker, Nai-Kui Liu, Shuang-Ni Yang, Xuejun Wen, Xiao-Ming Xu, Effect of filament density and extracellular matrix molecule precoating on neurite outgrowth on multifilament entubulation bridging device in vivo April 2014 The FASEB Journal vol. 28 no. 1 Supplement 729.2

Ying Yin, Weifeng Sun, Bin Zhang, Hua Cui**, Lingxiao Deng**, Ping Xie, Jian Zou, Effects of combining methylprednisolone with rolipram on functional recovery in adult rats following spinal cord injury.(2013) Neurochem Int Mar 15;62(7):903-912

Jian-Guo Hu, Xing-Jun Wu, Yi-Fan Feng, Gang-ming Xi, **Ling-Xiao Deng**, Zhen-Huan Wang, Rui Wang, Lin Shen, Jian-Sheng Zhou, He-Zuo Lü, The Molecular Events Involved in Oligodendrocyte Precursor Cell Proliferation Induced by the Conditioned Medium from B104 Neuroblastoma Cells. Neurochemical research.2013January 1;38:601-609

**Ling-Xiao Deng**, Jian-Guo Hu, Nai-Kui Liu, George Smith, Xuejun Wen, Xiao-Ming Xu, GDNF modifies astrogliotic responses at graft-host interfaces allowing robust axonal regeneration into Schwann cell-seeded guidance channels grafted into hemisected adult rat spinal cords Exp Neurol, 2011 Jun;229(2):238-250.(**Cover paper**)

**Lingxiao Deng**, Xiaofei Wang, Chandler Walker, Xiaoming Xu, Animal Models of Acute Neurological Injuries II, Humana Press, 2009,Chapter 38,39

W. Lee Titsworth , Xiaoxin Cheng, Yan Ke , **Lingxiao Deng** , Kenneth A. Burckardt , Chris Pendleton , Nai-Kui Liu, Hui Shao , Qi-Lin Cao, Xiao-Ming Xu, Differential

expression of sPLA(2) following spinal cord injury and a functional role for sPLA(2)-IIA in mediating oligodendrocyte death, Glia. 2009 Nov 1;57(14):1521-37

Hu J, **Deng L**,Wang X, Xu XM, Effects of extracellular matrix molecules on the growth properties of oligodendrocyte progenitor cells in vitro.J Neurosci Res. 2009 Oct;87(13):2854-62

Jian-Hua Lin, **Ling-Xiao Deng**, Zhao-Yang Wu, Lei Chen, Li Zhang, Pilose antler polypeptides promote chondrocyte proliferation via the tyrosine kinase signaling pathway, Journal of Occupational Medicine and Toxicology. 2011,6:27

Zhu Xia, Lin Jianhua, **Deng Lingxiao**, A preliminary clinical outcomes of Paclitaxel in treating soft-tissue sarcoma, Chinese Journal of Bone Tumor and Bone Disease (in Chinese), 2008-04

**Deng Lingxiao**, Expression of c-fos gene associated with the proliferation and differentiation of cartilage, Chinese Journal of Clinical Rehabilitation (in chinese), 2006

Lin Jianhua, **Deng Lingxiao**, The mechanism of antler polypeptides promoting chondrocyte proliferation, Chinese Journal of Orthopaedic Trauma( in chinese), 2007

Lin Jianhua, CHEN Xiaodong, **Deng Lingxiao**, Observation of replicative senescence of rat chondrocytes in vitro, Chinese Journal OF Reparative And Peconstructive Surgery( in chinese), 2007

**Academic services**

Invited as a reviewer for:

1) International Journal of Neuroscience

2) Cell transplantation

3) Journal of Neurorestoratology

4) Journal of Pain Research

5) Plos One

6) JSM Neurosurgery and spine

7) Biomedical and Environmental sciences

8) Neural Negeneration Research

9) International Journal of Ophthalmology

10) Journal of Visualized Experiments

11) Chinese Journal of Orthopaedic Trauma

13) Chinese Journal of Reparative and Reconstructive Surgery

## Professional Societies

2012-present Member, Society for Neuroscience

2012-present Member, Sigma Xi, The scientific research society

2013-present Member, American Association of Anatomists