Universitat Autònoma de Barcelona

Changing the Intrinsic Growth Capacity of motor and sensory neurons to promote axonal growth after injury

The role of FGF2 in axonal regeneration

Presented by

Ilary Allodi

Group of Neuroplasticity and regeneration,
Faculty of Medicine, July 2012

axregen
axonal regeneration, plasticity and stem cells

MARIE CURIE ACTIONS

Changing the Intrinsic Growth Capacity of motor and sensory neurons to promote axonal growth after injury

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Ilary Allodi

ACADEMIC DISSERTATION

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Supervised by

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Dimenticare chi siamo, alle volte,

ci permette di inciampare in una nuova coscienza;

ridere del presente

ed imbastire nuovi orizzonti.

IA

To M.P. who turn me into a researcher

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INTRODUCTION

SPECIFICITY OF PERIPHERAL NERVE REGENERATION: INTERACTIONS AT THE AXON LEVEL

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ABSTRACT

Peripheral nerves injuries result in paralysis, anesthesia and lack of autonomic control of the affected body areas. After injury, axons distal to the lesion are disconnected from the neuronal body and degenerate, leading to denervation of the peripheral organs. Wallerian degeneration creates a microenvironment distal to the injury site that supports axonal regrowth, while the neuron body changes in phenotype to promote axonal regeneration. The significance of axonal regeneration is to replace the degenerated distal nerve segment, and achieve reinnervation of target organs and restitution of their functions. However, axonal regeneration does not always allows for adequate functional recovery, so that after a peripheral nerve injury, patients do not recover normal motor control and fine sensibility. The lack of specificity of nerve regeneration, in terms of motor and sensory axons regrowth, pathfinding and target reinnervation, is one the main shortcomings for recovery. Key factors for successful axonal regeneration include the intrinsic changes that neurons suffer to switch their transmitter state to a pro-regenerative state and the environment that the axons find distal to the lesion site. The molecular mechanisms implicated in axonal regeneration and pathfinding after injury are complex, and take into account the cross-talk between axons and glial cells, neurotrophic factors, extracellular matrix molecules and their receptors. The aim of this review is to look at those interactions, trying to understand if some of these molecular factors are specific for motor and sensory neuron growth, and provide the basic knowledge for potential strategies to enhance and guide axonal regeneration and reinnervation of adequate target organs.

Key words: axonal regeneration, peripheral nerve, Schwann cell, neurotrophic factors, extracellular matrix, motor neuron, sensory neuron.

1. INTRODUCTION

Injuries to the peripheral nerves result in loss of motor, sensory and autonomic functions conveyed by the involved nerves. After a nerve injury, transected fibers distal to the lesion are disconnected from the neuronal body and undergo Wallerian degeneration, thus, leaving the peripheral organs denervated. In parallel, a series of molecular and cellular changes known as retrograde reaction and chromatolysis occur at the soma of axotomized neurons. Wallerian degeneration serves to create a microenvironment distal to the injury site that favors axonal regrowth, while retrograde reaction leads to metabolic changes necessary for regeneration and axonal elongation. The functional significance of axonal regeneration is to replace the distal nerve segment lost during degeneration, allowing reinnervation of target organs and restitution of their corresponding functions. Through this sequence of events, injured axons of the peripheral nervous system are able to regenerate and reinnervate their target organs.

After axonotmesis, where the connective sheaths of the nerve are preserved and only the axons are injured, functional recovery is usually good. In contrast, after neurotmesis (nerve transection), when the endoneurial tubes loss their continuity, axons are often misdirected and reinnervate incorrect target organs even if refined repair is applied (Bodine-Fowler *et al.*, 1997; Molander and Aldskogius, 1992; Valero-Cabre and Navarro, 2002). Thus, although the amount of axonal regeneration can be considerably high, the lack of selectivity of axon-target reconnection leads to a poor functional recovery. Indeed, only a low percentage of adult patients regain normal function after complete transection and surgical repair of a major peripheral nerve. Appropriate and inappropriate targets can be reinnervated by axotomized neurons. For example, efferent motor axons may be misdirected to sensory end organs, and cutaneous afferents to motor endplates or sensory end organs of inappropriate modality or location. Thus, function will be degraded or permanently lost, depending on the severity of mismatch. As surgical nerve repair techniques cannot be further refined, there is a need for new and improved strategies to enhance specific axon regeneration following nerve injuries (Lundborg, 2003). Tissue specificity, i.e. preferential growth of axons towards a

distal nerve stump rather than to other tissues, has been documented (Politis *et al.*, 1982). Fascicular specificity, the preferential regeneration through the original nerve fascicle, was suggested in early studies but not confirmed by further investigations. Target organ specificity, or adequate reinnervation of each type of end organ (muscle, sensory receptor, …) by axons that originally served that organ, is less than perfect, although preferential reinnervation has been observed (Figure 1). However, the mechanisms through which motor and sensory axons specifically reinnervate their corresponding targets are still poorly understood. Some authors defend a preferential muscle reinnervation by motor axons, the so-called preferential motor reinnervation (Brushart, 1988, 1993; Brushart *et al.*, 1998). Pruning of the axons that reinnervated an erroneous target may contribute to improve the specificity of regeneration (Madison *et al.*, 1996). Expression of the peptide L2/HNK1 (Martini *et al.*, 1992) in motor but not sensory Schwann cells, or the presence of NCAM and polisyalic acid in the regenerative motor axons (Franz *et al.*, 2005) can also mediate this preferential motor reinnervation, although other authors argue that the key point for preferential attraction of axons to their targets is the expression of trophic factors by the own target organ and the distal stump (Madison *et al.*, 2007).

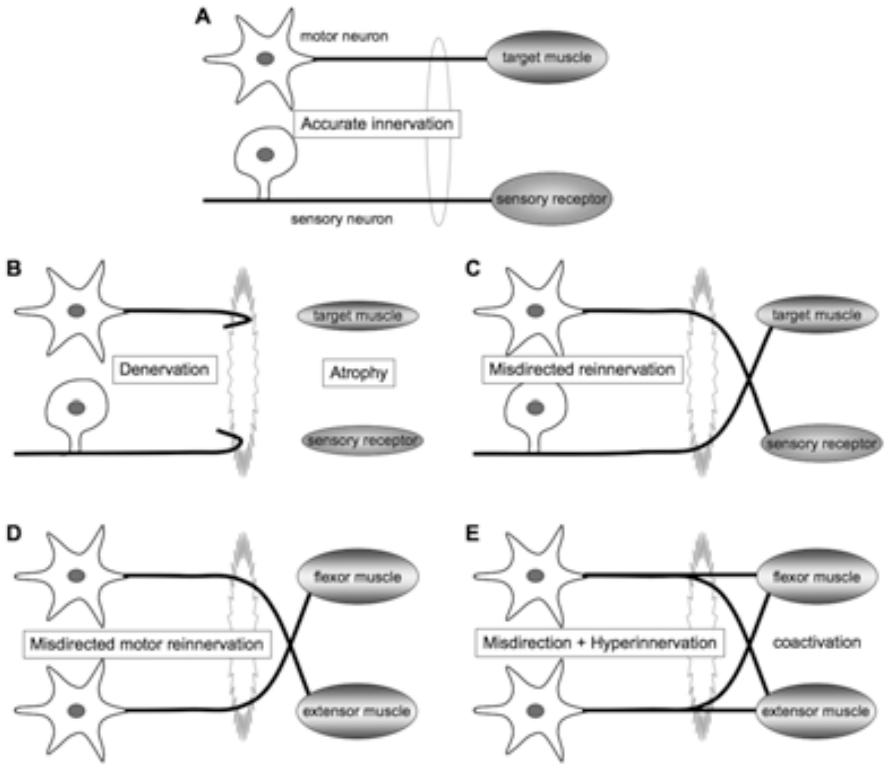


Fig. 1. Problems derived from the lack of specificity of peripheral nerve regeneration, which disrupt the normal accurate innervation of motor and sensory targets (**A)**. **B**: Abortive regeneration leads to chronic target denervation and atrophy. **C**: Misdirected regeneration of axons to inappropriate targets. **D**: Misdirected regeneration of axons to functionally inappropriate muscle targets. **E**: Hyperinnervation of muscle targets by regenerating axons of several different neurons.

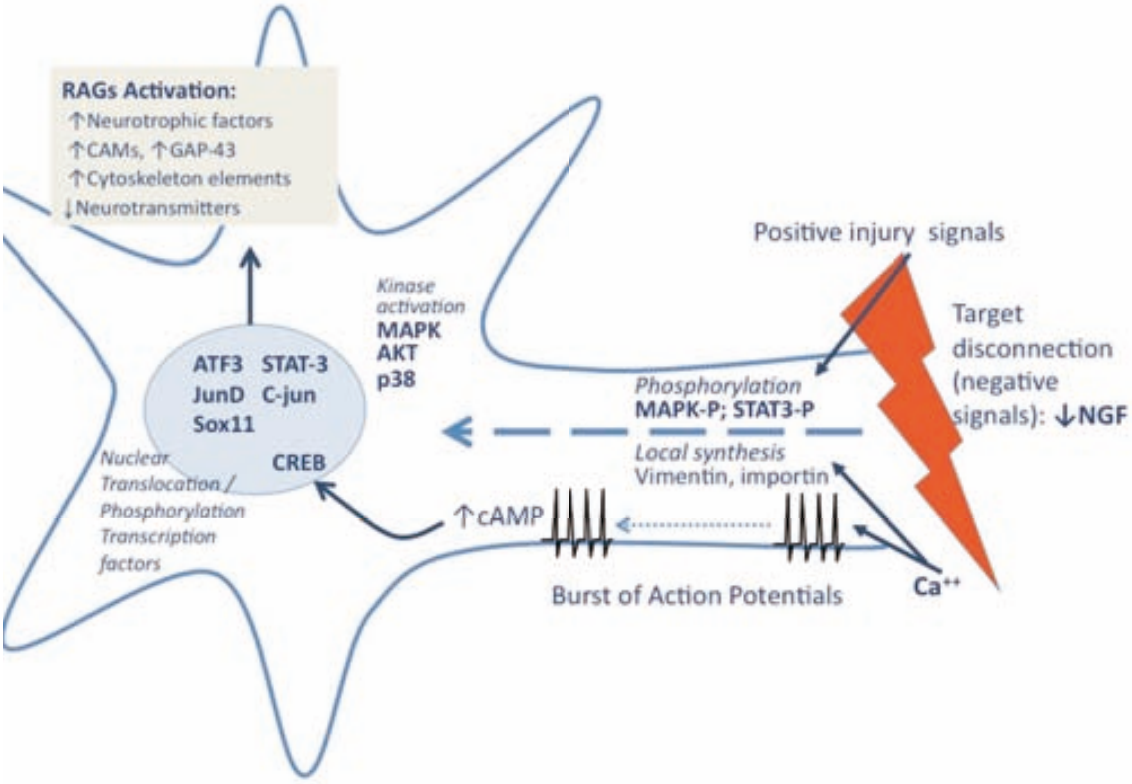
Since the mechanisms that control axon regeneration are diverse and complex, it is important to take all of them into account before designing new strategies that may improve specific reinnervation. After the lesion, the injured neurons suffer important changes to switch their neuro-transmitter state to a pro-regenerative state (Plunet *et al.*, 2002). The environment that the axons find distal to the lesion site is also determinant for successful regeneration. Neurotrophic factors secreted by Schwann cells or target organs modulate axonal attraction, and the creation of a favorable pathway in the distal nerve stump allows the growing axons to adhere and elongate. The cross-talk between axons and their immediate environment is thus essential in determining the intrinsic capacities for regeneration and reinnervation. Therefore, it is important to deeper understand the molecular interactions between neurotrophic factors, their receptors, adhesion molecules and extracellular matrix elements in the regenerative process. These clues are involved in cell chemotaxis, migration and axonal elongation, and their signaling pathways can promote cell survival or apoptosis. The focus of this work is to review those interactions, trying to understand whether any factors can be specific for motor and sensory axons, and how can they help to induce specific reinnervation of target organs.

2. NEURONAL CHANGES TRIGGERED BY AXONAL INJURY

Neurons have an intrinsic growth capacity during the embryonic stage, which is repressed upon the adult transition to allow proper synaptic development. However, after axotomy, neurons switch again from a transmission state to a growth state, with changes in the expression of genes that encode for transcription factors (Herdegen *et al.*, 1991; Leah *et al.*, 1991; Schwaiger *et al.*, 2000), which in turn regulate the expression of genes involved in cell survival and

neurite outgrowth (for reviews see Navarro *et al.*, 2007; Raivich and Makwana, 2007). This switch is essential in the capacity of neurons to regenerate; therefore the neuronal reaction is stronger after peripheral than central injuries, in which regeneration is poor and limited (Rossi *et al.*, 2007). The increased intrinsic growth capacity of injured peripheral neurons is manifested experimentally by the conditioning lesion paradigm (McQuarrie *et al.*, 1977). Axotomy of a peripheral neuron previous to the test lesion, "primes" the neuron, switches it on to a regenerative state and, thus, it will regenerate faster after receiving the second injury. Since the effect may require time for gene transcription (Smith and Skene, 1997), the conditioning lesion is effective if applied from 2 to 14 days before the test lesion.

Signals responsible for the initiation and maintenance of the regenerative neuronal response include a variety of mechanisms acting at sequential time phases (Figure 2). The burst of action potentials initiated at the injury site and the disruption of the axonal transport are key points to trigger the growth capacity of neurons after axotomy (for reviews see Abe and Cavalli, 2008; Hanz and Fainzilber, 2006; Rishal and Fainzilber, 2010). At the lesion site, entrance of extracellular sodium and calcium to the injured axoplasm triggers action potentials that will be the first signals to warn the soma of the axonal injury and will provoke chromatolytic changes in the cell body (Mandolesi *et al.*, 2004) mediated by rapid elevation of intracellular calcium and cAMP. On the other hand, the injury also disrupts the retrograde transport flow of signals from normal innervated targets, providing negative signals that inform the soma of the disconnection. Therefore, the reconnection has to be linked to recovery of the lost signals to allow proper synaptic development. For example, transport of endogenous nerve growth factor (NGF) decreases after injury (Raivich *et al.*, 1991) and it is considered an important negative signal to inform the neuron that its axon has been disconnected from its target. For this reason, artificial application of NGF to axotomized sensory neurons interferes with the axotomy-like changes observed in the cell body and delays axonal regrowth (Gold, 1997). Finally, there are also positive signals arriving to the soma by retrograde transport. Activated proteins, termed "positive injury signals", are endogenous axoplasmic proteins that undergo post-translational modifications at the lesion site upon axotomy, and then


RAGs Activation:
↑Neurotrophic factors
↑CAMs, ↑GAP-43
↑Cytoskeleton elements
↓Neurotransmitters
Positive injury signals
Target disconnection (negative signals): ↓NGF
Kinase activation
MAPK
AKT
p38
ATF3
STAT-3
JunD
C-jun
Sox11
CREB
Phosphorylation
MAPK-P; STAT3-P
Local synthesis
Vimentin, importin
Nuclear Translocation / Phosphorylation Transcription factors
↑cAMP
Burst of Action Potentials
Ca++

Regeneration Associated Genes (RAGs), inducing the increased production of GAP43 and BDNF among other factors.

The different signals induced by the axonal injury lead to changes at the neuronal body. Among them, increase of intraneuronal cAMP after injury seems crucial to guarantee successful regeneration of the neurons even in non-permissive environments. cAMP can regulate axon attraction or repulsion by guidance cues from the environment (Domeniconi and Filbin, 2005; Filbin, 2003). For example, low levels of cAMP convert the attractive effects of NGF and brain derived neurotrophic factor (BDNF) into repulsion. In the case of NT3, levels of cGMP but not cAMP, regulate its responsiveness (Song *et al.*, 1997). On the other hand, the decrease in cAMP levels in mature neurons when compared to embryonic or postnatal cells, explains the lower capacity of mature neurons to regenerate (Cai *et al.*, 2001). Downstream effectors of cAMP are polyamines, with a strong stimulatory effect on neurite outgrowth both in vitro and in vivo (Wong and Mattox, 1991). Another downstream effector transducing the neuronal changes due to cAMP is the mammalian sterile 20-like kinase-3b (Mst3b), a serine protein-kinase which is phosphorylated by PKA (Zhou *et al.*, 2000). The in vivo inactivation of Mst3b reduces the density of regenerating sensory axons a few days after axotomy (Lorber *et al.*, 2009).

A key point in the changes that neurons suffer after injury is the activation of mitogen associated protein kinase cascades. Some activated intracellular signaling enzymes, such as MAPK, JNK and ERK are known to play an important role in triggering neurite outgrowth (Lindwall and Kanje, 2005; Perlson *et al.*, 2005), whereas other kinases, as AKT and p38, have been recently described to exert early signaling responses (Michaelevski *et al.*, 2010), although their role is still controversial. In parallel, there is nuclear localization and phosphorylation of different transcription factors, such as c-Jun, junD, ATF3, Sox11 and STAT3 (Fujitani *et al.*, 2004; Jankowski *et al.*, 2009; Mason *et al.*, 2011; Nadeau *et al.*, 2005; Schwaiger *et al.*, 2000). Activation of these transcription factors will lead to changes in gene expression of the injured and regenerating neurons, with an increase of actin, growth associated tubulin isotypes and growth associated

protein GAP-43, and a decrease of neurofilament proteins (Hoffman and Cleveland, 1988; Hoffman *et al.*, 1987; Skene and Willard, 1981), ion channels and proteins involved in the neurotransmission machinery (Costigan *et al.*, 2002; Jankowski *et al.*, 2009; Mason *et al.*, 2003; Tsujino *et al.*, 2000).

Another possible regulator of the JAK/STAT pathway recently described that promotes axon regeneration is mTOR. Activation of the mTOR pathway is also essential for the growth capacity of peripheral neurons after injury. Blockade of the upstream inhibitors of mTOR, TSC1/TSC2 complex, mimics the conditioning lesion effect (Abe *et al.*, 2010; Hay and Sonenberg, 2004; Park *et al.*, 2008). In contrast, mTOR is not activated in central neurons, a fact that may contribute to their reduced regenerative response, since forced activation of mTOR in retinal ganglion cells or corticospinal neurons promotes their axonal growth (Liu *et al.*, 2010; Park *et al.*, 2008). The control of protein synthesis by mTOR is important in the ability of neurons to regenerate, however its expression after injury seems to be transient and it returns to baseline levels 3-4 days after injury, suggesting that a prolonged expression of mTOR might enhance axonal regeneration. Indeed, mTOR over-expression increases the levels of GAP43 after injury, but a chronic over-expression of mTOR can affect the accuracy of target reinnervation (Abe *et al.*, 2010). Interestingly, another upstream inhibitor of mTOR, PTEN, seems to play an important role in the intrinsic capacity of peripheral neurons to regenerate, since its inhibition promotes axon growth independently of mTOR activation (Christie *et al.*, 2010).

Some of the genes over-expressed after injury by neurons are related to increased production of trophic factors, that can act in an autocrine or a paracrine manner. After axotomy, both motor and sensory neurons increase the expression of BDNF (Kashiba and Senba, 1999; Kobayashi *et al.*, 1996) and FGF-2 (Grothe and Unsicker, 1992; Huber *et al.*, 1997; Madiai *et al.*, 2003), NGF is only increased in sensory neurons (Gu *et al.*, 1997; Shen *et al.*, 1999), and the expression of NT-3 does not change (Shen *et al.*, 1999). The expression of different receptors for neurotrophic factors is also differentially regulated after axotomy (see below).

Another relevant change in the neuronal phenotype after axotomy is the downregulation of neurotransmitters and transmitter-related proteins. However,

there are also marked changes in the expression of neuropeptides in the axotomized neurons, which vary in different types of neurons and contribute to neural signaling after injury (Hökfelt *et al.*, 2000). Thus, motor neurons characteristically show a rapid and long-lasting increase in calcitonin gene-related peptide (CGRP) immunoreactivity (Borke *et al.*, 1993), whereas CGRP, as well as substance P (SP) are down-regulated after injury in small and medium size primary sensory neurons (Dumoulin *et al.*, 1991; Noguchi *et al.*, 1990; Shadiack *et al.*, 2001; Villar *et al.*, 1991), due to interruption of target-derived NGF to the neuronal body (Eriksson *et al.*, 1997). However, CGRP and SP appear to increase at the regenerating front, where they are synthetized at the tip of the growing axons (Li *et al.*, 2004; Toth *et al.*, 2009; Zheng et al. 2008). Blocking CGRP synthesis at the axonal level with siRNA and blocking its receptor on Schwann cells were found to impair nerve regeneration and Schwann cell recruitment during neurite growth (Raivich *et al.*, 1992; Toth *et al.*, 2009). Other peptides such as vasoactive intestinal polypeptide, galanin and neuropeptide Y, which are normally expressed at low levels in sensory neurons, are markedly increased in those neurons (Villar *et al.*, 1989; Villar *et al.*, 1991; Wakisaka *et al.*, 1991), and transiently upregulated also in motor neurons (Zhang *et al.*, 1993) after axotomy. These changes seem to be involved in regenerative and/or compensatory processes following nerve damage.

3. BIOLOGY OF AXONAL ELONGATION: THE GROWTH CONE

The regeneration of a sectioned axon involves the transformation of a stable axonal segment into a highly motile tip, called growth cone, that senses the surrounding environment and leads the elongation of the regenerating axon. It is important that growth cones elongate following the endoneurial tubes of the distal nerve to eventually reinnervate their peripheral target organs. When growth cones do not reach the distal stump they may sprout within the proximal stump forming a neuroma. In the distal stump, denervated Schwann cells proliferate on the basal membrane of the endoneurial tubes and form columns, the so-called bands of Büngner, over which growth cones advance (Bunge *et al.*, 1982). Each regenerating axon may give rise initially to over 10 axonal sprouts (Witzel *et al.*, 2005), but the number of branches decreases with time in the distal segment, as sprouts that do not make peripheral connections undergo atrophy and eventually disappear,

whereas axons reinnervating target organs mature and enlarge in size. Nevertheless, even several months after injury, regenerating neurons may support multiple sprouts, branching from the level of the lesion, in the distal nerve stump. This fact explains the higher counts of nerve fibers in the distal stump compared to the real number of proximal fibers that regenerate across the lesion (Horch and Lisney, 1981; Jenq and Coggeshall, 1985; Mackinnon *et al.*, 1991).

The orientation of the advancing tip is guided by gradients of neurotrophic and neurotropic factors produced mainly by non-neuronal cells (Figure 3). This process is also known as chemotaxis. The molecular cues that the regenerating axon will find at the lesion site and into the distal nerve stump can have both chemoattractive and chemorepulsive properties, and can be diffusible or membrane-bound. Indeed, the different signals may create a permissive environment for axonal growth or constitute a molecular barrier that impairs axonal elongation (for reviews see Dickson, 2002; Mueller, 1999; Song and Poo, 1999; Tessier-Lavigne and Goodman, 1996; Wen and Zheng, 2006).

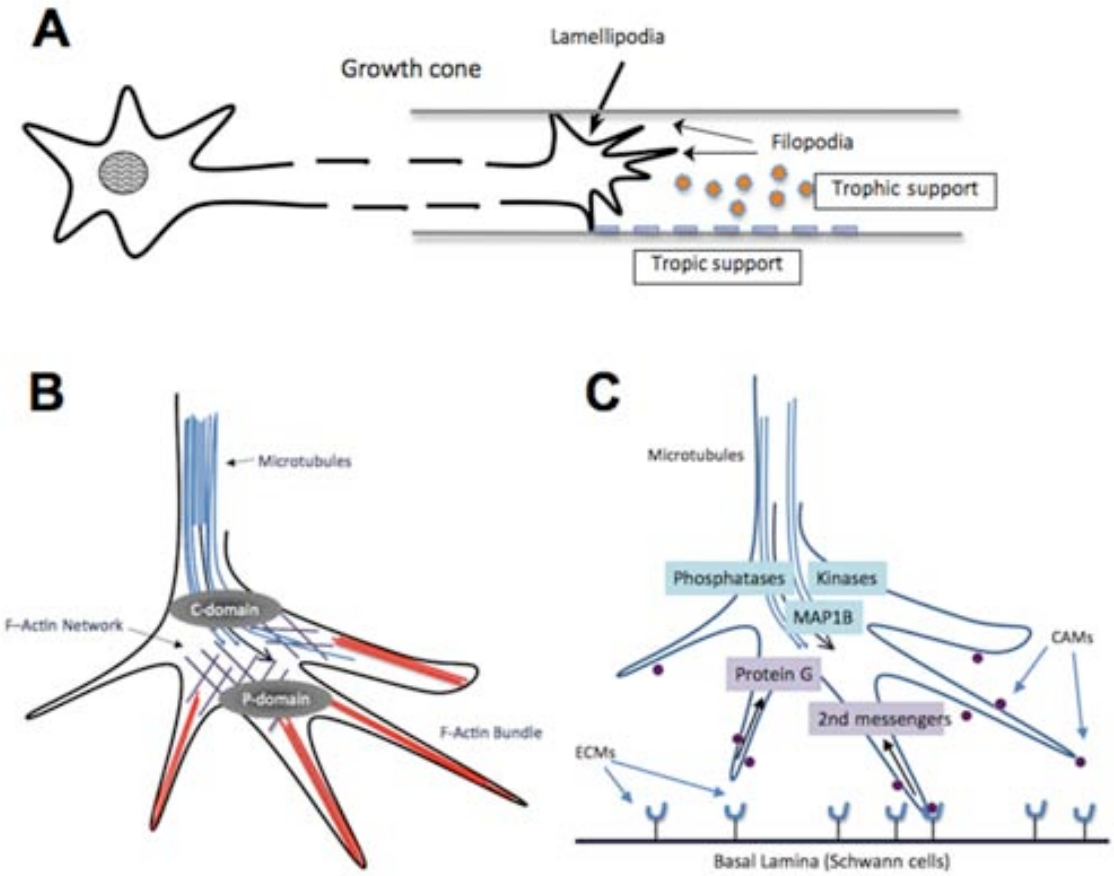


Fig. 3. **A**. Neurotrophic and neurotropic factors are needed during peripheral nerve regeneration and determine the response of the growth cone, which extends lamellipodia and filopodia depending on the different attractive and repulsive gradients present in the extracellular environment. Neurotrophic factors are diffusible molecules like NGF, GDNF, BDNF or NT3, while neurotropic factors are substrate-bound cues such as laminin, fibronectin and collagen or cell adhesion molecules, that maintain hemophilic interactions. **B**. Filopodia and lamellipodia are the mobile path-finder parts of the growth cone. When filopodia encounter a permissive substrate, growth cone receptors bind to ECM molecules. These receptors are coupled to F-actin fibers that re-organize the cytoskeleton and lead growth cone protrusion. F-actin polymerization pushes the membrane forward at the protrusion domain, while microtubules already stabilized at the central domain localize and polymerize depending on actin network. **C**. The extracellular matrix influences the state of the regenerating neurons and determines the response of the growth cone. Interactions between substrates and their receptors change the intracellular levels of second messengers, as for example cAMP, in the growth cone. Interactions between ligands and CAMs activate small GTPases that play a fundamental role in growth cone motility and cytoskeleton remodeling.

The growth cone advances by three main steps: protrusion, engorgement and consolidation (Mortimer *et al.*, 2008). The growth cone shows three functionally specific regions: a central domain (C-domain), a transition zone (T-zone) and a peripheral domain (P-domain) (Bouquet and Nothias, 2007). The T-zone is placed between the microtubule-rich C-domain and the actin-rich P-domain. Actin polimerization triggers the protrusion of growth cone membrane and this facilitates the delivery of new microtuble segments at the P-domain, as well as their stabilization along actin bundles. This allows the displacement of the C-domain forward (engorgement) and the further consolidation of the nascent axon. Addition of soluble tubulin into the distal ends of microtubules that enter the C-domain leads to microtubule polymerization, whereas high concentrations of myosin at the T-zone lead to the contraction of the actin network, inducing formation of an actin-filament arc. The most distal part of the growth cone, the P-domain bears lamellipodia, membranous protrusions from which extend several expansions called filopodia, mainly formed by actin filament bundles. Equilibrium between actin polymerization and depolymerization generates constant protrusion forces, thus allowing filopodia to act as the "tentacles" of the growth cone, pulling and pushing to explore the microenvironment.

Cytoskeleton proteins of regenerating peripheral axons undergo qualitative and quantitative changes similar to those of growing axons during development (Hoffman and Cleveland, 1988). Transection of motor and sensory fibers increases the levels of tubulin isoforms, actin and peripherin, and decreases the levels of neurofilaments that regulate axon caliber. The enhanced metabolic requirements of the growing axons are sustained by an increased synthesis of different components at the neuron body that will be anterogradely transported to the fiber tip (Hoffman and Lasek, 1980; McQuarrie *et al.*, 1989), although there is also local synthesis and degradation of proteins in the axon (Verma *et al.*, 2005; Willis and Twiss, 2005). During the final consolidation phase, new microtubules are assembled in the C-domain, probably mediated by the activity of microtubule- and actin-associated proteins.

Factors that may be involved in the control of microtubule assembly in growth cones include calcium, cyclic nucleotides, post-translational modification of tubulin and microtubule-associated proteins (MAPs) (Gordon-Weeks and Mansfield, 1992). When the growth cone has moved on and microtubules have become incorporated into the neurite cytoskeleton, post-translational modifications of tubulin increase the stability of microtubules to depolymerization (Schulze *et al.*, 1987). The stabilization of microtubules in a permanent cytoskeleton is also dependent on a set of structural proteins that can be classified as MAP (MAP1-MAP5) and tau proteins; both classes induce polymerization of tubulin and remain bound to the newly formed microtubules. Tau proteins bind to several tubulin molecules simultaneously, thereby enhancing tubulin polymerization. MAPs act similarly, but they can cross-link the microtubule to other cell components. All known MAPs are highly phosphorylated proteins, and thus substrates for phosphorylating enzymes and GAPs, particularly GAP-43. GAP-43 expression is closely related with periods of active growth cone function, and its levels are dramatically increased within regenerating nerves after axonal injury (Benowitz and Lewis, 1983; Skene, 1989).

The contribution of cytoskeletal proteins to axonal regeneration is crucial (Lewis and Bridgman, 1992). In fact, despite the diversity of signaling pathways that can lead to or inhibit axonal growth, ultimately any factor that alters the

growth capacity of neurons implies changes in the cytoskeleton components (for reviews see Lowery and Van Vactor, 2009; Tang, 2003). The small GTPases of the Rho family (being RhoA, Rac1and Cdc42 the most well-known) integrate the upstream signaling cues for the downstream cytoskeleton rearrangements in a spatial-specific manner, reproducing at the cytoplasmatic level, the extracellular environmental changes. Receptor complexes present at the growth cone can recruit GTPases, which are needed to control the contractile capability dependent on actin-myosin interactions (Luo, 2002). Although numerous signalling transduction molecules can convey guidance information, the Rho-family and their effectors, such as Rho-associated kinases (ROCKs) among others, are probably the key points in the intracellular signaling cascades that promote regeneration (Tang, 2003). Activation and regulation of these molecules and their effectors is complex, and different GTPases can be activated by the same guidance cues. On the other hand, different trophic molecules, as for example NGF, and the extracellular matrix molecule laminin, regulate neurite outgrowth acting coordinately on the same GTPase (Rankin *et al.*, 2008). Nowadays, it is considered that the spatial-temporal localization and activation of small GTPases by different trophic molecules is essential for their functional outcome (Pertz *et al.*, 2008).

Guidance cues of the external environment also play a main role in the elongation of the growth cone. Guidance cues interact with specific receptors on the surface of the growth cone, and trigger a cascade of cytoplasmic events that eventually lead to the cytoskeletal rearrangement associated with oriented neurite extension. For example, cell adhesion molecules (CAMs) mediate the adhesion of filopodia to tropic molecules of the extracellular matrix (Letourneau and Shattuck, 1989). Moreover, lipid rafts (see below) present at the growth cone amplify guidance cue signals (Fujitani, 2005; Guirland *et al.*, 2004); neurotrophins, for instance, can exert their chemoattractive gradient acting through these microdomains.

During development, axons use at least two types of cues as they grow along the spinal nerves and into peripheral nerves. First, they respond with differential attraction between different types of axons, an effect mediated by changes in expression of several CAMs. Second, axons can also be guided by

molecular cues, either attractive or repulsive, coming from the local environment of the limb. Both motor and sensory axons are specified with respect to their peripheral targets during development, thus their axons have to respond to a set of guidance signals as they travel through the spinal plexus, the peripheral nerve trunk and successive branching points to an adequate target. Motoneurons project axons out of the central nervous system to muscle targets located at the periphery. Distinct pools of motoneurons, corresponding to fast/slow and flexor/extensor subclasses seem to be specified at embryonic stages (Landmesser, 2001). During development, motoneurons are able to grow their axons to appropriate muscles, in response to sets of guidance cues, presumably inhibitory, provided by non-myogenic cells within the limb. The decision of a motor axon to project to dorsal or ventral muscles (functionally antagonists) in the limb is controlled in part by signaling through several ephrins and their receptors at the plexus level (Schneider and Granato, 2003). Muscular sensory axons grow slightly later than motor axons and they become adjacent and fasciculate along motor axons innervating the same muscle. In contrast, developing cutaneous sensory axons bundle together, and at short distance from the dorsal root ganglia (DRG), axons that project along individual cutaneous nerves are already restricted in their position (Honig *et al.*, 1998). The achieved pattern of peripheral projections has to be maintained throughout life for an adequate functional control of skeletal muscles, as well as for the accurate localization and perception of stimuli affecting cutaneous or muscular sensory receptors. It has been reported that members of the three main families of guidance molecules (Netrins, Semaphorins and Slits) play key roles as molecular barriers or promoters for sensory axon growth during development (Del Río *et al.*, 2004). For motor axons, molecules such as T-Cadherin, HGF/SF as well as Ephrins have been involved during their development (Gavazzi, 2001; Krull and Koblar, 2000). However, despite the similarities of axonal growth during development and post-injury regeneration, the regulation and participation of the former molecules during peripheral nerve regeneration and selective sensory and motor axon outgrowth remain unclear. In addition, the growth cone integrates the information of several extracellular signals and convergent intracellular pathways. Indeed, numerous *in vitro* growth cone turning assays indicated that intracellular kinases (e.g., ERK1/2, PI3K, GSK3 (Liu and Snider,

2001) and signalling cascades activated by axonal guidance receptors are modulated by cyclic nucleotides cAMP and cGMP (Song and Poo, 1999). For example, elevated cGMP levels may counteract the inhibitory effects of Sema3A on sensory neurons (Schmidt *et al.*, 2002).

Several studies have reported that motor and sensory fibers respond differently after nerve lesions (Hoke *et al.*, 2006; Lago *et al.*, 2007). CAMs differentially expressed by sensory or motor Schwann cells may play a role for preferential guidance. Preferential reinnervation is thought to be due to collaboration between the regenerating axons and the appropriate pathway (Brushart, 1993; Madison *et al.*, 1996), although attempts to promote specific axon guidance using biochemical methods had modest success until now. Moreover, a detailed study of factors able to induce specific regeneration of motor and sensory axons is currently not available.

4. REGENERATION ALONG THE DISTAL NERVE STUMP

In order to achieve successful regeneration, neurons have to survive and to activate the re-growing program that will lead to axonal elongation and associated plastic changes of their network. This program is influenced by interactions between the neurons and the neighboring glial cells, mediated by cell- and substrate-adhesion molecules and their receptors, and by several trophic factors secreted into the extracellular space. A number of key players in this regenerative process have been identified, but the relationship between molecular events, particularly the triggering of gene expression and the corresponding cascade of signalling pathways, is still incompletely understood.

The environment that the axons find distal to the lesion site is crucial for their regeneration and thus, it is important to create a favorable milieu for axonal outgrowth. An intact peripheral nerve does not support axonal growth as effectively as a degenerated nerve, due to the presence of inhibitory factors for regeneration (Mueller, 1999; Tang, 2003), such as chondroitin sulfate proteoglycans (CSPGs) of the extracellular matrix and myelin-associated inhibitors of regeneration (Mukhopadhyay *et al.*, 1994). These molecules, upregulated after nerve injury, show neurite-inhibitory activity (Braunewell *et al.*, 1995; Shen *et al.*, 1998; Zuo *et al.*, 1998) and contribute in delaying axonal growth following the

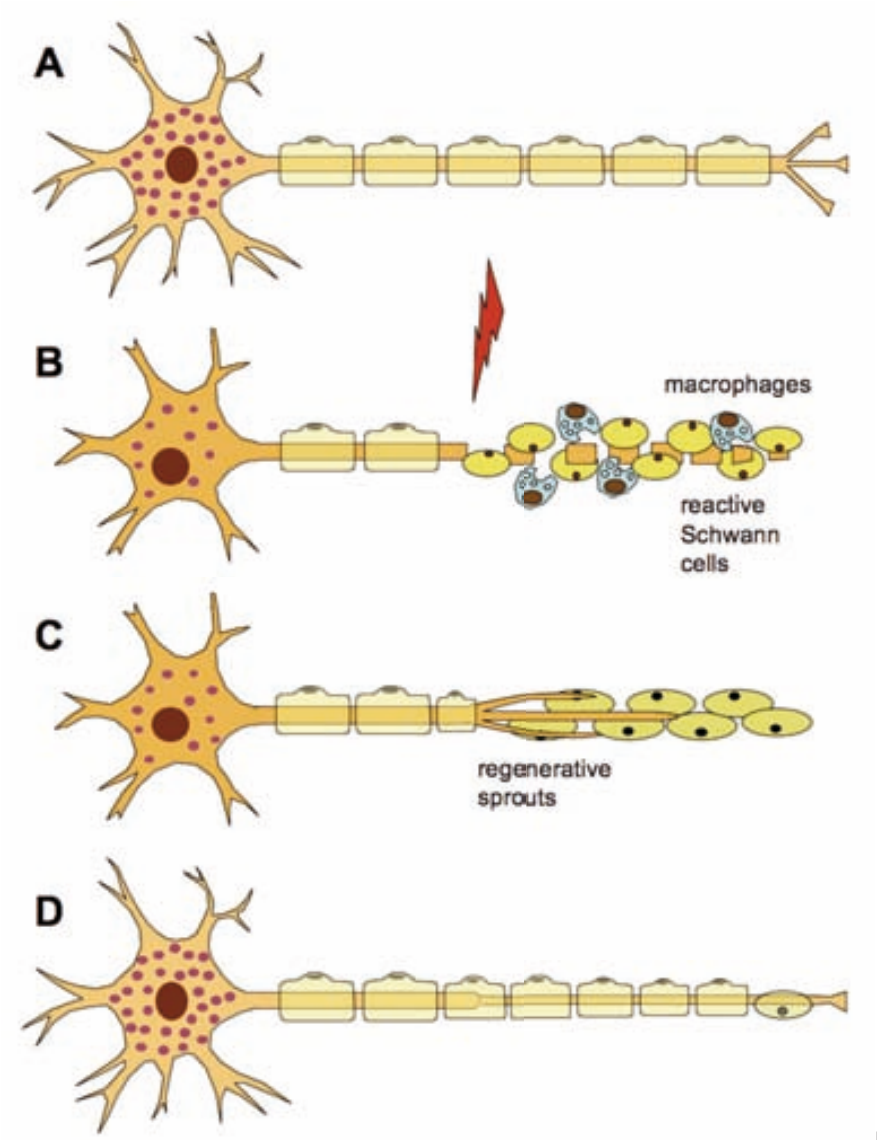

A
B
macrophages
reactive
Schwann
cells
C
regenerative
sprouts
D

Fig. 4. Degeneration and regeneration after peripheral nerve injury. **A**: Normal neuron and nerve fiber. **B**: Wallerian degeneration. The axotomy results in fragmentation of the distal axon and myelin sheaths. Schwann cells proliferate and macrophages invade the distal nerve segment and phagocyte degrading materials. **C**: Schwann cells in the distal segment line up in bands of Büngner. Axonal sprouts advance embedded in the Schwann cells and attracted by gradients of neurotrophic factors. **D**: Axonal reconnection with end organs and maturation and remyelination of the nerve fiber.

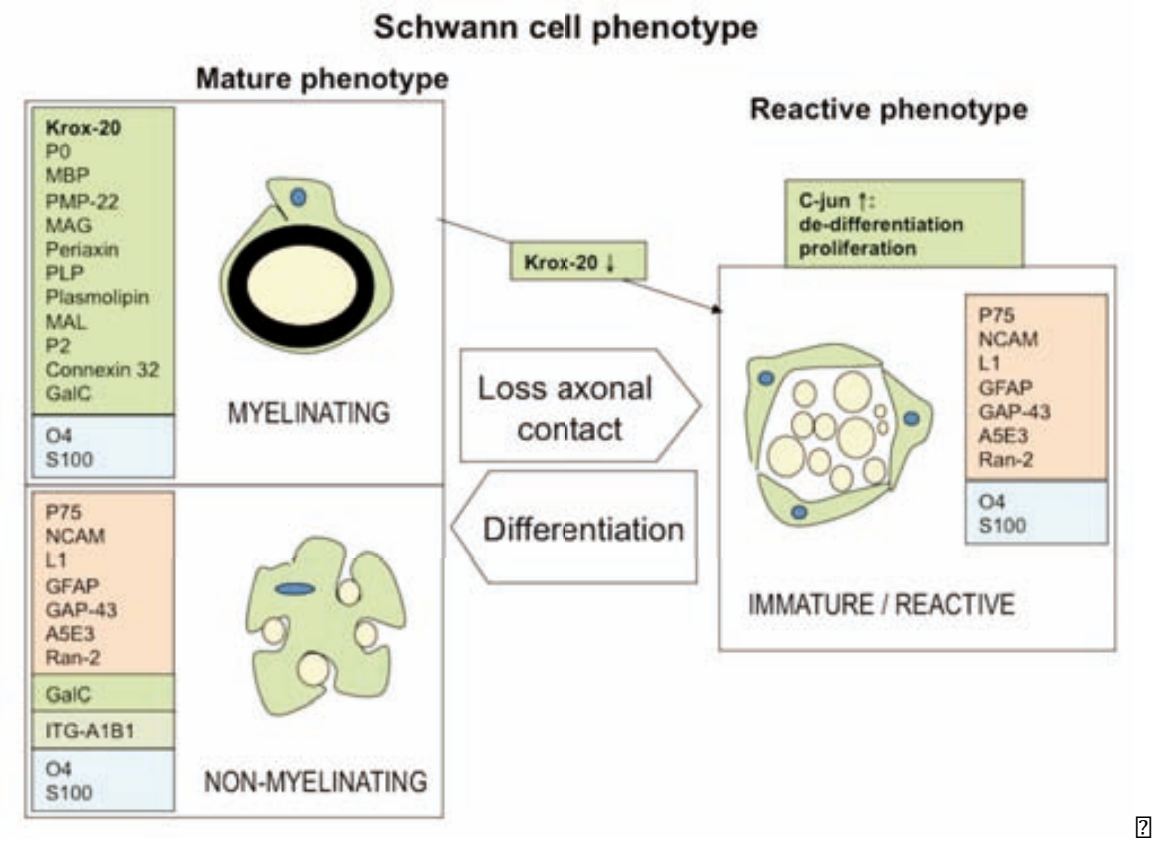
Regenerating axons have to cross the injury site prior to reaching the distal stump. After axonotmesis, as produced by a nerve crush, there is only disruption of the axons but the perineurium and the endoneurial tubules are preserved, so the axons usually reach the distal stump with no major delays. However, after complete nerve transection, when the continuity of the connective tissue is lost and elongation towards the distal nerve is not allowed, a neuroma usually forms at the severed nerve end, which is frequently associated with significant pain and dysesthesia. Regenerative axonal sprouts continue to grow blindly within a dense collagenous and fibroblastic stroma in the neuroma. In contrast, if the continuity of the nerve is re-established by means of a direct suture of the stumps, axons can readily elongate along the distal degenerating nerve, although they have to cross the scar tissue at the rejoined site that imposes some delay. When primary suture is not possible, a bridge constituted by an autologous nerve graft is an adequate alternative (Lundborg *et al.*, 2004). An important impediment for a good recovery after nerve transection is the loss of the normal fascicular architecture of the peripheral nerve after the surgical repair, with axons growing dispersed in numerous mini-fascicles (Lago and Navarro, 2006). Consequently, the topographical peripheral projections of motor and sensory neurons are distorted and functional recovery is hampered (Gramsbergen *et al.*, 2000; Valero-Cabre and Navarro, 2002; Valero-Cabre *et al.*, 2004). The type of nerve graft used has been related to the level of recovery; motor nerve grafts have been found to allow more robust regeneration and better functional outcome than sensory nerve grafts (Moradzadeh *et al.*, 2008; Nichols *et al.*, 2004), possibly related to the larger endoneurial tubes of motor nerves. Another study provided evidence that regeneration of motor axons was promoted at earlier times by a motor nerve graft (from ventral spinal root), whereas reinnervation of sensory pathways was slightly

improved in the presence of a sensory graft (from dorsal root) (Lago *et al.*, 2007). Findings by Redett *et al.* (2005) showed that motoneurons maintain more regenerating collaterals in cutaneous than in muscular distal nerves. Such difference could be due either to increased collateral formation in cutaneous nerve or to increased collateral pruning in muscle nerve. In either instance, they indicate that motor and sensory pathways have distinct functional identities that regulate the regenerative sprouting of axons. Nevertheless, the potential differential contribution of Schwann cells in either motor and sensory nerves has been also suggested.

5. CHANGES IN SCHWANN CELLS AFTER NERVE LESIONS

Schwann cells are derived from the neural crest and their precursors are able to proliferate until they differentiate to mature myelinating and non-myelinating phenotypes. Interestingly, mature Schwann cells have the remarkable capacity to reverse their phenotype and dedifferentiate when they lose contact with axons. Therefore, after a peripheral nerve injury, the molecular markers characteristic of myelinating and non-myelinating Schwann cells are downregulated (Figure 5). In particular, expression of myelin markers decreases dramatically as a consequence of axonal degeneration distal to the injury site, whereas markers of immature and non-myelinating Schwann cells are re-acquired (Scherer, 1994; Scherer and Salzer, 1996). The loss of the transcription factor Krox-20 after nerve section or axonotmesis (crush) probably allows the cells to resume their constitutive c-Jun expression, which will help the glial cells to de-differentiate and proliferate (Parkinson *et al.*, 2008). The phenotype of reactive Schwann cells resembles the one of immature Schwann cells and they form a permissive substrate for regeneration. When these cells regain contact with the axons, they re-differentiate again (Jessen and Mirsky, 2008).

Between day 1 and 5 after injury, Schwann cells start proliferating and their peak of activation occurs around day 3 and then decreases during the following weeks (Bradley and Asbury, 1970). This proliferation plays a key role during Wallerian degeneration since, in coordination with macrophages, Schwann cells


Schwann cell phenotype
Mature phenotype
Reactive phenotype
Krox-20
P0
MBP
PMP-22
MAG
Periaxin
PLP
Plasmolipin
MAL
P2
Connexin 32
GalC
O4
S100
MYELINATING
P75
NCAM
L1
GFAP
GAP-43
A5E3
Ran-2
GalC
ITG-A1B1
O4
S100
NON-MYELINATING
Krox-20 ↓
Loss axonal contact
Differentiation
C-jun ↑:
de-differentiation
proliferation
P75
NCAM
L1
GFAP
GAP-43
A5E3
Ran-2
O4
S100
IMMATURE / REACTIVE

factor (GDNF) and insulin-like growth factor-1 (IGF-1). Interestingly, the time course of expression after the injury is different for each factor. In contrast, Schwann cells down-regulate the production of myelin proteins, as well as some other trophic factor, as for example CNTF (Rabinovsky *et al.,* 1992).

As regenerating axons enter the distal portion of the nerve, they are guided haptotactically along the Schwann cell substratum by binding of trophic factors to their own receptors. The close apposition of regenerating axons and Schwann cells facilitates the receptor-mediated, intercellular transfer of neurotrophic factors. Once Schwann cells regain the contact with axons, the expression of neurotrophic factors and their receptors is suppressed. This mechanism creates a dynamic gradient where the highly activated Schwann cells are located distally into the degenerated nerve stump. This gradient of substrate-adsorbed neurotrophic factors would help to maintain the proper directionality of axonal regeneration. When the neuronal axons reinnervate target organs, Schwann cells return to a quiescent state (Taniuchi *et al.*, 1988). The regenerating axons that are advancing into the endoneurial tubes give signals to the Schwann cells making them turn to a mature phenotype. Interestingly, the regenerating axons originated by myelinated neurons instruct the Schwann cells to form myelin, while unmyelinated fibers remain unmyelinated independently of the origin of the Schwann cells they encounter in the distal pathway (Politis and Spencer, 1981).

Besides the myelinating or unmyelinating phenotype, Schwann cells from motor and sensory nerve branches appear to express different molecular markers that may contribute to the capacity of some axons to specifically regenerate towards appropriate pathways and reinnervate corresponding target organs. It was shown that Schwann cells associated to motor but not to sensory neurons differentially express the L2/HNK1 carbohydrate epitope (Martini *et al.*, 1992). Interestingly, after injury it seems that the expression of L2/HNK1 by motor associated Schwann cells is lost, but if these cells recover contact with regenerating motor axons, they show a stronger tendency to express L2/HNK1 than Schwann cells formerly associated with sensory axons, although they also enter in contact with motor axons (Martini *et al.*, 1994). On the other hand, NCAM-positive cells have been localized exclusively in sensory nerve fascicles, and they

have been identified as non-myelin forming Schwann cells of sensory unmyelinated fibers (Saito *et al.*, 2005). Both markers appear distinctly by about three weeks postnatally, suggesting that they play a role during development of nerve fasciculation. However, HNK-1 and NCAM immunoreactivity is lost within weeks/days after nerve transection in motor and sensory fascicles, respectively (Saito *et al.*, 2005), although it may reappear after recontact with the appropriate axons (Martini *et al.*, 1994). This fact suggests that Schwann cells dedifferentiate after injury and lose the expression of specific markers decisive for their phenotype, but they retain some of their acquired properties.

Regarding the neurotrophic profile, Hoke *et al.* (2006) observed that Schwann cells of sensory and motor (ventral roots) nerves exhibit differing growth factor profiles at baseline and, interestingly, respond differently during denervation and when reinnervated by cutaneous or motor axons. mRNAs for NGF, BDNF, vascular endothelial growth factor (VEGF), IGF-1 and hepatocyte growth factor were expressed vigorously by denervated and reinnervated cutaneous nerves but minimally by ventral roots, whereas mRNAs for GDNF and pleiotrophin were increased to a greater degree in motor than in cutaneous nerves. Such differences were maintained during Wallerian degeneration, although they tended to decline with time when reinnervation was induced by the wrong type of axons (Hoke *et al.*, 2006). Immunohistochemical analyses of the expression of neurotrophins have shown that Schwann cells around intact sensory axons of the dorsal root and cutaneous saphenous nerve exhibit a higher expression of NGF, BDNF and NT-3 than those around intact motor axons. In contrast with intact nerves, distal segments of transected saphenous nerve and muscular femoral nerve displayed much higher intensity of immunostaining for neurotrophins, but at similar levels when compared with each other (Dubovy, 2004). These results indicate that upregulation of neurotrophins in the reactive Schwann cells of the injured peripheral nerve provides similar conditions for the growth of both sensory and motor axons, suggesting that trophic factors may not contribute significantly to the sorting of different types of axons during nerve regeneration. Further investigations addressed to enhance the selective phenotype of motor or sensory Schwann cells, by engineering them to overexpress certain neurotrophic

factors or membrane adhesion molecules, might help to elucidate the above discrepancies and promote selective axonal regeneration.

The key role of active Schwann cells in axonal regeneration is evidenced by the poor functional recovery achieved when using acellular nerve grafts to repair nerve resections (Hall, 1986). Similarly, the slow decrease in the capacity of Schwann cells to maintain an active pro-regenerative phenotype with time explains the limited capacity of chronically denervated nerves to sustain axon regeneration, and reveals the importance of early nerve repair and strategies to accelerate regeneration (Gordon *et al.*, 2003). After severe injuries of proximal nerves, that requiere long periods of time for regeneration, the poor motor recovery reached has been related to the decreased capacity of Schwann cells to provide support for regeneration (Sulaiman and Gordon, 2000), together with the regression growth state of the chronically axotomized neuron (Fu and Gordon, 1995a) and the atrophy of the denervated muscle (Fu and Gordon, 1995b; Gordon *et al.*, 2011; Ma *et al.*, 2011). Interestingly, a recent work points out the importance of terminal Schwann cells of the motor endplate in failed reinnervation of chronically denervated muscles. After a window of opportunity, motor axons that reach the target organ fail to reinnervate the endplate. In contrast, sensory axons are able to reinnervate the skin in a similar degree both at early or later stages. Schwann cells of the motor endplate could become non-permissive for regeneration after long periods of denervation and prevent functional synapses of the motor axons with the muscle (Ma *et al.*, 2011).

6. NEUROTROPHIC FACTORS. EFFECTS ON DIFFERENT NEURONS AFTER INJURY

Large quantity of studies depicts the important role of neurotrophic factors in determining neuronal survival both during development and after injury. Under physiological conditions, non-neuronal cells synthesize and secrete several trophic factors, needed for maintenance of the homeostatic state of intact neurons. Schwann cells and several peripheral tissues (muscle, skin, etc.) have a basal expression of neurotrophic factors, which play biological functions in promoting survival and maintenance of sensory, motor and autonomic peripheral neurons (Gordon, 2010; Lewin and Barde, 1996; Raivich, 1994). After nerve injury the

expression of those neurotrophic factors increases, in an attempt to support the survival and growth of regenerating axons and reinnervation of denervated target organs. The changes in the distal nerve follow different patterns for different trophic factors; thus, there is an initial up-regulation of some of them, like NGF, BDNF and GDNF, whereas others, as NT-3 and CNTF, are down-regulated. Their levels return to normal with regeneration, but in case of chronical denervation the distal nerve stump may continue to express neurotrophic factors for at least 6 months following injury (Michalski *et al.*, 2008), although other studies point the failure of Schwann cells to maintain high levels of trophic factors after 2 months of chronic denervation (Gordon, 2009).

6.1. Neurotrophins

The neurotrophin family includes NGF, BDNF, and neurotrophins NT-3, and NT-4/5. Among them, NGF is the most studied one. It is known to act specifically on a subpopulation of small primary sensory and on sympathetic neurons (Levi-Montalcini, 1987). It seems to be expressed at similar levels in intact cutaneous and motor nerves, but after injury there is an up-regulation in denervated sensory dorsal roots to a larger extent than in motor ventral roots (Hoke *et al.*, 2001). In adult animals, NGF is needed for collateral sprouting of nociceptive and sympathetic axons into denervated skin, but does not affect large sensory axons (Diamond *et al.*, 1987; Gloster and Diamond, 1992). In contrast, application of NGF after axotomy delays the onset of regeneration (Gold, 1997), probably by reducing the neuronal body response to injury (Mohiuddin *et al.*, 1999), although without compromising the rate of subsequent regeneration. On another hand, focal administration of NGF or NT-3 to adult axotomized nerves inside a blinded impermeable chamber effectively prevented sensory neuronal loss, which mainly affects small DRG neurons (Rich et al., 1987; Groves et al., 1999).

Another neurotrophin, BDNF, is differently expresssed in motor and sensory intact nerves, and after injury, it can be detected also at higher concentrations in cutaneous nerves than in ventral roots (Hoke *et al.*, 2006). The role of BDNF in nerve regeneration is controversial, although the presence of its receptor trkB seems crucial to sustain axonal regeneration (Boyd and Gordon, 2001). Deprivation of endogenous BDNF impairs axonal growth and myelination

(Zhang *et al.*, 2000), whereas local infusion of BDNF improved nerve regeneration in neural conduits (Vögelin *et al.*, 2006). Interestingly, Boyd and Gordon (2001) described no effects on axonal regeneration when BDNF was administered acutely after a cut and suture of the sciatic nerve, although it enhanced regeneration in a dose dependent manner when applied after chronic axotomy (Boyd and Gordon, 2003).

Neurotrophin-3 was shown to be present at higher concentration in cutaneous sensory than in motor nerves in baseline conditions, but no great differences between roots were found after injury (Hoke *et al.*, 2006). NT-3 is present in adult skeletal muscles and has a trophic role on motoneurons and primary sensory neurons innervating muscles. Its trophic action on motoneurons was shown in vitro (Braun *et al.*, 1996), whereas in vivo it seems to exert a selective action on type 2b fast muscle fibers (Sterne *et al.*, 1997b). On the other hand, other studies (Ernfors *et al.*, 1993; Airaksinen *et al.*, 1996) confirm its important role for the survival of proprioceptive and mechano-receptive sensory neurons. Moreover, NT-3 can be found in trigeminal, cervical and lumbar spinal ganglia (Hory-Lee *et al.*, 1993; Zhou and Rush, 1995). It is interesting to highlight that in chicken embryos, NT-3 responsive sensory neurons extend neurites well both on laminin and fibronectin coated coverslips, whereas NGF responsive neurons grow better on laminin. Thus, differences seem to exist in substrate preference between NGF and NT-3 responsive sensory neurons (Guan *et al.*, 2003).

Focal delivery of BDNF, NT-3 or NT-4 into fibronectin guides revealed that NT-4 preferentially improved the functional reinnervation of slow motor units, whereas BDNF and NT-3 showed less effects on regenerating motoneurons (Simon *et al.*, 2003). In another study, exogenous administration of NT-3 was selectively beneficial for the reinnervation of 2b fast muscle fibers (Sterne *et al.,* 1997b), suggesting that different growth factors might preferentially influence different type of motoneurons. The use of specific growth factors could also be useful to selectively guide axons from a mixed population of DRG neurons. By using a compartmentalized delivery of NGF and NT-3 in vitro, Lotfi *et al.* (2011) tried to preferentially enhance growth of nociceptive and proprioceptive subsets of sensory neurons, respectively. They found that the NGF channel attracted

nociceptive axons, that elongated longer when compared to saline or NT-3 channels, whereas there were more proprioceptive fiber branches in the NT-3 channels. However, the authors failed to corroborate these findings in vivo.

The specific receptors for neurotrophins are called Trks, and are a family of protooncogene receptors, which mediate their effects on distinct populations of DRG neurons from E15 onward and in some motoneurons as well. TrkA is the high affinity receptor for NGF and it is expressed in subpopulations of primary sensory neurons, especially in the small ones (Bennett *et al.*, 1996a). Interestingly the expression of this receptor is higher in visceral afferents than in cutaneous afferents (Mu *et al.*, 1993). The high affinity receptor for BDNF and NT-4/5 is TrkB, normally found in spinal motoneurons and also in mid-size primary sensory neurons (Klein *et al.*, 1989, 1992; Mu *et al.*, 1993). After axotomy, motoneurons increase the expression of TrkB (Ernfors *et al.*, 1993; Kobayashi *et al.*, 1996; Piehl *et al.*, 1994). TrkC, the high affinity receptor for NT-3, is present in spinal motoneurons and in a subpopulation of large diameter primary sensory neurons (Lamballe *et al.*, 1991; Mu *et al.*, 1993). In addition, it is important to consider that neurotrophins have also a low affinity receptor P75, whose expression increases mainly after injury. The role of this receptor is controversial as it can exert also pro-apoptotic functions. Following peripheral nerve injury, neurotrophin receptors are upregulated on the distal portion of the nerve, in denervated Schwann cells and in growth cones of regenerating axons (Raivich and Kreutzberg, 1994), although differentially regulated. Thus, reduced levels of TrkB and TrkC receptors are detected in Schwann cells of the distal injured nerve (Funakoshi *et al.*, 1993), whereas TrkA has been found to decrease in the DRG neurons perikarya, but increase at the regenerating nerve front (Webber *et al.*, 2008). After axotomy there is an increased transport of all the neurotrophins dependent on neurotrophin binding to the P75 and Trk receptors in sensory neurons, but only to P75 that is upregulated in motoneurons (Curtis *et al.*, 1998).

6.2. Glial derived neurotrophic factor

GDNF belongs to the TGFα-1 superfamily and has a trophic effect on sensory, motor and autonomic neurons (Buj-Bello *et al.*, 1995; Ebendal *et al.*, 1995; Henderson *et al.*, 1994a; Trupp *et al.*, 1995; Matheson *et al.*, 1997; Baloh *et al.*,

1998). Two weeks after birth, some of the small-diameter non-peptidergic neurons of the DRG (a subpopulation that can be identified as lectin IB4 positive) lose their sensitivity to NGF and become GDNF sensitive (Bennett *et al.*, 1996a; Molliver *et al.*, 1997). The IB4-labeled sensory neurons have an impaired intrinsic axonal regeneration capacity and higher vulnerability to axonal injury than the NGF-dependent sensory neurons, even if exposed to GDNF supply (Leclere *et al.*, 2007). In contrast, overexpression of GDNF in motoneurons has profound positive effects on neuronal survival after axotomy (Zhao *et al.*, 2004) and prevents axotomy-induced ChAT decrease (Henderson *et al.*, 1994a; Yan *et al.*, 1995)

Although it has been shown that GDNF levels are higher in cutaneous nerves than in ventral roots in intact animals (Hoke *et al.*, 2006), this factor is similarly up-regulated both in injured ventral and dorsal roots (Hoke *et al.*, 2001). This up-regulation of GDNF after injury triggers also the up-regulation of its receptor (GFRα-1) suggesting its important role for neurotrophic support (Höke *et al.*, 2000). In fact, several studies showed that GDNF enhances regeneration of both motor and sensory axons. For example, sustained delivery of GDNF to the injury site in vivo by a synthetic nerve guide allowed regeneration of both sensory and motor axons over long gaps to a significantly higher number than with NGF delivery (Fine *et al.*, 2002). In DRGs, GDNF delivery enhances the conditioning injury effect, although only at low GDNF concentrations (Mills *et al.*, 2007). Furthermore, GDNF expression decline was associated with impaired regeneration after long-term denervation (Hoke *et al.*, 2002), whereas GDNF applied to the proximal stump of chronically sectioned nerves increased the number of motoneurons that were able to regenerate their axons (Boyd and Gordon, 2003). GDNF has also important effects on Schwann cells, and when applied at high doses in adult rats induces proliferation of Schwann cells and the myelination of axons, even the normally unmyelinated ones (Höke, 2003). This fact suggests that GDNF can mediate axon-glial interactions. Schwann cell-derived GDNF is taken up by sensory and motor neurons and transported anterogradely along the axons for release from terminals, where it may act on glial cells expressing its receptor GFR.

The GDNF receptor complex is composed of the ligand binding receptors GFRα and the signal-transducing domain RET (Treanor *et al.*, 1996; Bennett *et al.*,

2000; Trupp *et al.*, 1996). GDNF seems to bind preferentially GFRα-1, while GFRα-2, GFRα-3 and GFRα-4 are supposed to bind preferentially neurturin, persephin and artemin. GDNF forms a high-affinity complex with GFRα-1, and then this complex recruits Ret, induces phosphorylation of specific tyrosine residues of Ret and activates intracellular signaling. One third of primary sensory neurons that bind to lectin IB4 do not express Trk receptors but the GDNF specific receptor Ret (Lin *et al.*, 1993). GFRα-1 and RET are expressed in subpopulations of both small and large diameter DRG neurons, while GFRα-2 and GFRα-3 are found only in small diameter primary sensory neurons (Bennett *et al.*, 2000). GFRα-1 is also expressed in glial cells (Trupp *et al.*, 1997). Dramatic changes are found after axotomy in the expression of these receptors: GFRα-1 and Ret are greatly increased in large diameter neurons, whereas GFRα-3 increases in small diameter cells, and, in contrast, GFRα-2 is reduced after injury.

6.3. Fibroblast growth factor

Among the 23 members of the FGF family, FGF-2 has been shown to be the most important contributor to nerve regeneration (Grothe and Nikkhah, 2001). Different isoforms of this trophic factor are expressed in adult nervous tissue by glial cells and different neuronal populations, and play a key role in signal transduction in central and peripheral nervous systems (Ornitz and Itoh, 2001). Moreover, some FGF-2 isoforms seem to be differentially regulated during development and also after injury (Giordano *et al.*, 1992; Grothe, 2000; Meisinger *et al.*, 1996) and differential effects of FGF-2 isoforms have been reported. Transplantation of Schwann cells overexpressing the 21-23 kDa FGF-2 supported sensory recovery through a long gap injury, whereas the 18 kDa isoform inhibited myelination of regenerated axons (Haastert *et al.*, 2006). In contrast, in cultured DRG neurons both low- and high molecular weight FGF-2 isoforms increase the number of axonal branch from control rats. Although it does not promote neurite elongation, FGF-2 significantly enhances the response to a preconditioning lesion (Klimaschewski *et al.*, 2004). In vivo, FGF-2 is up-regulated both at the lesion site and in neuron bodies after nerve lesion. Whereas transgenic mice overexpressing FGF-2 showed greater number of regenerating axons after injury by regulating Schwann cell proliferation (Jungnickel *et al.*, 2006), mutant mice lacking FGF-2 had

significantly increased axon and myelin size but no difference in the number of regenerated fibers (Jungnickel, 2010). In fact, FGF-2 acts also on Schwann cells, as it was reported to stimulate their mitogenesis and proliferation (Davis and Stroobant, 1990).

Four different high affinity tyrosine transmembrane receptors have been described to which FGF-2 binds with different affinities (Ornitz *et al.*, 1996). The main receptors in the nervous system, FGFR 1-3, are up-regulated after injury (Grothe *et al.*, 1997) and *in vitro* studies found enhanced sensory neuron outgrowth after FGFR-1 overexpression, mediated by activation of extracellular signal-regulated kinase (ERK) and Akt pathways (Hausott *et al.*, 2008). Moreover, FGF seems to maintain interactions with heparin and heparan sulfate protoglycan (HSPG) (Ornitz, 2000) that increase the affinity of the FGF-FGFR complex (McKeehan *et al.*, 1998; Szebenyi and Fallon, 1999).

6.4. Insulin-like growth factors

IGF-1 and IGF-2 are related to the endogenous regulation of repair and regeneration processes. Both isoforms were reported to support motoneuron development as well as survival after axotomy when administered at the injury site (Pu *et al.*, 1999). In vitro, addition of IGF-1 to the medium elicited neurite growth of sensory neurons, through activation of the PI3-kinase pathway (Kimpinski and Mearow, 2001). Moreover, exogenous application of this neurotrophic factor enhances the speed of axonal regeneration (Kanje *et al.*, 1989; Glazner *et al.*, 1993) and improves functional recovery (Emel *et al.*, 2011). Some studies support the concept that IGF-1 promotes motor branches regeneration as it enhances motoneuron sprouting *in vitro* and *in vivo* (Caroni and Grandes, 1990), and it is up-regulated in ventral roots after injury (Hoke *et al.*, 2006). Injured nerves show increased expression of IGF-1 and IGF-2 as well as IGF binding proteins (IGFBP) 4 and 5, whereas IGFBP-6 mRNA is strongly upregulated in spinal motoneurons after lesions, suggesting a special relevance for these neurons (Hammarberg *et al.*, 1998). On the other hand, the expression of IGF-1 is known to decrease during aging and continuous application of this trophic factor improved axonal regeneration and muscle reinnervation in aging animals (Apel *et al.*, 2010).

6.5. Neuregulins

Alternative splicing of the *NRG1* gene gives at least 16 different products (Falls, 2003; Steinthorsdottir *et al.*, 2004) of which the most studied are neu differentiation factor (NDF), heregulin, acetylcholine receptor inducing activity (ARIA) and glial growth factor (GGF). NDF and heregulin are known to play a role in growth and differentiation activities in breast epithelial cells, and ARIA and GGF are found mainly at the neuromuscular junction and at Schwann cells during development (Corfas, 1993; Falls, 2003; Holmes *et al.*, 1992; Marchionni *et al.*, 1993; Wen *et al.*, 1992; Wen *et al.*, 1994).

GGF is expressed by sensory, motor and sympathetic neurons (Marchionni *et al.*, 1993; Chen and Ko, 1994). It has specific effects on Schwann cells, mediated via heterodimers of erbB2, erbB3 and erbB4 receptors, which seems to be important for the interactions between these glial cells and neurons. Following nerve lesion, GGF mRNA increases in DRG neurons, and expression of erbB2 and erbB3 receptors in glial cells of the distal nerve stump is also upregulated (Li *et al.*, 1997) indicating a coordinated response to axotomy between axon and Schwann cells (Carroll *et al.*, 1997). GGF promotes Schwann cell proliferation and maturation when regenerative axons have reinnervated target organs (Birchmeier and Nave, 2008; Falls, 2003; Raivich, 1994). GGF was shown to be a promising enhancer for glial cell proliferation when compared with NGF, as exogenous application of GGF after facial nerve anastomosis promotes Schwann cell migration and decreases the amount of myelin debris (Yildiz *et al.*, 2011).

Neuregulin1 effect through erbB signaling is necessary for Schwann cell differentiation and myelination, establishment of neuromuscular junctions and also for normal sensory function development (Chen *et al.*, 2006). Neuregulins may act as an axonally derived signal with trophic effect on denervated Schwann cells, facilitating their supportive role in axonal regeneration. In addition, neuregulins produced by Schwann cells themselves may be partially responsible for Schwann cell proliferation during Wallerian degeneration, via autocrine or paracrine mechanisms (Carroll *et al.*, 1997). After sciatic nerve crush, neuregulin1 ablation resulted in a slower rate of regeneration, severe defects in remyelination, and abnormalities in neuromuscular junction reinnervation (Fricker *et al.*, 2011).

6.6. Pleiotrophin

Pleiotrophin (PTN), also called heparin binding neurotrophic factor, is a heparin binding secreted protein. PTN is expressed in the nervous system during development and seems to trigger postsynaptic clustering of acetylcholine receptors (Peng *et al.*, 1995). Two days after nerve injury there is an up-regulation of PTN, that reaches a peak after 7 days and returns back to baseline levels 3 months after injury (Mi *et al.*, 2007). Immunohistochemical analysis showed PTN in nonneuronal cells in distal nerve segments, including Schwann cells, macrophages and endothelial cells, but not in axons (Blondet *et al.*. 2005). High concentrations of this trophic factor were found in intact ventral roots, whereas they were low in intact cutaneous nerves. After injury it was dramatically up-regulated in ventral roots, PTN being one of the neurotrophic factors more markedly overexpressed in axotomized motoneurons (Hoke *et al.*, 2006). PTN application in spinal cord organotypic cultures promoted neurite elongation of motoneurons. Moreover, PTN increased axonal regeneration but not survival of avulsed spinal motoneurons (Chu *et al.*, 2009). However, PTN applied to the lesioned peripheral nerve in vivo impaired muscle reinnervation (Blondet *et al.*, 2006).

6.7. Osteopontin

Osteopontin (OPN) is a matricellular glycoprotein, strongly expressed by macrophages after injuries of the central but not in the peripheral nervous system (Küry *et al.*, 2005), a fact that could explain why OPN is supposed to inhibit axonal growth only in the central nervous system. On the other hand, OPN is expressed by myelinating Schwann cells of intact peripheral nerves. One to 4 days after axotomy, OPN levels transiently increase in the distal nerve stump and there is a downregulation thereafter, reaching a minimum 14 days after the lesion (Jander *et al.*, 2002). This event suggests a role of such glycoprotein in peripheral nerve regeneration, but this hypothesis needs further investigation to be corrobarated.

6.8. Neuroactive cytokines

Some studies indicate that ciliary neurotrophic factor (CNTF) can be considered an “injury factor” released by glial cells after axotomy (Adler, 1993). It

is known to be present in normal adult peripheral nerves, produced by the Schwann cells. Overexpression of CNTF in Schwann cells increases the expression of myelin proteins and activates their differentiation in vitro and in vivo (Homs *et al.*, 2011). Released CNTF following nerve lesion may bind to the CNTF receptor a-ret, that is localized mainly in neurons and exerts a paracrine effect promoting cell survival and axonal growth (Hu *et al.*, 2005). CNTF also promotes survival of motoneurons in neonatal animals following axotomy (Sendtner *et al.*, 1990). This neuroactive cytokine seems to play an important role in guiding motorneuron reinnervation and in promoting the sprouting of their axons (Siegel *et al.*, 2000). Indeed, systemic CNTF administration enhanced muscle fiber reinnervation and intramuscular nerve branching (Ulenkate *et al.*, 1994). However, in the screening done by Hoke *et al.* (2006) CNTF was expressed at similar levels in intact cutaneous and motor nerves and its levels were similar in both sensory and motor axons after injury as well. In fact, viral-mediated overexpression of CNTF in Schwann cells enhanced the regenerative responses of both sensory and motor neurons (Homs *et al.*, 2011).

The leukemia inhibitory factor (LIF) has an in vitro activation similar to CNTF in sympathetic neurons. It shares the signaling pathway with CNTF and it is considered that the roles of both cytokines are overlapping (Ip *et al.*, 1992). Schwann cells are the main producer of LIF, that it is retrogradly transported by a subpopulation of small diameter neurons in DRG; most of them are positive for IB4, while the remaining are positive for TrkA and CGRP (Curtis *et al.*, 1998; Thompson *et al.*, 1997). Following nerve transection, LIF is up-regulated by Schwann cells at the injury site. *In vivo* studies suggest a role of LIF in promoting motor pathway regeneration, since its administration after nerve injury increases muscle mass and muscle contraction force (Tham *et al.*, 1997). Moreover, motor regeneration is impaired in LIF knockout mice (Kurek *et al.*, 1997). On the other hand, LIF seems to be essential for sympathetic (Rao *et al.*, 1993) and sensory (Ekström *et al.*, 2000; Cafferty *et al.*, 2001) neuron survival and regeneration after injury.

Interleukin-6 (IL-6) and its receptor are up-regulated by neurons after injury (Streit *et al.*, 2000) and the combined over expression of both elements

enhances nerve regeneration (Hirota *et al.*, 1996). IL-6 also plays a role in the conditioning lesion effect, since in IL-6 -/- mice there was a lack of GAP43 upregulation after a preconditioning injury and no enhancement of regeneration (Cafferty *et al.*, 2004). However, the role of IL-6 in the conditioning lesion effect is controversial, since other authors defend that IL-6 is sufficient but not necessary to mimic the conditioning lesion effect on axonal growth (Cao *et al.*, 2006). Moreover, the exogenous application of FGF-2 (low molecular weight isoform) strongly enhanced the mRNA levels of IL-6 and its receptor in Schwann cells, as both trophic factors are supposed to play a key role in the early reaction of Schwann cells to peripheral nerve injury (Grothe *et al.*, 2000).

Although several experiments have demonstrated the usefulness of specific neurotrophic factors in nerve regeneration, it is becoming more apparent that the survival of sensory neurons and motoneurons depends on multiple neurotrophic factors acting synergistically or in a defined sequence (Terenghi, 1999). Furthermore, the possible preferential effects of some neurotrophic factors on regeneration of certain subpopulations of neurons have not been fully demonstrated in comprehensive comparative studies.

7. EXTRACELLULAR MATRIX AND ADHESION MOLECULES

Cell adhesion molecules (CAMs), extracellular matrix (ECM) molecules and guidance molecules maintain an important role during axonal regeneration, by exerting attraction/repulsion cues to the tip of the re-growing axon. Thanks to the receptors present at the growth cone site, the axons can respond to the different cues (Huber *et al.*, 2003). For instance, chemoattraction is the consequence of a gradient that the growth cone interprets as a “signal” and converts into motion, giving rise to a well defined machinery in which ECM and CAM bindings are fundamental.

7.1. ECM components

The ECM is part of the extracellular environment in animal tissues, and it is composed of different kinds of glycoproteins, proteoglycans and also non-proteoglycan polysacharides (as hyaluronic acid). It has mainly structural functions, providing support and maintaining cellular regulation. Many cells are

able to bind the extracellular matrix and this cell-to-ECM adhesion is regulated by specific ECM receptors that are present on the cell surface.

In the peripheral nerve, Schwann cells and fibroblasts produce the components of the endoneurial ECM under the control of axons. The molecules present at the ECM may have stimulatory or inhibitory cues for axonal regeneration. Therefore, in an intact adult nerve, the "inhibitory" component predominates to prevent collateral sprouting, whereas after injury the ECM undergoes changes to favor a pro-regenerative environment. The ECM components can be divided into two main categories: proteoglycans and glycoproteins. The latter can be divided into collagen and non-collagenous molecules (for example tenascin, laminin and fibronectin) (Carbonetto *et al.*, 1987).

Proteoglycans, such as chondroitin sulfate proteoglycans (CSPG), are found in the peripheral nerve, where they can inhibit the growth-promoting activities of other extracellular matrix components (Zuo *et al.*, 1998). For example, they inhibit growing of the axons on laminin by interfering with integrin signaling (Hamel *et al.*, 2008). Degradation of CSPG in the distal stump increases regeneration (Krekoski et al. 2001; Zuo et al., 2002) of both motor and sensory neurons (Udina *et al.*, 2010). After injury, CSPGs are cleaved by activated matrix metalloproteinases (Ferguson and Muir, 2000; Zuo *et al.*, 1998) (Zuo *et al.*, 2002) and this allows axonal regeneration, creating a permissive environment. On the other hand, glycosaminoglycans (GAGs) have an enhancing role on *in vitro* neuritogenesis and *in vivo* nerve regeneration and muscle reinnervation. Moreover, GAGs are supposed to maintain interaction with IGF-1(Gorio *et al.*, 1998).

Among the glycoproteins, three of them have an important role in axonal regeneration and their effects have been proved both *in vivo* and *in vitro*: collagen (Babington *et al.*, 2005), fibronectin (Gardiner *et al.*, 2007) and laminin (Werner *et al.*, 2000).

Collagens are trimeric molecules formed by three α chains (for review see Gordon and Hahn, 2010) and can be divided into several sub-families according to

the type of structure they form: fibrillar (types I, II , III, V, XI), facit-fibril associated collagens with interrupted triple helices (types IX, XII, XIV), short chain (types VIII, X), basement membrane (type IV) and other kinds with a different conformation (types VI, VII, XIII). Several studies showed the important role of collagens in axon pathfinding and in synaptic connection and maintenance (Fox, 2008).

Fibronectins are dimers formed by two almost identical monomers; in vertebrates, different kinds of isoforms are present due to alternative mRNA splicing. Fibronectin exists in two conformations: soluble and insoluble, the soluble fibronectin is one of the most abundant components of blood plasma and it is produced by hepatocytes, while the insoluble cellular fibronectin is incorporated in the double layer membrane of many cells types (Pankov and Yamada, 2002). In the nervous system fibronectin is secreted by Schwann cells and also by fibroblasts (Baron-Van Evercooren *et al.*, 1986; Chernousov and Carey, 2000). It forms a fibrillar network and maintains interactions with collagen IV and laminins, promoting in this way cell proliferation and differentation. Moreover, fibronectin binds mainly integrins but contains also domains for fibrin, collagen, heparin and syndecan (Mao and Schwarzbauer, 2005). Fibronectin is up-regulated immediately after peripheral nerve injury (Lefcort *et al.*, 1992); embryonic isoforms of this ECM molecule were found to be up-regulated after injury and they seem to promote regeneration in different ways (Lefcort, 1996; Mathews and Ffrench-Constant, 1995; Vogelezang *et al.*, 1999). However, *in vitro* studies demonstrated that fibronectin stimulates less neurite outgrowth in primary sensory neurons than laminin (Gardiner *et al.*, 2007).

Laminins are heterotrimers consisting of α, β and γ chains. In vertebrates five α, three β and three γ chains have been found and these subunits can form 45 different laminin isoforms with a tissue-specific localization. The different isoforms are usually identified by changes in the α subunit: α1 chain is expressed by developing and some adult epithelial cells; α2 is mainly found in Schwann cells and basement membranes of striated muscles; α3 is expressed in the epidermis; α4 can be found in a variety of tissues, as endothelium, smooth muscles, fat cells, Schwann cells, neuromuscular junction and bone marrow, and α5 is widely expressed in embryonic, developing and adult tissues (epithelia, endothelia,

muscles, neuromuscular junctions) (Durbeej, 2010; Hallmann *et al.*, 2005; Miner, 2008; Schéele *et al.*, 2007; Tzu and Marinkovich, 2008). Some laminin isoforms are now known to play a fundamental role in nerve regeneration. In the peripheral nervous system, two different kinds of laminins are mainly present, containing α2 and α4 chains, both synthetized by Schwann cells (Wallquist *et al.*, 2002), while α5 can be detected in the sensory end organs (Caissie *et al.*, 2006). Together with a β1 and a γ1 chain, these α subunits form laminin 2 (α2β1γ1), laminin 8 (α4β1γ1) and laminin 10 (α5β1γ1), respectively. The main receptors for these isoforms are integrins α1β1, α2β1, α6β1, α7β1 and sydecans.

After nerve injury the levels of laminin 8 increase in the proximal stump, with a peak of up-regulation at 3 days and normalization around 42 days after the lesion, a fact that hihglights the important role that this isoform probably plays in the early dynamics of nerve regeneration. On the other hand, laminin 2 is up-regulated after injury and this increase lasts longer than 42 days (Wallquist *et al.*, 2002). The same study proposes a correlation between the expression of cytokines by macrophages and the up-regulation of laminins. During the early stages of nerve regeneration, there is a coordinated temporal expression of laminin α4 and integrin α6 subunits in relation to Schwann cell proliferation, while laminin α2 and integrin α7 subunits are over-expressed at later stages, playing a more direct function on axonal regeneration itself. Although separate studies point out that spinal motoneurons preferr to grow on substrates containing α2 laminin (Wallquist, 2005) whereas sensory neurons extended neurites preferentially on α4 laminin (Fried *et al.*, 2005), no conclusive data supports the specificity of laminins for some populations of axons. Furthermore, there is not a differential pattern of expression of both isoforms α2 and α4 in motor and sensory roots (Plantman *et al.*, 2008).

Tube repair is an alternative technique to the classical suture or autograft repair of transected peripheral nerves, but it also offers a unique method to investigate and manipulate the local environment of axonal regeneration. Regeneration in tubular guides is dependent on the formation of a connective cable that bridges the gap between the nerve stumps. Inside the tube with the attached nerve stumps at the ends, an initial fibrin clot is formed, and provides a guiding

surface for the ingrowth of fibroblasts and Schwann cells migrating from both proximal and distal stumps (Liu, 1992; Williams *et al.*, 1983). The intratubular cable is then enriched with ECM components, mainly collagen fibrils longitudinally oriented, fibronectin and laminin. Regenerating axons then grow from the proximal nerve, directed by the Schwann cells in the intratubular connective cable. In vivo studies that used components of the naturally formed ECM, such as fibrin, fibronectin, hyaluronate, collagen and laminin-containing gels to prefill the lumen of the tube guide, have reported enhancement of peripheral nerve regeneration with respect to empty guides (Williams, 1987; Bailey *et al.*, 1993; Chamberlain *et al.*, 1998; Madison *et al.*, 1988; Labrador *et al.*, 1998). On the contrary, application of anti-laminin antibody or anti-laminin/collagen receptor to grafts or tubes inhibits axonal regeneration, whereas anti-fibronectin antibody has no effect (Wang *et al.*, 1992). In comparative studies, it was found that axonal growth for longer distances and higher levels of target reinnervation occurred with a laminin matrix with respect to collagen, fibrin and hyaluronate containing gels (Labrador *et al.*, 1998; Navarro *et al.*, 1996). On the other hand, by using an aligned collagen or laminin matrix, the rate and the direction of axonal elongation is improved due to contact guidance with the fibrils aligned along the tube axis, mimicking the natural orientation of the endoneurial tubules (Ceballos *et al.*, 1999; Chamberlain *et al.*, 1998; Verdu *et al.*, 2002). However, most of the studies investigating the influence of ECM components in vivo have focused on enhancing axonal regeneration across long gaps, and little is known on the differential effects in vivo regarding selective interaction with sensory and motor axons.

7.2. CAM components

Different CAMs can be found in the nervous system and are well-known for their key role in binding molecules of the ECM and the surface of other adjacent cells. Cadherins, members of the immunoglobulin superfamily (IgSF), and integrins mediate the interactions between the ECM and other CAMs present on other cells.

7.2.1. Cadherins

Cadherins (calcium-dependent adherent proteins) mediate cell to cell adhesion and consist in a superfamily with almost 50 different members (Angst *et*

al., 2001). The different cadherin subfamilies present different structures; the prototype contains single-pass transmembrane proteins, five cadherin repeats in the ectodomain and a highly conserved cytoplasmic region (Juliano, 2002). Among all the different subtypes, N-cadherin is the most studied in the nervous system, where it plays an important role in axon outgrowth and fasciculation and in synaptic formation and consolidation (Ranscht, 2000). These adhesion molecules contain two different domains, the juxtamembrane domain (JMD) and the C-terminal catenin-binding domain (CBD) that can bind to catenins and form cadherin-catenin complex.

7.2.2. Ig CAMs

Among the different members of the immunoglobulin (Ig) super family the most studied in the nervous system are the neural CAMs (NCAMs). These molecules are characterized by the presence of Ig-like domains, bind mainly in a homophilic manner and trigger different processes, such as axon pathfinding and target recognition. NCAM seems to mediate neurite outgrwth through at least two main different pathways: FGFR phosphorilation and Fyn association. Several studies indicate that the disruption of these interactions affect MAPK activation and the consequent neurite outgrowth (Jessen *et al.*, 2001; Niethammer *et al.*, 2002; Saffell *et al.*, 1997). Moreover, the polysilated form of NCAM is known to play a crucial role in regeneration of motor nerve branches (Franz *et al.*, 2005) and in promoting preferential reinnervation of muscles, as motor neurons in NCAM (-/-) mice reinnervate motor and cutaneous pathways with equal preference. Polysialic acid (PSA) is a carbohydrate attached to the glycoprotein backbone of the NCAM molecule, and it is implicated in nervous system development. Besides, PSA seems to be differently upregulated in motoneurons after injury; in fact, some pools of motoneurons show a high increase in PSA expression and this seems to trigger the preferential motor reinnervation of the quadriceps femoris muscle (Franz *et al.*, 2008). to Repair of the transected femoral nerve by a tube guide containing a PSA mimetic peptide enhanced Schwann cell proliferation, remyelination of regnerated axons and locomotion recovery. These effects were likely mediated by NCAM through its interaction with FGFR (Mehanna *et al.*, 2009).

Other types of CAMs, as for example L1 and L2, are known to affect axonal regeneration. However, their role is controversial since L1 ablation in Schwann cells was shown to enhance functional recovery after peripheral nerve injury, suggesting an inhibitory role of this molecule in Schwann cell proliferation after nerve damage (Guseva *et al.*, 2009). On the other hand, L2 epitope interacts with the adhesion molecule HNK-1, a marker of motor Schwann cells. It has been suggested that HNK-1 participates in motor axon regeneration (Mears *et al.*, 2003; Vrbova *et al.*, 2009) and influence preferential motor reinnervation (see above).

7.2.3. Integrins

Integrins are glycosylated heterodimers formed by α and β subunits, which are mainly implicated in focal adhesion and focal complexes formations (cell-to-basal lamina interactions). Integrins are non-covalently associated type I transmembrane proteins which have a cytoplasmatic (β) and an extracellular (α) domain (van der Flier and Sonnenberg, 2001; Hynes, 2002). In vertebrates, 18 α and 8 β subunits have been described and these can form 24 different integrin complexes (Takada *et al.*, 2007). Depending on their functions and interactions, integrins can be grouped into different sub-families (Table 1). Certain integrin subunits are ligand-specific, while some ECM molecules as laminin and fibronectin can bind to different integrins (Hynes, 2002). Integrins are also receptors for members of the Ig superfamily, thus mediating cell-to-cell adhesion. The integrin β1 family is composed of 12 different heterodimers; the β1 subunit provides the bound to actin cytoskeleton in each of this heterodimers, while the α subunit determines the ECM molecule-ligand specificity. Schwann cells express three different integrins: α1β1, α6β1 and α6β4, which are known to bind both laminin 2 and 8. Integrin α1β1 seems to be expressed only by immature Schwann cells, while α6β1 is expressed also during adulthood, although it is thought that its main function could be induction of myelination during development (Wallquist *et al.*, 2002).

Integrins have both mechanical and chemical functions at the same time and are involved in outside-in and inside-out signaling. Integrins control Rho protein activation and translocation of Rac1 and Cdc42 to the plasma membrane

(Del Pozo *et al.*, 2002), especially in specific membrane domains called lipid rafts (see below). Many integrins can form complexes with receptor protein tyrosine kinases (RPTKs) (Giancotti and Ruoslahti, 1999; Yamada and Even-Ram, 2002)(Table 1).

After axotomy, neurons overexpress α4β1, α5β1, α6β1 and α7β1 integrins (Ekström *et al.*, 2003; Kloss *et al.*, 1999; Vogelezang *et al.*, 2001; Wallquist, 2004; Werner *et al.*, 2000) that are important to promote axonal regeneration (Figure 6). Thus, embryonic and neonatal neurons can grow on inhibitory environments due to the elevated expression of integrins among other intrinsic factors (as elevated cAMP, see above) when compared to mature neurons (Lemons and Condic, 2008).

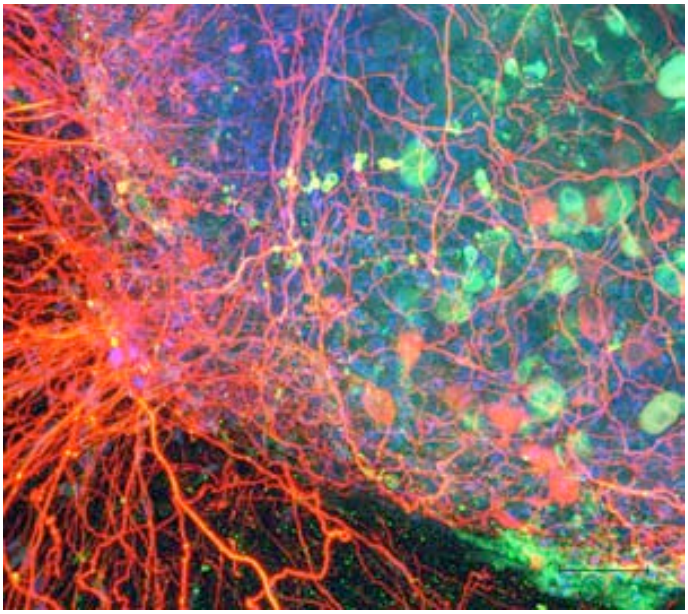


Fig 6. In vitro expression of integrin β1 (green) in regenerating sensory neurons of a DRG explant embedded in a collagen type I 3D matrix. The concentration of certain ligands influences the expression of integrin receptors and the elongation of neurites. Phosphorylated neurofilament – neuronal marker in red, DAPI – nuclear marker in blue.

Integrin subunit α7 is considered crucial for axonal regeneration in peripheral nerves. For example, deletion of the gene that encodes for α7 drastically reduces the speed of motor axon regeneration and delays target reinnervation (Werner *et al.*, 2000). Similarly, the poor regenerative response observed in knock out mice of c-jun could be related to the lack of up-regulation of α7 integrin subunits in these animals after injury (Raivich *et al.*, 2004). In agreement with these observations, over-expression of α7 subunit in injured neurons increases axon regeneration (Condic, 2001). The conditioning lesion effect also depends on integrin over-expression by sensory neurons. Increased neurite growth in DRG

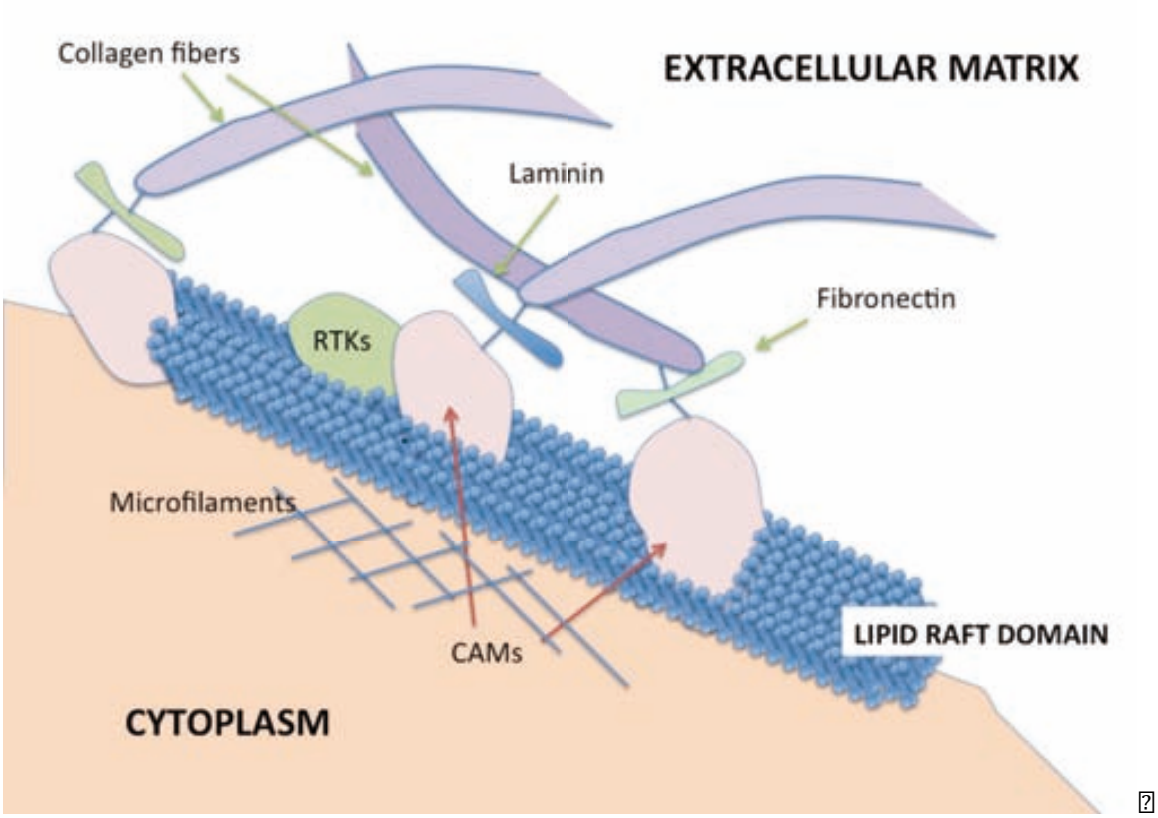
neurons primed by a previous axotomy is mediated by α7 integrin in matrigel or collagen substrates (Ekström *et al.*, 2003). In contrast, over-expression of α5 integrin mediates the enhancement of neurite outgrowth of conditioned neurons on fibronectin (Gardiner *et al.*, 2007).

The intracellular signals triggered by the the interaction between ECM and integrins also influences the cellular response to soluble growth factors and cytokines. In fact, integrin-mediated cell adhesion not only initiates direct signals but also modulates transmission of signals downstream of growth factor receptors (Del Pozo *et al.*, 2004; Del Pozo *et al.*, 2002; Schwartz and Ginsberg, 2002). Integrins, as other cell adhesion molecules, are necessary partners of growth factors and cytokine receptors (Giancotti and Tarone, 2003). For example, integrin β1 is involved in transmembrane signaling of GDNF, since the GDNF receptor GFRα-1 can form complexes with this integrin subunit after administration of GDNF (Cao *et al.*, 2008), and GDNF can also increase the integrin levels (Chao *et al.*, 2003; Funahashi *et al.*, 2003; Grabham and Goldberg, 1997).

8. INTERACTIONS BETWEEN NEUROTROPHIC FACTORS, RECEPTORS AND CAMS AT LIPID RAFTS

Interactions between cell adhesion molecules, growth factors and cytokines are mainly performed in specific plasma membrane domains called lipid rafts, an important structure rich in receptors that can be found at the growth cones.

The membrane bilayer in eukaryotic cells is mainly composed of three different classes of lipids: glycerophospholipids, sphingolipids and sterols; these kinds of lipids allow proteins to associate to the membrane thanks to their different properties. However, the lipid distribution in the cell membrane is asymmetrical and the composition and the physical state of certain lipid domains differ from the rest of the bilayer mosaic. Lipid rafts are rich in cholesterol and sphingolipids, and their state reveal a dynamic structure where cholesterol maintains a liquid order thanks to interaction with sphingolipids. A general marker of lipid rafts is the ganglioside GM1. Some lipid rafts are enriched with caveolin, and they are called caveloae microdomains. Among the proteins that accumulate in the lipid rafts are src-family kinases and G-proteins (Simons and


Collagen fibers
EXTRACELLULAR MATRIX
Laminin
Fibronectin
RTKs
Microfilaments
CAMs
LIPID RAFT DOMAIN
CYTOPLASM

present in the lipid rafts (Poteryaev *et al.*, 1999; Trupp *et al.*, 1997) and it recruits RET after GDNF administration. Thus, any corruptive manipulations of the lipid raft blocks RET recruitment (Tansey, 2000).

TrkB is translocated at the lipid rafts after BDNF administration but its translocation has been related to short-term BDNF actions, as synaptic transmission and plasticity, whereas it does not affect neuronal survival (Suzuki *et al.*, 2004). Similarly, Guirland *et al.* (2004) described a selective implication of lipid rafts in some of the functions of BDNF when examining its chemoattractant effect using a growth cone turning assay (Lohof *et al.*, 1992). Disruption of lipid rafts by depletion or sequestration of cholesterol only affected the growth cone turning assay, while the growth cone rate of extension mediated by BDNF was not altered. Thus, it seems that the lipid rafts can be mostly involved in specific behaviors of the cells. When the stability of lipid rafts was perturbed, by affecting GM1 gangliosides, BDNF effects on growth cone turning responses and elongation were abolished; therefore, it seems that gangliosides have an important role in BDNF signaling pathway, as reported also in other studies (Ferrari *et al.*, 1995; Pitto *et al.*, 1998). Repulsion from inhibiting molecules, such as netrin-1 and semaphorin 3A, was also blocked after the disruption of lipid rafts. Guirland and colleagues (2004) looked at TrkB receptor behavior and observed that, after BDNF administration just on one side of the growth cone, the receptors were distributed in an asymmetric way and were co-localized with gangliosides GM1 at the side of the growth cone that was exposed to the neurotrophic factor gradient. Therefore, the asymmetrical distribution of the lipid rafts in the cell may allow BDNF to elicit different effects under different conditions on the same neurons or just in certain parts of the cell (Guirland *et al.*, 2004). On the other hand, the presence of P75 receptor in the lipid raft has an inhibitory role in the translocation of TrkB into the raft mediated by BDNF (Suzuki *et al.*, 2004). The activated P75 receptor can also be localized in lipid rafts after NGF administration (Higuchi *et al.*, 2003). Since in this study they used mouse cerebellar neurons and E18 rat hippocampal neurons that are known to express P75 but not TrkA, the results suggest that NGF induces translocation of phosphorilated P75 to lipid rafts in neurons that express only P75 as an NGF receptor.

Besides its role in growth factor signaling transduction, lipid rafts play also a role in cell adhesion mechanisms: many adhesion molecules such as TAG-1 (transiently expressed axonal glycoprotein-1), NCAM-120 (neural cell adhesion molecule), Thy-1 and F3/contactin can be found in the lipid rafts and are GPI-anchored. Integrins have also been found into lipid rafts in fibroblasts, T cells and neurons (Ichikawa *et al.*, 2009; Palazzo *et al.*, 2004)

Thus, lipid rafts are key points where adhesion molecules and growth factors can interact and cross-talk. For example, the Rho family of small GTPase plays a central role in integrating signals from integrins and growth factors. Interestingly, the presence of Rho and Rac in lipid rafts depends on integrins. Integrins maintain lipid rafts in the plasma membrane, inhibit internalization of Rac binding sites and regulate Rho-mediate microtubule stabilization (Palazzo *et al.*, 2004). Integrins can also control signal pathways other than Rho, like ras/Erk, JNK, PI-3-kinase, FAK and src family kinases, by raft internalization. ERK activation by integrins would be important in situations with low concentration of growth factors, where growth depends on co-stimulation of ERK by integrins and growth factors.

It is important to take into account the role of these structures in nerve regeneration when looking at the axonal rewiring, as they are known to make a specialized signaling platform in the plasma membrane, and therefore can transduce signals different from those in the non-raft membrane (Anderson and Jacobson, 2002; Simons and Toomre, 2000; Suzuki *et al.*, 2004). Moreover, lipid rafts are a platform for protein complexes able to promote specific and short-lasting behaviors, for example, from neurotrophic factors and adhesion molecule signals, which will be affected by disruption of these microdomains.

9. CONCLUSIONS

Rewiring the peripheral nervous system after nerve injury has been considered an easier issue to manage when compared to the central nervous system repair. However, several mechanisms underlying nerve regeneration are not fully known yet. Several molecules are known to play an important role in axonal regeneration, both at the neuronal and at the distal nerve levels, but the

way they interact between each other is still not clear. This issue reduces the possibilities of achieving optimal regeneration, also in terms of target organ specific reinnervation. The present review presents an overview of the factors that are playing a major role during peripheral nerve regeneration, taking in consideration the interactions between axons, glial cells and the extracellular matrix molecules. The switch of the gene machinery of the neurons to a pro-regenerative state triggers axon sprouting and elongation along the endoneurium, where Schwann cells have proliferated. Unfortunately, the topographic distribution of nerve fascicles and of motor and sensory fibers is disrupted after transection injuries of the nerve (Badia *et al.*, 2010; Lago and Navarro, 2006), a fact that largely affects the accuracy of target organ reinnervation. Therefore, attempts to directing axons during re-growth and appropriate pathfinding will be crucial for improving functional recovery after severe nerve lesions. For such attempts, it is important to consider the interactions maintained between neurotrophic factor receptors and cell adhesion molecules, since their reciprocal influence is known to direct the tip of the axon during outgrowth. The emerging concept is to attempt re-wiring at the microdomain level, where neutrophic factor receptors and cell adhesion molecules are interacting and activating the regenerative machinery under the influence of the extracellular environment. Promising strategies to promote specific axon regeneration could be bio-engineered grafts to bridge the lesion gap (Rodriguez *et al.*, 2000; Navarro *et al.*, 2003; Marino *et al.*, 2009), which combine the best substrates for regeneration, enriched with Schwann cells and funcionalized to over express selected trophic factors (Tannemaat *et al.*, 2008; Mason *et al.*, 2011).

Table 1. Types of integrins and their main specifications. For complete review see (Barczyk *et al.,* 2010).

β1 Family	**ECM components & NT factor relations**	**Cell type expression**
α1β1	Collagens/Sema7A VEGF/TGFβ/PDGF/MCP-1/IL-8	Endothelial cells/Fibroblasts
α2β1	Collagens/E-cadherin/Endorepellin VEGF	Endothelial cells/Fibroblasts
α3β1	Laminins/CSPG4	Epithelial cells
α4β1	Fibronectin/VCAM1	Endothelial cells
α5β1	Fibronectin/Endostatin	Fibroblasts
α6β1	Laminin	Epithelial cells and glia
α7β1	Laminin	Muscle cells and neurons
α8β1	Fibronectin/Vitronectin/Nephronectin	Kidney and ureter bud epithelium
α9β1	Fibronectin/VCAM1/Cytotactin/ Osteopontin/Tenascin-C/VEGF	Endothelial cells
α10β1	Collagens	Chondrocytes
α11β1	Collagens PDGF	Fibroblasts
αVβ1	Fibronectin/Vitronectin	T-cells

ABBREVIATIONS

AKT/PKB: protein kinase B

BDNF: brain derived neurotrophic factor

cAMP: cyclic adenosine monophosphate

CAMs: cell adhesion molecules

Cdc42: cell division control protein 42 homolog

cGMP: cyclic guanosine monophosphate

ChAT: choline acetyltransferase

CGRP: calcitonin gene-related peptide

CNTF: ciliary neurotrophic factor

CSPG; chondroitin sulfate proteoglycan

DRG: dorsal root ganglia

ECM: extracellular matrix

ERK: extracellular signal-regulated kinase

FGF: fibroblast growth factor

FGFR**:** fibroblast growth factor receptor

GAP: growth associated protein

GDNF: glial cell-derived neurotrophic factor

GFR: glial cell derived neurotrophic factor family receptor

GGF: glial growth factor

GSK3b: glycogen synthase kinase 3 beta

HGF/SF: hepatocyte growth factor/scatter factor

HNK1: human natural killer 1

HSPG: heparan sulfate protoglycan

IB4: isolectin B4

IGF-1: insulin-like growth factor 1

IL-6: interleukin 6

JNK: c-Jun N-terminal kinase

LIF: leukemia inhibitory factor

mTOR: mammalian target of rapamycin

MAP: microtubule-associated protein

MAPK: Mitogen-activated protein (MAP) kinase

NCAM: neuronal cell adhesion molecule

NGF: nerve growth factor

NRG1: neuregulin 1

NT-3: neurotrophin 3

OPN: osteopontin

P38: mitogen-activated protein kinase 38

P75: low-affinity nerve growth factor receptor/P75 neurotrophin receptor

PKA: protein kinase A

PMR: preferential motor reinnervation

PNS: peripheral nervous system

PTEN**:** phosphatase and tensin homolog

PTN: pleiotrophin

RET: proto-oncogene - receptor tyrosine kinase

Rho: ras homolog gene family

Sema 3A: semaphoring 3A

SP: substance P

STAT3: signal transducer and activator of transcription 3

TGFα-1: transforming growth factor alpha 1

Thy-1/CD90: cluster of differentiation 90

TrK: receptor tyrosine kinase

TSC: tuberous sclerosis protein

VEGF: vascular endothelial growth factor

OBJECTIVES

OBJECTIVES

Functional recovery is still poor after peripheral nerve lesions, mainly due to insufficient axonal regeneration and to misrouted growth and reinnervation of the target organs. The lack of specificity of nerve regeneration, in terms of motor and sensory axons growth, pathfinding and target matching, is one of the main shortcomings for recovery. The molecular mechanisms involved in nerve regeneration after injury are complex, including the interrelation between axons and glial cells, neurotrophic factors, extracellular matrix molecules and their receptors.

The main aims of this thesis are:

- to investigate potential molecular factors that may selectively promote regeneration of motor and sensory neurons, and

- to provide basic tools for strategies to enhance and guide axonal regeneration and selective reinnervation of adequate target organs.

The specific objectives of this thesis are:

1) To set up a 3D *in vitro* model that mimics the permissive peripheral nervous system environment. This model allowed the comparison of motor and sensory neuron regeneration using a collagen matrix as a support for neurites outgrowth,
2) To modify the environmental conditions of the 3D culture models by adding different trophic factors in the matrix, in order to assess the differential effects on neuritogenesis of spinal sensory and motor neurons. Several neurotrophic factors were tested,
3) To correlate the effects of selected neurotrophic factors *in vitro* with the changes in their expression in motor and sensory nerve branches after injury,

4) From the results of the previous objectives, to investigate in detail the possible mechanisms of action through which fibroblast growth factor 2 (FGF-2)promotes motoneuron neuritogenesis,

5) To produce a lentiviral vector (LV) that allows for FGF-2 over-expression. The effects of the LV-FGF2 were characterized *in vitro* and *in vivo*, and the bio-activity of FGF-2 secreted by infected cells tested on motor and sensory neurons using our *in vitro* model,

6) To comparatively assess the capabilities of Schwann cells and olfactory ensheathing cells, two glial populations that support axonal regeneration, for promoting neurite elongation and branching of sensory and motor neurons.

CHAPTER 1

IN VITRO COMPARISON OF MOTOR AND SENSORY NEURON OUTGROWTH IN A 3D COLLAGEN MATRIX

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Abstract

In this work we set up an in vitro model, based on organotypic cultures of spinal cord slices and dorsal root ganglia explants from P7 rats, embedded in a collagen matrix and cultured under the same conditions. As specific reinnervation of end-organs is still an unresolved issue in peripheral nerve research, we characterized a model that allows us to compare under the same conditions motor and sensory neuron regeneration. RT97 labeling was used to visualize the regenerating neurites that extended in the collagen gel from both motor neurons in the spinal cord slices and sensory neurons in the DRG explants after a few days in vitro. By adding different neurotrophic factors in the collagen matrix, we evaluated the reliability of DRG and spinal cord preparations. Moreover, we also set up a co-culture with dissociated Schwann cells to further mimic the permissive environment of the peripheral nerve. Thus, these *in vitro* models can be useful tools to investigate mechanisms for the selective regeneration of sensory and motor neurons, which can be translated into *in vivo* models.

Key words: axonal regeneration, organotypic culture, motor neurons, sensory neurons, neurotrophic factors, Schwann cells.

Introduction

After peripheral nerve injury transected axons can regenerate and reinnervate denervated targets. Although neurons are able to regenerate their axons along the distal degenerating nerve after a nerve transection, reinnervation of target organs and functional recovery is often deficient because axons regenerate at random and can aberrantly reinnervate targets that do not match their original function. Specificity of reinnervation is a key issue to improve functional recovery after peripheral nerve injuries, but not much is known about this phenomenon and how it could be improved. Neurotrophic factors and extracellular matrix molecules are potential promoters of axonal regeneration for certain neuronal population (Huber *et al.*, 2003), but a direct comparison between the effects of different factors on sensory and motor regenerative capabilities has not been fully addressed. On the other hand, a differential expression of molecules in motor and sensory nerves has been reported, for example, motor axons express polysialic acid-neural cell adhesion molecule (PSA-NCAM) during regeneration (Franz et *al.*, 2005), while motor Schwann cells present L2/HNK-1 (Martini *et al.,* 1992). Moreover, a different profile of trophic factor expression between motor and sensory nerves has been described (Hoke *et al.,* 2006). These evidences support the contention that motor and sensory axons may respond differently depending on the environment and on the intrinsic neuron regenerative capabilities. Differences in these capabilities have already been observed between subpopulations of sensory neurons in vitro (Leclere *et al.*, 2007) (Kalous and Keast, 2010).

In vitro models offer certain advantages over *in vivo* studies since interpretation of results may be easier and more conditions can be assessed. However, such studies have mainly focused on sensory neurons (Tucker and Mearow, 2008), since dissociated neurons or whole explants of dorsal root ganglia (DRG) from adult animals can be easily cultured. In contrast, motor neurons are more complex to manage *in vitro*. This fact limits a direct comparison of the growth capabilities of these two neuronal populations and their response to different cues. Dissociated motoneuron cultures are usually from embryonic origin. However, embryonic neurons have an intrinsic capacity to grow that adult neurons

lack. Adult dissociated motoneurons can survive only in the presence of serum in the medium, or in serum-free media with muscle extracts and cAMP (Montoya *et al.,* 2009*)*. However, addition of these factors can interfere with the results of the experiments when testing other factors or conditions. Postnatal motoneurons can be also studied on organotypic cultures of spinal cord slices. Young postnatal rats (around P7) are commonly chosen since the cytoarchitecture of the spinal cord is already established, neuronal viability is still high and there is no dependence of the target organ (Delfs *et al.*, 1989; Stavridis *et al.*, 2005). Successful organotypic cultures of adult mice have also been reported when supplemented with serum (Krassioukov *et al.,* 2002). The organotypic spinal cord slice has been mainly used to study motoneuron survival and not often motor axon outgrowth (Guzman-Lenis *et al.,* 2009a). In these preparations, neurites usually grow in or around the spinal cord (Bonnici and Kapfhammer, 2008; Guzman-Lenis *et al.*, 2009b).

The aim of this work was to set up an *in vitro* model allowing a reliable comparison of motor and sensory neurons outgrowth in control and manipulated environments, where different molecules with potential capacity to promote specific outgrowth of sensory or motor neurons can be assayed. We focused on neurotrophic factors as they are known for their capability in attracting axons (Terenghi, 1999) and we made a screening in order to find possible guiding cues for motor and sensory axons. We modified two well known *in vitro* methods: the organotypic spinal cord slice and the DRG explant, embedding both samples into a 3D collagen matrix, which creates a permissive environment for neurite elongation (Tucker *et al.*, 1996). Collagen gels can be easily manipulated maintaining the same concentration, density and pH and allow to add molecules to assess changes in neurite outgrowth related to the modified environment. Because Schwann cells are known to play an important role during peripheral nerve regeneration (Jessen and Mirsky, 1999), we also set up a co-culture with primary Schwann cells, to look at their interactions with the regenerating neurites.

Materials and Methods

Spinal cord slices and DRG explants cultures

Sprague-Dawley OFA rats of postnatal day 7 were decapitated and their spinal cord (lumbar and high sacral segments) and DRG (low thoracic and lumbar) were dissected, placed in cold Gey's balanced salt solution (Sigma) enriched with 6 mg/ml glucose, and cleaned from blood and meningeal debris. Spinal cords were then cut with a McIlwain Tissue Chopper into 350 μm thick slices.

A volume of 450 μl of rat tail type I collagen solution (BD Biosciences) at a concentration of 3.4 mg/ml, was mixed with 50 μl of 10X basal Eagle's medium (Gibco) and 2 μl of 7.5% sodium bicarbonate solution (Tucker *et al.,* 1996). The final concentration of the collagen gel was thus 3.05 mg/ml. Single drops of 30 μl were deposited on poly-D-lysine (PL, 1μg/ml, Sigma) coated coverslips, which were placed in Petri dishes or 24-well multidishes (Iwaki, Asahi Technoglass, Chiba, Japan) and kept in the incubator at 37°C and 5% CO_2 for two hours to induce collagen gel formation.

Spinal cord slices and DRG explants were then placed on the gelled collagen droplets and covered by a second drop of 30 μl of the same collagen solution. The embedded samples were placed again in the incubator for 45 minutes before adding Neurobasal medium (NB, Invitrogen), supplemented with B27 (Invitrogen), glutamine and penicillin/streptomycin (Sigma). The medium volume delivered into Petri dishes and wells was 1.5 ml and 0.5 ml respectively. After one day in culture, the medium of spinal cord cultures was removed and changed by a penicillin/streptomycin free medium. DRG explants were cultured for 2 days, and spinal cord slices for 4 days.

Schwann cells culture and purification

Schwann cells were isolated from two months old Sprague-Dawley rats, following a previously reported method (Verdu et al., 2000). Sciatic nerve segments were stored in cold Gey's balanced salt solution (Sigma) and cleaned from connective tissue. Nerves were enzymatically dissociated in 1 ml Ca^{2+} and Mg^{2+} free Hank's medium (Sigma) with 0.25% trypsin (Sigma), 1mg/ml collagenase A (Sigma) and 1 mg/ml DNAse (Roche), followed by mechanical dissociation. Samples were then centrifuged at 900 rpm for 7 minutes. The cells

were resuspended and seeded onto poly-D-lysine coated Petri dishes (Iwaki, Asahi Technoglass, Chiba, Japan) and incubated with 2 ml of DF-10S medium. After 5 days, cells were enzymatically detached with 0,25% trypsin (Sigma). For immunopurification, cells were incubated with mouse monoclonal primary antibody anti-nerve growth factor receptor (NGF-Receptor, p75, 1:1000, Chemicon), and then with goat secondary antibody anti-mouse IgG conjugated with microbeads (Miltenyi). Finally, labeled cells were separated in a MACS column (Miltenyi) placed within a magnetic field.

Cells were transfected with a lentivirus-GFP vector (a generous gift of Dr. Joost Verhaagen, Netherlands Institute for Brain Research). Lentiviral vectors were added to the dish at a multiplicity of infection of 1:100 for 48 h. Transduced Schwann cells were used when high levels of transgene expression were achieved. Expression of the reporter gene GFP was directly visualized under an inverted fluorescence microscope (Olympus CKX41). An adequate amount of Schwann cells suspension in DF10S medium was gently mixed into the collagen matrix to get a final concentration of $50x10^3$ or $10x10^3$ Schwann cells in each volume of collagen matrix used to embed spinal cord organotypic slices and DRG explants.

Immunohistochemistry

Spinal cord cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes, and DRG cultures for 15 minutes. Samples were then incubated for 48 hours with primary antibodies: mouse RT97 (1:200, Developmental Studies Hybridoma Bank), mouse SMI32 (1:2500, Sternberger Monoclonals Inc.), chicken anti-NF200 (1:1000, Millipore), rabbit anti-growth associated protein-43 (GAP43, 1:1000, Chemicon), rabbit anti-cleaved caspase-3 (1:200, Cell Signalling Technology), rabbit anti-S100 (1:200, Immunostar), rabbit anti-fibronectin (1:400, Sigma), mouse anti-Thy.1 (1:300, Abcam), rabbit anti-GFP (1:200, Invitrogen). After three hours washing, the sections were incubated overnight with secondary antibodies Cy3 conjugated donkey anti-mouse (1:200, Jackson IR) or FITC conjugated goat anti-rabbit (1:200, Vector). For biotin amplification, samples were incubated overnight with biotin anti-rabbit antibody (1:200, Vector) and then, after washes, for two hours with Cy2 streptavidin (1:200,

Jackson IR). Samples were then mounted on slides with glycerol supplemented with 10% Moviol and 0.6% DABCO containing DAPI marker (1:1000, Sigma).

Colocalization of SMI32, RT97, NF200 and GAP43, and qualitative analysis for cleaved caspase-3, S-100, fibronectin, Thy.1 and GFP expression were done with 3D images taken with a SP5 confocal microscope (Leica).

TUNEL staining

After 4 days in vitro, spinal cord organotypic slices were fixed in 4% paraformaldehyde in PBS, incubated for 10 minutes in 4% Proteinase K (Millipore) diluted in PBS, and then a TUNEL Apoptosis Detection Kit (Upstate-Millipore; Catalog No. 17-141-FITC) was used following the manufacturer recommendations.

Motoneuron survival

In order to assess the potential contribution of the different conditions tested on neuronal survival that might influence the measures of axonal growth, we quantified the number of surviving and regenerating motoneurons in the spinal cord organotypic slices. Slices were stained with RT97 and SMI32. The number of motoneurons was counted for at least six samples per condition under a SP5 confocal microscope (Leica). Only cells with morphology corresponding to motoneurons and a clearly visible nucleus were counted in each section.

Neurotrophic factors assay

A screening for the effects of different neurotrophic factors (Table 1) was made by adding into the collagen matrix 10 ng/ml of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), or glial cell line-derived neurotrophic factor (GDNF). Neurotrophic factors were added to 50 μl of 10X basal Eagle's medium and then kindly mixed with the collagen solution with a pipette.

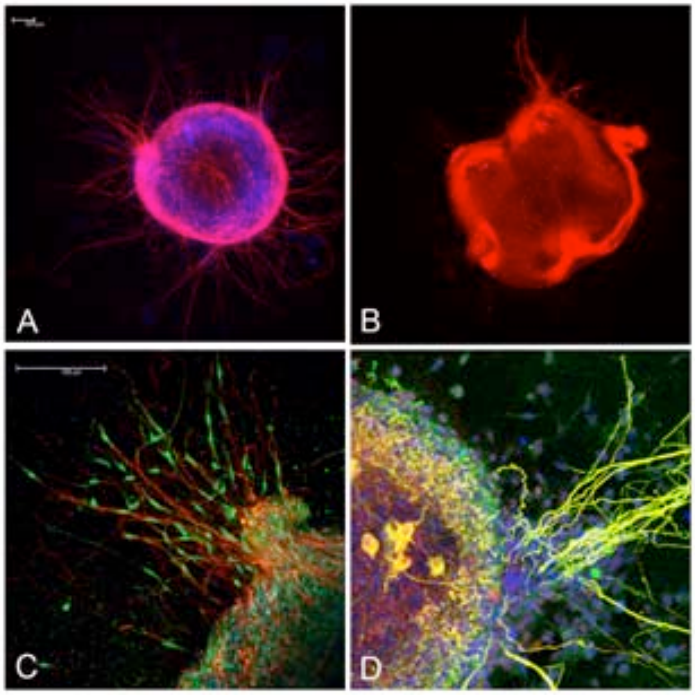

Figure 1. Neurite outgrowth from sensory and motor neurons in a 3D collagen matrix. DRG explant after two days in vitro (A) and spinal cord slice after four days in vitro (B) stained with RT97, a marker for phosphorylated neurofilaments. At higher magnification it can be observed the migration of Schwann cells (marked with S100β, in green) into the collagen matrix associated with the growing neurites (labeled with RT97, in red) in a spinal cord slice (C). Detail of motoneurons and their regenerating neurites co-labeled with RT97 (in red) and Neurofilament 200 (in green) in a spinal cord slice (D). The nuclear marker DAPI is shown in blue. Bars = 100 µm.

Data analysis

Spinal cord and DRG cultures were stained with anti-neurofilament antibody RT97 to label neurons and the growing neurites. Microphotographs for quantitative analysis were taken at 20× with a digital camera (Olympus DP50) attached to the microscope (Olympus BX51), acquired in Adobe Photoshop CS4 to automatically photomerge them, and analyzed with the aid of ImageJ software (NIH, available at http://rsb.info.nih.gov/ij/). The length of the longest neurite in the cultures was measured for 20 samples per condition. For the arborization area, the microphotographs were transformed to a gray scale, 8-bit image, and quantification was assessed after defining a threshold for background correction using ImageJ software.

Statistical analysis was performed using Sigma Stat 3.5 software. All results are given as the mean±SEM. One-way ANOVA, with post-hoc Holm-Sidak method (multiple comparisons versus control group) where necessary, was used to statistically analyze neurite outgrowth and branching pattern. T-test was performed to compare motor and sensory percentages of elongation and arborization for each trophic factor. A p value lower than 0.05 was considered significant.

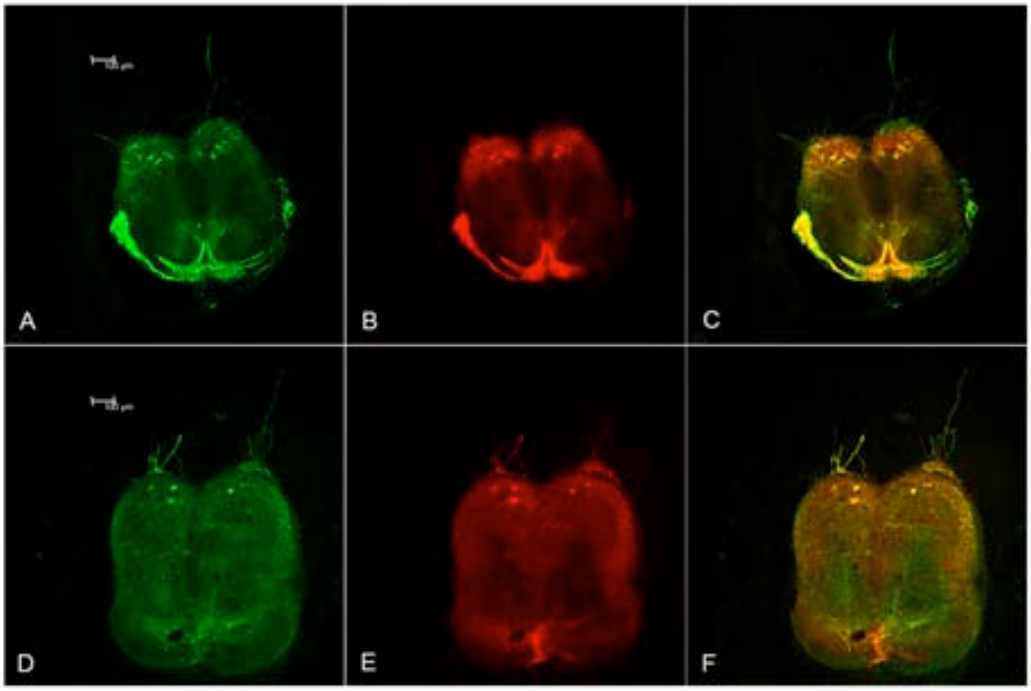

Figure 2. Different expression of phosphorylated and non-phosphorylated neurofilaments in motoneurons after 4 days in culture. The panmarker of neurofilaments NF-200 (green, A and D) stains both somas and neurites of motoneurons, whereas the non-phosphorylated form (SMI32, red, B) stains the somas but not the neurites (no merge in the neurites, C). In contrast, the phosphorylated form stains the somas of the regenerating neurons and their neurites (RT97, red, E; see the merge in F). Bars = 100 µm.

Results

Spontaneous neurite outgrowth in the collagen matrix

By using RT97 labeling we observed that the 3D collagen matrix allowed the extension of neurites from both motor neurons in the spinal cord slices and sensory neurons in the DRG explants after a few days in vitro (Fig. 1). Thus, the

collagen matrix used creates a permissive environment, in which neurites elongate radially from both tissues. The pattern of neurite growth was different depending on the tissue. Motor neurites emerged mainly from the ventral horn of the spinal cord, following the remaining stump of the spinal root and, at the same time, S100-positive glial cells and fibronectin/Thy1 positive cells migrated outside the slice interacting with and giving structural support to the newborn neurites (Fig. 1C). In DRG explants non-neuronal cell migration also supported neurite outgrowth, but the neurites extended around the whole ganglion. Primary sensory neurons appeared to have a higher intrinsic capacity of outgrowth (1078±79 μm elongation and 9148±2218 μm^2 arborization) in these conditions compared with spinal motoneurons (374±66 μm elongation and 180±56 μm^2 arborization). For this reason we maintained in culture the DRG explants for two days and the spinal cord slices for four days.

Confocal microscopy investigations showed that regenerating neurons and their neurites were stained with RT97, while SMI32 stained all motoneuron somas but not the neurites, and the pan-marker for neurofilaments NF200 labeled all the neurons (regenerating and not) and also the neurites (Fig. 2). The counts of SMI32 positive neurons revealed that, after 4 days in vitro, there was an average of 65.5±7.5 motoneurons in a 350 thick slice. This neuronal loss is probably due to the proximal axotomy performed during harvesting of the samples and the limited oxygenation of the culture (Gahwiler et al., 1997). On the other hand, the number of RT97 positive neurons was lower than that of SMI32 positive ones (34.8±4.7), thus suggesting that only a proportion of the surviving motoneurons turn to a regenerative state (Fig. 3C). These observations were corroborated by co-localization analysis with GAP43 (Fig. 3A-B), which revealed that RT97/GAP43 have a Person's correlation of 0.81, while SMI32/GAP43 have a correlation of 0.47. Therefore, we used RT97 labeling to measure neurite growth, since RT97-positive neurons seem to be in a regenerative state. Investigations with cleaved caspase 3 and TUNEL did not show signs of apoptosis in neither SMI32 positive nor RT97 positive neurons (Fig. 4).

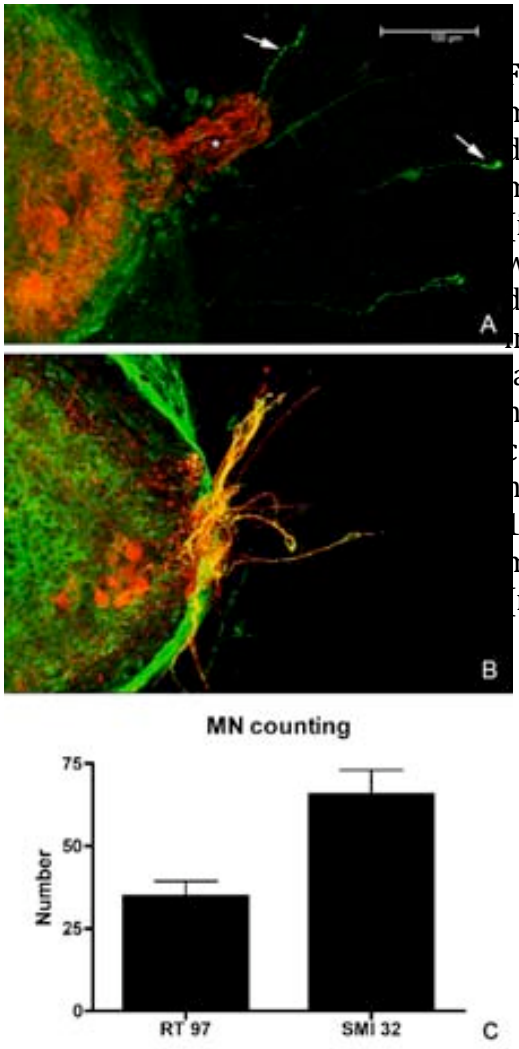

Figure 3. Detail of the regenerating motoneurons in spinal cords cultured for 4 days. (A) SMI32 (red) labels the soma of motoneurons but not the neurites (indicated with arrows), that are labeled with GAP43 (green). SMI32 stains the degenerating neurofilaments still present in the roots *. (B) In contrast, there is co-labeling of the phosphorylated neurofilament (RT97, red) and the growth cone marker GAP43 (green) in the neurites of motoneurons (yellow). Bar = 100 μm. (C) Number of RT97 positive motoneurons versus SMI32 positive ones (n=7).

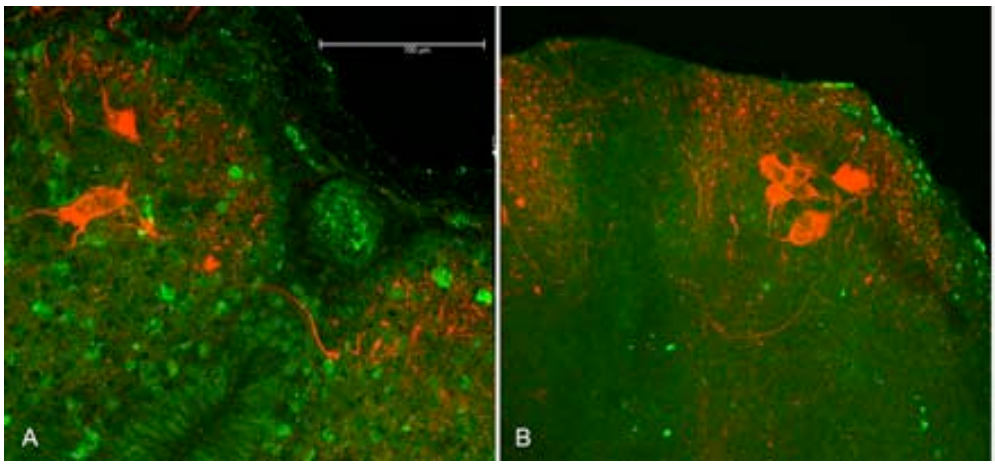

Figure 4. Assessment of motoneuron death after 4 days in culture. (A) RT97 labeled motoneurons (red) do not colocalize with caspase 3 (green). (B) SMI-32 labeled motoneurons (red) do not colocalize with TUNEL positive cells (green). Bars = 100 μm.

Neurotrophic factors assay

We manipulated the environmental conditions of the organotypic cultures by adding different neurotrophic factors (Table 1) to the collagen matrix and comparing the results with control conditions for both types of cultures (Fig. 5). We added GDNF, BDNF, NGF or NT-3 to assess the reliability of the in vitro models, to study the pattern of axonal regeneration and to screen the specificity of different factors for motor or sensory neurite growth. To be sure that the possible differences in neuron survival after the addition of neurotrophic factors were not affecting outgrowth measures, we counted the number of RT97 positive neurons (Fig. 5E). These results showed that the average numbers of regenerating motoneurons were not significantly different between any condition from controls. We then compared the effects of these factors on regeneration measuring neurite length and arborization. BDNF enhanced motoneurons outgrowth, NGF had a positive effect only on sensory neurons, while GDNF promoted neurite outgrowth in both types of neurons (Fig. 6, Table 2). NT-3 did not seem to promote motor and sensory neuron outgrowth, although it enhanced the degree of arborization in DRG.

To compare the effects of each factor on spinal cord and DRG explants, the values of neurite length and amount of arborization were normalized as the percentage of the corresponding control values (Fig. 7). Addition of GDNF and BDNF to the collagen matrix increased about 100% the maximal motor neurite length, whereas the other neurotrophic factors did not cause a significant effect. Regarding the amount of neurite branching, BDNF showed a marked increase of about 5-fold, whereas NT3 and GDNF had lower positive effect. On DRG explants, addition of NGF and GDNF increased by 60% the sensory neurite length, and BDNF and NT-3 caused a lower increase (about 20%). GDNF and NGF significantly enhanced neurite arborization of sensory neurons. When comparing the differential effects of the trophic factors on both DRG and spinal cord explants, we found that NGF exclusively promoted growth of sensory neurites, whereas BDNF selectively enhanced motor neurite outgrowth.

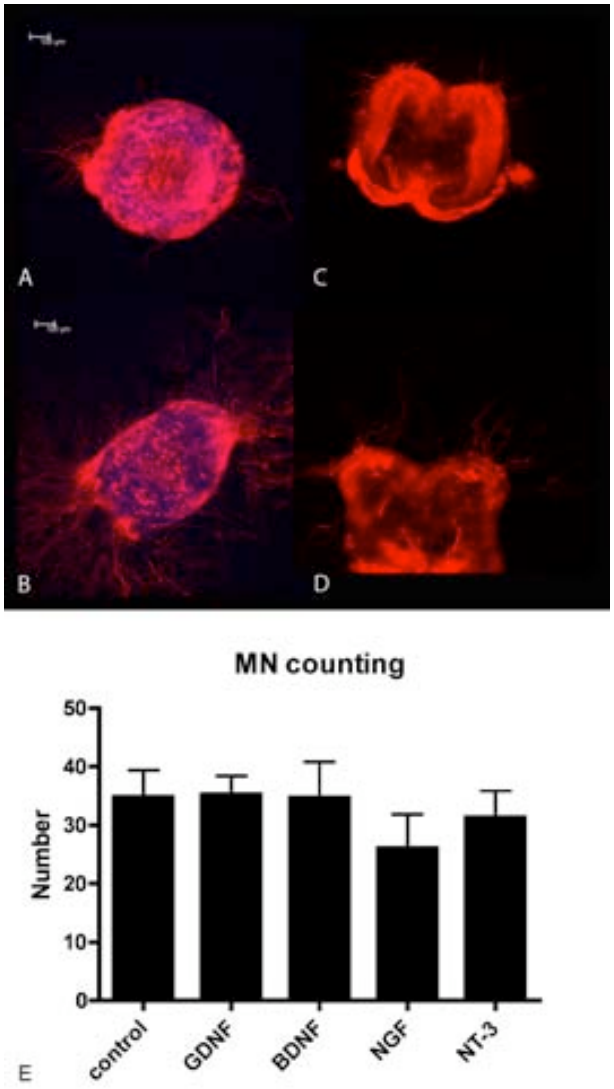

Figure 5. Representative images of DRG explants (left) in the collagen matrix in control conditions (A), and with addition of 10 ng/ml of NGF (B) after 2 days in culture. Representative images of spinal cord slices (right) in the collagen matrix in control conditions (C) and enriched with 10 ng/ml BDNF (D) after 4 days in vitro. Bars = 100 µm. (E) The graph is showing the number of motoneurons present in the organotypic slices in each condition. Only RT97 positive motor neurons present in the ventral horn were counted. A number of at least 6 slices per condition was analyzed.

Co-culture of explants with Schwann cells

Schwann cells morphology and viability was not affected in our *in vitro* models at concentrations of $50x10^3$ and $10x10^3$. The cells showed good migration within the collagen matrix. Moreover, in the $50x10^3$ cell condition, Schwann cells became confluent after four days in vitro. Confocal microscopy observations allowed to see that glial cells were integrated in the matrix and guided the growing neurites (Fig. 8).

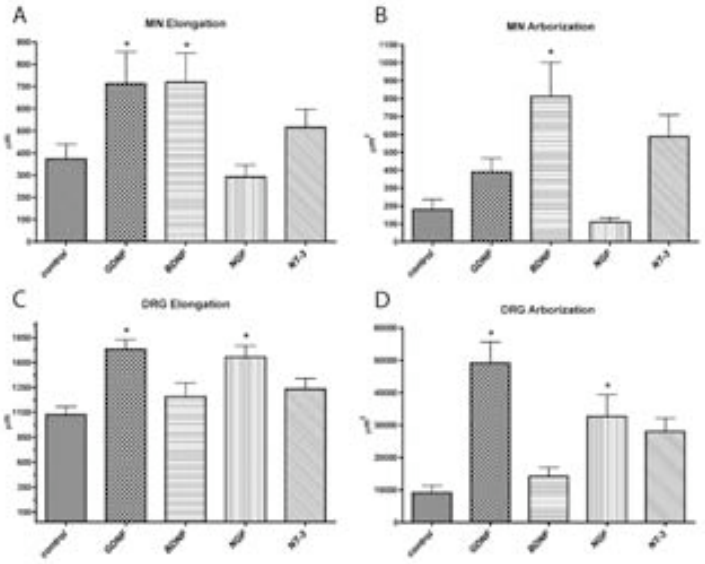

Figure 6. Effect of neurotrophic factors added to the collagen matrix on length (A, C) and arborization (B, D) of neurites from motoneurons in spinal cord slices after 4 days (A, B) and from sensory neurons in DRG explants after 2 days in vitro (C, D). GDNF and BDNF significantly enhance motoneuron outgrowth. GDNF promotes also outgrowth of primary sensory neurons whereas NGF promotes both neurite elongation and arborization of sensory neurons.

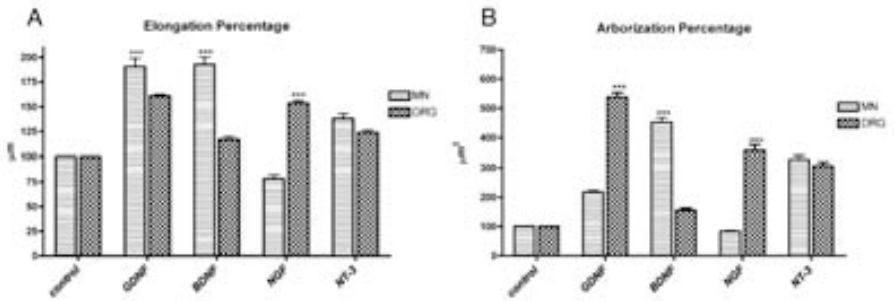

Figure 7. Comparison between motor and sensory neurite outgrowth (expressed as percentage of control conditions) in different neurotrophic factor enriched environments. Quantification of neurite length and arborization reveals that NGF has specific effects on sensory neurons, while BDNF has a significant effect on motoneuron outgrowth, and GDNF enhances regeneration of both kinds of neurons. Asterisks indicate significant differences in outgrowth between motor and sensory preparations ($p<0.005$, t test).

Discussion

In this study we successfully cultured postnatal motor and sensory neurons in spinal cord organotypic slices and DRG explants respectively. Both cultures were embedded in a collagen matrix and cultured under the same conditions, with neurobasal enriched medium without serum or general trophic factors, thus allowing for a reliable comparison of the response of motor and sensory neurons to the addition of different neurotrophic factors. The collagen gel is an adequate matrix to support axonal growth and can be easily manipulated to modify the extracellular ambiance, by seeding it with molecules or cells. In fact, both motor and sensory neurons extended neurites in the matrix in basal conditions. In addition, a collagen matrix is also an adequate scaffold to fill artificial nerve guides to repair an injured nerve *in vivo*, thus our *in vitro* findings may be easily translated to *in vivo* models (Midha *et al.,* 2003; Rodriguez *et al.,* 1999).

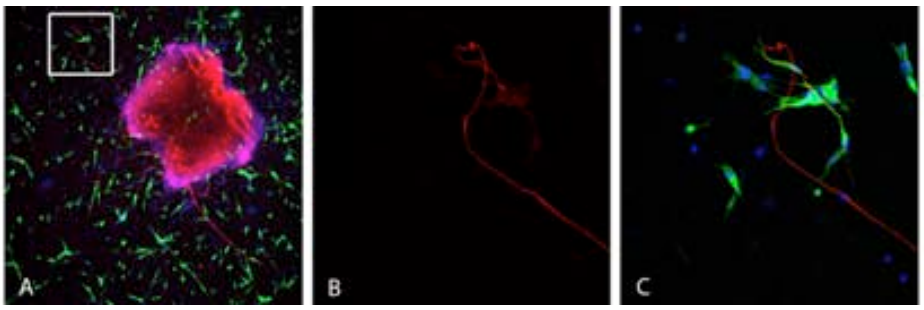

Figure 8. Representative image of a spinal cord organotypic slice co-cultured with GFP positive Schwann cells at $50x10^3$ concentration (A). Detail of a neurite growing in the collagen matrix, labeled with RT97 (B), and merge showing the interaction with the exogenous Schwann cells (C). DAPI is used for labeling the cell nuclei.

The organotypic neural cultures are multicellular *in vitro* models, in which neurons remain embedded in their natural environment in contact with glial cells and maintain interneuronal connections (Crain and Peterson, 1967; Stoppini *et al.*, 1991). Since there is a preservation of the tissue cytoarchitecture and the interactions between neurons, glia and extracellular matrix, the general response to each single neurotrophic factor is maintained (Gahwiler *et al.*, 1997). Therefore, organotypic cultures can give a closer indication to *in vivo* responses than

dissociated cell cultures, where the disruption of the normal relationship with supporting cells may affect the neuronal phenotype.

The age of the cultured neurons is a key point to take into account for the reliability of the model. Embryonic neurons have a higher intrinsic capacity to grow than adult neurons, and adult neurons need trophic factor support to survive (Montoya *et al.,* 2009). In contrast, spinal cord explants from P7 rats can be maintained for relatively long-term on media without additional growth factors (Acosta *et al.*, 2001; Guzman-Lenis *et al*., 2009a; Lowry *et al*., 2001; Rakowicz *et al.*, 2002). Moreover, some intrinsic promoters of regeneration (as cAMP levels or integrin expression) are already normalized to the adult levels at this postnatal time (Domeniconi and Filbin, 2005; Lemons and Condic, 2008).

Although both motor and sensory neurons were able to extend neurites in the collagen matrix (see Fig. 1), primary sensory neurons showed a stronger growth capacity than motor ones (Table 2). For this reason, we decided to measure DRG explants cultured for two days, to have a limited basal outgrowth to which compare situations with enhanced neurite growth. In contrast, organotypic slices were cultured for 4 days, a time at which the slices showed some neurite outgrowth in basal conditions.

Another important point to take into account is which marker to use for labeling the neurons and their neurites. DRG have a complex and heterogeneous population of sensory neurons, whereas the main population of neurons in the spinal cord ventral horn is the alfa-motoneurons that innervate the skeletal muscles. Since alfa motoneurons have large myelinated axons, we also focused on the subpopulation of myelinated sensory axons of the DRG. Indeed, myelinated sensory axons are related to the most complex somatosensory functions (fine touch, proprioception), which need an accurate reinnervation to sustain good functional recovery after injury (Verdu and Navarro, 1997). Both myelinated motor and sensory neurons can be labeled by neurofilament markers. Despite previous works describing the non-phosphorylated form (SMI-32) as a marker of regenerating axons in the sciatic nerve (Pestronk *et al.,* 1990; Tsuda *et al.,* 1997); we found that SMI32 labeled all the motoneurons somas but not their neurites in the cultures (Fig. 2). In contrast, RT97, that recognizes phosphorylated

neurofilaments, stained only regenerating motoneurons and also their newborn neurites *in vitro*. Double staining with GAP43 (Fig. 3), which labels growing neurites and regenerating axons, revealed almost complete colocalization between RT97 and GAP43, while SMI32 did not colocalize with GAP43 at the neurites. Motoneurons were not labeled by RT97 antibody at 0 days in vitro, as it seems that phosphorylated neurofilaments are expressed only after injury (Liu *et al.,* 2005; Penas *et al.*, 2009). Thus, RT97 can be an adequate marker during the early regeneration period, whereas after several days *in vitro*, motor neurites express again non-phosphorylated neurofilaments (Guzman-Lenis *et al.*, 2009b; Mi *et al.*, 2007). Interestingly, sensory neurons expressed phosphorylated as well as non-phosphorylated neurofilaments at 0 days in vitro, suggesting a different pattern of neurofilament phosphorylation between sensory and motor fibers.

After setting up the cultures, we wanted to corroborate the reliability of our models to compare the effects of different factors on both motor and sensory neurite outgrowth. It is well-known that NGF is a specific factor for a subpopulation of primary sensory and sympathetic neurons (Levi-Montalcini, 1987), and its specific receptor TrkA is mainly present in peptidergic sensory neurons (Bennett *et al.*, 1996a). Around 20% of peptidergic TrkA-positive neurons are also neurofilament positive (Averill *et al.*, 1995), thus the increased outgrowth we observed exclusively in DRG explants was expected. On the other hand, GDNF is known to have a trophic effect on DRG, motor and autonomic neurons (Baloh *et al.*, 1998; Bennett *et al.*, 1998; Buj-Bello *et al.*, 1995; Ebendal *et al.*, 1995; Henderson *et al.*, 1994; Kotzbauer *et al.*, 1996; Matheson *et al.*, 1997; Trupp *et al.*, 1995), and therefore it was able to promote outgrowth in both our *in vitro* preparations. BDNF receptor TrkB is widely expressed in spinal motoneurons and it can also be found in mid-size primary sensory neurons (Henderson *et al.*, 1994; Mu *et al.*, 1993). Since it has been reported that BDNF increases in sensory nerves after injury (Hoke *et al.*, 2006) and expression of TrkB increases in motoneurons after axotomy (Ernfors *et al.*, 1993; Kobayashi *et al.*, 1996; Piehl *et al.*, 1994), we expected that BDNF enhanced both motor and sensory outgrowth. However, the increase in neurite elongation was only significant in spinal cord slices and not in DRG explants. Despite the positive effect of NT-3 on general outgrowth, its addition to the cultures did not enhance significantly neither arborization nor elongation in

both preparations when compared with NGF and BDNF. NT-3 significantly enhanced arborization of primary sensory neurons, an effect that can be explained by the predominant expression of its specific receptor (TrkC) in large sensory neurons (Henderson *et al.*, 1993).

The findings of this study allow us to propose that these models can be useful to study the effect of diffusible molecules and drugs on the axonal regeneration capabilities of different types of peripheral neurons. The model is also appropriate to investigate the actions of different cell types that can be exogenously introduced in the collagen matrix. We show that Schwann cells responded in an appropriate way in the tested conditions, so that the in vitro model can be used to obtain information about the action of genetically modified cells, as for example, Schwann cells over-expressing certain neurotrophic factors (Madduri *et al.*, 2009).

Table 1. Summary of the neurotrophic factors used for the in vitro screening.

Neurotrophic Factor	Molecular Weight	Provider	ng/ml	Reference
Human GDNF	15 kDa (two monomers)	Peprotech	10	(Salie, 2005)
Human BDNF	27.0 kDa	Peprotech	10	(Salie, 2005)
Human NGF-beta	13.5 kDa (two monomers)	Peprotech	10	(Salie, 2005)
Human NT-3	13.6 kDa (two monomers)	Peprotech	10	(Salie, 2005)

Table 2. Results of the measurements of neurite length and arborization for motor and sensory neurons in organotypic cultures maintained in a collagen gel matrix with the different neurotrophic factors added in the gel. Values are mean ± SEM of measurements made on at least 3 cultures for each condition. Values indicated with * are statistically significant vs control.

Trophic Factor	Neurite Length (μm)		Neurite Arborization (μm^2)	
	Motor	Sensory	Motor	Sensory
Control	374 ± 66	1078 ± 80	180 ± 56	9148 ± 2219
BDNF	720 ±130 *	1259 ± 137	813 ± 190 *	14149 ± 2834
b-FGF	750 ± 83 *	1277 ± 70	1757 ± 378 *	22081 ± 2569
CNTF	441 ± 60	1163 ± 66	510 ± 114	17012 ± 2229
GDNF	712 ± 144 *	1734 ± 97 *	383 ± 77	49188 ± 6472 *
IGF-1	448 ± 69	1125 ± 65	425 ± 144	16049 ± 2135
NGF	292 ± 54	1654 ± 116 *	150 ± 28	32686 ± 6723 *
NT-3	516 ± 82	1335 ± 107	589 ± 120 *	28029 ± 4178
PTN	261 ± 60	1122 ± 64	110 ± 23	11632 ± 3964

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FGF-2 LOW MOLECULAR WEIGHT (18 kDa) SELECTIVELY PROMOTES NEURITOGENESIS OF MOTOR NEURONS IN VITRO

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Abstract

In this study we screened in vitro the different capabilities of trophic factors with promising effect on specific reinnervation of target organs after peripheral nerve regeneration. Trophic factors which promoted in vitro neuritogenesis of sensory and motor neurons were up-regulated in Schwann cells obtained from axotomized sensory and motor branches respectively. We found that FGF-2 (18 kDa) was the trophic factor that exerted the most selective effect in promoting neurite outgrowth of spinal motoneurons both in terms of elongation and arborization. The mechanism underling this effect in neuritogenesis seems related to FGF-2 enhancing the interaction between FGFR-1 and PSA-NCAM. The interaction of these two receptors is important during early stages of neuritogenesis and pathfinding, while integrin alpha7B subunit seems to play a role during neurite stabilization.

Key words: axonal regeneration, motor neuron, sensory neuron, *in vitro* cultures, neurotrophic factors, cell adhesion molecule, FGFR-1.

Introduction

After a peripheral nerve injury, the degree and the quality of functional recovery depend on the successful regeneration of injured axons along the distal nerve stump and the specific reinnervation of denervated target organs with an appropriate anatomical and physiological pattern. Both the intrinsic capacity of neurons to switch to a pro-regenerative state and the trophic environment of the distal nerve are key factors influencing the outcome of nerve regeneration. Tropic and trophic cues play important roles in the guidance of axons through the degenerated distal nerve towards the targets. Growing axons respond to secreted trophic factors and interact with Schwann cells and extracellular matrix components by means of different cell adhesion molecules (CAMs). These interactions are also dependent on trophic factor receptors. However, the molecular mechanisms that can guide the selective and separate regeneration of motor and sensory axons are not well known. It was proposed that secretion of different trophic factors by Schwann cells of motor and sensory branches might promote selective regeneration. An elegant work of Hoke et al. (2006) demonstrated that motor and sensory Schwann cells have different phenotypes and that they express different trophic factors. Several studies reported specific actions of individual growth factors on nerve regeneration (Sendtner *et al.*, 1996; Ulenkate *et al*., 1994; Ernfors *et al*., 1994; Munson and McMahon, 1997). However, there are no comprehensive analyses comparing the capability of different trophic factors in promoting sensory and motor regeneration, probably related to the expression of receptors in different populations of neurons.

The aim of this work is to provide clues on the potential selective effects of trophic factors to promote axonal growth from motor and sensory neurons. By using an *in vitro* method previously set up in our laboratory (Allodi *et al.,* 2011a), we screened several trophic factors that could promote selective regeneration, and whether these factors were differentially up-regulated in ventral (motor) and dorsal (sensory) spinal roots after injury. We also studied how these trophic factors modulate the expression of integrin alpha7B, a CAM component essential for regeneration (van Kesteren *et al.,* 2011) and thus a good marker for neurons in pro-regenerative state.

We found that FGF-2 (18 kDa) promoted motor but not sensory neurite outgrowth *in vitro* and it was more markedly up-regulated in motor than sensory branches after injury. A previous study already suggested that this low-molecular weight form of FGF-2 could promote motoneuron regeneration (Haastert *et al*., 2006), whereas the high molecular weight (21/23 kDa) enhanced sensory target reinnervation *in vivo*. The effects of FGF-2 in the nervous system are mainly exerted through FGF receptor 1 (FGFR-1) (Ford-Perriss *et al.,* 2001), which also interacts with the polysialylated form of NCAM (Grothe and Nikkhah, 2001). Interestingly, the expression of PSA-NCAM seems to favor specific motor reinnervation (Franz *et al.,* 2005). Therefore, we further studied the mechanism through which FGF-2 could enhance motoneuron outgrowth in our model by focusing on the interactions of this trophic factor with its receptor FGFR-1 and PSA-NCAM.

Material and methods

Spinal cord slices and DRG explants cultures

Spinal cord slices and DRG explants of postnatal day 7 Sprague-Dawley rats were cultured as detailed in a previous work (Allodi *et al.,* 2011a). Briefly, lumbar and high sacral spinal cord segments and low thoracic and lumbar DRG were dissected and cleaned from blood and meningeal debris. A volume of 450 μl of type I collagen solution (BD Biosciences) at a concentration of 3.4 mg/ml, was mixed with 50 μl of 10X basal Eagle's medium (Gibco) and 2 μl of 7.5% sodium bicarbonate solution (Tucker et al., 1996). The final concentration of the collagen gel was 3.05 mg/ml. Single drops of 30 μl were deposited on poly-D-lysine (PL, 1μg/ml, Sigma) coated coverslips, which were placed in Petri dishes or 24-well multidishes (Iwaki, Asahi Technoglass, Chiba, Japan) and kept in the incubator at 37°C and 5% CO_2 for two hours to induce collagen gel formation.

Spinal cord slices and DRG explants were then transferred on the gelled collagen droplets and covered by a second drop of 30 μl of the same collagen solution. The embedded samples were placed again in the incubator for 45

minutes before adding Neurobasal medium (NB, Invitrogen), supplemented with B27 (Invitrogen), glutamine and penicillin/streptomycin (Sigma). The medium volume delivered into Petri dishes and wells was 1.5 ml and 0.5 ml respectively. DRG explants were cultured for 2 days, and spinal cord slices for 4 days.

Neurotrophic factors assay

A screening for the effects of different neurotrophic factors (Table 1) was done by adding into the collagen matrix 10 ng/ml of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor 1 (IGF-1), 20 ng/ml of interleukin 6 (IL-6), 50 ng/ml of basic fibroblast growth factor (FGF-2) or 100 ng/ml of pleiotrophin (PTN). Neurotrophic factors were added to 50 μl of 10X basal Eagle's medium and then mixed with the collagen solution with a pipette.

Inhibition assay in spinal cord organotypic slices

Inhibitors were added into the collagen matrix at the following concentration: 500 nM of PD 173074 (Parke Davis, Ann Arbor, MI, USA) (Mehanna et al., 2009), a specific inhibitor of FGFR-1, 200 μg/ml of EndoNeuraminidase (an enzyme that degrades the terminal polysialic acid of PSA-NCAM) as recommended by the producer (AbCys, Paris, France), and 50 μg/ml of anti-integrin alpha7 antibody (Gardiner et al., 2007). PD 173074 was dissolved in DMSO and then diluted in 10X DMEM, while the other two inhibitors were diluted directly in the medium. After 4 days in vitro spinal cords were stained with RT97 antibody and quantitative analysis was done as described below.

Analysis of neurite outgrowth

Spinal cord and DRG cultures were stained with anti-neurofilament antibody RT97, in order to label neurons and their growing neurites.

Microphotographs for quantitative analysis were taken at 20X with a digital camera (Olympus DP50) attached to the microscope (Olympus BX51), acquired in Adobe Photoshop CS4 to automatically photomerge them, and analyzed with the aid of ImageJ software (NIH, available at http://rsb.info.nih.gov/ij/). The length of the longest neurite in the cultures was measured for at least 15 samples per condition. For the arborization area, the microphotographs were transformed to a gray scale, 8 bit image, and labeled area was assessed after defining a threshold for background correction using ImageJ software.

Statistical analysis was performed using Prism 4 software. All results are given as the mean±SEM. One-way ANOVA, with post-hoc Bonferroni test (multiple comparisons among all the groups) where necessary, was used to statistically analyze neurite outgrowth and branching. T-test was used to compare motor and sensory percentages of elongation and arborization for each trophic factor. A p value lower than 0.05 was considered significant.

Schwann cell culture

Schwann cells were obtained from postnatal days 7 and 21 Sprague-Dawley rats, as previously described (Verdu *et al.,* 2000). Ventral and dorsal roots from thoracic, lumbar and sacral levels were dissociated and kept in cold Gey's balanced salt solution (Sigma) and cleaned from meninges and connective tissue. Roots were dissociated in 1 ml Ca^{+} and Mg^{+} free Hank's medium (Sigma) with 0.25% trypsin (Sigma), 1 mg/ml collagenase A (Sigma) and 1 mg/ml DNAse (Roche), followed by mechanical dissociation. Cells were then centrifuged at 900 rpm for 7 minutes, resuspended and seeded onto poly-D-lysine coated coverslips insert into 24 well plates (Iwaki, Asahi Technoglass, Chiba, Japan) and incubated with 0.5 ml of DF-10S medium. Cells were kept in vitro for 3 and 7 days, and medium was changed every three days.

Proliferation assays

Spinal cord slices were plated as previously explained. One day after seeding, 20 μM BrdU (Sigma) was added to the culture medium. Organotypic cultures were cultured for 3 days and then fixed with 4% paraformaldehyde in PBS for half an hour for immunohistochemistry.

For proliferation assay, Schwann cells were plated at a density of 100.000 cells/ml on poly-D-lysine substrate. BrdU was then added to the medium 1 h after treatment administration. After 1 day *in vitro,* cells were fixed with 4% paraformaldehyde in PBS with 0.4% picric acid for 15 minutes.

Real-time PCR

Two months old rats were sacrificed by decapitation after deep anesthesia, and low thoracic, lumbar and sacral roots were dissected and maintained in RNA-later solution (Qiagen, Barcelona, Spain). Dorsal and ventral roots were kept in vitro for 4 and 7 days in DF 10S medium in a non-coated Petri dish. Roots were cleaned of connective tissue to avoid fibroblast and epithelial cell contamination. Medium was changed every 3 days. This method was used to mimic the degeneration that occurs after lesion and to keep the Schwann cell population as pure as possible.

Samples used for integrin mRNA quantification were obtained by dissection of the ventral horn of the spinal cord. Dissection was performed in naive animals and at 3 and 8 days after lesion, in these cases only the ipsilateral ventral horn was analyzed.

The samples were processed for mRNA analysis following the manufacturer instructions. The total RNA of intact, 4 and 7 days predegenerated roots was extracted with RNeasy mini kit (Qiagen), including a DNase step (RNase free DNase set, Qiagen, Barcelona, Spain). Then, 1 μg of RNA was reverse-transcribed using 10 μmol/L DTT, 200 U M-MuLV reverse transcriptase (New England BioLabs, Barcelona, Spain), 10 U RNase Out Ribonuclease Inhibitor (Invitrogen), 1μmol/L oligo(dT) and 1 μmol/L of random hexamers (BioLabs, Beverly, MA, USA). The reverse transcription cycle conditions were 25°C for 10 min, 42°C for 1 h and 72°C

for 10 min. We analyzed the mRNA expression by means of specific primer sets (Table 2) of the following neurotrophic factors: NGF, NT-3, BDNF, GDNF, FGF-2; cytokines: IL-6 and LIF, and integrins: alpha5, alpha7 and alpha9. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) expression was used to normalize the expression levels of the different genes of interest. Gene-specific mRNA analysis was performed by SYBR-green real-time PCR using the MyiQ5 real-time PCR detection system (Bio-Rad Laboratories, Barcelona, Spain). We previously fixed the optimal concentration of the cDNA to be used as template for each gene analysis to obtain reliable CT (threshold cycle) values for quantification. Three samples were used per condition and each one was run in duplicate. The thermal cycling conditions comprised 3 min polymerase activation at 95°C, 40 cycles of 10 s at 95°C for denaturation and 30 s at 62°C for annealing and extension (60°C in the case of NGF, NT-3, alpha5, alpha7 and alpha9 genes), followed by a DNA melting curve for determination of amplicon specificity. CT values were obtained and analyzed with the BioRad Software. Fold change in gene expression was estimated using the CT comparative method ($2^{-\Delta\Delta CT}$) normalizing to GADPH CT values and relative to control samples. Results are expressed as mean ± SEM. Statistical analysis was made by two-way ANOVA, considering days *in vitro* and type of roots as indipendent variables.

Immunohistochemistry

Spinal cord cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, 0.1 M, pH 7.4) for 30 minutes, and DRG explants and dissociated Schwann cells for 15 minutes. Samples were then incubated for 48 h (explant cultures) or over-night (dissociated Schwann cells) with primary antibodies: mouse RT97 (1:200, Developmental Studies Hybridoma Bank), rabbit anti-S100 (1:200, Immunostar), rabbit anti-laminin (1:1000, Sigma), rabbit anti-fibronectin (1:400, Sigma), rabbit anti-alpha 7B integrin (1:500, a generous gift of Prof. Guido Tarone, Turin University), rabbit anti-alpha 5 integrin (1:500, Millipore), mouse anti-PSA NCAM (1:100, Millipore), mouse anti-NCAM (1:200, Millipore), rabbit anti-FGFR-1 (1:400, AbCam), sheep anti-BrdU (1:200, Fitzgerald). After washing, the sections were incubated overnight (explants) or for

one hour (dissociated cells) with secondary antibodies Cy3 conjugated donkey anti-mouse (1:200, Jackson IR), FITC conjugated goat anti-rabbit (1:200, Vector), Alexa 680 conjugated goat anti-mouse (1:500, Invitrogen) or Alexa 594 conjugated donkey anti-sheep (1:500, Invitrogen).

To look at FGFR-1 expression in motor and sensory branches, two months old rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in PBS. Ventral and dorsal roots were carefully harvested under a dissection microscope, post-fixed overnight and cryoprotected in 30% sucrose at 4°C. Roots were cut in 15 μm longitudinal sections with a cryostat and collected onto gelatin-coated glass slides. Slices were incubated overnight with primary antibody against FGFR-1 (1:400, AbCam), and after washes, with secondary antibody (FITC 1:200, Vector).

Western Blot

Culture slices were homogenized in modified RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate (Sigma), 0.2% SDS, 100 mM NaCl, 1mM EDTA) containing protease inhibitor cocktail (10 μl/ml, Sigma). The homogenate was then centrifuged at 13000 rpm and the obtained supernatant was processed for protein concentration determination using the BCA protein assay (Pierce, Rockford, IL, USA). Equal amount of protein (30 – 50 μg depending on the detected protein) were separated on 10% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad Laboratories, Barcelona, Spain). The membranes were then incubated for blocking with 25% BSA (Sigma) in TBS plus 0.05% tween-20 (TBST) for 30 minutes and incubated with primary antibodies anti-PSA-NCAM (1:500, Millipore), anti-NCAM (1:1000, Millipore), anti-integrin alpha7 (1:500, a gift of Prof. G. Tarone, University of Turin), anti-FGFR-1 (1:400, AbCam) and anti-β-actin (1:10000, Sigma) at 4°C overnight. Horseradish peroxidase-coupled secondary antibody incubation was performed for 1 h at room temperature after 3 washes in TBST. Blots were developed using the Immobilon Western chemiluminescent HRP substrate (Millipore). Signals were analyzed by band densitometry with the Gene

Snap and the Gene Tools software in a Gene Genome apparatus (Syngene, Cambridge, UK).

Results

Neurotrophic factor screening results

Different neurotrophic factors were added into the collagen matrix to enrich the environment of the cultures. GDNF, BDNF, NGF and NT-3 were tested to corroborate previous findings (Allodi *et al.,* 2011a). We expanded the screening to other neurotrophic factors, including CNTF, IL-6, IGF-1, FGF-2 and PTN (Fig. 1). The effects of each trophic factor were assessed by measuring neurite length and arborization.

In our conditions, PTN did not increase motoneuron or sensory neuron outgrowth, compared to control conditions, at concentrations of 100 ng/ml (Fig. 1) and 200 ng/ml (data not shown). Similarly, no differences in neurite elongation were found when adding IL-6 compared to controls both in DRG and motor neurons (data not shown). NT-3, IGF-1 and CNTF did not significantly affect neuronal growth (Fig. 1).

In order to compare the effects of each growth factor on spinal cord and DRG explants, the values of neurite length and amount of arborization were normalized as the percentage of the corresponding control values (Fig. 1). Addition of GDNF, BDNF and FGF-2 to the collagen matrix increased about 100% the maximal motor neurite length, whereas the other neurotrophic factors did not cause a significant effect. Regarding the amount of motor neurite branching, FGF-2 showed a marked increase of about 10-fold, BDNF about 5-fold, whereas NT3, CNTF, IGF1 and GDNF had lower positive effect. On DRG explants, administration of NGF and GDNF increased by 60% neurite length, and BDNF and NT3 caused a lower increase (about 20%). GDNF and NGF significantly enhanced neurite arborization of sensory neurons. When comparing the differential effects of the trophic factors on both DRG and spinal cord explants, we found that NGF

exclusively promoted growth of sensory neurites, whereas FGF-2 and BDNF selectively enhanced motor neurite outgrowth.

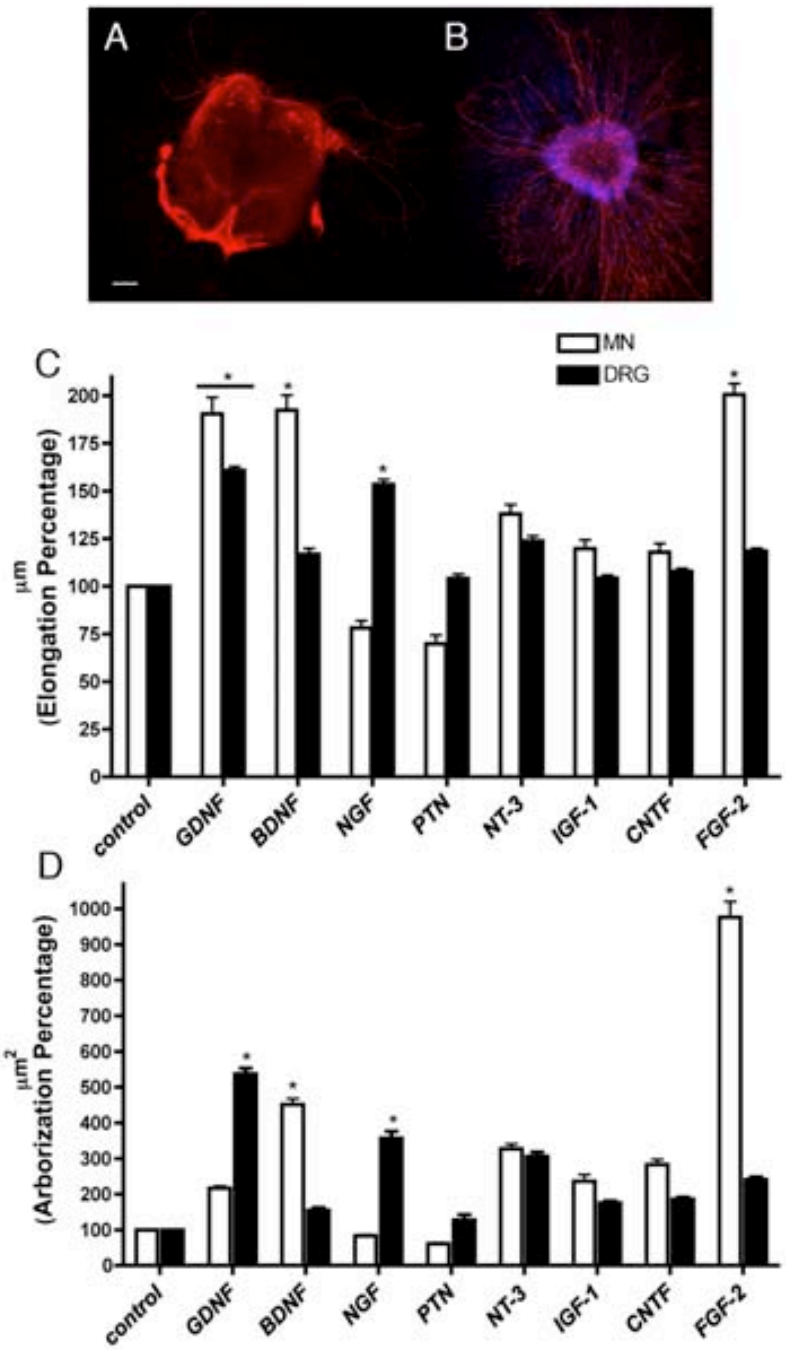

Figure 1. Representative images of motoneuron outgrowth when FGF-2 was added into the culture (**A**) and of DRG neuritogenesis after NGF administration (**B**). RT97 (phosphorylated heavy and medium neurofilament) in red, DAPI (nuclear marker) in blue. Bar = 100 μm. Comparison between motor and sensory neurite outgrowth (expressed as percentage of control conditions) after administration of different trophic factors into the collagen matrix of DRG explants (DRG, white columns) and spinal cord organotypic cultures (MN, black columns) (**C, D**). Quantification of neurite length (**C**) and arborization (**D**) reveals that NGF has

specific effect on sensory neurons, while BDNF and FGF-2 have significant effect on motoneuron outgrowth, and GDNF enhances regeneration of both kinds of neurons. * $p<0.001$ motor vs. sensory neurons (t test).

Real-time PCR results for neurotrophic factors in dorsal and ventral roots

In order to assess the differential expression in motor and sensory nerves, the mRNA levels of different neurotrophic factors in ventral and dorsal spinal roots were investigated by real-time PCR at baseline, and at 4 and 7 days of *in vitro* degeneration. The trophic factors were chosen depending on the *in vitro* results obtained in the previous study with the recombinant proteins. No marked differences were found at baseline in trophic factors expression between dorsal and ventral roots (data not shown). However, mRNA expression changed over time *in vitro*. The expression of NGF and NT-3 increased slightly in dorsal roots though not significantly compared to ventral roots or baseline values (Fig. 2). On the other hand, GDNF showed a significant up-regulation in both roots during *in vitro* degeneration, but with a different time pattern; in dorsal roots there was a 11-fold increase at 4 days and then a slight decrease at 7 days, while in ventral roots there was a mild increase at 4 days, and a significant 16-fold increase after 7 days ($P<0.001$). BDNF showed higher expression in ventral roots, and it was significantly up-regulated when compared to sensory branches both at 4 and 7 days *in vitro*. On the other hand, FGF-2 expression was up-regulated in both types of roots, but about twice in motor (5.5 times, $P<0.01$) than in sensory roots (3.5 times, $P<0.05$) (Fig. 2).

The expression of two cytokines, IL-6 and leukemia inhibitory factor (LIF), which are supposed to be related to FGF-2 expression (Grothe *et al*., 2000), was also assayed. mRNA expression of IL-6 was markedly up-regulated in ventral roots, reaching an increase of 600-fold after 4 days, while it declined to baseline levels at 7 days. On the other hand, LIF is known to be up-regulated after injury and to promote muscle regeneration (Tham *et al*., 1997). For this reason, we looked at its mRNA levels after injury, and found that it was largely up-regulated at 7 days, with a higher increase in ventral roots of around 15-fold (Fig. 2). Due to the interesting findings obtained from the *in vitro* assay and the real-time PCR analysis, we

decided to further investigate the mechanism of action of FGF-2 in motoneuron neuritogenesis.

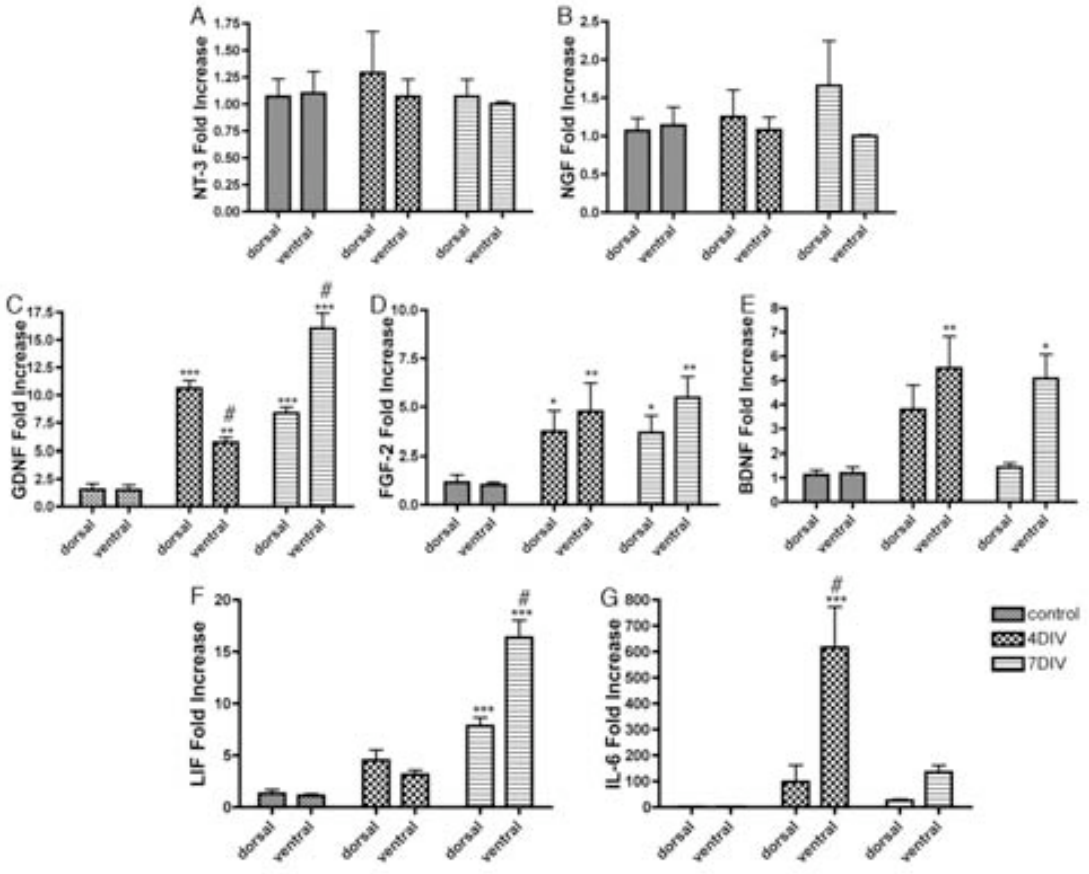

Figure 2. mRNA expression of trophic factors in degenerating ventral and dorsal roots. Spinal roots were pre-degenerated for 4 and 7 days *in vitro* (DIV) and their RNA was screened for five trophic factors (NGF, NT-3, BDNF, GDNF and FGF-2) and two cytokines (IL-6 and LIF). * indicates significant differences in expression among time (* $p<0.05$, ** $p<0.005$, *** $p<0.001$). # indicates differences between motor vs. sensory branches. BDNF was up-regulated in ventral roots (**E**), IL-6 only at 3 DIV (**G**), while FGF-2 was increased in both roots but more marked in ventral ones (**D**). GDNF was increased in both sensory and motor branches, but with a different timing (**C**). LIF increase was significant only at 7 DIV and more marked in ventral than in dorsal roots (**F**).

FGFR-1 expression in neurons and Schwann cells

To study if the expression of FGFR-1, the main receptor of FGF-2 in the nervous system, may be different in Schwann cells associated to motor and sensory axons, we performed immunohistochemestry of FGFR-1 in naive dorsal and ventral roots. Unexpectedly, we found no differences (Fig. 3). Then, we

analyzed the expression of FGFR-1 in Schwann cells obtained from dorsal and ventral roots at 1, 4 or 15 days in vitro; no changes were detected between the two types of roots, neither after FGF-2 administration in the medium (Fig. 3).

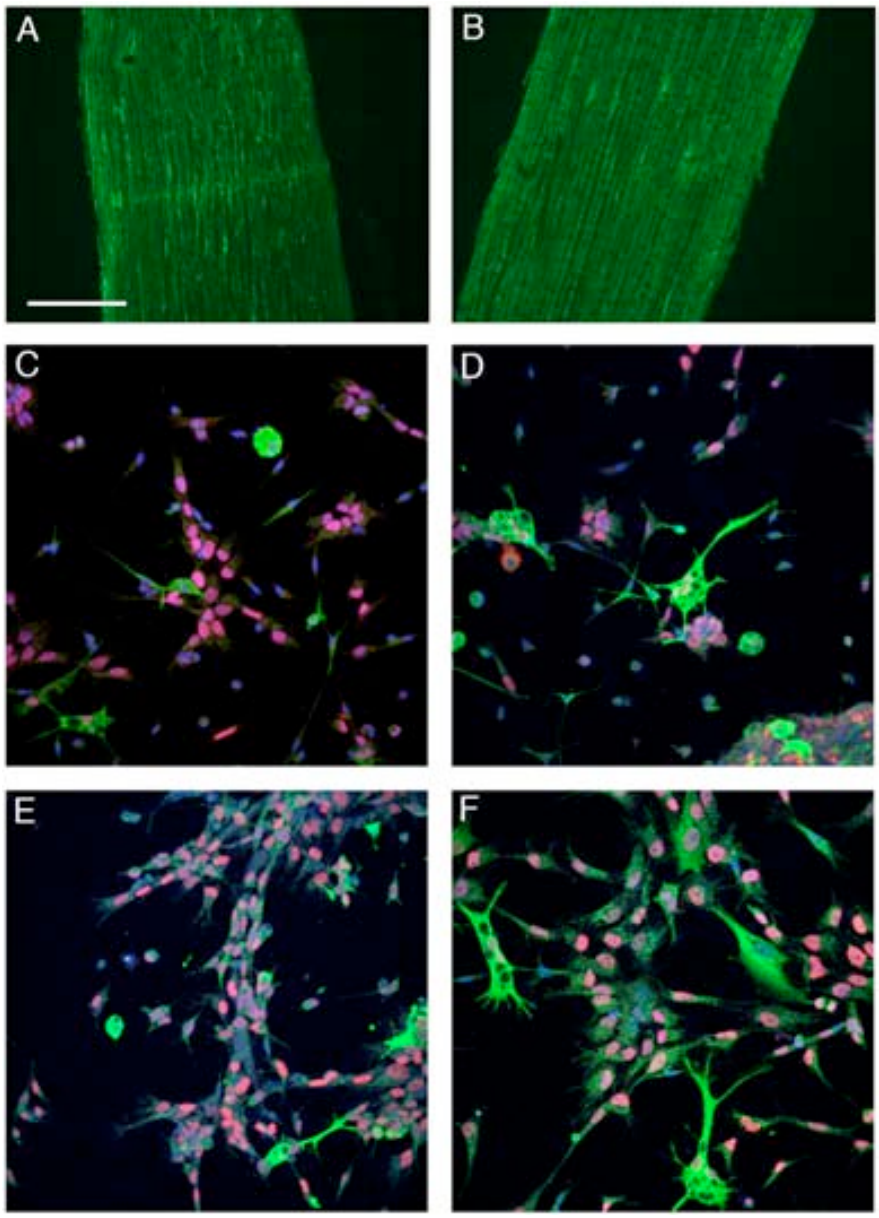

Figure 3. Immunohistochemestry for FGFR-1 performed on dorsal (**A**) and ventral (**B**) roots. No differences are present in receptor expression in naïve animals, FGFR-1 in green. Expression of FGFR-1 in dissociated Schwann cells obtained from dorsal (**C**) and ventral (**D**) roots after 4 days in culture. Quantification by image analysis did not show significant differences between dorsal and ventral roots in control and after FGF-2 administration (**E, F**). As expected, FGF-2 addition enhanced Schwann cell proliferation. FGFR-1 green, BrdU red and DAPI blue. Bar = 100 μm.

On the other hand, we found some differences in the expression of FGFR-1 between spinal cord and DRG cultures. Sensory neurons expressed this receptor in somata and neurites, while in spinal cord slices FGFR-1 was mainly present in Schwann cells and also in the somata of motoneurons but with lower intensity (Fig. 4). Interestingly, neurites elongating from motoneurons did not express the receptor (Fig. 4). These findings suggest that the FGFR-1 may play a different role during sensory and motor axons regeneration.

When double staining for FGFR-1 and BrdU was performed in spinal cord slices, we observed that proliferating Schwann cells do not express FGFR-1 (Fig. 4). This observation suggests that the mature Schwann cells trigger the FGF-2 effect and support neurites outgrowth of motoneurons.

However, when Western blot quantification was assessed on spinal cord slices in control and FGF-2 conditions, we could not observe an increase of the receptor, probably because its expression is dependent on the regenerative state of neurons and Schwann cells and not on the amount of FGF-2 (Fig. 4). For this reason, we decided to investigate if FGF-2 could exert its effect influencing the interaction between its receptor and neuronal cell adhesion molecules during axonal growth.

Integrin alpha 7B expression in sensory and motor neurons

Since several studies demonstrated the importance of alpha7 integrin in DRG neurons during regeneration, but less is known about motoneurons, we wondered of axotomized motoneurons *in vivo* also show an upregulation of this subunit. We measured its mRNA expression after injury and compared it with the expression of two other integrin receptors: alpha5 and alpha9. We observed a significant up-regulation of alpha7B and alpha5 subunits, which reached 1.65 and 2.25 increases respectively ($P<0.05$) 3 days after axotomy (Fig. 5). On the other hand, alpha9 was slightly downregulated after injury, as already shown (Andrews et al., 2009).

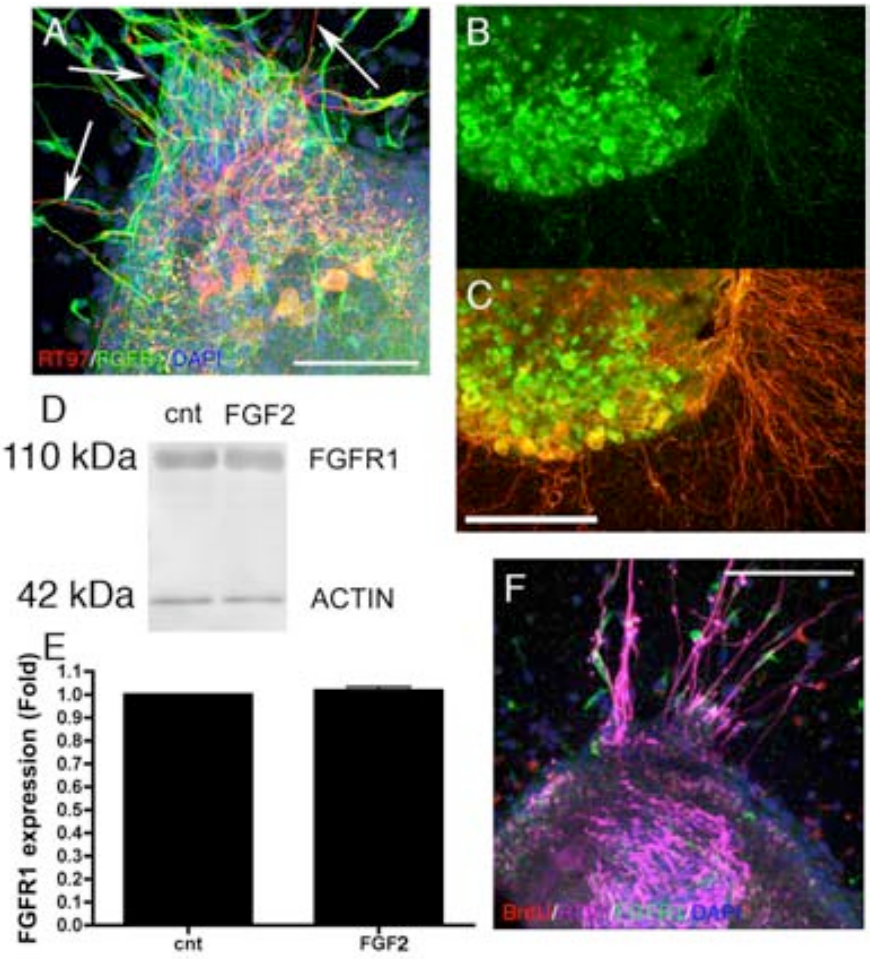

Figure 4. Immunohistochemestry for FGFR-1 performed on spinal cord slices and DRG explants. In the spinal cord, FGFR-1 is mainly found in Schwann cells and motoneurons (**A**). Arrows indicate neurites which are not stained for FGFR-1 (**A**). In DRG explants FGFR-1 stains both neurons and neurites (**B**). Co-labeling with RT97 in DRG explants for corroboration (C). Western blot analysis for FGFR-1, image **D** shows FGFR-1 bands at 110 kDa and beta-actin at 42 kDa. The quantification reveals that no differences in spinal cord slices between control and FGF-2 enriched medium (**E**). (**F**) FGFR-1 expression in proliferating Schwann cells in spinal cord slices. BrdU positive cells do not co-label with FGFR-1, thus the receptor is mainly found in mature Schwann cells, which are the ones sustaining the outgrowth. BrdU in red, RT97 in purple, FGFR-1 in green, DAPI in blue. Bars = 100 μm.

Since one of the objectives of our study was to check if the expression of alpha 7B integrin could be modulated by trophic factor administration, we performed immunohistochemistry for this subunit in our organotypic spinal cord slice and DRG explant cultures. We found that it was exclusively expressed in sensory and motor neurons (Fig. 5) and that Schwann cells migrating from both

kinds of explants were the main provider of laminin, the ligand of alpha7 integrin (Fig. 6). To label both motor and sensory neurons and their neurites we used the phosphorylated form of neurofilament 200 (RT97) (Allodi *et al.,* 2011), only present in myelinated neurons. In motoneurons, this subunit was also expressed both in somata and neurites (Fig. 5). On the other hand, since DRG have a heterogeneous neuronal population, we also labeled the subpopulation of peptidergic sensory neurons with CGRP. Alpha7B was expressed in both RT97 and CGRP positive neurons (Fig. 6).

Since we measured outgrowth of just RT97 positive neurons, we quantified the number of RT97/alpha7B positive neurons in control conditions and after addition of growth factors (BDNF, GDNF, NGF, NT-3 and FGF-2) in both spinal cord slices (at 4 days *in vitro*) and DRG explants (at 2 days *in vitro*). Only trophic factor which significantly enhanced neuritogenesis were taken in consideration in this study. For comparisons, the neuronal expression of integrin alpha7B values was normalized as the percentage of RT97 positive neurons. The administration of different trophic factors did not change the proportion of RT97 positive DRG neurons expressing integrin alpha7B. Similarly, in regenerating motoneurons, the expression or distribution of alpha7B integrin was not affected by the addition of BDNF, GDNF, NGF and NT-3. In contrast, addition of FGF-2 led to a decrease in the proportion of alpha7B positive neurons co-labeled with RT97 (Fig. 7).

Integrin expression in motoneurons treated with FGF-2

The slight but significant decrease in alpha7B integrin expression under addition of FGF-2 compared with control cultures indicates that regenerating motoneurons synthesize alpha7B integrin to elongate their neurites, but in the presence of FGF-2 its expression is mostly found in neurites (Fig. 7). Quantification by Western blot revealed a decrease, although not significant, of alpha7B integrin (Fig. 7). Due to the lower expression at 4 days, we investigated if FGF-2 could accelerate the peak of expression of this subunit. However, at 2 days *in vitro* its expression was still very low (Fig. 7). We further studied if other integrin subunits could be up-regulated after FGF-2 administration. We focused on the alpha5

subunit (receptor for fibronectin), since a previous study (Tomatis *et al.,* 1999) revealed that it is up-regulated when alpha7 is down-regulated. Moreover, a consistent migration of Thy1/fibronectin positive cells was seen after FGF-2 administration (Fig. 7). In contrast to what was expected, alpha5 was not detected in motoneurons, neither in the soma nor in neurites (Fig. 7).

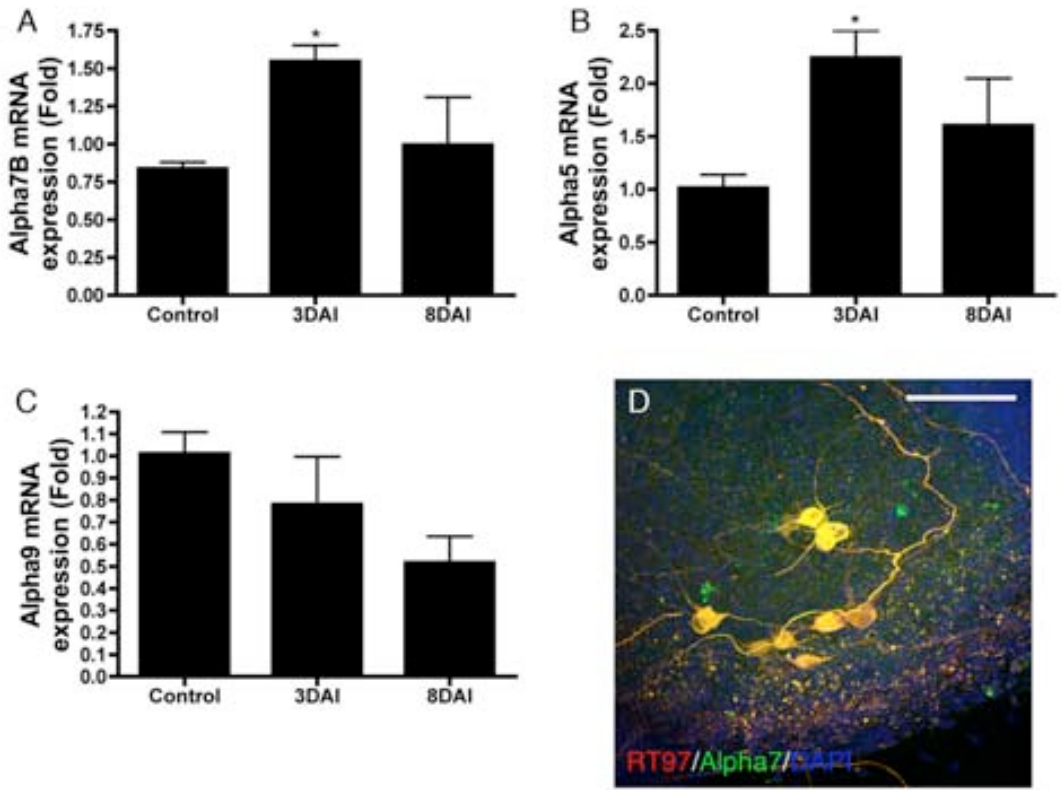

Figure 5. mRNA expression of alpha7B, alpha5 and alpha9 integrins at baseline and at 3 and 8 days after axotomy (**A, B, C**). Both alpha7 (A) and alpha5 (B) mRNA expression increase at 3 days, whereas mRNA levels of alpha9 (C) show a non significant trend to decrease after injury. * indicates significant differences in expression compared to baseline ($p<0.05$, t test). Immunohistochemistry performed on spinal cord slices *in vitro* confirms alpha7B up-regulation in motoneurons (**D**). Alpha7B in green, RT97 in red, DAPI in blue. Yellow color indicates co-labeling for alpha7B and RT97. Bar = 100 μm.

PSA-NCAM role in motoneuron regeneration and interaction with FGFR-1

Immunostaining of spinal cord slices revealed that NCAM is present in motoneuron somata and neurites, and in Schwann cells, whereas its polysialylated form is present only in neurites (Fig. 8). Neurites that expressed the heavy chain neurofilament did not stain for PSA-NCAM, suggesting that this cell adhesion

molecule is playing a role mainly at the front of regeneration and at early time points. On the other hand, the heavy chain neurofilament positive neurites were the ones that expressed the alpha7B integrin subunit in our model. Moreover, PSA-NCAM was found mainly in regenerating neurites strictly related with the FGFR-1 expression in the adjacent Schwann cells accompanying these neurites (Fig. 8).

By Western blot we compared the levels of PSA-NCAM in control conditions and after FGF-2 administration in spinal cord slices, and found a higher but not significant expression of this cell adhesion molecule when FGF-2 was added in the culture (Fig. 8).

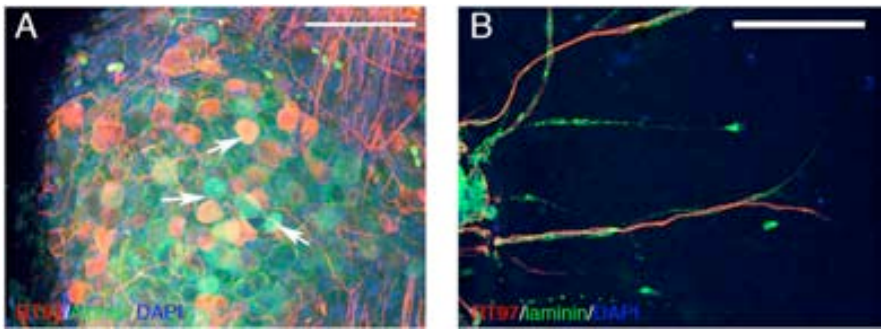

Figure 6. Confocal image showing alpha7B expression in DRG neurons (**A**). The integrin subunit was mainly found in large and medium DRG neurons (indicated by arrows) at 2 DIV. RT97 in red, alpha7 in green and DAPI in blue. Laminin staining reveals that the Schwann cells migrating from the DRG explants and the spinal cord slices are the main supplier of this extracellular matrix molecule (**B**). Laminin in green, RT97 in red, DAPI in blue. Bars = 100 μm.

Inhibition assay results

Three different kinds of inhibitors were used to study the role of FGFR-1, PSA-NCAM and integrin alpha7B in motor neurite outgrowth. EndoNeuraminidase, an enzyme that degrades the terminal polysialic acid of PSA-NCAM, induced a dramatic decrease of motor outgrowth, reducing neurite elongation by 75% (89±15 μm compared to 312±70 μm in controls, $P<0.01$). This inhibition was even more marked, of 87%, after administration of FGF-2 (50 ng/ml) (Fig. 9). Moreover, in the presence of EndoNeuraminidase, neurite growth cones showed an aberrant button-like morphology, typically associated with growth cone elongation inhibition (Fig. 9). Similarly, the other two inhibitors affected neurite elongation

but not sprouting. Administration of PD 173074, a specific inhibitor of FGFR-1, significantly decreased neurite elongation by 70% (96±12, P<0.05) when compared to control conditions. Neurite outgrowth after addition of anti-alpha7B antibody, that blocks alpha7B integrin, was decreased by 45% (172±33 μm), and the effect of this inhibitor was more marked if combined with FGF-2 administration, reducing outgrowth to 77%. When FGF-2 was added to the spinal cord culture, we observed that the regenerating effect was inhibited when either of the cell adhesion molecules, PSA-NCAM and alpha7B integrin, were inactivated, thus suggesting that FGF-2 role is strongly dependent on CAMs.

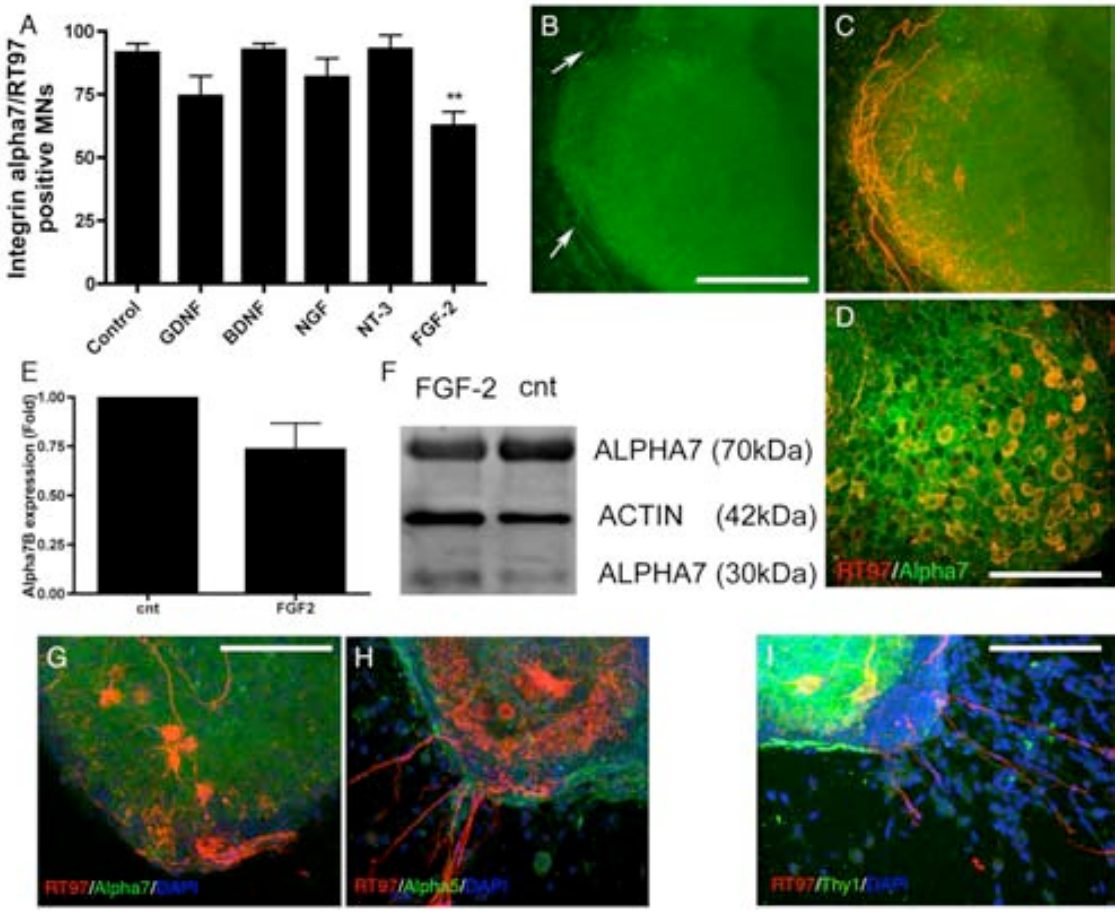

Figure 7. Quantification of integrin alpha7B expression in neurons. Neurons positive for RT97 and alpha7B were counted, and the values were normalized as the percentage of RT97 positive neurons. Among the different neurotrophic factors, only FGF-2 modified the proportion of alpha7B positive neurons (** p<0.005 vs. control) (**A**). Expression of alpha7B in spinal cord slices after 4 DIV (indicated by the arrows) (**B**). The integrin subunit was detected only in neurites co-stained for RT97 **(C**). No differences were found in DRG, where alpha7B was present in large and medium neuronal populations (**D**). Western blot quantification of alpha7B in spinal cord slices in control and FGF-2 conditions. A

lower expression of alpha7B was found after FGF-2 administration (**E**). Bands at 70 and 30 kDa were found after reduction with beta-mercapto-ethanol (**F**). **G,** Alpha7B expression at 2 DIV, alpha7B in green, RT97 in red and DAPI in blue. **H,** immunohistochemestry for alpha5 integrin. No expression in motoneurons was found even with the addition of FGF-2. Alpha5 in green, RT97 in red and DAPI in blue. **I**, Confocal image of the ventral horn showing neurites elongation and Thy1 positive cell migration after FGF-2 administration. Thy1 in green, RT97 in red and DAPI in blue. Bars = 100 μm.

Discussion

Role of trophic factors in specific motor and sensory outgrowth

In this study, we first made a thorough screening of the effect of different neurotrophic factors on motor and sensory neuritogenesis *in vitro*. By culturing spinal cord slices and DRG explants under the same conditions (Allodi *et al*., 2011a), we could reliably compare the response of motor and sensory neurons to the addition of neurotrophic factors. We could corroborate the effect of well known neurotrophins on specific regeneration (NGF effect on sensory neurons, BDNF effect on motoneurons), and found a new target for motoneuron regeneration, FGF-2.

The specific effects of some trophic factors on motor or sensory regeneration might be correlated with the different expression of these trophic factors after injury by Schwann cells from motor and sensory nerves. GDNF, which promotes both motor and sensory outgrowth *in vitro* at similar levels, was also similarly up-regulated in ventral (motor) and dorsal (sensory) spinal roots. In contrast, up-regulation of BDNF and FGF-2, that mainly promote motor outgrowth, was higher in ventral than in dorsal roots. We also found a consistent increase of the cytokines IL-6 and LIF in ventral roots compared to dorsal roots. Both cytokines have been related to FGF-2 function (Grothe *et al.,* 2000; Tham *et al.,* 1997). Indeed, IL-6 and LIF were up-regulated at different time points, suggesting complementary roles. However, as already described by Grothe et al (2000), IL-6 did not promote regeneration by itself.

In agreement with previous studies (Hoke *et al.,* 2006), our results indicate that, after injury, Schwann cells formerly associated with motor or sensory axons

have different phenotypes and express different trophic factors that would favor specific regeneration. However, in the context of a mixed nerve and taking into account that denervated Schwann cells switch to an immature state and lose their mature phenotype (Hoke *et al.,* 2006; Martini *et al.,* 1994), the different expression of factors between motor and sensory Schwann cells may not guarantee specific regeneration.

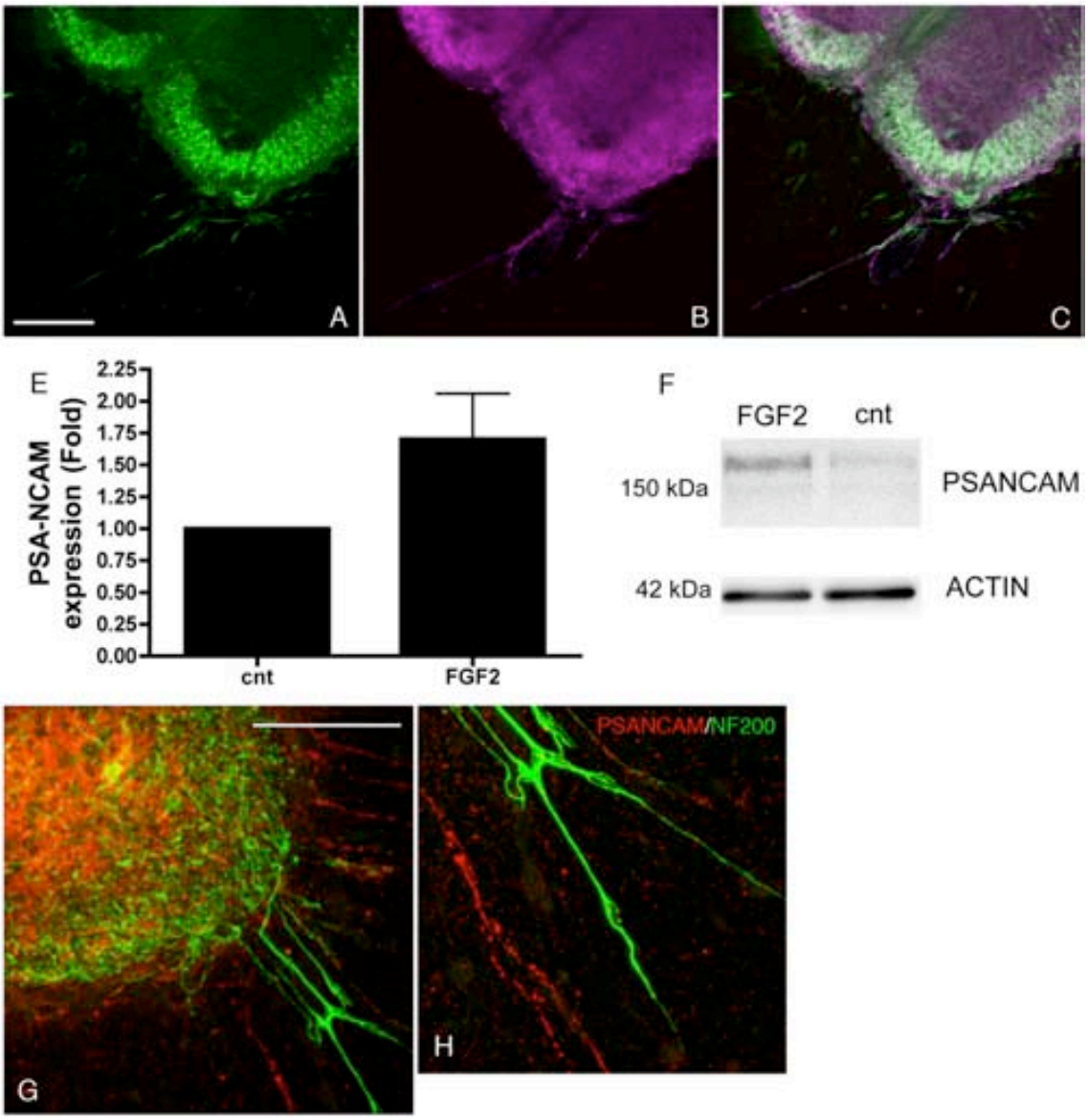

Figure 8. FGFR-1 is mainly found in Schwann cells supporting neurite elongation (**A**), while PSA-NCAM is expressed only in motoneuron neurites (**B**). **C,** co-staining for PSA-NCAM and FGFR-1. **D**, PSA-NCAM quantification by Western blot in control and FGF-2 enriched cultures (**E**). Bands from 150 to 300 kDa were analysed (**F**). The increase of the cell adhesion molecule is consistent but not significant. **G,** Neurites stained for PSA-NCAM do not co-localize with neurofilament 200 positive

ones. Image **H** shows in detail the differences in expression. PSA-NCAM in red, NF200 in green. Bars = 100 μm.

Integrin expression after injury and their role in neuritogenesis

Integrins are glycosylated heterodimers formed by α and β subunits, which are mainly implicated in focal adhesion and focal complexes formations. These CAMs are known to form complexes with receptor protein tyrosine kinases (RPTKs) (Giancotti and Ruoslahti, 1999; Yamada and Even-Ram, 2002). For instance, integrins can modulate the transmission of signals from growth factor receptors (Schwartz and Ginsberg, 2002). The β1 integrin family members have been shown to be important for neurite outgrowth *in vitro* (Condic and Letourneau, 1997). Among the alpha subunits, integrins alpha7 and alpha5 seem to play also an important role during nerve regeneration (Werner *et al.,* 2000; Gardiner *et al.,* 2005, Gardiner *et al.,* 2007). In this study, we focused on the alpha7B isoform that is the splicing variant present in the nervous and muscular systems (Velling *et al.,* 1996; Barczyk *et al.,* 2010). The alpha7B integrin was found to be up-regulated in regenerating motor and sensory neurons. Although this subunit is not expressed in all the sensory neurons (Leclere *et al.,* 2007), we found its expression in a large population of CGRP and neurofilament 200 positive neurons and in their neurites, both in control cultures and after trophic factor administration. On the other hand, all the regenerating motoneurons, labeled with phosphorylated neurofilament (RT97), expressed the alpha7B isoform. None of the trophic factors added, with the only exception of FGF-2, changed the proportion of RT97+ neurons expressing this integrin.

Effects of FGF-2 in motor neurite outgrowth

When focusing on the trophic factors that selectively promote motor regeneration, FGF-2 seems the most promising, showing the highest increases on neurite elongation and arborization of motoneurons. FGF-2 is known to play an

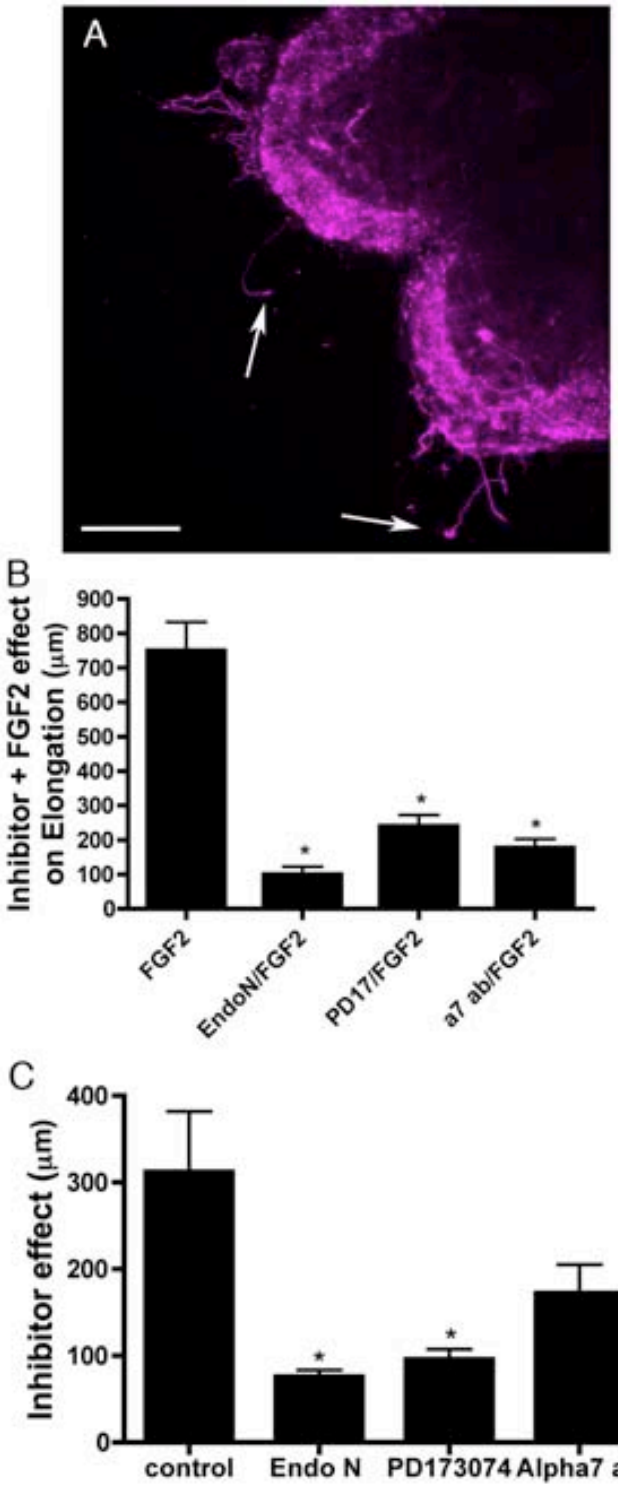

Figure 9. Role of alpha7B integrin, PSA-NCAM and FGFR-1 in the neurotrophic effects of FGF-2. **A,** Motoneuron outgrowth after degrading the polysialylated form of PSA-NCAM by EndoNeuraminidase. Neurite sprouting was not affected by the enzyme, but the elongation was impaired and the front of regeneration showed an aberrant button-like morphology (indicated by the arrows). Bar = 100 μm. **B,** Histogram showing the effects of alpha7B integrin blockade, EndoNeuraminidase administration and inhibition of FGFR-1 by PD 173074 on motoneuron elongation when FGF-2 was added into the matrix. **C**, Graph showing the role of the same inhibitors in control spinal cord organotypic cultures (* p<0,05).

important role during motoneuron development (Liu *et al.,* 2001) and also after peripheral nerve injury (Haastert *et al.,* 2006). The work of Haarsten et al (2006) suggested that the low molecular form of FGF-2 could promote motor regeneration, whereas the higher molecular form would contribute in sensory regeneration. However, this study did not focus on the possible mechanism of

action of the two forms on specific regeneration. Therefore, we wanted to further elucidate how FGF-2 enhances so markedly neurite outgrowth of motoneurons *in vitro*.

Interestingly and in contrast to the other trophic factors, FGF-2 administration decreased the expression of alpha7B integrin, investigated by immunohistochemistry and by Western blot. To discard that FGF-2 was accelerating alpha7B expression at early time points, we also evaluated the expression of this subunit at 2 days and found a low expression level. Nevertheless, FGF-2 could be affecting other integrin subunits. Since after FGF-2 administration we observed increased migration of Thy-1/fibronectin positive cells from the spinal cord slices into the matrix, we assessed the levels of alpha5 integrin. Alpha5 is also known to play an important role during neuronal regeneration (Gardiner *et al.,* 2007), and preferentially binds to fibronectin, whereas alpha7 is a specific receptor for laminin. Moreover, alpha5 subunit is known to undergo up-regulation when alpha7 is down-regulated (Tomatis *et al.,* 1999). However, no expression of alpha5 was found in motoneurons at 4 days, while it was present in other kind of cells, mainly Schwann cells and fibroblasts migrating into the matrix.

All these results and the presence of alpha7B integrin at the neurites (mainly in those that express heavy chain neurofilaments) suggest a role of this receptor during neurite elongation and stabilization. Moreover, when blocking alpha7B in the cultures, the enhanced capacity of FGF-2 to promote motoneuron elongation was reduced by about 80%. Therefore, integrin alpha7B functionality seems necessary for the actions of FGF-2 on motor outgrowth.

In order to further investigate FGF-2 activity, we also looked at the receptors through which it could be acting. In agreement with previous studies, we found that FGFR-1 was neither up-regulated during regeneration (Grothe *et al.,* 2006), nor increased by the addition of FGF-2. Since proliferating Schwann cells did not show FGFR-1 immunostaining, it seems that FGFR-1 is not up-regulated in Schwann cells reactive after nerve injury. Nevertheless, this receptor is needed for FGF-2 effects, since its blockade also decreases neurite outgrowth.

It is important to consider the interaction maintained between NCAM and FGFR-1 during neuritogenesis (Niethammer *et al.,* 2002) and after peripheral nerve injury (Mehanna *et al.,* 2009). Moreover, Franz et al (2005) showed that the expression of the polysialylated form of NCAM in axons is needed to sustain preferential motor regeneration. In agreement with this observation, we found that PSA-NCAM was essential for motor outgrowth, both in control and in FGF-2 conditions. In fact, EndoNeuraminidase administration markedly reduced neurite outgrowth in spinal cord slices. By Western blot, we observed a slight increase in the expression of PSA-NCAM in the ventral spinal cords after FGF-2 treatment. When studying its localization by immunohistochemistry, we could observe that it was only expressed in neurites at early stages of elongation, whereas when neurites became neurofilament 200 positive, they did not express PSA-NCAM anymore. This fact suggests that PSA-NCAM would be important in early stages of motoneuron regeneration. Moreover, PSA-NCAM positive neurites did not express FGFR-1, but were intimately associated to Schwann cells expressing FGFR-1, which were supporting their elongation. Thus, Schwann cells are the main suppliers of FGFR-1 that allows the interaction with axonal PSA-NCAM and enhances neuritogenesis. We hypothesize that FGF-2 administration probably exerts its main effect by favoring PSA-NCAM and FGFR-1 interactions, enhancing in this way early neurite elongation of motoneurons.

It is important to keep in consideration that in an *in vivo* situation, when there is a marked up-regulation of FGF-2 at the distal nerve stump, motoneurons will already have an important source of this factor. Moreover, it has been already shown that addition of FGF-2 in a tube repair model enhances motor regeneration (Haastert *et al.,* 2006). Thus, overexpression of FGF-2 in motor branches can overcome the mild differences found for this neurotrophic factor between motor and sensory branches and facilitate preferential motor regeneration.

Table 1. Neurotrophic factors used for the *in vitro* screening of effects on motor and sensory neurons outgrowth.

Neurotrophic Factor	Molecular Weight	Provider	ng/ml	Reference
Human GDNF	15 kDa (two monomers)	Peprotech	10	Salie and Steeves, 2005
Human BDNF	27.0 kDa	Peprotech	10	Salie and Steeves, 2005
Human NGF-β	13.5 kDa (two monomers)	Peprotech	10	Salie and Steeves, 2005
Human PTN	15.3 kDa	Sigma	100/200	Mi *et al.,* 2007
Human NT-3	13.6 kDa (two monomers)	Peprotech	10	Salie and Steeves, 2005
Human CNTF	22.7 kDa	Peprotech	10	Salie and Steeves, 2005
Human IGF-1	7.6 kDa	Peprotech	10	Salie and Steeves, 2005
Human b-FGF	17.2 kDa	Peprotech	10/50	Klimaschewski et al., 2004
Human IL-6	26 kDa	Peprotech	20	Cao *et al.,* 2006

Table 2. List of primer sets used for real time PCR.

Gene Name	Accession number	Primer sequence 5'-3'	Product size (bp)
NGF	XM_227525.5	Forward: ACCTCTTCGGACACTCTGGA Reverse: GTCCGTGGCTGTGGTCTTAT	168
NT-3	NM_031073.2	Forward: GATCCAGGCGGATATCTTGA Reverse: AGCGTCTCTGTTGCCGTAGT	182
GDNF	NM_019139.1	Forward: CCAGAGAATTCCAGAGGGAAAGGT Reverse: TCAGTTCCTCCTTGGTTTCGTAGC	124
BDNF	NM_012513	Forward: ATCCCATGGGTTACACGAAGGAAG Reverse: GTAAGGGCCCGAACATACGATTG	98
FGF-2	NM_019305.2	Forward: GGTCACGGAAATACTCCAGTTGGT Reverse: AAACAGTATGGCCTTCTGTCCAGG	98
LIF	NM_022196.2	Forward: AAGTTGGTCGAGCTGTATCGGATG Reverse: TTGAGTTTGATCTGGAGGCTCACG	113
IL-6	NM_012589.1	Forward: ATCTGCCCTTCAGGAACAGCTATG Reverse: ACTTGTGAAGTAGGGAAGGCAGTG	110
Itga 5	NM_001108118.1	Forward: CCCTACCAAATCCTGCCTC Reverse: AGGATGGCGAGGATGATG	122
Itga 7	NM_030842.1	Forward: GCAGCAGTTCAAGGAGGAG Reverse: TTGGGACAGGAGAAGTTAGG	166
Itga 9	XM_002727107.1	Forward: TTTCTGCTGCTGGCTGTG Reverse: TCCCAAGCCTCTTCATTCTC	107
GAPDH	XM_573304.3	Forward: AATTCAACGGCACAGTCAAGGC Reverse: TACTCAGCACCGGCCTCACC	116

CHAPTER 3

CHARACTERIZATION OF LENTIVIRAL VECTOR OVEREXPRESSING FGF-2 – A PROMISING TOOL TO PROMOTE MOTONEURON REGENERATION

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Abstract

FGF-2 is a trophic factor expressed by glial cells and different neuronal populations. Interestingly, addition of FGF-2 to spinal cord and dorsal root ganglia (DRG) explants increases specifically outgrowth of motor neurons but not of sensory neurons. With the aim to further explore the potential capacity of FGF-2 to selectiveley promote motor regeneration in vivo, we produced a lentiviral (LV) vector to overexpress FGF-2. We verified the biological activity of the virus infecting Schwann cells, fibroblasts and 293T cells. At 7 days post-infection, there was an overexpression of this trophic factor in 293T cell cultures. In contrast, when infecting Schwann cells, FGF-2 levels did not increase in the culture medium but in the lysated cells, thus suggesting that FGF-2 was within the extracellular matrix and not secreted into the medium. The increase in secretion was seen at 9 days after infection. Addition of cultured Schwann cells infected with FGF-2 into a collagen matrix embedding spinal cords or DRG significantly increased motor neurite growth but not sensory outgrowth when compared to co-cultures with LV-GFP, thus demonstrating that the LV construct was as effective as direct addition of the trophic factor to selectively promote motor neuron growth. By injecting the LV construct direclty into the sciatic nerve in vivo, we corroborated the localization of the secreted FGF-2 in the basal lamina of Schwann cells. Levels of FGF-2 from homogenated sciatic nerves one week after injection of 1µl LV-FGF-2 were higher than from nerves injected with vehicle or LV-GFP. Therefore, the LV vector can be used in vivo to verify our in vitro results and further study the capacity of FGF-2 to enhance motor nerve regeneration.

Key words:

FGF-2, Schwann cell, fibroblast, lentiviral vector, in vitro DRG explant and spinal cord organotypic slices

Introduction

After peripheral nerve injury, axotomized neurons are able to regenerate. Denervated Schwann cells of the distal stump dedifferentiate, proliferate and provide support to axonal regeneration, by secreting trophic factors and expressing several cell adhesion molecules. Eventually, axons will be able to grow into this permissive environment, find the peripheral target organs and reinnervate them. Therefore, after complete nerve transection, reconnection of the two nerve stumps is mandatory, so axons can regenerate through the scar tissue to the distal stump. The use of an autograft to connect both stumps when direct suture is not possible is the gold standard repair technique. However, even when axons are able to reach the distal nerve stump, they can misroute and easily reinnervate an incorrect target organ. In these cases, although regeneration may be good, functional recovery is quite limited. Artificial nerve guides, although widely used in experimental models, are still below the success of autografts. Tubulization with guides containing different matrix and glial cells have been used experimentally. The functionalization of this guides, by engineered glial cells to overexpress trophic and tropic factors, may improve their permissive regenerative capacities and more closely resemble the autograft. Schwann cells genetically modified to secrete neurotrophic factors (Tannemaat *et al.,* 2008) have been proved to be a feasible tool to enhance peripheral nerve regeneration. In fact, gene therapy is a useful tool to study the effect of certain therapeutic protein over-expression. The use of viral vectors is a promising technique to engineer cells (Mason *et al*., 2011). Previous problems due to vector-mediated immune responses and neurotoxicity of the virus itself have been overcome with the development of lentiviral (LV) vectors (Naldini *et al.,* 1996), which are able to infect dividing and non-dividing cells. On the other hand, the limitations of functional recovery after nerve transection, due to axonal misrouting and non-specific reinnervation, might be overcome by the overexpression of factors that selectively enhance motor or sensory axons regeneration. *In vitro* studies performed in our laboratory revealed an enhancing effect of fibroblast growth factor 2 (FGF-2) (low molecular weight isoform - 18 kDa) on motor but not sensory neuritogenesis. This trophic factor is secreted by fibroblasts and Schwann

cells and it is known to be kept into the extracellular matrix, in contrast to other neurotrophic factors (Ornitz and Itoh, 2001).

As described by an *in vivo* work of Haastert K et al in 2006, the low molecular weight isoform of FGF-2 can be a promising trophic factor to enhance motor axon regeneration. Thus, the aim of this study was to produce a LV vector which allow the overexpression of FGF-2. *In vitro* characterization was assesed to corroborate previous findings (Allodi *et al.,* 2011b), in order to use this vector to overexpress FGF-2 in an *in vivo* animal model of peripheral nerve injury and repair.

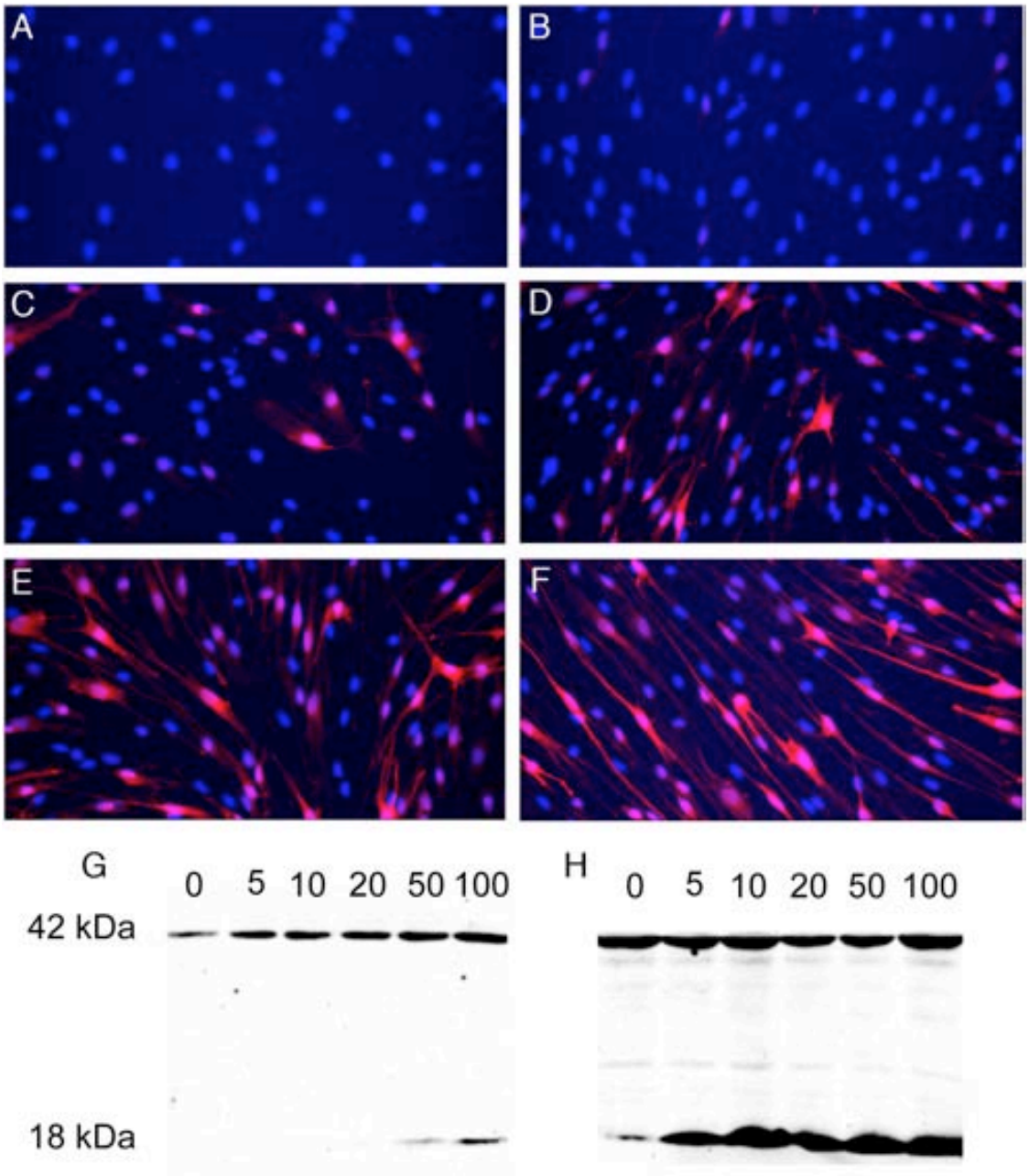

Figure 1. Confocal micrographs of cultured Schwann cell taken at 40x. Immunostaining for FGF-2 in red and DAPI (nuclear marker) in blue reveals the different expression of the trophic factor depending on the multiplicity of infection (MOI) used to infect the cells *in vitro*. (**A)** MOI of 0, (**B**) MOI of 5, (**C**) MOI of 10, (**D**) MOI of 20, (**E**) MOI of 50 and (**F**) MOI of 100. (**G**) and (**H**) show respectively Western blots of lysated Schwann cells and 293T cells after 7 days *in vitro*. Bands at 42 (actin) and 18 (FGF-2) kDa were detected. FGF-2 expression was found at MOI of 50 and 100 in Schwann cells.

Material and Methods

LV vector production

The LV vector encoding FGF-2 (low molecular weight isoform – 18 kDa) was produced using the pRRL-MCS vector containing the Woodchuck post-transcriptional regulatory element (Brun *et al.,* 2003). The NheI/XmaI FGF-2 cDNA excised from pCI-bFGF (a generous gift of Prof. Claudia Groethe, University of Hannover) was cloned into a XbaI/MscI opened LV transfer vector. cDNA was under the control of the human cytomegalovirus (CMV) promoter. The LV vector was sequenced to verify the sequence and orientation of the insert.

Lentiviral vectors were generated as described previously (Naldini *et al.,* 1996). pRRL-MCS encoding FGF-2 (20 µg), the VSV-G envelope protein vector pMD.G.2 (7 µg) and the viral core-packaging construct pCMVdeltaR8.74 (13 µg) were co-transfected in 293T cells with Isocove's Modified Dulbecco's Medium (IMDM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin/streptomycin and 2 mM glutamine. The next day, medium was replaced and cells were incubated for 24 h. Medium was harvested and spun at 176 ×*g*. Supernatant was filtered through a 0.22 µm cellulose acetate filter and centrifuged at 80,000 ×*g* for 2.5 h. The supernatant was discarded and the pellet resuspended in 0.1 M sodium phosphate buffer pH 7.4 in saline (PBS), aliquoted and stored at − 80 °C. The titer of the LV vector stock was evaluated by infecting 293T cells upon serial dilution and determining the number of transducing units per ml (TU/ml) by immunocytochemistry. 293T cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and then incubated with primary antibody against FGF-2 (Upstate – Millipore, 1:200) for 2h at room temperature. After washes, cells were incubated

with rabbit anti-mouse HRP secondary antibody (Dako, 1:100) for one hour and reaction was developed with the VIP kit (Vector Laboratories). The cell counting gave a titer in the order of 10^9 TU/ml. For additional titering, viral vector stocks were analyzed with p24 content (ng/ml) with an ELISA assay (ZeptoMetrix Corporation, 0801111). The ratio between the TU/ml and p24 content of the LV-FGF2 stock was used and the final titer was $2x10^9$.

Schwann cells culture

Dissociated Schwann cells were obtained from P21 rats, as described by Verdu et al., 2000 (Verdu *et al.,* 2000). Sciatic nerves were dissociated and kept in cold Gey's balanced salt solution (Sigma) and cleaned from connective tissue. Nerves were cut into small pieces and dissociated in 1 ml Ca^+ and Mg^+ free Hank's medium (Sigma) with 0,25% trypsin (Sigma), 1 mg/ml collagenase A (Sigma) and 1 mg/ml DNAse (Roche), followed by mechanical dissociation. Cells were than centrifuged at 900 rpm for 7 minutes, resuspended and seeded onto poly-D-lysine coated coverslips inserted into 24 well plates or into 12 well plates (Iwaki, Asahi Technoglass, Chiba, Japan) and incubated in IMEM supplemeted with 10% FCS, 100 U/ml penicillin/streptomycin and 2 mM glutamine. The purity of the cultures was of about 70% of Schwann cells, whereas the rest of the cells were mainly fibroblasts. The next day, LV-FGF2 was added at a multiplicity of infection (MOI) of 0, 20, 50 and 100. Cells were kept at 37° C and 5% CO2. Subsequently, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and stained for FGF-2 as described before. Images were taken with a confocal laser scanning microscope (SP5, Leica).

LV-FGF2 expression in vitro

293T and Schwann cells were passaged to be 60-70% confluent the next day, when were infected with LV-FGF2. The LV was added at different multiplicity of infection - MOI (0, 5, 10, 20, 50 and 100). Medium was changed after 24 h. To provide conditioned medium containing FGF-2 derived from LV vector, cells were cultured for 7 days, and the medium collected at 2, 3, 5 and 7 days. Conditioned medium was collected and frozen at -20° C for further assays. Then, it was centrifuged to remove detached cells. After 7 days in vitro, cells were lysated into

RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate (Sigma), 0.2% SDS, 100 mM NaCl, 1mM EDTA) containing protease inhibitor cocktail (10 μl/ml, Sigma), and the protein suspension obtained was included in the analysis. Part of the proteic extract of medium and lysated cells was separated on 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Whatman, Dassel, Germany). The membranes were then incubated for blocking with 25% BSA (Sigma) in TBS plus 0.05% tween-20 (TBST) for 30 minutes and incubated with primary antibodies anti FGF-2 and anti β-actin (1:10000, Sigma) at 4°C overnight. After that, membranes were incubated with secondary antibodies against mouse IgG (1:3000, BioRad) for one hour and the reaction was developed by Immobilon reagent (Millipore) and analyzed with GeneSnap software.

A further assay was conducted on Schwann cells until day 15 post-infection. Conditioned medium was collected at 3, 5, 7, 9, 11, 13 and 15 days. The cells were lysated as described previously and included into the proteic analysis. The concentration of FGF-2 was measured using FGF-2 ELISA kit (R&D, DuoSet DY233) according to the manufacturer's instructions. This kit detects both human (endogenous) and rat (transgenic) FGF-2.

Spinal cord organotypic slices and DRG explants co-culture

Cultures of spinal cord slices and DRG explants were prepared as described previously (Allodi et al. 2011a). Schwann cells were cultured for four days and then transfected with FGF-2 or GFP expressing LV vector using a MOI of 50. Medium was refreshed after one day and the cells kept under 37° C and 5% CO2 conditions for three more days. Then cells were trypsinized, centrifuged and re-suspended in 1 ml of medium. Cells were counted with the help of a Neubauer chamber and a number of $10x10^3$ or $50x10^3$ was kindly mixed with the collagen solution. Spinal cord slices and DRG explants were placed within the collagen matrix and kept in culture for 4 days. Pictures were taken with a confocal microscope (SP5, Leica) and neurite elongation and arborization measured as previously described (Allodi *et al.,* 2011a).

Quantification of LV-FGF2 expression in vivo

For *in vivo* quantification of LV-FGF2 expression, the LV construct was injected in the sciatic nerves of rats and the transgene expression was analysed 1 week later. Two months old Sprague Dawley rats were anesthetized with 90 mg/kg ketamine – 10mg/kg xilacine. Sciatic nerves were exposed under a dissection microscope and 1 μl of $2x10^9$ TU of LV-FGF2 vector solution was injected into the nerve using a glass capillary with an 80 μm diameter tip attached to a 10 μl Hamilton syringe. The same volume of saline was injected in the contralateral side as sham condition. Fast green (Sigma) was previously added into the solutions at a final concentration of 0.5% to visualize solution spread during injection. The point of injection was marked with a stitch of 10-0 nylon suture. The wound was sutured and disinfected.

Sciatic nerves of 5 animals were harvested at 1 week post-surgery, 1 cm proximally from the microsuture. From the nerve that received saline solution, a piece of 1 cm obtained 5 cm away from the injection site was used as a further control condition. All the segments were snap frozen on dry-ice and stored at -80° C. For FGF-2 quantification by ELISA, the frozen nerves were grounded with a mortar on dry-ice and suspended in 250 μl of buffer containing 137 mM NaCl, 20 mM Tris/HCl pH 8.0, 10% glycerol, 0.1% Tween-20, 0.5 mM sodium orthovanadate, 1% Nonidet P40 substitute and complete protease inhibitor (Roche, Germany). Samples were then vortexed for 30 seconds, centrifuged and the supernatant stored in 50 μl aliquots at -20° C. The concentration of FGF-2 was measured with ELISA kit (R&D, DuoSet DY233) according to the manufacturer's instruction and used to calculate total content in pg/cm nerve segment. The detection limit was 1,5 ng/ml.

Immunohistochemestry

Two other rats were anesthetized, the sciatic nerves exposed and injection of the LV-FGF2 or saline was performed as explained previously. One week later, animals were deeply anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde in PBS. Ipsilateral and controlateral sciatic nerves were carefully harvested and cryoprotected in 30% sucrose at 4°C. Nerves were

cut in longitudinal sections of 15 μm thickness with a cryostat (Leica) and collected onto gelatin-coated glass slides. Slices were incubated overnight with primary antibody mouse anti FGF-2 (Upstate – Millipore, 1:200) and rabbit anti-laminin (1:1000, AbSerotec), and after washes, with secondary antibody (FITC anti rabbit, 1:200, Vector, and Cy3 anti mouse, 1:200, Jackson Laboratory).

Statistical analysis

Analysis for statistical differences was performed with Prism software. One and two way ANOVA with Bonferroni posthoc test were used. A p value lower that 0.5 was considered significant.

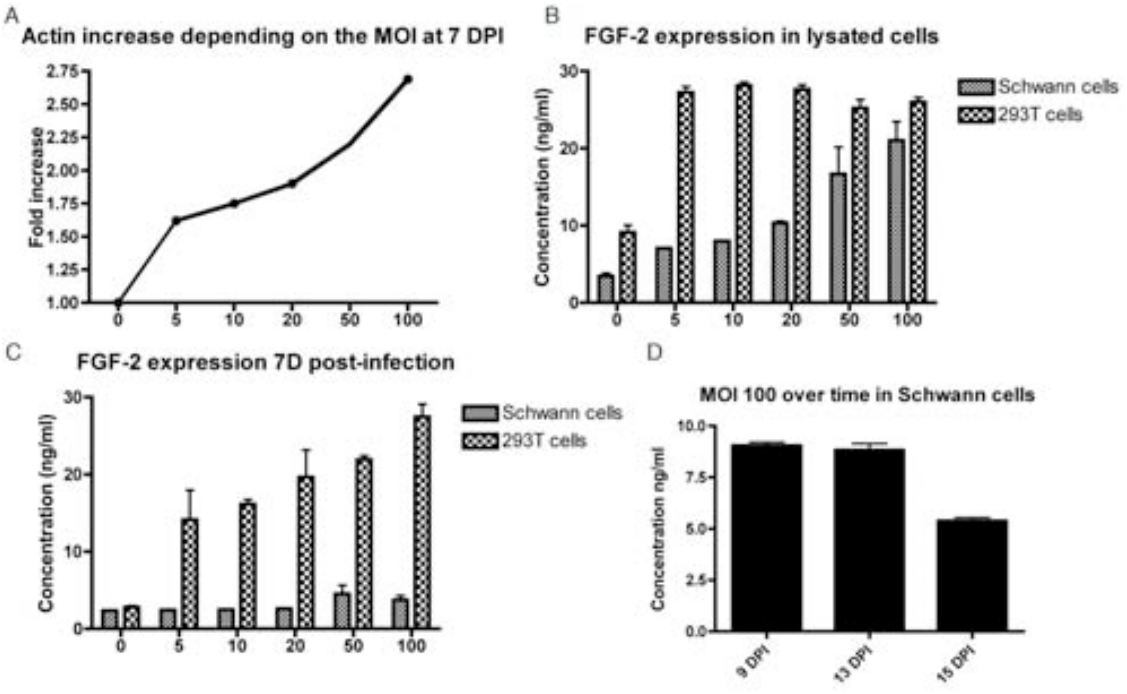

Figure 2. (**A**) Quantification of actin fold increase in lysated Schwann cells. The same amount of cell lysated solution was added in all the Western blot conditions. Actin increased depending on MOIs (in the x axis), thus indicating that cell proliferation was dependent on the amount of viral particles per number of cells. This effect was not observed in 293T cells. (**B**) Expression of FGF-2 quantified by ELISA in lysated Schwann and 293T cells at 7 days post infection. The graph shows that in 293T cells the plateau was reached with a MOI of 5 at 7 days. In Schwann cells, the amount of FGF-2 increased at MOI of 20, but only with a MOI of 100 it reached the same level observed in 293T cells. (**C**) Quantification of FGF-2 present into the culture media by ELISA. A higher amount of FGF-2 is secreted by 293T cells than by Schwann cells after 7 days post infection. Also in this case the secretion depends on the MOIs. (**D**) Levels of FGF-2 reach 9 ng/ml at 9 days post infection and remain unchanged until day 15 post infection.

Results

Characterization of LV-FGF2 vector in vitro

By manual titering and p24 ELISA quantification we estimated that the titer of the LV-FGF2 was $2x10^9$. We determined if cells infected by LV-FGF2 with different MOIs were producing FGF-2 by immunohistochemestry, ELISA and Western blot. Immunohistochemical labeling showed that expression of FGF-2 in Schwann cells and 293T cells depended on the different MOIs. There was increased cell proliferation, morphological elongation and boundary formation in Schwann cells infected with the FGF-2 lentiviral vector (Figure 1). By ELISA, in 293T cells infected by LV-FGF-2, we observed a high secretion of FGF-2 in the medium at 7 days (Figure 2) already at low MOIs. In contrast, the secretion of FGF-2 by Schwann cells was low and only significantly increased when using MOI of 100. 293T cells always secreted higher amount of FGF-2 than Schwann cells. On the other hand, the concentration of FGF-2 present in the lysated cells at 7 days was similar in both cells at MOI of 100. In Schwann cell cultures, FGF-2 levels reached a peak of 9 ng/□l at 9 days that was maintained at 13 days and slightl reduced therafter (Figure 2).

In vitro biological activity of LV-FGF2

The biological activity of LV-FGF2 transfected Schwann cells was analyzed using a co-culture preparation. Schwann cells overexpressing FGF-2 were mixed in the collagen matrix embedding spinal cord organotypic slices and DRG explants. Schwann cells infected with LV-eGFP were used as control. We found that cells overexpressing FGF-2 promoted neurite elongation and arborization of motoneurons, whereas addition of the same cells did not have any significant effect on DRG neuron outgrowth (Figure 3). When compared to e-GFP we observed that FGF-2 overepression enahnced more than 40% ($p<0.05$) neurite elongation, while arborization was increased more than 55% ($p<0.5$) (Figure 3).

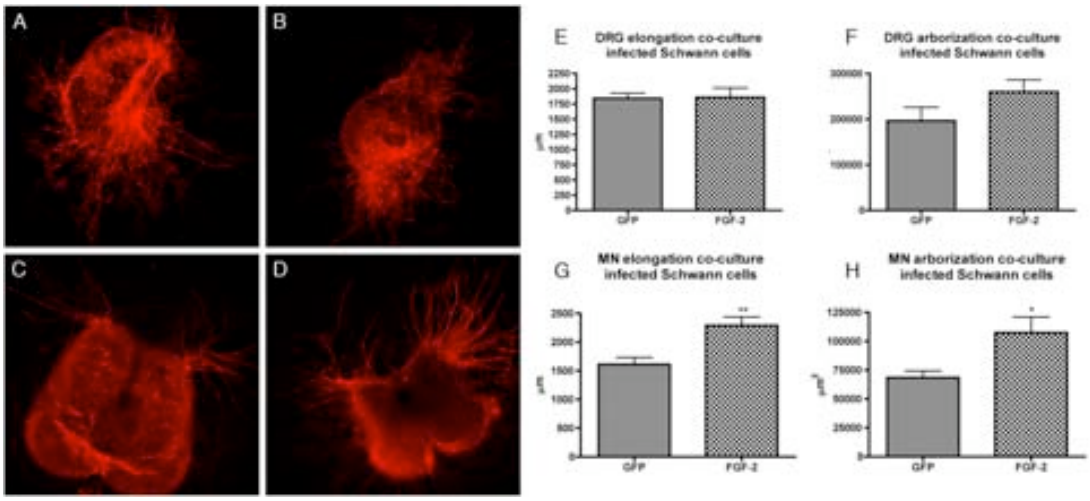

Figure 3. (**A**) Outgrowth of DRG neurons co-cultured with Schwann cells infected by LV-eGFP. (**B**) Co-culture of DRG and Schwann cells infected by LV-FGF2. Both LV vectors were used at a MOI of 50. RT97 (marker for phosphorylated heavy chain neurofilament) in red. (**C**, **D**) Representative confocal images of motoneuron outgrowth in co-culture with Schwann cells infected with LV-eGFP and LV-FGF2 respectively. Graphs indicate the quantification of neurite elongation and arborization of sensory (**E** and **F**) and motor neurons (**G** and **H**). Motoneuron outgrowth was significantly enhanced by LV-FGF2 infected Schwann cells, compared to LV-eGFP (* $p<0.05$; ** $p<0.01$). In contrast, sensory outgrowth was not affected by FGF-2 secretion.

Trangene expression in vivo

After injection of LV-FGF2 in the sciatic nerve, levels of FGF-2 were determined by ELISA and compared with those found in intact and saline-injected nerves. The amount of FGF-2 was significantly increased 7 days after the injection ($p<0.001$), reaching a concentration of 15.4 ± 2.5 ng per cm of nerve. At the same time point, immunohistochemestry performed on longitudinal sections of the nerve revealed high immunoreactivity for FGF-2 (Figure 4). Secreted FGF-2 co-labeled with laminin, indicating its localization at the basal lamina of the Schwann cells, in contact with the axons (Figure 4).

Discussion

In this study we characterized the biological activity of a LV vector for the expression of FGF-2. In a previous *in vitro* study (Allodi *et al.,* 2011b) we found that

this factor has a promising effect on motoneuron neuritogenesis. Therefore, gene therapy to induce FGF-2 over-expression at the injured nerve appears as a potential tool to enhance motor regeneration after peripheral nerve lesions. Thus, we decided to produce a lentiviral vector because LVs are known to transduce Schwann cells and fibroblasts in the rat sciatic nerve with a high efficiency (Eggers *et al.,* 2008; Tannemaat *et al.,* 2008).

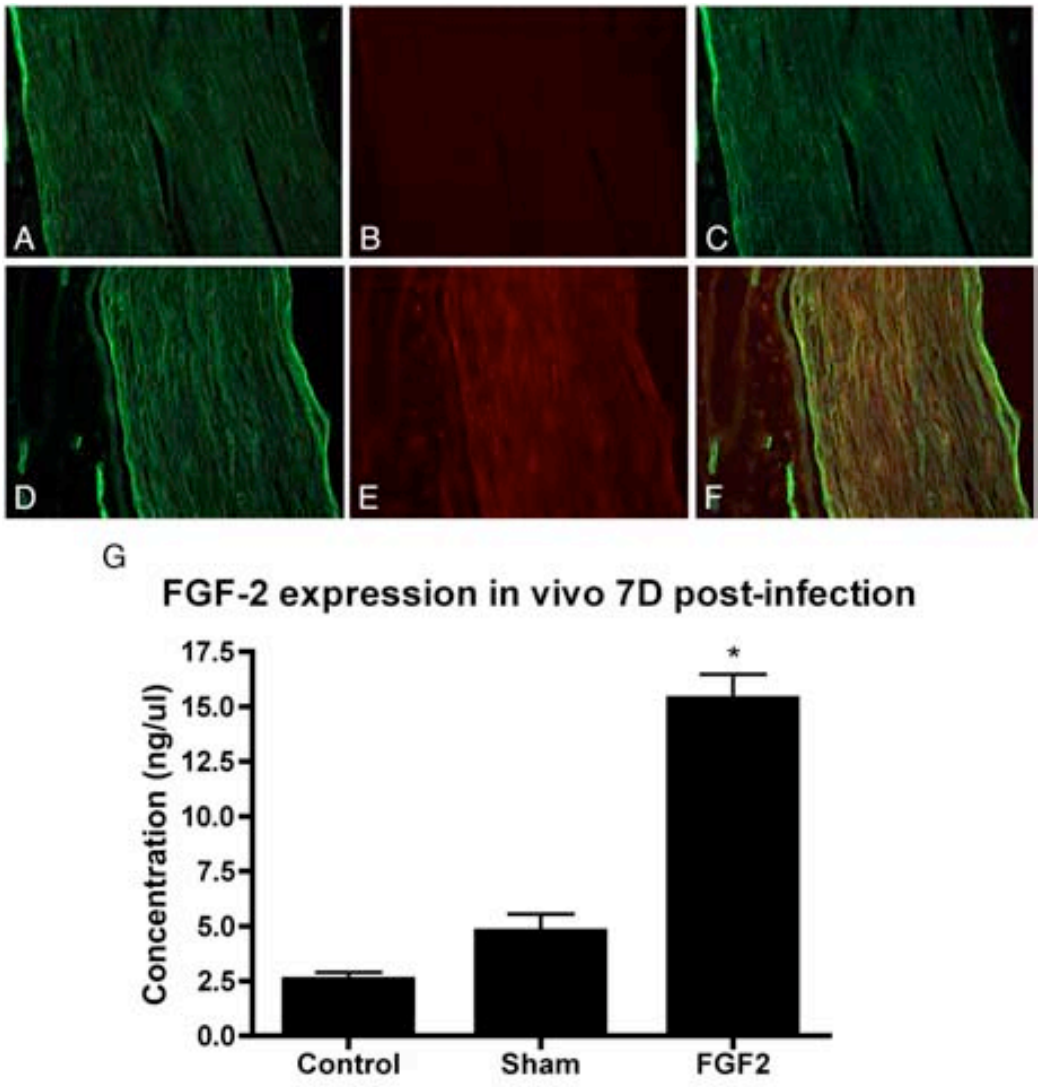

Figure 4. Laminin expression in the nerve after injection of saline (**A**) and LV-FGF2 (**D**) solutions. Immunolabeling for FGF-2 one week after infection (**B** and **E**) showed higher FGF-2 immunoreactivity in the LV-FGF-2 (**B**) than in the LV-eGFP infected nerves (**E). C** and **F** show mergeing of laminin and FGF-2 labeling. Histogram in **G** shows quantification of FGF-2 in a control nerve and 7 days after saline injection (sham) or LV-FGF-2 infection. (* $p<0.05$).

The LV-FGF-2 construct was able to infect 293T cells and induced an overexpression of this factor in the culture medium. However, since the final goal of our study was to transfect Schwann cells, we also studied the effects of the vector on these glial cells *in vitro*. We cultured dissociated cells from sciatic nerves, thus obtaining a culture mainly composed of Schwann cells, with a low percentage of fibroblasts. We chose a non-pure culture because it closely mimiks the in vivo situation, as both kind of cells are present in the nerve and both may be infected by the virus in case of direct application into the nerve. The LV vector infection induced changes in Schwann cell proliferation and morphology. The amount of FGF-2 expressed by these cells reached the plateau after 9 days and this level was kept until day 15 post infection. However, levels of FGF-2 from lysated cells increased already at 7 days *in vitro,* and were dependent on the number of viral particles per cell. Interestingly, the levels of FGF-2 secreted to the medium was very low in Schwann cells cultures compared to 293T cells. This low amount of FGF-2 in the medium suggests that the secreted factors is kept at the basal lamina of Schwann cells (Ornitz and Itoh, 2001). In fact, *in vivo* testing revealed co-labeling of the secreted FGF-2 and laminin, the main component of the basal lamina (Figure 4). Since secretion of FGF-2 by Schwann cells has this peculiarity, it is important to guarantee that the entrapment of the factor in the basal lamina is efficient to sustain axonal growth. Previous studies in our laboratory have shown that the presence of FGF-2 in a collagen matrix specifically enhances neurite outgrowth of motor but not sensory neurons (Allodi *et al.,* 2011b). Spinal cord organotypic slices and DRG explants were co-cultured with Schwann cells infected with the LV-FGF2. In this case we observed that the effect of FGF-2 produced by infected Schwann cells produced the same results, i.e. promoted neuritogenesis in motor neurons, that when the human recombinant protein was added to the culture. On the other hand, FGF-2 had no significant effect on sensory neuron outgrowth.

To corroborate that LV-FGF2 was able to infect Schwann cells in an *in vivo* model, we injected the solution containing the virus in adult rat sciatic nerves. The expression of FGF-2 was significantly increased at 7 days post infection compared to intact and vehicle injected nerves.

All these studies indicate the possibility of using LV-FGF2 in peripheral nerves after injury to promote motoneuron regeneration. *In vivo* studies are now needed to corroborate the *in vitro* findings. It is important to note that the LV-FGF2 can be applied directly into an injured nerve or used to engineer isolated cells then added in artificial nerve conduits (Rodríguez *et al.,* 2000; Haastert-Talini *et al.,* 2010, McGrath AM *et al.,* 2012). In the last case, it will improve the capability to sustain regeneration over long gaps. Interestingly, it can also be injected into chronic denervated nerves, i.e. long after the injury was produced. In this case, gene therapy may improve the regenerative capabilities of these nerves, usually poor since Schwann cells decrease their trophic factors secretion after long periods of denervation. In fact, chronic denervation often occurs after severe injuries, and it is one of the main limitations of a succesful motor recovery (Gordon T *et al.,* 2011). Chronic denervation of the most distal branches is almost inevitable in proximal injuries, as the regenerative front may need months to reach these branches. Application of the LV vector into the distal motor branches could reduce the effects of chronic denervation and also favour the attraction of motor axons towards the right pathway.

CHAPTER 4

FGFR-1 DIFFERENTIALLY DETERMINES OLFACTORY ENSHEATHING GLIA AND SCHWANN CELLS NEURITOGENESIS SUPPORT IN DRG EXPLANTS AND SPINAL CORD ORGANOTYPIC SLICES

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Abstract

In this work we compare the abilities of olfactory ensheathing cells (OEC) and Schwann cells in sustaining *in vitro* motor and sensory neuritogenesis. Co-cultures of these glial cells with DRG explants and spinal cord organotypic slices were set up. Due to the maintained cytoarchitecture of the spinal cord *in vitro*, astrocytes and endogenous Schwann cells were also present. For this reason, when OEC were added into the spinal cord culture, we found cell clusters and motoneuron outgrowth inhibition. On the other hand, OEC significantly increased neurite outgrowth in DRGs, where astrocytes were absent. Previous studies demonstrated that this mechanism is probably due to the FGFR1-FGF2-HSPG complex. In fact high levels of HSPG were found into the boundary formations, and this can explain the chemorepellent role of the cell cluster on neurite outgrowth. Boundary formations in spinal cord and OEC co-culture were overcome by FGFR-1 blockage. After FGFR-1 inhibition motoneuron neuritogenesis was comparable to controls.

Key words: Schwann cells, Olfactory Enshealting cells, DRG explant, spinal cord organotypic slice, FGFR-1, HSPG

Introduction

Peripheral axonal regeneration after an injury depends on the intrinsic growth capability of the injured neuron, but also on the presence of proliferating Schwann cells (SC) into the distal stump. SC of the injured nerve switch to a proliferative state, and their trophic secretions and the molecules present on their extracellular matrix (Navarro *et al.,* 2003) facilitate injured axon regrowing. In contrast, most of the glia present in the central nervous system does not show supportive trophic and tropic capabilities after lesion. Among other factors, this explains why regeneration is almost absent after central nervous injuries (Huebner and Strittmatter, 2009). An exception is the spontaneous regeneration of olfactory axons, mainly due to the special glia of this system, the olfactory ensheathing glia (Doucette, 1984). In fact, olfactory ensheathing cells (OEC) and SC are known to share genotypic and phenotypic properties (Pollock *et al.,* 1999, Ramon-Cueto and Valverde, 1995, Smith *et al.,* 2001, Wewetzer *et al.,* 2002). Therefore, these two types of glial cells are promising candidates for cell therapy after CNS and PNS injuries (for reviews see Radtke *et al.,* 2011, Richter and Roskams, 2008, Hernandez *et al.,* 2011).

After peripheral nerve injuries, transplants of SC are useful to enhance the poor capabilities of artificial nerve guides to sustain regeneration across long gaps (Navarro *et al.,* 2003). Transplants of OEC also enhance regeneration and functional recovery after peripheral nerve transection (Verdú *et al.,* 1999, You *et al.,* 2011, Guerout *et al.,* 2011). However, the potential different capabilities of both Schwann cells and olfactory glia to sustain motor and sensory outgrowth has not been adressed in the literature.

Two main subtypes of SC are present in the peripheral nervous system; myelinating ones and non-myelinating ones. Moreover, SC accompanying motor and sensory axons show different phenotypes, and express different trophic factors after injury (Hoke *et al.,* 2006). However, these cells lose their specific phenotype after denervation. On the other hand, OEC are normally associated to unmyelinated sensory axons of the olfactory nerve. Therefore, it is of interest to study if both types of glial cells can differentially sustain regeneration of different

types of axons. Consequently, it is important to take into account how these cells respond to the different environments when they are transplanted. For this reason, we co-cultured SC and OEC with DRG explants and spinal cord slices (Allodi *et al.,* 2011a), with the aim of assessing the capacity of SC and OEC to enhance neuritogenesis of sensory and motor neurons. We observed that OEC were more effective than SC in sustaining sensory outgrowth in DRG explants. In contrast, SC but not OECs were able to sustain motoneuron outgrowth. Moreover, we demonstrated that the different ability of OEC to sustain regeneration on DRG and spinal cord explants was due to the absence/presence of central glia respectively.

Material and Methods

Schwann cells culture

SC were isolated from the sciatic nerves of P21 Sprague-Dawley rats, as previously described (Verdú *et al.,* 2000). Sciatic nerve segments were stored in cold Gey's balanced salt solution (Sigma) and cleaned from connective tissue. Nerves were enzymatically dissociated in 1 ml Ca^{2+} and Mg^{2+} free Hank's medium (Sigma) with 0.25% trypsin (Sigma), 1mg/ml collagenase A (Sigma) and 1 mg/ml DNAse (Roche), followed by mechanical dissociation. Samples were then centrifuged at 900 rpm for 7 minutes. The cells were resuspended and seeded onto poly-D-lysine coated Petri dishes (Iwaki, Asahi Technoglass, Chiba, Japan) and incubated with 2 ml of DF-10S medium in 5% CO_2 at 37ºC. After 5 days, cells were enzymatically detached with 0.25% trypsin (Sigma).

Olfactory Ensheating cell culture

OECs were obtained from primary cultures of olfactory bulbs from P21 Sprague-Dawley rats. The bulbs were aseptically removed and stored in Hank's balanced salt solution (HBBS) with calcium and magnesium at 4 ºC. The meningeal layer was stripped off with a fine forceps, and tissue was enzymatic and mechanically dissociated. Cells were recovered by centrifugation in Dulbecco's minimum essential medium nutrient mixture F-12 Ham (DMEM) and seeded onto

25 cm^2 flasks coated with poly-L-lisine and incubated in 5% CO_2 at 37ºC. Culture medium was DMEM supplemented with 10% fetal calf serum. Cells were kept in culture at least for 7 days. Then the cells were immunopurified by microbeads pre-coated with anti-p75NGFR antibodies that recognize the OECs. Purity of both glial cell cultures was at least 75%.

Cell purification and transfection with LV-eGFP

For immunopurification, cells were incubated with mouse monoclonal primary antibody anti-nerve growth factor receptor (NGF-Receptor, p75, 1:1000, Chemicon), and then with goat secondary antibody anti-mouse IgG conjugated with microbeads (Miltenyi). Finally, labeled cells were separated in a MACS column (Miltenyi) placed within a magnetic field.

Cells were transfected with a lentivirus-GFP vector. Lentiviral vectors were added to the dish at a multiplicity of infection of 100 for 24 h. Transduced SC and OEC were used when high levels of transgene expression were achieved. Expression of the reporter gene GFP was directly visualized under an inverted fluorescence microscope (Olympus IX71). An adequate amount of cell suspension in DF10S medium was kindly mixed into a collagen solution to get a final concentration of 10^4 glial cells in each volume of collagen matrix used to embed spinal cord organotypic slices and DRG explants.

Fibroblasts culture

Fibroblasts were obtained from P21 Sprague-Dawley rat sciatic nerves. Nerves were dissociated and kept in cold Gey's balanced salt solution (Sigma). Epineuria were taken with the help of microscissors and a fine forceps, and then enzymatically dissociated in 1 ml Hank's salt solution Ca2+ and Mg2+ free with the addition of 0.25% Trypsin, 1mg/ml Collagenase A and 1 mg/ml of Dnase-I. Enzymatic digestion was kept at 37°C for 1 h, mixing every 15 minutes. Then tissue was mechanically dissociated with the help of a Pasteur pipette and 13 ml of DF10S medium were added to stop the enzymatic reaction. After centrifugation at 900 rpm for 10 minutes, the pellet was resuspended in 1 ml DF10S medium and

cells were counted in a Neubauer chamber. Cells were plated at a concentreation between 350 and 500 cells/mm^2. Fibroblasts were cultured at 37°C and 5% CO2, with changes of medium every 3 days.

Spinal cord slices and DRG explants cultures

Spinal cord slices and DRG explants of postnatal day 7 Sprague-Dawley rats were cultured as detailed in a previous work (Allodi *et al.,* 2011a). Briefly, lumbar and high sacral spinal cord segments and low thoracic and lumbar DRG were dissected and cleaned from blood and meningeal debris. A volume of 450 ml of type I collagen solution (BD Biosciences) at a concentration of 3.4 mg/ml, was mixed with 50 ml of 10X basal Eagle's medium (Gibco) and 2 ml of 7.5% sodium bicarbonate solution (Tucker *et al.,* 1996). Single drops of 30 ml containing a cell suspension were deposited on poly-D-lysine (PL, 1mg/ml, Sigma) coated coverslips, which were placed in Petri dishes or 24-well multidishes (Iwaki, Asahi Technoglass, Chiba, Japan) and kept in the incubator at 37°C and 5% CO_2 for two hours to induce collagen gel formation.

Spinal cord slices and DRG explants were then transferred on the gelled collagen droplets and covered by a second drop of 30 ml of the same collagen solution. The embedded samples were placed again in the incubator for 45 minutes before adding Neurobasal medium (NB, Invitrogen), supplemented with B27 (Invitrogen), glutamine and penicillin/streptomycin (Sigma). The medium volume delivered into Petri dishes and wells was 1.5 ml and 0.5 ml respectively. In co-culture assays both DRG explants and spinal cord slices were cultured for 4 days, to assure the same trophic and tropic support given by the cells.

FGFR-1 inhibition assay

PD 173074 (Parke Davis, Ann Arbor, MI, USA), a specific inhibitor of FGFR-1 (Mehanna *et al.,* 2009) was used at a concentration of 500 nM. PD 173074 was dissolved in DMSO and then diluted in 10X DMEM. The medium was gently mixed with the collagen solution and the cell suspension. Droplets of matrix were added

to coverslips coated with poly-D-lysine and let gelled as previously described. Spinal cord and DRG were placed on the gelled droplets, covered with the same solution and cultured for 4 days.

OEC conditioning medium assay

OEC conditioned medium was added into the collagen matrix solution and in the culture medium as well. An amount of 10 ml was mixed with the collagen solution, to allow direct contact with the tissue explants. Then, a further amount of 500 µl was added to the spinal cord organotypic slice medium, while 170 µl were added to DRG explant medium.

Immunohistochemistry

Spinal cord cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes, and DRG cultures for 15 minutes. Samples were then incubated for 48 hours with primary antibodies: mouse RT97 (1:200, Developmental Studies Hybridoma Bank), chicken anti-NF200 (1:1000, Millipore), rabbit anti-GFP (1:200, Invitrogen), rabbit anti-GFAP (1:500, Dako), mouse anti-GFAP (1:500, Sigma), rabbit anti heparan sulfate protoglycan (1:100, Acris) and mouse anti nerve growth factor receptor (1:200, Millipore). After three hours washing, the sections were incubated overnight with secondary antibodies Cy3 conjugated donkey anti-mouse (1:200, Jackson IR), Alexa 488 donkey anti-mouse (1:200, Invitrogen), FITC conjugated goat anti-rabbit (1:200, Vector), Cy5 donkey anti mouse (1:200, Jackson IR) or DyLight 405 donkey anti chicken (1:200, Jackson IR). For biotin amplification, samples were incubated overnight with biotin anti-rat antibody (1:200, Vector) and then, after washes, for two hours with Texas red streptavidin (1:200, Vector). Samples were then mounted on slides with glycerol supplemented with 10% Moviol and 0.6% DABCO. DAPI staining was used where needed (1:1000, Sigma). Colocalization and qualitative analysis were done with 3D images taken with a SP5 confocal microscope (Leica).

Data analysis

Spinal cord and DRG cultures were stained with anti-neurofilament antibody RT97 to label neurons and the growing neurites. Microphotographs for quantitative analysis were taken at 20× with a digital camera (Olympus DP50) attached to the microscope (Olympus BX51), acquired in Adobe Photoshop CS4 to automatically photomerge them, and analyzed with the aid of ImageJ software (NIH, available at http://rsb.info.nih.gov/ij/). The length of the longest neurite in the cultures was measured for 20 samples per condition. For the arborization area, the microphotographs were transformed to a gray scale, 8-bit image, and quantification was assessed after defining a threshold for background correction using ImageJ software.

Statistical analysis was performed using Prism 4 software. All results are given as the mean±SEM. One-way ANOVA, with post-hoc Bonferroni method (multiple comparisons versus control group), was used to statistically analyze neurite outgrowth and branching pattern. A p value lower than 0.5 was considered significant.

FGF-2 quantification by ELISA

Conditionated medium was collected from Schwann cells and OEC confluent cultures just before using them for co-culture assay, and frozen at -20° C for further investigation. The medium was thawed and centrifuged to revome detached cells. The concentration of FGF-2 was measured with an ELISA kit (R&D, DuoSet DY233) according to the manufacturer's instruction and used to calculate total content in the medium. The detection limit was 1.5 ng/ml.

Results

Spinal cord co-culture

Fibroblasts were added into the collagen matrix as control condition, as they were found to allow neurite outgrowth similar to the spontaneous outgrowth

in the collagen matrix alone (data not shown). SC enhanced significantly motoneuron arborization compared to fibroblasts and OEC conditions, almost a 70% more than the control condition (Figure 1). On the other hand, an inhibitory effect of OEC on motoneuron outgrowth was mainly seen on neurite elongation (OEC 94.4±89.8 mm; SC 578.7±42.5 mm; fibroblasts 458.5±76.8 mm) (Figure 1). By immunohistochemestry OEC stained by anti-GFP were found to form clusters in the matrix, inhibiting the outgrowth of neurites, which followed the direction towards the dorsal horn. This pattern differs from the normal motor neurite outgrowth observed in control cultures and in the presence of SC (Figure 1).

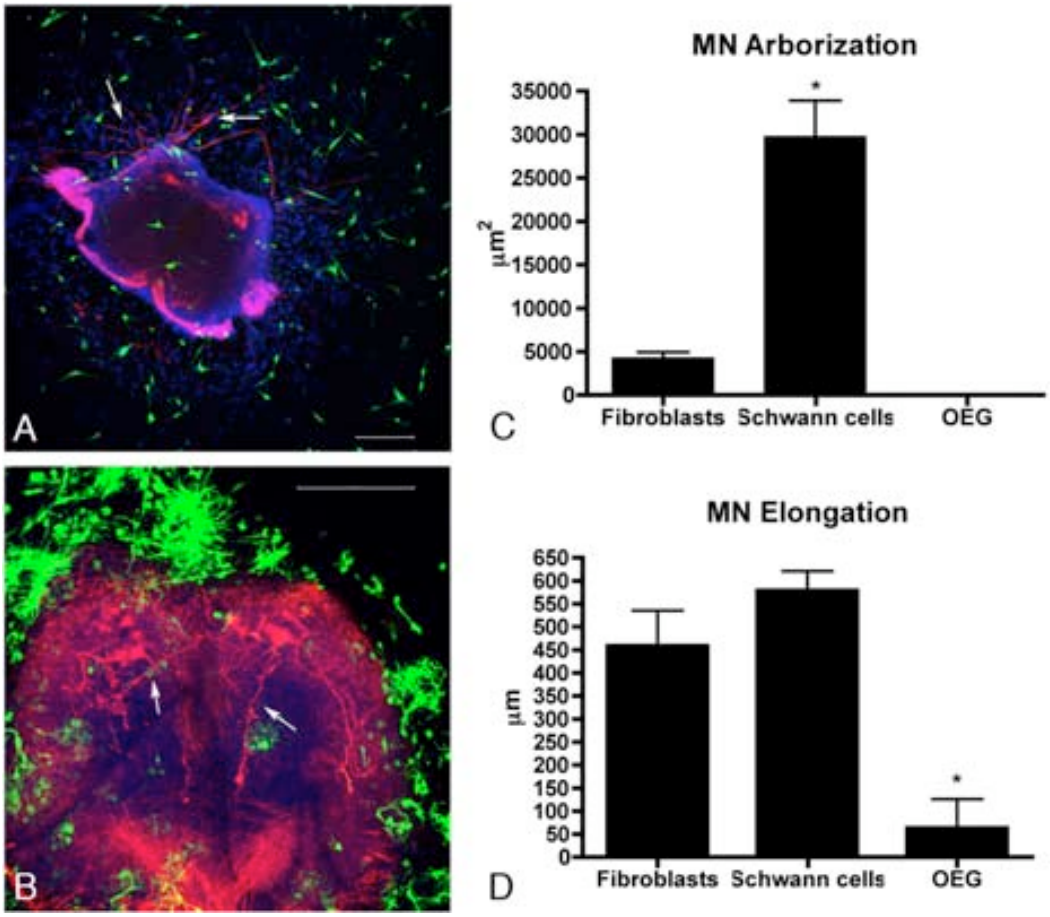

Figure 1. Confocal pictures show motoneuron outgrowth when co-cultured with $10x10^3$ Schwann cells (arrows indicate neurite elongation into the matrix) (**A**) or Olfactory Ensheating cells (**B**). Cell clusters can be seen in OEC co-culture in front of the ventral horn. Arrows indicate neurite elongation into the spinal cord slice, avoiding contact with clusters (**B**). RT97 in red, GFP in green and DAPI in blue. Bar = 100 μm (**A**) and 200 μm (**B**). Histograms show motoneuron arborization (**C**) and elongation (**D**) when co-cultured with the three different cell types. Fibroblasts are used as control condition. (*$p<0.05$).

DRG co-culture

Fibroblast co-culture was used also as control condition. Addition of SC to the collagen matrix did not enhance significantly neurite outgrowth in DRG explants (Figure 2); the average sensory neurite elongation was 1428±244 mm and the arborization area 10422±2441 mm^2. On the other hand, OEC had a marked effect on neurite elongation that reached 2250±267 mm, a 2.5 fold increase compared to the control condition (Figure 2).Immunohistochemical labeling showed that the OEC had a different morphology when co-cultured with DRGs than with spinal cord organotypic cultures (Figure 2). No boundary formations were found in the DRG co-culture and the OEC morphology was more similar to the Schwann cells (Figure 2).

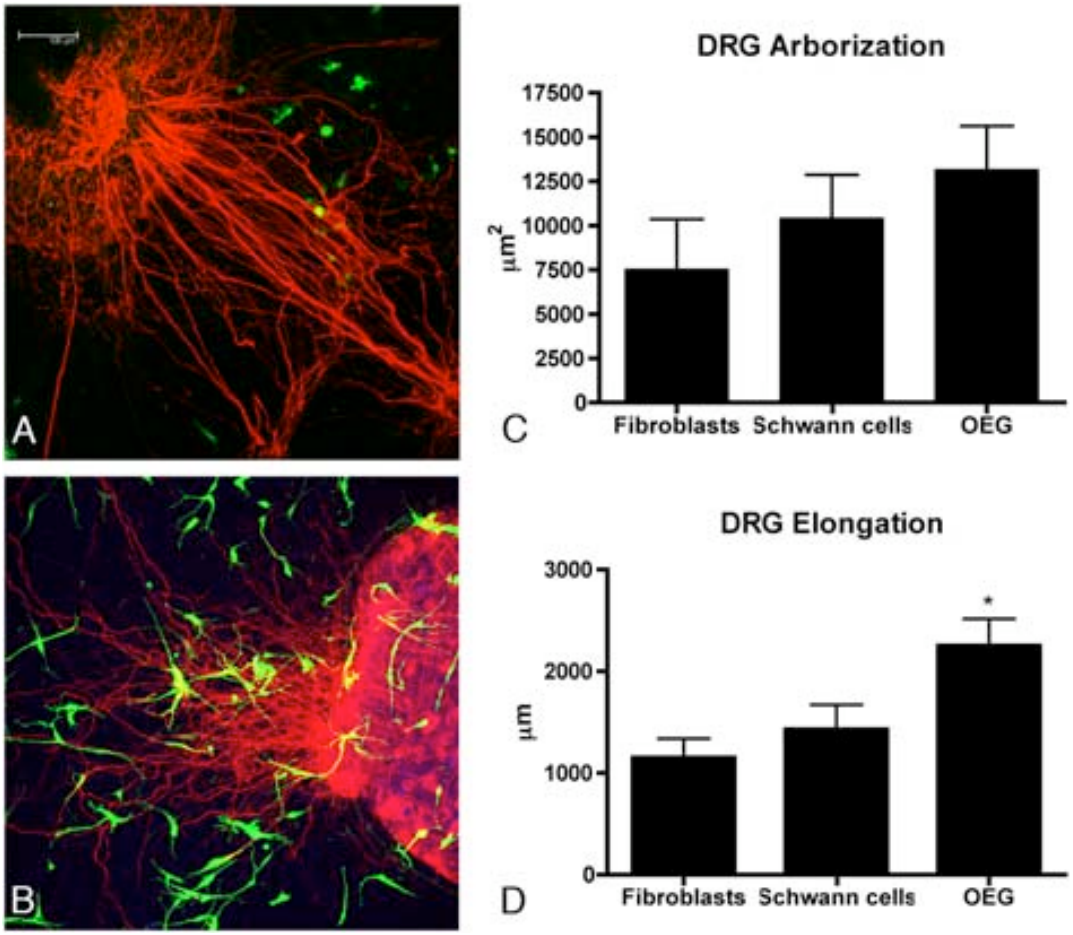

Figure 2. Representative pictures of DRG neuron outgrowth when co-cultured with SCs (**A**) and OECs (**B**). In both situations neurites grow into the matrix and no cluster formations are present. RT97 in red, GFP in green and DAPI in blue. Bar = 100 µm. Graphs indicate neurite arborization (**C**) and elongation (**D**) when fibroblasts, SCs and OECs are added into the matrix. (*$p<0.05$).

HSPG interaction in OEC boundary formation

Immunostaining for GFAP in the co-cultures of spinal cord slice and OEC demonstrate that astrocytes play a role in boundary formations. GFAP positive cells and negative for GFP were found within the boundary formation (Figure 3), where also SC were present. Moreover, the OEC that formed the boundaries had a reactive morphology and were strongly immunostained by anti-HSPG antibody (Figure 3).

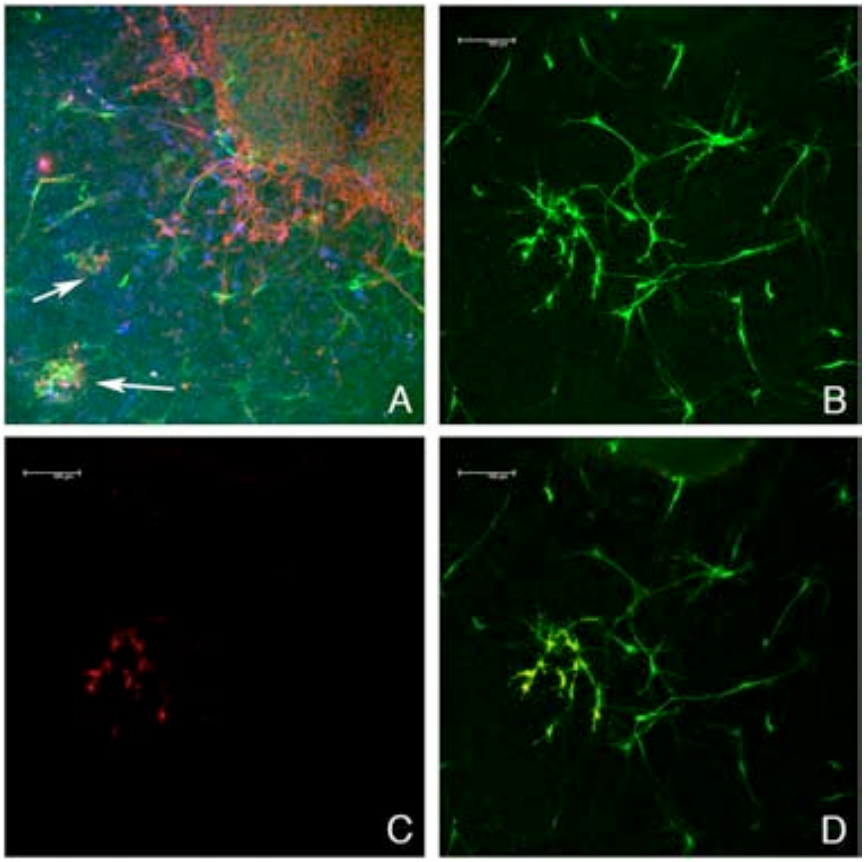

Figure 3. (**A**) spinal cord organotypic slice co-cultured with $10x10^3$ OECs. Arrows indicate cluster formations. OEC were previously labeled with LV-GFP. Neurites are not elongating into the collagen matrix. GFAP in red, GFP in green, NF200 in blue. (**B**) rapresentative picture of a cell cluster. (**C**) staining for HSPG strongly marks cells formating the cluster. (**D**) merge. GFP in green, HSPG in red. Bars = 100 μm.

Conditioned medium assay

Conditioned media were collected from OEC and SC confluent cultures. The amount of FGF-2 present in the medium was quantified by ELISA. No significant

differences were found between both media, which contained an amount of FGF-2 around 50 pg/ml.

As Schwann cells are already present in both *in vitro* models, due to the root remains present in the tissue explants, we only added the OEC conditioned medium to the co-culture. In fact, previous studies in our laboratory demonstrated that Schwann cells present in the tissue cultures migrated into the collagen matrix, giving structural support to the neurites. The OEC conditioned medium did not increase neurite outgrowth (data not shown), that followed the normal pattern without boundary formations observed (Figure 4).

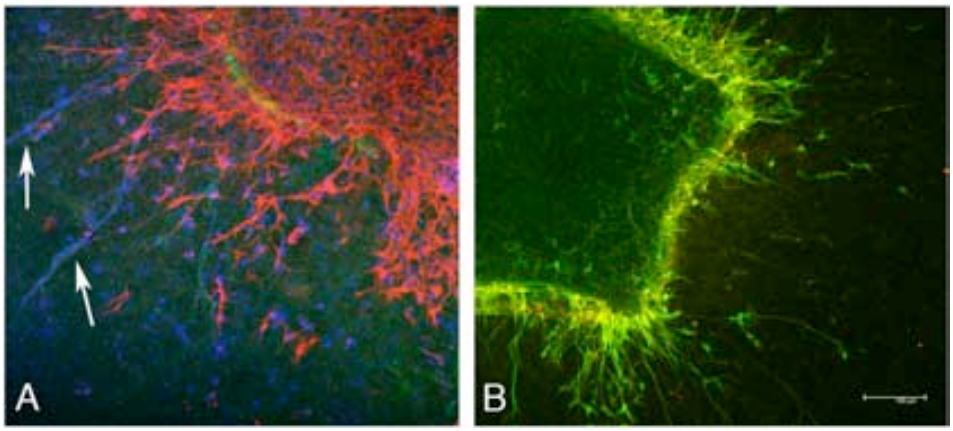

Figure 4. Spinal cord organotypic slices after 4 DIV cultured with the addition of OEC conditioned medium into the collagen matrix and the culture medium. (**A**) Arrows show neurites elongation into the matrix. Astrocytes and Schwann cells are migrating into the collagen matrix, and no boundary formations are present. GFAP in red, p75 in green, NF200 in blue. (**B**) Immunostaining for GFAP in green and HSPG in red, low expresion of HSPG can be seen into the culture. Bar = 100 μm.

FGFR-1 role in OEC boundary formation

To assess FGFR-1 role in OEC and astrocytes boundary formation in the spinal cord culture model, we added PD 173074, a specific inhibitor of FGFR-1 to the collagen matrix. When the inhibitor was applied, no boundary formations were observed and the GFAP reactivity was reduced in the spinal cord. Interestingly, motoneurons were able to extend neurites into the collagen matrix, with values of elongation and arborization similar to the ones seen in control conditions (Figure 4).

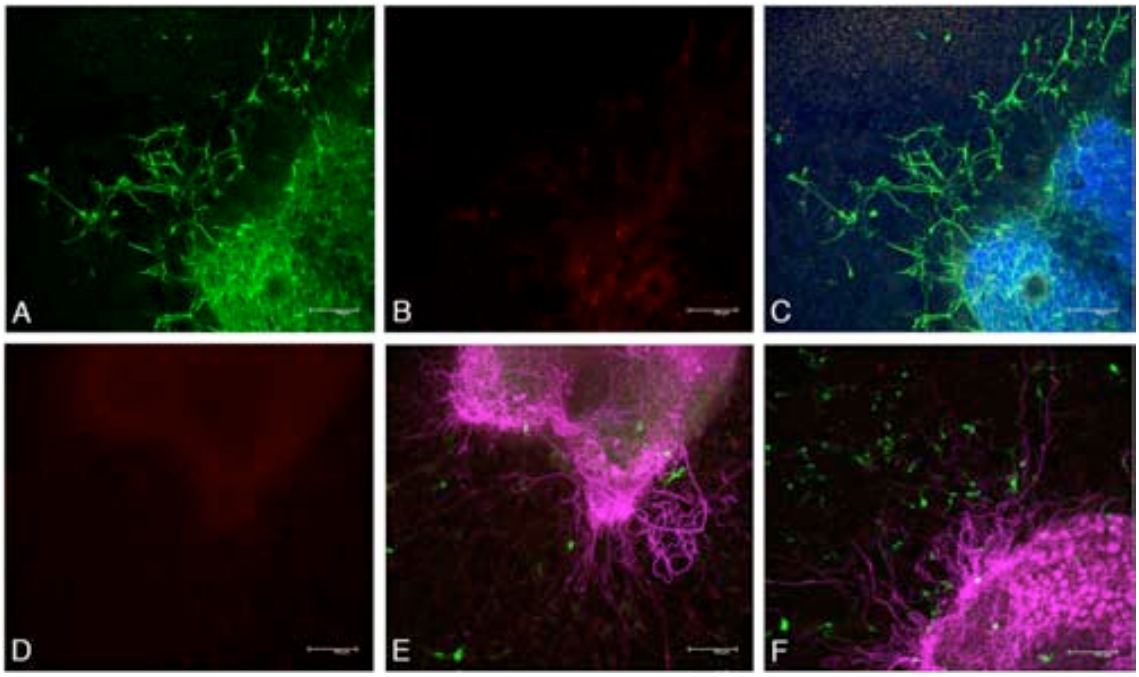

Figure 5. Motoneurons co-cultured with OEC after the addition of PD 173074, a FGFR-1 specific inhibitor. No cluster formations are present (**A**). HSPG immunoreactivity is reduced (**B**). GFAP in green, HSPG in red. (**C**) Merge, DAPI neuronal marker in blue. (**D**) FGFR-1 reduced immunoreactivity after PD 173074 treatment. (**E**) Motoneuron neuritogenesis after PD 173074 addition in spinal cord and OEC co-culture. After FGFR-1 inhibition, motor neurites are able to grow into the matrix. (**F**) FGFR-1 inhibition is not affecting DRG neurite elongation. HSPG in red, GFP in green and RT97 in purple. Bars = 100 μm.

Discussion

SC and OEC are considered promising targets for cell transplant to repair the injured central or peripheral nervous system, since they are able to support axonal regeneration and neuronal survival, thanks to their ability to provide trophic and tropic support to axotomized neurons (Wewetzer *et al.,* 2002; Li *et al.,* 2012). Both these regeneration-promoting glial cells share many phenotypical characteristics but other remarkable differences. Their capabilities for the repair of injuries in the central nervous system have been compared in studies focused on spinal cord injuries (Lakatos *et al.,* 2000; García-Alías *et al.,* 2004; Pearse *et al.,* 2007), but little is known about their intrinsic differences for interaction with axons in the peripheral nervous system. When comparing the effects of these two types of glial cells on neurite outgrowth, we observed that OEC significantly enhanced sensory outgrowth in DRG explants, whereas the effects of SC were less

marked, thus suggesting that OEC might have a higher capability to sustain regeneration of sensory axons. In contrast, when OEC were added into the collagen matrix embedding spinal cord slices, the normal outgrowth of motor neurites observed in control cultures was significantly reduced. Addition of SC, on the contrary, greatly increased the elongation of motoneurons axons.

The differential effect of OEC to promote sensory but inhibit motor outgrowth can be related to the type of explant used more than to the type of axons. DRG contain the somas of primary sensory neurons and satellite cells, and Schwann cells from dorsal roots. Satellite cells are considered SC-like, and thus, peripheral glia. In contrast, in the spinal cord slices, besides some SC from the ventral roots, motoneuron somas are accompanied with central glia: astrocytes, microglia and oligodendrocytes. Therefore, in a co-culture of cells and spinal cord slices we are not just evaluating the effects of the exogenous implanted cells on neurite growth but also how these cells interact with the endogenous glia of the central nervous system. This fact may largely explain why OEC do not have the same trophic capabilities shown in DRG explants when added to the spinal cord slices. In contrast, SC maintained the ability to sustain neurite elongation in spinal cord slices. In fact, despite both glial cells share several properties, their behavior diverges depending on the environment (Li *et al.,* 2012). The differences seem mainly due to extracellular matrix receptors and cell adhesion molecules that allow their interactions with the microambiance. *In vivo* and *in vitro* studies demonstrated that SC and OEC maintain different interactions with astrocytes (Lakatos *et al.,* 2000; Li *et al.,* 2012; Santos-Silva *et al.,* 2007).

When OEC are added to the culture, in presence of SC and astrocytes, they appear to have a reactive response, that depends on the interaction of FGF-2 and HSPG, acting through FGFR-1 (Santos-Silva *et al.,* 2007). In our study we observed that OEC also cluster in contact with astrocytes and SC, in contrast to the behavior seen in DRG explants without astrocytes. In fact, motoneurons elongate their neurites around the spinal cord and not into the collagen matrix, as normally occurring (Allodi *et al.,* 2011a), suggesting that the clustering of OEC constitute a chemorepulsive barrier for axonal regeneration. We show that the clusters are enriched in HSPG, well-know inhibitors of regeneration (Fernaud-Espinosa *et al.,*

1998). The boundary clustering and the inability of OEC to sustain motor outgrowth is not related to secreted factors by the OECs themselves, since the conditioned medium had no inhibitory effects on motoneuron outgrowth. This fact suggests that clustering formation is due to the interaction between OEC, SC and central glial cells. In fact, blockade of FGFR-1, reduces the formation of these clusters, and allows motoneuron neurite growth into the matrix. These results are in agreement with the findings of Santos-Silva et al (2007), who suggested that FGFR-1 has an important role in OEC boundary formations in presence of Schwann cells and astrocytes, influencing OEC migration in the central nervous system.

On the other hand, we observed that OEC enhanced the outgrowth of sensory neurites from the DRG explants to a higher degree than SC. These differences may suggest that these two glial populations may be good candidates to improve regeneration of different types of nerve fibers.

General Discussion

Summary of this thesis

Peripheral nerve regeneration is a challenging field nowadays; several studies revealed that the enhancement or the inhibition of certain cell pathways can modulate the regenerative capabilities of neurons after injury (Udina et *al.,* 2010; Christie *et al.,* 2010). After axotomy, positive and negative signals coming from the injury site turn neurons to a pro-regenerative state, activating the regenerative machinery. Expression of regeneration associated genes produces downstream changes affecting mainly cytoskeleton organization, small GTPases activation, tyrosin kinase and cell adhesion molecule receptor recruitment at the front of regeneration (van Kesteren *et al.,* 2011). However, neuronal activation needs to concert its activity with the extracellular environment in order to get a positive regenerative outcome.

In fact, both chemoattractive and chemorepulsive molecules can be found at the injury site, but, thanks to the degenerative response that occurs in the peripheral nerve after lesion, the environment becomes permissive and favours regeneration, in contrast to what happens in the CNS (Huebner and Strittmatter, 2009). Nevertheless, the outcome of peripheral nerve regeneration is far from completely positive, as regenerating axons can undergo misrouting along the distal nerve pathways and branching points, and therefore reinnervate mismatching sensory and motor target organs.

Thus, the aim of this thesis was to study the specific effect of certain trophic factors in enhancing motor or sensory regeneration, in order to chemoattract axons and, maybe, guide them again to the appropriate type of end organs. Due to the large amount of existing trophic factors, we decided first to make a thourough screening assay of their effects *in vitro*. For this reason, we set up an *in vitro* model that allowed the comparison of motor and sensory neuron pattern of regeneration. DRG explants and spinal cord organotypic slices were embedded in a 3D collagen matrix and cultured for a few days to look at the early stages of axonal regeneration. DRG have been frequently used as a regenerative *in vitro* model, both as explants and as dissociated cells, due to their capability of elongating neurites in culture, also when the samples are obtained from postnatal and adult animals (Owen and Egerton, 2012). On the other hand, postnatal

motoneurons are more difficult to culture. Embryonic motoneurons are mainly used for this aim, but their survival is strongly dependent on trophic support and cAMP levels (Montoya-Gacharna *et al.,* 2012). For this reason, we modified the spinal cord organotypic culture to obtain an *in vitro* model allowing to study neurite growth in postnatal motoneurons. The collagen matrix used represents a permissive environment for both sensory and motor neurite growth, and it is easy to manipulate by mixing trophic factors or inhibiting peptides during collagen gel formation (Tucker *et al.,* 1996). Moreover, we could demonstrate that their addition into the matrix actually influenced neuronal regeneration in a reproducible way.

In spite that spontaneous sprouting of motoneurons into the collagen matrix occurred, we observed that not all the motoneurons present in the slices presented the same intrinsic regenerative capability. In fact, two main subpopulations were detected: one pro-regenerative and the other quiescent. The pro-regenerative motoneurons expressed typical regenerative markers (phosphorylated heavy chain neurofilament, GAP43, integrin alpha7), which were not present in the other subpopulation. Nonetheless,cell death typical markers were not found in the quiescent motoneurons, indicating a comparable survival of both groups.

Several neurotrophic factors were tested in the *in vitro* models: NGF, BDNF, NT-3, GDNF, CNTF, FGF-2, IGF-1, PTN and IL-6. Some of them were found to enhance neurite outgrowth. We were able to corroborate the specific effect of NGF on sensory neurons (Levi-Montalcini, 1987) and of BDNF on motoneurons. Moreover, we showed non-specific but strong promotion of neuritogenesis exerted by GDNF in both kind of neurons. On the other hand, we demonstrated the selective effect of FGF-2 (low molecular weight isoform – 18 kDa) on motoneuron regeneration, as already proposed by previous studies (Haastert *et al.,* 2006). mRNA analysis performed from motor and sensory nerve branches during degeneration after injury showed that the trophic factors which were found to promote regeneration *in vitro* were also upregulated after injury, with the only exception of NGF, as already known in the literature (Gold, 1997).

Then, we investigated the mechanisms through which FGF-2 was promoting regeneration of motor axons. Its action was triggered by the interaction maintained between its main receptor present into the nervous system (FGFR-1) and PSA-NCAM, at least during the early stages of regeneration. Further assays showed that its specific role is extremely dependent on cell adhesion molecules interactions. In fact, when alpha7B integrin subunit was inhibited, the action of FGF-2 was significantly decreased. This is probably due to the important role maintained by alpha7B during neurite stabilization.

Because of the selective role of FGF-2 in promoting motoneuron regeneration, we decided to produce a lentiviral vector to over-express this trophic factor, which might be used also *in vivo* during long periods of time. A LV vector was chosen as our target cells for infection were Schwann cells and fibroblasts, and the LV vector is known to positively transduce both kind of cells in the rat sciatic nerve with high efficiency (Eggers *et al.,* 2008; Tannemaat *et al.,* 2008). The *in vitro* and *in vivo* characterization of the LV-FGF2 demonstrated that the virus was functional; in fact, by ELISA assay we found an increased amount of the protein with respect to control and sham conditions. We tested the biological activity of the FGF-2 produced by infected Schwann cells in our *in vitro* models, and observed that it reproduced the positive results obtained with the human recombinant protein on motoneuron neuritogenesis. The *in vitro* observations and the increased FGF-2 amount found *in vivo* after the infection suggest that LV-FGF2 can be a promising tool to enhance FGF-2 expression *in vivo*, in order to promote regeneration of motor axons after peripheral nerve injury.

A study of the specific effect of Schwann cells and olfactory ensheathing cells on sensory and motor neuron regeneration was also included in the study. The aim of this work was to find a target cell for specific regeneration, in order to optimize cell transplantation after peripheral nerve injury. Nowadays, the autologous graft is considered the best repair method after peripheral nerve transection, when direct suture cannot be performed due to the long gap formed by the lesion (Lundborg, 2000). The endoneurial environment created mainly by Schwann cells and fibroblasts present in the graft allows nerve regeneration and axonal pathfinding. Hovewer, artificial guides can mimic the autograft model and

provides a suitable containement that can be filled by transplanted cells previously engineered to over-express molecules able to enhance regeneration. For these reasons, we compared two kind of cells (Schwann cells and OECs) in our *in vitro* model, which are supposed to share several phenotypical and genotypical characteristics (Pollock *et al.,* 1999, Ramon-Cueto and Valverde, 1995, Smith *et al.,* 2001, Wewetzer *et al.,* 2002), and have been found to sustain peripheral nerve regeneration (Verdú *et al.,* 1999, Rodríguez *et al.,* 2000, Udina *et al.,* 2004, Radtke *et al.,* 2011). We observed that Schwann cell and OECs exerted differential effects when co-cultured with DRG explants and spinal cord organotypic slices. Schwann cells were found to significanlty improve motoneuron regeneration, whereas their effect was not significant on sensory neuron outgrowth. On the other hand, DRG neuritogenesis was enhanced by OECs. Surprisingly, OECs were found to play a chemo-repellent role in co-cultures with spinal cord slices, probably due to the maintained cytoarchitecture of the spinal cord. In fact, the presence of OECs, astrocytes and Schwann cells in the same culture induced cell clustering in front of the ventral horn, impeding neurite elongation. These boundary formations are known to be dependent on the FGFR1-heparan sulfate protoglican-FGF2 complex (Santos-Silvas *et al.,* 2007). Therefore, the inhibition of FGFR-1 diminished such cell boundary formations and restores normal motoneuron outgrowth into the collagen matrix. These observations indicates how important are the environmental conditions during regeneration, and how cell interactions can revert a permessive substrate.

In chapters 2 and 4 we demonstrated how the same receptor, FGFR-1, can promote or inhibit motoneuron regeneration, depending on the interactions maintained with the surrounding environment, further indicating that the regeneration outcome depends on how neurons, glia and the extracellular environment interplay.

From a "specific regeneration" to a "topographic regeneration"

Unfortunately, not all the neurons have the same abilities of spontaneous axonal regeneration. As we observed in our *in vitro* models, there are consistent

differences in the intrinsic growth capability of neuronal populations. Thus, we are able to differenciate three DRG sensory subpopulations and several motoneuron pools, depending on their position into the spinal cord and likely the type of muscle they innervate. Indeed this is not a novel observation; the fact that several neuronal types differ in behavior during degeneration and regeneration is not new (Navarro *et al.,* 1994). Nowadays, it seems clear that further studies are needed in order to understand which transcripition factor activation and their downstream effectors can play a role in these processes. Genetical differences among the subpopulations might exert a "switch" in some kind of neurons that are intrinsically prepared to regenerate. The intrinsic growth capacity of neurons can be manipulated using gene therapy techniques to silence or re-express target genes inhibiting or playing an important role during regeneration, as demostrated by several studies (Abe *et al.,* 2010; Ma *et al.,* 2011).

Studies coming from several areas of research underline differences in genetic machinery, transcription factor activation and interaction with the extracellular environment of motoneuron pools and their implication in development (Dasen *et al.,* 2008) and degeneration (Hendlund *et al.,* 2010). The same event was observed during regeneration both in motor (Franz *et al.,* 2008) and sensory (LeClere *et al.,* 2007) neurons. Thus, accurate analyses of neuronal phenotype and genotype should be done to further investigate how to promote specific reinnervation, depending on the characteristics of neuronal subpopulations.

"Topographic regeneration" should complement the expression of "selective regeneration", as the central aim of our investigations is to re-connect proximal (neurons) and distal (end organs) targets, following a determined topographic order that was created during development and disrupted after lesion (Lago and Navarro, 2006; Badia *et al.,* 2010). Actually, also during development, one of the most critical aspects of the organization of the neuromuscular system is the process through which motoneurons create connections with their muscle targets (Dasen and Jessell, 2009). The Hox transcriptional regulatory network is known to play a fundamental role in motoneuron specification and in topographic connectivity (Dansen *et al.,* 2005). Moreover, Hox (*Hoxc9 and Hoxc10*) gene

expression in the lumbar ventral horn spinal cord was shown to be dependent on FGF-2 gradients during development (Liu *et al.,* 2001). For this reason, regarding our findings of FGF-2 on motoneuron regeneration, it is important to remind that lumbar and sacral segments of the spinal cord were used in the experimental design; the same part of the spinal cord that is dependent on FGF-2 gradient for topographic specification during development. In our opinion this is an example of how regeneration can recapitulate developmental circumstances and use it to favor its own positive outcome.

The three main players – where to act

When referring to "topographic regeneration and reinnervation", we should take into account that three main components are reciprocally interacting: the neuronal subpopulation, the axon that re-create the connection and that is directly in contact with the extracellular environment, and the target organ itself. In the previous paragraph, the neuronal potential differences were considered, while little was said about axon-environment interactions and the role of target organs.

The regenerative machinery triggers cytoskeletal re-organization and causes neuronal sprouting, which lead to growth cone formation and axonal elongation (Bradke *et al.,* 2012). Growth cone formation requires elevated calcium levels, transport of material at the front of regeneration and a complex re-organization of the membrane and cell surface molecules (Bradke *et al.,* 2012). During its protrusion, the growth cone enters in direct contact with the extracellular environment and turns in response to chemoattractive and chemorepulsive cues (Lowery and Van Vactor, 2009). The main substrate for regenerative axons is formed by proliferating Schwann cells, which are highly dividing cells without a proper phenotype (Jessen and Mirsky 2002; Hoke 2006). Trophic factor secretions and extracellular matrix molecule components present on those cells can modulate growth cone response and facilitate nerve regeneration (Jessen and Mirsky, 1999). For this reason, several studies have attempted to get engineered Schwann cells in order to further sustain regeneration (Haastert *et al.,* 2006, Napoli *et al.,* 2011), and improve axonal rewiring.

On the other hand, the absence of the trophic support given by the target organ is one of the main signals that triggers the regenerative machinery. The interruption of retrograde transport from the periphery to the somas, switchs on the neurons, whose axons will sprout and elongate in order to reach their previous connections. However, after severe lesions, even if the regenerating axons reinnervate target organs, these have usually undergone atrophy and are not able to give trophic support as before the lesion (Gordon *et al.,* 2011). Moreover, several weeks after lesion, terminal Schwann cells present at the end organs do not facilitate synaptogenesis anymore, as they undergo differentiation and, after a certain time of denervation, start expressing some inhibiting extracellular matrix molecules as Sema3A (Ma *et al.,* 2011). All these events are strongly marked after chronic denervation (Gordon *et al.,* 2011). For all these reason, it is important to promote faster nerve regeneration, to avoid distal atrophy (Ma *et al.,* 2011).

New therapies – how to act

Several strategies have been set up and ameliorated in the last years to overcome the limiting variables which affect peripheral nerve regeneration. Among all these strategies we consider especially promising cell and gene therapies. After peripheral nerve injury, it is not so important cell replacement but structural and trophic support, to limit neuronal death and promote faster and more accurate axonal regeneration (Radtke *et al.,* 2011). For this reason, cell therapy in the peripheral nervous system has mainly focused on three different cell kinds: Schwann cells (Rodriguez *et al.,* 2000), OECs (Verdú *et al.,* 1999; Guerout *et al.,* 2011) and mesenchymal stem cells (McGrath *et al.,* 2012). In fact, all of them are known to support axonal regeneration both structurally and trophically (Hernandez *et al.,* 2011). As suggested previously, these cells can be used to fill an artificial nerve guide and mimic the autograft situation (Rodriguez *et al.,* 2000; Udina *et al.,* 2004). Despite autografts are still considered the gold standard for nerve repair, a considerable amount of research has been conducted to improve artificial nerve guides (Deumens *et al.,* 2010). Moreover, these guides give the opportunity of adding neurotrophic factors or transplanting cells

previously engineered *in vitro,* in order to increase the focal presence of promising factors to improve regeneration.

While cell therapy can suitably be applied at the injury site in case of nerve injury, gene therapy treatments can be administrated to all the three main players acting during nerve regeneration. Although gene therapy is still raising several doubts related to safety issues (Manson *et al.,* 2011), it is a good strategy to study the role of potentially interesting genes. Those genes can be cloned into different types of vectors, which allow selective cell type transduction, showing the effect of the target gene in an *in vivo* model. This represents a benefit that would be difficult to achieve in the absence of this technique. In our study we used a lentiviral vector because other kind of safer viruses, like the adeno-associated viral (AAV) vectors, are less effective in infecting Schwann cells, even if recent studies have demonstrated the contrary (Homs *et al.,* 2011). Direct injection of the LV vector into the nerve leads to a high expression of the target gene in Schwann cells and fibroblasts (Tannemaat *et al.,* 2007). Unfortunately, LV vectors might cause side effects in transduced cells with the possibility of insertional mutagenesis (Hargrove *et al.,* 2008). On the other hand, AAV vectors are able to transduce neurons (Mason *et al.,* 2010) with a high efficency, and for this reason they can be considered a promising tool to modify gene expression in sensory and motor neurons.

Even when these two novel therapies are still presenting several limits and give rise to ethical issues, we think these are extremelly challenging and their application will improve over time.

Conclusions

CONCLUSIONS

1. The *in vitro* models developed, based on organotypic spinal cord slices and DRG explants from postnatal animals, permit a reliable comparison of motor and sensory neurons regeneration. The easy manipulation of the microenvironmental conditions allows to study the effects on neuritogenesis of specific treatments, by either addition of diffusible molecules or introduction of cells in the collagen matrix.
2. *In vitro* neuronal outgrowth is dependent on the trophic factor support. Specific effects on sensory and motor regeneration were measured after trophic factor addition to the collagen matrix.
    a. On spinal cord cultures, addition of FGF-2, GDNF and BDNF to the collagen matrix increased motor neurite length, whereas the maximal effect on motor neurite branching was detected for FGF-2.
    b. On DRG explants, administration of NGF and GDNF caused the highest increase of neurite length, and significantly enhanced neurite branching of sensory neurons.
    c. When comparing the differential effects of neurotrophic factors, we found that NGF exclusively promoted growth of sensory neurites, whereas FGF-2 and BDNF selectively enhanced motor neurite outgrowth.
3. The neurotrophic factors that promoted enhancing neuritogenesis effects *in vitro* were found to be up-regulated in motor or sensory nerve branches after injury; the most relevant being:
    a. The expression of NGF and NT-3 increased slightly in dorsal roots.
    b. GDNF showed a significant up-regulation in dorsal and ventral roots.
    c. BDNF and FGF-2 showed higher expression in ventral than in dorsal roots.
4. FGF-2 (low molecular weight isoform – 18 kDa) selectively promotes motoneuron regeneration *in vitro*, and its effects are triggered by FGFR-1/PSA-NCAM interactions at least during early phases of regeneration, while alpha7B

integrin subunit plays a key role during neurite stabilization. FGF-2 trophic action seems to be dependent on cell adhesion molecule interaction.

5. Transfection by means of a Lentiviral vector over-expressing FGF-2 (18 kDa isoform) leads to an increased amount of this trophic factor *in vitro* and *in vivo*. Co-culture of Schwann cells infected with LV-FGF2 with DRG explants or spinal cord organotypic slices mimics the effect previously observed with the recombinant protein, with a selective enhancement of motoneuron neuritogenesis.

6. Schwann cells and OECs promote axonal growth in our *in vitro* models, but with a different outcome depending on the environmental conditions. Specific effects were found in co-cultures with sensory and motor neurons, with an inhibitory effect on motoneurons produced by OECs, due to the preserved cytoarchitecture of the spinal cord slice. The presence of astrocytes, Schwann cells and OECs leads to boundary formations which are chemorepulsive for neurites and seem to be caused by interactions between FGFR-1 and HSPGs.

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LIST OF PUBLICATIONS

Peer Reviewed Publications

1. Allodi I, Guzmán-Lenis MS, Hernàndez J, Navarro X, Udina E. (2011) In vitro comparison of motor and sensory neuron outgrowth in a 3D collagen matrix. *Journal of Neuroscience Methods*, 198(1):53-61

2. Allodi I, Udina E, Navarro X. (2012) Specificity of peripheral nerve regeneration: interaction at the axon level. *Progress in Neurobiology* (In press)

Under Revision

1. Allodi I, Casals-Díaz L, González-Pérez F, Navarro X, Udina E. FGF-2 low molecular weight (18 kDa) selectively promotes neuritogenesis of motor neurons in vitro.

2. Allodi I, Torres-Espin A, Hernandez J, Roet K, Navarro X, Udina E. FGFR-1 differentially determines olfactory ensheathing glia and Schwann cells neuritogenesis support in DRG explants and spinal cord organotypic slices.

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