Universitat Autònoma de Barcelona

Functionalizing artificial nerve guides to promote regeneration and recovery after peripheral nerve injuries

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INDEX

	Page
INTRODUCTION	**9**
Peripheral nervous system	11
Extracellular matrix in the peripheral nervous system	13
ECM components	13
Peripheral nerve injuries	16
Clinical relevance of peripheral nerve injuries	17
Classification of peripheral nerve injuries	18
Degeneration and regeneration following peripheral nerve injuries	19
Neuronal response	19
Wallerian degeneration	20
Axonal regeneration	21
ECM role in axonal regeneration	24
Reinnervation of the target organs	25
Limitations of functional recovery after peripheral nerve injuries	25
Surgical repair strategies	27
Chitosan	31
Functionalizing artificial nerve conduits	31
ECM-based strategy for functionalizing artificial nerve guides	32
Other 3D- scaffolds for functionalizing artificial nerve guides	34
Growth factors-based strategy for functionalizing artificial nerve guides	36
Cell therapy-based strategy for functionalizing artificial nerve guides	38
Immunosuppression	42
Conclusions	43
OBJECTIVES	**45**
CHAPTER I	**49**

CHAPTER II **63**

CAHPTER III **75**

CHAPTER IV **95**

DISCUSSION **121**

Summary of results and discussion 123

Role of ECM molecules on selective neuronal regeneration 124

Artificial nerve guides for peripheral nerve regeneration 127

Functionalizing nerve guides with ECM molecules 128

ECM-based scaffolds to support cell transplantation 131

CONCLUSIONS **135**

REFERENCES **139**

ABBREVIATIONS **163**

ACKNOWLEDGEMENTS **167**

ANNEXES **171**

INTRODUCTION

INTRODUCTION

Peripheral nervous system

The peripheral nervous system (PNS) is the part of the nervous system outside the brain and the spinal cord, whose main function is to connect the central nervous system (CNS) with the muscles, organs and skin to allow for complex movements and behaviors. The human PNS consists of 12 pairs of cranial nerves (that establish connections in the head and upper body), 31 pairs of spinal nerves (establish connections in the head and the rest of the body) together with their ganglia, and the peripheral nerves. Peripheral nerves are composed of motor axons of lower motoneurons (whose soma is located in the ventral horn of the grey matter of the spinal cord and in the motor nuclei of cranial nerves in the brainstem), sensory axons from primary sensory neurons of the dorsal root ganglia or sensory nuclei of cranial nerves, and autonomic axons, that innervate their target organs: skeletal muscles, sensory receptors or visceral muscles and glands, respectively.

Primary sensory neurons transduce different stimuli into electrical signals that will be carried from the periphery to the CNS. A wide range of cutaneous and subcutaneous receptors specifically respond to tactile, nociceptive, thermal and itching stimuli. Proprioceptors located in the muscles, joints and other deeper structures control the length, tension and position of the muscles. Specialized tactile and proprioceptive neurons have large axons and thick myelin sheaths (Aα and Aβ fibers), whereas protopatic tactile, thermoceptive and nociceptive neurons are of small size (Aδ and C-unmyelinated fibers). Whereas neurons innervating cutaneous receptors project mainly to the skin via cutaneous nerves, neurons than carry the sensory information of the muscle (as proprioceptive neurons) project to the muscles or tendons via muscular nerves.

Lower motoneurons innervating skeletal muscles are also called alfa motoneurons, due to the size of their axons (Aα). There are also smaller motoneurons, γ motoneurons that innervate the intrafusal fibers of the muscle spindle, and play an important role in adjusting the sensitivity of these sensory organs.

Peripheral axons are always coated by Schwann cells. Schwann cells myelinate thick axons whereas the smaller axons are unmyelinated (Jessen & Mirsky, 2005). Myelin acts as an insulator of high resistance and low capacitance, so the ion current

moves from node to node (saltatory conduction) increasing the conduction speed, where the nerve impulse jumps between the spaces between the nodes of Ranvier. Conduction velocity in the unmyelinated axons is lower due to the absence of myelin and the impulse travels down the whole unmyelinated axon.

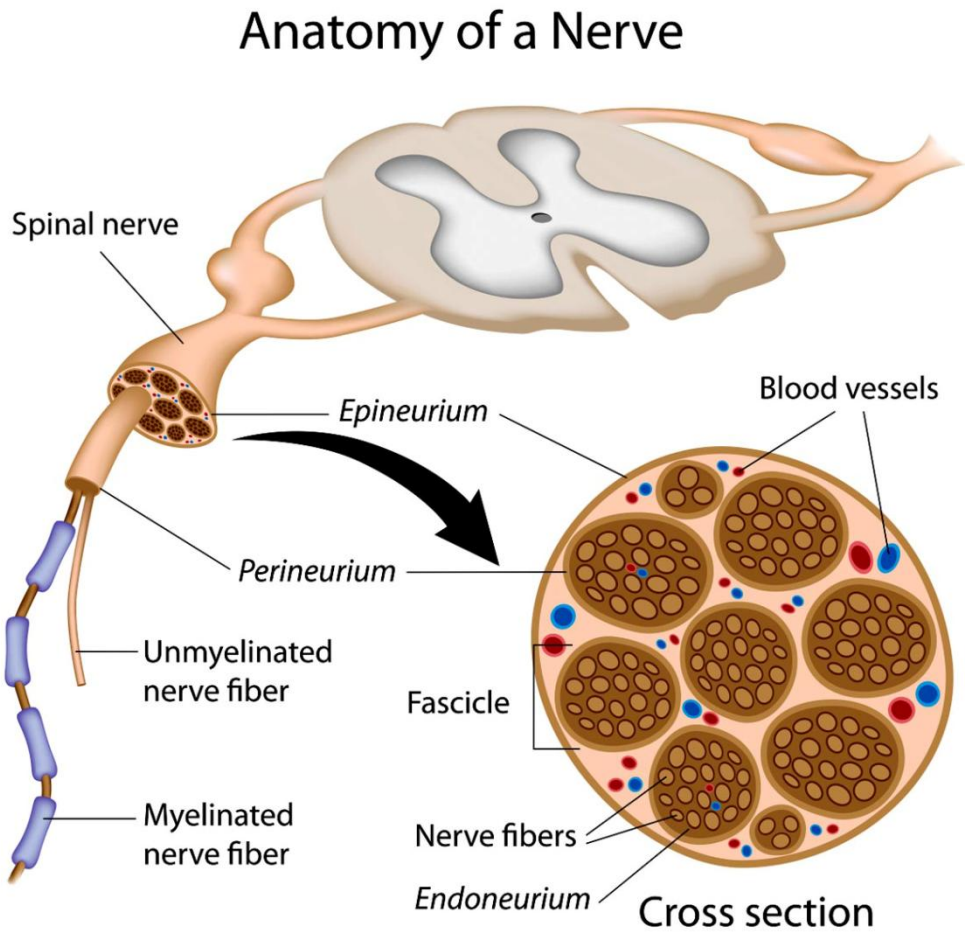

Figure 1: Anatomy of the peripheral nerve. Peripheral nerves are formed by bundles of axons from lower motoneurons, sensory neurons of the dorsal root ganglia and from postganglionar autonomic neurons. The epineurium is the outer connective tissue layer that encloses the whole peripheral nerve. Inside the epineurium there is fat tissue, arteries and veins. Perineurium is the connective tissue layer that encloses the nerve fibers organized into nerve fascicles. Each nerve fiber is protected by the endoneurium. The endoneurial tubes contain the axons and the Schwann cells that support these axons. In the case of myelinated axons, these Schwann cells are the responsible to form the myelin sheaths.

Peripheral nerves consist of bundles of axons each one surrounded by the endoneurium, a layer of delicate connective tissue which contains the endoneurial fluid (equivalent to the cerebro-spinal fluid in the CNS). The endoneurium is composed of collagen fibrils which run longitudinally along the nerve fibers, between the basal lamina of the Schwann cells and the perineurium, and serves to support capillary blood

vessels. The perineurium is the connective tissue around a nerve bundle or fascicle. It is denser than the endoneurium and is composed of several layers of flattened fibroblasts surrounded by basal lamina. The epineurium is the outermost covering of a peripheral nerve, surrounding and connecting the nerve bundles in a single beam. It is composed by dense connective tissue, mainly of longitudinal collagen fibers, fibroblasts, mast cells and fat. Collagen fibers prevent the stretch of the nerves, avoiding injuries during movement of the body parts. The blood vessels supplying the nerves are found in the epineurium. They branch and penetrate to the perineurium. However, endoneurium has poor vascularization, and nutrition of the axon mainly depends on the exchange of substances by diffusion from the perineurial capillaries.

Extracellular matrix in the peripheral nervous system

(Extracted from Gonzalez-Perez, et al. 2013).

The ECM is a physiological integrative matrix of complex molecular nature, where axons and supportive cells are immersed. The ECM is a three-dimensional network arranged in the intercellular space, which includes proteins and carbohydrates synthesized and secreted by the cells. It is present in the interstitial spaces of all tissues, playing important roles in cell migration, proliferation and differentiation, and providing structural support and regulating intercellular communications. It contributes to mechanical tissue properties, allows the cells to form tissues, serving to cell communication, and forms paths where cells can move. In the peripheral nerve, the ECM is found in the basal lamina of Schwann cells and the endoneurium.

The basal lamina produced by the Schwann cells, may be considered as a layer of the ECM, mainly composed of collagen type IV, laminin, fibronectin and nidogens (Bannerman et al., 1986; Baron-Van Evercooren et al., 1986; Bryan et al., 2012). After injury, besides the degenerative process behold in the distal stump, basal lamina tubes remain as scaffolds where proliferative Schwann cells align forming the bands of Bünger.

ECM components

The ECM is composed of a complex network of secreted proteins, glycoproteins, proteoglycans and non-proteoglycan polysaccharides. The first group of components, the glycoproteins, can be classified into collagen and non-collagenous molecules.

- Collagens are a superfamily of trimeric molecules composed of three identical triple helical α chains that define tissue structures (Brown & Phillips, 2007; Gordon & Hahn, 2011). Up to 26 different types of collagens have been described, that are divided into different groups according to the structures they form. The main subfamilies are fibril-forming collagens (type I, II, III, V, XI), collagens banded-fibrils associated (IX, XVI, XIX, XXI, XXII), networking collagens (IV, VI, VIII, X), transmembranous collagens (XIII, XXIII, XV), endostatin precursor collagens (XV, XVII), and other collagens. The collagen types most relevant for peripheral nerve regeneration are described later. However, it is interesting to highlight the importance of those related to fibril formation (collagen type I) and the basement membrane (collagen type IV).

Among the ECM non-collagenous molecules of glycoprotein origin, the most important are laminins and fibronectin.

- Laminins: are the major proteins of the ECM, participating in cell differentiation, migration, and adhesion activities. They are an active part of the natural scaffolding which structure the tissues. They are mainly found in the basal lamina. Laminins are heterodimers of α, β and γ chains, and 18 different types have been described to date (Durbeej, 2010). The trimers are named according to the composition of the different chain types, but usually it is the α chain that identifies the isoform. Secreted by Schwann cells, laminin-2 (α2, β1, γ1) and laminin-8 (α4, β1, γ1) are found in the peripheral nerves (Wallquist et al., 2002), whereas laminin-10 (α5, β1, γ1) can be detected in sensory end organs (Caissie et al., 2006). Laminin is the adhesive component that gives the regenerative promoting capability to basal lamina scaffolds after nerve injury (Wang et al., 1992) and has been shown to promote neuritogenesis *in vitro* (Agius & Cochard, 1998).
- Fibronectin is the other major component of non-collagen glycoproteins of the ECM (Singh et al., 2010). It forms a fibrillar matrix similar to collagen and mediates cell binding. Fibronectin is a dimer existing in different isoforms because of alternative splicing generation. Totally, 12

isoforms for mice and 20 for humans have been described. At first, soluble fibronectin is produced by hepatocytes, being found in the blood plasma. The insoluble form is incorporated into the membrane of many cells. In the nervous system, it is synthesized and secreted by Schwann cells and fibroblasts ((Baron-Van Evercooren et al., 1986; Chernousov & Carey, 2000). Te important relations that it maintains with collagen type IV and laminins and with fibril formation make fibronectin an interesting candidate for scaffolding in nerve regeneration (Brown & Phillips, 2007).

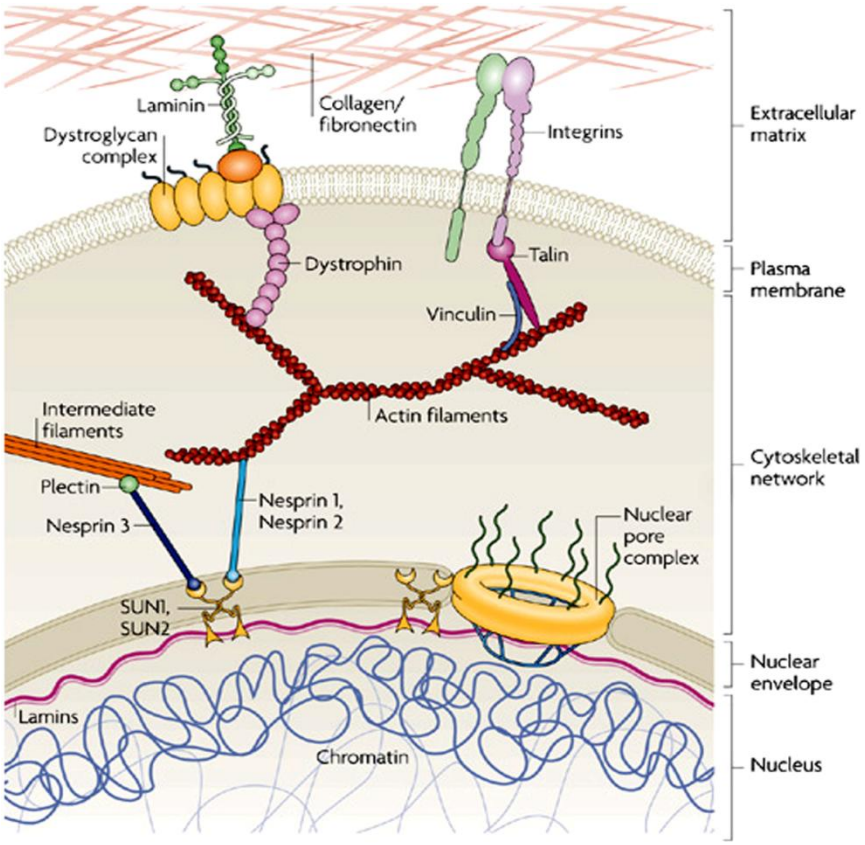

Figure 2: Extracellular matrix is a complex molecular network that includes laminins, proteoglycans, fibronectin and collagens. They can also bind to other signaling molecules in the extracellular milieu and guide the growth of regenerating axons. (Extracted from Jaalouk & Lammerding, *Nature Reviews Molecular Cell Biology*, 2009).

There are other non-collagen glycoprotein molecules of the ECM although they are probably no related to axonal regeneration after nerve injury. Nidogen-1 (also called entactin) forms non-covalent unions with laminin and collagen type IV and may play its

role as a pro-migratory factor for adult Schwann cells (Lee et al., 2007). On the other hand, vitronectin binds to collagen and glycosaminoglycans (GAGs) (heparin), acting as a regulatory molecule controlling cell adhesion (Schvartz et al., 1999).

The GAGs are carbohydrate polymers that are covalently bound to glycoproteins in their native state, forming proteoglycans. These molecules include heparin, keratin, chondroitin, dermatan, and their respective sulfates (Rutka et al., 1988). Proteoglycans are formed by a GAG linked to a hydroxyl group of certain amino acids (serine and threonine) or a core molecule which is also linked to hyaluronic acid. Proteoglycans like chondroitin sulfate proteoglycans (CSPGs) create an inhibitory environment by neutralizing the growth-promoting activities of other ECM elements (McKeon et al., 1995; Muir et al., 1989). CSPG has been found in the peripheral nerve where it may inhibit the growth-promoting activity of endoneurial laminin (Zuo et al., 1998a; Zuo et al., 1998b).

Fibrin is not associated with the mature tissue structure but it is a key factor in the repair strategy of the ECM components. It will form a provisional mesh after damage that will be later replaced by the mature components of the ECM secreted by invading cells. In the presence of thrombin, fibrinogen polymerizes into fibrin to form a dense meshwork of fibers (Brown & Phillips, 2007; McKee et al., 1970). In fact, formation of a fibrin cable between the two stumps when a cap nerve is repaired by a tube is needed to guarantee successful axonal regeneration.

Peripheral nerve injuries

Peripheral nerve injuries (PNIs) often cause structural alterations and loss of motor, sensory and autonomic functions, acute or progressive, of the areas innervated by the injured nerve fibers (Seddon et al., 1943). These injuries cause deficits of varying degrees depending on the severity of the lesion, which is usually disabling for the patient.

Although peripheral axons are able to regenerate when the circumstances and the environment allow it to happen, functional recovery is not guaranteed (Allodi et al., 2012). The severity of the lesion would determine the final recovery grade.

After the lesion, neurons that survive suffer phenotypic changes addressed to promote regeneration of their injured axons, whereas the distal nerve undergoes Wallerian degeneration, to clear from myelin and cellular debris the distal path and to

create a permissive environment for regeneration. Eventually, regenerating axons will reinnervate the target organs and function can be recovered. Complementary to the process of regeneration, collateral sprouting of intact axons in closer areas can cover denervated areas. Structural and functional reorganization of neural circuits can also compensate part of the functional loss. However, all these mechanisms are limited and not always will translate on a correct functional recovery. It should not be forgotten that not only regeneration of injured axons but also an adequate reinnervation of the target organs is necessary for a correct functional recovery.

Clinical relevance of peripheral nerve injuries

PNI is a major clinical and public health challenge. Worldwide, more than one million people are affected by PNIs. In the United States of America (USA), about 20 million Americans suffer from peripheral nerve injury, caused by trauma and medical disorders (Lundborg, 2003) resulting in approximately $150 billion spent in annual health-care (Taylor et al., 2008). Additionally, in Europe, more than 300,000 PNIs are reported annually (Ciardelli & Chiono, 2006). Severe injuries have devastating effects on patients' quality of life. Typical symptoms are sensory and motor function deficits that can result in complete paralysis and anesthesia of the affected limb, and development of neuropathic pain (Siemionow & Brzezicki, 2009).

Injuries to peripheral nerves, plexuses and roots represent 5% of patients seen in civilian trauma centers (Robinson, 2000), caused due to motor vehicle accidents, falls, domestic violence, sport activities, etc. (Ciaramitaro et al., 2010; Fox & Mackinnon, 2011). Additionally, some nerve damages are due to carpal tunnel syndrome or secondary to diabetes (Aboonq, 2015; Vital et al., 1983). The clinical significance, prognosis and treatment of PNIs depend on the site and extent of the injury. The majority of PNIs occur in the upper limb.

After the injury, there is a loss of motor, sensory and autonomic function of the denervated territory. Damage of motor axons leads to paralysis or paresis (weakness) of the affected muscles; accompanied with loss of muscle tone, atrophy, fibrillations and fasciculations. Besides the loss of sensory perception due to affectation of primary sensory neurons, patients also can refer positive symptoms, as hyperalgesia, allodyinia and paresthesia. On amputees, syndrome of phantom limb is common and can refer to pain on the amputated zone.

Classification of peripheral nerve injuries

The insults that can damage a peripheral nerve are very diverse (mechanical, thermal, biochemical, ischemic stresses, etc.). PNIs can be classified according to the morphology of the lesion, their therapeutic requirements and their prognosis (Sunderland, 1951) in five different injury grades:

- Grade 1: small alterations in myelin without disruption of the axon continuity. There is a temporary loss of motor and sensory function due to local blockage of the nerve conduction, usually by compression. This grade of lesion results in temporary damage of the myelin sheath but leaves the axon intact. Thus, Wallerian degeneration does not occur. It is the less severe lesion. Also corresponds to the *neurapraxia* on Seddon's classification (Seddon, 1942).
- Grade 2: The axon continuity is disrupted, but the endoneurium remains intact. There is preservation of endoneurial tubes and Schwann cell basal lamina. Wallerian degeneration occurs distally to the lesion. Complete recovery is expected over months. Also corresponds to the *axonotmesis* on Seddon's classification (Seddon, 1942).
- Grade 3: The axon and endoneurium continuity is disrupted. The perineural tissue is maintained. Motor and sensory recovery is expected from 60% to 80% over months.
- Grade 4: axons, endoneurium and perineurium are disrupted. The epineural tissue is maintained.
- Grade 5: Complete section of the nerve. There is a total disruption of the connective tissue; nerve stumps suffer a physical retraction. In this case, no recovery is expected without surgical repair. It corresponds to the *neurotmesis* on Seddon's classification (Seddon, 1942).

Prognosis is good in PNIs when the endoneurium remains intact. However, disruption of this structure results in the loss of the established pathways for the regenerating axons to their target organs, and in these situations functional recovery is compromised. From the third grade of lesion, surgical intervention is mandatory. Nerve fibers of transected nerves may regenerate spontaneously, but regeneration is limited by the length of the gap between the two stumps, the formation of a neuroma and the scar tissue (Grinsell & Keating, 2014).

Table 1: Nerve injury classification in increasing severity.

Sunderland	Seddon	Characteristics
Grade 1	*Neurapraxia*	Localized damage of myelin
Grade 2	*Axonotmesis*	Damage to axons but endoneurium remains intact.
Grade 3	*Axonotmesis*	Damage to axons and endoneurium
Grade 4	*Axonotmesis*	Damage to axons, endo- and perineurium
Grade 5	*Neurotmesis*	Complete damage to all elements including epineurium

Degeneration and regeneration following peripheral nerve injuries

After PNIs, axons distal to the site of lesion are disconnected from the neuronal body and degenerate. The proximal part of the axon that remains attached to the soma has the capacity of regenerate if it finds a suitable environment that supports this process. The functional significance of regeneration is to replace the distal segment loss during degeneration, allowing reinnervation of target organs and the restitution of lost functions. The main events occurring after PNIs are explained below.

Neuronal response

Neurons have an intrinsic growth capacity during the embryonic stage which is repressed during the adult transition to permit a suitable synaptic development (Allodi et al., 2012). However, after axotomy, the success of nerve regeneration depends primarily on the capacity of the injured neurons to survive, and to switch from a neurotransmitter to a regenerative phenotype. Neurons will express genes that encode for transcription factors which regulate other genes involved in cell survival and neurite growth (Navarro et al,, 2007).

The neuronal retrograde reaction represents the metabolic changes which are necessary for regeneration (Grinsell & Keating, 2014). The signals responsible for the initiation and maintenance of the proregenerative fate of axotomized neurons are finely orchestrated. At the lesion site there is a rapid influx of extracellular calcium and sodium ions to the injured axoplasm which causes an electrical response, inducing a

high frequency burst of action potentials that propagate retrogradely (Lunn et al., 1990). Then, the neuronal body will suffer from chromatolysis with dissolution of Nissl bodies. The injury also disrupts retrograde transport of signals from target organs, acting as a negative signaling for the soma. In parallel, at the site of injury, some proteins undergo post-translational modifications and through retrograde transport, that involves local synthesis of carrier proteins (i.e. vimentin) (Hanz et al., 2003). These positive signals will also reach the soma.

Wallerian degeneration

Following injury of an axon, the distal part is separated from the cell body and eventually it will undergo Wallerian degeneration, a well characterized molecular and cellular event to clear the debris of the distal segment (Zochodne, 2012), thus creating a permissive environment that will allow axons to re-grow back to their targets. process that requires guidance signals different from those originated during developmental stages (Dudanova & Klein, 2013). Wallerian degeneration initiates about 24-48 hours after nerve injury and can last for 1-2 weeks following a proximo-distal progression. The degenerative end products are eliminated by the cooperative action of SCs and infiltrating macrophages. SCs dedifferentiate to a progenitor-like state and divide in their basal lamina (Cattin et al., 2015). These cells down regulate expression of myelin proteins (i.e myelin basic protein or myelin protein zero) and enhance neurotrophic factor and GAP-43 production. Denervated SCs are able to phagocyte myelin debris to some extent; however, recruitment of macrophages is necessary to complete the phagocytosis of myelin and axonal debris. This clearance is important, since myelin debris can contain neurite outgrowth inhibitors (i.e. myelin-associated glycoproteins). Resident macrophages also produce cytokines and chemokines that attract blood-derived monocytes (Dubový, 2011). From 2 to 3 days after injury there is an important infiltration of macrophages into the degenerating nerve. The dedifferentiated SCs and the macrophages line up within the endoneurial tubes to form the bands of Bünger, that later provide support for regenerating axons.

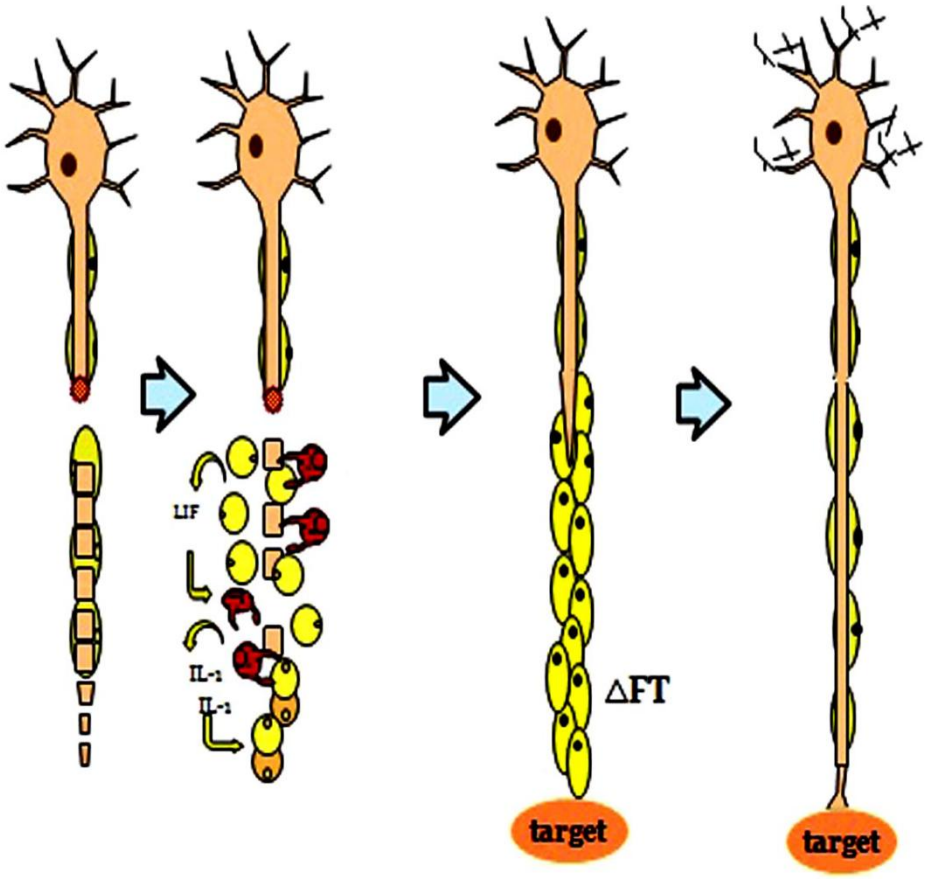

Figure 3: Schematic representation of the events of degeneration and regeneration after peripheral nerve injury. After axonal disruption, fragmentation of distal axon and myelin occurs. Macrophages and Schwann cells phagocyte the degraded debris. The neuron switches from a neurotransmitter state to a pro-regenerative state. Proliferating Schwann cells in the distal stump will create a permissive environment. Fine sprouts emerge from the proximal axon and elongate in association with the proliferating Schwann cells which guide the re-growing axons to their targets. (Extracted from Navarro et al. *Progress in Neurobiology*, 2007).

Axonal regeneration

The peripheral neuron is able to initiate a regenerative response for at least 12 months after injury. On the proximal segment, the axons degenerate back to the adjacent node of Ranvier, the site of subsequent axonal re-growth. The surrounding SCs dedifferentiate towards a progenitor-cell like phenotype, to support and guide the regenerating axons to their target organs. The rate of axonal regeneration is about 1-2 mm/day in mammals. Proximal to the lesion, growth cones emerge from the injured axons, induced by local factors. In the absence of a guiding structure, the re-growing axons form a neuroma composed of immature axonal sprouts and connective tissue (Siemionow & Brzezicki, 2009). In contrast, if the re-growing axons reach the distal

nerve and find a suitable environment, they would elongate within the endoneurial tubes in association with the SCs and the basal lamina. An appropriate relationship between the re-growing axons and the trophic and tropic cues provided by the reactive SCs and the ECM within the degenerated nerve stump is essential for a successful regeneration.

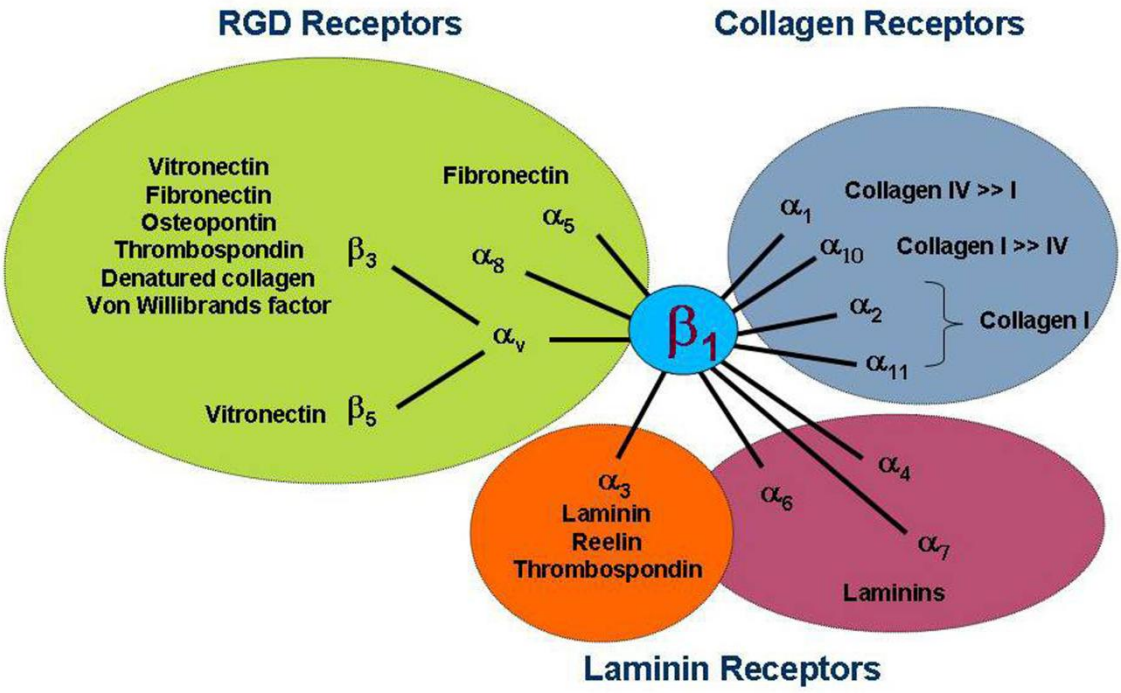

Figure 4: ECM molecules and their integrin receptors. Integrins are glycosylated heterodimers formed by α and β subunits. B subunit bounds to actin cytoskeleton and the α subunit determines the specificity to the ECM molecule adhesion.

Neurotrophic and neurotropic factors are needed during peripheral nerve regeneration and determine the response of the growth cone. The ability of neurons to interact with these cues is due to the expression in its membrane of different of cell adhesion molecules (CAMs). Among these CAMs, integrins are the ones that mainly mediate growth cone-ECM interactions (Hynes, 2002). Integrins are glycosylated heterodimers formed by α and β subunits. The integrin β1 subfamily is composed of integrins with a β1 subunit, which bounds to actin cytoskeleton, and the α subunit, which determines the specificity to the ECM molecule adhesion. Thus, integrin α1, α3, α6, and α7 can interact with laminin, whereas α5 interacts with fibronectin. Moreover, laminin and fibronectin have the characteristic of binding to other ECM components. For instance, laminin interacts with nidogens, agrin, perlecan, fibulin-1, heparin, and sulfatides (Holmberg & Durbeej, 2012; Rudenko et al., 2001), whereas fibronectin binds to collagen, fibril, and heparin sulfate proteoglycans (Hörmann, 1982). SCs will

also secrete neurotrophic factors (i.e. NGF, BDNF, NT-3, NT-4/5, GDNF.), growth factors (i.e. b-FGF, IGF) and cytokines (i.e. CNTF, Il-1, Il-6, TNF-α) which stimulates and control axonal regeneration.

Filopodia and lamellipodia are the mobile path-finder parts of the growth cone. When filopodia encounters a permissive substrate, growth cone receptors are able to bind to the ECM. These receptors re-organize the cytoskeleton and lead growth cone protrusion. Microtubules and actin filaments are responsible for the neurite elongation and growth cone motility controlled by the intracellular concentrations of calcium (Mattson & Kater, 1987). After peripheral nerve transection, motor and sensory fibers are capable to increase the levels of tubulin, actin and peripherin, and decrease the level of neurofilaments. GAP-43 is a protein associated to the basal lamina of the growth cones which, when phosphorylated, allow the extension and ramification of the growth cone (Van Lookeren Campagne et al., 1989). GAP-43 is phosphorylated by microtubule-associated proteins (MAPs), which indeed are able to induce polymerization of tubulin molecules simultaneously.

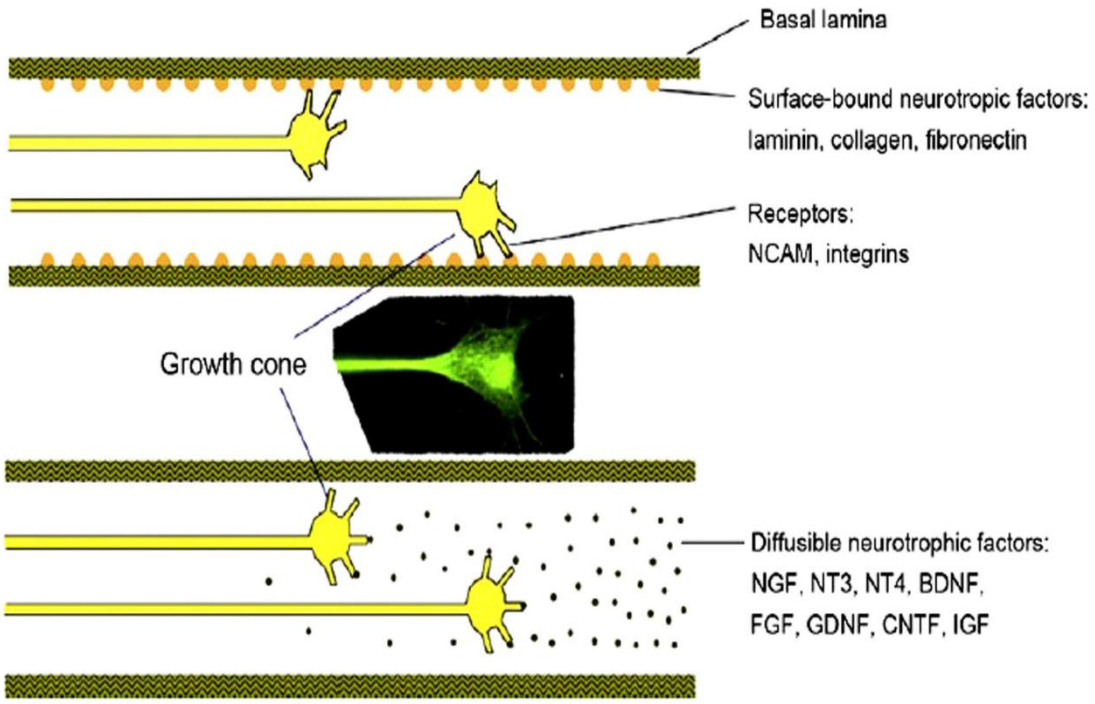

Figure 5: Trophic and tropic support for the axonal growth cone. The molecular cues that the regenerating axons find at the distal stump can be diffusible neurotrophic factors or membrane-bound neurotropic factors, as the ECM components. (Extracted from Gonzalez-Perez et al., *International Reviews in Neurobiology*, 2013).

ECM role in axonal regeneration

(Extracted from Gonzalez-Perez, et al. 2013).

Interactions of regenerating axons with ECM are key factors in the regenerative process. It is well known that dissociated sensory neurons show longer neurite extension on laminin and fibronectin-coated substrates compared to poly-L-lysine-coated surfaces (Rogers et al., 1983). When comparing the ability of different ECM components, laminin coated surfaces sustained better neurite outgrowth than vitronectin, collagen IV, fibronectin, or collagen I (Plantman et al., 2008; Wood & Willits, 2009). Laminin is also a preferred substrate for Schwann cells that extend and acquire a better morphology on laminin-coated rather than fibronectin-coated substrates (Palm & Furcht, 1983). When focusing on the different isoforms of laminin, DRG neurons grow better on laminin-1 and laminin-10 coated surfaces. Interestingly, when nerve growth factor (NGF) was added to the culture, there was a marked increase of neurite elongation of laminin-2 and laminin-8 in comparison to laminn-1 and laminin-10 coated surfaces, which seemed to sustain a neurotrophic-independent growth (Plantman et al., 2008).

Sulfated proteoglycans have inhibitory effects when studying neurite extension on explant cultures from DRG (Ughrin et al., 2003). Some authors claimed that proteoglycans inhibit neurite growth acting at the cone elongation (Snow et al., 2002) but others relate this inhibition to the effect of sulfated proteoglycans on the neuron soma (Kuffler et al., 2009).

When studying neurite elongation from DRG explants in 3D cultures, longer neurites were found in matrigel (a laminin-containing gel) that in collagen type I gel (Tonge et al., 1997), although the results might be affected by additional factors present in matrigel. In another study, explants of DRG grew better on laminin gels compared to fibronectin, collagen type I, or hyaluronic gels (Deister et al., 2007), supporting the findings from 2D cultures. On the other hand, different populations of sensory neurons may have different substrate preferences. Thus, embryonic proprioceptive sensory neurons grow similarly on fibronectin and laminin, whereas cutaneous ones prefer laminin (Guan et al., 2003). The inhibitory effect of the proteoglycans has also been proved in the 3D cultures (Tonge et al., 1997). When using 3D cultures, not only the components of the matrix but also the density should be taken into account. For example, when comparing different concentrations of an inert agarose gel, neurite extension was inversely correlated with the porous diameter that decrease with the

concentration of the gel (Balgude et al., 2001). Similarly, longer neurite extension was seen in lower concentration collagen gels (Willits & Skornia, 2004).

In contrast to the widely studied role of the ECM components on primary sensory neuron outgrowth *in vitro*, motoneuron outgrowth *in vitro* has been hardly analyzed. This fact is probably related to the technical difficulties of culturing primary spinal motoneurons compared to DRG neurons. Spinal cord slices embedded in a 3D matrix appear as an adequate model to investigate the role of ECM molecules on motor neurite outgrowth.

Reinnervation of the target organs

Usually not all the axons will be able to reinnervate the original targets. However, once the axons successfully reach the endoneurial tubes in the segment distal to the injury site, these have greater possibilities to find their distal targets. End organs, such as muscles, are able to produce chemical messengers that act as attracting factors. However, functional recovery will mainly depend on reinnervation of the motor axons to their specific muscle fibers. Motor functional outcome may also be impaired by associated sensory deficits, particularly those affecting proprioception. Sensory recovery is also modality specific, and cross-reinnervation is common (erroneous connections to different types of receptors). Collateral reinnervation by undamaged axons is limited to temporal and spatial constrains and it is generally helpful to recover protective pain sensibility and motor strength of partial denervated muscles.

Limitations of functional recovery after peripheral nerve injuries

There are several factors contributing to poor long-term functional recovery after PNIs. Early repair results in improved functional outcomes (Mackinnon, 1989). Time is crucial. For motor recovery, muscle reinnervation must occur in a timeframe between 12-18 months, before irreversible motor end plate degeneration. In contrast, sensory regeneration does not seem so time dependent, at least for gross sensibilities (Bester et al., 1998). Therefore, the level at which the injury is caused is also important. Distal injuries (i.e. median nerve in the wrist in humans) will require lesser amount of days than proximal injuries (i.e. brachial plexus injury) to reinnervate target organs, since the distance for the regeneration axons to cover is shorter. During this period, neurons remain axotomized and the target organ denervated, and eventually they can become atrophied. Age is also a key factor for regeneration. Re-growth of peripheral axons takes

longer in older individual than in young ones. The processes of myelin clearance, re-growth of axon sprouts and axon maturation become better orchestrated and synchronized in younger than in adult organisms (Vaughan, 1992).

Besides these general factors that can limit axonal regeneration and reinnervation, one of the main limiting for a good functional recovery is the lack of specificity of reinnervation. In severe PNIs, when the endoneurial tubes loss their continuity, axons are often misdirected and reinnervate incorrect target organs (Valero-Cabré & Navarro, 2002). For instance, motor axons may grow into a cutaneous branch or vice versa. Although axons are able to grow and survive in the foreign territory for an extended period of time, functional recovery is impaired. It is proposed that motor axons preferentially regenerate through their original terminal nerve branch in a process called "preferential motor reinnervation" (PMR) (Brushart, 1988). When muscle contact is denied but the cutaneous branch remains intact to the skin, motor axons grow towards the cutaneous branch, suggesting that regeneration is also dependent on the accessibility (Robinson & Madison, 2005).

It becomes essential to understand the cross-talk between the axons and their immediate environment to modulate the capacity of axons to regenerate and reinnervate specific organs. Neurotrophic factors and extracellular matrix proteins are able to serve as guidance cues by triggering cytoplasmic cascade events directed to arrange cytoskeleton, thus orienting neurite extension and attraction.

During development, motor and sensory axons are able to find their optimal peripheral targets by responding to a set of guidance signals. Axons travel through the spinal plexus, the peripheral nerve trunk and finally branch to their targets (Allodi et al., 2012). Motoneurons during development are capable to distinguish between flexor/extensor muscles and fast/slow muscular fibers. This is mainly controlled by a set of transcriptional factors and signaling pathways such as ephrins, cadherins, HGF/SF (Gavazzi, 2001; Krull & Koblar, 2000). On the other hand, sensory axon regeneration and reinnervation towards specific receptors is controlled by other transcriptional factors and signaling pathways such as netrins, semaphorins and slits (Del Río et al., 2004). However, although much information is recapitulated from developmental stages, little is known about selectivity during sensory and motor regeneration after PNIs in the adult.

Interestingly, SCs from motor and sensory branches express different molecular markers that may help re-growing axons to find their suitable target. For instance, L2/HNK1 expression is different between motor and sensory branches of the femoral nerve after a crush lesion (Martini et al., 1992). Indeed, myelin-forming SCs differentiate into HNK1 positive cells (motor myelin forming SCs) and HNK1 negative cells (sensory myelin forming SCs) (Saito et al., 2005). Furthermore, SCs from motor and sensory branches express different levels of specific growth factors. mRNA levels for NGF, BDNF, VEGF or IGF were highly expressed in sensory but not motor axons after injury. On the other hand, mRNA levels of GDNF or pleitrophin showed a higher expression in motor than in sensory branches (Höke et al., 2006).

Neurons require target-derived trophic support for survival, concept known as trophomorphism. This concept may play an important role during PMR, since regenerating axons will be able to choose one of both branches (muscular or cutaneous) depending on the trophic support present on both sites (Burnett & Zager, 2004). It has already been described that trophic factors can differentially influence growth of motor and sensory axons *in vitro* (Allodi et al., 2011) and *in vivo* (Boyd & Gordon, 2003). For instance, FGF-2 has been demonstrated to preferentially promote neuritogenesis of motor neurites *in vitro* (Allodi et al., 2013).

Additionally, extracellular matrix (ECM) components and cell adhesion molecules (CAMs) may act as tropic cues by exerting attracting/repulsing effects to the tip of the re-growing axon, mainly mediated by integrin interaction. It has been shown that laminin may preferentially acts on the elongation of presumptive cutaneous sensory neurons whereas fibronectin preferentially acts on presumptive proprioceptive sensory neurons during embryonic stages (Guan et al., 2003). However, how the ECM molecules modulate preferential outgrowth of motor and sensory axons postnatally remains unclear.

Surgical repair strategies

After peripheral nerve transection (grade 3-5 Sunderland), surgical repair is mandatory to facilitate that severed axons grow into the distal degenerating nerve. The microsurgical intervention reconnects the proximal and distal stumps, trying to match individual fascicles to facilitate the appropriate navigation of growing axons to their targets. However, matching of proximal and distal endoneurial tubes is impossible, and thus, misdirection of axons to a wrong distal path is common after these types of lesions

(Gonzalez-Perez et al., 2013). Repair techniques will vary depending on the distance in which proximal and distal stumps are separated.

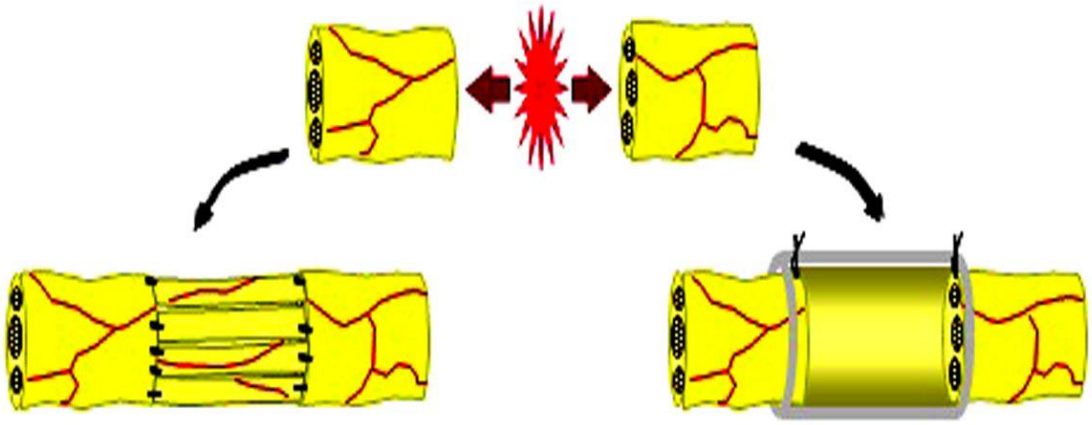

Figure 6: Repair strategies after severe peripheral nerve lesions. When the distance between the nerve stumps does not allow direct suture, a bridge is needed to re-unite the nerve ends. Autograft repair is considered the gold standard technique in the clinical practice to repair transections of peripheral nerves. Therefore, strategies to improve the design of artificial nerve guides and their internal milieu is needed in order to offer a real alternative to the autograft repair in the clinics.

- End-to-end repair: when the distance between proximal and distal stumps is small (not greater than 0.5-2 cm in humans), the coaptation of the nerve ends can be solved by direct epineural suture, as long as tension-free repair is possible (Bell & Haycock, 2012). It is a simple technique which cause minimal traumatism associated to the surgical intervention. After removing the scarred part of the injured tissue, fascicular patterns and longitudinal surface vessels will help to guarantee accurate re-organization by suturing individual fascicles (Brushart et al., 1983).
- Autograft: when the gap to be bridged increases and tension-free coaptation is not guaranteed, the interposition of a nerve graft is considered the "gold standard technique" for the repair of severe PNIs (Lundborg, 1988). In humans, this repair technique requires the harvest of nerve tissue from the sural nerve or other nerves of lower functional hierarchy (i.e. medial antebrachial nerve) (Isaacs, 2010). The harvested autologous nerve grafts undergo Wallerian degeneration and provide a mechanical guidance for the re-growing axons. The major number of fibers in the proximal part of the graft and the decrease in number of these

distally would facilitate axonal branching in the proximal suture but limit the number of fibers regenerating into the distal segment. Therefore, nerve grafts are usually reversed in orientation to maximize the number of axons that successfully regenerate trough the graft (Grinsell & Keating, 2014). Autografting repair fulfils the criteria for an ideal natural conduit, since these are able to provide a permissive scaffold for SCs to migrate and accompany the re-growing axons. However, this method implies donor site morbidity, with high risk of neuroma formation in this zone (Moore et al., 2009), limitations related to the amount of nerve that can be harvested, etc. Additionally there is an inevitably fascicle mismatch at the repair site. It should be mentioned that only 40-50% patients receiving these grafts achieve a useful degree of functional recovery (Lee & Wolfe, 2000).

- Allograft: human cadaveric nerve allografts solve autograft disadvantages related to donor site morbidity and supply. Injured patients which receive these allografts are immunosuppressed to avoid the response of T-cells and major histocompatibility complex against donor SCs (Moore et al., 2009). To overcome the problem of immune rejection, nerve allografts can be decellularized by chemical, enzymatic or irradiation degradation processes (Karabekmez et al., 2009). A decellularized allograft made by AxoGen Inc. (Alachua, Fl, USA) is commercially available, have received the approval from the Food and Drug Administration (FDA) and has already reached clinical trials (Brooks et al., 2012).
- Conduits: as an alternative to nerve grafts, conduits have been though to repair peripheral nerve defects. Artificial nerve conduits should mimic the natural environment found in a degenerating distal stump or an autograft (Deumens et al., 2010; Doolabh et al., 1996). Tube repair emerged as an alternative repair method. The regenerative process through tubular guides differs from that occurring in a degenerating segment. It starts with the formation of a fibrin cable (rich in fibroblasts, fibronectin, macrophages, leucocytes, eritrocytes) (Lundborg et al., 1982) which provides a guiding surface for the ingrowth of fibroblasts, blood vessels and SCs. Then, the fibrin cable is substituted by collagen and laminin secreted by fibroblasts and SCs respectively, which fills the lumen of the conduit and allow the regenerating axons to grow from the proximal to the distal stump (Deumens et al., 2010; Gonzalez-Perez et al., 2013). Failure of the

formation of the fibrin cable will compromise regeneration through the conduit (Yannas et al., 2007).

Success of regeneration in a neural guide is mainly linked to the material properties and the physical characteristics of the conduit, and the length of the gap to be bridged. The improvement of some parameters such as the internal diameter of the conduit, permeability of the outer wall of the conduit, re-absorbable materials, etc. will help to improve the final outcome.

Conduits can be classified into different categories (Deumens et al., 2010).

- Autogenous biological conduits: usually made of vein or arterial conduits and soft tissues (i.e. muscle and tendons) (Konofaos & Ver Halen, 2013). Their main advantages are that these are harvested from the patient and therefore are immunologically compatible. Additionally, these tissues contain fine orientated microstructure that act as guidance channels.
- Non-autogenous biological conduits: usually made from collagen type I, III or IV. Advantages to collagen-based conduits include its adhesive properties for different cell types, its fibrilar structure, and its facility to be isolated and purified. Furthermore, they can be degraded into non-toxic products resulting in minimal foreign body response (Ruszczak, 2003). One commercially available collagen conduit is Neuragen® (Integra Life Sciences Co., Plainsboro, NJ, USA) which has received approval for FDA.
- Non-resorbable conduits: include silicone and Gore-Tex. Silicone has been used in many medical applications because of its biocompatibility, biodurability, elastic properties and chemical and thermal stability. It has already been tested in humans to repair PNIs (Lundborg et al., 1997). However silicone has been demonstrated to present unwanted effects such as fibrous foreign body reaction and axonal compression during regeneration which leads to a secondary surgical intervention.
- Re-sorbable conduits: there is a second generation of conduits with improved physical properties. These properties include the decrease in their mechanical strength, their biocompatibility and biocompatibility, their permeability to allow exchange of oxygen and nutrients, etc. In these category we can highlight conduits made from:

- Polyglycolic acid (PGA): Neurotube® (Synovis Micro Companies Alliance, Birmingham, Al, USA) is a commercially available product.
- Polylactic acid (PLA): usually combined with caprolactone, which have been demonstrated to behave better than silicone for the repair of PNIs (Navarro et al., 1996). Nuerolac® (Polyganics, Groningen, Netherlands) is a commercially available product.
- Poly lactide-co-glycolide acid (PLGA).

Chitosan

During the last years, a natural biopolymer named chitosan (derivative of chitin) has increased in interest for biomedical and tissue engineering applications. Chitin can be gained from the exoskeleton of arthropods, cuticles of insects, walls of fungi and shells of crustaceans. It is the second most abundant organic source on earth after cellulose (Freier et al., 2005a). Chitosan is comprised of β(1-4) linked D-glucosamine and N-acetyl-D-glucosamine subunits with a degree of acetylation (DA) that can range from 0-60% (Freier et al., 2005b). Chitosan displays biocompatibility, biodegradability, low toxicity and structural similarity to natural glycosaminoglycans. Recent *in vitro* studies have demonstrated the suitability of chitosan membranes for survival of SCs (Yuan et al., 2004) and differentiated neuronal cells (Freier et al., 2005b). Recent technological improvements have overcame the poor mechanical strength of chitosan tubes increasing their potential use to be used as conduits for the repair of PNIs (Haastert-Talini et al., 2013). Chitosan nerve guides have recently received the CE-certified for medical devices and are currently commercially available as Reaxon®.

Functionalizing artificial nerve conduits

All clinically available conduits are hollow tubes, and in the clinical practice, their use is limited to the repair of small gaps. However, extensive research is continuously addressed to improve the internal milieu of the nerve guides, by mimicking the natural environment found in a degenerated distal nerve; so nerve guides could become a real alternative to autograft in the repair of severe peripheral nerve defects. Therefore, it seems mandatory the functionalization of scaffolds to fill the inner lumen of the conduit in order to mimic the natural endoneurium of the peripheral nerve tissue. Thus, addition of ECM-based scaffolds, neurotrophic factors and support cells may help to improve the final outcome of artificial nerve devices.

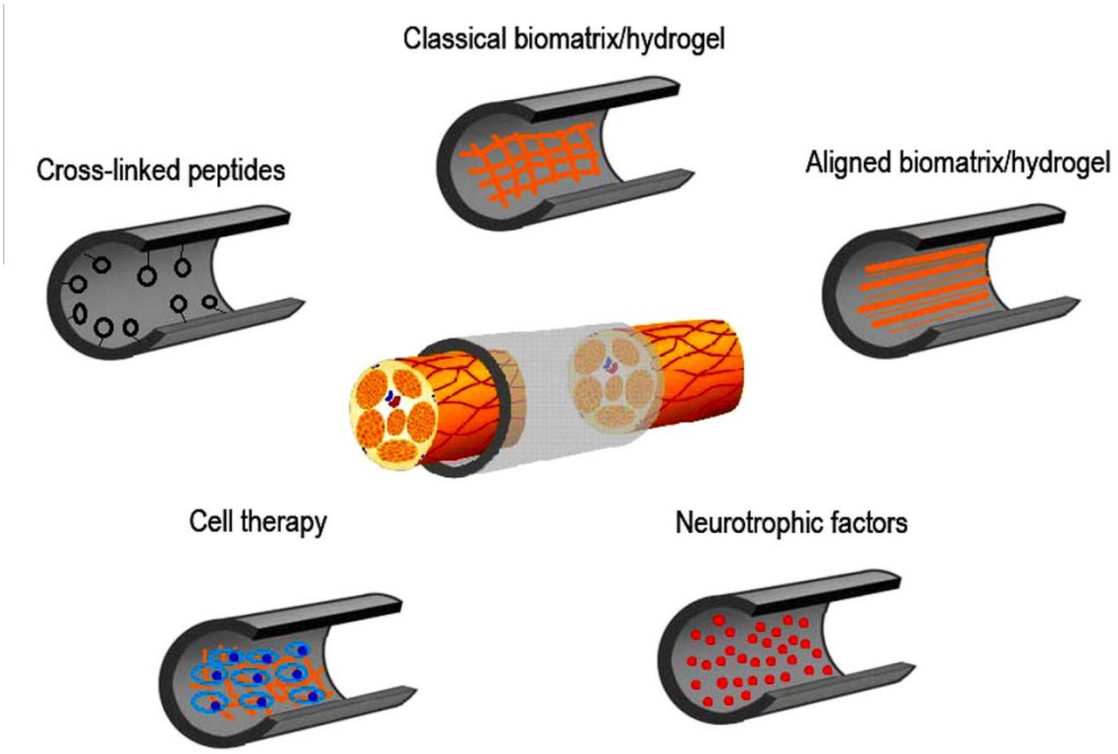

Figure 7: Schematic representation of the possibilities of functionalizing artificial nerve guides. When designing a nerve guide, physical properties of the tube and the design of the lumen of the conduit must be taken into account. For long gaps, introduction of scaffolds and supporting cells into the lumen may improve the final outcome. (Modified from Daly et al., *Journal of the Royal Society Interface*, 2011).

ECM-based strategy for functionalizing artificial nerve guides

(Extracted from Gonzalez-Perez, et al. 2013).

In vivo, the role of the ECM components in nerve regeneration has been widely demonstrated in nerve graft experimental models. Even when acellular nerve grafts are used, axons are able to regenerate through the scaffolds of basal lamina (Hall, 1997). By using anti-laminin antibodies, it was demonstrated that the capability of basal lamina to sustain axon growth was highly dependent on laminin. Anti-fibronectin antibody reduced neurite outgrowth, but did not influence the capability of axons to grow through the basal lamina (Wang et al., 1992). In similar models, the inhibitory role of proteoglycans was reduced when enzymes that degrade CSPG were applied to the distal stump or a graft, accelerating regeneration of motor and sensory axons (Krekoski et al., 2001; Udina et al., 2010).

Besides the studies that evaluate the role of different ECM in the distal stump of the injured nerve, the effects of these molecules have also been studied when used to fill

artificial nerve guides. Moreover, addition of these substrates to a hollow tube can increase the capability of the tube to sustain regeneration, and thus, it is a first step toward engineering an artificial guide that mimics the autograft. In fact, the sole addition of plasma, an important source of fibrin, into a silicone tube increased the gap length permissive for regeneration (Williams et al., 1987). On the other hand, collagen-based (Chamberlain et al., 1998; Labrador et al., 1998) and laminin-based matrices (Bailey et al., 1993; Labrador et al., 1998) have been successfully used to enhance axonal regeneration in nerve guides. Prefilling tubes with collagen or laminin containing gels improved regeneration through long gaps, whereas for short gaps, where regeneration is usually successful, addition of matrices into the tubes did not benefit the final outcome when compared to saline-filled tubes (Labrador et al., 1998; Valentini et al., 1987). When comparing the effects of different ECM components, laminin-containing gel performed slightly better than collagen I and hyaluronate gels on long gaps (Labrador et al., 1998). Combinations of ECM components have been tested in attempts to provide complex neurotropic support. The number of regenerated axons was higher in a gel mixture of collagen, laminin, and fibronectin compared to the control group (Chen et al., 2000). Similarly, a combination of laminin and fibronectin used to fill silicone tubes improved regeneration through long gap in rats (Bailey et al., 1993). Fibronectin was also tested with laminin in double-coated collagen fiber bundles inserted into collagen tubes. The results were better when using un-coated collagen fibers, revealing that laminin and fibronectin together may act positively on axonal growth (Tong et al., 1994).

As in 3D cultures, it is important to take into account the concentration of the matrix used, since the gel substrate, even if containing neurotropic agents, may impair the regenerative process by physically impeding the diffusion of factors, the migration of cells, or the elongation of axons. Lower concentrations of agarose, which forms a gel whose pore size decreases as concentration increases, were more permissive for regeneration trough a silicone tube than higher ones (Labrador et al., 1995), demonstrating the importance of density of the matrix used to guarantee axonal growth. Similar results were obtained when using collagen, laminin and hyaluronate gels (Labrador et al., 1998; Tona et al., 1993).

Nerve regeneration using gel-filled tubes across long gaps usually remains inferior to that obtained with nerve autografts. A relevant difference accounts by

endoneurial tubules in nerve grafts offering a mechanical guide to the regenerating axons, while gels filling tubes do not provide local direction for axons and may even impede the ingrowth on non-neuronal cells and axons. By using a longitudinally aligned ECM gel, the rate and direction of axonal elongation should improve due to contact guidance with the fibrils aligned along the tube axis. This option has been reported for tubes prefilled with magnetically or gravitationally aligned collagen gel, matrigel (Ceballos et al., 1999; Dubey et al., 1999; Verdú et al., 2002), and fibrin matrix (Dubey et al., 2001), and with collagen-GAG matrix (Chamberlain et al., 1998). Conduits containing longitudinally orientated extruded collagen microfibers and collagen scaffolds with orientated micropores, fabricated by directional ice-crystal formation, have been reported to allow regeneration over quite long gaps in the rat sciatic nerve (Yoshii et al., 2003). When fibronectin-orientated strands were introduced in nerve conduits to repair the rat sciatic nerve, axonal regeneration and Schwann cell recruitment were better than when using freeze-thawed muscle grafts (Whitworth et al., 1995). Fibronectin mats have been developed as orientated substrates that are capable of improving longitudinal migration of neurites and Schwann cells.

In conclusion, an adequate exogenous matrix designed to promote nerve regeneration within a nerve guide should have neuritotropic activity, be diluted in order to provide wide-enough pores for cellular and axonal migration, and also longitudinally orientated pathways that mimic the endoneurial tubules of the nerve.

Other 3D scaffolds for functionalizing artificial nerve guides

(Extracted from Gonzalez-Perez, et al. 2013).

As an alternative to biological/classical ECM-based scaffolds, several bioartificial polymers have been used to promote and guide axonal regeneration inside a neural guide. Such polymers can be introduced as a gel, coated directly into the internal wall of the tube of forming frameworks (like filaments or sponges used to enrich the internal architecture of the guide).

Gelatin is a natural-origin protein derived from collagen that maintains its inert properties, and can be functionalized by cross-linking techniques (Ciardelli & Chiono, 2006). Another natural polymer is chitosan, a polysaccharide obtained from chitin. Its molecular structure is similar to GAGs in the ECM. It can be introduced into the lumen of a tube as nano/microfibers that can be functionalized with laminin and trophic factors

(Patel et al., 2007) or combined with cells (Wang et al., 2009) to enhance nerve regeneration. Alginate is a biodegradable polysaccharide with repeat units of mannuronic acid and glucuronic acid. It has been used in the form of freeze-dried sponge to promote axon regeneration across a long gap in the cat sciatic nerve (Suzuki et al., 1999). Alginate also received attention as a slow-release hydrogel for the controlled supply of trophic factors. Other natural polymers, with potential to support nerve regeneration, are silk fibroin (Yang et al., 2007) and agarose (Labrador et al., 1995).

Synthetic polymers can also be used to form intratubular scaffolds that should combine the biocompatible characteristics of the natural polymers with improved mechanical and chemical performance. They also have to be surgically practicable, immunocompatible, and allow the diffusion of nutrients and growth factors to supply the re-growth of the injured nerve. Polymers can be presented as hydrogels when having high water content. Some examples of synthetic scaffolds used in peripheral nerve regeneration studies include, among others, poly(ethylene glycol) (Scott et al., 2010; Shepard et al., 2012), poly(lactide-co-glycolide) (Subramanian et al., 2009), polycrapolactone (Daud et al., 2012), and poly(N-isopropylacrylamide-co-acrylic acid) (Newman et al., 2006) in the form of hydrogels or fibers.

Recent developments of nanotechnology propose the use of fibrous scaffolds at the nanoscale level for the artificial replacement of basal lamina in tissue-engineered nerve grafts. Nanotubes can be produced from various materials, such as carbon, synthetic polymers, DNA, proteins, lipids, silicone, and glass. Moreover, they can serve as an extracellular scaffold, filling a hollow nerve conduit, to guide directed axonal growth (Cao et al., 2009; Olakowska et al., 2010). Surfaces with nano-sized topographies, electrospinned nanofibers, or replicas of the ECM with nanoresolution were found to guide neurite outgrowth from sensory and autonomic ganglia in the culture (Kanje & Johansson, 2011). *In vivo* studies showed that conduits filled with aligned polymer nanofibers resulted in better functional recovery than hollow conduits (Neal et al., 2011). These studies provide the basis for the use of nanofibers combined with molecular constituents of the ECM to enhance nerve conduits.

The performance of either synthetic tubes or scaffolds can be improved by functionalizing them with specific cues. These cues are usually motifs from ECM components with special effects on cell migration, attachment, and proliferation. Tubes

can be directly functionalized by linking cues to the internal wall of the conduit. For instance, a laminin-2 motif has been reported to have a positive effect on nerve regeneration when it as used to coat a PLGA guide (Seo et al., 2012). Also, a laminin-1 motif TATVH was proposed to promote neurite outgrowth and cell attachment on coated tubes (Nickels & Schmidt, 2012).

ECM peptides are being increasingly used for functionalizing hydrogels. Based on the results of *in vitro*studies, laminin cues have been the most widely used sequences for improving hydrogel performance on nerve regeneration. For instance, a laminin-1 sequence, the IKVAV pentapeptide, has been shown to promote axonal elongation on central and peripheral neurons (Bellamkonda et al., 1995; Tashiro et al., 1989). YIGSR is another pentapeptide used to graft agarose inert gels (Borkenhagen et al., 1998; Yu et al., 1999) and collagen polymers (Newman et al., 2006). The results revealed a significant increase in neuronal extension on YIGSR-coated gels in comparison to controls. Other motifs from laminin isoforms 1 and 2 have not been directly proved to enhance nerve regeneration.

Fibronectin cues have also been proposed for peripheral nerve repair. The GRGDS amino acid sequence regulates cell adhesion (Rutka et al., 1988), and it is probably the most studied shot sequence from fibronectin, although the RGD sequence is also found in other ECM components such as laminin-1 or tenascin (Meiners & Mercado, 2003). The GRGDS sequence has been used to functionalize hydrogels. For instance, gellan gum, which is a polysaccharide from bacterial origin, functionalized with this fibronectin sequence improved axonal growth *in vitro* (Luo & Shoichet, 2004) and survival and proliferation of neural stem/progenitor cells (Silva et al., 2012).

Nowadays, it is accepted that to design an artificial nerve guide as efficient as a nerve graft, it is important to combine different approaches. After designing the best conduit, addition of neurotropic cues to the luminal space, either as a matrix or by functionalizing a gel, may improve axonal growth. Certainly, this growth can be further enhanced by introducing supportive cells or trophic factors.

Growth factors-based strategy for functionalizing artificial nerve guides

Diffusible factors have been widely used in the promotion of regeneration after PNIs. Their effects on promoting neuronal survival and neurite outgrowth, proliferation and differentiation of non-neuronal cells, etc. have been previously mentioned. As for

the design of the nerve conduit, neurotrophic factors can be directly added to the matrix or through a more complex delivery system. The delivery of these factors has become a challenge due to the short half-life of them. Different scaffolds have been used for the release of growth factors, including fibronectin mats, collagen or micro/nano particles. The main advantage of a matrix-based system is that the internal structure of the tube is closer to that observed in a peripheral nerve. However, delivery systems are preferred, since prolonged and controlled diffusion of factors increase their effect. The gradients of growth factors can be manipulated and incorporated over the length of the nerve tube to attract the regenerating axons and sustaining their growth (de Ruiter et al., 2009). As an alternative to micro/nano spheres, the administration of cDNA sequences coding for these growth factors can be incorporated in viral vectors to transfect neurons or SCs which can sustain the diffusion of neurotrophic factors in time, increasing their lifespan and their effect (Hendriks et al., 2007).

The most widely studied neurotrophic factors with positive effects on nerve regeneration after injury are described below.

- Nerve growth factor (NGF): It was the first isolated growth factor, and probably the most studied one. It acts preferentially on the population of small primary sensory neurons and on sympathetic neurons (Levi-Montalcini, 1987). Although the expression of the factor seems to be equally distributed on cutaneous or muscular branches, after a peripheral injury there is a higher up-regulation in denervated sensory dorsal roots (Allodi et al., 2012). The application of NGF on silicone chambers demonstrated to promote neuronal survival of DRG neurons (Otto et al., 1987) and increased the number of myelinated fibers (Rich et al., 1989).
- Brain derived neurotrophic factor (BDNF): after a peripheral injury, higher levels of this neurotrophin are detected in cutaneous branches than in muscular ones (Höke et al., 2006). Infusion of BDNF in nerve tubes improved nerve generation and reduced automutilation in the rat (Vögelin et al., 2006). In contrast, when BDNF was acutely administered after end-to-end repair, no effects were observed (Boyd & Gordon, 2001).
- Fibroblast growth factor (FGF): this factor preferentially stimulates motoneurons regeneration *in vitro* (Allodi et al., 2013).Diffusible FGF on a

collagen matrix (Cordeiro et al., 1989) or released from synthetic guide channels (Aebischer et al., 1989) increased the number of regenerated myelinated fibers.

- Glial-derived neurotrophic factor (GDNF): has trophic effect on sensory, motor and autonomic peripheral neurons (Allodi et al., 2012). Although higher levels are observed in cutaneous than in muscular branches (Höke et al., 2006), after PNI, GDNF is indistinctly up-regulated (Höke et al., 2002). Sustained release of GDNF by synthetic guidance channels increased the number of retrograde labeled motoneurons and sensory neurons compared to control group (Fine et al., 2002). When GDNF was applied in the proximal stump of chronically denervated nerves, higher number of motoneurons regenerated (Boyd & Gordon, 2003).
- Neurotrophin-3 (NT-3): this neurotrophin is present in higher concentrations in cutaneous than muscular branches (Höke et al., 2006). It is highly associated with the Tyrosine kinase receptor C (TrkC). NT-3 is present in adult skeletal muscles and proprioceptive sensory neurons (Braun et al., 1996). When NT-3 and NGF were used to functionalize peripheral nerve interfaces, NGF compartments attracted an increased number of CGRP+ pain fibers whereas NT-3 compartments attracted large-diameter axons (Lotfi et al., 2011).
- Neurotrophins-4/5 (NT-4/5): focal delivery of NT-4 into fibronectin guides demonstrated to improve functional reinnervation of small motor units (Simon et al., 2003).

Cell therapy-based strategy for functionalizing artificial nerve guides

Another key element to enhance the regenerative capability of artificial nerve guides is the introduction of cells into these guides, and these cells can became an alternative to the prolonged delivery of growth factors. Among other cell types, SCs and stem cells represent the classical cellular support for nerve regeneration. The therapeutic role of these cells has been previously studied in the repair of PNIs. Their high tissue repair activity may reside in their capacity of cell replacement in the lesion site, the prolonged production of growth factors, the synthesis of components of the ECM, the modulation of the immune system, etc. (Wang et al., 2015). To functionalize a tube with cells, the number of cells that should be transplanted is crucial. Between 400,000-500,000 cells were sufficient to ensure a stimulating effect on the elongation and myelination of the re-growing axons in a 18 mm gap in the rat sciatic nerve (Ansselin et al., 1997). These

transplanted cells may be incorporated in a vehicle or carrier, like proteins of the ECM, which constitute an attempted replica of the natural endoneurium of healthy peripheral nerves. However, the best vehicle in which different cell types have to be incorporated is still unknown. Many studies have used Matrigel (laminin-containing gel from Engelbreth-Holm-Swarm mouse sarcoma, enriched with collagen IV, heparin sulfate proteoglycans, entactin and other growth factors) in a variety of transplantation paradigms within the PNS or CNS (Hood et al., 2009). However, the approval of the FDA for a scaffold based on a xenogeneic tumor cell line remains unclear. In fact, the possibility that transplanted cells lead to tumor formation is a major concern for their clinical use.

The main cell types used for peripheral nerve regeneration are described below.

- Schwann cells: these are probably the most used cells to modify the lumen of a tube for the repair of PN defects. SCs are the principal supporting cells of the PNS becoming the responsible of guiding and myelinating regenerating axons. The main advantage of these cells for the repair of peripheral nerve defects is their potential autologous supply, avoiding the problem of immune rejection. However, the autologous supply of SCs requires to isolate resident SCs via peripheral nerve biopsy prior to their incorporation into cellular prosthesis (Hood et al., 2009). This isolation via nerve biopsies evokes additional morbidity of the donor nerve, similar to autograft repair. Moreover, their clinical application is limited due to the extended time required to culture and expand these cells *in vitro*. Moreover, SCs lose rapidly their phenotypic characteristics (i.e. myelinating properties) *in vitro.* (Kingham et al., 2007).

  Transplanted SCs in vein grafts allow regeneration over a 25 mm gap in the rat, larger than previously reported (10 mm to 15 m) (Foidart-Dessalle et al., 1997).

  Furthermore, SCs have also been used to bridge a 6 mm defect in the mouse sciatic nerve (Rodriguez et al., 2000). In this study, transplants of autologous SCs in poly(l-lactide-co-epsilon-caprolactone) guides resulted in slightly lower levels of reinnervation than autografts, but higher recovery and number of regenerated fibers reaching the distal nerve than transplants of isologous and syngeneic SCs. Similar results were obtained when comparing allogenic vs autologous SCs embedded in alginate hydrogel to bridge a 10 mm gap in the rat sciatic nerve (Mosahebi et al., 2002).

- Stem cells: these are an attractive source of cells for nerve repair. Similarly to SCs, stem cells are able to provide a prolonged supply of growth factors and modulate the inflammatory response (Ribeiro et al., 2013). The use of stem cells avoid cell source related problems since self-renew of these cells in the tissue is high-rated and possess multi-potent differentiation properties (Pittenger et al., 1999). Although use of stem cells to repair PNI has not reached clinical trials yet, the benefits of these cells in experimental models have been observed both *in vitro* and *in vivo*. The great growth rate of these cells may facilitate the formation of bank of cells to be used as reservoirs for clinical use, overcoming the main disadvantage of SC. One of the main features of stem cells is the ability of them to transdifferentiate. That means that stem cells are able to reprogram and differentiate not only in mesenchymal lineages, but also into non-mesenchymal lineages (i.e. astrocytes, myocardium, endothelial cells, neurons and SCs) (Keilhoff et al., 2006). This SC-like phenotype could be able to guide and myelinate axons, resembling the major characteristics of the glial cells of the PNS. By treating mesenchymal stem cells (MSC) with SC mitogenic and differentiating factors (i.e. bFGF, PDGF, forskolin), the transdifferentiated cells are capable to express glial markers (i.e. S100, GFAP, p75, etc.) (Kingham et al., 2007). In vivo, the maintenance of the transdifferentiated cells may be controlled by the local microenvironment and local cytokines and growth factors of the devitalized muscle, although transdifferentiation state of transplanted cells may be diluted by cell division.

  Stem cells from different sources have been used for peripheral nerve regeneration.

  - Bone Marrow Mesenchymal Stem Cells (BM-MSCs): they are non-hematopoietic cells which reside in the bone marrow. They mainly differentiate into cell lineages from mesodermal origin such as muscle, bone, cartilage, etc. (Baksh et al., 2004). By aspiration of the bone marrow, these cells can be obtained. These cells exhibit a great plasticity that can adjust to the requirements needed for peripheral nerve transplantation Direct transplant of MSC to an injured nerve increases survival of motor and sensory axons and improves axonal outgrowth by increasing the number of myelinated fibers and the production of neurotrophic factors (i.e. GDNF) (Marconi et al., 2012).

MSCs have also been previously used to seed grafts in rats. These studies showed beneficial effects of these cells by means of electrophysiological and histological tests (Keilhoff et al., 2006; Wang et al., 2015).

- Adipose tissue Mesenchymal Stem Cells: as an alternative, the adipose tissue becomes a promising source of multi-potent stem cells, adipose-derived stem cells (ADSC). Their phenotypic characteristics and gene expression profiles are similar to those exhibited by bone marrow derived MSC (De Ugarte et al., 2003). The frequency of MSC obtained from the bone marrow is approximately 1 in 100,000 cells (Muschler et al., 2001) when compared to 2 in 100 obtained by lipoaspirating the human fat (Strem et al., 2005). Human adipose tissue is a great deposit of subcutaneous fat, where ADSC can be easily isolated. It requires minimally invasive surgical procedures and involves: washing of the adipose tissue, enzymatic digestion, centrifugation and removal of red blood cells. The resultant fraction contains not only stem cells, but also preadipocytes, endothelial cells, macrophages, fibroblasts, etc. However, the stem cells rapidly adhere to the plastic substrate and proliferate (Kingham et al., 2014).

  ADSC have been demonstrated to potentiate peripheral nerve regeneration *in vivo*. ADSCs in fibrin nerve conduits enhanced axonal regeneration when compared to hollow tubes (di Summa et al., 2010).

  Furthermore, it increased levels of regenerated-associated gens such as GAP-43 (Kingham et al., 2014).

  Moreover, systemic administration of ADSC one week after sciatic nerve crush in mice increased axonal sprouting and reduced the inflammatory infiltrated cells after injury, followed by an increased expression of GDNF in the injured nerves (Marconi et al., 2012).

- Skeletal muscle-derived stem cells (Sk-MSCs): they are derived from the skeletal muscle interstitium. As they are multi-potent stem cells they are capable to differentiate into mesodermal cells (skeletal muscle cells, vascular smooth muscle cells, pericytes, endothelial cells, etc.) and ectodermal cells (i.e. SCs). Sk-MSCs express neurotrophic growth factors (i.e. NGF, GDNF, bFGF, etc). When transplanted *in vivo* in an

acellular conduit, they increase the number of myelinated fibers compared to control group (Tamaki et al., 2014).

- Olfactory Ensheathing Cells (OECs): these cells can be derived from the olfactory mucosa, olfactory nerve and the outer nerve layer of the olfactory bulb. They are the glial cells of the olfactory system, residing not only in the PNS but also in the CNS. They promote neural regeneration in a wide variety of lesions of the nervous system (Radtke et al., 2011). Harvesting OECs from the olfactory mucosa is an easy alternative for autologous transplantation for nerve repair with low risk of immune rejection. OECs are similar to SCs in the main functions. They are able to phagocyte degenerating axons and produce guidance to the regenerating ones. They can also produce a great variety of growth factors, enhancing the survival and promoting the elongation of the new re-growing axons. OECs sustained regeneration when transplanted in the rat to solve a 15 mm sciatic nerve defect (Verdú et al., 1999).

Immunosuppression

Immunosuppressant agents are widely used in the clinical practice as selective suppressors of cell immunity, to prevent organ rejection after transplantation. Some of these drugs (i.e. cyclosporine A (CsA) or FK506) have been previously tested in humans. CsA, FK506 and rapamicin inhibit the function of immunophilins and, trough blocking or activating several intracellular pathways, they can exert neuroprotective effects in different experimental models of ischemia, Parkinson's disease and excitotoxic insults (Butcher et al., 1997; Gerard et al., 2010)

Immunophilins are a well studied protein family which act as receptors of these immunosuppressant drugs. Immunophilins are cytoplasmatic proteins implicated in a wide variety of biological functions (i.e. protein folding, protein transport, formation of protein complexes, stabilization of membrane channels etc.) (Kuffler, 2009). Interestingly, FK506 not only is able to prevent graft rejection, but also promote axon regeneration in experimental models (Udina et al., 2004). Its dual effect is highly interesting, since it can be used to avoid the rejection when peripheral injuries are repaired with allogenic grafts or neural guides containing transplanted cells, and also facilitate axonal regeneration.

- Cyclosporine A: is a cyclic peptide from fungi origin which binds to a class of immunophilins called cyclophilins. The role of CsA in nerve regeneration has

not been clearly identified. When CsA was administered to rats to avoid rejection of a decelullarized donor graft to bridge a 20 mm nerve defect, CsA therapy prevented any histological evidence of rejection and supported axonal regeneration trough nerve allografts. In comparison, in the same study, FK506 treatment correlated with a significant increase in number of fibers compared to both untreated and CsA-treated animals (Brenner et al., 2008).

- FK506: also kwon as tacrolimus. It is a calcineurin phosphatase inhibitor, inhibiting T-cell proliferation. The receptors for FK506 belong to the family of FK506-binding proteins (FKBP). FK506 is a FDA-approved immunosuppressant agent used to avoid organ rejection after transplantation. The effect of FK506 on peripheral nerve regeneration is due to a calcineurin-independent mechanism (i.e. not involving GAP-43 phosphorylation) (Kuffler, 2009). Experimental studies have shown that systemic administration of FK506 enhances regeneration by increasing the number of myelinated and unmyelinated fibers, after crush (Lyons et al., 1995), transection (Jost et al., 2000), chronic dennervation (Sulaiman et al., 2002) or after autograft or allograft repair (Udina et al., 2004). As an alternative to systemic administration of FK506, topical FK506 administration directly to the zone of peripheral nerve injury via conduit device is a potential method in order to minimize the side effects and risks of systemic administration (Toll et al., 2011).

Conclusions

In this introduction we have discussed the various steps that are involved in the design of an ideal conduit for peripheral nerve repair. In the first step, the choice of the biomaterial results crucial to the final outcome. The desired material needs to be permeable and flexible with controlled degradation rates and limited swelling. Second, there is the need to functionalize the conduit by means of a scaffold that closely mimics the natural environment found in the distal degenerating nerve. Various components of the ECM can be added in different ways (i.e. matrices, filaments, channels or hydrogels). The scaffolds can be further enriched by transplantation of different cell types to support peripheral nerve regeneration, in which immune rejection can be prevented by the administration of immunosuppressant agents.

OBJECTIVES

OBJECTIVES

Despite that regeneration can occur after peripheral nerve lesions, functional recovery is still poor due to an insufficient axonal regeneration and misdirection of re-growing axons regarding their specific target organs. Therefore, strategies aimed to enhance axonal growth and specifically direct regenerating axons will improve functional recovery after nerve injuries. Furthermore, although many efforts in tissue engineering are being addressed to improve quality material and internal environment of nerve conduits, autograft repair is still considered the "gold standard" technique for the repair of severe nerve injuries. Mimicking the natural environment of a degenerated distal nerve when designing a composite nerve guide may increase the potential of this alternative repair technique over limiting gaps.

The main aims of this thesis are:

- To investigate the potential of ECM molecules that may selectively promote regeneration of motor and sensory neurons, and therefore become improved scaffolds for peripheral nerve repair.
- To design the optimal artificial nerve graft to promote peripheral nerve regeneration through a long gap in the peripheral nerve.

The specific objectives of this thesis are:

- To compare the differential effect of ECM molecules on regeneration of motor and sensory neurites in a 3D *in vitro* model.
- Corroborate *in vivo* the preferential effect of ECM molecules on neurite elongation showed *in vitro*.
- To study the potential of chitosan hollow tubes to promote regeneration of peripheral nerves in a limiting gap of 15 mm in the rat sciatic nerve.
- To incorporate ECM-based scaffolds to the chitosan conduits to improve the quality of peripheral nerve regeneration in a limiting gap of 15 mm in the rat.
- To design the optimal artificial nerve graft by combining tissue-engineering and cell therapy to improve the quality of nerve regeneration in a limiting gap of 15 mm in the rat.

CHAPTER I

Substratum preferences of motor and sensory neurons in postnatal and adult rats

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Substratum preferences of motor and sensory neurons in postnatal and adult rats

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Abstract

After peripheral nerve injuries, damaged axons can regenerate but functional recovery is limited by the specific reinnervation of targets. In this study we evaluated if motor and sensory neurites have a substrate preference for laminin and fibronectin in postnatal and adult stages. In postnatal dorsal root ganglia (DRG) explants, sensory neurons extended longer neurites on collagen matrices enriched with laminin (~50%) or fibronectin (~35%), whereas motoneurons extended longer neurites (~100%) in organotypic spinal cord slices embedded in fibronectin-enriched matrix. An increased percentage of parvalbumin-positive neurites (presumptive proprioceptive) vs. neurofilament-positive neurites was also found in DRG in fibronectin-enriched matrix. To test if the different preference of neurons for extracellular matrix components was maintained *in vivo*, these matrices were used to fill a chitosan guide to repair a 6-mm gap in the sciatic nerve of adult rats. However, the number of regenerating motor and sensory neurons after 1 month was similar between groups. Moreover, none of the retrotraced sensory neurons in DRG was positive for parvalbumin, suggesting that presumptive proprioceptive neurons had poor regenerative capabilities compared with other peripheral neurons. Using real-time PCR we evaluated the expression of α5β1 (receptor for fibronectin) and α7β1 integrin (receptor for laminin) in spinal cord and DRG 2 days after injury. Postnatal animals showed a higher increase of α5β1 integrin, whereas both integrins were similarly expressed in adult neurons. Therefore, we conclude that motor and sensory axons have a different substrate preference at early postnatal stages but this difference is lost in the adult.

Introduction

The ability of peripheral nerves to sustain regeneration is mainly due to the presence in the distal stump of Schwann cells of an increased supply of trophic factors and a substratum rich in extracellular matrix (ECM) components, such as laminin, fibronectin and type IV collagen (Baron-Van Evercooren *et al.*, 1982; Rogers *et al.*, 1983; Gardiner *et al.*, 2007). The interaction between neurons and ECM components depends mainly on integrins, a family of heterodimeric receptors that mediate axonal guidance, adhesion and migration. Integrins are highly expressed in growth cones, both during development and after injury (Lemons & Condic, 2008; Gardiner, 2011). ECM components are also rapidly up-regulated after nerve injury. Therefore, successful axonal regeneration is dependent on the interactions between ECM in the environment and integrins expressed by axons (Wakatsuki *et al.*, 2014).

However, even if injured axons are able to regenerate along the distal nerve stump, functional recovery is limited by the lack of specific reinnervation of target organs. After severe nerve injuries, with discontinuity of endoneural tubes and connective layers, non-specific reinnervation is the rule, due to misrouting of growing axons through non-appropriate nerve branches. It remains unclear whether injured axons have any intrinsic mechanism providing them with a preference for a certain pathway or target organ (Madison *et al.*, 2009). It has been proposed that trophic factors can differentially influence growth of motor and sensory axons *in vivo* (Boyd & Gordon, 2003) and *in vitro* (Allodi *et al.*, 2011), whereas Schwann cells from motor and sensory nerves present a different profile of neurotrophic factor expression (Höke *et al.*, 2006). However, the exact role of the differential expression of trophic factors after injury is not clear. Whether the different components of the ECM might preferentially promote growth of subtypes of neurons remains poorly addressed in the literature. Interestingly, in chick embryos, proprioceptive and cutaneous sensory neurons express different types of integrins, which determine its preference for fibronectin or laminin (Guan *et al.*, 2003). Presumptive proprioceptive neurons extend neurites similarly on fibronectin and laminin, and highly express fibronectin receptors, such as integrin α5β1, whereas presumptive cutaneous neurons prefer to grow on laminin, and show higher levels of integrin α7β1, which mainly binds to laminin.

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The levels of integrins highly expressed in embryonic neurons are reduced in the adult (Lemons & Condic, 2008), but after peripheral nerve injuries integrin expression is up-regulated again (Wallquist *et al.*, 2004; Eva & Fawcett, 2014). However, it is not clear whether different types of neurons, by expressing different types of integrins, show some substrate preference as in developmental stages. For example, because proprioceptive neurons have to grow towards targets into the muscles, along muscular nerves, motor axons might have similar preferences for fibronectin than proprioceptive neurons.

The aim of this study was to evaluate if peripheral sensory axons maintain a substratum preference postnatally as well as in adulthood, so fibronectin may maiuly sustain neurite outgrowth of motor and proprioceptive neurons, whereas growth of cutaneous sensory neurons may be mainly favoured by laminin. We also evaluated if the substratum preference of different neurons was related to the expression of different integrins.

Materials and methods

All experimental procedures were approved by the ethics committee of the Universitat Autònoma de Barcelona and were in accordance with the European Community Council Directive.

In vitro experiments

For *in vitro* testing, Sprague-Dawley rats of postnatal day 7 ($n = 21$) were decapitated whereas rats of 21 days ($n = 9$) were killed via an intraperitoneal injection of pentobarbital. Immediately, the lumbar and sacral spinal cord and lumbar dorsal root ganglia (DRG) were dissected and placed in cold Gey's balanced salt solution (Sigma, St Louis, MO, USA) enriched with 6 mg/mL glucose and cleaned of meninges, blood and roots. Spinal cords were cut using a McIlwain tissue chopper (Stoetling co., Wood Dale, IL, USA) into 350-μm-thick slices.

Matrix preparation and orgnaotypic cultures

A volume of 450 μL of rat tail type I collagen solution (BD-Biosciences, Two Oak Park, Bedford, MA, USA) at a concentration 3 4 mg/mL was mixed with 50 μL of 10× Eagle's medium (Gibco, Waltham, MA, USA) and 2 μL of 7.5% sodium bicarbonate solution. Euriched matrices were prepared by addition of 50 μL (10% final volume) or 100 μL (20% final volume) of fibronectin (BD Biosciences) or laminin type I (Sigma) at 1 mg/mL. Control collagen matrices received the same amount of phosphate-buffered saline (PBS).

Single 30-μL drops of each matrix were deposited on poly-D-lysine- (1 μg/mL, Sigma) coated round coverslips in 24-well Petri dishes, and then kept in the incubator at 37 °C and 5% CO_2 for 2 h to allow matrix gel formation. Spinal cord slices and DRG explants were placed on top of the gelled drop and covered by a second 30-μL matrix drop. The embedded samples were kept again in the incubator for 45 min before adding Neurobasal medium (Invitrogen, Carlsbad, CA, USA), supplemented with B27 (Invitrogen), glutamine and penicillin/streptomycin (Sigma). After 1 day in culture the medium was changed. Due to the different intrinsic capacity of both types of tissues to grow in the culture, we maintained spinal cord slices for 4 days and DRG explants for 2 days (Allodi *et al.*, 2011). As adult neurons also have a reduced capacity to grow compared with postnatal neurons, DRG explants from postnatal day (p) 21 animals were kept in culture for 4 days.

Analysis of neurite outgrowth

Spinal cord and DRG cultures were fixed with 4% paraformaldehyde in PBS for 30 min. Samples were washed three times for 20 min each in PBS and incubated for 48 h in PBS triton 0.3% with blocking serum at 1.5% and anti-neurofilament antibody NF-200 (1 : 1000; EMD Millipore, Billerica, MA, USA) to label growing neurites into the gelled matrices; DRG were also incubated with anti-parvalbumin (1 : 1000; Swant, Marly, Switzerland) antibody to label proprioceptive sensory neurites. The samples were then washed three times for 1 h each with PBS triton and the sections were incubated overnight with PBS triton 0.3% with blocking serum at 1.5% and filtered secondary antibodies Alexa 488 conjugated goat anti-chicken (1 : 1000, Life BioScience, Oakleigh, Australia) and Cy3 conjugated donkey anti-rabbit (1 : 200, Jackson Immunoresearch, West Grove, PA, USA). The samples were allowed to dry and mounted in Moviol.

To analyse the length of neurite extension, photomicrographs of the cultures were taken at 10 × with a digital camera (Olympus DP50) attached to the microscope (Olympus BX51), acquired in Adobe Photoshop CS and automatically photomerged. Using ImageJ software (NIH, Bethesda, MD, USA) resolution parameters were fixed and neurites were followed from the ventral horn (spinal cord) or ganglion boundary (DRG) to their ending projections. Neurite outgrowth measurements were normalized as the percentage of neurite outgrowth compared with the mean growth on the collagen controls used in parallel in each experiment.

A macro for ImageJ designed to automatically count neurites crossing defined distances from the explant was used to quantify the number of growing neurites (Torres-Espín *et al.*, 2014). The program recognizes the body of the organotypic culture and, after applying a set of manual thresholds, it draws concentric shapes from the body, each separated by 25 μm. The density of neurites that cross the defined distances was measured automatically. As large numbers of neurites were present at 25 μm from the explant on postnatal DRG, hindering the automatic detection of individual processes, we started the measures at 50 μm from the ganglion in these conditions. The number of neurites growing from spinal cord slices and DRG explants is presented as the absolute number of neurites that crossed the defined concentric circles and compared with control samples grown on collagen matrices.

The longest three neurites of each explant (spinal cord slices and lumbar DRG; eight per condition) were measured in three separate cultures.

In vivo experiments

For the *in vivo* experiments, adult female Sprague-Dawley rats (9 weeks old; 220 270 g) were used. The animals were housed with free access to food and water at room temperature of 22 ± 2 °C. Surgeries were performed under ketamine/xylacine anaesthesia (90/10 mg/kg i.p.). The right sciatic nerve was exposed and cut. Repair of the injured nerve was performed with a chitosan tube (8 mm) leaving a 6-mm gap between proximal and distal stumps; the tubes were filled with different combinations of biomatrices. Animals were distributed into three groups ($n = 4$) according to the biomatrix contained in the tube: one group with collagen type I matrix at 2 mg/mL; a second group with collagen type I matrix supplemented with 20% (v/v) laminin, and a third group with collagen type I matrix supplemented with 20% (v/v) fibronectin. At 31 days after injury, animals were anaesthetized again, and the right regenerated sciatic nerve was exposed and cut 8 mm distal to the distal end of

the conduit and the proximal stump dipped in Fluorogold solution (5%; Fluorochrome Inc., Denver, CO, USA) placed in a vaseline pool for 1 h. After 1 week, animals were killed by a intraperitoneal injection of pentobarbital and perfused with 4% paraformaldehyde. L4 and L5 DRG and the lumbar segment of the spinal cord containing the motoneuron sciatic pool were harvested.

Counting of backlabelled neurons

DRG and spinal cord from perfused animals were kept cryopreserved in PBS-sucrose 30%. With a cryostat, DRG were cut at 15 μm thickness and spinal cord at 40 μm, and serially mounted on gelatin-coated slides, which were kept in the dark to avoid fading of the labelling. The number of counted labelled sensory and motor neurons was corrected using the Abercrombie correction factor to avoid duplicate counting of neurons (Abercrombie, 1946). For identification, the proprioceptive sensory neurons were labelled with anti-parvalbumin (1 : 1000, Swant) and the myelinated neurons with anti-NF-200 (1:1000, EMD Millipore).

Real-time PCR

Spinal cord slices and DRG explants cultured for 2 days within the different matrices were processed for mRNA analysis. Freshly extracted samples were used as controls (day 0). Adult Sprague Dawley rats were anaesthetized and the right sciatic nerve transected. Two days after injury, animals were anaesthetized again and L4 and L5 DRG and the lumbar segment of the spinal cord were harvested and the samples processed for mRNA analysis. Samples were kept at 80 °C in RNA-latter solution (Qiagen, Barcelona, Spain). The total RNA of spinal cord slices and DRG grown in collagen control and in fibronectin- or laminin-enriched matrices was extracted with an RNeasy mini kit (Qiagen), including a DNase step (RNase free DNase set; Qiagen). Then, 1 μg of RNA was reverse-transcribed using 10 μmol/L dithiothreitol, 200 U M-MuLV reverse transcriptase (New England BioLabs, Ipswich, MA, USA), 10 U RNase Out Ribonuclease Inhibitor (Invitrogen), 1 μmol/L oligo(dT) and 1 μmol/L of random hexamers (New England BioLabs). The reverse transcription cycle conditions were 25 °C for 10 min, 42 °C for 1 h and 72 °C for 10 min. We analysed the mRNA expression of integrins α5 and α7 by means of specific primer sets. The primer sequences 5′ 3′ were: Itga 5: Fw, CCCTACCAAATCCTGCCTC; Rv, AGGATGGVGAGGATGATG; Itga 7: Fw, GCAGCAGTTCAA GGAGGAG; Rv, TTGGGACAGGAGAAGTTAGG

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 cycle threshold (CT) values for quantification. Three samples per condition were used and each one was run in duplicate. The thermal cycling conditions comprised 3 min of 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, followed by a DNA melting curve for determination of amplicon specificity. CT values were obtained and analysed with Bio-Rad 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. For each condition, 24 explants were grouped and the expression of the integrins was analysed in three different cultures.

Data analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0 (Graphpad Software Inc., La Jolla, CA, USA). Statistical comparisons between conditions for the analysis of neurite extension in spinal cord slices and DRG explants were made by one-way analysis of variance (ANOVA) followed by Bonferroni post-test. Statistical comparison between conditions for the analysis of neurite density was made by two-way ANOVA for repeated measurements, followed by Bonferroni post-test. Statistical comparisons of the expression of integrins α5 and α7 on spinal cord slices and DRG explants and of the number of neurons retrolabelled from the *in vivo* study were made by one-way ANOVA followed by Bonferroni post-test. Differences were considered significant at $P < 0.05$.

Results

Neurite outgrowth on spinal cord slices and DRG explants of postnatal rats

By using NF-200 staining, the outgrowth of motor and sensory neurites from spinal cord slices and DRG explants of p7 rats was observed in the 3D matrices (Fig. 1). Addition of laminin type I in the matrix increased the elongation of sensory neurites both at 10% (145.90 ± 7.49; Bonferroni $t = 4.217$, $P = 0.0006$) and at 20% (v/v) (151.55 ± 5.56; Bonferroni $t = 5.214$, $P = 0.0001$) compared with collagen controls. Outgrowth of sensory neurites was also longer in matrices enriched with 20% fibronectin (135.43 ± 5.24; Bonferroni $t = 3.871$, $P = 0.0025$) and laminin type II (131.07 ± 4.46; Bonferroni $t = 3.355$, $P = 0.0154$) but not at fibronectin 10% (111.09 ± 3.74; Bonferroni $t = 1.192$, $P = 0.89$) nor at laminin type II 10% (108.03 ± 3.36; Bonferroni $t = 0.8956$, $P = 0.97$) (Fig. 2A). In contrast, motor neurites elongated longer in fibronectin-enriched matrices than in control collagen matrices at 10% (199.75 ± 14.47; Bonferroni $t = 8.348$, $P = 0.0001$) and 20% (199.76 ± 9.66; Bonferroni $t = 8.349$, $P = 0.0001$). Addition of either laminin type I at 10% (100.64 ± 8.71; Bonferroni $t = 0.045$, $P = 0.99$) and 20% (v/v) (79.22 ± 10.88; Bonferroni $t = 1.313$, $P = 0.99$) or laminin type II at 10% (88.34 ± 5.88; Bonferroni $t = 0.976$, $P = 0.99$) and 20% (v/v) (92.61 ± 5.94; Bonferroni $t = 0.636$, $P = 0.99$) did not increase neurite growth of motor neurites in spinal cord slices compared with control collagen matrices (Fig. 2B).

We then evaluated the number of neurites emerging from the spinal cord slices and DRG explants embedded in matrices enriched with laminin type I and fibronectin at 20% (v/v). The number of sensory neurites emerging from DRG explants embedded on laminin type I-enriched matrices was significantly higher (two-way ANOVA: $F_{2,300} = 76.16$, $P = 0.0001$) from 150 to 375 μm from the limit of the ganglia, compared with control collagen matrices. Although the number of neurites was higher in laminin- than in fibronectin-enriched matrices, no significant differences between these two groups were found at any distance (Fig. 2C). The number of motor neurites emerging from spinal cords embedded on fibronectin-enriched matrix was significantly higher (two-way ANOVA: $F_{2,240} = 25.98$, $P = 0.0001$) from 50 to 175 μm from the limit of the spinal cord, than in samples embedded on control collagen matrices and laminin type I-enriched matrices. Further away, although numbers were still higher in the fibronectin-enriched matrices, no significant differences were found (Fig. 2D).

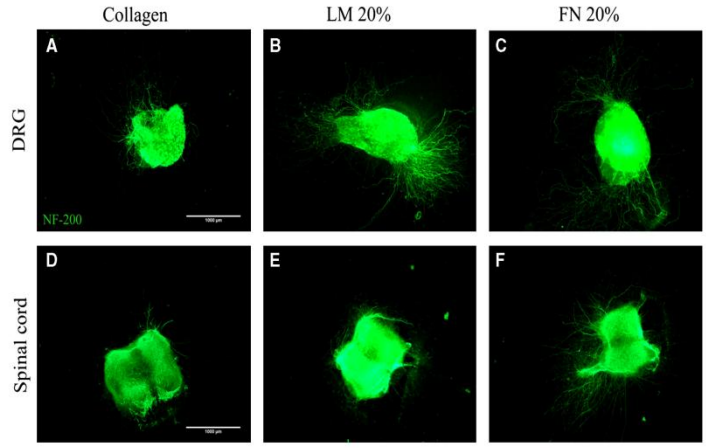

FIG. 1. Representative images of p7 DRG explants and spinal cord slices grown on different extracellular matrices. Neurofilament-labelled neurites from DRG explants (top panels) cultured for 2 days and from spinal cord slices (bottom panels) cultured for 4 days, embedded in collagen type I (A, D), laminin-enriched (LM; B, E) and fibronectin-enriched matrices (FN; C, F). Scale bar = 1000 μm.

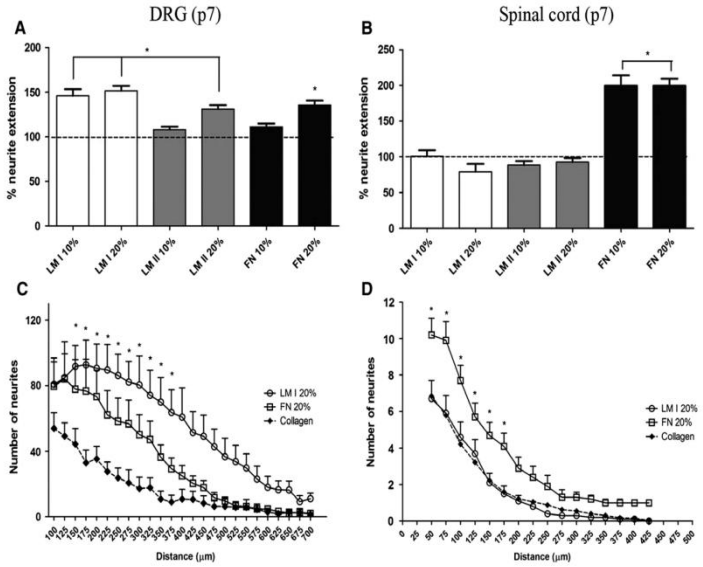

FIG. 2. Neurite outgrowth of postnatal sensory and motor neurons on different extracellular matrices *in vitro*. Length of the longest neurites growing from p7 spinal cord slices (A) and DRG explants (B) cultured in collagen matrices enriched with laminin (LM) type I, type II or fibronectin (FN) at 10 or 20% (v/v). Values are expressed as percentage vs. control collagen type I matrices. Measurements were made on independent cultures per condition. Quantification of the number of neurites that reached concentric distances from the spinal cord (C) and the DRG (D) counted using a semi-automatic method. $*P < 0.05$.

Neurite outgrowth of parvalbumin-positive sensory neurons in postnatal rats

Whereas motor neurite elongation and arborization on spinal cord slices was increased in the fibronectin-enriched matrices, the elongation and arborization of sensory neurites seemed to be enhanced in both laminin type-I and for the fibronectin-enriched matrices at high concentration (20%, v/v). To determine if laminin type I and fibronectin act on different sensory neuron populations, we labelled the DRG explants against parvalbumin, a marker of proprioceptive sensory neurons (Inoue *et al.*, 2002; Levanon *et al.*, 2002) (Fig. 3). The percentage of parvalbumin-positive neurites vs. all neurofilament-positive neurites in the DRG explants was significantly increased (two-way ANOVA: $F_{2,300} = 36.67$, $P = 0.0001$) from the first studied distance 100 μm (32.33 ± 19.00) to 225 μm (17.67 ± 9.16) in the fibronectin-enriched matrices compared with samples grown on control collagen matrices (12.25 ± 6.55 and 3.00 ± 2.45) and laminin type I-enriched matrices (14.56 ± 6.68 and 7.98 ± 6.89) (Fig. 3M). However, the absolute numbers of parvalbumin-stained neurites were similar between fibronectin- and laminin-enriched matrices. Thus, there was a higher number of neurofilament-positive neurites in laminin- than fibronectin-enriched matrices.

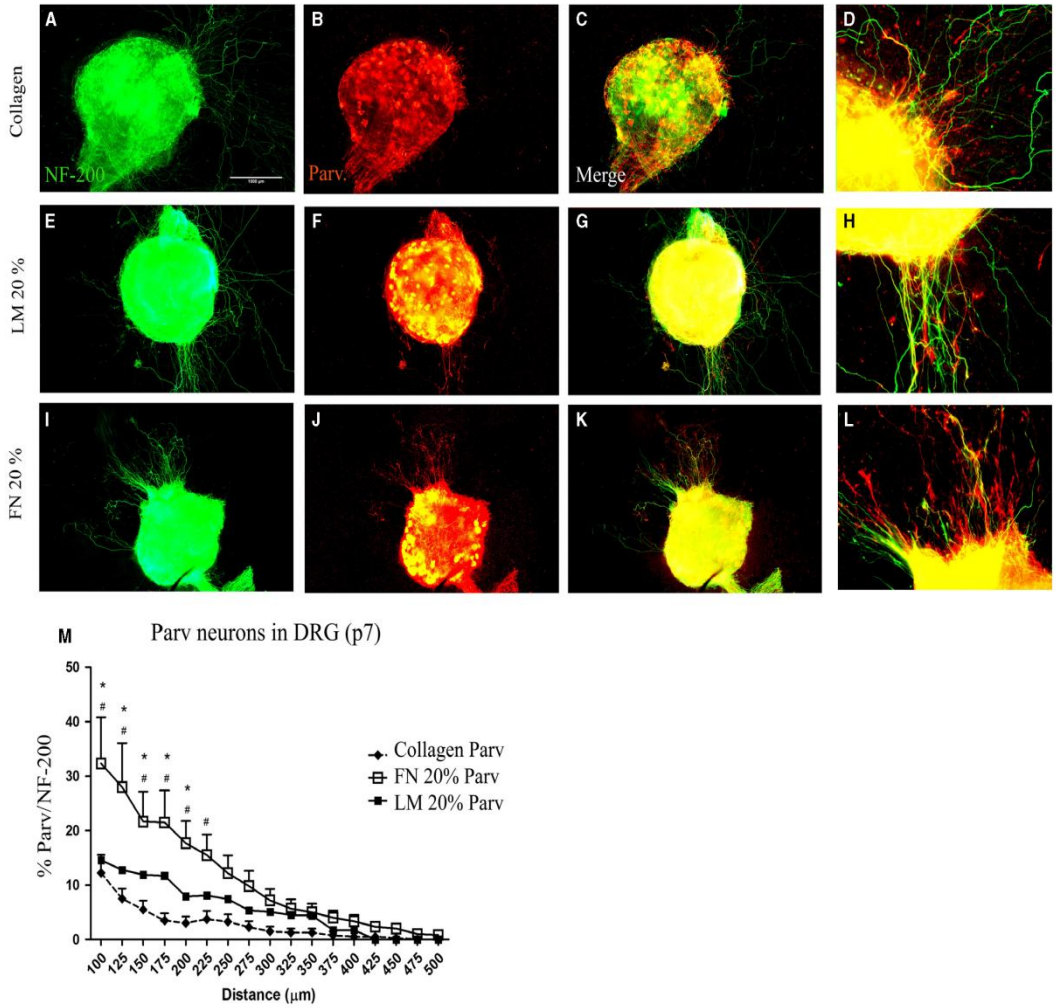

FIG. 3. Representative images of p7 DRG explants grown in a control collagen matrix (top panels), laminin-enriched matrix (middle panels) and fibronectin-enriched matrix (bottom panels). Samples were immunostained against NF-200 (green; A, E and I) to label neurites from myelinated neurons, and against parvalbumin (red; B, F and J) to label proprioceptive neurites. Merged (C, G and K) and magnifications of the merged images (D, H and L). Scale bar = 1 000 μm. Quantification of the percentage of parvalbumin (Pv)-stained postnatal sensory neurons vs.the total neurofilament (NF)-stained sensory neurons that grew to specific distances from the DRG explant body in the different extracellular matrices (M). *$P < 0.05$ vs laminin; #$P < 0.05$ vs collagen.

Neurite outgrowth from DRG explants of p21 rats

Neurite outgrowth in DRG explants of p21 animals was also analysed in laminin type I- and fibronectin-enriched matrices at the highest concentration (20%, v/v) and compared with control collagen matrices after 4 days *in vitro*. As observed in p7 DRG samples, sensory neurites extended longer on laminin-enriched matrices (182.01 ± 24.14%) vs. control collagen matrices (100 ± 13.46%, Bonferroni $t = 8.056$, $P = 0.0001$) and compared with fibronectin-enriched samples (139.44 ± 18.45%, Bonferroni $t = 3.681$, $P = 0.0014$). Neurite extension was also higher in fibronectin-enriched matrices (Bonferroni $t = 3.842$, $P = 0.0008$) than in control collagen matrices (Fig. 4A). The number of sensory neurites emerging from DRG embedded on laminin-enriched matrices was increased significantly (two-way ANOVA: $F_{2,375} = 179.2$, $P = 0.0001$) from the first defined distance (50 μm) to 350 and 450 μm compared with fibronectin-enriched or collagen control matrices, respectively. Further from these distances, we did not observe significant differences between groups of cultures, although the tendency of laminin to favour neurite growth was maintained (Fig. 4B).

We analysed the percentage of parvalbumin-positive neurites, and thus proprioceptive sensory neurons, in DRG explants of p21 rats. NF-200-positive neurites were abundantly observed in the matrices, but parvalbumin-positive neurites were scarcely detected (Fig. 5). The percentage of parvalbumin-positive neurites with respect to the neurofilament-positive neurites was markedly reduced from p7 to p21 animals. Therefore, in collagen control matrices, less than 1% of the neurofilament-positive neurites were also parvalbumin positive in p21 DRG, and about 12% in p7 DRG. Although, the percentage of parvalbumin-positive neurites was higher in fibronectin- and laminin-enriched matrices compared to control collagen matrices in p21 animals, this was not accompanied by significant changes (Fig. 5M).

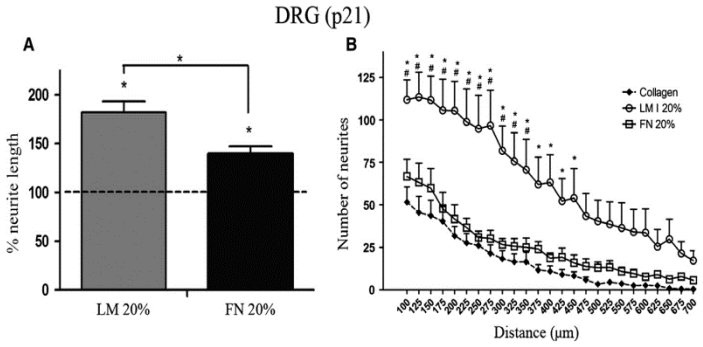

FIG. 4. Neurite outgrowth of adult sensory neurons on different extracellular matrices *in vitro*. Length of the longest neurites of p21 DRG explants (A), expressed as percentage vs. values obtained in control collagen matrices. Quantification of the number of neurites that grew to specific distances from the DRG explant (B). **P* < 0.05 vs laminin; #P < 0.05 vs collagen. FN, fibronectin; LM, laminin.

Preference of motor and sensory axons for different ECM in vivo

We analysed if motor and sensory axons of adult rats have preferential growth *in vivo* on laminin- or fibronectin-enriched matrices, by repairing a resection of the sciatic nerve with a chitosan guide (6-mm gap) filled with the different matrices. Fluorogold was applied 8 mm distal to the distal suture to label the number of regenerating neurons 31 days after the lesion (Fig. 6A and B). The number of motoneurons that had regenerated in the rats with laminin- (808 ± 122, Bonferroni $t = 0.672$, $P = 0.67$) and fibronectin-enriched matrices (819 ± 176, Bonferroni $t = 0.722$, $P = 0.72$) in the conduit showed no significant differences from the number of motorneurons in the collagen-based matrix (662 ± 208) (Fig. 6D). The number of regenerated labelled sensory neurons at 31 days followed a similar pattern, being higher in laminin- (3457 ± 1017, Bonferroni $t = 0.905$, $P = 0.99$) and fibronectin-enriched matrices (4182 ± 110, Bonferroni $t = 1.549$, $P = 0.43$) compared with the collagen control matrix (2438 ± 778) but without significant differences between groups (Fig. 6C).

DRG samples were also processed for immunohistochemistry to label parvalbumin-positive neurons. However, co-localization between parvalbumin-positive neurons and fluorogold-labelled neurons was not observed at 19 days (data not shown) or at 31 days post-lesion (Fig. 7).

Expression of integrins α5β1 and α7β1 in DRG and spinal cord neurons

By semiquantitative reverse transcriptase PCR, we evaluated the expression of α7β1 integrin, a laminin receptor, and α5β1 integrin, a fibronectin receptor, on L4 and L5 DRG and spinal cord 2 days after sciatic nerve injury *in vivo*. Both integrins were up-regulated after injury compared with the contralateral non-injured site both in DRG (α5: 2.60 ± 0.78, Bonferroni $t = 3.394$, $P = 0.012$; α7: 2.69 ± 0.57, Bonferroni $t = 3.585$, $P = 0.0081$) (Fig. 8A) and in the spinal cord (α5: 2.78 ± 0.08, Bonferroni $t = 4.100$, $P = 0.0028$; α7: 3.86 ± 0.67, Bonferroni $t = 6.588$, $P = 0.0001$) (Fig. 8B).

When analysing the expression of these integrins *in vitro*, we found that α5β1 was more markedly up-regulated than α7β1 integrin in p7 animals in both DRG (Bonferroni $t = 4.720$, $P = 0.0019$) and SC (Bonferroni $t = 4.720$, $P = 0.0001$) explants cultured for 2 days when compared with day 0 samples. In DRG explants, α5β1 was increased 30.26 ± 7.21-fold (Bonferroni $t = 5.923$, $P = 0.0003$) and α7β1 4.86 ± 0.99-fold (Bonferroni $t = 2.981$, $P = 0.017$) (Fig. 8C). In spinal cord slices, α5β1 was increased 8.45 ± 0.16-fold (Bonferroni $t = 38.21$, $P = 0.0001$) but not α7β1 (0.78 ± 0.16-fold) (Bonferroni $t = 1.128$, $P = 0.87$) (Fig. 8D). We also analysed if the addition of fibronectin or laminin in the collagen matrices affected the expression of integrins observed in control collagen matrices. The addition of fibronectin or laminin to the matrix changed the expression of integrins α5β1 and α7β1. Although both integrins were up-regulated in both cases in the DRG culture, they were less up-regulated compared with collagen (α5β1in DRG laminin vs. collagen: Bonferroni $t = 11.57$, $P = 0.0001$; fibronectin vs. collagen: Bonferroni $t = 16.47$, $P = 0.0001$; α7β1 in DRG laminin vs. collagen: Bonferroni $t = 9.814$, $P = 0.0001$; fibronectin vs. collagen: Bonferroni $t = 14.16$, $P = 0.0001$) (Fig. 9A and B).

In contrast, the addition of fibronectin or laminin to the matrix did not change the expression of these integrins in the spinal cord slices compared with control collagen matrix (α5β1 in SC laminin vs. collagen: Bonferroni $t = 1.157$, $P = 0.80$; fibronectin vs. collagen: Bonferroni $t = 0.964$, $P = 0.99$; α7β1 in SC laminin vs. collagen: Bonferroni $t = 0.143$, $P = 0.99$; fibronectin vs. collagen: Bonferroni $t = 0.400$, $P = 0.99$) (Fig. 9C and D).

Discussion

In this study we have analysed whether motor and sensory neurons have substrate preference when growing in a three-dimensional (3D) culture model. Motoneurons from postnatal organotypic spinal cord slices extended longer neurites in fibronectin-enriched matrices, whereas sensory neurite outgrowth from early postnatal DRG was enhanced when either laminin or fibronectin were added to the culture. When analysing subpopulations of sensory neurons, we observed that parvalbumin-positive, presumptive proprioceptive neurons preferentially grew on fibronectin at early postnatal age. However, this differential preference decreased at later ages (p21). *In vivo*, adult motor and sensory neurons do not maintain the substratum preference observed in postnatal stages.

Substrate preference of different types of peripheral neurons

We have used a 3D culture model, previously set up in our laboratory (Allodi *et al.*, 2011), based on organotypic spinal cord slices and DRG explants that allow a comparison of motor and sensory neurite outgrowth. In a previous study we injected a retrotracer in

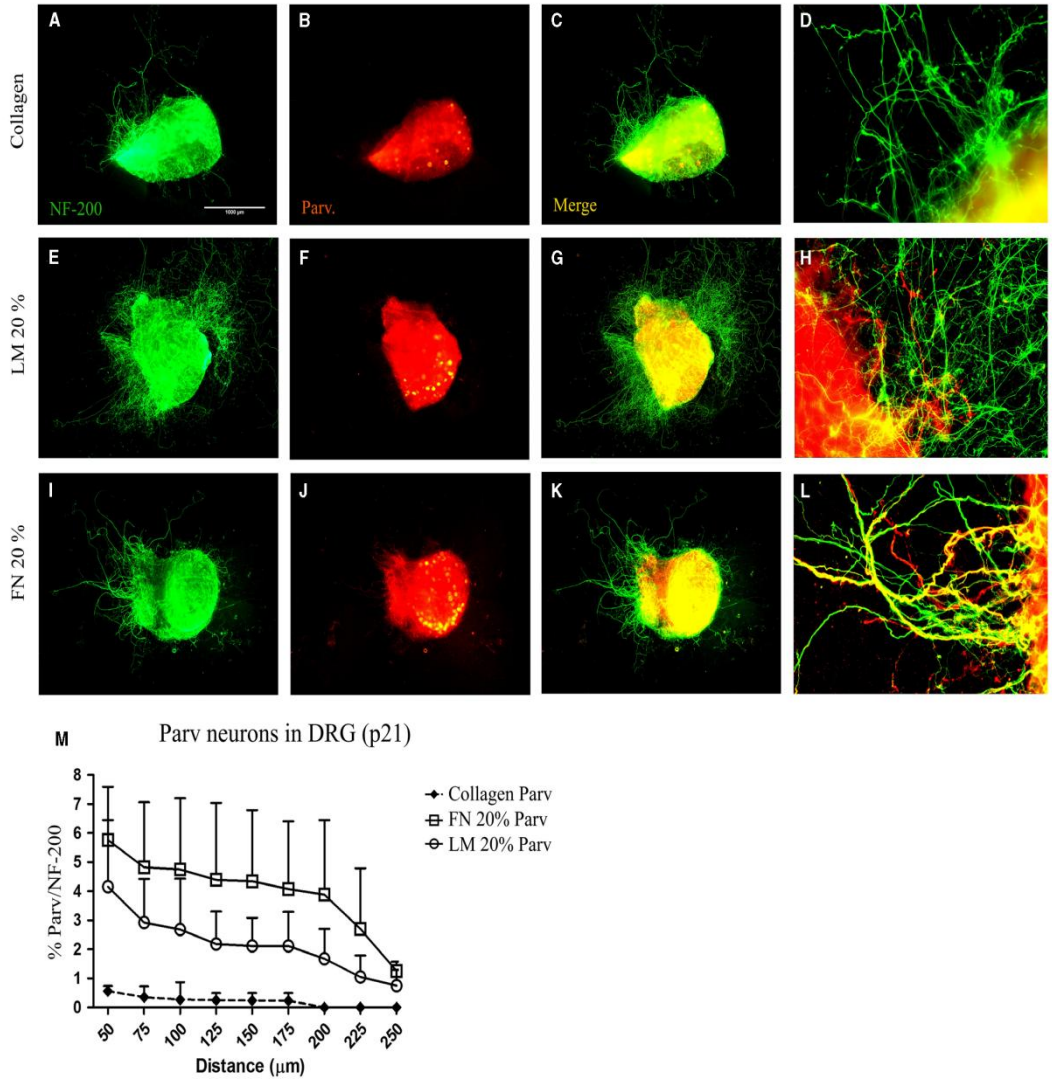

FIG. 5. Representative images of DRG explants of p21 rats grown on different extracellular matrices. Samples were immunostained against NF-200 (green; A, E and I) to label neurites from myelinated neurons, and against parvalbumin (red; B, F and J) to label proprioceptive neurites. Merged (C, G and K) and magnifications of the merged images (D, H and L) of cultures in control collagen matrices (top panels), laminin-enriched matrices (middle panels) and fibronectin-enriched matrices (bottom panels). Scale bar = 1000 μm. Quantification of the percentage of parvalbumin-positive neurons vs. the total neurofilament-labeled sensory neurons that grew to specific distances from DRG explants of p21 rats in different extracellular matrices (M). FN, fibronectin; LM, laminin; Parv, parvalbumin.

the muscles of pups 2 days before harvesting of the samples to corroborate that the neurites we were evaluating in this setting were from motoneurons and not from other neurons of the spinal cord (Allodi *et al.*, 2013). An adequate culture to evaluate motor growth is important, as motoneurons have been rarely evaluated *in vitro*. In contrast, DRG neurons, either in dissociated cultures or as explants, have been widely used in the literature. This is due to the demanding protocol needed to culture adult motoneurons (Montoya *et al.*, 2009), and the inconvenient use of embryonic neurons to evaluate regeneration. In contrast, when using organotypic spinal cord cultures, postnatal animals can be used. Unfortunately, this culture is not feasible from adult animals. Therefore, to compare the regenerative preferences of adult motor and sensory neurons, we used an *in vivo* model, complemented with *in vitro* DRG explants.

In the different studies we used a matrix based on type I collagen. Both fibronectin and laminin can bind to all types of collagen. Fibronectin is known to bind more actively to type III collagen (Engvall *et al.*, 1978) whereas laminin binds more actively to type IV collagen (Charonis, 1985), but they have no preferential binding for type I collagen, and therefore the effect of both ECM molecules on motor and sensory regeneration is probably independent of the interactions between the ECM molecules.

We observed that addition of laminin or fibronectin to the collagen based matrix had different effects on neurite outgrowth in p7

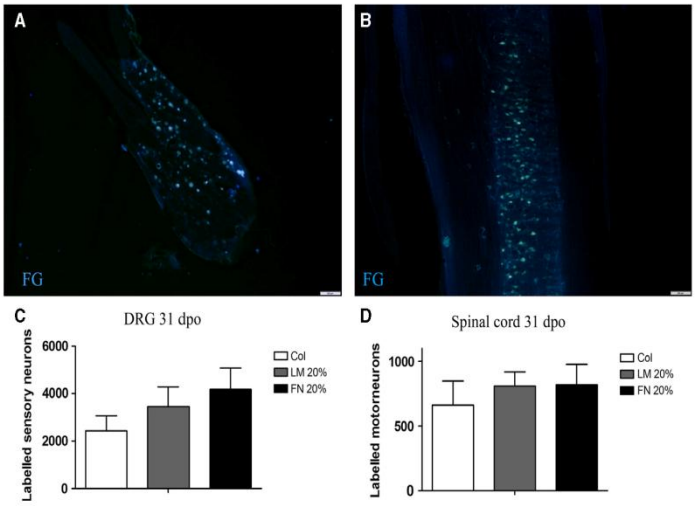

FIG. 6. Regeneration of adult motor and sensory axons in matrices enriched with laminin or fibronectin *in vivo*. Representative micrographs of retrolabelled sensory neurons in the DRG (A) and motoneurons in the spinal cord (B) from a rat after sciatic nerve resection and repair with a chitosan conduit filled with a collagen gel. Counts of regenerated sensory neurons (C) and motoneurons (D) in the groups of rats repaired with a chitosan conduit filled with different extracellular matrices. Fluorogold retrotracer was applied 8 mm from the distal suture 31 days after sciatic nerve injury and repair. dpo, days post-operation; FN, fibronectin; LM, laminin.

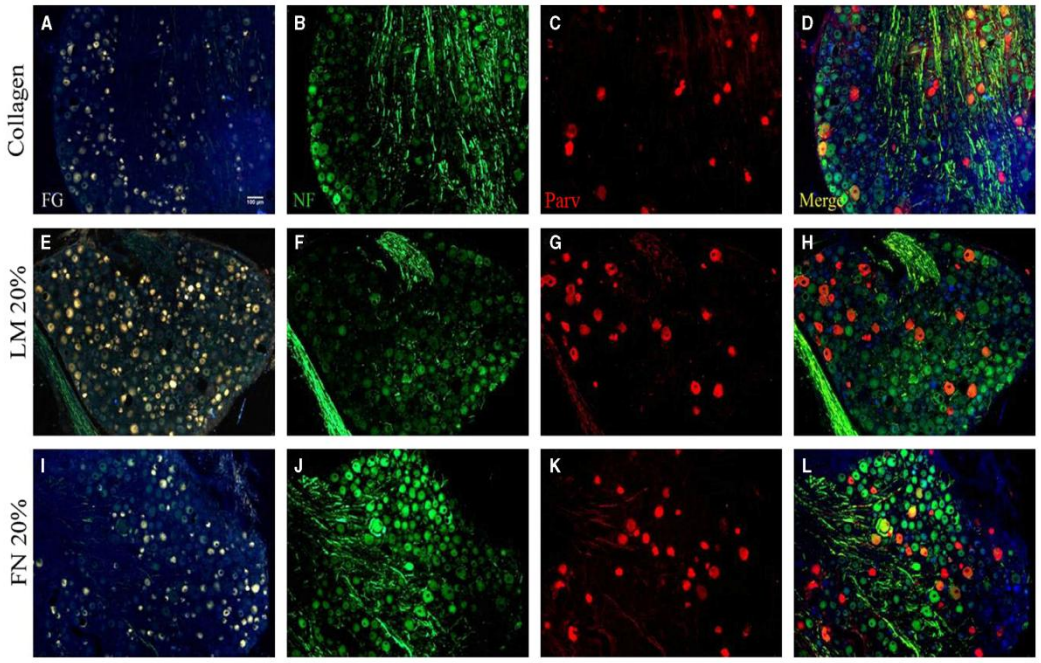

FIG. 7. Regenerative proprioceptive sensory neurons *in vivo*. Representative images of DRG sections from rats with a sciatic nerve resection repaired with a chitosan conduit filled with collagen type I matrix (top panels), laminin-enriched matrix (middle panels) and fibronectin-enriched matrix (bottom panels). Thirty-one days after the surgery, the regenerated neurons were labelled with Fluorogold (in yellow; A, E and I). The same sections were immunostained against NF-200 (green) to label myelinated sensory neurons (B, F and J) and parvalbumin (red) to label proprioceptive neurons (C, G and K). In the merged images (D, H and L) no co-labelling of parvalbumin and Fluorogold can be observed, indicating that the regenerating sensory neurons were not showing a proprioceptive phenotype. Scale bar = 100 μm. FN, fibronectin; LM, laminin.

postnatal cultures. Addition of fibronectin mainly enhanced growth of motor neurites, whereas laminin had stronger effects on sensory neurites. However, the neural population in DRG is heterogeneous (Usoskin *et al.*, 2014). Mechanoreceptive tactile and proprioceptive neurons have large axons and thicker myelin sheaths (Aα and Aβ fibres), whereas protopatic tactile, thermoceptive and nociceptive

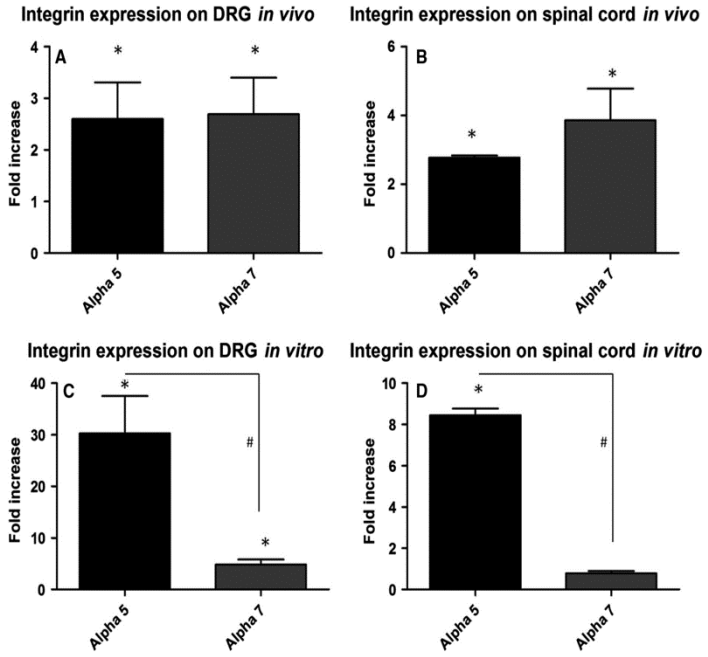

FIG. 8. Integrin expression in adult motor and sensory neurons after axotomy. mRNA expression of integrins α5β1 and α7β1 *in vivo* after sciatic nerve injury, in samples of DRG (A) and spinal cord (B) relative to the non-injured contralateral side values. Expression of integrins α5β1 and α7β1 *in vitro* in p7 DRG explants (C) and spinal cord slices (D) embedded in collagen type I after 2 days in culture, compared with day 0 samples. $^{*}P < 0.05$ vs. non-injured animals. $^{\#}P < 0.05$ for differences between integrins.

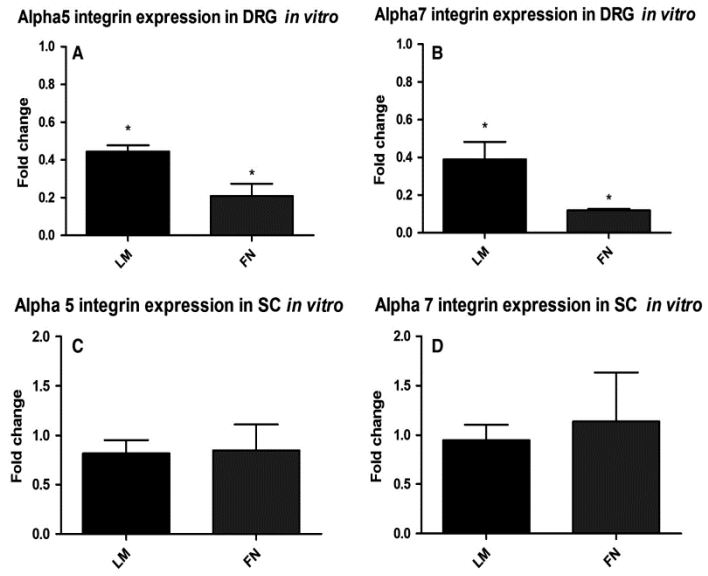

FIG. 9. Changes of integrin expression in neurons exposed to ECM molecules. mRNA expression of integrin α5β1 (A, C) and α7β1 (B, D) in p7 spinal cord slices (A, B) and DRG explants (C, D) maintained in culture in matrices enriched with fibronectin and with laminin compared with control collagen matrix. $^{*}P < 0.05$.

neurons are small (Aδ and C-unymelinated fibres). Tactile neurons project mainly to the skin through cutaneous nerves, whereas proprioceptive neurons reach their target, into muscles or tendons, through muscular nerves. During development, fibronectin is highly expressed in the pathway that proprioceptive neurons follow, which extends ventrally from the DRG towards the motor axons.

Fibronectin is expressed at early stages, when cutaneous sensory neurons have not yet extended their axons, suggesting a different ability of both subtypes of sensory neurons to interact with ECM cues (Guan *et al.*, 2003).

In our study we wanted to evaluate if proprioceptive neurons had different substrate preference from other myelinated neurons. To label the population of myelinated neurons we used NF-200, a marker for neurofilament heavy chain. We used parvalbumin to label proprioceptive neurons, as it is well accepted that ~90% of the parvalbumin-positive neurons represent proprioceptive sensory neurons which project to skeletal muscle (Honda, 1995). Therefore, we estimated the growth of presumptive proprioceptive neurons (parvalbumin+) and the growth of presumptive cutaneous myelinated neurons (neurofilament+, parvalbumin−). We assumed that most neurofilament-positive neurons that were not parvalbumin positive would be large tactile afferents from the skin, although they can also be thin myelinated fibres, present in both cutaneous and muscular branches. Interestingly, neurons from p7 animals show a similar preference for laminin and fibronectin than those described at embryonic stages (Guan *et al.*, 2003). The absolute number of proprioceptive sensory neurites was similar in fibronectin- and laminin-enriched cultures, whereas non-parvalbumin neurons preferred laminin. When using p21 DRG explants we observed that parvalbumin-positive neurons grew similarly in fibronectin- and laminin-enriched matrices. However, the proportion of proprioceptive sensory neurons was dramatically reduced compared with that found in DRG of p7 animals.

When analysing the effects *in vivo* in adult rats, we found that either laminin- or fibronectin-enriched matrices placed within a nerve conduit slightly increased the number of regenerating motor and sensory neurons when compared with control collagen matrices, although the differences did not reach significance. When analysing the populations of regenerated sensory neurons, we did not observe co-localization between the retrotracer fluorogold and parvalbumin, indicating that none of the regenerating neurons was proprioceptive. Therefore, these data suggest a slow regeneration capacity of adult proprioceptive neurons compared with other types of sensory neurons.

These results were consistent with our results *in vitro*, in which adult parvalbumin-positive neurons showed poor neurite growth compared with postnatal ones. This is an unexpected finding as it is assumed that the regenerative abilities of the different populations of peripheral neurons are similar, even when large sensory neurons survive better after peripheral nerve injuries than small neurons (Tandrup *et al.*, 2000). When comparing sensory neurons from cutaneous and muscular branches, it has been described that neurons from cutaneous branches are more sensitive to nerve injury, although both types of populations have a similar regenerative ability (Welin *et al.*, 2008). However, in that study the authors did not separately evaluate the different sub-populations of sensory neurons from muscular nerves that also contain small myelinated and unmyelinated axons. Moreover, we cannot discard that proprioceptive neurons reduce the expression of parvalbumin after axotomy *in vivo*.

Expression of integrins α7β1 and α5 β1 in different populations of peripheral neurons after axotomy

Several integrin subunits, including integrins α5 and α7 of the β-1 integrin family, are up-regulated in sensory and motor neurons during peripheral nerve regeneration (Lefcort *et al.*, 1992; Ekström *et al.*, 2003; Wallquist *et al.*, 2004; Gardiner *et al.*, 2007), and an increased expression of integrin receptors after peripheral nerve injury is correlated with successful regeneration (Previtali *et al.*, 2001). Integrins mediate neuronal outgrowth on ECM molecules (De Curtis, 1991; Reichardt & Tomaselli, 1991; Letourneau *et al.*, 1994; Gardiner, 2011). The distribution of the integrin receptors for the ECM components and the regulation of the receptor expression or their function in different classes of neurons may generate unique profiles of affinity for ECM molecules contributing to characteristic patterns of growth cone migration and neurite extension (Letourneau *et al.*, 1994). During development, differences in the subtypes of integrin expression have been correlated with changes in the prospective fate of the sensory neurons. This different expression of integrin subtypes comes prior to target innervations and may be one of the earliest markers for sensory neuron fate (Guan *et al.*, 2003). Integrin α5, receptor for fibronectin, is more markedly expressed in proprioceptive sensory neurons, whereas expression of α7β1 integrin, receptor for laminin, is higher in cutaneous neurons.

Because motor and sensory neurons had a substratum preference at p7, these two populations of neurons might have a different pattern of expression of integrins α5β1 and α7β1. We found an up-regulation of both types of integrins after 2 days in culture, this increase being lower in spinal cord slices than in DRG explants. This up-regulation was expected, as it is known that many integrins are up-regulated after peripheral nerve injury. Among them, α7β1 seems to be crucial for sensory (Ekström *et al.*, 2003; Gardiner *et al.*, 2005) and motor regeneration (Werner *et al.*, 2000), whereas the role of α5 is more controversial. Gardiner *et al.* (2007) showed that α5β1 was not up-regulated in axotomized sensory neurons, although it played a role in neurite extension after a preconditioning injury. However, they checked levels of integrins 7 days after injury, whereas we evaluate the expression of this integrin 2 days after injury. It is possible that this integrin has a fast up-regulation and levels normalized after 7 days. On the other hand, differential expression of integrins in subpopulations of neurons is also controversial, with some authors pointing that α7β1 can be highly expressed in small but not large sensory neurons (Werner *et al.*, 2000), and others stating that α7β1 is similarly expressed in all size sensory neurons (Wallquist *et al.*, 2004).

During development, cutaneous and proprioceptive sensory neurons express both integrins, but it is the relative amount of particular integrins that determines its ability to interact with fibronectin (Guan *et al.*, 2003). Therefore, when comparing expression of α7β1 and α5β1 integrins in motor and sensory neurons in p7 cultures, we observed that although both types of neurons expressed higher levels of α5β1 than of α7β1 integrins, the proportion was slightly higher in spinal cord (10×) than in DRG (6×). In contrast, these proportions were not maintained in the adult animal measured *in vivo*. We cannot exclude that the differences in integrin expression between adult and postnatal neurons are due to the different environment in the two settings used. *In vitro* conditions might change the expression of integrins compared with the *in vivo* situation. However, if we compare the relationship between both integrins, we can observe higher levels of α7 integrin than of α5 integrin in the adult spinal cord, a fact that can explain why motor axons lose their preference for fibronectin in adult stages. In DRG, similar amounts of both integrins were found. On the other hand, integrin increase induced by the injury was lower in adult than in postnatal DRG. It is well known that there is an age-related decrease in integrin levels in sensory neurons that correlates with the lower ability of adult neurons to regenerate (Condic, 2001). In contrast, expression of integrins, mainly α7β1, was lower in postnatal spinal cords than in adult cords. However, it is important to note that, in organotypic cultures, the manipulation of DRG is easier than for spinal cord which has to be sectioned, a process that may affect the integrity and viability of some motorneurons (Guzmán-Lenis *et al.*, 2009; Allodi *et al.*, 2011). Therefore, the lower increase of integrin

expression can be due to the reduction in the number of available motoneurons at 2 days compared with basal conditions. In the adult animal, where survival is not compromised, integrin expression would be higher. Even with this limitation, the relative amounts of integrins indicate that, after injury, postnatal motoneurons up-regulate α5β1 10-fold compared with α7β1, whereas adult motoneurons express slightly more α7β1 than α5β1.

When focusing on the effects of the enrichment of the matrices with ECM on the expression of the two integrins, we found that fibronectin and to a lesser extent laminin reduced the up-regulation of the two integrins compared with samples grown on collagen type I control matrices. Therefore, it seems that over exposure of neurons to ECM molecules reduces the up-regulation of α5β1 and α7β1 integrins, although levels are still higher than basal. However, it is known that in a laminin-rich environment, a combined increase in cAMP and integrin activation can lead to growth cone collapse (Lemons & Condic, 2008). By contrast, in non-permissive substrata, increased expression of integrins leads to neurite outgrowth *in vitro* (Condic, 2001) and *in vivo* (Leclere *et al.*, 2007). Therefore, it is important that neurons establish a substratum-specific balance of integrin expression, integrin activation and cAMP to be able to grow in different environments (Lemons & Condic, 2008). Thus, neurons exposed to very permissive substratum, such as our collagen matrices enriched with laminin and fibronectin, where integrins are highly activated, would need lower over-expression of these receptors to successfully grow.

Conclusions

In this study we show that motor and sensory axons have a different ECM substrate preference at early postnatal stages. Motor neurites grow better in fibronectin- than in laminin-enriched matrices. Proprioceptive sensory neurons, which accompany motor axons into muscular branches, also grow better on fibronectin than other myelinated sensory neurons that grow better on laminin. However, this difference on neuron subtype selectivity to different ECM molecules is not observed in adult neurons. Postnatal and adult axotomized neurons also present a different pattern of expression of integrins. The greater increase in α5β1 vs. α7β1 in young neurons is lost in the adult, when both integrins are overexpressed similarly.

Conflict of interest

No competing financial interests exist.

Acknowledgments

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Abbreviations

CT, cycle threshold; DRG, dorsal root ganglia; ECM, extracellular matrix.

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CHAPTER II

Tubulization with chitosan guides for the repair of long gap peripheral nerve injury in the rat

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TUBULIZATION WITH CHITOSAN GUIDES FOR THE REPAIR OF LONG GAP PERIPHERAL NERVE INJURY IN THE RAT

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Biosynthetic guides can be an alternative to nerve grafts for reconstructing severely injured peripheral nerves. The aim of this study was to evaluate the regenerative capability of chitosan tubes to bridge critical nerve gaps (15 mm long) in the rat sciatic nerve compared with silicone (SIL) tubes and nerve autografts (AGs). A total of 28 Wistar Hannover rats were randomly distributed into four groups ($n = 7$ each), in which the nerve was repaired by SIL tube, chitosan guides of low (~2%, DAI) and medium (~5%, DAII) degree of acetylation, and AG. Electrophysiological and algesimetry tests were performed serially along 4 months follow-up, and histomorphometric analysis was performed at the end of the study. Both groups with chitosan tubes showed similar degree of functional recovery, and similar number of myelinated nerve fibers at mid tube after 4 months of implantation. The results with chitosan tubes were significantly better compared to SIL tubes ($P < 0.01$), but lower than with AG ($P < 0.01$). In contrast to AG, in which all the rats had effective regeneration and target reinnervation, chitosan tubes from DAI and DAII achieved 43 and 57% success, respectively, whereas regeneration failed in all the animals repaired with SIL tubes. This study suggests that chitosan guides are promising conduits to construct artificial nerve grafts. © 2014 Wiley Periodicals, Inc. Microsurgery :300–308, 2015.

Peripheral neurons have the ability to regenerate and reinnervate target organs when there is a suitable environment to do so, thus allowing some degree of recovery of the lost functions, depending upon severity of the injury and quality of the repair.[1,2] On the other hand, after complete nerve transection, surgical repair is mandatory to reunite the two stumps to facilitate regeneration. When the distance to be bridged does not allow direct suture between stumps, the interposition of a nerve graft is used as the gold standard technique in clinical practice. The use of autografts (AGs) has some disadvantages, such as the sacrifice of a healthy nerve of the subject affected, the mismatch between the injured nerve and the grafts, and the limited source of donor nerves. As an alternative to AGs, the use of biogenic conduits[3,4] or artificial guides has been proposed.[5,6] However, the success of regeneration when using artificial nerves guides is limited by the length of the gap (less than 15 mm in the rat).[7] Strategies focused on altering the characteristics of the guidance tubes to increase the ability to sustain axon regeneration, have been attempted to overcome the gap limitation.[5,8]

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The cross-sectional dimensions of the tube[9,10] and the materials, in which the tubes are constructed are other factors that also affect the final outcome. Initial studies used nondegradable silicone (SIL) tubes[11] but now-a-days it is considered that the ideal material should be biocompatible, have sufficient mechanical stability, be flexible, be porous to facilitate the incorporation of nutrients, and degrade into nontoxic products to prevent long-term body reaction.[12] Indeed, nerve guides made from collagen (NeuraGen™),[13] polyglycolic acid (Neurotube™),[14] and polylactide caprolactone (Neurolac™)[15] have been approved for clinical use. However, for the moment nerve guides have been approved and tested in humans only for the repair of relatively short gap injuries.

Among the different materials experimentally tested to improve the results obtained by available nerve guides, chitosan is a promising alternative.[16,17] Chitosan is a polymer derived from chitin, a molecule obtained from the exoskeleton of arthropods, shellfish, and cell wall of fungi,[18] and fulfills the characteristics above indicated to construct nerve guides. Some *in vivo* studies reported the benefits of chitosan scaffolds and guides in peripheral nerve repair. However, most of these studies combined the chitosan-based guide with internal fillers, such as neurotrophic factors,[19,20] molecules or peptides from the extracellular matrix[21,22] and supporting cells,[23,24] in non-critical peripheral nerve gaps. Recent technological improvements overcame the poor mechanical strength of chitosan tubes, which was one of the main factors limiting their use as single material for nerve guides. In this study, we aimed to evaluate the capabilities of hollow chitosan tubes to sustain regeneration when used to repair a critical 15 mm sciatic nerve gap in rats, and compare their outcome to standard SIL tubes and the ideal nerve

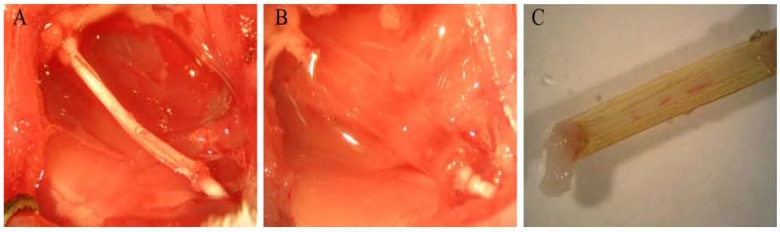

Figure 1. Representative images of a sciatic nerve resected and repaired with an autograft (AG) of 15 mm (A), or with a hollow DAII chitosan tube of 19 mm leaving a 15 mm gap (B). Regenerated nerve found 4 months after repairing the sciatic nerve with a hollow DAII chitosan tube (C).

AG. We tested two types of chitosan conduits with different degrees of acetylation, whose characteristics in terms of biocompatibility and adequacy for nerve repair have been recently described.[17]

MATERIALS AND METHODS

Animals

A total of 28 female Wistar Hannover rats, aged 3 months were used in the experiment. The animals were housed in plastic cages, maintained at 22°C with a 12 hour light/dark cycle and allowed free access to water and food. The experimental procedures were approved by the Ethical Committee of our institution and followed the rules of the European Communities Council Directive.

Experimental Design and Surgical Procedure

Animals were randomly distributed into one of four experimental groups according to the type of repair: SIL repaired animals (n = 7), chitosan of low degree of acetylation (~2%) (DAI) (n = 7), chitosan of medium degree of acetylation (~5%) (DAII) (n = 7), and AG (n = 7). The chitosan tubes manufacturing and characteristics were the same as reported in a previous study.[17]

All surgical procedures were performed with aseptic operating conditions and under anesthesia with ketamine/xylazine (90 mg/kg and 10 mg/kg i.p., respectively). Chitosan tubes were immersed in saline solution 20 minutes before implantation to reduce the strength of the tube. Under a dissecting microscope, the sciatic nerve was exposed and cut 6 mm distal to the exit of the gluteal nerve, and a nerve segment of 6 mm was resected. The distal and proximal stumps were fixed by two epineural 10-0 sutures into the ends of the implanted tube leaving a 15 mm gap (Fig. 1). All the tubes used had a length of 19 mm, and an internal diameter of 2 mm. Once implanted, the tubes were filled with sterile physiologic saline solution. For the AG group, the sciatic nerve was cut at the same level explained above and 15 mm distally. The nerve segment resected was flipped and sutured to bridge the gap with two 10-0 sutures at each side. The muscle plane was then sutured with reabsorbable 5-0 sutures, the skin with 2-0 silk sutures, and the wound was disinfected. Animals were treated with amitriptyline for preventing autotomy.[25]

Electrophysiological Tests

Functional reinnervation of target muscles was assessed at 7, 30, 60, 90, and 120 days postoperation (dpo). Animals were anesthetized with pentobarbital (40 mg/kg i.p.). The sciatic nerve was stimulated by transcutaneous electrodes placed at the sciatic notch, and the compound muscle action potential (CMAP) of tibialis anterior and plantar muscles was recorded using monopolar needle electrodes, placing the active one in the muscle belly and the reference in the fourth toe.[26] During the tests, the rat body temperature was maintained by means of a thermostated warming flat coil. The amplitude and the latency of the M-wave were measured. The contralateral limb was used as control.

Functional Evaluation of Sensory Recovery

The threshold of nociceptive responses to mechanical and thermal stimuli was evaluated on both hindpaws by means of algesimetry tests at 7, 21, 45, 60, 90, 92, and 120 dpo. For both tests, the lateral area (innervated by tibial and sural nerves, both being branches of the sciatic nerve) of the plantar surface was tested.[27] The contralateral paw of each rat was tested as control each day, to overcome possible variations between testing conditions. Sensibility to mechanical stimuli was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers 30 minutes before the experiment for habituation. The mechanical nociceptive threshold was taken as the mean of three measurements per paw region, and expressed as the force (in grams), at which rats withdrew their paws in response to the stimulus. A cutoff force was set to 40 g, at which stimulus lifted the paw with no response. Thermal sensibility was assessed by using a plantar algesimeter (Ugo Basile, Comerio, Italy). The beam of a projection lamp was focused onto the hindpaw plantar surface pointing at the lateral side. The thermal nociceptive threshold was taken as the mean of three

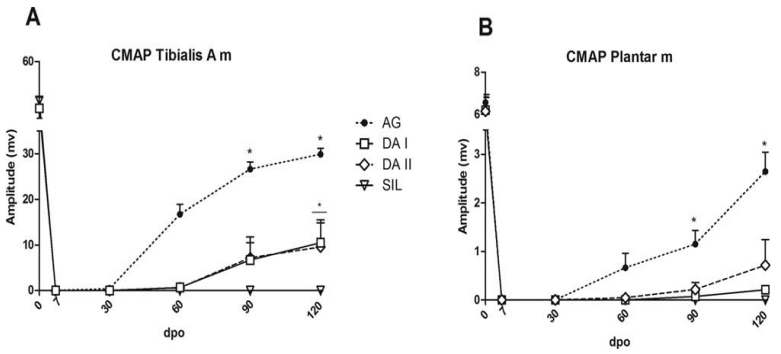

*Figure 2. Mean amplitude of the compound muscle action potential (CMAP) of tibialis anterior (A) and plantar muscles (B) of the injured hind limb of the rats during 4 months after sciatic nerve lesion and repair. *P < 0.05 AG vs. DAI, DAII, and SIL; *P < 0.05 DAI and DAII vs. SIL.*

trials, and expressed as the latency (in seconds) of paw withdrawal response. A cutoff time was set at 20 seconds to prevent tissue damage. All the values are presented as percentage of response with respect to the contralateral non-injured paw.

Histology and Morphometry

Four months after the injury, animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline solution (0.1*M*, pH = 7.4). After perfusion, the regenerated nerves were harvested and postfixed in 3% paraformaldehyde and 3% glutaraldehyde phosphate-buffered solution. The nerves were postfixed in osmium tetroxide (2%, 2 hours, 4°C), dehydrated through ascending series of ethanol, an embedded in Epon resin. Nerves were sectioned using an ultramicrotome (Leica). Semithin sections (0.5 μm) were stained with toluidine blue and examined by light microscopy. Images of the whole sciatic nerve were acquired at 10× with a digital camera, while sets of images chosen by systematic random sampling of squares representing at least 30% of the nerve cross-sectional area were acquired at 100× from mid and distal parts of the tube or graft. Measurements of the cross-sectional area of the whole nerve, and counts of the number of myelinated fibers, were performed by using Image software (National Institutes of Health). Morphometrical analysis was made using a protocol previously described[28] to obtain measurement of regenerated fibers and axons diameter, myelin thickness, and the *g*-ratio.

Muscle Weight

Once the animals were perfused, tibialis anterior and gastrocnemius muscles were dissected. The muscles were kept in a tube placed in an incubator at 37°C for 2 days to allow them to dry, and then weighted.

Statistics

Results are expressed as mean ± SEM. Statistical comparisons between groups and intervals for algesimetry and electrophysiological tests results were made by two-way ANOVA for repeated measurements, followed by Bonferroni post hoc test. Statistical analysis of histological results was made by one-way ANOVA followed by Bonferroni post test. Differences were considered significant if $P < 0.05$.

RESULTS

Muscle Reinnervation

Nerve conduction tests performed 1 week after sciatic nerve injury demonstrated complete denervation of the hindlimb muscles. At 30 dpo, four of six rats in the AG group had evidence of reinnervation in the tibialis anterior muscle, whereas in the groups with chitosan tubes, the first CMAPs were recorded at 60 dpo in two of seven animals. The CMAPs progressively increased in amplitude and were recorded in higher number of animals over time. At the end of follow up (120 dpo), reinnervation of the tibialis anterior muscle was observed in all the animals in the AG group, in three of seven rats repaired with the chitosan tubes and in none of the rats repaired with SIL tube. When comparing the mean CMAP amplitude between groups at the end of follow up (all the animals included in the analysis), the AG group showed a mean amplitude (29.90 ± 1.40 mV) significantly higher than SIL (0 ± 0 mV; $P < 0.01$), DAI (10.50 ± 5.00 mV; $P < 0.01$), and DAII (9.54 ± 5.75 mV; $P < 0.01$) groups (Fig. 2A). Significant differences were also found between the two groups repaired with chitosan guides compared to SIL group ($P < 0.05$), but no differences ($P > 0.05$) were observed between DAI and DAII groups.

At the more distal interosseous plantar muscle, the first CMAPs were recorded as small polyphasic potentials at 60 dpo in all the animals of the AG group, and in two animals of the DAII group, whereas the first signs of plantar reinnervation appeared at 90 dpo in rats of the DAI group. After 4 months follow-up, plantar muscle reinnervation was detected in all the animals in the AG

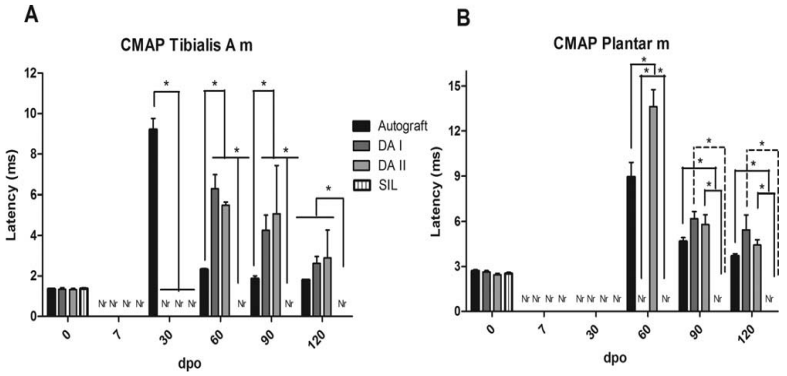

*Figure 3. Mean latencies of the tibialis anterior (A) and plantar muscles (B) CMAP recorded in the regenerated rats during the 4 months follow-up. *P < 0.05.*

group, in three of seven rats in group DAI, in two of seven rats in group DAII, and in none of group SIL. Mean CMAP amplitude in group AG was significantly higher (2.65 ± 0.49 mV) compared to SIL (0 ± 0 mV, $P < 0.01$), DAI (0.212 ± 0.10 mV, $P < 0.01$), and DAII (0.716 ± 0.53 mV, $P < 0.01$) groups (Fig. 2B). Significant differences were not observed within the tube-repaired groups ($P > 0.05$). When CMAPS were recorded, the latency of the waves was considerably longer than normal during the first stages of reinnervation and tended to shorten with time toward normal values. At the end of follow-up, latencies were 1.81 ± 0.03 ms in the AG group, 2.63 ± 0.31 ms in group DAI and 2.89 ± 1.38 ms in group DAII for the tibialis anterior muscle, and 3.69 ± 0.18 ms in group AG, 5.44 ± 0.89 ms in group DAI, and 4.43 ± 0.25 ms in group DAII for the plantar muscle (Fig. 3B).

Recovery of Nociceptive Sensibility

Withdrawal responses to mechanical stimuli, evaluated by means of the Von Frey test, showed that animals had no responses in the denervated paw until 30 dpo, and therefore they were penalized with a cut off value of 40 g. From 60 to 90 dpo most rats showed withdrawal responses at lower stimulus intensity than in the contralateral side. After elimination of the saphenous nerve at 90 dpo, measurements made at120 dpo showed that all the rats of the AG group had withdrawal responses to mechanical stimuli (17.35 ± 7.16 g at 120 dpo) at lower intensity than in the contralateral paw (27.28 ± 1.76 g, $P < 0.05$). Mean values in the chitosan tubes groups were higher (34.12 ± 4.34 g, $P < 0.05$ for DAI; 31.45 ± 4.26 g, $P < 0.05$ for DAII), due to failed regeneration of some animals that did not respond to mechanical stimuli. Values of the subset of rats that had reinnervated were similar to the values observed in AG rats. None of the animals of the SIL group responded (40.0 ± 0.0 g, $P < 0.05$) indicating absence of sensory reinnervation of the hindpaw (Fig. 4A).

Withdrawal responses to heat stimulation in the plantar test showed similar results than the ones observed for the Von Frey test. Denervated paws did not respond to the hot stimuli on the sciatic lateral region until 45 dpo. At 120 days withdrawal latencies in the AG group (12.75 ± 1.38 seconds) were similar to the contralateral paw (12.36 ± 0.76 seconds, $P > 0.05$). After saphenous nerve cut, some of the animals of the chitosan groups did not respond to heat stimuli, due to lack of reinnervation in the sole, thus resulting in slightly higher mean values (16.80 ± 2.19 seconds, $P > 0.05$ for DAI; 16.87 ± 1.66 seconds, $P > 0.05$ for DAII). None of the rats repaired with SIL tubes withdrew the paw when applying the heat stimuli (20.0 ± 0.0 seconds, $P < 0.05$) (Fig. 4B).

Histological Results

Macroscopic examination of the injured nerves after the 4 months follow-up showed that all the AG repaired nerves had good regeneration, whereas three of the seven rats in the DAI group and four of the seven rats in the DAII group presented a regenerative cable inside the tube. The regenerated nerve had a compact appearance and occupied the center of the tube lumen (see Fig. 1C). The nerves were surrounded by a thick, homogeneous connective layer, with no signs of inflammatory reaction. The size of the regenerated sciatic nerve found in the AG group was larger than the regenerated cables found in both chitosan groups. There was no regenerative cable in any of the rats of the SIL group.

Transverse sections of the regenerated nerves taken at the midpoint of the graft of the tube and at the distal segment were analyzed under light microscopy (Figs. 5 and 6). To compare the absolute number of myelinated fibers, regenerated and non-regenerated rats were included in the statistical analysis. Non-regenerated animals were given null value. The mean number of

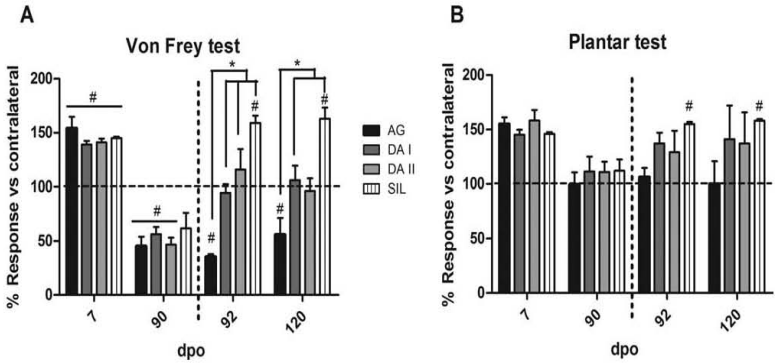

*Figure 4. Mechanical (A) and thermal (B) algesimetry test results. Values were expressed as percentage of withdrawal response to mechanical stimulus (A) and thermal stimulus (B) applied to the lateral side of the injured paw versus the withdrawal response in the uninjured paw. *P < 0.05 for differences between groups. #P < 0.05 for differences between groups and the baseline. Horizontal dotted lines represent the normalized baseline values. Vertical dotted lines indicate when the saphenous nerve was cut.*

myelinated fibers at the midpoint of the graft or the tube was higher in the AG group (14,409 ± 1,564; $P < 0.01$) compared to both chitosan tube groups (DAI: 2,275 ± 1,159; DAII: 2,265 ± 1,534). Significant differences were observed between the chitosan-repaired groups and the SIL group ($P < 0.01$) (Fig. 7A). These differences were also observed at 3 mm distal to the end of the graft or the tube, where the estimated number of myelinated fibers in the AG group (6,865 ± 295; $P < 0.01$) was significantly higher than in both chitosan tube groups (DAI: 1,971 ± 1,013; DAII: 1,644 ± 984). Significant differences were again observed between chitosan groups and the SIL group ($P < 0.01$) (Fig. 7B). When taking into account only the animals, in which a regenerated nerve was found after 4 months postinjury, the number of myelinated fibers was still higher in the AG group compared to both chitosan groups at the mid level (DAI: 5,308 ± 1,161; DAII: 6,218 ± 2,328) and at the distal level (DAI: 4,598 ± 1,070; DAII: 3,837 ± 1,602), but differences were not significant ($P > 0.05$).

The regenerated nerves at the mid level had a larger cross-sectional area in the AG group than in the chitosan tube repaired animals (AG: 0.427 ± 0.173 mm^2; DAI: 0.089 ± 0.056 mm^2; DAII: 0.149 ± 0.095 mm^2) ($P < 0.05$). However, the density of the myelinated fibers was similar in the three groups (AG: 40,714 ± 4,538 axons/mm^2; DAI: 51,886 ± 2,530 axons/mm^2; DAII: 38,877 ± 3,371 axons/mm^2) ($P > 0.05$). At the distal level, the AG group presented a higher density of myelinated fibers per section (32,805 ± 1,686 axons/mm^2) compared to

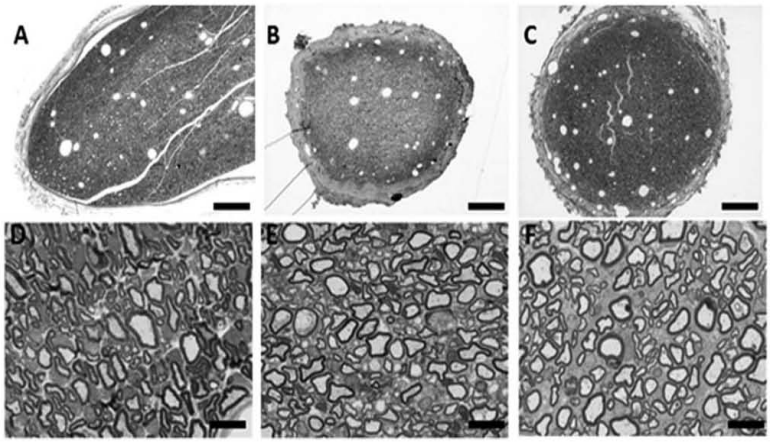

Figure 5. Micrographs of semithin sections of the regenerated nerve taken at the midpoint of the graft or tube 4 months after sciatic nerve resection and repair from a representative animal of group AG (A, D), and one of the animals that regenerated in group DAI (B, E), and DAII (C, F). General appearance of the regenerated nerves (A–C); bar = 100 μm. Higher magnification of the regenerated nerves (D–F); bar = 10 μm.

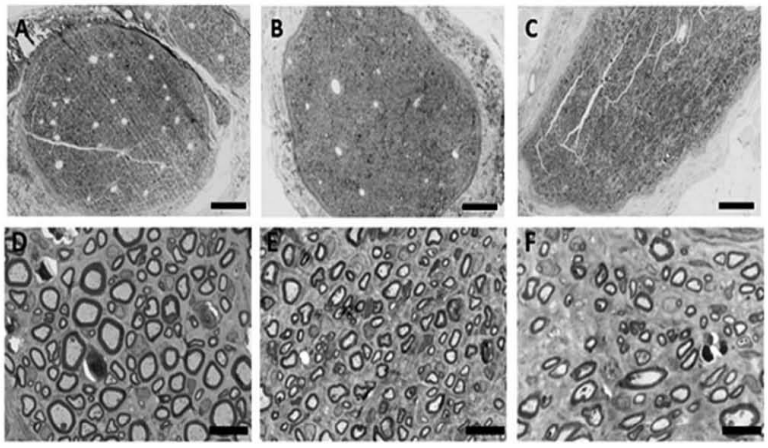

Figure 6. Micrographs of semithin sections of the regenerated nerve taken 3 mm distally to the graft or tube 4 months after sciatic nerve resection and repair from a representative animal of group AG (A, D), and one of the animals that regenerated in group DAI (B, E) and DAII (C, F). General appearance of the regenerated nerves (A–C); bar = 100 μm. Higher magnification of the regenerated nerves (D–F); bar = 10 μm.

both chitosan groups (DAI: 16,865 ± 2,506 axons/mm^2; DAII: 18,399 ± 4,636 axons/mm^2) ($P < 0.05$).

Morphometrical analysis performed at the midpoint of the graft or tube showed that the mean values of the diameter of the regenerated axons were not significantly different ($P > 0.05$) between the AG group (2.12 ± 0.16) compared to DAI (1.9 ± 0.08) and DAII (2.27 ± 0.19) groups. The myelin thickness of the regenerated axons showed no differences ($P > 0.05$) between the AG group (0.59 ± 0.03) and DAI (0.49 ± 0.01) and DAII (0.49 ± 0.03) groups. Regarding the *g*-ratio of the regenerated axons, no significant differences ($P > 0.05$) were observed between the AG group (0.63 ± 0.01) and the DAI (0.64 ± 0.01) and DAII (0.68 ± 0.03) groups (Fig. 8).

Muscle Weight

Both tibialis anterior and gastrocnemius muscles of the AG group had higher weight (56.3 ± 4.66; 62.82 ± 3.57 g; $P < 0.05$) than in animals repaired with chitosan tubes of the two degrees of acetylation (DAI: 35.89 ± 1.56; 45.94 ± 0.72 g; DAII: 35.49 ± 4.37; 52.98 ± 3.79 g, respectively), corroborating a lower degree of reinnervation.

DISCUSSION

In this study, we have investigated the capability of hollow chitosan tubes to sustain axonal regeneration when used to repair a critical 15 mm gap resection of

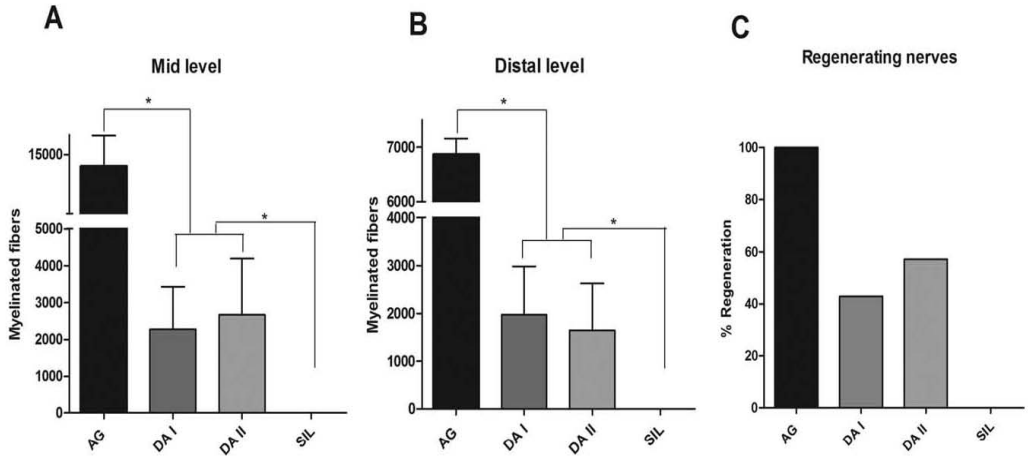

*Figure 7. Number of regenerated myelinated fibers found in the tibial nerve at the mid-tube or graft (A) and 3 mm distal (B) in AG, DAI, DAII, and SIL groups. Animals with no regenerated nerve were also included (with values of 0) in the calculation. *P < 0.05. Percentage of regenerated nerves found at 120 dpo (C).*

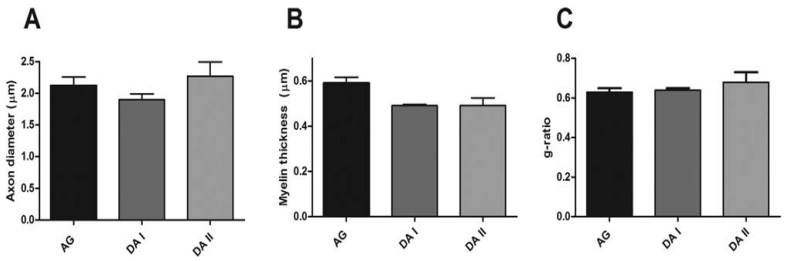

Figure 8. Morphometrical results of axon diameter (A), myelin thickness (B), and g-ratio (C) of the regenerated nerves found at the midpoint of the graft or the tube in groups AG, DAI, and DAII. No significant differences were found between groups.

the sciatic nerve in rats, compared with repair by standard SIL tubes and the gold standard autologous graft. In contrast to AGs, in which all the cases presented effective regeneration and target reinnervation, only three of seven and four of seven animals of groups DAI and DAII, respectively presented a regenerated nerve inside the guide at the end of follow up, whereas regeneration failed in all the animals repaired with SIL tubes. These failures were expected, since the main limitation of nerve guides is the distance between the stumps that may be bridged. As the distance increases, regeneration and functional outcome decrease and eventually fail.[29] In rats, the limiting distance, at which a simple nerve guide cannot sustain regeneration is considered 15 mm.[7] With SIL tubes, no axons reached the distal segment in a 15 mm defect, whereas axons readily crossed a gap up to 10 mm. Over time, the characteristics and the quality of the nerve guides have been improved by research on biomaterials with the aim of sustaining regeneration over such critical long-gap[8]. The advantages of these guides are that, being artificial, there is no need to sacrifice a healthy donor nerve from the patient, and they reduce surgical time for repair.[30] In addition, nerve guides may be advantageous since they may reduce the fibrous entrapment of the injured nerve at the suture site[31,32] and the problems related to noncorrect alignment of nerve fascicles.

Among other biomaterials, chitosan has emerged as an interesting polymer for peripheral nerve bridging. Chitosan has been proved as a suitable biomaterial for medical and pharmaceutical applications because of its compatibility, nontoxicity, and biodegradability.[17,33] Furthermore, chitosan-based tubes are easy to handle and their transparency facilitates surgical manipulation and suturing of the nerve stumps in place. Chitosan has also been used as an scaffold, in the form of freeze-dried sponge[34] and micro/nanofiber mesh,[35] serving as an internal filler of the lumen of the tube that can also be combined with other biomaterials or grafted cells.[36] Furthermore, chitosan tubes offer the possibility of modifying their inner surface to mimic the nerve-guiding basal lamina present in nerve grafts, by coupling small peptides derived from extracellular matrix components, such as laminin and fibronectin. By influencing cell adhesion and migration, axonal growth and vascularization of the regenerating cable,[37] these extracellular matrix molecules can potentiate the role of tubes in repairing long peripheral nerve defects.[38,39] Chitosan-based materials have already been used for repairing long gap nerve injuries in rats. In previous studies a mix of polypyrrole/chitosan composite[40] or the combination of chitosan tubes with cross-linked peptides[38] resulted in enhancement of nerve regeneration, but the efficacy of the chitosan material alone to improve nerve regeneration has not been previously reported.

In the current study, we used hollow chitosan tubes of two different degrees of acetylation, controlled during the manufacturing process that may affect the degradation process of the tube once implanted. We chose these degrees of acetylation since higher ones have been shown to be affected by faster degradation and lower mechanical stability.[17] Although, the success of regeneration was lower when using chitosan tubes than when using AGs, these tubes showed considerably better results than the standard control SIL tube. Indeed, the rate of successful regeneration and the levels of reinnervation achieved are among the highest reported for a hollow nerve guide alone over the critical 15 mm long gap in the rat sciatic nerve model.

To evaluate the success of regeneration and target reinnervation, we used functional and electrophysiological techniques that allowed us to follow the evolution of motor and sensory recovery over time. By means of algesimetry tests, we evaluated the responses to mechanical and thermal stimuli of the denervated hind paw. Since confounding responses can be due to collateral sprouting of the intact saphenous nerve, we cut this nerve after tests performed at 90 dpo and repeated the measurements at 92 and 120 dpo to guarantee that the responses observed were exclusively due to reinnervation by the regenerated sciatic nerve.[27] We found withdrawal responses in all the animals of the AG group and some of the chitosan tube groups. In fact, all the animals with evidence of paw reinnervation displayed a mechanical

withdrawal threshold lower than the control, indicative of hyperalgesia.[41] All the SIL tube repaired animals failed to respond to noxious stimuli. We also performed serial electrophysiological tests to evaluate the degree of muscle reinnervation by regenerating motor axons. Muscle reinnervation started earlier and achieved higher levels in the rats repaired by AG than in those repaired with chitosan tubes. These differences are not unexpected, since regeneration in tube repair depends on the initial formation of a new extracellular matrix bridge, over which fibroblasts and Schwann cells migrate and form a new nerve structure.[42] This implies a delay in onset of axonal elongation, and failure of regeneration if the nerve stumps do not provide enough promoting elements inside the tube, as occurred in the long gap repaired with SIL tubes. The histological study corroborated the functional findings explained above. Only in the animals with evidence of reinnervation a regenerated nerve was found inside the tube. The regenerated sciatic nerves in the AG group were larger and had a higher number of myelinated fibers than in the chitosan tubes groups. Although, the mean size of the myelinated fibers was similar between animals repaired with AG and with the chitosan guides, the myelin sheath was slightly thicker in axons of the AG group. This could be due to the faster onset of regeneration in AGs compared to tubulization, where the formation of the fibrin cable slows the initial phase of regeneration.[43]

CONCLUSION

The current study provides novel proof that chitosan-based tubes are good candidates for an artificial nerve guide, allowing nerve regeneration across a critical long gap in a significant number of cases.

ACKNOWLEDGMENTS

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CHAPTER III

Stabilization, rolling and addition of other ECM proteins to collagen hydrogels improves regeneration in chitosan guides for long peripheral nerve gaps in rats.

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Stabilization, rolling and addition of other ECM proteins to collagen hydrogels improves regeneration in chitosan guides for long peripheral nerve gaps in rats.

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Abstract

BACKGROUND: Autograft is still the gold standard technique for the repair of long peripheral nerve injuries. The addition of biologically active scaffolds to the lumen of the conduits to mimic the endoneurium of peripheral nerves may increase the final outcome of artificial nerve devices. Furthermore, the control of the orientation of the collagen fibers may provide some longitudinal guidance architecture providing a higher level of meso-scale tissue structure.

OBJECTIVE: The aim of this study is to evaluate the regenerative capabilities of chitosan conduits enriched with ECM-based scaffolds to bridge a critical gap of 15 mm in the rat sciatic nerve.

METHODS: The right sciatic nerve of female Wistar Hannover rats was repaired with chitosan tubes functionalized with ECM-based scaffolds fully hydrated or stabilized and rolled to bridge a 15 mm nerve gap. Recovery was evaluated by means of Electrophysiology and algesimetry tests and histological analysis 4 months after injury.

RESULTS: Stabilized constructs compared to fully hydrated scaffolds enhanced success of regeneration. Moreover, fibronectin-enriched scaffolds increased muscle reinnervation and number of myelinated fibers compared to laminin-enriched constructs.

CONCLUSION: A mixed combination of collagen and fibronectin may be a promising internal filler for neural conduits for the repair of peripheral nerve injuries, and the stabilization of them may increase the quality of regeneration over long gaps.

Keywords: chitosan, extracellular matrix, fibronectin, laminin, peripheral nerve, regeneration.

Running head: ECM in peripheral nerve regeneration

Introduction

The use of artificial nerve conduits has emerged as an alternative to the classical autologous graft repair to bridge gaps in continuity of peripheral nerves after severe injuries (Deumens et al., 2010; Doolabh et al., 1996; Gu et al., 2011). Nerve conduits avoid some of the main problems associated with autograft repair, such as the additional surgical intervention to obtain the graft material, the mismatch between the injured nerve and the graft, and the limited source of donor nerves. However, the success of regeneration when using artificial nerve guides is limited by the length of the gap, the amount of regeneration being poor for gaps longer than a critical length. Such critical gap length is dependent on the size of the nerve and the species (Yannas and Hill, 2004). Thus, when using standard silicone or plastic tubes, nerve regeneration occurs in the rat sciatic nerve if the gap is 10 mm or less but fails in most cases with 15 mm gaps (Lundborg et al., 1982; Williams et al., 1983), whereas in the mouse sciatic nerve regeneration occurs in all cases with a 4 mm gap but fails with gaps 6 mm or longer (Madison et al., 1987; Butí et al., 1996; Gómez et al., 1996).

Current advances in the use of artificial nerve conduits to repair severe peripheral nerve lesions aim to create an internal milieu that mimics the natural microstructure found in a normal nerve (Deumens et al., 2010). An explored direction is the addition of biologically active scaffolds to the inner lumen that mimics the endoneurium of peripheral nerve tissue. The extracellular matrix (ECM) plays an important role in the proliferation and migration of Schwann cells, that guide the regenerating axons and their myelination (Rutka et al., 1988; Gonzalez-Perez et al., 2013) after nerve damage. Among other ECM components, laminin and fibronectin have a fundamental role in guiding the re-growth of damaged axons. Laminin and fibronectin have been both implicated in the regeneration of peripheral neurons *in vitro* (Baron-VanEvercooren et al., 1982; Gardiner et al., 2007) and *in vivo* (Bailey et al., 1993; Chen et al., 2000; Labrador et al., 1998; Liu et al., 2009; Madison et al., 1988).

However, the incorporation of ECM-based scaffolds into nerve conduits has to take into account the matrix composition, density and the orientation of the fibrils, all factors that may influence the final outcome of the

regeneration (Labrador et al., 1998; Verdú et al., 2002). Cell and ECM alignment is a common feature of biological tissues, with anisotropy being critical to function in many instances (Georgiou et al., 2013). The control of the orientation and direction of collagen fibers have been demonstrated to increase the invasion of neurites and Schwann cells from dorsal root ganglia cultured on the gel surface compared to unaligned collagen gels *in vitro* (Dubey et al., 1999). The range of approaches to achieve anisotropic engineered tissues include the use of aligned fibers, patterned surfaces, electrical and magnetic fields, mechanical gradient loadings or physical and chemical cues. Aligned cellular collagen-based hydrogels have been recently used to bridge peripheral nerve defects (Georgiou et al., 2013) reporting regeneration of peripheral nerves in a critical gap model of 15 mm in rats. In the experiments described here, we have compared the capability to support regeneration across a non permissive 15 mm long defect of the sciatic nerve in the adult rat of chitosan conduits pre-filled with collagen I based matrices enriched with either laminin or fibronectin and delivered as simple fully-hydrated hydrogels or stabilized by plastic compression and rolled to provide some longitudinal guidance architecture. Regardless of the cell and fibril level architecture, the meso-level anisotropy of the substrate may improve regeneration, since the matrix will serve to distribute the collagen fibers in a 3D space, and the anisotropic fibers will provide a 2D surface for regenerating axons (Bellamkonda 2006). By means of functional and morphological methods we assessed the effects of stabilized and rolled collagen-based matrices enriched with laminin or fibronectin to sustain nerve regeneration, migration of Schwann cells and axonal growth.

Methods

Animals

Adult female Wistar Hannover rats (Janvier), weighing 220-250 g, were used in the experiment. The animals were housed in plastic cages and maintained under standard conditions with free access to water and food. The experimental procedures were approved by the Ethical Committee of our institution and followed the rules of the European Communities Council Directive.

Preparation of conduits prefilled with extracellular matrix

A solution of 800 µl of rat tail type I collagen (BD-Biosciences) at a concentration of 3-4 mg/ml was mixed with 50 µl of 10x Eagle's medium (Gibco) and 2 µl of 7.5% sodium bicarbonate. For enriched matrices, 200 µl (20% final volume) of human fibronectin (BD Biosciences) at 1 mg/ml or laminin type I (Sigma) at 1mg/ml (concentration of the solution) were added separately to the collagen type I mixed solution. The collagen concentration was then corrected to 2 mg/ml with extra PBS.

The manufacturing and characteristics of chitosan tubes were reported in a previous study (Gonzalez-Perez et al., 2015a). For the preparation of fully hydrated, the chitosan tubes were carefully filled with the matrix preparations and kept in the incubator at 37ºC for at least 30 minutes to allow matrix gel formation prior to implantation.

For the preparation of stabilized rolled hydrogels, the same matrix solutions were used to fill rectangular ABS moulds following the method described previously for cellular gels (Phillips and Brown, 2011). One ml of the mixture was added to each mould and integrated with tethering mesh at opposite ends. Tethered gels were covered with PBS and the moulds kept in the incubator at 37ºC for 24 hours then gels were separated from the tethering mesh and rapidly stabilized using plastic compression by placing them on an absorbant pad. The resulting sheets were rolled (approximately 15 mm length) and placed into a longitudinally opened chitosan tube, which was closed with glue prior to implantation.

Experimental design and surgical procedure

For the study of regeneration over a critical sciatic nerve gap (15 mm), 37 rats were used. The animals were randomly distributed and assigned to one of five experimental groups: in which the chitosan conduits contained collagen I matrix (n=7, COL), laminin-enriched matrix (n=7, LM), fibronectin enriched matrix (n=8, FN), laminin-stabilized matrix (n=7, LM-St) and fibronectin-stabilized matrix (n=8; FN-St).

All surgical procedures were performed by the same researcher under aseptic conditions. Rats were anesthetized by intraperitoneal injection of ketamine/xylazine (90/10 mg/kg). Under a dissecting microscope the right sciatic nerve was exposed and cut 6 mm distal to the exit of the gluteal nerve, and a segment of 6 mm was resected.

The distal and proximal stumps were fixed by two epineural 10-0 sutures into each end of the chitosan tube, leaving a 15 mm gap. All the tubes used had a length of 18 mm and an internal diameter of 2 mm. The muscle plane was then sutured with re-absorbable 5-0 sutures, the skin with 3-0 silk sutures, and the wound was disinfected with povidone iodine. Animals were treated with amitriptyline to prevent autotomy (Navarro et al., 1994).

For the study of Schwann cell migration 20 female Wistar Hannover rats were randomly distributed in the same experimental conditions (n=4 per group). We used a short sciatic nerve gap of 6 mm repaired with chitosan tubes of 9 mm in length. The operations were performed as described above.

Electrophysiological tests

Functional reinnervation of target muscles was assessed for the long gap study at 30, 60, 90 and 120 days post-operation (dpo). Animals were anesthetized with pentobarbital (40 mg/kg i.p.). The sciatic nerve was stimulated by needle electrodes placed at the sciatic notch, and the compound muscle action potential (CMAP) of tibialis anterior, gastrocnemius and plantar muscles was recorded using monopolar needle electrodes, placing the active one in the muscle belly and the reference in the fourth toe. During the tests, the rat body temperature was maintained by means of a thermostatic warming flat coil. The amplitude and the latency of the CMAP were measured. The contralateral limb was used as control.

Functional evaluation of sensory recovery

The threshold of nociceptive responses to mechanical and thermal stimuli was evaluated in the long gap study by means of algesimetry tests at 7, 21, 30, 60, 90, 120 and 122 dpo. For both tests, the lateral area (innervated by tibial and sural nerves, branches of the sciatic nerve) of the plantar surface was tested. To eliminate the possible confounding effect of collateral sprouting of the saphenous nerve (Cobianchi et al., 2014), this nerve was sectioned after 120 dpo tests, and the tests repeated at 122 dpo. The contralateral paw of each rat was tested as control, to overcome possible variations between testing conditions. Sensibility to mechanical stimuli was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). The mechanical nociceptive threshold was taken as the mean of three measurements per paw, and expressed

as the pressure (in grams) at which rats withdrew their paws in response to the stimulus. A cutoff force was set to 40 g at which stimulus lifted the paw with no response. Thermal sensibility was assessed by using a plantar algesimeter (UgoBasile, Comerio, Italy). The beam of a projection lamp was focused onto the hindpaw plantar surface pointing at the lateral side. The thermal nociceptive threshold was taken as the mean of three trials, and expressed as the latency (in seconds) of paw withdrawal response. A cutoff time was set at 20 s to prevent tissue damage. All the values of the functional evaluation of sensory recovery are presented as percentage of the response in the contralateral non-injured paw.

Histology and morphometry

Four months after the injury, animals were deeply anesthetized and perfused transcardiacally with 4% paraformaldehyde in phosphate-buffered saline solution (0.1M, pH=7.4). After perfusion, the regenerated nerves were harvested and postfixed in 3% paraformaldehyde - 3% glutaraldehyde phosphate-buffered solution. The nerves were postfixed in osmium tetroxide (2%, 2 h, 4°C), dehydrated through ascending series of ethanol, and embedded in Epon resin. Nerves were sectioned using an ultramicrotome (Leica). Semithin sections (0.5 μm) were stained with toluidine blue and examined by light microscopy. Images of the whole cross-section of the sciatic nerve were acquired at 10× with a digital camera (Olympus DP50) attached to the microscope (Olympus BX51), while sets of images chosen by systematic random sampling of squares representing at least 30% of the nerve cross-sectional area were acquired at 100× from segments at the mid of the tube and 3 mm distal to the tube. Measurements of the cross-sectional area of the regenerated nerve, as well as counts of the number of myelinated fibers, were carried out by using ImageJ software (National Institutes of Health).

Assessment of Schwann cell migration

For the study of Schwann cell migration, 12 days after nerve section and tube repair leaving a 6 mm gap, animals were deeply anesthetized and perfused transcardiacally as above. The tubes were harvested and postfixed in 4% paraformaldehyde. 30 μm thick longitudinal sections of the regenerating cable were cut with a cryostat (Leica). Samples were incubated for 48 h with rabbit antibody against S100 (1:100, Immunostar) to label Schwann cells and

anti-NF-200 (1:1000, Millipore) to label regenerating axons. After washes, the sections were incubated for two hours with biotinylated IgG (1:200, Life Bioscience) and incubated overnight with secondary antibodies goat anti-rabbit conjugated with Alexa 488 and Alexa 594 (1:200, Life BioScience). To analyze Schwann cell migration, microphotographs of the regenerative front and Schwann cells were taken at 4× with a digital camera, acquired in Adobe Photoshop CS and photomerged. Using ImageJ software, resolution parameters were fixed and Schwann cells were followed from the proximal to the distal part of the implanted tube. The distance of the regenerated axonal front and the percentage of area occupied by migrating Schwann cells was calculated and compared.

Statistical analysis

Results are expressed as mean ± SEM. Statistical comparisons between groups and intervals for algesimetry and electrophysiological tests results were made by two-way ANOVA for repeated measurements, followed by Bonferroni post-hoc test. Statistical analysis of histological results and Schwann cell migration were made by one-way ANOVA followed by Bonferroni post-test. Differences were considered significant if $P<0.05$.

Results

Muscle reinnervation

Nerve conduction tests performed one month after sciatic nerve injury demonstrated complete dennervation of the hindlimb muscles. Initial evidence of reinnervation of the tibialis anterior and gastrocnemius muscles was found at 60 dpo in some animals of each group, with CMAPs of small amplitude. The CMAPs progressively increased in amplitude and were recorded in more animals over time, with a similar pattern for both muscles (Fig. 1A,B). At the end of the follow-up (120 dpo), reinnervation of the tibialis anterior and the gastrocnemius muscles was observed in 3 of 7 animals in the COL group, 2 of 7 in the LM group, 5 of 8 in the FN group, 4 of 7 in LM-St group, and 6 of 8 in the FN-St group. Significant differences ($P<0.05$) were observed at the final time point between the FN-St group and the COL and LM groups. Both FN and LM-St groups performed better than the LM group ($P<0.01$) (Fig. 1A,B).

When CMAPs were recorded, their latency was considerably longer than normal during the first stages of reinnervation, and tended to shorten

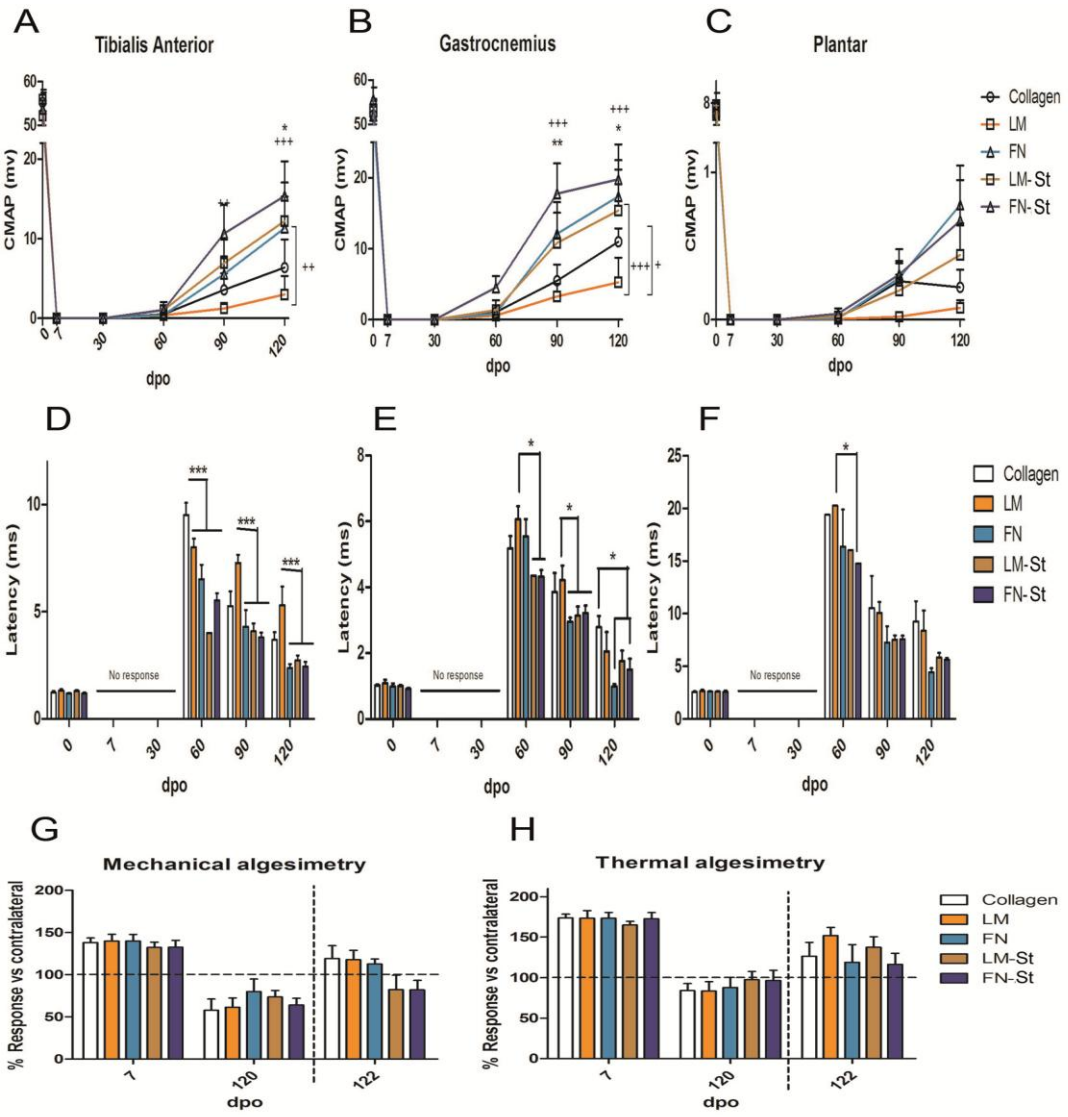

Figure 1: Mean amplitude of the compound muscle action potential (CMAP) of tibialis anterior (A), gasctrocnemius (B) and plantar muscles (C) of the injured himblimb of the rats during 4 monts after sciatic nerve lesion and repair. *P<0.05 fibronectin vs laminin enriched groups; $^{+}$P<0.05 fibronectin vs collagen group. Mean latencies of the tibialis anterior (D), gastrocnemius (E) and plantar (F) CMAPs recorded in the regenerated rats during the 4 months follow-up. *P< 0.05. Mechanical (G) and thermal (H) algesimetry test results. Values are expressed as percentage of withdrawal threshold to mechanical and thermal stimuli applied to the lateral side of the injured paw versus the contralateral uninjured paw. Horizontal dotted lines represent the normalized baseline values. Vertical dotted lines indicate when the saphenous nerve was cut.

with time toward normal values. At the end of the follow-up, the latencies of the waves recorded on tibialis anterior and gastrocnemius muscles were significantly shorter in the FN, FN-St and LM-St groups than in the LM and COL groups (Fig. 1D,E).

At the more distal plantar interosseous muscles, onset of reinnervation was also

found at 60 dpo. In this case, the CMAPs were of very small amplitude (less than 0.05 mV). At the end of the follow-up (120 dpo), evoked CMAPs of the plantar muscle were observed in 3 of 7 animals in the COL group (CMAP amplitude 0.22 ± 0.16 mV), 2 of 7 in the LM group (0.08 ± 0.08 mV), 5 of 8 in the FN group (0.78 ± 0.37 mV), 4 of 7 in the LM-St group (0.44 ± 0.28 mV) and 5 of 8 in the FN-St group (0.67 ± 0.37 mV), but no significant differences were observed between groups (Fig. 1C).

The plantar CMAP latency averaged 9.25 ± 2.51 ms in the COL group, 8.37 ± 2.52 ms in the LM group, 4.45 ± 0.59 ms in the FN group, 5.83 ± 0.60 ms in the LM-St group and 5.64 ± 0.21 ms in the FN-St group at the end of the follow-up, without significant differences between groups (Fig. 1F).

Recovery of nociceptive sensibility

Withdrawal responses to mechanical stimuli, evaluated with the Von Frey test, demonstrated absence of responses during the first 30 dpo, and therefore they were penalized with a cut off value of 40 g. From 60 to 120 dpo most of the rats showed withdrawal responses in the injured paw at lower stimulus intensity than in the contralateral side, indicating some degree of hyperalgesia. After elimination of the saphenous nerve at 120 dpo, withdrawal responses to mechanical stimuli in rats of the COL group (119.01 ± 20.48g), the LM group (117.69 ± 15.91g), and the FN group (112.53 ± 8.87g), were slightly higher, but not significantly different ($P>0.05$) than in the contralateral side, whereas both LM-St (82.17 ± 21.17g) and FN-St (81.96 ± 13.84g) groups were slightly lower, but not significantly different compared to the contralateral paw (Fig. 1G), suggesting increased skin reinnervation in these two groups.

Withdrawal responses to heat stimulation in the plantar test showed similar evolution than those observed for the Von Frey test. Animals had no response to heat stimuli on the denervated paw during the first 30 dpo. From 60 to 120 dpo, most rats showed withdrawal responses at lower stimulus intensity than in the contralateral side. When the saphenous nerve was cut, the withdrawal time of the injured paw at 122 dpo in rats of the COL group (126.37 ± 22.72s), LM group (151.93 ± 14.15s), FN group (118.97 ± 30.97s), LM-St group (137.49 ± 16.07s) and FN-St group (116.29 ± 16.97s) were higher, although not significantly different than in the contralateral intact side (Fig. 1H).

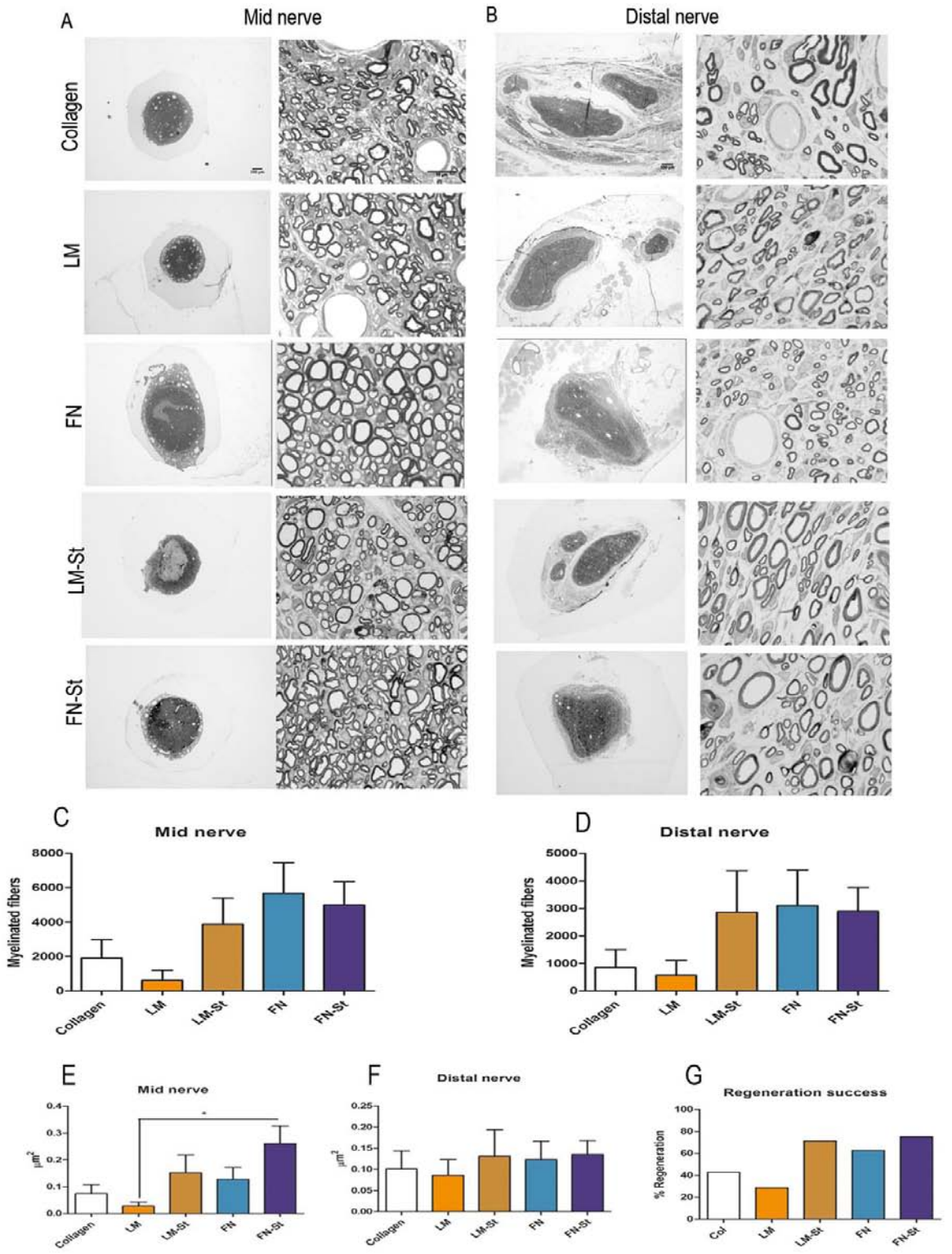

Figure 2: Micrographs of semithin sections of the regenerated nerve taken at the midpoint of the tube (A) and 3 mm distal to the tube (B) 4 months after sciatic nerve resection and repair from a representative animal of collagen group, laminin group, fibronectin group, laminin stabilized group and fibronectin stabilized group. The whole regenerated nerve is shown in the left panels; scale bar = 500 µm. Higher magnification of the regenerated nerves is shown in the right panels; scale bar = 10 µm. Estimated number of regenerated myelinated fibers in the regenerated nerve at the mid-tube (C) and 3mm distal (D) to the tube 4 months after sciatic nerve resection and repair. Cross-sectional area of the nerve at mid-tube (E) and 3 mm distal (F) levels. Animals with no regenerated nerve were also included (with null values) in the calculation. * $P<0.05$. Plot representing the percentage of regenerated nerves found at 120 dpo (G).

Histological results

Macroscopic examination of the injured nerves after the 4 months follow-up showed that 3 of 7 animals in the COL group, 2 of 7 in the LM group, 5 of 8 in the FN group, 4 of 7 in the LM-St group and 6 of 8 in the FN-St group presented a regenerated cable inside the chitosan tube. The regenerated nerve had a compact appearance and occupied the center of the lumen. The nerves were surrounded by a thick connective layer, without signs of inflammatory reaction (Figs. 2A,B). Transverse sections of the regenerated nerves taken at the midpoint of the tube and at the distal segment were analyzed under light microscopy. To compare the absolute number of myelinated fibers, regenerated and non-regenerated rats were included in the statistical analysis, giving a null value to non-regenerated animals. The mean number of myelinated fibers at the midpoint of the tube was higher in the FN group (5,673 ± 2,501) followed by FN-St (4,999 ± 1,922), LM-St (3,882 ± 1,985), COL (1,909 ± 1,521) and LM (614 ± 838) groups (Fig. 2C), without significant differences between groups due to high variability. The same pattern was observed in sections taken 3 mm distal to the end of the tube, where the number of myelinated fibers was higher in the FN group (3,098 ± 1,813) followed by FN-St (2,894 ± 1,218), LM-St (2,860 ± 1,990), COL (857 ± 904) and LM (568 ± 774) groups (Fig. 2D), without significant differences.

When analyzing the cross-sectional area of the regenerated nerve in the tube, the FN-St group had the largest area (0.26± 0.09 μm^2), followed by LM-St group (0.15± 0.09 μm^2), FN group (0.13± 0.06 μm^2), COL group (0.08± 0.05 μm^2) and LM group (0.03± 0.02 μm^2), with significant differences between the FN-St versus LM group ($P<0.05$). Distally, the same order was found for the size of the nerve, but without significant differences between groups (Fig. 2E,F).

Schwann cell migration

Immunohistochemical labeling of the regenerating cable formed inside the tube at 12 dpo (Fig. 3A) revealed that the distance covered by the regenerating front, labeled against neurofilament heavy chain, was longer in the FN-St (5,214 ± 124 µm) and LM-St groups (4,778 ± 198 µm), followed by the FN (4,037 ± 495 µm) and LM (3,675 ± 668 µm) horizontally polymerized groups, and finally the COL group (1,906 ± 954 µm), with significant differences between the FN-St versus the COL group ($P<0.05$) (Fig. 3B). Similarly, the

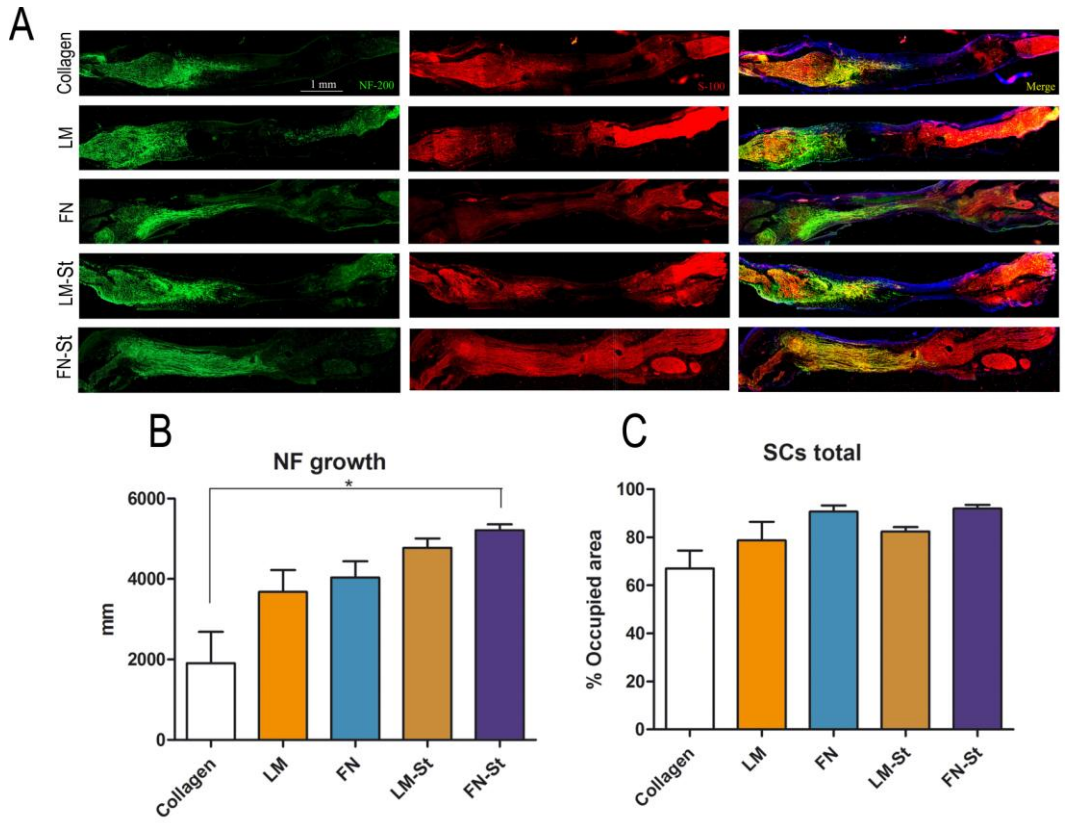

Figure 3: Representative images of longitudinal sections of sciatic nerves from rats injured and repaired with a chitosan conduit leaving a 6 mm gap in the collagen, laminin, fibronectin, laminin stabilized and fibronectin stabilized groups. 12 days after the surgery, regenerated axons were labeled with NF-200 (left panels), Schwann cells were labeled with S-100 (central panels), and the images were photomerged (right panels) with DAPI (blue). Scale bar = 1 mm (A). Length of the front of axonal growth from the proximal end of the tube (B) and percentage of the nerve cable area occupied

area occupied by Schwann cells, labeled for S-100, was larger in the FN-St group (91.98 ± 1.37 %), followed by the FN (90.66 ± 3.34 %), LM-St (82.48 ± 1.61 %), LM (78.68 ± 9.87 %) and COL groups (67.03 ± 9.23 %), without significant differences between them (Fig. 3C).

Discussion

In this study, we have compared the effect of the addition of laminin or fibronectin in a collagen type I based matrix, both incorporated either as fully-hydrated hydrogel fillers or stabilized and organized into longitudinally orientated structures, within chitosan conduits to sustain axonal regeneration across a critical model of 15 mm gap resection of the sciatic nerve in rats. Our results show that addition of fibronectin in the collagen matrix enhanced nerve regeneration, and that stabilization and organization of the hydrogels into

longitudinally orientated structures further increased the cases in which regeneration occurred over the 15 mm long gap. Furthermore, we investigated the effect of those matrices at short term, and found that the stabilization and organization of the initial matrix increased Schwann cell migration and axonal growth.

Here we have used a chitosan tube that was already proven to be more effective than the standard silicone tube for supporting axonal regeneration across limiting gaps in rats (Gonzalez-Perez et al., 2013). However, the percentage of success in this model is still far from the 100% success of an autograft, the gold standard repair technique to bridge long peripheral nerve gaps. Therefore, there is the need to further improve the regenerative capability of these guides. Regeneration in tubular guides is dependent on the formation of an initial fibrin matrix which has to bridge the gap formed between the stumps. This fibrin cable provides a guidance surface to fibroblasts, blood vessels and Schwann cells to migrate from proximal and distal nerve stumps to populate the cable and sustain the advance of the regenerating axons (Williams et al., 1983). Therefore, prefilling the tube with scaffolds can favor Schwann cell migration and facilitate regeneration across these conduits when the formation of the fibrin cable is limited.

In this work we have used a collagen type I hydrogel matrix as a base, that has previously been shown to allow axonal growth in 3D *in vitro* assays (Allodi et al., 2011). It is easy to manipulate and can be enriched with different extracellular matrix molecules, such as fibronectin or laminin, in a mixture that is permissive for regeneration both *in vitro* and *in vivo* (Gonzalez-Perez et al., 2015b). Previous works have already shown that pre-filling a nerve conduit with ECM components supports axonal growth (Bailey et al., 1993; Chen et al., 2000; Labrador et al., 1998; Madison et al., 1988), although the regeneration promoting capacity remains inferior compared to an autograft (Rodriguez et al., 2000). This limitation may be due to the composition, the density of the matrices and the lack of alignment of their components (Madison et al., 1988; Labrador et al., 1995) that may interfere with the migration of Schwann cells and the directed growth of axons along the conduit lumen. The longitudinal alignment of an ECM gel within a nerve conduit attempts to mimic the natural geometry of the endoneurial tubes in

nerve grafts. Many efforts during the last years have focused on the development of novel tissue engineering techniques and biomaterials that can confer orientation upon cells and ECM, including the use of gradients, electrical and magnetic fields, and cellular self-alignment in response to tension in tethered collagen gels (Verdú et al., 2002: Dubey et al., 1999; Ceballos et al., 1999). In addition to the regeneration support and guidance resulting from micro and nanoscale alignment of cells and collagen fibrils in the latter studies (Georgiou et al, 2013; Georgiou et al., 2015) rolling sheets of stabilized collagen hydrogel inside a conduit provides an additional level of meso-scale tissue structure than obtained from simply filling the conduit lumen with a fully hydrated hydrogel. Here we have isolated this variable and directly compared the extent of nerve regeneration that results from using simple hydrogel fillers versus the same materials stabilized using plastic and rolled to provide some tissue architecture. The results indicate that the more organized matrix structure is beneficial in promoting regeneration even in the absence of self-aligned therapeutic cells and the accompanying nanofibrillar anisotropy of the collagen matrix.

Interestingly, we found that addition of fibronectin to the intratubular matrix increased the proportion of animals that regenerated, enhanced motor reinnervation and number of myelinated axons than the addition of laminin or the collagen matrix alone. Furthermore, when stabilized rolled gels were incorporated in the tube, the regeneration was further improved; in the FN-St group the proportion of animals that presented a regenerative cable after 4 months increased to 75% of the rats, whereas in the LM-St group it was 57%. Both laminin and fibronectin have been used to promote nerve regeneration and provide support in long nerve gaps using various different approaches. A previous study compared the addition of laminin and/or fibronectin in tubular devices to repair long nerve gaps (Bailey et al., 1993). The authors filled silicone tubes with saline solution containing these ECM proteins, and found that the incidence of cable formation that bridged the gap was similar in all groups, although combination of both molecules increased the number of regenerating axons (Bailey et al., 1993). In contrast, we have found that the addition of fibronectin within a collagen-based matrix increased the percentage of regeneration across a critical gap

compared to the laminin-containing matrix. These differences could be due to the composition and topographical conformation of the fibrils constituting the matrix used to fill the tube, which are important factors determining the rate of axonal regeneration (Madison et al., 1988; Labrador et al., 1995; Balgude et al., 2001; Willits and Skornia, 2004).

Since aligned type I collagen facilitates migration of Schwann cells *in vitro* compared to unaligned collagen gel (Dubey et al., 1999), and fibronectin seems to play plays an important role in migration of Schwann cells *in vitro* and into neural guides (Mosahebi et al., 2003), we wanted to evaluate migration of Schwann cells from the proximal and the distal stump. For that experiment we used a shorter gap, in order to guarantee the formation of the fibrin cable in all the animals. We observed that the regenerating axon front extended to longer distances in fibronectin and laminin stabilized rolled hydrogel groups compared to fully hydrated hydrogel matrices. Schwann cell migration was not significantly different between groups at 12 days using this gap length. However, the trend observed in the short gap between fibronectin and laminin groups may become a relevant difference in the long gap model and thus, the slight enhancement of Schwann cell migration into the tube may be decisive to sustain regeneration in a critical gap. Indeed, fibronectin aligned fibers were shown to provide an orientating cue for migrating fibroblasts and Schwann cells, and for neurite elongation (Ahmed and Brown, 1999). Besides its effects on Schwann cell migration, the beneficial effects of fibronectin in nerve conduits can also be due to its supportive effect on viable Schwann cells (Mosahebi et al., 2003)..

Conclusion

The present study shows the importance of the conformation and organization of hydrogel matrix components on promoting regeneration after severe peripheral nerve injuries. Stabilization and rolling of collagen-based matrices, and enrichment with other ECM proteins improved the quality and quantity of the nerve repair process. The fibronectin-collagen stabilized rolled construct seems a promising candidate to be used as internal filler of tubular nerve conduits, allowing regeneration across a critical long gap in a significant number of cases, by facilitating formation of the fibrin cable, Schwann cell migration and growth of regenerating axons through the neural conduit.

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CHAPTER IV

Aligned Mesenchymal Stem cells and Schwann cells on laminin or fibronectin matrices guarantee regeneration in a critical size defect of 15 mm in the rat sciatic nerve

Gonzalez-Perez F, Hernández J, Heimann C, Phillips JB, Udina E, Navarro X. Aligned Mesenchymal Stem cells and Schwann cells on laminin or fibronectin matrices guarantee regeneration in a critical size defect of 15 mm in the rat sciatic nerve. Manuscript.

Aligned Mesenchymal Stem cells and Schwann cells on laminin or fibronectin matrices guarantee regeneration in a critical size defect of 15 mm in the rat sciatic nerve

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ABSTRACT

Artificial nerve guides are developed to become a real alternative to autograft in the repair of peripheral nerve defects. However, the use of nerve conduits is limited by the length of the gap, being the success of regeneration compromised at long gaps. Current efforts are addressed to functionalize these conduits altering their internal milieu to recreate the natural microstructure or natural endoneurium of healthy nerves. Alignment of cellular and extracellular matrix components becomes crucial. Recent studies demonstrate the promising effect of generating anisotropic tethered cellular constructs to promote peripheral nerve regeneration. In this study we have combined different proteins of the extracellular matrix with two different cellular types, Mesenchymal Stem cells and Schwann cells subjected to alignment, to promote regeneration over a critical sized gap of 15 mm in the rat sciatic nerve. To prevent rejection of allogenic transplanted cells, animals were treated with the immunosuppressant FK506 (1 mg/kg s.c.) during the first two months of follow up. Functional analysis demonstrates that SC-aligned scaffolds results in a 100% regeneration success when used to bridge a 15 mm nerve defects in rats. On the other hand, animals repaired with MSC-aligned constructs were close to 90% of success, whereas acellular bridges reached only 75% regeneration success. These results revealed that combination of chitosan conduits with ECM enriched gels embedding pro-regenerative cell grafts is an available strategy that may be a good alternative to the use of autografts to repair long nerve gaps in the clinical practice.

Keywords: mesenchymal stem cells, Schwann cells, peripheral nerve regeneration, chitosan, alignment, laminin, fibronectin

Running head: Cellular aligned hydrogels to bridge peripheral nerve defects

Introduction

The use of artificial nerve guides can be an alternative to autograft for the repair of peripheral nerve defects. However, the length of the gap to be bridged determines the success of axonal regeneration after tube repair of transected nerves. Regenerating axons are able to bridge tubes implanted in the rat peripheral nerve if the gap is inferior to 10 mm, but not in longer gaps. We have previously demonstrated that the use of chitosan tubes may be a promising material to be used to solve critical peripheral nerve lesions (Gonzalez-Perez et al., 2015). However, repair with this guides never reached the 100 % success of autograft repair.

Tissue-engineered alternatives for the design of complex artificial nerve guides seek to mimic spatial and molecular phenotype of natural microenvironment of an autograft, resembling the original tissue structure, geometric design and composition (Boecker et al., 2015). Then, pre-filling the tubes with extracellular matrix components (Verdú et al., 2002a) or addition of cells into these constructs has been demonstrated to enhance axonal regeneration.

Schwann cells (SCs) have been the principal choice to improve regeneration through neural conduits. However, Mesenchymal Stem cells (MSCs) (especially isolated from the bone marrow or the adipose tissue) have emerged as an important alternative due to their higher potential clinical application (Guo et al., 2015; Hsu et al., 2013; Keilhoff et al., 2006). The therapeutic role of SCs (Fansa & Keilhoff, 2004) and MSCs (Boecker et al., 2015) has been studied previously in the repair of peripheral nerve injuries. Their high tissue repair activity may explain their capacity of cell replacement, production of growth factors, extracellular matrix synthesis, immune modulation, etc. (Wang et al., 2015).

In addition, alignment of cellular and extracellular constituents within the conduit has been shown to provide cell-level guidance and facilitate axonal re-growth when incorporated to hollow nerve conduits. The incorporation of guiding elements such as microchannels, nano-fibers of extracellular matrix components to direct axonal re-growth have been widely used (Bozkurt et al., 2009). Tethered aligned gels emerge as an alternative for guiding re-growing axons after peripheral nerve injuries (Georgiou et al., 2013). However, when

combining cell therapy with scaffolds to further improve the capabilities of neural guides, is important to take into account the interactions between the grafted cells and the scaffolds where these cells are embedded. The use of collagen-based scaffolds allows the further addition of other ECM molecules like laminin (Verdú et al., 2002b) and fibronectin (Chen et al., 2000). It remains extremely challenging to dissect the cooperative influence of multiple ECM parameters on cell behavior (Rape et al., 2015) when evaluating cellular scaffolds within conduits to repair nerve defects.

In the present work, we have compared the capability of nerve guides containing SCs or MSCs to support regeneration across a limiting peripheral nerve defect of 15 mm in the adult rat. The conduits were made of chitosan and pre-filled with collagen type I based scaffolds enriched with either fibronectin or laminin and SCs or MSCs tethered aligned. Since the cells were allogenic, animals were treated with the immunosuppressant FK506 (1 mg/kg s.c.) during the first two months of follow up to avoid immune rejection of transplanted cells. By means of functional and morphological methods we assessed the effects of these cellular aligned constructs to sustain regeneration over a critical sized gap during 4 months follow-up.

MATERIALS AND METHODS

Animals

Adult female Wistar Hannover rats (Janvier), weighting 220-250 g, were used in the experiment. The animals were housed in plastic cages and maintained under standard conditions with free access to water and food. The experimental procedures were approved by the Ethical Committee of our institution and followed the rules of the European Communities Council Directive.

Mesenchymal Stem cell culture

Primary cultures of MSC were set up from p21 female Wistar Hannover rats. The rats were euthanized using lethal anesthesia (Dolethal). From each animal, tibias and femurs were dissected, placed in cool PBS and epiphysies removed. The diaphyses of bones were flushed with PBS using a syringe and the marrow was homogenized. The extract was filtered through 70 µm nylon mesh and recovered by centrifugation for 10 min at 1,500 rpm. The pellet was re-suspended in growth medium α-MEM with L-glutamine (Life Technologies,

Grand Island, NY), supplemented with 20% heat-inactivated fetal bovine serum (FBS, Lonza, Verviers, Belgium), 2 mM L-glutamine (Life Technologies) and 100 units/mL penicillin-streptomycin (Life Technologies, 100X), and plated in 100 mm culture dishes (Iwaki, Asahi Technoglass, Chiba, Japan) at a density of 5 x 10^6 cells/cm^2. After 24 h, the supernatant containing non-adherent cells was removed and fresh medium was added. When the cell culture was near confluence, the cells were detached using PBS with 0.05% trypsin (Life Technologies) and 0.04% EDTA (Sigma, St. Louis, MO) and re-plated at 5,000 cells/cm^2.

Schwann cell culture

Dissociated Schwann cells were prepared from p21 female Wistar Hannover rats. Sciatic and median nerves were dissociated and kept in cold Gey's balanced salt solution (Sigma, St. Louis, MO) supplemented with 6 mg/mL glucose during dissection and cleaned from connective tissue. Nerves were cut into small pieces and stripped off from the epineurial sheaths. The pieces were dissociated in 10% collagenase (Invitrogen), 10% trypsin (Life Technologies) and 10 % DNase (Invitrogen) in Hanks solution (without Ca-Mg) (Invitrogen) and incubated for 30 minutes at 37 ºC, shaking every 10 minutes. Then, the nerves were finally dissociated by pipetting thoroughly the pieces of the explant. The action of the trypsin was stopped by Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco) and 100 units/mL penicillin/Streptomycin (Life Technologies). The cells were centrifuged 5 minutes at 900 rpm and plated in 35 mm culture dishes (Greiner Bio-one), previously pre-treated with 10 µL/mL poly-D-Lysine (Sigma, St. Louis, MO), in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco) and 100 units/mL penicillin/Streptomycin (Life Technologies) at a density of 1 x 10^6 cells/cm^2. 24 h after the supernatant containing non-adherent cells was removed and fresh medium was added. When the cell culture was near confluence, the cells were detached using PBS with 0.05% trypsin (Life Technologies) and 0.04% EDTA (Sigma, St. Louis, MO) and re-plated at 1,000 cells/cm^2.

MTT assay

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma

Chemical Co., St. Louis, MO), was used to evaluate mitochondrial activity as an indirect measure of cell survival. 8,000 Mesenchymal Stem cells and Schwann cells were cultured on 24-well plates previously treated with poly-D-lysine 10 µL/mL, laminin (5 µg/cm^2) or fibronectin (5 µg/cm^2) for 72 h. Then, 0.15 mg/mL of MTT was added and the cells were incubated for 45 minutes at 37 ªC. The formazan crystals were dissolved in 200 µL of dimethyl sulfoxide (DMSO) and 100 µL were passed to 96-well plates. The optimal density was determined with a microculture plate reader (Bio-teck) at 570 and 620 nm and analyzed with KcJunior software.

BrDU proliferation assay

To analyze proliferation of Mesenchymal Stem cells and Schwann cells, these were cultured in glass coverslips pre-treated with poly-D-lysine 10 µL/mL, laminin (5 µg/cm^2) or fibronectin (5 µg/cm^2) for 48 h at 8,000 cells per well (24-well plate). 1 µLof BrdU was added to the cultures at a concentration of 20µM. One day after, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes. The fixed cells were then treated with HCl at a concentration of 2M and left for 20 minutes at 37 ºC. Afterwards, the pH was re-equilibrated with TBS to 8.5 and incubated overnight with primary antibodies to stain BrdU (1:500, Fitgerald) and Scwhann cells with S-100 (1:100, Immunostar) After several washes, the cells were incubated for 2 h with biotinylated IgG (1:200, Life Bioscience) and incubated overnight with secondary antibody anti-sheep 594 Alexa and Spreptavidin 488 conjugated secondary antibodies (1:200, Life Bioscience) and mounted with mowiol containing DAPI. To label MSC, we used Cd90 conjugated with FITC (1:100, BD Pharmigen).

Preparation of cellular constructs

For the preparation of the extracellular matrix, a volume of 800 µL of rat tail type I collagen solution (BD Bioscience) at a concentration of 3-4 mg/mL was mixed with 100 µL of 10X Eagle's medium (Gibco) and 4 µL of 7.5% sodium bicarbonate solution. These matrices were enriched with 200 µL (20% final volume) of fibronectin (BD Biosciences) or laminin type I (Sigma) at 1 mg/mL and kept in ice.

For the preparation of the cellular constructs, Mesenchymal Stem cells and Schwann cells were trypsinized and centrifuged (MSC at 1,500 rpm for 10 minutes; SC at 900 rpm for 5 minutes),

and re-suspended in 1 mL of medium. Cells were counted using a Neubauer chamber. Then, appropriate volume of medium containing cells was centrifuged to get 750,000 cells. The pellet containing the 750,000 cells was re-suspended in 1 mL of appropriate matrices.

1 mL of Mesenchymal Stem cells and Schwann cells at 750,000 cells/mL were aligned within tethered collagen-based gels enriched with either fibronectin or laminin at 20% V/V in rectangular ABS moulds according to methods described previously (Phillips & Brown, 2011). The moulds were integrated with tethering mesh of nylon at opposite ends before setting at 37 ºC for 15 minutes. Tethered gels were immersed in culture medium and incubated at 37 ºC in a humidified incubator (5% CO_2) overnight to allow alignment to develop. Acellular matrices were submitted to the same procedure. The resulting constructs were separated from the nylon mesh using a scalpel and placed on absorbent pads to stabilize. Then, the matrices were rolled and placed in chitosan tubes of 19 mm length that had been longitudinally cut. The tubes were sealed with glue and transferred into eppendorfs containing medium prior to animal transplantation.

Experimental design and surgical procedure

For the *in vivo* experiments, adult female Wistar Hannover rats (10 weeks old; 220-250 g) were used. The animals were housed with free access to food and water at room temperature of 22 ± 2 ºC. All surgical procedures were performed with aseptic operating conditions and under ketamine/xylacine anesthesia (90/10 mg/kg i.p.). The tubes were filled with different combinations of matrices and/or cells. Animals were distributed into six experimental groups: aligned fibronectin 20% and 750,000 MSC (n=6; FN-MSC); aligned fibronectin 20% and 750,000 SC (n=7; FN-SC); aligned laminin 20% and 750,000 of MSC (n=7; LM-MSC) and aligned laminin 20% and 750,000 SC (n=8; LM-SC); stabilized and rolled FN enriched construct (n=4; FN-St) and stabilized and rolled LM enriched constructs (n=4; LM-St) which served as controls.

Under a dissecting microscope, the right sciatic nerve was exposed and cut 6 mm distal to the exit of the gluteal nerve, and a nerve segment of 6 mm was resected. The distal and proximal stumps were fixed by two epineural 10-0 sutures into the ends of the implanted tube leaving a 15 mm gap. All the tubes

had a length of 19 mm, and an internal diameter of 2 mm. The muscle plane was then sutured with re-absorbable 5-0 sutures, the skin with 2-0 silk sutures and the wound was disinfected. Animals were treated with amitriptyline to prevent autotomy (Navarro & Butí, 1994).

Once the animals were operated, they received a subcutaneous injection of the immunosuppressant FK506 (Fujisawa Pharmaceuticals, Osaka, Japan) at 2 mg/kg diluted in saline solution. Rats we given daily subcutaneous injection of 1mg/kg of FK506 diluted in saline solution for up to two months. In order to prevent possible infections, all the rats were treated with 500 mg/L amoxicillin (Normon, Madrid, Spain) in drinking water during all the follow-up.

Electrophysiological tests

Functional reinnervation of target muscles was assessed at 7, 30, 60, 90 and 120 days postoperation (dpo). Animals were anesthetized with ketamine/xylacine (90/10 mg/kg i.p.). The sciatic nerve was stimulated by transcutaneous electrodes placed at the sciatic notch, and the compound muscle action potential (CMAP) of tibialis anterior, gastrocnemius and plantar muscles was recorded using monopolar needle electrodes, placing the active one in the muscle belly and the reference in the fourth toe (Asensio-Pinilla et al., 2009). During the tests, the rat body temperature was controlled an maintained with a thermostated warning flat coil. The amplitude and the latency of the M-wave were measured. The contralateral limb was used as control.

Functional evaluation of sensory recovery

The threshold of nociceptive responses to mechanical and thermal stimuli was evaluated on both hindpaws by means of algesimetry tests at 7, 30, 60, 90, 120 and 122 dpo. For both tests, the lateral area (innervated by tibial and sural nerves, both being branches of the sciatic nerve) of the plantar surface was tested (Cobianchi et al., 2014). The contralateral paw of each rat was tested as control, to overcome possible variations between testing conditions. Sensibility to mechanical stimuli was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers 30 min before the experiment for habituation. The mechanical nociceptive threshold was taken as the mean of three measurements per paw region, and expressed as the force (in grams) at which rats withdrew their paws in

response to the stimulus. A cutoff force was set to 40 g at which stimulus lifted the paw with no response. Thermal sensibility was assessed by using a plantar algesimeter (Ugo Basile, Comerio, Italy). The beam of a projection lamp was focused onto the hindpaw plantar surface pointing at the lateral side. The thermal nociceptive threshold was taken as the mean of three trials, and expressed as the latency (in seconds) of paw withdrawal response. A cutoff time was set at 20 s to prevent tissue damage. All the values are presented as percentage of response with respect to the contralateral non-injured paw.

Histology and morphometry

Four months after the injury, animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline solution (0.1M, pH=7.4). After perfusion, the regenerated nerves were harvested and postfixed in 3% paraformaldehyde - 3% glutaraldehyde phosphate-buffered solution. The nerves were postfixed in osmium tetroxide (2%, 2 h, 4°C), dehydrated through ascending series of ethanol, an embedded in Epon resin. Nerves were sectioned using an ultramicrotome (Leica). Semithin sections (0.5 μm) were stained with toluidine blue and examined by light microscopy. Images of the whole sciatic nerve were acquired at 10× with a digital camera, while sets of images chosen by systematic random sampling of squares representing at least 30% of the nerve cross-sectional area were acquired at 100× from mid and distal parts of the tube or graft. Measurements of the cross-sectional area of the whole nerve, as well as counts of the number of myelinated fibers, were carried out by using Image software (National Institutes of Health).

Survival of cells

Cellular constructs of MSC and SC were prepared as described above. For the analysis of cell survival *in vivo*, 150,000 MSC or SC were incorporated into matrices of collagen rat tail enriched with fibronectin 20%. Then 1 mL of the matrix containing 150,000 cells was incorporated in the ABS mould with the tether nylon mesh and was aligned overnight. Next day, matrices were stabilized, rolled and cut into 7.5 mm length. These matrices were used to fill 8 mm chitosan tubes. Two adult female Wistar Hannover rats (10 weeks old; 200-250 g) were anesthetized with ketamine/xylacine anesthesia (90/10 mg/kg i.p.) and the

right sciatic nerve was exposed and cut. The proximal and distal stumps were bridged with 8 mm length chitosan tubes, of an internal diameter of 2 mm, filled with collagen tethered constructs enriched with fibronectin and 75,000 MSC or SC.

72 h post operation, the animals were sacrificed with lethal anesthesia (Dolethal). The tubes were extracted and the matrices fixed with 4 % paraformaldehyde in phosphate-buffered saline solution for 2 h. The constructs were cut longitudinally with a cryostate in 50 µm serial sections.

To analyze survival and alignment of MSC and SC, we labeled the samples with primary antibodies to stain CD90-FITC for MSC (1:100, BD Pharmigen) or S-100 (1:100, Immunostar) for SC. SC were incubated for 2 h with biotinylated IgG (1:200, Life Bioscience) and incubated overnight with Spreptavidin 488 conjugated secondary antibodies (1:200, Life Bioscience). MSC were incubated for 2h with the CD90-FITC antibody. Cells were mounted in mowiol containing DAPI.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis comparisons between groups for MTT assay and histological results was made by one-way ANOVA followed by Bonferroni post test. Statistical comparisons between groups and intervals for algesimetry and electrophysiological tests results were made by two-way ANOVA followed by Bonferroni post test. Differences were considered significant if $P < 0.05$.

RESULTS

Susceptibility of SCs and MSCs to different ECM

Cell survival on different ECM coatings was examined by MTT after 72 h in culture (Fig. 1). When analyzing mitochondrial activity in SCs, we observed a significant increase of cells grown on laminin coated surfaces (119.89 ± 4.46; $P < 0.0001$) compared to the ones grown on fibronectin (108.57 ± 2.37) or poly-D treated ones (100.00 ± 1.92). Similar, cells cultured on fibronectin had higher mitochondrial ($P < 0.0001$) activity compared to the ones cultured on poly-D lysine (Fig. 1).

When analyzing cell survival of MSCs in different ECM coatings, we observed a significant increase of cells grown on fibronectin coated surfaces (121.76 ± 5.40) compared to poly-D treated ones (100.00 ± 6.04; $P < 0.0001$), but not with laminin coated ones (110.76 ± 9.24; $P > 0.05$) (Fig. 1).

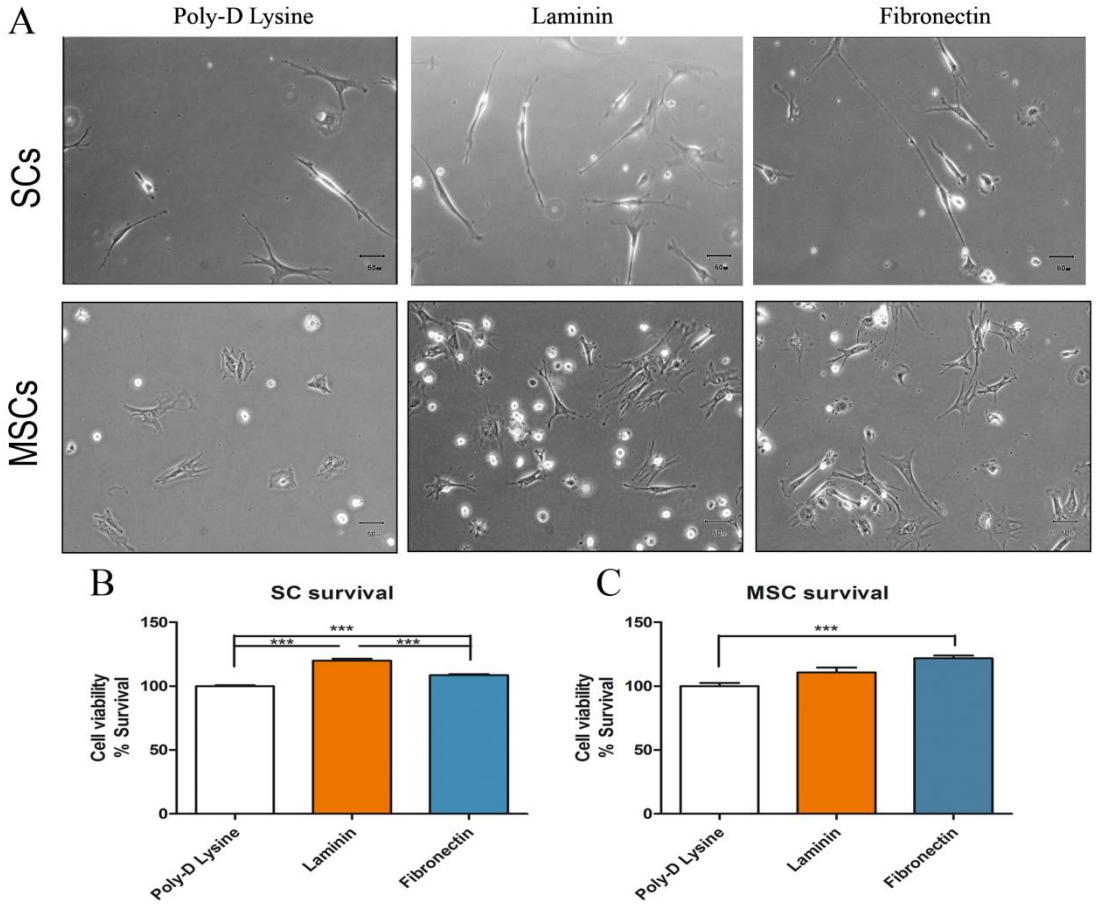

Figure 1: Representative images of cultured Schwann cells (upper panels) or Mesenchymal Stem cells (lower panels) on poly-D lysine, laminin and fibronectin coatings (A). MTT assays of dissociated Schwann cells (B) and Mesenchymal Stem cells (C) cultured for 72 h. * < 0.05; ** < 0.001; *** < 0.0001.

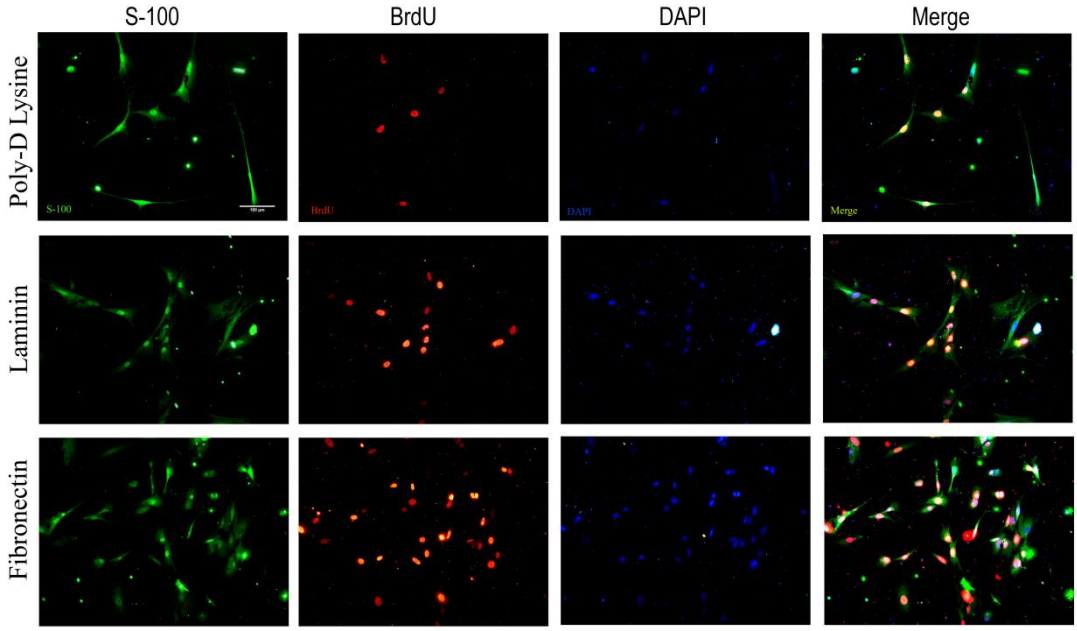

Figure 2: *In vitro* proliferation of Schwann cells grown on poly-D lysine, laminin and fibronectin coated surfaces after 72 h in culture. BrdU was incorporated in the media after 48h in culture and cells were fixed 24 h after. Schwann cells were labeled with S-100 (green), BrdU (red) and DAPI (blue). Scale bar = 100 µm.

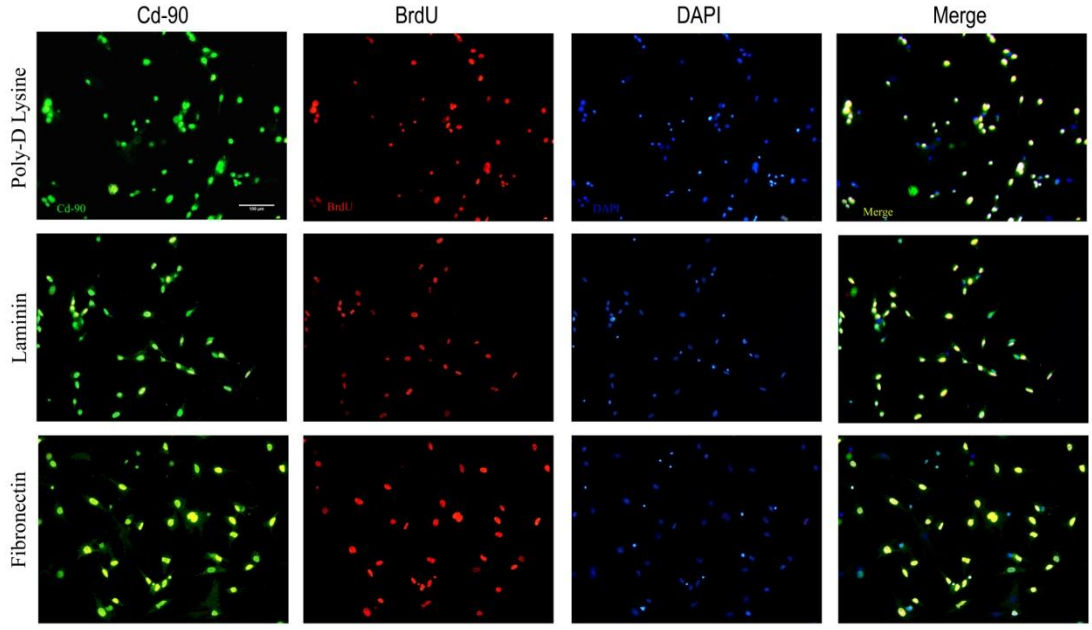

Figure 3: *In vitro* proliferation of Mesenchymal Stem cells grown on poly-D lysine, laminin and fibronectin coated surfaces after 72 h in culture. BrdU was incorporated in the media after 48h in culture and cells were fixed 24 h after. Mesenchymal Stem cells were labeled with Cd90 (green), BrdU (red) and DAPI (blue). Scale bar = 100 µm.

Proliferation activity of SCs and MSCs

By BrdU staining, we evaluated cell proliferation on different coatings. After 72 h in culture, SCs (Fig. 2) and MSCs (Fig. 3) proliferated similarly on the different coatings.

Muscle reinnervation

Nerve conduction tests performed one month after sciatic nerve injury demonstrated complete dennervation of the hindlimb muscles. Initial evidence of reinnervation of the tibialis anterior and gastrocnemius was found at 60 dpo in some animals of each group, with CMAPs of small amplitude. The CMAPs progressively increased in amplitude and were recorded in the majority of animals over time, with a similar pattern for each muscle. At the end of follow up (120 dpo), reinnervation of the tibialis anterior and gastrocnemius muscles was observed in 3 of 4 animals in the FN-St group, 3 of 4 in the LM-St group, 5 of 6 in the FN-MSC group, 6 of 7 in the LM-MSC group, 7 of 7 in the FN-SC group and 8 of 8 in the LM-SC group. Significant differences at the tibialis anterior muscle ($P<0.0001$) were observed at the final time point between FN-SC and LM-SC groups vs the acellular stabilized constructs (FN-St and LM-St). Significant differences ($P<0.05$) were also observed at the tibialis anterior muscle between the FN-SC and LM-SC group vs the MSC cellular

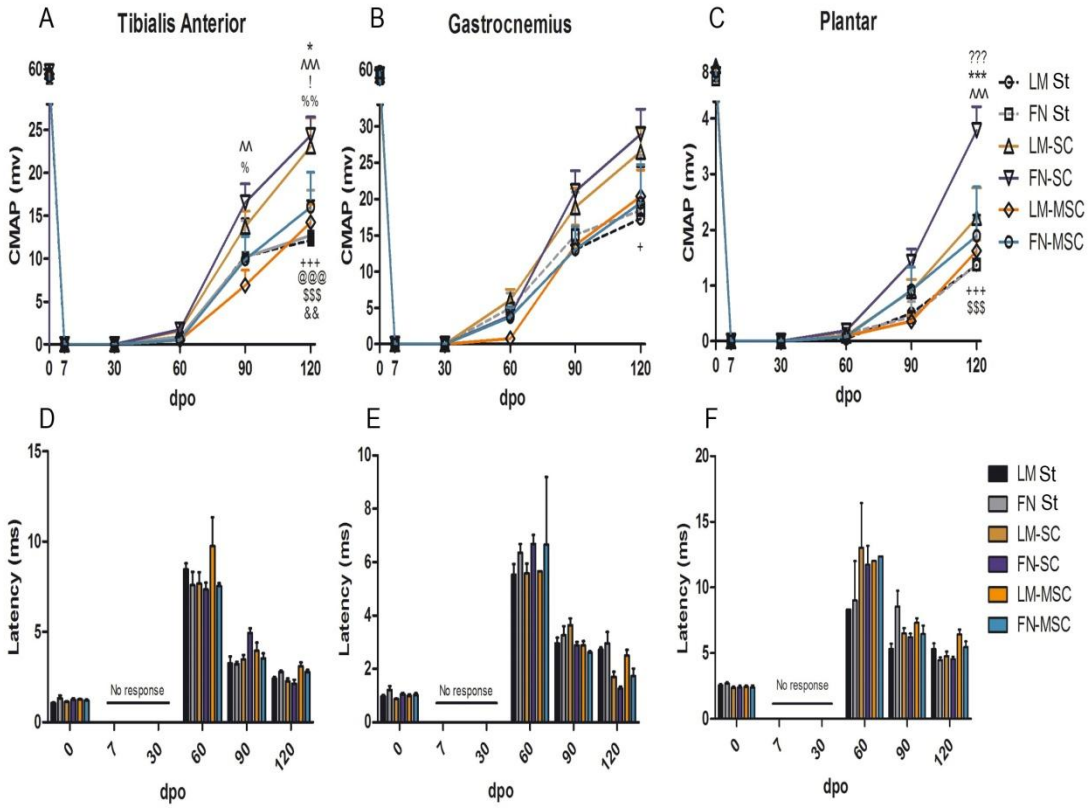

Figure 4: Mean amplitude of the compound muscle action potential (CMAP) of tibialis anterior (A), gasctrocnemius (B) and plantar muscles (C) of the injured himb limb of the rats during 4 monts after sciatic nerv lesion and repair. $^{+}$< 0.05; $^{+++}$ < 0.0001 between FN-SC and LM-St groups, $^{@@@}$ < 0.0001 between LM-SC and LM-St groups, $^{\$\$\$}$ < 0.0001 between FN-SC and FN-St groups, $^{\&\&}$ < 0.001 between LM-SC and FN-St groups, $^{\%\%}$ < 0.001 between LM-SC and LM-MSC groups, $^{!}$ < 0.05 between LM-SC and FN-MSC groups, $^{\wedge\wedge\wedge}$ < 0.0001 between FN-SC and LM-MSC groups, * < 0.05; *** < 0.001 between FN-SC and FN-MSC groups, and $^{???}$ < 0.001 between FN-SC and LM-SC groups.

constructs (FN-MSC and LM-MSC) groups (Fig 4). Significant differences at the final time point in the gastrocnemius muscle (P<0.05) were only observed between the FN-SC and the LM-St groups.

Latencies of CMAPs were considerably longer than normal during the first stages of reinnervation, and tended to shorten with time toward normal values. At the end of the follow-up, the latencies of the waves recorded on tibialis anterior and gastrocnemius muscles were similar between groups (Fig. 4).

At the more distal plantar interosseous muscles, some animals already presented signs of reinnervation at 60 dpo. In these cases, CMAPs were of very small amplitude (less than 0.5 mV). At the end of the follow-up (120 dpo), evoked potentials of the plantar muscle were observed in 3 of 4 animals in the FN-St group (1.37 ± 0.87 mV), 3 of 4 in the LM-St group (1.37 ± 0.90 mV), 5 of 6 in the FN-MSC group (1.89

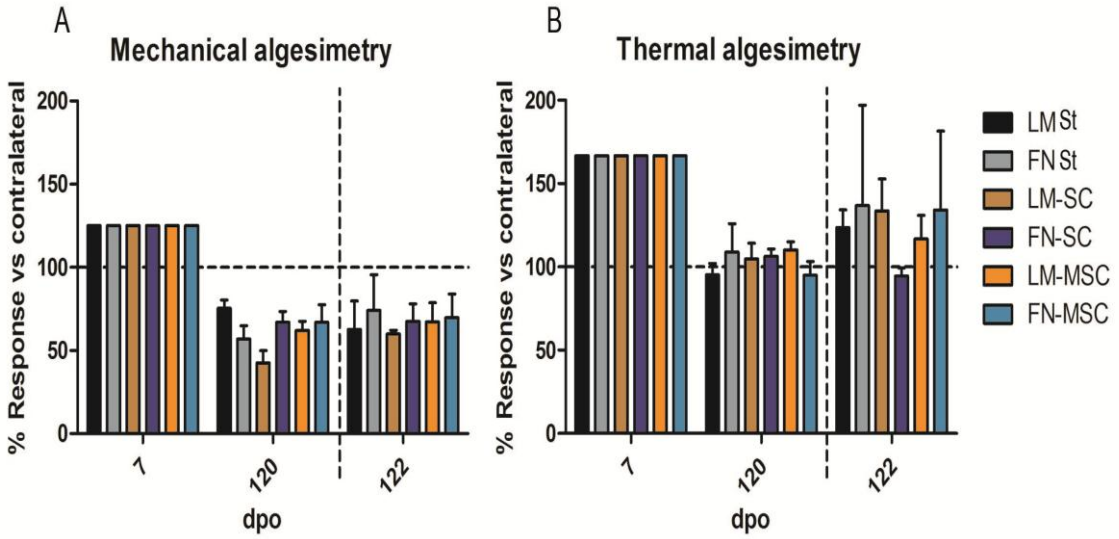

Figure 5: Mechanical (A) and thermal (B) algesimetry test results. Values were expressed as percentage of withdrawal response to mechanical stimuli and thermal stimulus applied to the lateral side of the injured paw versus the withdrawal response in the uninjured paw. Horizontal dotted lines represent the normalized baseline values. Vertical dotted lines indicate when the saphenous nerve was cut.

± 0.99 mV), 6 of 7 in the LM-MSC group (1.62 ± 0.25 mV), 7 of 7 in the FN-SC group (3.78 ± 0.56 mV) and 8 of 8 in the LM-SC group (2.22 ± 0.71 mV). Significant differences ($P<0.0001$) were observed between the FN-SC and the rest of the groups (Fig. 4).

The plantar CMAP latency of the waves registered followed a similar pattern than the one described in the tibialis anterior and gastrocnemius muscle, and were shortened with time toward normal values during the progression of the regeneration process and without significant differences between groups.

Recovery of nociceptive sensibility

Withdrawal responses to mechanical and thermal stimuli were evaluated by means of the Von Frey test and the plantar test respectively. Absence of responses at the lateral part of the injured paws of the rats during the first 30 dpo demonstrated absence of reinnervation, and therefore they were penalized with a cut off value of 40 g (Von Frey) or 20 ms (Plantar test).

Withdrawal responses to mechanical stimuli obtained from 60 to 120 dpo demonstrated that the injured paw responded to lower intensity stimulus when compared to the contralateral side, indicating some degree of hyperalgesia (Fig. 5a). After elimination of the saphenous nerve at 120 dpo, the withdrawal responses to mechanical stimulus in the different groups compared to their contralateral side

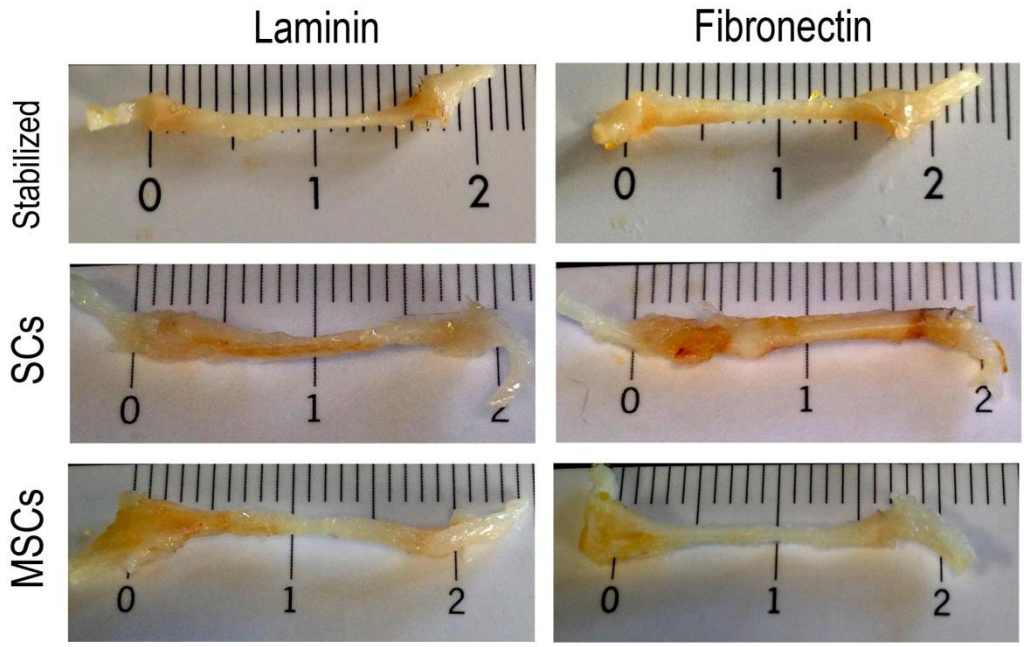

Figure 6: Representative images of regenerated cables found 4 mountsh after implantation inside the chitosan tubes (that have been retired) containing collagen matrices enriched with laminin or fibronectin either without cells (stabilized) or with SCs or MSCs.

were 74± 21g in FN-St group, 63 ± 15g in LM-St group, 70 ± 17 g in FN-MSC group, 67 ± 15g in the LM-MSC group, 68 ± 14g in the FN-SC group and 53 ± 3 g in the LM-SC group, without significant differences between them.

Withdrawal responses to heat stimuli in the plantar test showed similar evolution than those observed for the Von Frey test (Fig. 5b). Animals had no response to heat stimuli on the denervated paw during the first 30 dpo. From 60 to 120 dpo, most rats showed withdrawal responses at lower stimulus intensity than in the contralateral paw. When the saphenous nerve was cut, the withdrawal time of the injured paw at 122 dpo were 134± 60 s in the FN-St group, 124 ± 11s in the LM-St group, 134 ± 58s in the FN-MSC group, 117 ± 17s in the LM-MSC group, 95 ± 6s in the FN-SC group and 134 ± 25 s in the LM-SC group, without significant differences between them.

Histological results

Macroscopic examination of regenerated nerves after 4 months follow-up revealed that 3 of 4 animals in the FN-St and LM-St groups, 5 o 6 animals in the FN-MSC, 6 of 7 animals in the LM-MSC, 7 of 7 animals in the FN-SC and 8 of 8 animals in the LM-SC presented a regenerated cable inside the chitosan tube (Fig. 6). The regenerated nerve occupied the lateral parts of the chitosan conduit, growing around the non-degraded ECM-scaffold.

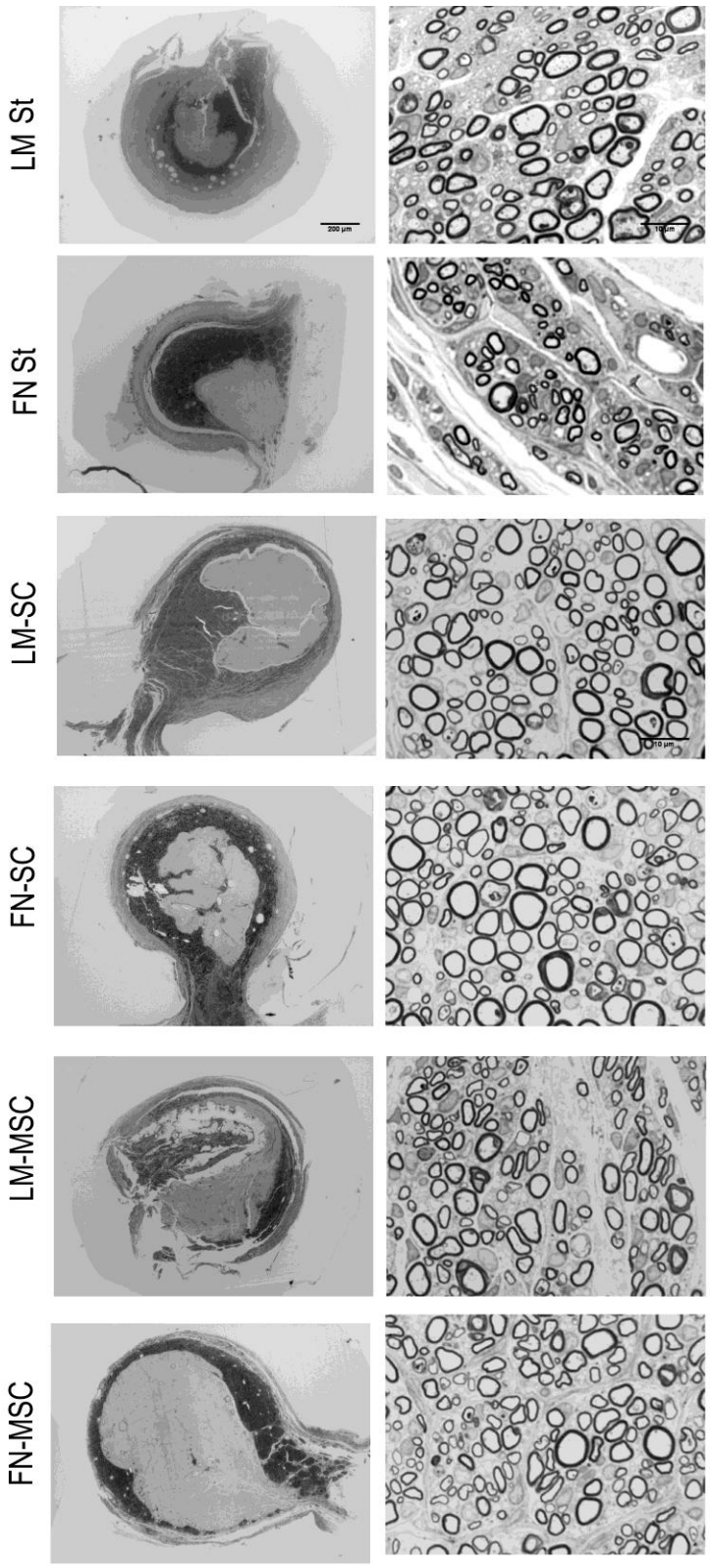

Figure 7: Micrographs of semithin sections of the regenerated nerve taken at the midpoint of the tube 4 months after sciatic nerve resection and repair from a representative animal of each group. General appearece of the regenerated nerves (left panels); scale bar = 100 µm. Higher magnification of the regenerated nerves (right panels); scale bar = 10 µm.

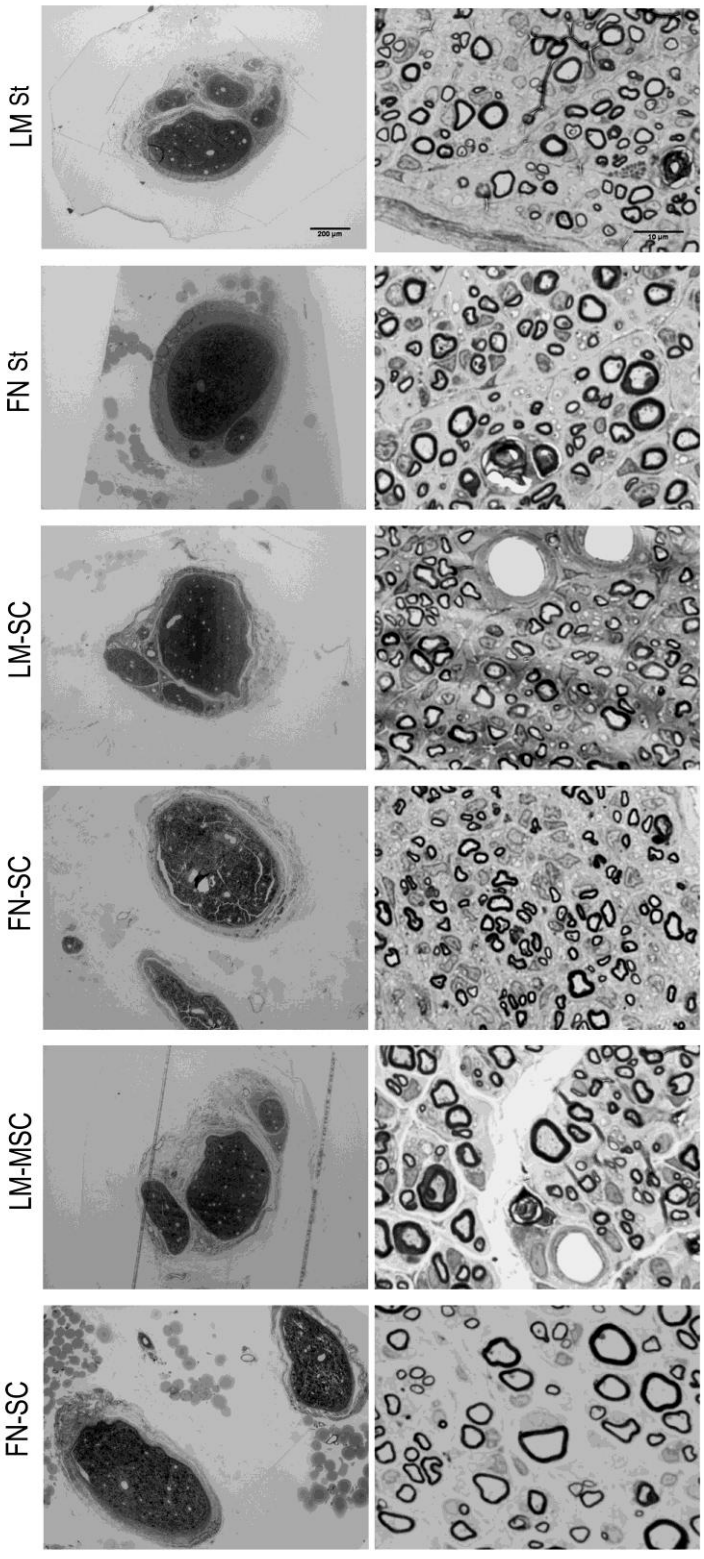

Figure 8: Micrographs of semithin sections of the regenerated nerve taken at the distal point of the tube 4 months after sciatic nerve resection and repair from a representative animal of each group. General appearece of the regenerated nerves (left panels); scale bar = 100 µm. Higher magnification of the regenerated nerves (right panels); scale bar = 10 µm.

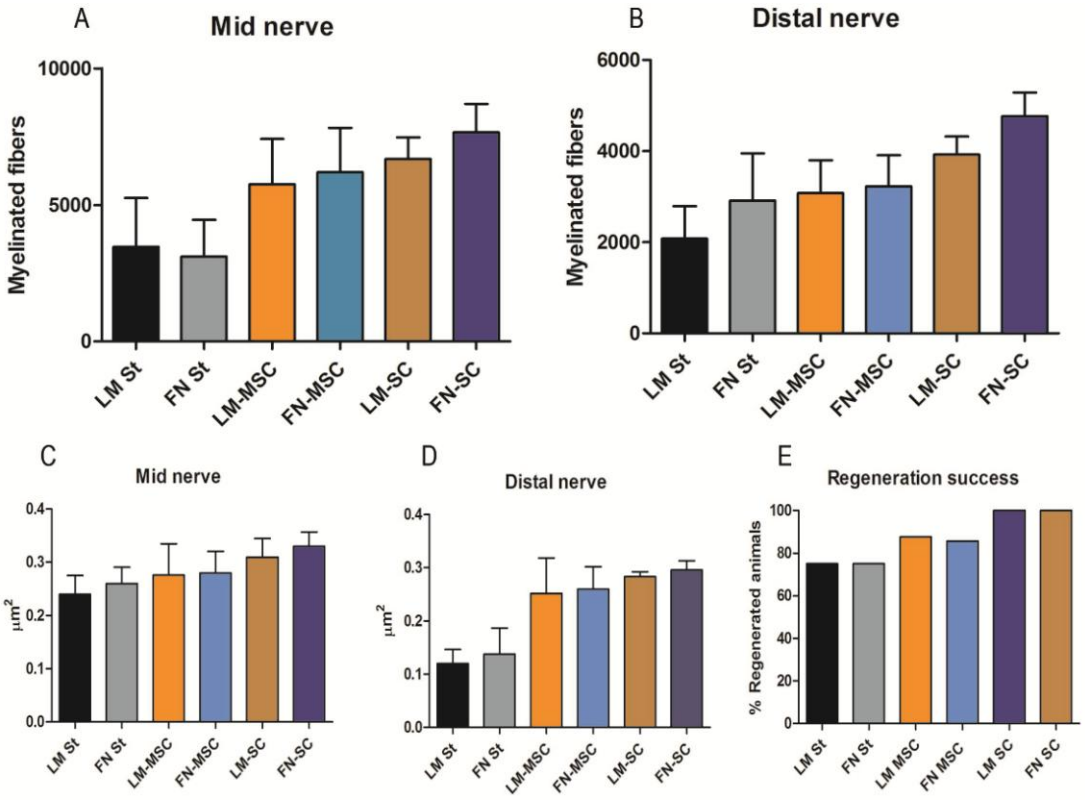

Figure 9: Estimated number of regenerated myelinated fibers in the tibial nerve at the mid-tube (A) and 3mm distal (B) 4 months after sciatic nerve resection and repair. Cross-sectional area of the the mid-tube (C) and 3 mm distal (D). Animals with no regenerated nerve were also included (with null values) in the calculation. * $P < 0.05$. Percentage of regenerated cables found at 120 dpo in the different experimental groups (E).

The nerves were surrounded by a thick, homogenous connective layer, with no signs of inflammatory responses.

Macroscopic appearance of regenerated nerves revealed a thicker cable in the FN-SC compared to other groups (Fig. 7-8). Transverse sections of the regenerated nerves taken at the midpoint of the tube and 3 mm distal to the distal suture were analyzed under light microscopy. To compare the absolute number of myelinated fibers, regenerated and non-regenerated rats were included in the statistical analysis, giving null values to non-regenerated animals. The mean number of myelinated fibers at the midpoint of the tube was higher in the FN-SC group (7,656 ± 1,379) followed by the LM-SC group (6,677 ± 1,119), the FN-MSC group (6,192 ± 1,999), the LM-MSC group (5,750 ± 2,198), the LM-St group (3,458 ± 1,795) and the FN-St group (3,105 ± 1,348), but without significant differences between groups. The same pattern was observed in the distal nerve, where the number of myelinated fibers was higher in the FN-SC group (4,771 ± 683) followed by the LM-SC group

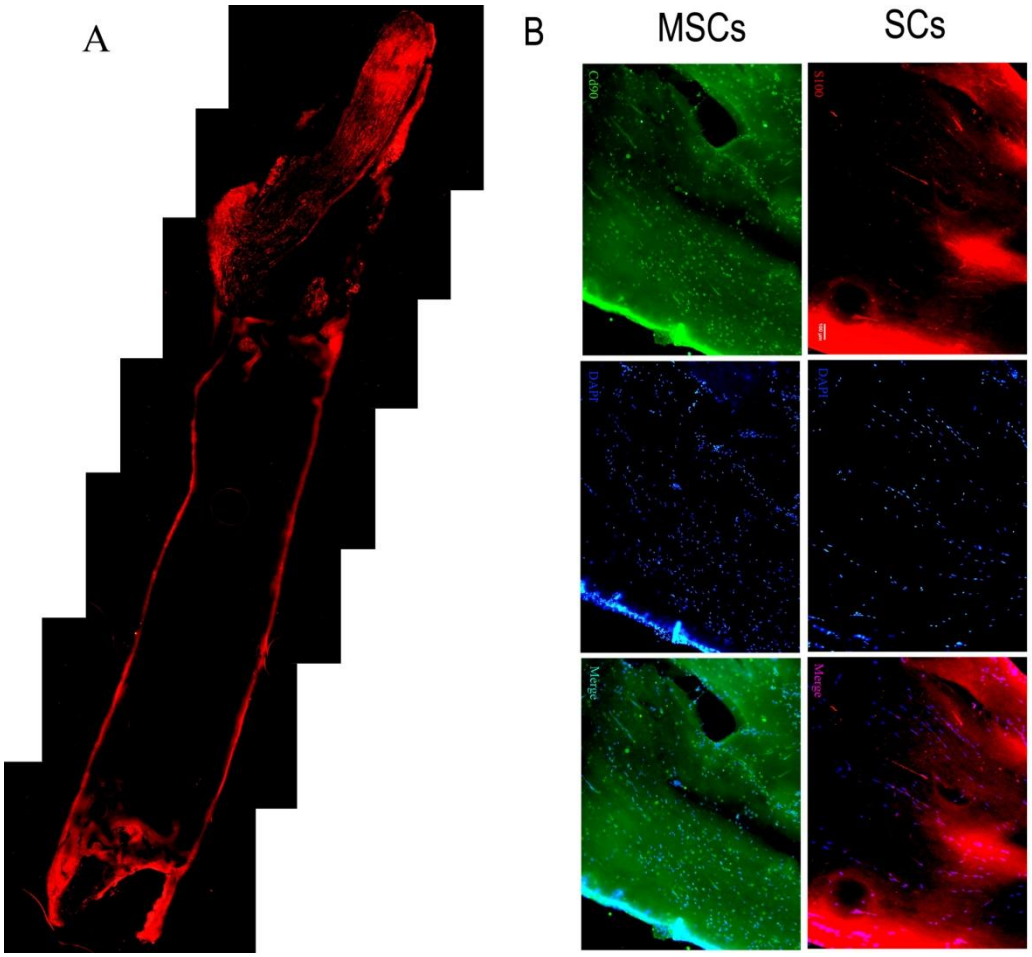

Figure 10: Micrographs of semithin sections of the regenerated nerve taken at the distal point of the tube 4 months after sciatic nerve resection and repair from a representative animal of each group. General appearece of the regenerated nerves (left panels); scale bar = 100 µm. Higher magnification of the regenerated nerves (right panels); scale bar = 10 µm.

(3,927 ± 524), the FN-MSC group (3,228 ± 907), the LM-MSC group (3,076 ± 962), the FN-St group (2,915 ± 1,037) and the LM-St group (2,079 ± 711) (Fig. 9), without significant differences between groups.

Cell viability within the constructs

SCs and MSCs were seeded in different matrices to bridge a 6 mm gap in the rat sciatic nerve. Cell viability and alignment was assessed 72 h post implantation. Seeded SCs labeled with S100 extended long processes and were aligned within the construct. However, MSCs, labeled with Cd90-FITC, were randomly distributed along the matrix and presented a circled shape (Fig. 10).

DISCUSSION

In this study we have compared the effect of using cellular aligned constructs of SCs or MSCs on collagen type I based matrix enriched with either fibronectin or laminin. These matrices were tested on chitosan conduits to

sustain axonal regeneration across a critical 15 mm gap resection of the sciatic nerve in rats. Our results show that addition of SCs to either fibronectin or laminin enriched scaffolds subjected to alignment guarantee regeneration of peripheral nerves in a 100% of cases.

Among other alternatives, the use of artificial nerve guides has been proposed to substitute autograft in the repair of severe peripheral nerve lesions. Material development of artificial nerve guides progressed during the last decades to obtain bio-degradable and bio-compatible devices for the repair of injured nerves (Deumens et al., 2010). In this study, we have used a chitosan device which has already been proven to be more effective than the standard silicone tube for supporting axonal regeneration across limiting gaps in rats (Gnavi et al., 2013; Gonzalez-Perez et al., 2015; Haastert-Talini et al., 2013). However, the percentage of success in this model is still far from the 100% of regenerated animals reached when using the autograft to bridge long peripheral nerve gaps. Therefore, there is the need to further improve the regenerative capabilities of the implanted neural guides. Regeneration in artificial conduits is highly dependent on the formation of an initial fibrin cable which serves as a guidance structure to fibroblasts, SCs and blood vessels to migrate and populate the cable and facilitate axonal regeneration (Williams et al., 1983). To further mimic the natural geometry and architecture of the endoneurial tubes in nerve grafts, cellular and extracellular components can be longitudinally aligned. Among different techniques (gradients, electrical fields, magnetic fields, nano-fibers, etc.), cells embedded in ECM-based hydrogels can be tethered aligned, to facilitate the recreation of the natural environment observed in the peripheral nerve tissues (Georgiou et al., 2013). The tension forces created by the nylon mesh at the opposite ends of the moulds generate a uniaxial strain in the gel in which the matrix become aligned (Phillips et al., 2005). In response to this tension, which is mainly dependent on the collagen concentration, the number of cells and the cell type, the collagen fibrils are able to effectively organize and orientate, conferring advantages to fully hydrated hydrogels.

The potential ability of cellular aligned constructs to promote neural regeneration *in vivo* was investigated using a rat sciatic nerve injury model. In

this study, we highlighted the potential use of collagen-based tethered gels, enriched with either fibronectin of laminin, combined with SCs or MSCs to bridge a critical sized gap of 15 mm in rats after peripheral nerve injuries. Animals were administered with the immunosuppressant agent FK506 for the first two months to avoid rejection of allogeneic donor transplanted cells. Since administration of FK506 has been demonstrated to enhance regeneration and functional recovery after peripheral nerve injuries (Udina et al., 2004), groups with acellular constructs were also treated with the drug. In these groups, the percentage of animals with a regenerating cable in the tube was of about 75% of success, similar to the results obtained in a previous study where animals were not treated with the immunosupressant (data not published). In contrast, addition of aligned cells to these constructs increased the success of axonal regeneration.

Both SCs and MSCs have been used to promote nerve regeneration and provide support in long peripheral nerve gaps. The potential ability of those cells rely mainly on populating the neural guide and continuously stimulate the production of neurotrophic factors (Levy et al., 2015).

Interestingly, we found that addition of SCs either to fibronectin or laminin-enriched constructs increased the proportion of animals that regenerated and enhanced motor reinnervation. When using SC-aligned hydrogels, the proportion of animals that presented a regenerated cable increased to 100% of cases, compared to the nearly 90% obtained when using MSC-aligned constructs. These differences may be determined by the different behavior of both these cells in the scaffolds. ECM molecules like laminin or fibronectin influence cell and tissue behavior by providing an adhesive substrate to anchorage-dependent cells. Therefore, these molecules can determine a variety of cell differentiation processes (Floren & Tan, 2015). For instance, Abdeen and colleagues demonstrated a positive correlation between fibronectin, stiffness and proangiogenic signaling of MSCs in 2D hydrogels (Abdeen et al.,, 2014). Indeed, in another study, the angiogenic marker PECAM was decreased in MSC when cultured on laminin soft matrices (Floren & Tan, 2015). On the other hand, SC proliferation was increased in laminin (Armstrong et al., 2007) and fibronectin (Baron-Van Evercooren., et al 1982) coated surfaces *in vitro*. In this study we also tested the commitment of MSCs

and SCs on laminin and fibronectin coated surfaces in vitro. As previously shown, MSCs survived better on fibronectin coated surfaces and SCs on laminin coated surfaces. However, in contrast to what was observed *in* vitro, SCs seeded in fibronectin aligned matrices provided better functional results than SCs seeded in laminin aligned matrices. Nevertheless, the positive effect of fibronectin on SC proliferation and migration have been already proved (Baron-Van Evercooren et al., 1982; Mosahebi et al., 2003) On the other hand, independently of the ECM matrix used to incorporate the cells within the aligned constructs, final outcome on animal recovery in groups with SCs was improved when compared to MSC groups. In fact, when we analyzed cell distribution on the 3D scaffolds, SCs appear to be positioned parallel to the aligned collagen fibrils, but MSCs acquire a rounded shape and seemed to appear randomly distributed. This random distribution was similar on laminin or fibronectin enriched scaffolds. Since not only single cells but also intercellular forces may play an important role on cellular alignment (Sanen et al., 2015), MSC density may be increased to facilitate the alignment of the collagen fibrils of the matrix. However, our results also point that SCs are better candidates that MSC to populate neural conduits to promote axonal regeneration in the PNS. SCs are the glial cells of the peripheral nervous system, which are the responsible of myelinating peripheral axons and guide them toward their target organs. However, their clinical use is limited. The acquisition of autologous SCs requires the extraction of a donor nerve and the consequent loss of its function. Moreover, to amplify the number of cells obtained, a long period of pre-operative cultivation is needed (Keilhoff et al., 2006). Furthermore, SCs lose rapidly their phenotypic characteristics *in vitro* (Kingham et al., 2007).

The notable results obtained in the constructs seeded with MSCs, where regeneration was guaranteed in a high proportion of cases (near to 90%) make MSCs a real alternative to seed artificial nerve conduits. MSCs are non-hematopoietic cells which reside in the bone marrow. They mainly differentiate into cell lineages from mesodermal origin such as muscle, bone, cartilage, etc. (Baksh et al., 2004). When MSCs have been directly transplanted to a injured nerve, these cells demonstrated to increase survival of motor and sensory axons, improved axonal outgrowth, increasing the number of

myelinated fibers and the production of neurotrophic factors (i.e. GDNF) (Marconi et al., 2012). MSCs have also been previously used to seed grafts in rats, improving electrophysiological and histological tests (Keilhoff et al., 2006; Wang et al., 2015). These cells have a high clinical potential since they are easy to be harvested from bone marrow biopsies and exhibit great plasticity and ability to differentiate towards multiple cell-lineages, allowing them to adjust to the requirements needed to promote axonal regeneration. For instance, MSCs may transdifferenitated to SC-like cell, acquiring the capacity of guiding and myelinating peripheral axons (Dezawa et al., 2001; Pan & Cai, 2012). Despite their potential, cell therapy for the repair of peripheral nerves using these cells still has not been investigated in clinical trials yet.

CONCLUSIONS

In experimental models, combination of cell therapy and tissue engineering caused a notable improvement in the number of regenerated animals and the degree of functional recovery achieved after repairing a critical sized nerve gap with a neural guide, turning tube repair as a real alternative to autograft in the repair of injured peripheral nerves. Although SC-seeded grafts are likely to become the best alternative to autograft in the repair of long peripheral nerve injuries, the higher potential of MSC-seeded grafts for the clinical use makes this cells a strong candidate.

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DISCUSSION

Summary of Results and Discussion

Severe peripheral nerve injuries lead to the disconnection between the neural soma and its target organ. This disconnection causes the loss of motor, sensory and autonomic control, which leads to a disabling situation for the patient. Regeneration can occur in the peripheral nervous system, but the clearance of the debris and the creation of a permissive environment at the distal nerve and the switch of the axotomized neuron to a pro-regenerative state are needed to facilitate adequate reinnervation and functional recovery.

In this thesis we aimed to further study the role of ECM molecules, focusing on laminin and fibronectin, on nerve regeneration, and their potential role to enhance regeneration when used to functionalize neural guides. For that in our first chapter we focused on the role of these ECM to promote selective regeneration of motor and sensory axons when used to enrich collagen-based scaffolds. We used organotypic spinal cord slices and DRG explants of early postnatal and young adults animals, and we contrasted our results in an *in vivo* model in the adult. Our results show that fibronectin enriched scaffolds increased the elongation of motor and sensory neurites whereas laminin mainly increased elongation of sensory ones, compared to collagen-based scaffolds *in vitro*. Furthermore, in young postnatal stages, fibronectin-enriched matrices preferentially promoted elongation of motor neurites and presumptive proprioceptive neurites, whereas laminin increased the elongation of presumptive cutaneous sensory neurites. This is important, since neuron type preference towards specific extracellular matrix components may facilitate the organization of regenerating axons in muscular or cutaneous nerve branches. However, this preferential activity was not observed in the adult.

Although the collagen-based matrices enriched with these two ECM was not able to promote selective regeneration of motor and sensory neurites in an *in vivo* model in the adult, we also wanted to test the potential of these matrices to be used as scaffolds to functionalize neural guides, as a feasible surgical alternative to the autograft in the repair of severe PNI. For the design of the best neural guide, we first evaluated the potential of chitosan-based guides to repair severe peripheral nerve defects (15 mm in the rat), where the general outcome achieved by this repair technique (degree of recovery, functional reinnervation and number of regenerated fibers) is still inferior to the autograft (considered the gold standard technique for the clinical practice) despite

assuming the loss of a secondary healthy nerve. As it is shown in the second chapter, repair with chitosan-based guides, that are biodegradable and absorbable, allowed about a 50% of the animals to regenerate, whereas repair with silicone conduits, the standard tube, resulted in complete failure during the regenerating process. Although these are promising results for a hollow conduit, the percentage of regenerated animals was still far from the 100% found in the autograft. Therefore, in our third chapter we implemented the intratubular content by introducing fibronectin and laminin enriched collagen scaffolds, both as fully hydrated hydrogels or stabilized and rolled. In the latter case, the percentage of animals that regenerated increased to a maximum of 75% in the group with tubes containing fibronectin-enriched, stabilized and rolled hydrogels. With the ultimate goal of mimicking what is occurring in a healthy nerve or the distal fragment of a degenerated nerve, in the fourth chapter we further functionalized the lumen of the tube by incorporating supporting cells to these matrices. We wanted to study whether these ECM-based scaffolds were suitable vehicles to incorporate support cells to the system. We harvested and cultured Schwann cells and Mesenchymal Stem cells which were embedded in a tethered-aligned collagen-based construct enriched with either fibronectin or laminin and implanted to the animals. To avoid rejection of the transplanted cells, we systemically treated the animals with the immunosuppressant agent FK506. When adding cells to the matrices, we achieved a degree of recovery comparable to the autograft model, since the percentage of regenerated animals were close to 90% of the Mesenchymal Stem cells repaired animals and of 100% of those repaired with SCs grafts.

Role of ECM molecules on selective neuronal regeneration

In vitro models offer certain advantages to *in vivo* studies since interpretation of results may be easier and more conditions can be assessed. However, most of these studies have focused on dissociated sensory neurons or DRG explants (Tucker & Mearow, 2008). In contrast, cultured motoneurons have been scarcely used, mainly due to the demanding protocol needed to culture them (Montoya et al., 2009). This fact limits a direct comparison between motor and sensory neuron population and their response to selective cues. In our experiments in Chapter 1, we used a 3D culture model which was previously set up in our laboratory based on organotypic spinal cord slices and DRG explants which allow to compare neurite outgrowth of motor and sensory neurons (Allodi, et al., 2011). After PNIs, although transected axons may be capable to

regenerate and reinnervate target organs, the reinnervation of the target organs may be incorrect due to the random regeneration of the injured axons. This aberrant regeneration leads to misrouting of growing axons through non appropriate nerve branches which affects functional recovery and the final outcome of nerve regeneration. For that reason, specificity of reinnervation is a key issue to be solved during peripheral nerve regeneration.

It remains unclear whether injured axons have any intrinsic mechanism which provides them preferential guidance or preference to certain pathways or targets (Madison et al., 2009). Differential expression of molecules in motor and sensory nerves has been previously reported. For instance, a different profile of neurotrophic factor expression between Schwann cells of motor and sensory nerves has been described (Höke et al., 2006). Moreover, in our lab, we demonstrated the potential role of the neurotrophic factor FGF-2 in the preferential promotion of motoneuron outgrowth *in vitro* (Allodi et al., 2013) and *in vivo* (Allodi et al., 2014). However, little is known about the role of tropic factors on neural guidance and preferential reinnervation.

We observed a great effect of fibronectin on promoting motoneuron and presumptive proprioceptive sensory neuron elongation in organotypic spinal cord slices and DRG explants of postnatal day 7 rats. On the other hand, laminin showed a higher effect on promoting elongation and arborization of other large myelinated sensory neurites, presumptive cutaneous ones. These results were in agreement to others found in the literature, where during development of chick embryos, fibronectin was highly expressed in the pathway that proprioceptive neurons follow and an increased expression of its receptor integrin α5β1 was observed; on the other hand presumptive cutaneous neurons preferred to grow on laminin coated surfaces rather than in fibronectin ones and these neurons expressed higher levels of its receptor integrin α7β1 (Guan et al., 2003).

However, when using DRG cultures obtained from postnatal day 21 rats, we observed a decrease in the proportion of proprioceptive sensory neurons compared to the ones observed at p7. Moreover, *in vivo*, the incorporation of laminin or fibronectin enriched collagen gels to a nerve conduit to repair a small peripheral nerve defect of 6 mm in the sciatic nerve in adult rats did not alter the number of regenerated motor and sensory neurons; when analyzing the population of presumptive proprioceptive sensory

neurons, we did not observe co-localization between the regenerated labeled neurons and parvalbumin expression, suggesting a slow regeneration rate of adult proprioceptive sensory neurons compared to other populations of sensory neurons.

To better understand the preferential role of fibronectin and laminin on guiding motor and sensory neurites during early postnatal stages and why this guidance is lost in the adult, we studied the expression of their integrin receptors. The levels of integrin receptors expressed during development are reduced in the adult (Lemons & Condic, 2008), but increased following peripheral nerve injuries (Eva & Fawcett, 2014; Wallquist et al., 2004), increase that it is related to successful regeneration (Previtali et al., 2001). It has been reported that the different affinity of integrin receptors to different ECM proteins may contribute to characteristic patterns of growth cone migration and neurite extension (Letourneau et al., 1994). As mentioned above, in the study of Guan and colleagues (Guan et al., 2003), the different expression of integrin receptors may be one of the earliest markers for sensory neuron fate. Consistent to previous data, our results in Chapter 1 corroborated an increase of integrin expression in both motor and sensory injured neurons *in vivo* and a marked increase in cultured spinal cord slices and DRG of p7 animals *in vitro*.

Furthermore, we found that in the samples embedded in the fibronectin or laminin enriched collagen gels, integrin expression was reduced compared to non-enriched gels, but still increased compared to basal levels. In our experiments, neurons exposed to a very permissive environment (ECM-enriched collagen gels) led to a lower expression of integrins, probably to avoid a cross activation of cAMP levels which can provoke growth cone collapse (Lemons & Condic, 2008). Interestingly, if we analyze the ratio of expression between both integrins, $\alpha5\beta1/\alpha7\beta1$, we can observe a higher expression in cultured spinal cord slices of p7 animals rather than *in vivo* spinal cords. These differences could explain why motor axons lose their preference to fibronectin in the adult.

Our results in Chapter 1 demonstrate that the potential capabilities of extracellular matrix proteins to control preferential motor and sensory neuron outgrowth is limited, since only when experiments where performed in early postnatal animals, different pattern of neurite elongation was found. On the other hand, *in vivo* studies in the adult rats showed that the difference in neuron subtype selectivity to different extracellular matrix molecules is lost.

Artificial nerve guides for peripheral nerve regeneration

After peripheral nerve transections, that causes the separation between the two nerve stumps, the lack of repair will lead to failure of regeneration and reinnervation. Therefore, in this case, the need of surgical repair is mandatory in order to reestablish the continuity of the two stumps. When the distance between the two stumps is small, their approximation and direct suture is sufficient to guarantee regeneration. However, when the distance increases and tension-free coaptation of the nerve is not possible, there is the need of the interposition of a bridge to re-unite these stumps. In this case, the gold standard technique is the autologous nerve graft. As an alternative, the gap can be bridged by using an artificial nerve guide.

However, the main limitation of nerve guides is the distance between the stumps that may be bridged. As the distance to be bridged increases, regeneration and functional outcome decrease and eventually fail. In fact, tube repair has been demonstrated to be as efficient as autograft in short peripheral nerve lesions both at histological level and functional recovery. However, in rats, the limited distance in which a hollow nerve guide cannot sustain regeneration is set at 15 mm (Lundborg et al., 1982).

The results obtained in Chapter 2 show that the autologous nerve transplant for the repair of a peripheral nerve injury of 15 mm in the rat allow the regeneration in a 100% of cases, when compared to the complete failure of regeneration in the animals repaired with silicone hollow conduits. Interestingly, chitosan hollow guides from two different degrees of acetylation showed considerably better results than standard silicone tubes, since near to 50% of operated animals regenerated. Although results at this step were far from the ones obtained with the autograft, the rate of successful regeneration and the levels of reinnervation achieved were the highest reported at that time for a hollow nerve guide over a critical sized gap of 15 mm in the rat sciatic nerve (see Gonzalez-Perez et al., 2014). The positive results obtained with chitosan guides may be related to the use of biological materials (i.e. chitosan) which permits a better integration of the implanted conduit in the connective tissues of the host, favoring the rapid vascularization and fibrin covering of the tubular wall. This enriched environment allows the formation of the fibrin cable and the subsequent cell migration. Furthermore, the biodegradation of biological-based conduits may avoid the mechanical damage due

to compression of the regenerated nerves in non-resorbable devices (Mackinnon & Dellon, 1990).

The degree of acetylation of chitosan alters the degradability rate of the tubes. Higher degree of acetylation (~20%) may result in the total degradation of the tube within 3-4 months, thus affecting to the final outcome, since cable formation over long peripheral nerve gaps may not be completed. On the other hand, first degree of acetylation of the chitosan tubes (~2%) might be comparable to a non-resorbable conduit, since degradation of the material is not expected during the first two years after implantation. For that reason, second degree of acetylation of chitosan devices (~5%) provided better results, since the physical dimensions and the stability of the tube is preserved during all the regenerative process and degraded shortly thereafter (Haastert-Talini et al., 2013).

The complete failure of nerve regeneration showed in silicone tube repaired animals may be related to: failure in the formation of the acellular fibrin cable, indispensable for the cellular migration from the nerve stumps; an insufficient infiltration from the stumps of the Schwann cells responsible to form the Bünger bands and their extracellular matrix; an insufficient input of trophic and tropic factors which stimulate the dedifferentiation, proliferation and migration on non-neuronal cells through the lumen of the tube, or the responsible cells which stimulate the neuron outgrowth. In this sense, our data corroborate the limited capacity of injured nerves to supply in an efficient manner these key elements. Despite the use of resorbable materials for the repair of peripheral nerve injuries which may allow the metabolic exchange (input of glucose and oxygen and output of waste products), functionalization of them results crucial to increase the success rate for bridging limited peripheral nerve gaps.

Functionalizing nerve guides with ECM molecules

Although the promising results when using hollow chitosan devices for the repair of long peripheral nerve gaps (~50% of success), our results were still far from the ones observed when using the autograft (100%), also evidenced for a later and inferior reinnervation, and a lower amount in the number of myelinated fibers after 4 months. Although chitosan tubes may favor regeneration by being permissive for the entrance of cells and the exchange of soluble regenerative promoting factors, they are still insufficient as an alternative to autograft repair. The nerve stumps may not be able

to generate the constituents of the initial connective bridge which would allow the infiltrating cells to migrate and guide the re-growing axons (Lundborg et al., 1982; Williams & Varon, 1985). Despite the formation of the fibrin cable (Williams et al., 1983), the fibrin net lacks neurotropic effect, and it only provides the physical support for the migration of Schwann cells and fibroblasts, which will degrade the fibrin and will replace it with a collagen matrix (Liu, 1992). For that reason, the modification of the properties of the intratubular space may enhance the regenerative capacity of tubular devices.

The main role of the ECM in the peripheral nerve is related to the maintenance of the normal function of peripheral axons, becoming essential for the proliferation and differentiation of Schwann cells which guide the growing fibers to their target organs (Rutka et al., 1988).

Collagen-based scaffolds have been widely used in the repair of peripheral nerve injuries as internal fillers of nerve conduits (Labrador et al., 1998; Rosen et al., 1990; Satou et al., 1986). Collagen gels have a neuritotropic effect and can form a 3D structure that allow cellular migration and regeneration of the re-growing axons; but it is important to take into account the density of the matrix, since axonal elongation may be inversely correlated with the concentration of the gel (Labrador et al., 1995; Labrador et al., 1998). Furthermore, other ECM molecules, such as laminin and fibronectin, are substrates of cellular adhesion. Axonal outgrowth strongly depends on the interaction between the membrane receptors of the re-growing axons and these molecules present in the basal lamina. As previously mentioned, the results in Chapter 1 indicate that the addition of fibronectin or laminin to collagen matrices enhances regeneration *in vitro* and *in vivo* in a short gap of 6 mm in the rat sciatic nerve, but differences in neuron type selectivity to different extracellular matrix proteins is gradually lost from postnatal to adult stages (Gonzalez-Perez et al., 2015).

Our results in Chapter 3 demonstrate that the enrichment of collagen scaffolds with fibronectin may improve the capability of chitosan guides to sustain regeneration over long gaps (15 mm) in the rat peripheral nerve, since increased functional recovery by means of electrophysiological test; an increased number of myelinated fibers and a greater percentage of regenerated animals was also observed.

Interestingly, matrices enriched with fibronectin performed better than the ones containing laminin. These differences were quite surprising. Probably, we used a

laminin type which was not appropriated for our *in vivo* model. Based in our results in Chapter I, where laminin type I from Engelbreth's murine sarcoma offered better results in terms of elongation and arborization of DRG explants of p7 animals than merosin (laminin-2), we decided to use it for the experiments *in vivo*. However, laminin type I is not found in the peripheral nervous system, in contrast to laminin-2 or laminin-8 (Caissie et al., 2006). For this reason the interaction between fibronectin and collagen may be more effective than the interaction between collagen and laminin type I, thus affecting fibril cable formation and cell migration during the regeneration process.

Not only the incorporation of ECM proteins but also the orientation of the fibrils is important for the final outcome of nerve regeneration (Verdú et al., 2002). The control of the orientation of collagen fibrils may facilitate the re-growth of injured axons. Among others, tethered aligned collagen constructs result in an easy manner to engineer anisotropic cellular constructs (Georgiou et al., 2013). Our results in Chapter 3 show that stabilization and rolling of collagen-based constructs improve nerve regeneration, since increased functional recovery by means of electrophysiological test, an increased number of myelinated fibers and a greater percentage of regenerated animals was observed compared to fully hydrated matrices. The alignment of matrices, by mimicking the natural orientation offered by endoneurial tubes in nerve grafts, enhances regeneration. The maximum success of regeneration was observed in fibronectin-enriched stabilized and rolled constructs (75% of cases). The meso-scale tissue structure achieved by stabilizing and rolling these constructs may provide and additional longitudinal guidance architecture which somehow facilitates the fibrin cable formation and speeds axonal regeneration. However, when comparing the capabilities of laminin and fibronectin enriched matrices to facilitate the fibrin cable formation and Schwann cell migration in a short gap of 6 mm, no differences were found (Chapter 3). However, it has to be taken into account that in this short gap, formation of the fibrin cable and Schwann cell migration always occurs. There was a non-significant trend in the fibronectin-enriched aligned constructs to enhance speed of axonal regeneration. However, these small differences can explain the good results for this construct in bridging longer peripheral nerve defects, where regeneration is highly compromised.

ECM-based scaffolds to support cell transplantation

Besides the incorporation of orientated structural components to fill the lumen of a neural guide, the incorporation of cellular support to these scaffolds may further enhance the regenerative capabilities of these guides. Transplants of Schwann cells and Mesenchymal stem cells promote peripheral nerve regeneration, mainly due to their capacities of replacement of distal Schwann cells, prolonged neurotrophic release, synthesis of extracellular matrix components and modulation of the inflammatory response (Wang et al., 2015).

The first aim of the fourth chapter was to analyze whether MSCs or SCs have preferential affinity to laminin or fibronectin substrates. We showed that both type of cells, when dissociated and cultured, were able to survive and proliferate either in laminin or fibronectin coated surfaces. However, SCs preferred laminin coated surfaces, since the number of survived cells after 72 h in culture was higher than in fibronectin or poly-D lysine coated surfaces. This was previously described in the literature since laminin is present at the basal lamina of these cells (Yu et al., 2009). On the other hand, MSCs survived better in fibronectin coated surfaces. However, when implanted *in vivo* SCs demonstrated higher regenerative capabilities than MSCs, independently of the ECM used to enrich the matrix. In fact, better results were obtained with SCs when incorporated in fibronectin-enriched scaffolds. Our results in this chapter demonstrate that Schwann cell-transplanted animals offer the best results, with better functional recovery, higher number of myelinated fibers and a complete success of regenerated animals (100%). On the other hand, Mesenchymal-stem cell-treated animals showed a regeneration success close to 90% of cases. In this sense, although Schwann cells reach the top values reported in this thesis, comparable to results obtained in the autograft, their potential clinical use is really limited. Morbidity of the donor nerve to obtain autologous supply of Schwann cells is comparable to the one provoked when using autologous nerve graft. In contrast, mesenchymal Stem cells are easy to be obtained and can be transdifferentiated towards Schwann cell-like phenotype, acquiring myelinating properties (Dezawa et al., 2001).

In order to avoid rejection of the transplanted cells, we treated the animals with the immunosuppressant agent FK506 for the first two months. However, FK506 for its own has been demonstrated to enhance regeneration and functional recovery in experimental models of peripheral nerve injuries (Udina et al., 2004). Therefore,

animals receiving acellular neural guides were also treated with FK506. The sole administration of this drug did not improve the regenerative capabilities of these neural guides, since the success of regeneration was similar to the one reported in the 3rd chapter, where the same type of acellular matrices were used.

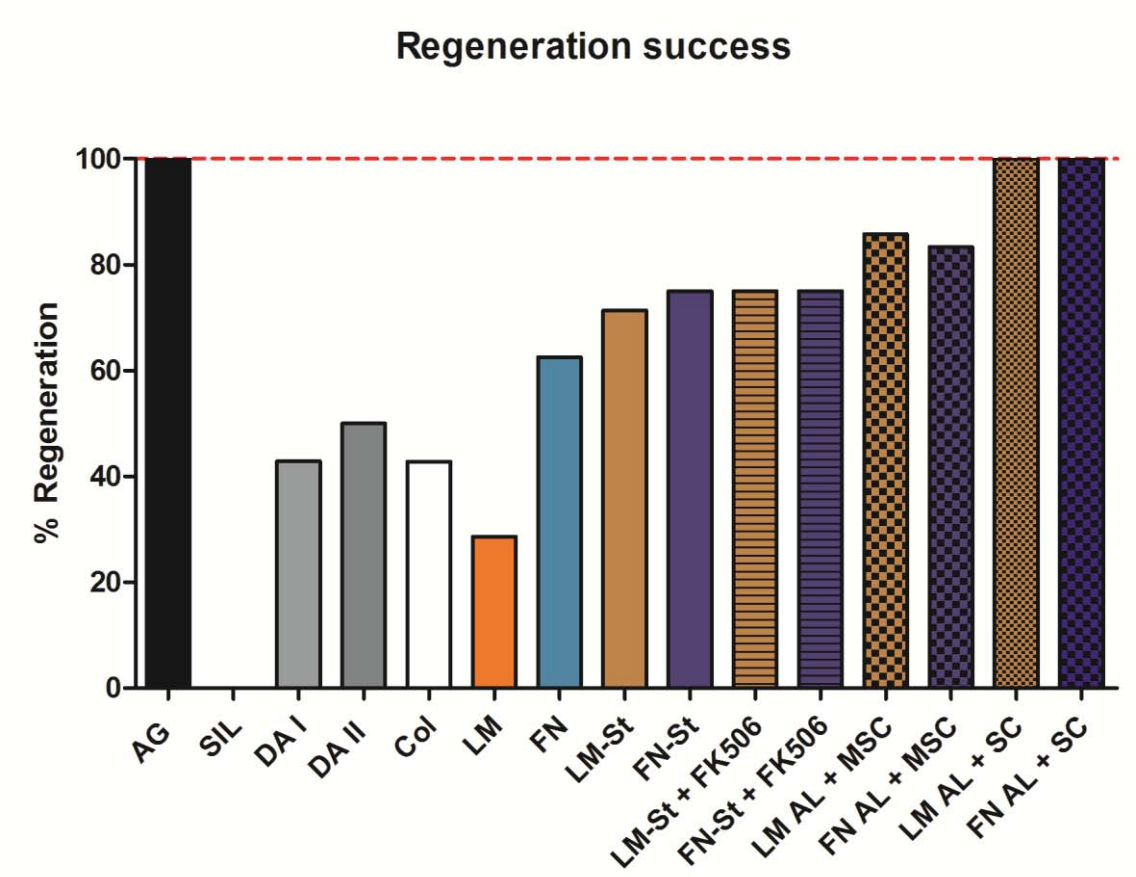

Figure 8: Overall representation of the percentage of regeneration success of all animals included in the experimental groups of the thesis. A defect of 15 mm in the rat sciatic nerve was repaired with an autograft (AG), a silicone tube (SIL) or a chitosan tube (DAI and DAII) empty or filled with different scaffolds.

Table 2: Results of the amplitude of CMAPs of the experimental groups included in the thesis.

	Tibialis Anterior														
	AG	SIL	DA I	DA II	COL	LM	LM-St	FN	FN-St	LM-St + FK506	FN-St + FK506	LM-AL + MSC	FN-AL + MSC	LM-AL + SC	FN-AL+ SC
CMAP (mv)	29.90 ± 1.40	0.00 ± 0.00	10.50 ± 5.00	9.54 ± 5.75	6.38 ± 4.25	3.00 ± 3.09	12.21 ± 6.40	11.34 ± 6.10	15.38 ± 5.75	12.13 ± 4.60	12.73 ± 5.22	14.25 ± 2.80	16.02 ± 4.61	23.08 ± 4.67	24.35 ± 2.76
	Plantar														
	AG	SIL	DA I	DA II	COL	LM	LM-St	FN	FN-St	LM-St + FK506	FN-St + FK506	LM-AL + MSC	FN-AL + MSC	LM-AL + SC	FN-AL+ SC
CMAP (mv)	2.65 ± 0.49	0.00 ± 0.00	0,212 ± 0.01	0.72 ± 0.53	0.22 ± 0.16	0.08 ± 0.07	0.44 ± 0.28	0.78 ± 0.38	0.67 ± 0.36	1.37 ± 0.89	1.37 ± 0.83	2.21 ± 0.75	1.89 ± 0.98	29.90 ± 1.49	3.78 ± 0.61

In conclusion, the combination of engineered aligned constructs and cell therapy is a feasible candidate of substituting autograft in the repair of long peripheral nerve gaps, since results obtained with these matrices in our limiting gap model in the adult rat were closer to the ones obtained when using an autograft.

CONCLUSIONS

CONCLUSIONS

1. The use of organotypic spinal cord slices and DRG cultures allows the study of preferential elongation of motor or sensory neurites within specific substrates.

2. When using p7 explants, fibronectin increased the elongation of motor neurites and proprioceptive sensory neurites, whereas laminin increased the elongation and arborization of other large myelinated sensory neurites (cutaneous).

3. The preferential role of fibronectin on presumptive proprioceptive sensory neurites found in p7 animals is partially decreased in p21 animals.

4. The preferential role of fibronectin on motor and proprioceptive sensory neuron outgrowth and of laminin on other large myelinated sensory axons found in p7 animals is lost in the adult *in vivo*.

5. The repair of severe peripheral nerve injuries in the rat (15 mm gap resection of the sciatic nerve) with autografts guarantee success of regeneration in all the cases, whereas the results obtained when using hollow neural guides are significantly lower

    a. The use of hollow silicone tubes to repair a 15 mm gap results in complete failure of the regeneration process.
    b. The use of resorbable and biodegradable hollow chitosan conduits substantially improves the success (near to 50%) of nerve regeneration and the rate of functional recovery achieved with respect to silicone hollow tubes. Chitosan tubes of the second degree of acetylation are the ones that offer the best results.

6. The incorporation of biological scaffolds based on ECM molecules into the lumen of tubular guides enhances the regenerative potential of these constructs.

    a. The incorporation of collagen-based matrix enriched with fibronectin increases the success of regeneration compared to laminin-enriched matrix after long gap peripheral nerve injury in the rat .
    b. Stabilization of collagen-based matrices results in an increase in the regeneration rate over long gap peripheral nerve injury compared to fully hydrated matrices.

7. The transplantation of Schwann cells or Mesenchymal Stem cells embedded in a collagen-based scaffold enriched with laminin or fibronectin and subjected to alignment substantially increase the success of regeneration and the degree of functional recovery compared to acellular scaffolds.

    a. The artificial graft containing Mesenchymal Stem cells reached 90% success in the regeneration process.

    b. The artificial graft containing Schwann cells allowed successful nerve regeneration in 100% of cases.

    c. Although transplantation of Schwann cells offers the best results, their use in the clinical practice may be limited due to the need of harvesting from a donor nerve. Mesenchymal Stem cells can be obtained easily and offer an alternative to the use of Schwann cells to repair severe peripheral nerve injuries.

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ABBREVIATIONS

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ADSC: Adipose-Derived Stem Cell

BDNF: Brain Derived Neurotrophic Factor

BM-MSC: Bone Marrow Mesenchymal Stem Cell

CNS: Central Nervous System

CSPG: Chondroitin Sulfate Proteoglycan

DA: Degree of Acetylation

DRG: Dorsal Root Ganglion

ECM: Extracellular Matrix

FDA: Food and Drug Administration

FGF: Fibroblast Growth Factor

FKBP: FK506-Binding Proteins

GAG: Glycosaminoglycan

GDNF: Glial-Derived Neurotrophic Factor

MSC: Mesenchymal Stem Cell

NGF: Nerve Growth Factor

OEC: Olfactory Ensheathing Cell

PGA: Polyglycolic acid

PLA: Polylactic acid

PLGA: Poly lactide co-glycolide acid

PMR: Preferential Motor Reinnervation

PNI: Peripheral Nerve Injury

PNS: Peripheral Nervous System

SC: Schwann Cell

Sk-MSC: Skeletal Muscle Mesenchymal Stem Cell

USA: United States of America

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ANNEXES

CHAPTER TEN

Extracellular Matrix Components in Peripheral Nerve Regeneration

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Contents

1. Introduction 258
2. Extracellular Matrix and Connective Layers of the Nerve 260
   2.1 Peripheral nerve microstructure 260
   2.2 ECM components 261
3. Role of the ECM Components in Axonal Regeneration 263
   3.1 *In vitro* 263
   3.2 *In vivo* 265
4. ECM Components in the Design of Artificial Biomatrices 267
Acknowledgments 270
References 270

Abstract

Injured axons of the peripheral nerve are able to regenerate and, eventually, reinnervate target organs. However, functional recovery is usually poor after severe nerve injuries. The switch of Schwann cells to a proliferative state, secretion of trophic factors, and the presence of extracellular matrix (ECM) molecules (such as collagen, laminin, or fibronectin) in the distal stump are key elements to create a permissive environment for axons to grow. In this review, we focus attention on the ECM components and their tropic role in axonal regeneration. These components can also be used as molecular cues to guide the axons through artificial nerve guides in attempts to better mimic the natural environment found in a degenerating nerve. Most used scaffolds tested are based on natural molecules that form the ECM, but use of synthetic polymers and functionalization of hydrogels are bringing new options. Progress in tissue engineering will eventually lead to the design of composite artificial nerve grafts that may replace the use of autologous nerve grafts to sustain regeneration over long gaps.

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1. INTRODUCTION

Injuries to the peripheral nervous system generally cause disconnection of the fibers distal to the site of injury from the neuronal soma. Consequently, the distal nerve undergoes degeneration, leaving the peripheral target organs denervated and, thus, functionally useless. In comparison to central axons, peripheral axons show a good capability to regenerate. This difference is partly due to not only the cellular and molecular changes occurring at the distal stump during Wallerian degeneration but also the intrinsic capabilities of the axotomized peripheral neurons to switch to a proregenerative state. Injured axons are able to elongate into the distal nerve stump if they find a permissive substrate, usually provided by tropic and trophic support from Schwann cells and connective cells (Fig. 10.1). Schwann cells that lose contact with axons switch to an immature proregenerative state, proliferating and secreting trophic factors with a temporo-spatial pattern that provides a regenerative-promoting terrain for the growing axons. The basal lamina remains in place in the endoneurium and helps to guide the axons toward distal target organs. Within the endoneurial tubules, proliferating Schwann cells form the aligned bands of Büngner. Eventually, regenerating axons will be able to reach the distal target organs and reinnervate

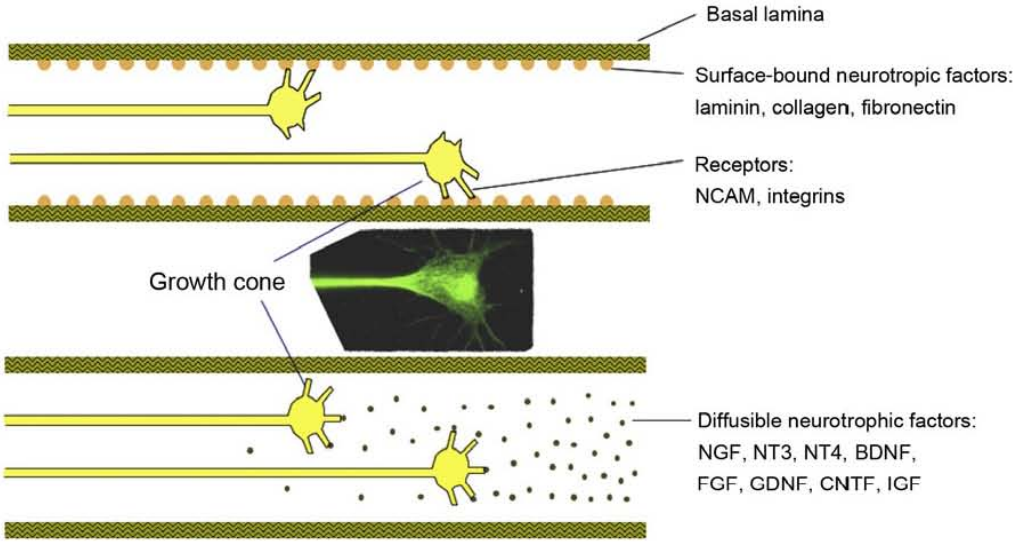

Figure 10.1 Trophic and tropic support for the axonal growth cone. The molecular cues that the regenerating axons find at the distal stump can be diffusible neurotrophic factors or membrane-bound neurotropic factors, as the ECM components laminin, fibronectin, and collagen.

them, thus allowing for the recovery of lost functions (for reviews see Allodi, Udina, & Navarro, 2012; Fu & Gordon, 1997; Navarro, Vivó, & Valero-Cabré, 2007).

After a peripheral nerve transection, surgical repair is mandatory to allow the severed axons to grow into the distal degenerating nerve. The microsurgical intervention reconnects the proximal and distal nerve stumps, trying to match individual fascicles to facilitate the appropriate navigation of growing axons to their targets. However, matching of proximal and distal endoneurial tubes is impossible and thus, misdirection of axons to a wrong distal path is common after these types of lesions. When direct suture is not possible due to loss of neural tissue, the interposition of a graft to reunite both stumps is needed. The interposition of an autologous graft is commonly used as the gold standard technique even if it causes the sacrifice of a noninjured nerve from the patient to be used as a graft. To reduce the secondary effects of autografting, alternatives to bridge the nerve gap have been investigated. Artificial nerve guides could be a good alternative to repair damaged nerves by mimicking the natural environment that the degenerating distal stump or autograft offers (Deumens et al., 2010; Doolabh, Hertl, & Mackinnon, 1996; Gu, Ding, Yang, & Liu, 2011; IJkema-Paassen, Jansen, Gramsbergen, & Meek, 2004). Tube repair has thus emerged as an alternative repair method. However, it is important to take into account that the regenerative process through tubular guides differs from that occurring in a degenerating nerve segment. Regeneration in a guide starts with the formation of a fibrin cable that bridges the gap between the transected nerve stumps. This fibrin cable provides a guiding surface for the ingrowth of fibroblasts, blood vessels, and Schwann cells that migrate from both stumps (Williams, Longo, Powell, Lundborg, & Varon, 1983). It is replaced by collagen secreted by the invading fibroblasts and laminin produced by the Schwann cells, in fibrils longitudinally oriented, and the tube lumen fluid is enriched by trophic factors. Then regenerating axons are able to grow from the proximal stump along the newly formed scaffold. Failure of the formation of the initial cable or limited supply of migrating cells into the tube due to excessive length of the gap lead to failure of regeneration (Yannas, Zhang, & Spilker, 2007). Prefilling the repair tube with components of the extracellular matrix (ECM) or with artificial scaffolds have been investigated to improve the regenerative potential of the guides, increasing the length of the gap that can be bridged by a nerve guide. Nowadays, efforts are addressed to improve the quality of artificial nerve guides to reach clinical application. To closely mimic the degenerating nerves, artificial nerve guides must offer

a proregenerative environment rich in Schwann cells, trophic factors, and components of the ECM that promote axonal regeneration.

2. EXTRACELLULAR MATRIX AND CONNECTIVE LAYERS OF THE NERVE

2.1. Peripheral nerve microstructure

In addition to bundles of axons, from spinal motor neurons, dorsal root ganglia (DRG), sensory neurons, and postganglionic autonomic neurons, the peripheral nerve is composed of three layers or compartments: epineurium, perineurium, and endoneurium. The epineurium is the outermost layer that delimits the nerve from the surrounding. It is composed of loose connective tissue and carries the blood vessels that supply the nerve. Delimiting each fascicle in the nerve there is the perineurium, a thin but dense sheath composed of flat perineurial cells and an outer layer of collagen fibers organized in bundles. The supporting connective tissue that fills each fascicle is the endoneurium, composed of fibroblasts, collagen, and reticular fibers and ECM, occupying the space between nerve fibers. The endoneurial tubes are composed of basal lamina sheets arranged in continuity around the axon–Schwann cell units and collagen fibrils.

The ECM is a physiological integrative matrix of complex molecular nature, where axons and supportive cells are immersed. The ECM is a three-dimensional network arranged in the intercellular space, which includes proteins and carbohydrates synthesized and secreted by the cells. It is present in the interstitial spaces of all tissues, playing important roles in cell migration, proliferation, and differentiation, and providing structural support and regulating intercellular communication. It contributes to mechanical tissue properties, allows the cells to form tissues, serving to cell communication, and forms paths where cells can move. In the peripheral nerve, the ECM is found in the basal lamina of Schwann cells and the endoneurium.

The basal lamina, produced by the Schwann cells, may be considered a layer of the ECM, mainly composed of collagen type IV, laminin, fibronectin, and nidogens (Bannerman, Mirsky, Jessen, Timpl, & Duance, 1986; Baron-Van Evercooren, Gansmüller, Gumpel, Baumann, & Kleinman, 1986; Bryan et al., 2012). After injury, besides the degenerative process behold in the distal stump, basal lamina tubes remain as scaffolds where proliferative Schwann cells align forming the bands of Büngner.

2.2. ECM components

The ECM is composed of a complex network of secreted proteins, glycoproteins, proteoglycans, and non-proteoglycan polysaccharides. The first group of components, the glycoproteins, can be classified into collagen and noncollagenous molecules.

Collagens are a superfamily of trimeric molecules composed of three identical triple helical α chains that define tissue structures (Brown & Phillips, 2007; Gordon & Hahn, 2011). Up to 26 different types of collagens have been described, that are divided into different groups according to the structures they form. The main subfamilies are fibril-forming collagens (types I, II, III, V, XI), collagens banded-fibrils associated (IX, XVI, XIX, XXI, XXII), networking collagens (IV, VI, VIII, X), transmembranous collagens (XIII, XXIII, XV), endostatin precursor collagens (XV, XVII), and other collagens. The collagen types most relevant for peripheral nerve regeneration are described in later sections. However, it is interesting to highlight the importance of those related to fibril formation (collagen type I) and the basement membrane (collagen type IV).

Among the ECM noncollagenous molecules of glycoprotein origin, the most important are laminins and fibronectins.

Laminins are major proteins of the ECM, participating in cell differentiation, migration, and adhesion activities. They are an active part of the natural scaffolding which structure the tissues. They are mainly found in the basal lamina. Laminins are heterotrimers of α, β, and γ chains, and 18 different types have been described to date (Durbeej, 2010). The trimers are named according to the composition of the different chain types, but usually it is the α chain that identifies the isoform. Secreted by Schwann cells, laminin-2 (α2, β1, γ1) and laminin 8 (α4, β1, γ1) are found in the peripheral nerves (Wallquist et al., 2002), whereas laminin 10 (α5, β1, γ1) can be detected in sensory end organs (Caissie, Gingras, Champigny, & Berthod, 2006). Laminin is the adhesive component that gives the regenerative-promoting capability to basal lamina scaffolds after nerve injury (Wang, Hirai, Shimada, Taji, & Zhong, 1992) and has been shown to promote neuritogenesis *in vitro* (Agius & Cochard, 1998).

Fibronectin is the other major component of noncollagen glycoproteins of the ECM (Singh, Carraher, & Schwarzbauer, 2010). It forms a fibrillar matrix similar to collagen and mediates cell-binding. Fibronectin is a dimer existing in different isoforms because of alternative splicing generation. Totally, 12 isoforms for mice and 20 for humans have been described. At

first, soluble fibronectin is produced by hepatocytes, being found in the blood plasma. The insoluble form is incorporated into the membrane of many cells. In the nervous system, it is synthesized and secreted by Schwann cells and fibroblasts (Baron-Van Evercooren et al., 1986; Chernousov & Carey, 2000). The important relations that it maintains with collagen type IV and laminins and with fibril formation make fibronectin an interesting candidate for scaffolding in nerve regeneration (Brown & Phillips, 2007).

The ability of cells to interact with both laminins and fibronectins is mainly due to the expression of the cell adhesion molecule integrins in their membrane (Hynes, 2002). Integrins are glycosilated heterodimers formed by α and β subunits. The integrin β1 subfamily is composed of integrins with a β1 subunit, which bounds to actin cytoskeleton, and the α subunit, which determines the specificity to the ECM molecule adhesion. Thus, integrin α1, α3, α6, and α7 can interact with laminin, whereas integrin α5 interacts with fibronectin. Expression of integrins on the growth cone determines the ability of the growing axon to interact with the ECM. Moreover, laminin and fibronectin have the characteristic of binding to other ECM components. For instance, laminin interacts with nidogens, agrin, perlecan, fibulin-1, heparin, and sulfatides (Holmberg & Durbeej, 2013; Rudenko, Hohenester, & Muller, 2001), whereas fibronectin binds to collagen, fibrin, and heparan sulfate proteoglycans (Hörmann, 1982).

There are other noncollagen glycoprotein molecules of the ECM although they are probably not related to axonal regeneration after nerve injury. Nidogen-1 (also called entactin) forms noncovalent unions with laminin and collagen type IV and may play its role as a promigratory factor for adult Schwann cells (Lee et al., 2007). On the other hand, vitronectin binds to collagen and glycosaminoglycans (GAGs) (heparin), acting as a regulatory molecule controlling cell adhesion (Schvartz, Seger, & Shaltiel, 1999).

The GAGs are carbohydrate polymers that are covalently bound to glycoproteins in their native state, forming *proteoglycans*. These molecules include heparan, keratin, chondroitin, dermatan, and their respective sulfates (Rutka, Apodaca, Stern, & Roseblum, 1988). Proteoglycans are formed by a GAG linked to a hydroxyl group of certain amino acids (serine and threonine) or a core molecule which is also linked to hyaluronic acid. Proteoglycans like chondroitin sulfate proteoglycans (CSPGs) create an inhibitory environment by neutralizing the growth-promoting activities of other ECM elements (McKeon, Höke, & Silver, 1995; Muir, Engvall, Varon, & Manthorpe, 1989). CSPG has been found in the peripheral nerve where it may inhibit the growth-promoting activity of endoneurial laminin

(Zuo, Ferguson, Hernandez, Stetler-Stevenson, & Muir, 1998; Zuo, Hernandez, & Muir, 1998).

Fibrin is not associated with the mature tissue structure but it is a key factor in the repair strategy of the ECM components. It will form a provisional mesh after damage that will be later replaced by the mature components of the ECM secreted by invading cells. In the presence of thrombin, fibrinogen polymerizes into fibrin to form a dense meshwork of fibers (Brown & Phillips, 2007; McKee, Mattock, & Hill, 1970). In fact, formation of a fibrin cable between the two stumps when a gap nerve is repaired by a tube is needed to guarantee successful axonal regeneration.

3. ROLE OF THE ECM COMPONENTS IN AXONAL REGENERATION

3.1. *In vitro*

To assess peripheral axon regeneration *in vitro*, the most used paradigm is the primary culture of DRG, either dissociated or as an explant. Tropic support of different molecules can be assessed by surface coating or by using 3D matrices (in which cells or explants are embedded) (Fig. 10.2).

It is well known that dissociated sensory neurons show longer neurite extension on laminin and fibronectin-coated substrates compared to poly-L-lysine-coated surfaces (Rogers, Letourneau, Palm, McCarthy, & Furcht, 1983). When comparing the ability of different ECM components, laminin-coated surfaces sustained better neurite outgrowth than vitronectin, collagen IV, fibronectin, or collagen I (Plantman et al., 2008; Wood & Willits, 2009). Laminin is also a preferred substrate for Schwann cells that extend and acquire better morphology on laminin-coated rather than fibronectin-coated substrates (Palm & Furcht, 1983). When focusing on the different isoforms of laminin,

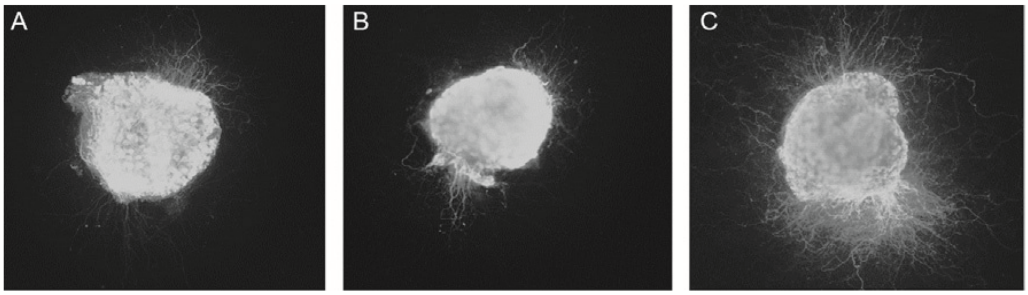

Figure 10.2 DRG explants cultured for 48 h in a 3D (A) collagen type I-containing gel, (B) fibronectin-containing gel, and (C) laminin type I-containing gel. Longer neurites can be observed in the matrix enriched with laminin type I.

DRG neurons grow better on laminin-1- and laminin-10-coated surfaces. Interestingly, when nerve growth factor was added to the culture, there was a marked increase of neurite elongation on laminin-2 and laminin-8 in comparison to laminin-1- and laminin-10-coated surfaces, which seemed to sustain a neurotrophic-independent growth (Plantman et al., 2008).

Sulfated proteoglycans have inhibitory effects when studying neurite extension on explant cultures from DRG (Ughrin, Chen, & Levine, 2003). Some authors claimed that proteoglycans inhibit neurite growth acting at the cone elongation (Snow, Smith, & Gurwell, 2002) but others relate this inhibition to the effect of sulfated proteoglycans on the neuron soma (Kuffler, Sosa, & Reyes, 2009).

When studying neurite elongation from DRG explants in 3D cultures, longer neurites were found in matrigel (a laminin-containing gel) than in collagen type I gel (Tonge et al., 1997), although the results might be affected by the additional factors present in matrigel. In another study, explants of DRG grew better on laminin gels compared to fibronectin, collagen type I, or hyaluronic gels (Deister, Aljabari, & Schmidt, 2007), supporting the findings from 2D cultures. On the other hand, different populations of sensory neurons may have different substrate preferences. Thus, embryonic proprioceptive neurons grow similarly on fibronectin and laminin, whereas cutaneous ones prefer laminin (Guan, Puthenveedu, & Condic, 2003). The inhibitory effect of the proteoglycans has also been proved in the 3D cultures (Tonge et al., 1997). When using 3D cultures, not only the components of the matrix but also the density should be taken into account. For example, when comparing different concentrations of an inert agarose gel, neurite extension was inversely correlated with the porous diameter that decreases with the concentration of the gel (Balgude, Yu, Szymanski, & Bellamkonda, 2001). Similarly, longer neurite extension was seen in lower concentration collagen gels (Willits & Skornia, 2004).

In contrast to the widely studied role of the ECM components on primary sensory neuron outgrowth *in vitro*, motoneuron outgrowth *in vitro* has been hardly analyzed. This fact is probably related to the technical difficulties of culturing primary spinal motoneurons compared to DRG neurons. Spinal cord slices embedded in a 3D matrix appear as an adequate model to investigate the role of ECM molecules on motor neurite outgrowth. Preliminary results from our laboratory indicate that motoneuron outgrowth is enhanced in matrices containing fibronectin more than in those containing laminin or collagen, suggesting that there may be a differential role of ECM molecules on different axonal populations.

3.2. *In vivo*

The role of the ECM components in nerve regeneration has been widely demonstrated in nerve graft experimental models. Even when nerve grafts are used, acellular axons are able to regenerate through the scaffolds of basal lamina (Hall, 1997). By using antilaminin antibodies, it was demonstrated that the capability of basal lamina to sustain axon growth was highly dependent on laminin. Antifibronectin antibody reduced neurite outgrowth, but did not influence the capability of axons to grow through the basal lamina (Wang et al., 1992). In similar models, the inhibitory role of proteoglycans was reduced when enzymes that degrade CSPG were applied into the distal stump or a graft, accelerating regeneration of motor and sensory axons (Krekoski, Neubauer, Zuo, & Muir, 2001; Udina et al., 2010).

Besides the studies that evaluate the role of different ECM in the distal stump of the injured nerve, the effects of these molecules have also been studied when used to fill artificial nerve guides. Moreover, addition of these substrates to a hollow tube can increase the capability of the tube to sustain regeneration and, thus, it is a first step toward engineering an artificial guide that mimics the autograft (Fig. 10.3). In fact, the sole addition of plasma, an important source of fibrin, into a silicone tube increased the gap length

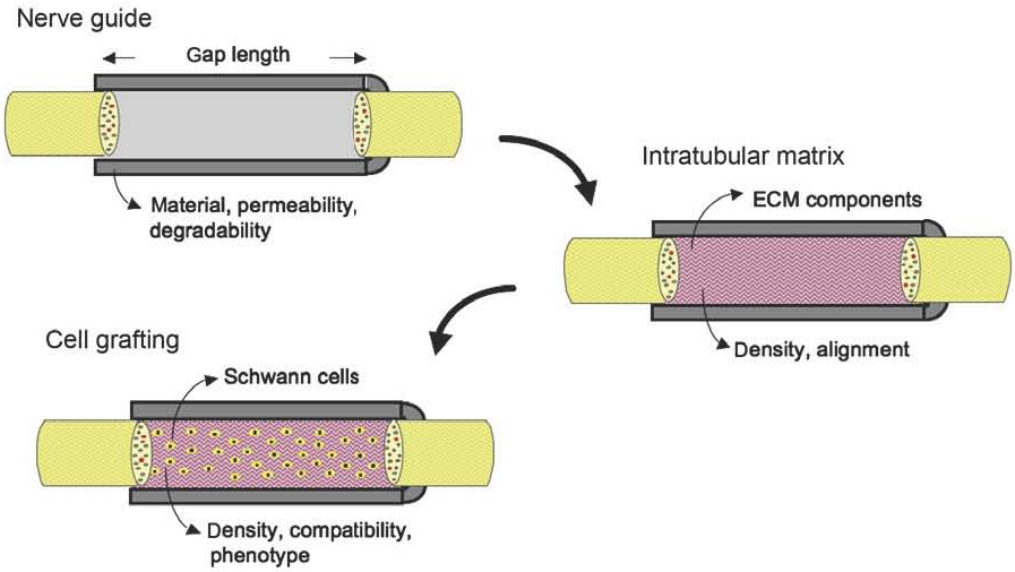

Figure 10.3 Schematic representation of the key points needed to engineer an artificial nerve graft. When designing a nerve guide, physical properties of the tube must be taken into account. For long gaps, introduction of matrices and supporting cells into the lumen improve the final outcome.

permissive for regeneration (Williams, Danielsen, Müller, & Varon, 1987). On the other hand, collagen-based (Chamberlain, Yannas, Arrizabalaga, et al., 1998; Chamberlain, Yannas, Hsu, Stritchartz, & Spector, 1998; Labrador, Butí, & Navarro, 1998; Rosen et al., 1990) and laminin-based matrices (Bailey, Eichler, Villadiego, & Rich, 1993; Labrador et al., 1998) have been successfully used to enhance axonal regeneration in nerve guides. Prefilling tubes with collagen- or laminin-containing gels improved regeneration through long gaps, whereas for short gaps, where regeneration is usually successful, addition of matrices into the tubes did not benefit the final outcome when compared to saline-filled tubes (Labrador et al., 1998; Valentini, Aebischer, Winn, & Galletti, 1987). When comparing the effects of different ECM components, laminin-containing gel performed slightly better than collagen I and hyaluronate gels on long gaps (Labrador et al., 1998). Combinations of ECM components have been tested in attempts to provide complex neurotropic support. The number of regenerated axons was higher in a gel mixture of collagen, laminin, and fibronectin compared to the control group (Chen et al., 2000). Similarly, a combination of laminin and fibronectin used to fill silicone tubes improved regeneration through long gaps in rats (Bailey et al., 1993). Fibronectin was also tested with laminin in double-coated collagen fiber bundles inserted into collagen tubes. The results were better than when using uncoated collagen fibers, revealing that laminin and fibronectin together may act positively on axonal growth (Tong et al., 1994).

As in 3D cultures, it is important to take into account the concentration of the matrix used, since the gel substrate, even if containing neurotropic agents, may impair the regenerative process by physically impeding the diffusion of factors, the migration of cells, or the elongation of axons. Lower concentrations of agarose, which forms a gel whose pore size decreases as concentration increases, were more permissive for regeneration through a silicone tube than higher ones (Labrador, Butí, & Navarro, 1995), demonstrating the importance of the density of the matrix used to guarantee axonal growth. Similar results were obtained when using collagen, laminin, and hyaluronate gels (Labrador et al., 1998; Tona & Perides, 1993).

Nerve regeneration using gel-filled tubes across long gaps usually remains inferior to that obtained with nerve autografts. A relevant difference accounts by the endoneurial tubules in nerve grafts offering a mechanical guide to the regenerating axons, while gels filling tubes do not provide local direction for axons and may even impede the ingrowth of non-neuronal cells and axons. By using a longitudinally aligned ECM gel, the rate and

the direction of axonal elongation should improve due to contact guidance with the fibrils aligned along the tube axis. This option has been reported for tubes prefilled with magnetically or gravitationally aligned collagen gel, matrigel (Ceballos et al., 1999; Dubey, Letourneau, & Tranquillo, 1999; Verdú et al., 2002), and fibrin matrix (Dubey, Letourneau, & Tranquillo, 2001), and with a collagen–GAG matrix (Chamberlain, Yannas, Arrizabalaga, et al., 1998; Chamberlain, Yannas, Hsu, et al., 1998; Chamberlain, Yannas, Hsu, Stritchartz, & Spector, 1998, 2000). Conduits containing longitudinally orientated extruded collagen microfibers and collagen scaffolds with orientated micropores, fabricated by directional ice-crystal formation, have been reported to allow regeneration over quite long gaps in the rat sciatic nerve (Bozkurt et al., 2012; Yoshii, Oka, Shima, Taniguchi, & Akagi, 2003). When fibronectin-oriented strands were introduced in nerve conduits to repair the rat sciatic nerve, axonal regeneration and Schwann cell recruitment were better than when using freeze-thawed muscle grafts (Whitworth, Brown, Doré, Green, & Terenghi, 1995). Fibronectin mats have been developed as oriented substrates that are capable of improving longitudinal migration of neurites and Schwann cells (Ahmed, Underwood, & Brown, 2003).

In conclusion, an adequate exogenous matrix designed to promote nerve regeneration within a nerve guide should have neuritotropic activity, be diluted in order to provide wide-enough pores for cellular and axonal migration, and also longitudinally oriented pathways that mimic the endoneurial tubules of the nerve.

4. ECM COMPONENTS IN THE DESIGN OF ARTIFICIAL BIOMATRICES

Further efforts are focused on the progress in tissue engineering and the improvement of artificial nerve conduits able to mimic the capabilities of autografts to sustain regeneration over long gaps. To form 3D scaffolds, besides the classical natural components of the ECM, several bioartificial polymers have been used to promote and guide axonal regeneration inside a neural guide. Such polymers can be introduced as a gel, coated directly into the internal wall of the tube or forming frameworks (like filaments or sponges used to enrich the internal architecture of the guide).

Gelatin is a natural-origin protein derived from collagen that maintains its inert properties, and can be functionalized by cross-linking techniques (Ciardelli & Chiono, 2006). Another natural polymer is chitosan,

a polysaccharide obtained from chitin. Its molecular structure is similar to GAGs in the ECM. It can be introduced into the lumen of a tube as nano/microfibers that can be functionalized with laminin and trophic factors (Patel, Mao, Wu, & Vandevord, 2007) or combined with cells (Wang et al., 2009) to enhance nerve regeneration. Alginate is a biodegradable polysaccharide with repeat units of mannuronic acid and glucuronic acid. It has been used in the form of freeze-dried sponge to promote axon regeneration across a long gap in the cat sciatic nerve (Suzuki et al., 1999). Alginate has also received attention as a slow-release hydrogel for the controlled supply of trophic factors. Other natural polymers, with potential to support nerve regeneration, are silk fibroin (Yang et al., 2007) and agarose (Labrador et al., 1995; Martin, Minner, Wiseman, Klank, & Gilbert, 2008).

Synthetic polymers can also be used to form the intratubular scaffolds that should combine the biocompatible characteristics of the natural polymers with improved mechanical and chemical performance. They also have to be surgically practicable, immunocompatible, and allow the diffusion of nutrients and growth factors to supply the regrowth of the injured nerve. Polymers can be presented as hydrogels when having high water content. Some examples of synthetic scaffolds used in peripheral nerve regeneration studies include, among others, poly(ethylene glycol) (Scott, Marquardt, & Willits, 2010; Shepard et al., 2012), poly-(L-lactic acid) (Ngo et al., 2003), poly(lactide-co-glycolide) (Subramanian, Krishnan, & Sethuraman, 2011), polycaprolactone (Daud, Pawar, Claeyssens, Ryan, & Haycock, 2012), and poly(*N*-isopropylacrylamide-co-acrylic acid) (Newman et al., 2006) in the form of hydrogels or fibers.

Recent developments of nanotechnology propose the use of fibrous scaffolds at the nanoscale level for the artificial replacement of basal lamina in tissue-engineered nerve grafts. Nanotubes can be produced from various materials, such as carbon, synthetic polymers, DNA, proteins, lipids, silicon, and glass. Moreover, they can serve as an extracellular scaffold, filling a hollow nerve conduit, to guide directed axonal growth (Cao, Liu, & Chew, 2009; Olakowska, Woszczycka-Korczyńska, Jędrzejowska-Szypułka, & Lewin-Kowalik, 2010). Surfaces with nano-sized topographies, electrospinned nanofibers, or replicas of the ECM with nanoresolution were found to guide neurite outgrowth from sensory and autonomic ganglia in culture (Kanje & Johansson, 2011). *In vivo* studies showed that conduits filled with aligned polymer nanofibers resulted in better functional recovery than hollow conduits (Neal et al., 2011). These studies provide the basis for the use of nanofibers combined with molecular constituents of the ECM to enhance nerve conduits.

The performance of either synthetic tubes or scaffolds can be improved by functionalizing them with specific cues. These cues are usually motifs from ECM components with special effects on cell migration, attachment, and proliferation. Tubes can be directly functionalized by linking cues to the internal wall of the conduit. For instance, a laminin-2 motif has been reported to have a positive effect on nerve regeneration when it was used to coat a PLGA guide (Seo et al., 2012). Also, a laminin-1 motif TATVH was proposed to promote neurite outgrowth and cell attachment on coated tubes (Nickels & Schmidt, 2012).

ECM peptides are being increasingly used for functionalizing hydrogels. Based on the results of *in vitro* studies, laminin cues have been the most widely used sequences for improving hydrogel performance on nerve regeneration. For instance, a laminin-1 sequence, the IKVAV pentapeptide, has been shown to promote axonal elongation on central and peripheral neurons (Bellamkonda, Ranieri, & Aebischer, 1995; Tashiro et al., 1989). YIGSR is another pentapeptide used to graft agarose inert gels (Borkenhagen, Clémence, Sigrist, & Aebischer, 1998; Yu, Dillon, & Bellamkonda, 1999) and collagen polymers (Newman et al., 2006). The results revealed a significant increase in neuronal extension on YIGSR-coated gels in comparison to controls. Other motifs from laminin isoforms 1 and 2 have not been directly proved to enhance nerve regeneration.

Fibronectin cues have also been proposed for peripheral nerve repair. The GRGDS amino acid sequence regulates cell adhesion (Rutka et al., 1988), and it is probably the most studied short sequence from fibronectin, although the RGD sequence is also found in other ECM components such as laminin-1 and tenascin-C (Meiners & Mercado, 2003). The GRGDS sequence has been used to functionalize hydrogels. For instance, gellan gum, which is a polysaccharide from bacterial origin, functionalized with this fibronectin sequence improved axonal growth *in vitro* (Luo & Shoichet, 2004) and survival and proliferation of neural stem/progenitor cells (Silva et al., 2012).

Nowadays, it is accepted that to design an artificial nerve guide as efficient as a nerve graft, it is important to combine different approaches. After designing the best conduit, addition of neurotropic cues to the luminal space, either as a matrix or by functionalizing a gel, may improve axonal growth. Certainly, this growth can be further enhanced by introducing supportive cells or trophic factors. However, another important key point is the need to mimic the guiding endoneurial tubes found in the degenerating distal stump of the nerve. This might be achieved by longitudinally orienting

scaffolds, to provide wide-enough channels for cellular and axonal ingrowth (Brown & Phillips, 2007). Further assays are needed to improve the microgeometry of such a bioartificial matrix with added cells in order to mimic the longitudinal axis offered by endoneurial tubules and aligned Schwann cells (forming bands of Büngner) in a degenerated nerve.

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