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Neuregulin1 expression following peripheral nerve injury and new tools to improve nerve regeneration

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ABSTRACT

Neuregulin1 (NRG1) is a growth factor belonging to the EGF-family, and it is a ligand for tyrosine-kinase receptors belonging to the ErbB family. NRG1 has different isoforms and it is involved in many biological processes. In the central nervous system it plays a role in neuronal migration during development; we demonstrated that two NRG1 isoforms (α and β) are expressed in the developing cortex and can stimulate *in vitro* the migration of neuronal progenitors.

In the peripheral nervous system NRG1 is involved in nerve development and regeneration after injury; we showed that, immediately after a nerve injury, the expression of soluble NRG1 and its isoforms α , β , a, and b, is highly up-regulated. Moreover, we observed that in degenerating injured nerves, without an immediate proper repair, the expression of soluble NRG1 is strongly down-regulated and Schwann cells undergo atrophy, suggesting that NRG1 lacking might explain Schwann cell impairment to support delayed repair. Indeed, when the repair time is delayed, the outcome is worse than after an immediate repair.

We hypothesized that the delivery of exogenous NRG1 might improve nerve regeneration when NRG1 is not sufficiently provided by endogenous Schwann cells, like in the delayed nerve repair or in hollow conduits used to repair a nerve gap. To this aim, we performed preliminary assays introducing inside a hollow chitosan tube, used to bridge proximal and distal stumps of an injured nerve, skeletal muscle fibres, which express and release soluble NRG1, showing that for immediately repaired short gaps its use is effective. These data suggest that the introduction of muscle fibres releasing soluble NRG1 might be a promising strategy to obtain a better outcome also when the repair is delayed in time.

Nevertheless, we must be aware that soluble NRG1 should be provided in a defined time-window and concentration: high amount of NRG1 stimulates Schwann cell survival and dedifferentiation, but also nerve myelination inhibition. Indeed, we demonstrated that soluble NRG1 isoforms are chronically over-expressed in the nerves of the inherited peripheral neuropathy Charcot-Marie-Tooth1A, a pathologic condition characterized by chronic demyelination and Schwann cell dedifferentiation, while after acute injury the NRG1 over-expression is transient.

ABSTRACT

In addition to the use of muscle fibres, we developed biomaterials that in the future might be functionalized to release soluble NRG1: we demonstrated, *in vitro*, that random and aligned gelatin and chitosan fibres, with their morphology, enhance Schwann cell proliferation, adhesion and migration, characteristics making them a suitable internal filler for nerve conduits.

SCOPE AND OUTLINE

The goals of this thesis were:

- (1) The evaluation of NRG1 expression levels in peripheral nerves under degenerative or regenerative conditions and the effects of delayed repair on nerve regeneration, paying attention to NRG1 role.
- (2) The development of a tool to deliver NRG1 consisting in a chitosan tube filled with fresh skeletal muscle fibres and the evaluation of its effect on nerve regeneration.
- (3) The biological evaluation of Schwann cell behaviour and axonal outgrowth on gelatin fibres with different diameters and the influences of gelatin or chitosan fibres with different orientation on it. Fibres were tested as internal filler for nerve guide.
- (4) The evaluation of NRG1 isoform expression levels in chronic injuries represented by an hereditary peripheral neuropathy.
- (5) The evaluation of the effect of stimulation with NRG1 α isoform on the migration of neuronal progenitors expressing different ErbB4 isoforms.

Chapter 1, corresponding to the **introduction**, provides a general view about peripheral nerve injury. In the first part, the peripheral nerve anatomy, the classification of peripheral neuropathies, the molecular basis of peripheral nerve degeneration and regeneration following traumatic events, and the involved growth factors are described, followed by the description of different approaches adopted to repair nerve injuries. More attention is being paid to tissue engineering, involved in the development of biomaterials, of biological or synthetic origins, used properly as nerve guide or as internal frameworks for empty tubes. The requirements that the different biomaterials need to satisfy and the fabrication techniques are also discussed. The last part of the introduction provides an overview on the involvement of Neuregulin (NRG1) in the central nervous system with a deeper attention on its role in neuronal migration.

To ensure peripheral nerve regeneration, a thorough knowledge of the molecular mechanisms underlying nerve degeneration and regeneration following traumatic events and the involved molecules, is required. Among them, I focused my attention on NRG1. In **chapter 2** the expression of different NRG1 isoforms and of their ErbB receptors was evaluated, at mRNA and protein level, in the distal stump of rat median nerve samples under regenerating conditions, after a crush injury and after a transection repaired with the end-to-end technique, and under degenerating condition. The obtained data demonstrated that the different NRG1 isoforms, accompanied by the ErbB receptors, are differently regulated, suggesting that NRG1 isoforms play a role in the early stages following nerve injury.

Tubulization is an available option to repair severe injuries with substance loss, but nerve guides, more than 3cm-long, require enrichments to provide an adequate microenvironment for nerve regeneration through long distances.

Among the internal frameworks of tubes, muscle fibres and electrospun fibres have been investigated in chapters 3-6.

Since NRG1 plays an important role in peripheral nerve regeneration, and nerve guides immediately after repair are hollow and lack Schwann cells releasing NRG1, its early delivery after injury might be a promising tool to improve nerve repair. This aim has been achieved by the introduction of fresh skeletal muscle fibres inside an hollow chitosan tube as discussed in **chapter 3**, where we analysed *in vitro* the soluble NRG1 isoforms expressed and released by the skeletal muscle and we verified *in vivo* its effectiveness to repair severe injury immediately repaired.

For what concerns electrospun fibres, as described in **chapter 4**, random gelatin fibres with different diameters (ranging from nano- to micro-scale) were produced from aqueous solutions, through electrospinning technique, by varying gelatin concentration and flow rate. Increasing gelatin concentration and flow rate resulted in increased fibre diameter allowing the preparation of fibres displaying different topography. *In vitro* tests were performed to investigate if the different diameters of fibres influences Schwann cell proliferation, adhesion, cytoskeleton organization, motility and migration, and axonal outgrowth that was tested on dorsal root ganglia

explants. The obtained data demonstrated that nano- or micro-fibres promote different processes, because of their different topography.

Besides the fibre diameter, fibre orientation can influence Schwann cell behaviour and axonal outgrowth. As discussed in **chapter 5**, gelatin random or aligned nano-fibres were obtained using a fixed or a rotating collector, respectively. The effect of fibre alignment on Schwann cells and on a sensory neuron-like cell line was tested by *in vitro* assays. The results demonstrate that the fibre alignment reduces cell proliferation, but induces a parallel orientation and growth of axons and Schwann cells.

Electrospun fibres can be prepared from different biomaterials of natural origins; in **chapter 6** are described nano-fibres made of gelatin or chitosan both with different orientation (random or aligned), to understand which tested condition better promotes nerve regeneration. *In vitro* analysis on Schwann cells, sensory neuron-like cell line and on dorsal root ganglia explants highlight the biocompatibility of both biomaterials and their potential use as substrates for nerve reconstruction.

Ineffective nerve regeneration can be due to a delay in the repair of peripheral nerve injury, in which the timing of intervention is fundamental for functional recovery. In **chapter 7**, the morphological and molecular changes occurring after a long-term denervation followed by a delayed repair and the concomitant levels of NRG1 and ErbB receptors were analysed. The results demonstrate that long-term degeneration negatively affects Schwann cells. Down-regulation of NRG1 might explain Schwann cell impairment and the poor outcome occurring after a delayed repair.

In **chapter 8** the expression level of NRG1 isoforms and ErbB receptors in sciatic nerves of a rat model of Charcot-Marie-Tooth 1A was evaluated. Charcot-Marie-Tooth 1A is the most common inherited peripheral neuropathy and it represents a condition of chronic injury. Here we demonstrated that soluble NRG1 isoforms are chronically over-expressed suggesting that in this pathologic condition a therapy based on NRG1 delivery (proposed by others) should be carefully reconsidered.

NRG1 has not only an important role in peripheral nerve regeneration, but also in neuronal migration in the central nervous system and it has been shown that NRG1 and its receptors are genes conferring susceptibility to schizophrenia. In **chapter 9**, the response of neuronal progenitors, expressing the different isoforms of the NRG1 receptor, ErbB4, following stimulation with NRG1 isoforms α or β , in terms of migration, and the expression of the different ErbB4 and NRG1 isoforms in the regions where neuronal migration occurs during development were analysed.

In **chapter 10**, corresponding to the **general discussion**, all the results of the thesis are summarized and discussed.

The chapters 2-9, which correspond to individual papers, do not follow a chronological order, but follow a logical order, as schematized in the figure at page 360 in the discussion section (Chapter 10).

To specify which has been my role in each paper, my personal contribution was reported at the end of each chapter, before references.

ABBREVIATIONS

ADAM17	A disintegrin and metalloprotease 17
ATF3	Activating transcription factor 3
BACE	β -site of amyloid precursor protein cleaving enzyme
BDNF	Brain-derived nerve growth factor
CIDP	Chronic inflammatory demyelinating polyneuropathy
CMT	Charcot-Marie-Tooth
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CRD	Cystein-rich domain
CS	Chitosan
dHMN	Distal hereditary motor neuropathy
DMSO	dimethyl sulfoxide
DSP	dibasic sodium phosphate
DRG	Dorsal root ganglia
ECM	Extracellular matrix
EGF	Epidermal growth factor
FDA	Food and Drug Administration
GBS	Guillain-Barré syndrome
GDNF	Glial-cell-line derived neurotrophic factor
GJB1	Gap junction protein beta 1
GFAP	Glial fibrillary acidic protein
GGF2	Glial growth factor-2
GL	Gelatin
GPTMS	γ -glycidoxypolytrimethoxysilane
HMN	Hereditary motor neuropathy
HMSN	Hereditary motor and sensory neuropathy
HPN	Hereditary peripheral neuropathy
HSAN	Hereditary sensory and autonomic neuropathy
HSN	Hereditary sensory neuropathy
ICD	Intracellular domain

ABBREVIATIONS

IGF	Insulin-like growth factor
IL-1	Interleukin 1
JM	Juxtamembrane
JNK	c-Jun N-terminal kinase
LGE	Lateral ganglionic eminence
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MCV	Motor conduction velocity
MFN2	Mitofusin 2
MGE	Medial ganglionic eminences
MPZ	Myelin protein zero
MSC	Mesenchymal stem cell
NGC	Nerve guidance channel
NGF	Nerve growth factor
NRG1	Neuregulin1
NT-3/4/5	Neurotrophin-3/4/5
NTF	N-terminus fragment
NTR	Neurotrophin receptor
OB	Olfactory bulb
OCP	Organic conducting polymer
OPC	Oligodendrocytes precursor cell
PCL	Poly(ϵ -caprolactone)
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PHB	Poly-3-hydroxybutyrate
PHEMA-MMA	Poly-2-hydroxyethyl methacrylate-methylmethacrylate
PI3K	Phosphatidylinositol-3-kinase
PLA	Poly(lactic acid)
PLGA	Poly(lactic acid-co-glycolic acid)
PLLA	Poly-(l-lactic acid)
PMP22	Peripheral myelin protein 22
PNS	Peripheral nervous system

ABBREVIATIONS

PVC	Polyvinyl chloride
RMS	Rostral migratory stream
SC	Schwann cell
SLC	Schwann-like Cell
SVZ	Subventricular zone
TACE	Tumour necrosis factor- α converting enzyme
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

CHAPTER 1

Introduction

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1. PERIPHERAL NERVE ANATOMY

Peripheral nerves represent the peripheral component of the nervous system that emerges from the central nervous system (CNS) and forms a complex arborisation, that expands throughout all body districts transmitting sensory or motor stimuli. Nerves originate from the spinal cord and are generally classified in two categories: the cranial nerves (12 pairs) and the spinal nerves (33 pairs). Except for the olfactory and the optic nerve, the morphology observed in all nerves is analogous (Figure 1).

The nerve fibre represents the smallest functional unit of nerves, composed by a single axon and its associated glial component. The classification methods of nerve fibres are different and based on various parameters as the function, the conduction velocity, the diameter and so on. Depending on glial cell/axon relationship, nerve fibres can be anatomically divided in unmyelinated or myelinated fibres. In the peripheral nervous system (PNS) the main glial component are Schwann cells (SC). In unmyelinated fibres, a single SC forms one sheath around multiple axons that result grouped by the SC. In the myelinated fibres, a series of SC embrace a single axon and the plasmalemma of each single cell wraps several times the axon forming a plurilamellar myelin sheath (Flores et al., 2000). The axon regions occupied by single SC are defined internodes and the space between two internodes represents the Ranvier's node, where the axon is uncovered by SC. The Ranvier's node length can range from 150 μm to 1500 μm , depending on the axon diameter. This unmyelinated portion permits the saltatory conduction of the impulses along the fibre. Normally, large diameter axons are myelinated, while small axons are unmyelinated.

Nerve fibres can also be classified, according to the type and the direction of the transported signal, in efferent or motor fibres, afferent or sensitive fibres and special sensory fibres (Lundborg, 2005). The efferent fibres transport motor signal from the CNS to the PNS; they can be divided in somatic fibres - controlling the voluntary movements of skeletal muscle - or visceral fibres - controlling the involuntary movements of cardiac tissue, smooth muscle and glands. The afferent fibres carry on sensory impulses from the periphery to the CNS. Among these type of fibres, the somatic fibres transport signals derived from receptors in skin, muscles and joints,

while visceral sensitive fibres connect receptors located into viscera to CNS. Meanwhile, the information from sense organs arrive to the CNS thanks to special sensory fibres.

Based on fibre type composition, peripheral nerve can be divided in three main categories: sensory, motor and mixed nerves.

Another way to categorize fibres is based on cell body size, axon length and conduction velocity. According to this classification, fibres are divided in: A fibres, myelinated fibres with a diameter of 3-22 μm ; B fibres, myelinated fibres with a diameter of 1.5-3 μm ; C fibres, unmyelinated fibres with small diameter (0.3-1.5 μm) (Lundborg, 2005).

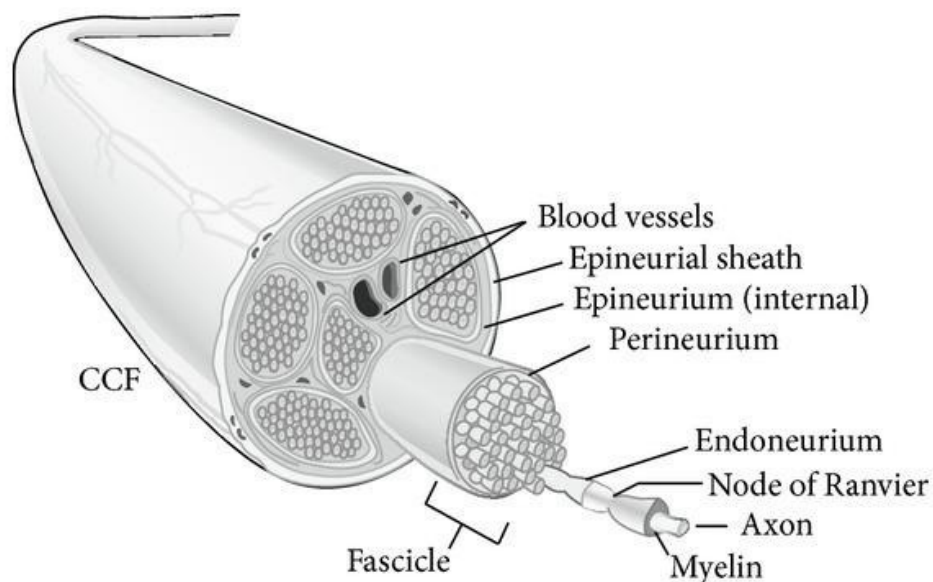


Fig. 1: Peripheral nerve anatomy (Siemionow and Brzezicki, 2009).

Besides the nerve fibre, the other main component of the peripheral nerve is the connective tissue, which sustains structurally the nerve and confers resistance to compression or stretch force. Three concentric connective layers are recognizable in nerves: the *endoneurium*, the *perineurium* and the *epineurium* (Lundborg, 2005, Geuna et al., 2009, Siemionow and Brzezicki, 2009). Each axon and its adjacent SCs are surrounded by the *endoneurium*, the innermost layer. This protective layer is

composed by loose connective tissue of collagen type I fibres that runs parallel to the axons, and a small number of fibroblasts and macrophages.

Groups of *endoneurium*-enveloped fibres are surrounded by a multilayer dense sheath, the *perineurium*, forming the nerve fascicles. The *perineurium* is given by alternating layers of collagen/extracellular matrix and flattened polygonal cells (perineurial cells) and is extremely resistant to pressure. The capillaries that pass through the *perineurium* continue into the *endoneurium*, and ensure the necessary oxygen and substance supplement to axons and SCs, representing an important component in the mechanism that regulates and maintains the endoneurial space (Diao and Vannuyen, 2000, Flores et al., 2000, Lundborg, 2005).

The outmost connective layer is the *epineurium*: it surrounds the nerve, defining its boundaries, and supports the inner nerve structure holding the fascicles. It is composed by dense collagen fibres, fibroblasts and adipocytes. Large blood vessels can be recognized in this layer, where they run parallel to fascicles and form numerous capillaries that, through the other connective layers, provide the oxygen and nutrients to each cellular component (Flores et al., 2000, Geuna et al., 2009, Siemionow and Brzezicki, 2009)

The spinal nerves extend from a nerve root generated from the lateral-ventral furrow of the vertebral canal and reside in the intervertebral foramina. The anterior root is composed by the axon from motor neurons located in the anterior horn of the spinal cord. While, a swelling of each posterior hood, called dorsal root ganglia (DRG), hold the soma of sensory neurons (pseudo-unipolar ganglion neurons) which project one T-shaped axon extending one branch to the peripheral target and the other branch to the spinal cord. DRG neurons are divided into distinct subpopulations, based on cell body morphology, sensory modalities and gene expression pattern (Price, 1985, Richner et al., 2014). According to the morphology, two main group are distinguished: large neurons, light with granular cytoplasm (A neurons) and small, dark neurons with dense cytoplasm (B neurons) (Lawson, 1979). Different sensory neuron subtypes require specific neurotrophic factors, for example distinct subpopulations of DRG neurons express TrkA, TrkB or TrkC receptors, responding to specific neurotrophins (Kashiba et al., 1995, Wright and Snider, 1995).

2. PERIPHERAL NEUROPATHIES

Peripheral neuropathies are the most frequent disorders of the peripheral nervous system (Tazir et al., 2014). They include all pathologic conditions that result in peripheral nerve damage compromising their functionality (Callaghan et al., 2015). Indeed, symptoms of this condition are weakness, numbness, tingling, and pain (Azhary et al., 2010). Peripheral neuropathies present a variegated and complex classification based on different criteria such as the functional impairment (motor, sensory, autonomic or mixed) or the topography (mononeuropathies, polyneuropathies, multifocal neuropathies, polyradiculopathies or ganglionopathies) (Hanewinckel et al., 2016). Another way to classify peripheral neuropathies takes into account the disorder etiopathogenesis. According to this view, peripheral neuropathies can be classified as hereditary, acquired, or idiopathic.

Clinicians define idiopathic a neuropathy when the cause of the disease can not be identified.

2.1. HEREDITARY PERIPHERAL NEUROPATHIES

Hereditary peripheral neuropathies (HPN) include hereditary motor and sensory neuropathies (HMSN), hereditary motor neuropathies (HMN) or distal hereditary motor neuropathies (dHMN) and hereditary sensory neuropathies (HSN) or hereditary sensory and autonomic neuropathies (HSAN) (Reilly, 2007, Tazir et al., 2014).

HMN or dHMN is a divergent phenotype of inherited neuropathies, characterized by a slowly progressive, length-dependent neuropathy, exclusively motor. Patients affected by HSN or HSAN, according to the mutated gene, may develop distinct symptoms that include congenital insensitivity to pain, distal lower limb sensory loss and neuropathic pain and pure autonomic dysfunctions (Pareyson et al., 2017).

Among the HPN, the most frequent are a spectrum of HMSN, also known as Charcot-Marie-Tooth (CMT). CMT prevalence is about 1 : 2500, with worldwide distribution. CMT are clinically and genetically a heterogeneous group of disorders,

indeed its classification is in a state of constant flux. In CMT disorder there is the involvement of both motor and sensory systems, with the development of motor and sensory neuropathy. Clinically, this pathology is characterized by distal muscle wasting and weakness, reduced tendon reflexes, impaired distal sensation and foot deformity (Reilly, 2007).

CMT disorders can be mainly classified in three categories, defined by their different motor conduction velocity (MCV) (Saporta, 2014):

- the demyelinating form (CMT1), $MCV < 35$ m/s
- the axonal form (CMT2), $MCV > 45$ m/s
- the intermediary form (CMTI), MCV between 35 and 45 m/s

Also, they can be distinguished by electrophysiology and nerve biopsies (Dyck and Lambert, 1968, Thomas and Calne, 1974, Harding and Thomas, 1980).

The majority (80%) of CMT cases belongs to the demyelinating form (CMT1) where the primary defect is an inability of SCs to properly form or maintain the axon myelination, resulting in a marked decrease in nerve conduction velocity and in a secondary degeneration of axons (Pareyson and Marchesi, 2009, Patzkó and Shy, 2011). Moreover, CMT1 pathologies appear in the first decade of life and show more severe sensory and motor impairment than CMT2 forms (McGrath, 2016).

Intermediate forms of CMT (CMTI) affect both glial and neuronal functions, highlighting the close relationship between these cell types (Tazir et al., 2014).

Subtype classification can also be based on the pattern of inheritance: demyelinating CMT1 and axonal CMT2 can be both inherited as autosomal dominant (more common) or recessive tracts. Moreover, CMT can also be an X-linked disorder (CMTX) (Harding and Thomas, 1980, Bergoffen et al., 1993, Birouk et al., 1998, Reilly, 2007).

Finally, CMT diseases can be further subdivided on the basis of the underlying genetic abnormalities. Genetically, more than 80 genes have been identified to result in a CMT phenotype (Stojkovic, 2016). About 90% of molecular diagnoses in western populations depend on mutations or rearrangements in peripheral myelin protein 22 (PMP22), gap junction protein beta 1 (GJB1), MPZ and mitofusin 2 (MFN2) in decreasing frequency order, while the mutations in the other genes seem rare

(Saporta et al., 2011, Murphy et al., 2012, Gess et al., 2013) (Figure 2). Mutations in GJB1 caused CMT1X, the most widespread form of CMTX. GJB1 encodes the gap junction protein connexin 32 and is responsible for 15% to 20% of CMT cases with a distinct molecular diagnosis (Bergoffen et al., 1993, Boerkoel et al., 2002, Saporta, 2014). For CMT2, that is associated with axon degeneration, causative mutations in at least 16 genes/loci were identified, although some mutations may also cause a dysmyelinating form of CMT. These mutations have been identified in 25-35% of CMT2 patients (Saporta et al., 2011, Murphy et al., 2012, Gess et al., 2013).

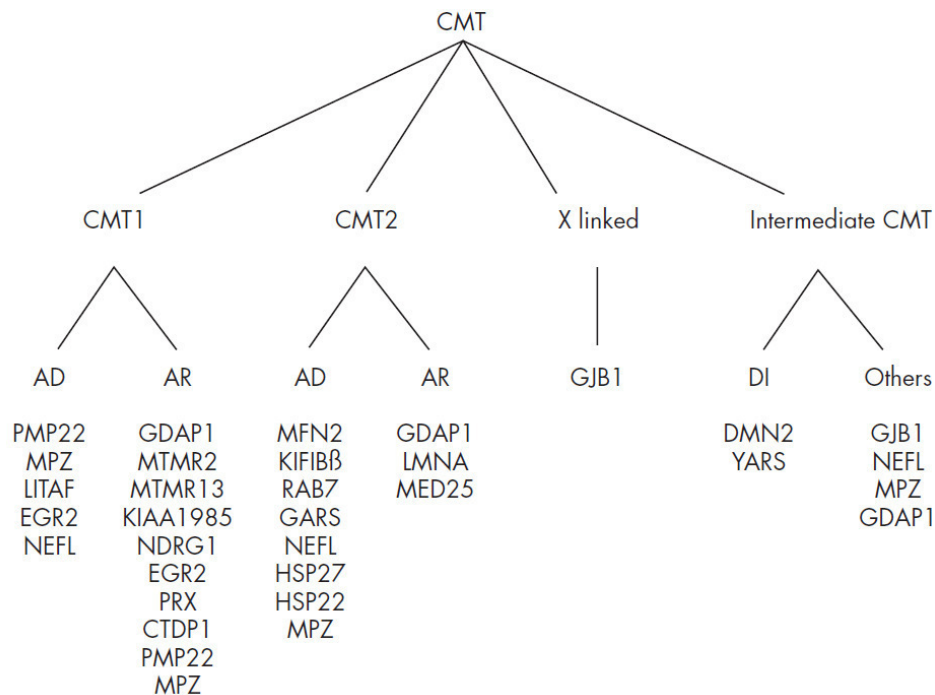


Fig. 2: Known causative genes of CMT. AD, autosomal dominant; AR, autosomal recessive; DI, dominant intermediate (Reilly, 2007).

The most common genetic defect for demyelinating CMT is duplication of the PMP22 gene, which causes excessive production of PMP22 (Patel et al., 1992, Hanemann et al., 1994, Yoshikawa et al., 1994). Indeed, up to 89% of CMT1 patients with a classic phenotype and a conduction velocity between 15 and 35 m/s tested positive for the PMP22 duplication (Saporta et al., 2011). The deletion of the same 1.4 Mb portion of chromosome 17, that is duplicated in CMT1A, causes hereditary neuropathy with liability to pressure palsies (HNPP). Other less frequent causes of CMT1 phenotype

include mutations in LITAF (CMT1C), EGR2 (CMT1D) and NEFL (CMT1F), which should be considered in patients who are negative for the most common genes associated with CMT1, such as MPZ (CMT1B)(Reilly, 2007, Baets et al., 2014, Saporta, 2014).

2.1.1 CHARCOT-MARIE-TOOTH1A

CMT1A is the most common hereditary peripheral neuropathy, it is a demyelinating disorder in which myelin dysfunction appears before axonal injury (Rummler et al., 2004). Neurological and clinical dysfunctions can be attributed to the loss or damage of motor and sensory neurons with a large diameter (Krajewski et al., 2000). It is caused by the heterozygous duplication of the chromosome 17p11.2-12, a portion that contains the PMP22 gene (Li et al., 2013), resulting in an increase of the protein quantity and peripheral nerve demyelination with secondary axonal loss. PMP22 expression is important for the correct myelination of peripheral nerves (Shapiro et al., 2006), indeed, PMP22, together with MPZ and MBP, participates in myelin sheath formation. It is an integral membrane glycoprotein of the internodal myelin (2-5% of the total proteins of the myelin in the PNS) (Snipes et al., 1992, Welcher et al., 1992).

In CMT1A, PMP22 is over-expressed, and this expression deregulation disrupts the organization of myelin; indeed CMT1A sural biopsies show onion bulbs that represent the excessive SC membrane processes around a myelinated nerve fibre that fail to form compact myelin (Gabreëls-Festen et al., 1995, Gabreels-Festen and R, 1999). This decompression of myelin leads to a significant reduction in nerve conduction that is typical of CMT1 disorders. The onion bulbs are the results of repetitive processes of demyelination and remyelination (Thomas et al., 1997, Robertson et al., 2002). Moreover, PMP22 over-expression may affect SC proliferation and survival (by inducing apoptosis) (Schneider et al., 1988) and may overwhelm protein degradation system leading to protein aggregate formation (Fortun et al., 2003, Fortun et al., 2006, Fortun et al., 2007). Researchers proposed different strategies to reduce PMP22 expression (Schenone et al., 2011), such as the

treatment with ascorbic acid (Passage et al., 2004) and the administration of selective antagonists of the progesterone receptor (Sereda et al., 2003). PMP22 expression reduction results in an improvement of the CMT1A phenotype. Another strategy to improve CMT1A symptoms is the promotion of the translocation of misfolded mutated PMP22 proteins from the endoplasmatic reticulum to the plasma membrane, that can be achieved by the oral administration of curcumin (Khajavi et al., 2007). Although different strategies are being investigated by researchers, no cure has yet been found (Pareyson and Marchesi, 2009, Patzkó and Shy, 2011).

2.2 ACQUIRED PERIPHERAL NEUROPATHIES

The development of acquired neuropathies can be attributed to different causes that determine different etiopathogenesis of the disease.

- Nutritional deficits: shortage of vitamins like vitamin B12 (McCombe and McLeod, 1984), B1, B6 (Hamel and Logigian, 2018) and D (Grim et al., 2017) can cause the development of neuropathy. Indeed, in neuropathic patients, the supplement of these vitamins may ameliorate the symptoms. Vitamin inadequate intake may be determined by dietary problems or by gastric or intestinal malabsorption.
- Exposure to toxic substances or drugs: neurotoxicity represents an important and sometimes preventable cause of neuropathy.

Toxic neuropathies include those produced by industrial toxins (organophosphates and organic solvents), heavy metals (thallium, arsenic, mercury and lead), drugs and alcohol (Weimer and Sachdev, 2009). Sensory nerves are more frequently affected (Karam and Dyck, 2015).

Drug-induced neuropathies involve different classes of medicines, among which chemotherapeutic agents (vinca alkaloids, taxanes, platinum derivatives), antimicrobials (such as fluoroquinolone and chloramphenicol) and cardiovascular drugs (such as amiodarone and statins) (Manji, 2011, Karam and Dyck, 2015).

Moreover, the chronic abuse of alcohol can be associated to peripheral neuropathy development, even if remains controversial whether alcohol abuse directly causes the pathology or if it causes vitamin deficiency that leads to the development of neuropathy (Koike and Sobue, 2006, Mellion et al., 2011).

- Complication of metabolic diseases: peripheral neuropathy is the most prevalent chronic complication of *diabetes mellitus* (Boulton et al., 2005, Sifuentes-Franco et al., 2017). Diabetic peripheral neuropathy includes sensory, motor, and autonomic neuropathy and the exact cause of the diabetic neuropathy development is still unclear, but different factors may occur, such as oxidative stress (Bodman and Dulebohn, 2017). Other pathologic conditions that may result in neuropathy development are hypothyroidism, end-stage renal disease and chronic liver disease (Azhary et al., 2010).
- Immune-mediated dysfunctions: the acquired immunologic neuropathies are a heterogeneous group of neuropathic conditions that result from immune system dysfunctions. The abnormal immune response produces antibodies against specific nerve antigens (Allen and Parry, 2015). The most known related neuropathies are the Guillain-Barré syndrome (GBS) and the Chronic Inflammatory Demyelinating Polyneuropathy (CIPD), that present many variants.

GBS is an acute immune-related polyneuropathy due to a molecular mimicry between bacterial antigens of a previous infection (*Campylobacter jejuni* infection) and peripheral nerve gangliosides (Fujimura, 2013). The anomalous immune response causes demyelination that could result in secondary axonal injury when pathology is more severe (Goodfellow and Willison, 2016). Otherwise, CIDP is not related to a previous infection and it is a chronic progressive or relapsing and remitting disease. Recently, IgG4 autoantibodies direct against proteins of the node of Ranvier were identified in some patients with CIDP (Querol and Illa, 2015, Querol et al., 2017), but other trigger factors associated with small disease subtypes of CIDP are still under investigation.
- Neoplasia: in patients with cancer, the development of neuropathy often represents a side effect of chemotherapy (Dalmau, 1999), but, neuropathy

may also derive from the direct infiltration of nerves by neoplastic cells or from indirect effects of the tumor (paraneoplastic syndrome). At least 15% of patients with cancer develops a paraneoplastic neuropathy. A neuropathy is defined as paraneoplastic when cancer-related immunological mechanisms are involved (like the presence of anti-Hu or anti-CV2/CRMP5 antibodies) (Rosenblum, 1993, Antoine and Camdessanche, 2013).

- Infectious agents: neuropathies caused by infectious agents are very frequent, principally in developing countries (de Freitas, 2007). The most common and frequently undiagnosed infectious neuropathy is caused by Varicella-zoster virus (Sindic, 2013), but also other infectious agents may lead to neuropathy development such as *Borrelia burgdorferi* (lyme syndrome), HCV and HIV (Sindic, 2013). HIV-induced neuropathies are the main neurological complication of HIV infection, even if the antiretroviral therapies can cause peripheral neuropathies that are indistinguishable from those directly caused by HIV (de Freitas, 2007). Moreover, the infection of nerve cells by *Mycobacterium leprae* causes leprosy-related neuropathies that lead to severe disabilities (Ooi and Srinivasan, 2004, de Freitas and Said, 2013).
- Trauma: compression of peripheral nerves can cause the development of neuropathies such as the carpal tunnel syndrome that is a very common pathologic condition due to the compression of the median nerve. Less frequent, but still common, is the ulnar nerve palsy determined by the compression of the ulnar nerve. Also, the radial nerve can be compressed (radial nerve palsy), but this condition usually resolves spontaneously (Hughes, 2008).

Nerve compression is only one of the possible consequences of a trauma; indeed, very often traumas, such as car accidents, cutting weapons, etc., cause peripheral nerve injuries, among which damages that determine nerve disruption.

The following paragraphs of the introduction refer to injured nerves following traumatic events, taking into account the cellular and molecular mechanisms

underlying the degeneration and regeneration of injured nerves, the involved growth factors and how to repair them through clinical approaches and tissue engineering.

3. PERIPHERAL NERVE INJURY CLASSIFICATION

Peripheral nerve lesions may result in partial or complete loss of motor or sensory function, leading to long-term disability (Grinsell and Keating, 2014).

More than 73.5% of nerve injuries occur in the upper extremities. Injury aetiology includes: crush, traction, penetrating injuries and ischemia, as well as electric shock and vibration (Robinson, 2000, 2004, Kouyoumdjian, 2006). Moreover, the development of neuropathic pain is frequently observed following nerve injury due to neuroma or scar tissue formation (Siemionow and Brzezicki, 2009). Thus, peripheral nerve injuries compromise the quality of life of affected people, with a consequently important socio-economic impact (Wiberg and Terenghi, 2003, Geuna et al., 2009, Siemionow and Brzezicki, 2009, Grinsell and Keating, 2014). Nerve injuries can be divided according to Seddon's classification (1943), in three categories: *neuroprassia*, *axonotmesis* and *neurotmesis*.

i) *Neuroprassia*, is an injury characterized by local damage of myelin and axon, without the interruption of *endoneurium* sheath. Although the axon continuity is preserved and the nerve does not undergo Wallerian degeneration (discussed later), the nerve does not transmit nerve impulses. This kind of lesion occurs following exposure to cold, heat, irradiation or electric shock, but it is most frequently due to mechanical stresses, such as compression, concussion or traction. Surgery is unnecessary and the recovery occurs within hours, days, or up to few months.

ii) *Axonotmesis* is a lesion in which axons are damaged or destroyed and lose their continuity, but without any alteration of connective tissue. Axon damage is usually due to nerve crushing, pinching or prolonged pressure. All the axon portions located after the site of injury undergo Wallerian degeneration, while the proximal stump of nerve can re-grow inside the intact endoneurial tubes. The transmission of pulses is compromised, as well as the functionality of the connected area of the body. The time of recovery depends on the severity degree of the lesion as well as the distance

of the injury site to the nerve target. Since connective tissue is intact, surgery is unnecessary.

iii) *Neurotmesis*, is a severe nerve injury. The nerve is completely destroyed and loses its continuity; also connective tissue is severely damaged. The proximal stump undergoes retrograde degeneration, while Wallerian degeneration occurs at the distal nerve stump. Due to the extended endoneurial tube disruption, spontaneous regeneration is slow and functional recovery does not easily occur; however, successful regeneration might be achieved by surgical intervention.

In 1951, Sunderland additionally divided *neurotmesis* injury in three categories with increasing level of severity (Figure 3). This classification identifies five degrees of nerve lesions. The first and the second degree match to *neuropraxia* and *axonotmesis*, respectively.

Injuries of third grade correspond to a *neurotmesis* with axon and *endoneurium* damaged, while *epineurium* and *perineurium* are intact.

In the fourth grade of nerve injury the internal structure of the nerve is completely destroyed, but its continuity is maintained by an undamaged *epineurium*. In the injury site, fibroblasts produce a fibrous scar. The presence of this scar inhibits axonal regeneration, contributing to the formation of a mass of disorganized fibres, known as neuroma-in-continuity. This type of injury requires surgery in order to remove the fibrotic scar and to promote regeneration.

The fifth-degree of injury is the most severe type of nerve damage, indeed it results in a complete transection of the nerve. The proximal and the distal stump of the nerve are physically separated, and surgical approach is required to obtain any functional recovery.

Since Sunderland's grades can be described only histologically, this classification has limited clinical utility. Moreover, the most nerve injuries are mixed, Mackinnon and Dellon included in Sunderland's classification, another type of injury which is a combination of all the degrees (grade 6) (Mackinnon, 1989).

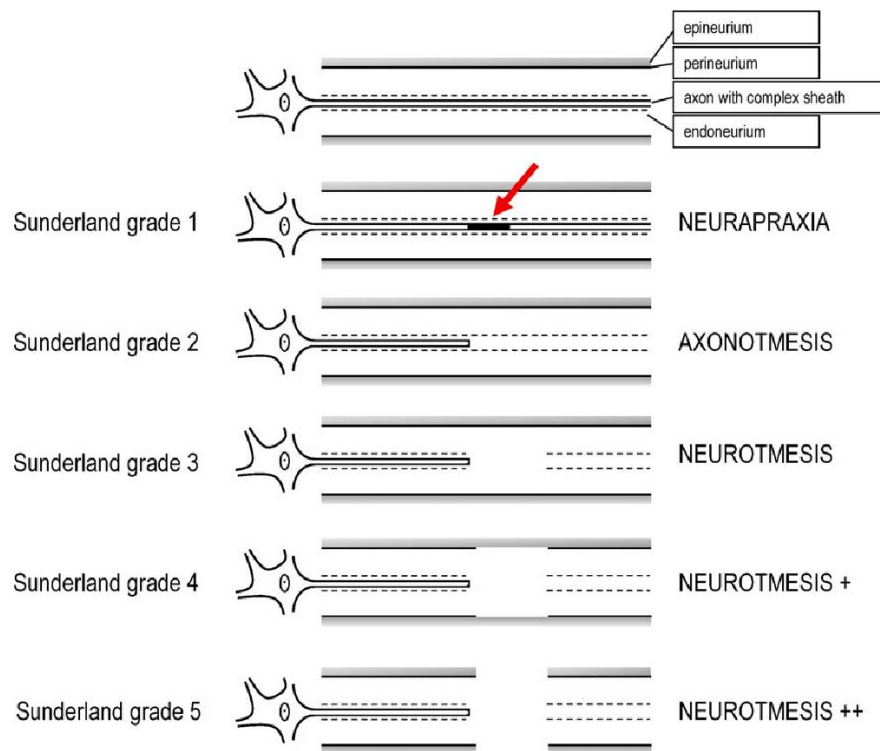


Fig. 3: Schematic representation of the five degrees of nerve injury in accordance with Sunderland's classification (Deumens et al., 2010).

4. CELLULAR AND MOLECULAR MECHANISMS IN PERIPHERAL NERVE DEGENERATION AND REGENERATION

Peripheral nerve injury induces several morphological and molecular changes at multiple levels: at the injury site (proximal and distal nerve stumps), at the cell body (dorsal and ventral roots) and at the level of the target organ (Figure 4). These changes start immediately and can be prolonged in time. Here, we describe the events that occur after *axonotmesis* or *neurotmesis*, in which immediately the synaptic transmission is lost and the target organ does not receive nerve signals.

During the early phase after a nerve injury (first few days), degenerative changes, including axon and myelin breakdown, started both proximally and distally to the injury site (Figure 4). The distal stump undergoes degeneration known as Wallerian degeneration (Figure 4A). This event was characterized for the first time by A. V. Waller (Waller, 1850), and is a sequence of events that results in the axon and myelin fragmentation and, finally, in nerve debris elimination. The increase in free intracellular Ca^{++} into axoplasm leads to the activation of calcium-sensible proteases, named calpains, which start the degradation of neurofilament proteins, microtubulin and other cytoskeletal components, leading to axon breakup in 48 hours (Coleman, 2005, Ma et al., 2013). SCs loose axon-contact, change their phenotype and start to degrade myelin (Figure 4B). The SC dedifferentiation is due to a change in gene expression, that occurs within 48 hours after nerve injury and implicates a down-regulation of myelination genes (MPZ, PMP22 and MBP) or gene related to node and internode organization (connexin 32 and E-cadherin) and the concomitant up-regulation of regeneration-associated genes (growth factor receptors, growth factor and adhesion molecules) (Hall, 2005, Deumens et al., 2010). Few days after the injury, activated SCs proliferate and later become aligned with the endoneurial tubes, forming the bands of Büngner, a supportive and guiding structure for regenerating axons (Figure 4C).

This structure physically supports regrowing axons and guides the regeneration through adhesion molecules and neurotrophic factors provided also by SCs (Höpker et al., 1999, Gordon, 2010, Richner et al., 2014, Madl and Heilshorn, 2015).

Among these neurotrophic factors, released by SCs, playing an essential role in the attraction of re-growing axons from the proximal nerve stump, there are: nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), neurotrophin 4 and 5 (NT-4 and 5), glial-cell-line derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor (IGF) and Neuregulin1 (NRG1) (Chen et al., 2001, Hall, 2001, Geuna et al., 2009, Deumens et al., 2010).

Moreover, SCs of the distal nerve stump are the main source of cytokines that attract macrophages. Indeed, few days after nerve injury, the resident *endoneurium* macrophages become activated and move to the injury site. Furthermore, the dysfunction of the blood-nerve barrier may lead to a consistent infiltration of haematogenous macrophages in the inner compartment of the nerve, with a subsequent massive myelin removal (Chen et al., 2015). Myelin clearance is one of the most important event for axon regrowth, indeed, when it does not occur properly, myelin components, like myelin associated glycoprotein (MAG), act as regeneration inhibitors. In addition to the receptor-dependent phagocytosis of axon and myelin debris, macrophages release also some mitogenic factors active on SCs and fibroblasts (Chen et al., 2015, Klein and Martini, 2016). Also other resident and infiltrated cells of the immune response play a role in the degeneration process. At the level of the distal stump, few hours from the injury, an increase in the levels of chemokines, cytokines, interleukin 1 (IL-1) and tumor necrosis factor (TNF) occur. In particular, blood vessel permeability increased by mast cells, facilitates macrophage migration to the injury site. Activated macrophages increase the IL-1 expression, stimulating the release of NGF and apolipoprotein E, that binds lipids derived from myelin and cells debris, directing them to SCs for degradation (Chen et al., 2001, Jessen and Mirsky, 2008). In addition, neutrophils are recruited from blood vessels, within the first week, and, together with lymphocytes, can lead to the development of neuropathic pain. The Wallerian degeneration is so associated with a strong inflammatory response (Gaudet et al., 2011).

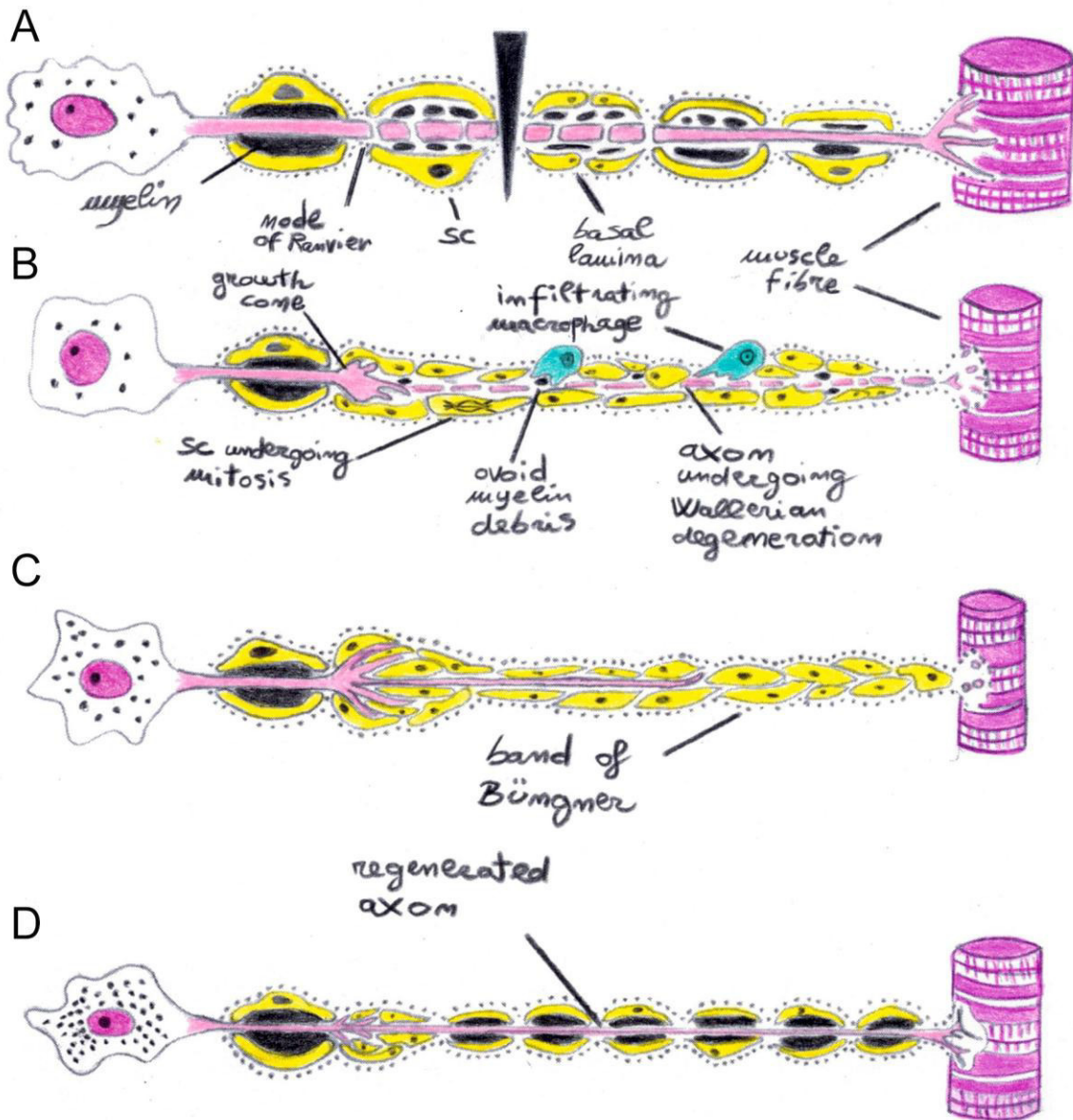


Fig. 4: Schematic representation of degenerative and regenerative events related to peripheral nerve injury.

Several hours after injury morphological changes occur also at the soma level, where information arrives through the retrograde signalling from proximal nerve stump (Rishal and Fainzilber, 2014). Morphologically, the nucleus of damaged neurons reach an eccentric position, the nucleolus becomes more evident, Nissl bodies disappear in the cytoplasm, cell volume increases and dendrites are retracted; these events are called "chromatolytic changes" (Hanz and Fainzilber, 2006) (Figure 4B).

To support the new activities (survival and growth), neurons modify their mRNA transcription profile with the up-regulation of proteins related to cell growth, like cytoskeletal components, transcription factors (c-Jun; c-Jun N-terminal kinase, JNK; activating transcription factor 3, ATF3) and growth factor receptors (Fornaro et al., 2008, Dahlin et al., 2009, Faroni et al., 2015).

Following a severe injury, the proximal nerve stump is involved in an initial retrograde degradation, consequently the axon retracts back to the first node of Ranvier, reduces its diameter, and then the neuronal regeneration process begins. Proximal stump maintains the contact with the soma, from which it receives resources through the fast and slow axoplasmic transport. Axon sprouting begins in different directions on basal lamina provided by SCs, guided by neurotrophic factors and adhesion molecules. Growth cones of attracted regrowing axons use these SC-made tubes as a growing substrate, extending at an average rate of 1-3 mm/day (Radtke and Vogt, 2009). At the end of the regeneration process, the interaction between maturing SCs and regrown axons in the distal nerve stump, induces myelin formation with significantly shorter internodes (Luís et al., 2007, Nave and Trapp, 2008, Deumens et al., 2010, Gordon et al., 2011) (Figure 4D).

The recovery from such changes can last from few weeks to months, depending on the re-establishment of the appropriate connections with the target organs. Failure of axotomized neurons to re-establish the appropriate connections may result in chronic chromatolytic state or even in their degeneration. Such conditions lead to prolonged or permanent denervation of the target organs, with consequent atrophy (i.e. skeletal muscle fibre atrophy). Since severe peripheral nerve injuries involve connective tissue scarring, axon regenerative attempts are often destined to fail and aberrant sprouting frequently occurs leading to neuroma development at the proximal nerve stump (Deumens, Bozkurt et al. 2010).

5. GROWTH FACTORS INVOLVED IN PERIPHERAL NERVE REGENERATION

Growth factors are small diffusible proteins and polypeptides produced and released by cells, or proteins and polypeptides anchored to the membrane, that act as signalling molecules regulating cell survival, migration, proliferation and differentiation, through paracrine, autocrine, juxtacrine or endocrine mechanisms. Usually, the signal transduction starts with the growth factor binding its receptor on the surface of the target cell. The specificity and the kind of signal is controlled both by the expression of the receptor and by the spatial/temporal release of growth factor. The localization of growth factors and their receptors has been extensively investigated in CNS and in PNS and numerous efforts have been made to understand growth factors role in neuronal tissue development and maintenance (Conover and Yancopoulos, 1997).

The production of neurotrophic growth factors and the expression of their receptors change considerably after nerve injury in the different nerve cell types (Boyd and Gordon, 2003, Richner et al., 2014) and is now accepted the idea that neurotrophic factors play an important role in peripheral nerve injury, influencing and controlling several aspects of nerve regeneration. This view is supported by several *in vitro* and *in vivo* studies about exogenous neurotrophic factors that promote neurite outgrowth, axon elongation, SC migration and axon remyelination.

Secretion of neurotrophic factors by SCs and denervated target organs modulates axon attraction, allowing the regrowing axons to elongate and to adhere, as well as SC differentiation and remyelination (Chen et al., 2007, Allodi et al., 2012, Richner et al., 2014). Increased secretion of these factors is fundamental to support axon survival and growth, allowing target organ reinnervation, and usually it returns to basal level at the end of the regeneration process. It is worth mentioning that sensory and motor neurons express different receptors and require different neurotrophic support for survival or growth (Richner et al., 2014).

The main studied growth factors are listed below, with a short description of their physiological role in nerve tissue and their role in peripheral nerve regeneration.

5.1. NEUROTROPHINS

In CNS and PNS neurotrophins are the group of factors that received most attention (Gordon, 2009). In mammals, neurotrophin family is composed by four members (NGF, BDNF, NT-3 and NT-4/5), all synthesized as precursor proteins and cleaved by convertases into small peptides of 13-15 kDa. The mature proteins are released as stable non-covalent homodimers and bind to two structurally unrelated receptors: the tropomyosin receptor kinase A, -B or -C (TrkA, -TrkB, and -TrkC) and the neurotrophin receptor p75 (NTR) (Yano and Chao, 2000).

- Nerve growth factor (NGF) is the first neurotrophin discovered by Rita Levi Montalcini and Viktor Hamburger in 1950, binding the TrkA receptors. This small protein promotes survival and differentiation of sensory and sympathetic neurons, neurite outgrowth and SC migration (Boyd and Gordon, 2003). Usually, NGF mRNA and protein are not detected in adult rat nerve, but its expression is induced after injury, in non-neuronal cells in the distal stump of transected nerves with a biphasic curve (Heumann et al., 1987a, Heumann et al., 1987b). Intriguingly, NGF is not expressed and not up-regulated after injury in motoneurons (Funakoshi et al., 1993, Escandon et al., 1994). NGF, released in silicone nerve conduit, increased the number of myelinated axons and the thickness of reformed myelin sheaths (Rich et al., 1989). The NGF delivery by microspheres inside chitin conduit shows similar results: improvement of maturation of myelinated fibres and of long-term functional recovery (Wang et al., 2014). Another controlled NGF release method has been successfully tested *in vivo* using various biomaterials, obtaining good nerve regeneration and functional recovery (Chang, 2009, Hsieh et al., 2011, Koudehi et al., 2014, Santos et al., 2016a).
- Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in CNS. Its action is mediated by TrkB, even if BDNF can bind with low affinity also p75 receptor. BDNF is expressed in DRG, in different subpopulations of neurons, as TrkA-positive cells and some TrkC-positive neurons. In undamaged motoneurons, BDNF is expressed at low levels, but it is rapidly up-regulated after injury, as well as in distal nerve stump (Meyer et al., 1992, Funakoshi et al., 1993,

Kobayashi et al., 1996). The role of endogenous BDNF after peripheral nerve injury is critical for the early induction of the regenerative response in sensory neurons (Geremia et al., 2010) and for motor neuron axonal regeneration and remyelination (Zhang et al., 2000, Boyd and Gordon, 2001, Wilhelm et al., 2012). Endogenous BDNF plays a role in controlling axon myelination, as underlined by negative effects given by anti-BDNF antibodies application which, in addition to reduce regenerated axon length and number of sensory neurons, reduces the number of myelinated axons (Zhang et al., 2000). Several strategies were investigated for BDNF delivery inside nerve conduits: a study in rat sciatic nerve injury showed that porous tubes of poly-2-hydroxyethylmethacrylate-methylmethacrylate (PHEMA-MMA) filled with collagen matrix releasing BDNF improved nerve regeneration in comparison to hollow tube or matrix alone (Midha et al., 2003). Furthermore, other studies using collagen conduit loaded with recombinant BDNF linked to laminin hydrogel for laryngeal nerve repair (Wang et al., 2016), or silicone tube filled with collagen matrix and microspheres releasing BDNF in rat sciatic nerve injury (Santos et al., 2016b) reported similar results.

- Neurotrophin-3 (NT-3) was discovered in 1990 and was found in brain, peripheral nerve as well as in kidney, lung, skeletal muscle and other organs (Hohn et al., 1990). The main source of NT-3 in peripheral nerve are satellite glial cells and SCs, while TrkC, NT-3 main receptor, is expressed by sympathetic and sensory neurons, as well as in SCs (Richner et al., 2014). In DRG or spinal cord neurons the expression of TrkC and NT-3 is down-regulated after severe injury (Boyd and Gordon, 2003). In the distal portion of the nerve, NT-3 mRNA levels decrease shortly after nerve injury and then return to normal level after 2 weeks (Funakoshi et al., 1993, Kohn et al., 1999). NT-3 is one of the most important factors for nerve regeneration and remyelination, by supporting denervated SC survival in the distal nerve stump. Indeed, in the absence of mature axons, SCs are able to survive, establishing an autocrine NT-3 long-term survival loop (Meier et al., 1999, Sahenk et al., 2008). The release of NT-3 by fibronectin matrix in 10 mm nerve gap increases the number of regenerated axons (Sterne et al., 1997). Porous PHEMA-MMA containing collagen matrix with NT-3 was used to repair 10 mm rat sciatic nerve gap, demonstrating that the enriched conduit led to better nerve

regeneration respect to hollow conduit or matrix without factor-filled tube (Midha et al., 2003). A poly(lactic acid-co-glycolic acid) (PLGA) nerve conduit combined with exogenous NT-3 has been shown to enhance axonal regrowth and to improve functional outcome after spinal cord injury (Fan et al., 2011).

- Neurotrophin-4/5 (NT-4/5) are the latest discovered neurotrophins, which exert their neurotrophic action promoting motor and sensory neuron survival (Schmalbruch and Rosenthal, 1995, Stucky et al., 2002), and axon growth (English et al., 2005). NT-4/5 bind preferentially TrkB. It has been shown that the expression of NT-4/5 decreases in motor neurons after nerve injury, that their mRNAs decline in distal nerve 6-12 hours after rat sciatic nerve injury, and then increase and became up-regulated after 2 weeks (Funakoshi et al., 1993). NT-4/5 are used to stimulate regeneration of peripheral nerves. Direct application of fibrin glue mixed with NT-4 on nerve injury site improves nerve regeneration, giving a higher number of regenerated axons, larger axonal diameter and myelin thickness (Yin et al., 2001). Another study reported that exogenous NT-4 exerts its effect principally on motor neurons innervating type 1 and type 2a muscle fibres (Simon et al., 2003). Furthermore, NT-4/5 are implicated in the positive effects given by treadmill training after peripheral nerve injury in mice (English et al., 2011).

5.2. NEUREGULINS

Neuregulins (NRGs) are a family of Epidermal Growth Factor (EGF)-like signalling molecules encoded by four genes (NRG1-4). Multiple isoforms are produced through alternative splicing of the different genes and are expressed in different tissues (Falls, 2003, Avramopoulos, 2017). NRG1 is one of the most extensively studied genes and through alternative splicing and different transcription start sites encodes at least 31 different isoforms (Falls, 2003, Mei and Xiong, 2008). All NRG1 isoforms are characterized in the extracellular region of the protein by the presence of an EGF-like domain, that is the responsible of the interaction with the receptor. The NRG1 isoforms can be further divided into NRG1 α and NRG1 β isoforms, according to the presence of a small domain downstream the EGF-like domain (Falls, 2003,

Edwards and Bottenstein, 2006), and into type a, b, and c isoforms, according to the exon present in the C-terminal tail (Falls, 2003, Gambarotta et al., 2013) (Fig. 5A). Isoforms differing in their N-terminus are grouped in six classes of NRG1, type I-VI. NRG1 type I, type II and type III have been found in several vertebrates, whereas NRG1 type IV is encoded only in mammalian and NRG1 V-VI are isoforms typical of primates (Chou and Ozaki, 2010). The NRG1 type I, II, IV and V possess an immunoglobulin (Ig)-like domain, absent in NRG1 type III and VI (N-terminal region directly connected to the EGF-like domain) (Mei and Xiong, 2008). This domain binds heparin and other glycosaminoglycans and can be a mechanism for limiting soluble NRG1 diffusion or creating a reservoir of the protein trapped in ECM. Moreover, NRG1 type III presents a unique folding, in fact both N- and C- terminals are located inside the cell (with the exception of NRG1 type III- β 3) due to a cystein-rich domain (CRD) and a second transmembrane domain. Downstream to the α or β domain, the following exon can be 1, ("2" means "missing exon"), 4 or 3, which contains a stop codon (Figure 5A). Despite most of NRG1 proteins are produced as transmembrane isoforms, to be later cleaved and released as active soluble proteins (except for type III that remains an active transmembrane protein), isoforms containing exon 3 (located downstream the EGF-like domain) are directly released and do not possess an intracellular domain (Figure 5B). The cleavage is given by the action of three type of transmembrane proteases: β -site of amyloid precursor protein cleaving enzyme (BACE); tumour necrosis factor- α converting enzyme (TACE), also referred as A disintegrin and metalloprotease 17 (ADAM17) and meltrin beta (also called ADAM19). The cleavage of different enzymes can result in different cellular signalling, as observed for NRG1 type III (Hu et al., 2006, Willem et al., 2006, La Marca et al., 2011) (Figure 5C).

The most widely studied NRG1 isoforms are subtypes I,II and III, because of their active role in the peripheral nervous system dynamics (Taveggia et al., 2010, Fricker and Bennett, 2011, Gambarotta et al., 2013).

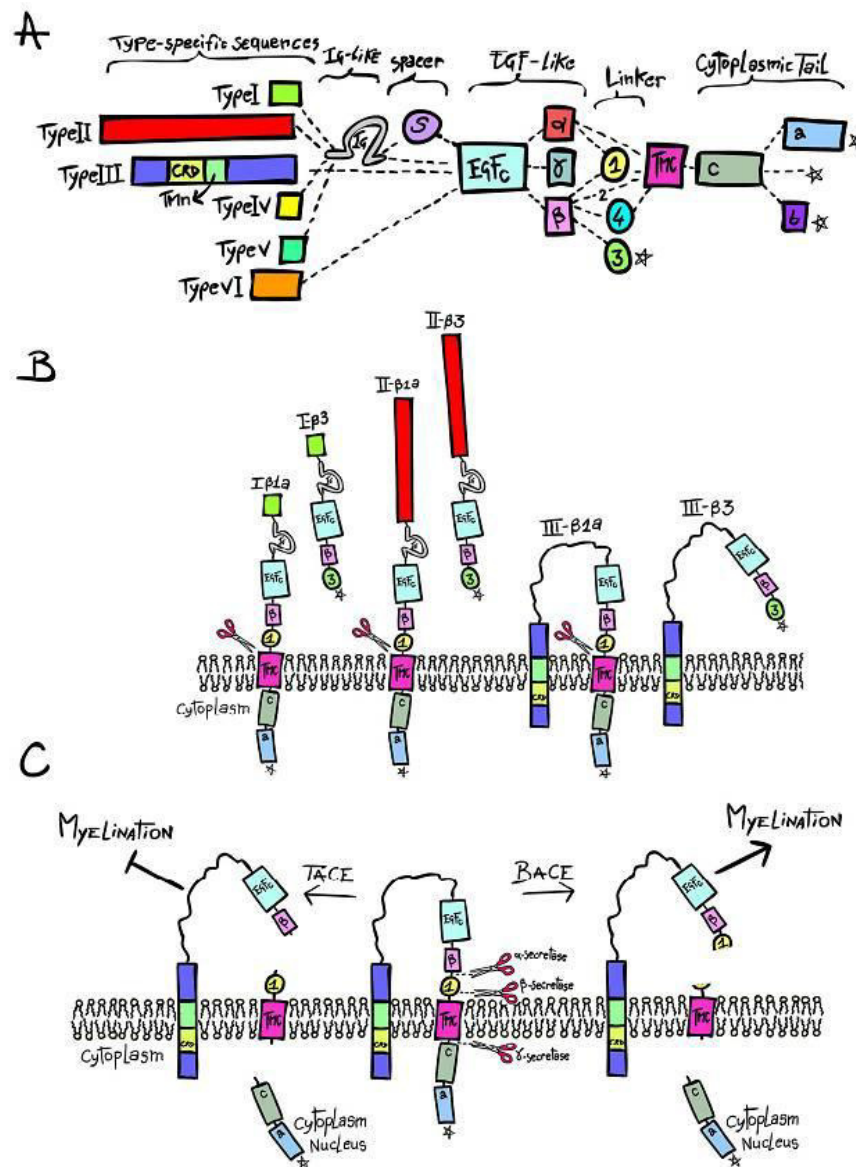


Fig.5: Schematic representation of NRG1 isoforms (Gambarella et al., 2013). (A) The six types of NRG1 differ for the N-terminal sequences. Moreover, the inclusion of several exons gives rise to various NRG1 isoforms. (B) After proteolytic cleavage the NRG1 isoforms type I, II (and IV, V, VI) that are produced as transmembrane precursor proteins, release a soluble active fragment, while transmembrane NRG1 type III remains transmembrane, but exposes the EGF-like domain. $\beta 3$ isoforms do not need proteolytic cleavage to be released or to expose the EGF-like domain. Some isoforms do not possess an intracellular domain and are directly released. (C) Example of different effects of the NRG1 processing by various secretases: NRG1 cleaved by BACE promotes myelination while the cleavage by TACE induces the opposite effect.

NRG1 signals through a family of single-transmembrane tyrosine kinase receptors, named ErbB proteins. ErbB receptor family members are ErbB1 (known also as EGF receptor), ErbB2, ErbB3 and ErbB4 (Britsch, 2007). The basic structure of ErbBs consists of an extracellular N-terminus and two cysteine-rich domains (CRD) for ligand binding, a transmembrane domain and a C-terminal tail containing the tyrosine kinase domain and binding sites for proteins involved in the signal transduction pathways.

Only ErbB3 and ErbB4 directly bind NRG1 (Marchionni et al., 1993, Carraway et al., 1997, Falls, 2003, Mei and Xiong, 2008). After ligand binding, ErbB receptors dimerize and the intracellular tyrosine residues are phosphorylated, becoming docking sites for adaptor proteins involved in several transduction pathways. While ErbB4 is an autonomous receptor, able to bind NRG1 and to form homodimers or heterodimers, ErbB3 kinase function is impaired and it can form only active heterodimers; ErbB2 does not bind NRG1, therefore it works as co-receptor (Figure 6). Thus, NRG1 can act on ErbB2-ErbB3, ErbB2-ErbB4 heterodimers and on ErbB4-ErbB4 homodimers. ErbB1 does not bind to NRG1, but it can signal through the heterodimerization with ErbB3 and ErbB4 that bind NRG1 (Mei and Xiong, 2008).

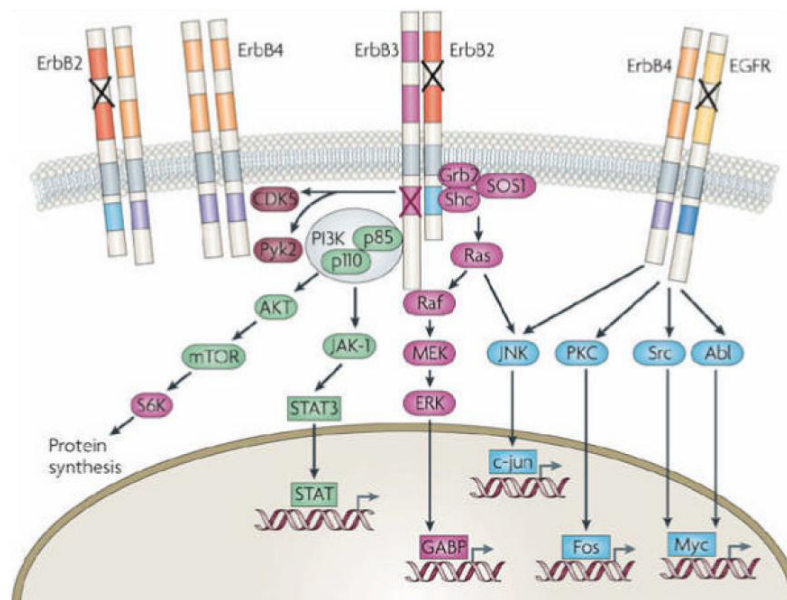


Fig. 6: Schematic diagram of ErbB dimerization and downstream signal activation. Each ErbB receptor owns two cysteine-rich domains in the extracellular region, a transmembrane domain, a short intracellular juxtamembrane region, a tyrosine kinase domain and a carboxy-terminal tail (Mei and Xiong, 2008).

In the peripheral nervous system, the NRG1/ErbB system is a determinant of the development of SC precursors (Castelnovo et al., 2017). Moreover, in the peripheral nerves, transmembrane NRG1 is expressed by the axon and interacts with ErbB2/ErbB3 heterodimer receptor expressed on the surface of SCs (Figure 7). Transmembrane NRG1 signals in a juxtacrine way and the interaction mediated by NRG1/ErbB system is the signal that stimulates SCs to deposit myelin layers around axons surface (Michailov et al., 2004, Taveggia et al., 2005). Moreover, it has been shown that the juxtacrine interaction can also signal in a reverse manner (“back-signalling”), mediated by the action of a γ -secretase enzyme, which causes the release of a fragment containing the intracellular domain (ICD) that can translocate into the nucleus and influence gene transcription (Bao et al., 2003, Bao et al., 2004, Chen et al., 2010).

Transmembrane NRG1 is required for the development of myelin and it controls myelin thickness (Michailov et al., 2004, Taveggia et al., 2005, Nave and Salzer, 2006, Fricker et al., 2011). Indeed, the myelin thickness depends on the amount of transmembrane NRG1.

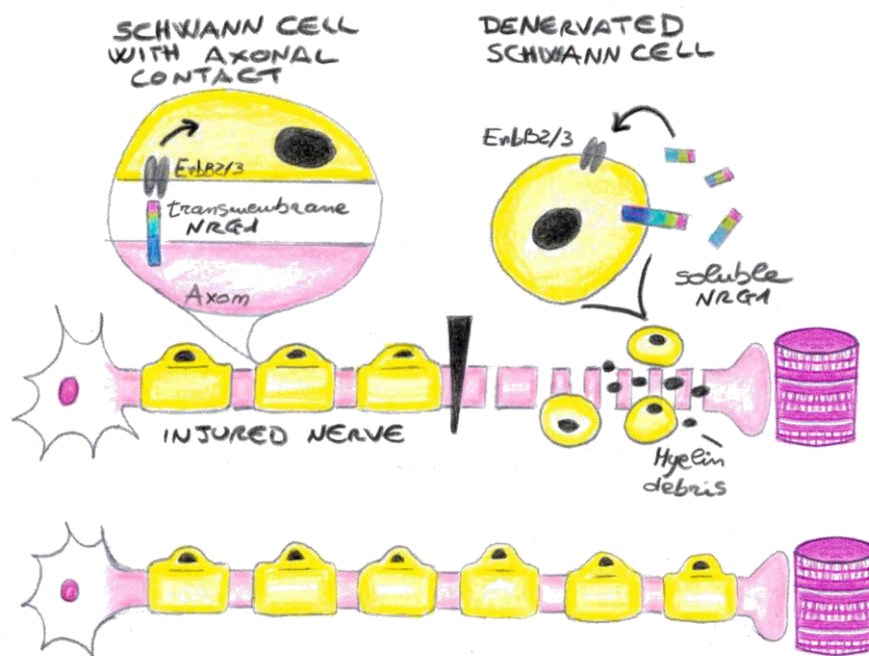


Fig. 7: In healthy nerves, transmembrane NRG1 controls myelination and inhibits soluble NRG1 expression in SCs. After nerve damage, SCs are detached and the axonal NRG1 signal is lost. By losing that connection, SCs start to express soluble NRG1 (Stassart et al., 2013, Mei and Nave, 2014).

Immediately after a nerve injury, SCs break away from the axon and start to express soluble NRG1 activating a paracrine and an autocrine loop that stimulates SC survival, proliferation and dedifferentiation (Birchmeier and Nave, 2008, Fricker and Bennett, 2011) (Figure 7). While soluble NRG1 plays a role during the early phases following a nerve injury, transmembrane NRG1 is involved in the remyelination process. Indeed, in animals lacking axonal transmembrane NRG1 the remyelination is impaired (Fricker et al., 2013), while animals over-expressing it show hypermyelination and an improved nerve regeneration after nerve injury (Michailov et al., 2004, Stassart et al., 2013).

Since NRG1 controls axon remyelination and SC behaviour, its use has been proposed as therapeutic approach for peripheral nerve repair (Gambarotta et al., 2013). NRG1 can be delivered in different ways, among which delivery through adeno-associated viral vector encoding it (Mancuso et al., 2016) or through different biomaterials as described below. Poly-3-hydroxybutyrate (PHB) conduits filled with alginate matrix containing recombinant NRG1 (type II) were used to bridge 4 cm nerve gap in rabbit peroneal nerve and the results showed an increase of SC number, an improvement of axonal regeneration and muscle reinnervation compared to matrix alone or empty tubes (Mohanna et al., 2005); injection at distal and proximal nerve stumps of adenoviruses expressing the recombinant EGF-like domain of NRG1 type I has been shown to promote nerve regeneration, as indicated by longer axon length and thicker calibres leading to improved sensory and motor functions (Joung et al., 2010); a 10 mm-gap bridge in rat sciatic nerve was performed with silicone tube filled with collagen gel containing adipose-derived regenerative cells releasing NRG1 and vascular endothelial growth factor (VEGF), resulting in the promotion of SC proliferation and migration (Suganuma et al., 2013); transfected SCs over-expressing NRG1 were implanted in rat after hemisection spinal cord injury with an ameliorated recovery, related to increased glial cell proliferation and protection from neuronal apoptosis (Zhang et al., 2010). Moreover, a gelatin hydrogel releasing NRG1 implanted in rat facial nerve resulted in faster muscular function recovery and higher density of large-diameter axon compared to no factor therapy or single NRG1 injection (Yasui et al., 2016). Release of NRG1 from poly(ϵ -caprolactone) (PCL) aligned nanofibres demonstrates *in vitro* the effectiveness of this functionalized

scaffold in promoting SC adhesion and directional growth compared with nanofibres without functionalization with NRG1 (Tonazzini et al., 2017). It has been shown that degenerating muscles express soluble NRG1, thus suggesting that muscles might be a tool for NRG1 delivery, as discussed later (Nicolino et al., 2003).

5.3. OTHER GROWTH FACTORS

- *Glial cell derived neurotrophic factor (GDNF)* belongs to the transforming growth factor (TGF) α -1 family. It was isolated from the cell culture supernatant of the rat glial cell line B49 and later it was described as a potent survival factor for motor neurons and midbrain dopaminergic cells (Lin et al., 1993, Henderson et al., 1994, Lin et al., 1994, Oppenheim et al., 1995). GDNF is not expressed in undamaged nerves (Naveilhan et al., 1997, Trupp et al., 1997), and the main source of this factor in nerves are SCs. GDNF receptors (c-RET and GFR- α) are expressed in motor and sensory neurons. SCs express only GFR- α receptor (Naveilhan et al., 1997). SC-derived GDNF is anterogradely transported along the axons and released at terminals of sensory and motor neurons, where it acts on glial cells expressing its receptor GFR- α (Allodi et al., 2012). Nerve injury induces in distal nerve stumps an increase of GDNF expression together with receptor up-regulation, suggesting an important trophic role of GDNF on nerve regeneration (Naveilhan et al., 1997, Höke et al., 2002).

Exogenous application of GDNF has been shown to improve motor axon regeneration (Qiao et al., 1995, Tajdaran et al., 2016); anyway, a single local dose produces a transient effect and elevated levels of GDNF generate trapping of regenerating axons and might induce the formation of nerve coils (Tannemaat et al., 2008, Santosa et al., 2013). Several studies investigated the release of GDNF inside nerve conduits and different strategies have been tested to control the factor release, as adenoviral (Sakamoto et al., 2000, Watabe et al., 2000) or lentiviral-mediated gene transduction (Eggers et al., 2013) of motor neurons and SCs; polymer-encapsulated cells producing GDNF (Shakhbazau et al., 2013); microsphere releasing GDNF (Wood et al., 2009, Kokai et al., 2010). Generally, the regeneration of both motor and sensory neurons was shown to be enhanced

by factor release in conduits, with a higher number of regenerated axons and improvement of functional recovery. Moreover, GDNF has been shown to regulate *in vitro* myelination (Zhang et al., 2009) and *in vivo* experiments demonstrated that exogenous GDNF increases the myelination of regenerating axons (Chen et al., 2017) and promotes SC proliferation (Höke et al., 2003).

- *Vascular Endothelial Growth Factor (VEGF)* is a powerful angiogenic factor, that stimulates endothelial cell migration and proliferation, blood vessel formation and increases vascular permeability (Neufeld et al., 1999, Holmes and Zachary, 2005, Kempton et al., 2009). VEGF family includes 5 members; among them, VEGF A (here indicated as VEGF) is the most studied. VEGF is an homodimeric glycoprotein that presents, thank to post-translational modifications, different isoforms characterized by different biochemical and biological properties (solubility, affinity for heparin, bioavailability) (Ferrara and Davis-Smyth, 1997). VEGF exerts its effect by binding to specific tyrosine kinase receptors: VEGFR1 and VEGFR2. VEGF exerts a trophic effect on primary cortical neurons and DRG, promoting neurite outgrowth (Sondell et al., 1999, Sondell et al., 2000, Jin et al., 2006). VEGF increases SC proliferation, and both neuron and satellite cell survival. Moreover, VEGF induces SC migration, and microglial cell migration and proliferation (Sondell et al., 1999, Schratzberger et al., 2000). Furthermore, VEGF is implicated in neuropathic pain and neuroprotection (de Almodovar et al., 2009, Hulse et al., 2014). Induction of VEGF expression has been described after traumatic spinal cord injuries and in DRG neurons after nerve injury (Sköld et al., 2000, Tang et al., 2009, Lindholm et al., 2017). It has been investigated for its role in nerve regeneration, given the close relationship between blood vessels and nerve fibres (Carmeliet and Tessier-Lavigne, 2005). Several studies reported that VEGF releasing nerve grafts or VEGF releasing matrix fillers (Hobson et al., 2000), or transplantation of transfected cells (Kempton et al., 2009) for the delivery of VEGF (Pereira Lopes et al., 2011) are all good strategies to enhanced nerve regeneration. An advantage of the VEGF use is its action on both vascular and nervous components in the nerve regeneration process.

6. CLINICAL APPROACHES FOR PERIPHERAL NERVE REPAIR

The primary purpose of nerve repair is the reinnervation of the target organs by guiding regenerating axons into the environment of the distal nerve with minimal fibre loss.

The functional and sensory outcome of peripheral nerve repair is variable, and influenced by many factors that have to be taken in consideration such as: the extent and location of the injury, timing of the surgery, surgical approach, proper alignment of nerve fascicles and patient co-morbidities (Dahlin, 2008, Battiston et al., 2009, Konofaos and Ver Halen, 2013).

About the timing of the surgery, when there are no other concomitant severe trauma, primary nerve repair is usually the best choice (within 72 hours up to 7 days) (Dvali and Mackinnon, 2003, Siemionow and Brzezicki, 2009). However, often an immediate repair is not possible and the nerve repair is delayed in time (secondary repair), until all other injured tissues are restored (Siemionow and Brzezicki, 2009). Often, after delayed nerve repair, functional recovery is impaired. The effectiveness of delayed nerve repair is limited by the viability of SCs and endoneurial tubes and by progressive muscle atrophy (Sulaiman and Gordon, 2000, Grinsell and Keating, 2014), making immediate repair the better choice when it is possible.

Peripheral nerve injuries need to be repaired by different surgical techniques, depending on injury severity and nerve gap length, as discussed below (Siemionow and Brzezicki, 2009, Grinsell and Keating, 2014).

6.1. DIRECT REPAIR

Direct repair is preferred when the gap between the two nerve stumps is absent or short (5 mm or less) and the two ends can be sutured with minimal tension. When the gap is longer, due to substance loss between the two nerve stumps, direct suture under tension results in very poor clinical outcome leading to the need to introduce graft materials to bridge the gap (Dvali and Mackinnon, 2003, Leach and Schmidt, 2005, Battiston et al., 2009, Scholz et al., 2009, Pfister et al., 2011). To obtain optimal nerve regeneration, nerve stumps must be precisely aligned and repaired with minimal tension and tissue damage. Better results are usually obtained when nerves are purely motor or sensory (Dahlin, 2008, Siemionow and Brzezicki, 2009, Konofaos and Ver Halen, 2013, Grinsell and Keating, 2014). The direct repair includes different surgical techniques: end-to-end repair, epineural sleeve repair and end-to-side repair.

- *End-to-end repair*

The surgeon reconnects the two free nerve stumps (end-to-end repair), suturing together the two nerve stumps through the epineurial tissue (“standard epineural repair technique”; Figure 8) or placing sutures into the interfascicular *epineurium* connecting groups of fascicles (“grouped fascicular repair technique”; Figure 9) or single fascicles (“fascicular repair”) (Flores et al., 2000, Siemionow and Brzezicki, 2009).

The main goal of epineural repair surgical technique is to obtain continuity of the nerve stumps without tension and with proper fascicular alignment.

The standard epineural repair is the commonly used technique, following partial nerve injuries with good fascicle alignment and without substance loss (Pfister et al., 2011).

The correct fascicular match can be confirmed by lining up the internal nerve fascicles and the surface epineural blood vessel patterns (*vasa nervorum*) (Siemionow and Brzezicki, 2009, Pabari et al., 2010, Grinsell and Keating, 2014).

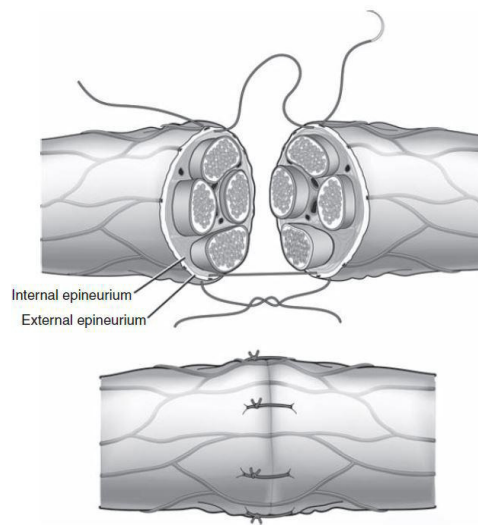


Fig. 8: Representation of the standard epineural repair technique, in which the internal and the external of the epineurium are involved in the sutures (Siemionow and Brzezicki, 2009).

Another used end-to-end repair technique is the grouped fascicle repair (Figure 9). Currently, this procedure is best applied in mixed nerves, where groups of fascicles can be easily identified (Dahlin, 2008, Siemionow and Brzezicki, 2009). This surgical practice requires interfascicular *epineurium* dissection, and direct matching and suturing of fascicular groups. Instead, in the fascicular repair technique, the single fascicles are sutured; this technique is not widely used anymore. The theoretical advantages given by better fascicle alignment through group fascicular and fascicular repair are offset by the number of sutures required (2-3 per fascicle), that imply more trauma and scarring tissue to the healing nerve (Dahlin, 2008, Siemionow and Brzezicki, 2009, Grinsell and Keating, 2014). Anyway, experimental studies on functional outcomes, demonstrate that group fascicular repair technique is not better than the standard epineural repair approach (Lundborg, 2000).

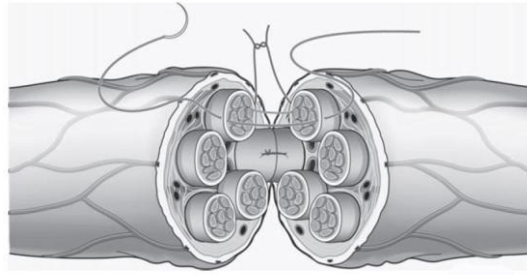


Fig. 9: Schematic illustration of the grouped fascicular repair. The groups of fascicles are sutured with stitches passing through the interfascicular epineurium (Siemionow and Brzezicki, 2009).

- *Epineural sleeve repair*

In epineural sleeve neuroraphy, the *epineurium* that covers the distal stump of the damaged nerve is rolled back creating an epineural sleeve, and 2-mm of nerve segment are removed (Figure 10). Epineural tissue sleeve on the distal nerve stump is sutured to the proximal nerve stump *epineurium*, providing a neuropermissive environment for regrowing axons, and guidance for regenerating nerve fibres (Siemionow and Brzezicki, 2009). The separation of the repair site from the surrounding tissue prevents the formation of scar tissue and fibrosis (Lubiatowski et al., 2008).

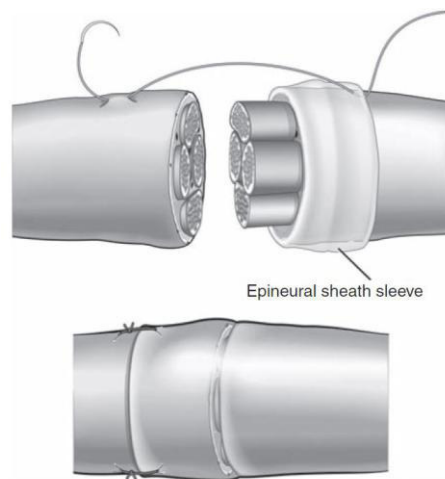


Fig.10: Schematization of epineural sleeve repair technique (Siemionow and Brzezicki, 2009).

- *End-to-side repair*

When the proximal nerve stump of the damaged nerve is unavailable, or a significant nerve gap exists, end-to-side repair technique is the way to repair this kind of injuries. The distal nerve stump of the injured nerve is coapted to the side of an healthy nerve. Collateral sprouting of healthy nerve fibres allows the reinnervation of the disconnected nerve. This technique is particularly useful in brachial plexus injuries or for facial nerve reconstruction in the clinical practice, and it allows target muscle reinnervation, without compromising the function of the donor nerve (Scholz et al., 2009, Siemionow and Brzezicki, 2009, Pabari et al., 2010).

6.2. NERVE GRAFTING

In case of nerve gaps that cannot be bridged without tension, a nerve grafting technique is required (Battiston et al., 2005, Dahlin, 2008, Grinsell and Keating, 2014). Depending on the origin of the material used for the graft, it is possible to distinguish between *autograft*, if the material is derived from the patient itself, and *allograft*, if the material comes from cadaver donors.

- *Nerve autograft*

For longer gap nerve damage, the standard approach used in clinic is the nerve autograft, which entails the suture of a nerve achieved from the patient himself (usually a sensory nerve, as the sural nerve) to bridge the gap of another more important nerve (as motor nerve) (Lee and Wolfe, 2000, Pabari et al., 2010). Generally, fragments from the sural or saphenous nerves are used since their loss do not lead to serious functional and sensory loss. Autologous nerve grafts provide mechanical support and a permissive and stimulating environment for nerve regeneration since they contain SCs, basal lamina, neurotrophic factors and adhesion molecules and reduce the onset of fibrosis (Lundborg, 2005).

Besides it remains the “gold standard” technique for peripheral nerve repair, the autologous nerve graft is associated with morbidity, including possible neuroma formation at the donor site (Lee and Wolfe, 2000, Lundborg, 2005, Siemionow

and Brzezicki, 2009). Furthermore, the calibre of donor nerve or its fibre composition often do not match perfectly for grafting, influencing negatively the functional outcome (Siemionow and Brzezicki, 2009, Daly et al., 2012).

Nerve grafts could be cable or single. The standard *cable grafting technique* (Figure 11) is used to obtain the maximal coverage of the damaged nerve cross-section area. Graft segments are interposed to the corresponding groups of fascicles of proximal and distal stumps, creating a direct regeneration pathway (Siemionow and Brzezicki, 2009, Grinsell and Keating, 2014).

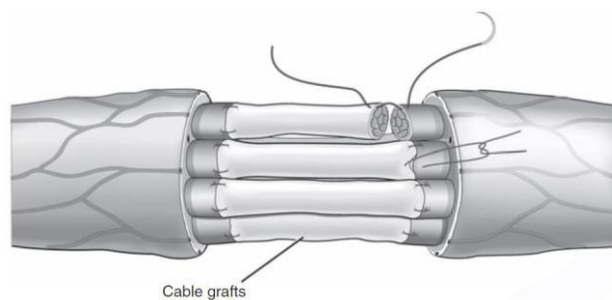


Fig. 11: Schematic representation of cable graft repair. Graft stumps are connected to the corresponding fascicles of proximal and distal stumps (Siemionow and Brzezicki, 2009).

In order to reduce donor site morbidity and to increase the amount of available material, single-fascicle nerve repair technique can be used. In this technique a single fascicle dissected from the donor nerve is coapted by single horizontal mattress suture. The central alignment of the graft is achieved, bridging proximal and distal stump and minimizing the foreign body reaction and fibrosis at the repair site (Figure 12) (Siemionow and Brzezicki, 2009).

During the last three decades, increasing efforts have been made to potentiate nerve autograft success, on one hand, and to seek new surgical alternative approaches, on the other hand.

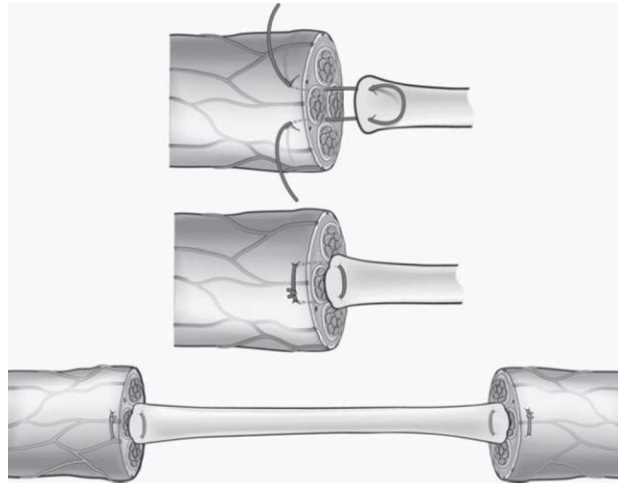


Fig. 12: Schematization of single fascicle nerve repair technique (Siemionow and Brzezicki, 2009).

- *Nerve allograft*

Regarding alternative technique to autograft, a valid option is the nerve allograft, in which the donor nerve derives from human cadaver. Nerve allograft avoids the donor site morbidity and is free from size limitation; theoretically it allows to replace the injured nerve drawing the same nerve type from the donor, providing a better recovery (Chu et al., 2008, Moradzadeh et al., 2008). Anyway, a concomitant immunosuppressant treatment is necessary, increasing the costs and the complexity of this technique. Currently, the allograft is the most used clinical approach for treating long gap nerve injury (up to 5 cm) (Safa and Buncke, 2016). For reducing nerve immunogenicity, various protocols have been tested to get an acellular nerve graft. Enzymatic digestion, detergent processing or irradiation are used to remove the cellular component and to maintain the internal structure and topography of the nerve, able alone to give physical support to cell invasion and axon elongation (Safa and Buncke, 2016). Intriguingly, the used protocol can affect nerve regeneration outcome (Jones et al., 2016). Avance® Nerve Graft (AxoGen, Alachua, FL, USA), a human prepared allograft, received the Food and Drug Administration (FDA) and is commercially available.

6.3. REPAIR WITH CONDUIT

Despite autograft technique is considered the “gold standard” to repair severe nerve injuries, multiple disadvantages are associated to this technique. The limited availability of donor tissue with its related morbidity, the potential formation of neuroma at the site of withdrawal, as well as frequent disappointing functional outcomes are the most recurrent issues (Ichihara et al., 2008). For these reasons, over the last three decades increasing attempts have been made to develop effective alternatives to the autologous nerve grafts such as the tubulization technique (Figure 13) that is one of the most promising surgical technique to bridge nerve gaps. This surgical procedure allows to repair severe nerve defects, by the enclosure of both damaged nerve stumps inside a tubular guide. These nerve conduits intent to connect nerve stumps, create a microenvironment in which neurotrophic and neurotropic factors can act, guiding the regenerating axons and protecting the nerve from the surrounding tissue (Sabongi et al., 2015). Success of the regeneration process depends on new extracellular matrix deposition inside the scaffold, which is used by vessels, fibroblasts and SCs to migrate and to form a new nerve structure (Siemionow and Brzezicki, 2009, Konofaos and Ver Halen, 2013, Grinsell and Keating, 2014). The use of hollow conduits is limited only for the clinical treatment of small nerve gaps up to few centimetres. For gaps longer than 3 cm, or in proximal nerve lesions, hollow nerve conduits are not considered an available option. To overcome this problem, researchers developed different kinds of enrichments for hollow tubes.

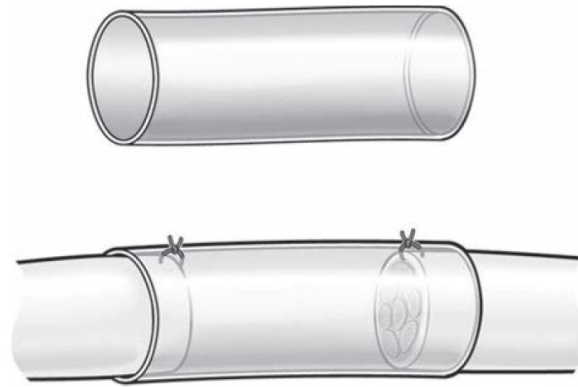


Fig. 13: Schematic representation of nerve repair through nerve conduit. The nerve stumps are pulled inside the tube and sutured to the tube ends (Siemionow and Brzezicki, 2009).

7. BIOMATERIAL SCAFFOLD TO REPAIR PERIPHERAL NERVE INJURY

7.1. NERVE GUIDE REQUIREMENTS

During the development of an ideal nerve guide, the choice of material and structure of the scaffold is a critical step. Indeed, the scaffold has to satisfy some requirements. The major biological and physicochemical requirements to suit are biomechanical properties, biocompatibility, biodegradability, permeability and mechanical strength (de Ruiter et al., 2009a, Gu et al., 2011).

- *Biocompatibility:*

In accordance with its original definition, biocompatibility is the ability of a biomaterial to support appropriate cell behaviour, including the promotion of molecular and mechanical signalling to enhance nerve regeneration, without eliciting any adverse effect on cells and tissues and/or inducing local or systemic immune-responses in the host organism (Williams, 1999). The biocompatibility could be evaluated considering

three main aspects: blood compatibility, histocompatibility and mechanical compatibility. Regarding blood compatibility, the biomaterial must not induce haemolysis, coagulation, blood components destruction, and thrombus formation.

Moreover, the scaffold should not induce toxic side effects on surrounding cells and tissues, resulting in cytotoxicity and inflammation response that may lead to immune rejection of the scaffold. Mechanical compatibility refers to mechanical properties of the scaffold towards nerve tissues (Ichihara et al., 2008, Gu et al., 2011).

- *Biodegradability:*

The degradation of a scaffold in a correct time-window is an important required feature. At the beginning, non-degradable materials, such as silicone and polyethylene, were used. These materials were not reabsorbed by tissues, making necessary a second surgical procedure to remove them (Deng et al., 2008).

In order to avoid long-term complications, the use of re-absorbable materials was gradually increased. The structure of the nerve scaffold must remain intact for the time needed by regenerating axons to regrow across the gap, and then to degrade gradually with minimal foreign body reaction (Ichihara et al., 2008, Gu et al., 2011, Konofaos and Ver Halen, 2013). Bioreabsorbable scaffolds that degrade quickly, fail to protect regrowing axons during regeneration. Early damages of the structure lead to fibrous scar formation inside the tube, impairing nerve regeneration (Dunnen et al., 1998).

- *Permeability:*

Regulated exchange between the intra/extra lumen environments is essential for the correct regeneration process, especially through synthetic nerve guides (Aebischer et al., 1988). Scaffold surfaces should be enough porous to support the regeneration process, ensuring diffusion of nutrients (glucose, oxygen, etc.), and growth factors from the external environment. Pores can prevent scar forming fibroblast infiltration (Ichihara et al., 2008, Gu et al., 2011, Konofaos and Ver Halen, 2013). Moreover, permeability may affect fibrin matrix formation during the initial stage of nerve regeneration (Zhao et al., 1993).

- *Mechanical strength*

A scaffold, to repair peripheral nerve, must be flexible enough to allow bending without kinking. It is important the right balance between flexibility and hardness: too unbending scaffolds are easy to cause dislocation and too flexible scaffolds fail to support axonal growth. The biomechanical properties of nerve guides depend from the nature of the material used for the scaffold preparation, the wall thickness, the lumen diameter and the nature of internal fillers (Ciardelli and Chiono, 2006, Gu et al., 2011).

7.2. BIOLOGICAL AND SYNTHETIC MATERIALS FOR NERVE CONDUITS

Several materials, both of biologic or synthetic origins, have been used to designed nerve guides. According to the material source, nerve conduits can be mainly classified in two categories: biological or synthetic nerve conduits.

7.2.1. BIOLOGICAL MATERIALS

Biological materials used to produce nerve scaffolds, take advantage of their immunological compatibility, with an optimal donor-host integration, and of their microstructure. Biological nerve guides can be made either of autogenous or non-autogenous materials.

AUTOGENOUS BIOLOGICAL MATERIALS

Among the different types of autogenous biological materials, skeletal muscle and blood vessels received most attention by researchers. Their natural topography, with oriented structures, could guide axon regeneration (Deumens et al., 2010, Konofaos and Ver Halen, 2013).

Veins or vessels: nerve conduits from the vascular system have been studied since the 20th century (Tang, 1995), because of the large material availability and for the reduced withdrawal side effects (non-immunogenic material). Good functional outcomes, particularly in the repair of small pure sensory digital nerve defects, were demonstrated in clinical applications (Chiu and Strauch, 1990). Vein tissue

composition is similar to that of the nerve, moreover the vein walls act as a barrier against scar growth and allow diffusion of the proper nutrients (Wang et al., 1993, Tseng et al., 2003). Finally, they also can provide a mechanical support for the regenerating axon (Sabongi et al., 2015). However, the effectiveness of veins as nerve conduit is still controversial. If applied to bridge long nerve gaps, the vein tends to collapse, leading to nerve compression and unsuccessful recovery. The vein collapse can be avoided by the enrichment of the venous tubes with internal fillers such as nerve tissue (Tang, 1995) or skeletal muscle (Brunelli et al., 1993, Raimondo et al., 2005, Battiston et al., 2009).

Skeletal Muscle: it is used because of the high donor site availability and in the low withdrawal morbidity degree. Moreover, extracellular matrix components and highly oriented basal lamina tubes are characteristic of the muscular tissue, providing a suitable substrate for regenerating axons (Battiston et al., 2007). Experimental studies in animals and humans reported encouraging results, demonstrating that both denaturated and fresh skeletal muscle could lead to successful nerve repair (Glasby et al., 1986, Norris et al., 1988), even superior if compared to end-to-end sutures (Pereira et al., 1991). In spite of the good experimental and clinical results, the main disadvantage of the use of skeletal muscle, is the risk that nerve fibres grow outside of the graft and create a neuroma. The axon regrowth in an inappropriate place impairs regeneration leading to poor clinical outcomes (Meek et al., 2004, Siemionow and Brzezicki, 2009, Deumens et al., 2010, Konofaos and Ver Halen, 2013).

Epineurial tube: it takes a potential advantage over other biological materials, due to its neural origin. Empty endoneurial tubes are obtained by pulling out the nerve fascicles from the harvested nerve fragment. Afterwards, the epineurial scaffold could be filled with supportive materials (cells, muscle, etc.) to avoid structure collapse (Siemionow and Brzezicki, 2009). Experimental studies suggest epineurial tubes as suitable conduits, for the repair of nerve defects, also with results similar to those obtained with the autologous grafts (Ayhan et al., 2000, Chatdokmaiprai et al., 2006, Lukaszuk et al., 2013). The low antigenicity of epineurial tissue could allow transplantation of allogenic epineurial conduits, avoiding immunosuppressive

treatments and the sacrifice of an healthy donor nerve (Siemionow and Brzezicki, 2009).

Tendon: Nerve grafts composed by tendon were proposed for the structural features of the tissue. The extracellular matrix components and the longitudinal arrangement of the collagen fibres are features favourable for axonal regrowth (Brandt et al., 1999). As for the muscular tissue, the main advantage of this technique is the high amount of available material, with limited associated morbidity (Nishiura et al., 2004). Moreover, experimental results show that the application of tendons, to treat peripheral nerve injuries, supports fibre regeneration across extended defects (Brandt et al., 1999, Brandt et al., 2002). Tendons can be used as conduits for gaps of 1 cm maximum (Belanger et al., 2016).

NON-AUTOGENOUS BIOLOGICAL MATERIALS

Considering the potential utility, a number of non-autogenous biological materials have been tested to bridge peripheral nerve gaps. Both allogenic (taken from the same species) and xenogenic (taken from different species) tissues were considered as an alternative to autogenous tissues.

Nerve allografts can be obtained from healthy donors or from cadavers. The allograft from living donor is limited in length, but allograft from cadaver is an alternative in complete or segmental nerve defects (Battiston et al., 2009, Ray and Mackinnon, 2010). Allograft nerves provide a functional extracellular matrix and sometimes viable SCs that are important for peripheral nerve regeneration (Carriel et al., 2014). However, the use of non-autogenous tissues presents some risk factors, such as the immunogenic mismatching. Indeed, long-term immune suppression is required to prevent graft rejection. To overcome the patient immune suppression, researchers developed strategies of cryopreservation of allografts before the implantation. This strategy reduces the immunogenicity of the graft and its effectiveness is confirmed in experimental and clinical studies (Fox et al., 2005, Ray et al., 2011, Squintani et al., 2013).

In recent years, decellularization has been described as an efficient method for the generation of non-autogenous scaffolds from native tissues and to avoid donor site

morbidity associated to nerve autograft (Badylak et al., 2011, Crapo et al., 2011, Oliveira et al., 2013). This technique removes cellular components from the tissue, but maintains intact the endoneurial collagen fibrils and the 3D architecture of the extracellular matrix (Deumens et al., 2010). Different protocols of decellularization were designed. Decellularization can be performed by chemical, physical and/or combined methods, and its effectiveness will depend of the organ size, cellular density and even more important of extracellular matrix composition and organization (Gilbert et al., 2006). Indeed, different nerve allografts derived from decellularization protocols show promising experimental results (Whitlock et al., 2009), and some of them were approved for clinical use in humans by the FDA (Kehoe et al., 2012). Decellularized non-autogenous materials as the human amniotic membrane and the rat small intestinal submucosa were used. Tubular rolls of both cited tissues can promote and support peripheral axon regeneration, leading to a functional recovery similar to groups repaired with the autograft technique (Mohammad et al., 2000, Hadlock et al., 2001, Szykaruk et al., 2012).

Further developments resulted in the study of decellularized tissue-based implants, in which the purified individual components of the extracellular matrix have been incorporated into nerve scaffold structure, because growing neuritis adhesion is mediated by specific receptor-ligand interaction, with the extracellular matrix proteins (collagen, fibronectin, laminin) and their appropriate Integrin receptors. In comparison to many synthetic polymers, scaffolds of natural origins take several advantages such as biocompatibility, biodegradability, non toxic degradation products and the minimal foreign body response induction (Carriel et al., 2014).

Collagen and others extracellular matrix components: Collagen is the major component of the extracellular matrix and it can be isolated from many biological tissues (e.g., tendon, skin, bone). Collagen is widely used as biomaterial because it provides good cell attachment sites, and is generally non-immunogenic (is quite highly conserved among mammalian species) (Khaing and Schmidt, 2012). Moreover, collagen scaffolds are stable and flexible (Kehoe et al., 2012). These characteristics render collagen one of the most used bioengineered materials in hard- and soft-tissue reconstruction (Rocha et al., 2002). Collagen type I is a very common protein in peripheral nerves. Mostly localized in the *epineurium* and basal

membranes, its role in promoting SC migration and proliferation during nerve repair is widely demonstrated (Lundborg, 2004, Chernousov et al., 2008, Battiston et al., 2009, Geuna et al., 2009, Carriel et al., 2011, Allodi et al., 2012, Gonzalez-Perez et al., 2013). Effectiveness of collagen nerve conduits to repair short nerve gap (5 mm) have been demonstrated since 1991 (Archibald et al., 1991). Studies demonstrated the biocompatibility of porcine collagen type I/III conduits, in which revascularization occurs and co-transplanted SCs survive inside the conduit (Keilhoff et al., 2003). Clinically, collagen-based nerve conduits were applied in the repair of upper limb nerve lesions including median, ulnar, radial and digital nerves, showing optimal outcomes. Instead, in large nerve gaps up to 5 cm, suboptimal results were observed (Bushnell et al., 2008, Lohmeyer et al., 2009, Taras et al., 2011). Other successful application of collagen-based nerve conduits, have been documented in repairing lingual and inferior alveolar nerve defects, with good sensory recovery (Farole and Jamal, 2008).

Despite these encouraging results, unsuccessful implantations has been reported. The conduit fast reabsorption rate seems to be the main failure reason. Indeed, these conduits were completely reabsorbed between 6 and 17 months, not allowing mechanical support to the regenerating axons over long time (Liodaki et al., 2013). Taking account of both positive and negative results, type I collagen-based nerve conduits are actually the most biocompatible nerve guides used in clinic, with outcomes comparable with the autograft technique in nerve repair up to 2 cm (Carriel et al., 2014).

In addition to the collagen, also other extracellular matrix (ECM) molecule-derived materials are used for the development of nerve guides. Fibronectin and laminin belong to this category. Fibronectin forms a fibrillar matrix similar to collagen; its interactions with collagen IV and laminin make fibronectin a suitable candidate for scaffold fabrication (Brown and Phillips, 2007, Gonzalez-Perez et al., 2013). The ability of fibronectin-made scaffolds to improve peripheral nerve regeneration have been demonstrated (Bailey et al., 1993, Whitworth et al., 1995). Particularly, the major effect of fibronectin is to promote SC survival and proliferation (Deumens et al., 2010). Thus, the maintenance of a large number of viable SCs is the best beneficial effect of fibronectin-made conduits (Mosahebi et al., 2003). Also laminin-made nerve

guides were used to bridge 10-mm long sciatic rat nerve gap, showing desired outcomes (Kauppila et al., 1993). Laminin is the first ECM protein expressed during the embryogenesis; it participates in cell differentiation, migration and adhesion and during the nerve regeneration process it can promote neuritis outgrowth both *in vitro* and *in vivo* (Deumens et al., 2010, Gonzalez-Perez et al., 2013).

Gelatin: gelatin is a natural biodegradable polymer obtained by the thermal denaturation of collagen. Animal derived gelatin has been widely investigated for medical application, for its peculiarity as biocompatible materials, its plasticity and adhesiveness. Gelatin is less expensive than collagen and, as for collagen, gelatin-based conduits have been also tested for nerve repair. Its solubility in water and the easy collapse of gelatin conduits make necessary some modification of its structure, using cross-linking agents (as glycidoxypolytrimethoxysilane (GPTMS) and genipin), resulting in the alteration of its physical and mechanical properties with a controlled degradation rate (Tonda-Turo et al., 2011). A biodegradable genipin-cross-linked gelatin-based conduit was used in sciatic nerve defect (10-mm gap) in rat with poor success, since after 8 weeks most of regenerated axons were not myelinated (Chen et al., 2005b). The same authors showed that an increase of conduit porosity can implement its performance, with good regeneration in the rat model, giving real prospective for future studies in longer defects (Chang et al., 2009). Moreover, gelatin-based neural scaffolds can be easily integrated with additional bioactive molecules, which can be gradually released during the scaffold degradation (Chen et al., 2005a). Various scaffolds have been prepared using gelatin combined with other materials (Binan et al., 2014, Koudehi et al., 2014, Vatankhah et al., 2014).

Chitosan and other natural polysaccharides: chitosan is a polymer of D-glucosamine and N-acetyl-D-glucosamine, obtained from full or partial N-deacetylation of chitin. This material is highly biocompatible, biodegradable and shows low toxicity. Moreover, chitosan is an attractive material to produce nerve guides because it can be easily blended with other materials or modified on its surface to better support axonal regrowth (Haastert-Talini et al., 2013, Wrobel et al., 2014). The biocompatibility of chitosan-based biomaterials with CNS and PNS cells, has been widely investigated with success (Yuan et al., 2004, Freier et al., 2005, He et al.,

2009, Simoes et al., 2011). Indeed, the study of chitosan-made tubes alone or in combination with other biomaterials in recent years, demonstrated the efficiency of such nervous guides for bridging peripheral nerve defects (Kim et al., 2008, Ao et al., 2011, Gnavi et al., 2013, Haastert-Talini et al., 2013, Biazar and Heidari Keshel, 2014, Nie et al., 2014, Gonzalez-Perez et al., 2015, Shapira et al., 2016). Recently, it has been also demonstrated that degradation products of chitosan, chito-oligosaccharides, can promote nerve regeneration (Zhao et al., 2017).

Both hyaluronic acid and alginate are polysaccharides used as biomaterials in peripheral nerve regeneration. Hyaluronic acid is non-immunogenic, biodegradable and its characteristics (e.g. hardness and degradation rate) can be easily modified (Khaing and Schmidt, 2012). It has an important role in tissue repair and could enhance peripheral nerve regeneration and prevent scar tissue formation (Özgenel, 2003). Alginate is commonly extracted from brown seaweed, and the potential of alginate-based nerve guide to promote nerve regeneration is demonstrated (Hashimoto et al., 2002). In particular, alginate is used as a filler for growth factor delivery to support nerve regeneration (Ohta et al., 2004, Pfister et al., 2007, Pfister et al., 2008).

Silk fibroin and natural proteins: Derived from natural silk, the application of silk fibroin in biomedical fields is increased in recent years, because of the unique material properties of high tensile strength, elasticity, low immunogenicity, biodegradability in a reasonable time and an easy functionalization (Gu et al., 2011, Kasoju and Bora, 2012, Belanger et al., 2016, Jao et al., 2016). In the field of peripheral nerve regeneration, silk fibroin has been used as biomaterial for nerve guides to clarify the biocompatibility with neural tissues *in vitro*, and for bridging nerve defects *in vivo* (Yang et al., 2007, Madduri et al., 2010, Sivak et al., 2014, Radtke, 2016). Just like silk fibroin, other natural proteins like keratins have also been tested as natural biomaterial for nerve scaffold (Apel et al., 2008, Sierpinski et al., 2008) or as filler to support peripheral nerve regeneration (Pace et al., 2013).

7.2.2. SYNTHETIC MATERIALS

In addition to naturally derived biopolymers, a wide range of synthetic materials have been studied and tested for the fabrication of artificial nerve guidance channels (NGCs). Currently, there are several FDA/Conformit Europe-approved synthetic conduits for peripheral nerve repair, including polyglycolic acid (PGA) and poly-DL-lactide-caprolactone (PCL), that are both biodegradable synthetic aliphatic polyesters (Kehoe et al., 2012). Biomaterials of natural origin tend to have weak mechanical properties, while the synthetic polymers possess many attractive chemical and physical properties that can be modified during the fabrication processes. Indeed, the possibility to fabricate scaffolds with desired customized properties, such as the degradation rate, is the main advantage over natural polymer-based nerve guides. In spite of the interesting engineering properties, such synthetic materials are often devoid of the bioactive molecules necessary to promote cell-substrate interaction. For this reason, nerve conduits made of synthetic materials often need to be functionalized by the incorporation of signalling molecules or internal fillers (Deumens et al., 2010, Gu et al., 2011, Konofaos and Ver Halen, 2013). Although synthetic biomaterials are suitable, they add expense to the surgical practice and increase the risk of foreign body reaction (Sabongi et al., 2015). Synthetic polymers should be divided in two big categories: non-biodegradable and biodegradable synthetic materials.

Non-biodegradable synthetic materials: historically were studied previously than biodegradable ones. Non absorbable materials can provoke foreign body response which can induce chronic inflammation with its consequences (e.g.; scar formation and nerve compression); in this case, a further surgery is required to remove the conduit (Belanger et al., 2016).

Among the non-biodegradable synthetic materials, silicone is one of the most studied and used in clinical practice, including for the repair of nerve injuries (Gu et al., 2011, Carriel et al., 2014). For many years, silicone-based nerve conduits have been the standard experimental model for peripheral nerve repair, showing encouraging results (Lundborg et al., 1982a, Lundborg et al., 1982b). Implantation of silicone-

based conduit in the median nerve to repair a short nerve gap (up to 1 cm) provides mechanical support to nerve regeneration, while the results were not as encouraging for larger nerve lesions (Braga-Silva, 1999, Lundborg et al., 2004). Even if silicon is considered an inert material, silicon-made nerve conduits are not biodegradable, thus foreign body reaction can occur, leading to the removal of the nerve guide.

Other materials: nerve conduits have also been fabricated using other non-biodegradable synthetic materials such as polylactic acid (PLA), polyvinyl chloride (PVC), polyethylene glycol (PEG), acrylic polymers, elastomer, and so on (Chiono et al., 2009, Deumens et al., 2010, Gu et al., 2011, Konofaos and Ver Halen, 2013, Carriel et al., 2014, Belanger et al., 2016).

Biodegradable synthetic materials: in order to overcome non-absorbable material associated disadvantages, researchers focused their attention on biodegradable synthetic materials. Like the synthetic non-absorbable materials, the physicochemical and biological properties can be customized according to the necessities. Moreover, these scaffolds can degrade within a reasonable time-window, and the degradation products are totally absorbed with mild foreign body reaction.

Among the several absorbable materials, aliphatic polyesters and co-polyesters have already been used in clinical application: PLA, PCL, PLGA, poly-(L-lactic acid) (PLLA), and PHB (Chiono et al., 2009, Deumens et al., 2010, Daly et al., 2012, Konofaos and Ver Halen, 2013). Polyglycolic acid (PGA) is a biodegradable polymer, and PGA-based conduits are currently the most applied nerve guides in the clinical and experimental practice (Konofaos and Ver Halen, 2013). Indeed, good results of regeneration and functional recovery were obtained repairing peripheral nerve defects with a PGA-made scaffold (Weber et al., 2000, Toba et al., 2002, Donoghoe et al., 2007). Among PGA-based guides, NeurotubeTM was the first approved nerve conduit for medical use in the human body (Belanger et al., 2016). Good results of nerve regeneration *in vivo* were also obtained by using conduits made by an amphoteric agmatine-deriving polyamidoamines hydrogel. Polyamidoamines are synthetic and biodegradable materials, usually used as hydrogels (Magnaghi et al., 2011).

A further option to obtain nerve guides with structural tailored characteristics, are synthetic copolymers (Siemionow et al., 2010). Several studies on PCL have been performed to test cytocompatibility, and the possibility to shape this copolymer in suitable tubular scaffolds, with encouraging results (Waddell et al., 2003, Bender et al., 2004, Ciardelli and Chiono, 2006). At the same time, poly(D,L-lactide)- ϵ -caprolactone (DLLA-E-CL) copolymer nerve guides have been widely studied with interesting results (Den Dunnen et al., 1996, Den Dunnen et al., 1998, Meek et al., 2001, Abbruzzese et al., 2004, Johnson and Soucacos, 2008), showing its suitability for the repair of short nerve gaps (Johnson and Soucacos, 2008).

8. TISSUE ENGINEERING FOR PERIPHERAL NERVE REGENERATION

As already discussed, artificial nerve guides may be used, instead of autografts, to enhance regeneration avoiding donor site morbidity and immune-rejection problems. Unfortunately, in spite of the spontaneous regeneration potential of peripheral nerve fibres, and of the scientific advancements in the management of small nerve defects, the clinical treatments of long nerve gaps are still unsatisfactory (Bushnell et al., 2008, Taras et al., 2011, Kehoe et al., 2012). Hollow tubes are deficient of the necessary supportive structures to provide an adequate microenvironment for regeneration through long distances, failing in clinical applications (Pabari et al., 2011). Especially, the production of extracellular matrix components during the early regeneration stages is inadequate, limiting SC migration into the lesion site, and consequently reducing the production of the trophic and topographical guidance structure (bands of Büngner) for regenerating axons. Due to the clinical limitation of nerve guides, and on the growing knowledge of the molecular and cellular mechanisms to improve peripheral nerve regeneration, several tissue engineering approaches have been developed (Battiston et al., 2009, Konofaos and Ver Halen, 2013, Carriel et al., 2014). The aim of these strategies is to support and improve the peripheral nerve regeneration process, especially in long nerve defects. In the last years, different strategies of nerve guide manipulation have been developed and

tested (Figure 14). Hollow nerve guides could be engineered and functionalized with structural components such as collagen and laminin-containing gels, internal frameworks, supportive cells, growth factors and conductive polymers (Battiston et al., 2009, de Ruiter et al., 2009a, Deumens et al., 2010, Pabari et al., 2011).

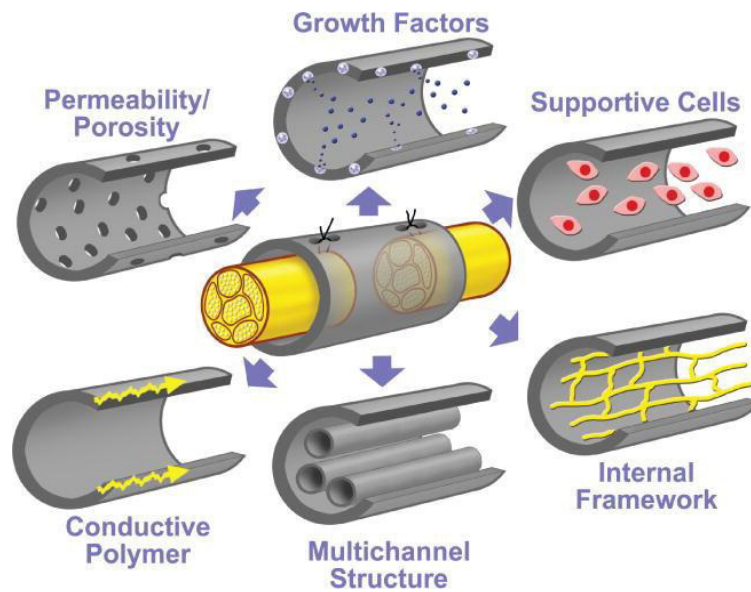


Fig. 14: Modifications of hollow tube for peripheral nerve injury repair (de Ruiter et al., 2009a).

Structural components: the extracellular matrix components are fundamental for SC migration and axon elongation and are able to modify cell behaviour. Therefore, researchers investigated several surface modification techniques, to work up biomimetic materials which mimic the properties of native extracellular matrix (Rahmany and Van Dyke, 2013). Presently, three different strategies are used to obtain biomimetic materials: (i) inclusion of bioactive molecules, later released; (ii) modification of material surface with ECM macromolecules or specific binding motifs; and (iii) nanoscale structural motif of the materials. Laminin, collagen and fibrinogen were used as biomaterial coating to facilitate cell attachment and migration (Lewandowska et al., 1992, Toba et al., 2002, Nakayama et al., 2007). Moreover, from extracellular matrix various binding motifs were isolated and immobilized on biomaterials in a sequence of short linear peptide to promote integrin-mediated cell adhesion (Figure 15). It is now clear that also the pattern of molecule disposition on

biomaterial influences the cell response and the outcome of nerve regeneration (Rahmany and Van Dyke, 2013).

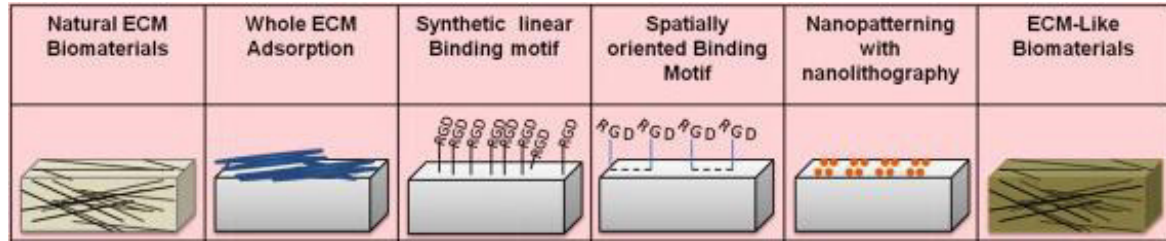


Fig. 15: Modulation of receptor-ligand interactions with both natural and functionalized biomaterials (Rahmany and Van Dyke, 2013)

Another structural improvement for synthetic conduits is enhancing the porosity of the material, with the presence of holes on nerve conduit wall (Rodríguez et al., 1999, Meek and Den Dunnen, 2009, Zhang et al., 2014). The size and the morphology of the holes need to be controlled, since macroporous materials (> 80% porosity) facilitate macrophage infiltration and let to increased fibrotic tissue in comparison to to intermediated porosity (70% porosity) (Chang et al., 2007). Moreover, large pores accelerate the hydrolysis of the absorbable conduits with easy obstruction of conduit lumen. Thanks to the improvements in nanofabrication field, we are now able to control, at microscale and nanoscale level, the size of the pores in nerve conduits. These lead to the production of asymmetric wall conduits with micropores inside and macropores in the outer layer, which allow to differently control the inflow and the outflow of the tube, promoting SC proliferation inside and blocking fibroblast proliferation outside (Chang and Hsu, 2006, Chang et al., 2007).

Internal frameworks: the lack of an internal structures in hollow conduit might be one of the causes of poor success in long gap repair. The introduction of appropriate internal filler or framework inside the conduit is an efficient strategy to improve the outcome of nerve regeneration (Deumens et al., 2010, Konofaos and Ver Halen, 2013, Carriel et al., 2014). The internal filler morphology must provide a suitable environment and mechanical support for migrating cell and regrowing axons. The internal structure may affect the physical properties of the nerve conduit, such as

permeability and flexibility, and reduce the total cross-sectional area available for nerve regeneration (de Ruiter et al., 2009a). Among surface functionalizations there are modifications such as the presence of regular or curvature features, surface hydrophobicity, surface roughness, surface charges, presence of grooves and ridges or presence of pits and pillars, cues that may affect cell behaviour (Harvey et al., 2013, Rahmany and Van Dyke, 2013).

A simple way to potentiate synthetic conduits is to fill the tube with fibrin, laminin, collagen and other extracellular matrix components provided as hydrogel. These hydrogels favourite cell invasion and axon elongation inside the conduit, and allow neurotrophic factor diffusion. Collagen-based hydrogel and other hydrogels have been used *in vivo* for nerve repair (Verdú et al., 2002, Katayama et al., 2006, Apel et al., 2008, Kalbermatten et al., 2008, de Ruiter et al., 2009b). Since the filler can slow down nerve regeneration physically obstructing the growth, it is necessary a fine control of distribution and density of the hydrogel itself. Currently, researchers are focused on mechanical qualities of the hydrogel, like material stiffness which seems to be able to modulate the tension exerted by neurites and growth cones influencing axon elongation and altering gene expression (Man et al., 2011, Koch et al., 2012). Multiple types of structures, as films, sponges, filaments, nano and microfibres, microchannels, have also been tested to control cell attachment, migration and proliferation inside nerve conduit (Figure 16).

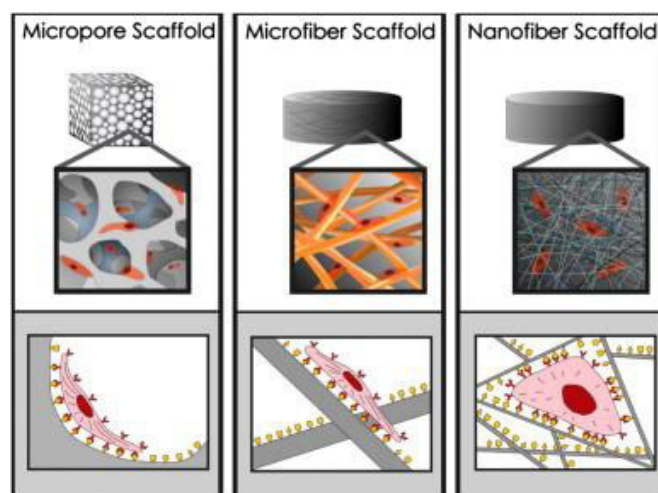


Fig. 16: Scaffold architecture affects cell adhesion.

A really promising strategy is the introduction of fibres, which act in the tubular lumen as physical support and guidance for SC migration. Collagen, gelatin, chitosan, PCL and other materials have been used to prepare fibres (Agarwal et al., 2008, Tonda-Turo et al., 2013, Tian et al., 2015, Zhang et al., 2016), preferentially through the electrospinning technique. Fibre size, topography and orientation affect cells behaviour (Tian et al., 2015). Moreover, hollow tubes can be enriched with internal fillers obtained from biological tissues such as denaturated (Alluin et al., 2009, Dornseifer et al., 2011) or fresh (Varejao et al., 2003a, Varejao et al., 2003b) skeletal muscle fibres.

Supportive cells: after nerve injury, different cells drive the complex process of peripheral nerve regeneration. As described above, cellular components (SCs, fibroblasts and macrophages) are essential to provide the physical and trophic support necessary for regeneration success. Taking account of their pivotal role, cell incorporation into nerve scaffolds is widely recognized as an attractive possibility to nerve conduit functionalization. Many studies demonstrated that autologous SCs seeded in a conduit can improve nerve regeneration (Rodríguez et al., 2000, Strauch et al., 2001, Sinis et al., 2005, Dahlin et al., 2007). Unfortunately, autologous or allogenic SCs are only viable after a long and difficult expansion time *in vitro*, making their clinical application arduous (Walsh and Midha, 2009, Daly et al., 2012). An interesting alternative to SCs are mesenchymal stem cells (MSCs) derived from bone marrow (Mantovani et al., 2010, Ladak et al., 2011, Sensharma et al., 2017). These stem cells can differentiate to Schwann-like Cells (SLCs) and could improve the nerve regeneration significantly (Fan et al., 2014). SLCs can be also obtained from adipose-derived stem cells (di Summa et al., 2010, Erba et al., 2010, Mantovani et al., 2010), making possible to obtain autologous SCs with less arduous procedures and from the patient itself avoiding immune-response problems. Finally, other stem cells such as embryonic (Cui et al., 2008), hair follicle (Fairbairn et al., 2015) and dental pulp-derived stem cells (Sasaki et al., 2011), have even been tested to improve peripheral nerve regeneration with interesting results.

Growth factors: several growth factors are released at the injury site after peripheral nerve injury, and trigger the complex process of nerve regeneration. The addition of

growth factors to the conduits can be useful for axonal regeneration, especially in case of large nerve defects. Indeed, nerve regeneration failure over long distance is principally due by the decay of growth factors released by SC and target organs (Höke et al., 2002). Since growth factors enhance nerve regeneration through several mechanisms, their controlled and prolonged release inside artificial nerve conduit is actually a goal in peripheral nerve tissue engineering (Bell and Haycock, 2011, Daly et al., 2012). Growth factors could be incorporated directly into the nerve guide (Wang et al., 2012, Tang et al., 2013), or by a delivery systems. A delivery system, such as hydrogels, sponges, fibres, microspheres and immobilization on nanoparticles is usually preferred to ensure a prolonged and controlled factor release (Wood et al., 2010, Zeng et al., 2014, Carballo-Molina and Velasco, 2015, Zhao et al., 2015, Johnson et al., 2016, Santos et al., 2016a, Yasui et al., 2016, Giannaccini et al., 2017). Several studies demonstrated that enrichment of nerve guides with neurotrophic factors such as NGF, GDNF, BDNF and NT-3, stimulates migration of cells and re-growing axons (Lee et al., 2003, Midha et al., 2003, Wood et al., 2009). Moreover, a combined functionalization of nerve guides with physical fillers and growth factors or with a combination of growth factors, provides a synergic effect strongly promoting nerve regeneration (Aszmann et al., 2002, Fine et al., 2002).

Conductive polymers: it has been demonstrated that electric stimulation promotes neurite outgrowth and glial cell proliferation *in vitro* (Kimura et al., 1998, Huang et al., 2010) and *in vivo* (Udina et al., 2008, Gordon et al., 2009). The incorporation of electrical conducting material in nerve conduit results difficult, but, recently, some advancements have been done using organic conducting polymers (OCPs). One of the first conductive polymers proposed for tissue engineering applications is polypyrrole, which is biocompatible and induces axon elongation *in vitro* and *in vivo* (Wang et al., 2004, Thompson et al., 2010, Xu et al., 2014). Besides, as other OCPs, polypyrrole is not biodegradable, so various studies explore its incorporation in biodegradable polymers. Recently a polypyrrole/chitosan-based nerve conduit has been used to repair a 15-mm gap in rats in combination with electrical stimulation, and great results were achieved (Huang et al., 2012). Since the electro-conductivity of conductive polymers declines after long time, researches are testing more stable electro-conductive materials.

8.1. FABRICATION TECHNIQUES

Different fabrication techniques allow the production of scaffolds for peripheral nerve repair with a specific three-dimensional structure and mechanical properties. Computational fluid dynamic design has been used to generate three-dimensional scaffolds, using a micro-fabrication process, suitable for microvasculature and a number of organ-specific constructs (Borenstein et al., 2007, Bhardwaj and Kundu, 2010, von der Mark et al., 2010). Biomaterial scaffolds can be prepared using both synthetic and natural materials with several techniques including: photolithography, molding, freeze-thawing and electrospinning.

Photolithography Recently, the development of new nanotechnologies such as photo- or electron-beam nanolithography, polymer demixing, nano-imprinting and compression molding, allow the production of biomimetic surfaces with a specific nano-pattern, eliciting tissue-specific cellular responses. The construction of a nano-scale geometrically defined microenvironment may influence the nano-topography-dependent mechanisms of integrin-mediated cell adhesion process (Borenstein et al., 2007).

Molding technique allows to obtain grafts at melt temperatures lower than those required by other fabrication methods. The scaffold is obtained using a special apparatus that produces matrix layers of the desired density and thickness. The limitations of this procedure reside in the low thoroughness to build the three-dimensional structure of the matrix and in the high costs (von der Mark et al., 2010).

Freeze-thawing is a procedure based on polymer solution freezing and lyophilization to obtain porous scaffolds without the aid of chemical molecules. This procedure limits the incorporation of growth factors or cells inside the scaffold. The freezing step leads to the formation of ice crystals. The following sublimation step contributes to porous structure formation. The size of the pores can be controlled by adjusting the freezing rate and the solution pH (Ciardelli and Chiono, 2006, Chiono et al., 2009, Tonda-Turo et al., 2011).

Electrospinning is a versatile technique employed to produce polymer fibres with diameters ranging from 2 nanometres to several millimetres starting from polymer solution of both natural and synthetic origins for several biomedical applications (Agarwal et al., 2008). This technique results in the production of fibres with a controllable diameter, pore structure and extremely high surface-to-volume area (Bhardwaj and Kundu, 2010, Chiono and Tonda-Turo, 2015). This technique exploits the electric field to produce thin fibres from polymer solutions or melts. Most of the polymers are dissolved in solvents before electrospinning. The polymer fluid is then introduced into the capillary tube. High voltage source is utilized to inject charges at a certain polarity into a polymer solution that is then accelerated towards a collector of opposite polarity (Liang et al., 2007, Sill and von Recum, 2008). When electric field is applied, Taylor cone formation occurs (see Figure 17) and the electrical forces overcome the surface tension forces resulting in solvent evaporation and fibre formation (Bhardwaj and Kundu, 2010). Electrospun fibres are organized into a three-dimensional network with morphological characteristics similar to those of the extracellular matrix, and this may result in the promotion of cell adhesion (Sill and von Recum, 2008, Rahmany and Van Dyke, 2013). Depending on the type of collector, fixed or rotating, random or aligned fibres, respectively, are produced (Figure 18) (Baji et al., 2010).

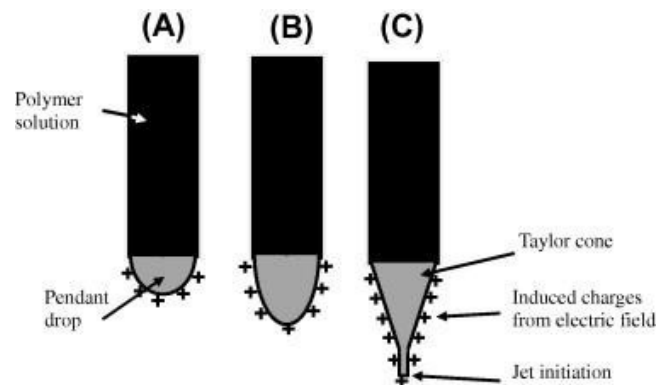


Fig. 17: Taylor cone formation: The electric field induces surface charges in the polymer solution (A). Elongation of the drop (B). The charge-charge repulsion lead to the Taylor cone formation (C) (Baji et al., 2010).

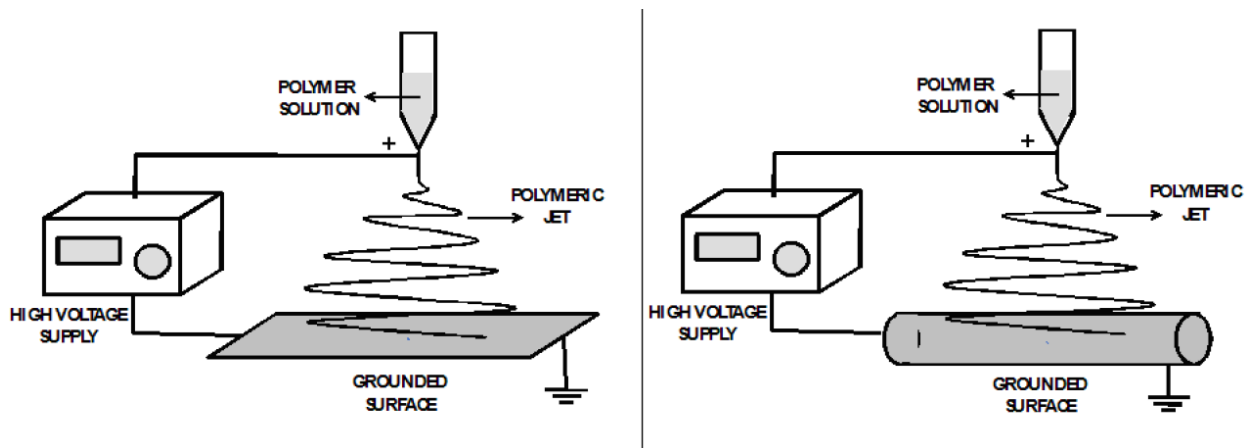


Fig.18: Random or aligned fibres can be obtained by changing the type of collector, random fibres were collected by fixed collector (on the left), while aligned fibres were produced in presence of rotating one (on the right) (Bizarria et al., 2014).

9. NEUREGULIN1/ERBB IN NEURONAL MIGRATION

NRG1 belongs to the family of Neuregulins, growth factors with a wide range of functions in the nervous system. NRG1 structure and function in the PNS was already discussed in section 5.2.

NRG1/ErbB signalling is important also in the CNS development and postnatal brain function. It is involved in many biological processes:

- NRG1 signalling is critical for the normal development of the CNS, it promotes the proliferation, migration and survival of neural precursors (Rio et al., 1997, Lai and Feng, 2004).
- NRG1/ErbB4 signalling is essential for excitatory synapse maturation and plasticity (Li et al., 2007).
- Release of NRG1 from neurons promotes the formation and maintenance of radial glial cells, which are necessary for the radial migration of neurons from the ventricular zones to the pial surface (Mei and Xiong, 2008).
- NRG1 is required for early fate determination, proliferation of oligodendrocytes precursor cells (OPCs) and the maturation of oligodendrocytes (Vartanian et al., 1999, Fernandez et al., 2000, Park et al., 2001).

- NRG1 action includes the ability to protect endothelial barrier functionality and to promote the survival of endothelial cells *in vitro* (Lok et al., 2009, Lok et al., 2012).

Interestingly, NRG1 is strongly involved in the migration of glial and neuronal cells in the CNS. Indeed, it controls the migration of the first wave of OPCs, acting as a chemoattractant for the migrating cells expressing ErbB4 (Ortega et al., 2012).

For what concern the neuronal migration, NRG1/ErbB4 signalling controls migration in different districts and in a different time-window. The balance between inhibitory and excitatory neurons in the cortex is required for the correct development of the CNS. During development, the excitatory neurons are generated in the *pallium* and migrate radially, while the inhibitory neurons are generated from the medial ganglionic eminences (MGE) of the *subpallium* and migrate tangentially to reach the cortex (Figure 19). During migration, inhibitory neurons express ErbB4 receptors and are driven in their migration by NRG1 that is expressed in the *pallium* and acts as a chemoattractive factor (Flames et al., 2004, Marín, 2013, Villar-Cerviño et al., 2015). Even if, also an alternative role of NRG1 as repellent was proposed (Li et al., 2012).

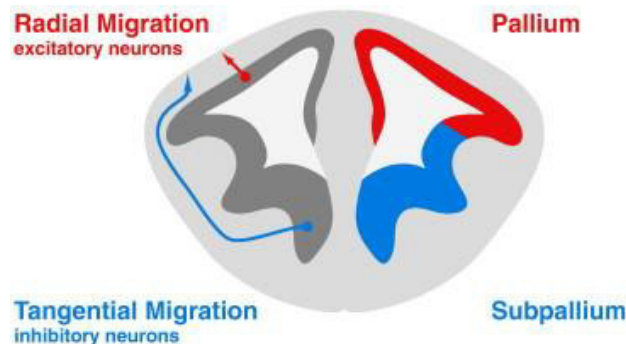


Fig. 19: The neuronal migration during cortical development (Birchmeier, 2009).

The expression of NRG1 in the cortex not only controls migration of GABAergic (inhibitory) neurons, but also the formation of the projections from the thalamus to the cortex. Indeed, in absence of NRG1, thalamocortical projections cannot be formed (López-Bendito et al., 2006).

NRG1 has also a role in migration during development and, more interestingly, during adult neurogenesis, that occurs mainly in the olfactory bulb (OB) and in the hippocampus. In the hippocampus NRG1 action is mediated by ErbB3. NRG1 administration in adult mice induces the proliferation of granule cell neurons in the ventral dentate gyrus (Mahar et al., 2011). Neurogenesis in the hippocampus is involved in stress response, antidepressant response and emotional behaviour (Mahar et al., 2014).

Otherwise, NRG1/ErbB4 signalling controls the migration of neuroblasts from the subventricular zone (SVZ) to the OB through the rostral migratory stream (RMS), a process that starts during development and continues throughout the whole life of the animal. Indeed, ErbB4 is expressed by the migrating neuroblasts subpopulation and some precursor cells in the SVZ and it was demonstrated that mice deficient for ErbB4 display an altered organization and orientation of the neuroblast chains and migration, as well as deficits in the differentiation and placement of the interneurons in the OB. (Anton et al., 2004, Ghashghaei et al., 2007, Birchmeier, 2009).

Among the ErbB receptors, ErbB4 is the predominant and widespread one in CNS. As described in section 5.2, ErbB4 is a tyrosine kinase receptor that can form both homodimers and heterodimers with other ErbB family members. Alternative splicing of ErbB4 gene determines the expression of four different ErbB4 isoforms: JMa-cyt1, JMa-cyt2, JMb-cyt1 and JMb-cyt2 (Mei and Xiong, 2008) (Figure 20) and *in silico* data demonstrated that there is a tissue-specific ErbB4 exon expression (Veikkolainen et al., 2011).

The alternative exclusive expression of the exon 15 or 16, determines the expression of juxtamembrane b (JMb) and juxtamembrane a (JMa) isoforms, respectively. JMa isoforms are sensitive to proteolytic cleavage elicited by the metalloprotease ADAM17/TACE with the consequent release of the extracellular domain (Ecto-ErbB4), followed by a second cleavage performed by a presenilin-dependent γ -secretase enzyme (Lee et al., 2002), which releases an ICD that can be translocated into the nucleus where it can affect gene transcription (Sardi et al., 2006). Isoforms containing the exon JMb are uncleavable. Moreover, in some embryonic and

malignant tissues was found the expression of the isoform JMc and JMd, that totally excluded or included both exon 15 and 16, respectively (Veikkolainen et al., 2011). The inclusion or exclusion of the exon 26, gives rise to cyt-1 or cyt-2 isoforms. Cyt-2 isoforms lack the exon 26, while in cyt-1 isoforms its expression confers to the receptor the ability to bind phosphatidylinositol-3-kinase (PI3K) and to activate the downstream pathway (Junttila et al., 2000); moreover, isoforms containing this exon are also bounded by the E3 ubiquitin ligase and are degraded faster than ErbB4 cyt2 isoforms (Sundvall et al., 2008).

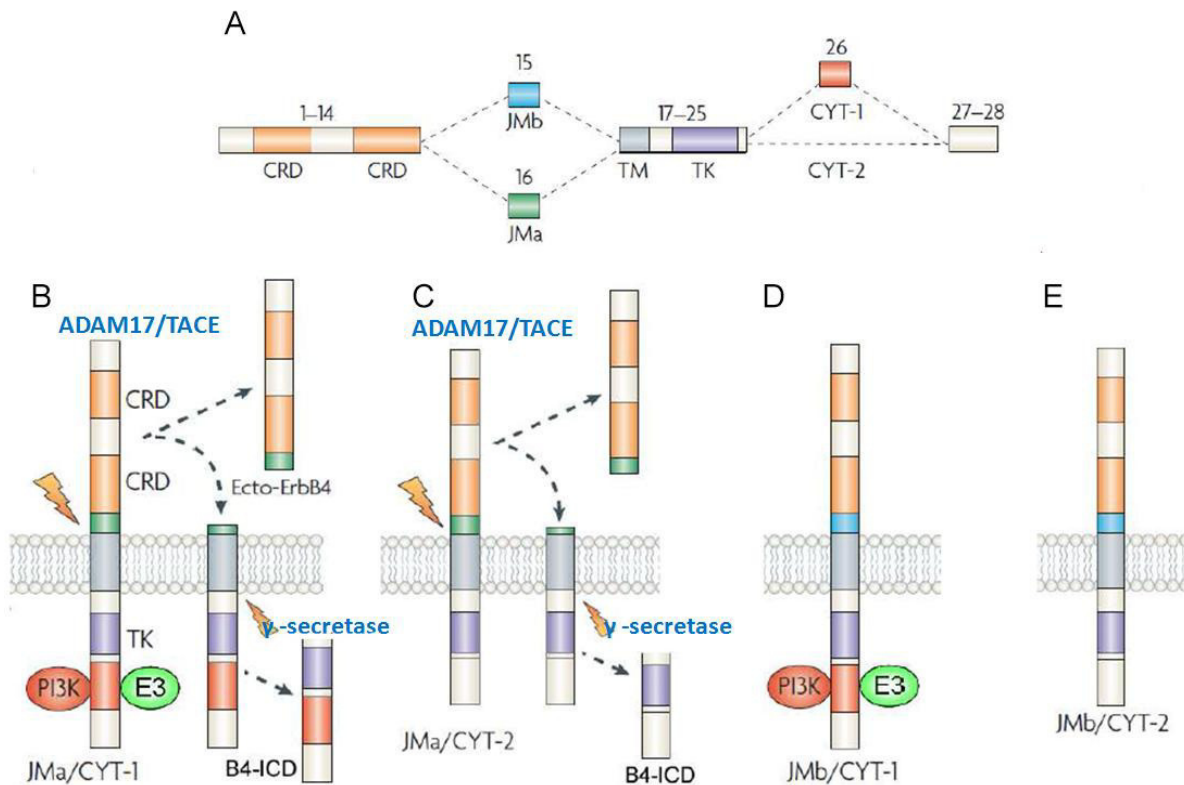


Fig. 20: ErbB4 gene (A), ErbB4 different isoforms: JMa-cyt1 (B), JMa-cyt2 (C) JMb-cyt1 (D) and JMb-cyt2 (E), modified from (Mei and Xiong, 2008).

From this information emerges that NRG1/ErbB4 signalling is important for brain development and its loss of function or perturbation in the system can cause deficits in the migration of interneurons, axon projection and synapse formation. Altered neurotransmission, migration and cortical function lead to psychotic symptoms and cognitive impairments associated to schizophrenia. Indeed, evidences from NRG1-hypomorphic and ErbB4-mutant mice support the idea that mutations in NRG1 and

ErbB4 have a role in the aetiology of schizophrenia and are considered genes conferring susceptibility to this pathology (Gerlai et al., 2000, Stefansson et al., 2002, Silberberg et al., 2006, O'Tuathaigh et al., 2007, Mei and Nave, 2014). Indeed, in schizophrenic patients reduced expression levels of NRG1 α were found, as well as elevated levels of NRG1 β (Bernstein et al., 2006, Bernstein and Bogerts, 2013) and of ErbB4 splice-variant isoforms containing the exons JMa and cyt-1 (Law et al., 2007). These data suggest that deeper studies about the role of NRG1 and ErbB4 isoforms may be necessary to better understand the mechanisms that underlie schizophrenia.

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CHAPTER 2

The Neuregulin1/ErbB system is selectively regulated during peripheral nerve degeneration and regeneration

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ABSTRACT

The peripheral nervous system has an intrinsic capability to regenerate, crucially related to the ability of Schwann cells (SC) to create a permissive environment, for example, through production of regeneration-promoting neurotrophic factors. Survival, proliferation, migration and differentiation of SC into a myelinating phenotype during development and after injury is regulated by different Neuregulin1 (NRG1) isoforms.

This study investigates the expression of different NRG1 isoforms and of their ErbB receptors in distal rat median nerve samples under regenerating conditions after a mild (crush) and more severe (end-to-end repair) injury and under degenerating condition. The expression of the NRG1/ErbB system was evaluated at mRNA and protein level, and demonstrated to be specific for distinct and consecutive phases following nerve injury and regeneration or the progress in degeneration. For the first time a detailed analysis of expression profiles not only of soluble and transmembrane NRG1 isoforms, but also of alpha and beta as well as type a, b, and c isoforms is presented. The results of mRNA and protein expression pattern analyses were related to nerve ultrastructure changes evaluated by electron microscopy.

In particular, transmembrane NRG1 isoforms are differentially regulated and proteolytically processed under regeneration and degeneration conditions. Soluble NRG1 isoforms alpha and beta, as well as type a and b, are strongly up-regulated during axonal regrowth, while type c NRG1 isoform is down-regulated. This is accompanied by an up-regulation of ErbB receptors. This accurate regulation suggests that each molecule plays a specific role that could be clinically exploited to improve nerve regeneration.

KEYWORDS

nerve injury, remyelination, NRG1/ErbB, biomolecular analysis, electron microscopy

INTRODUCTION

Following an injury to the peripheral nervous system (PNS), nerve fibres retain the capacity to regenerate. However, a complete repair is rarely achieved, leading to a partial or total loss of motor, sensory and autonomic functions.

Peripheral nerve degeneration and regeneration involve complex processes, such as morphological and physiological modifications, variation in cellular composition, and changes in gene expression and signal transduction. Investigating these mechanisms on a molecular level is therefore important to identify factors promoting successful nerve repair, especially axonal regrowth, remyelination, and target reinnervation.

It is well known that peripheral glia cells, Schwann cells (SC), are the crucial element to create an environment supportive for axonal regeneration. Thus, recognizing signals that control the SC response in nerve regeneration and degeneration is of great interest. One of the most important factors regulating SC action is Neuregulin1 (NRG1), which is not only involved in survival, proliferation, differentiation, and migration of SC during development, but is also required for axon guidance by SC and remyelination after nerve injury in adulthood (Taveggia et al., 2010, Fricker and Bennett, 2011, Pereira et al., 2012, Salzer, 2012, Gambarotta et al., 2013, Heermann and Schwab, 2013, Gambarotta et al., 2014b).

The gene coding for NRG1 is very large and, through the use of different promoters and alternative exon splicing, a vast variety of distinct transcripts and protein isoforms are generated (Adlkofer and Lai, 2000). According to their structure and signalling activity, NRG1 isoforms can be classified as soluble or transmembrane. Soluble isoforms (type I/II) are produced as pro-NRG1 anchored to the cell membrane. Following a proteolytic cleavage, a soluble fragment is released into the extracellular environment for paracrine or autocrine signalling. Transmembrane isoforms (type III) signal in a juxtacrine manner. The NRG1 isoforms can be further divided into alpha and beta isoforms, according to the presence of a small domain downstream the epidermal growth factor (EGF)-like domain, and into type a, b, and c isoforms, according to the exon present in the C-terminal tail (Falls, 2003).

NRG1 signalling is transduced in SC by ErbB2/ErbB3 heterodimers (Garratt et al., 2000, Yarden and Sliwkowski, 2001). NRG1 isoforms have different spatiotemporal patterns of expression providing different roles during myelination and nerve repair. When expressed by axons, transmembrane NRG1, determines their myelination status and the myelin sheath thickness, both during development and nerve regeneration (Michailov et al., 2004, Taveggia et al., 2005). In contrast, autocrine signalling via soluble NRG1 is not necessary for developmental myelination, but for SC survival, redifferentiation and remyelination after nerve injury (Stassart et al., 2013).

In this study, changes in mRNA and protein expression of NRG1, ErbB receptors, and myelin basic protein (MBP) during early peripheral nerve regeneration and degeneration were elucidated. Therefore, adult rat median nerves were crushed or end-to-end repaired (regenerating conditions) or left unrepaired after transection (degenerating condition), and distal nerve segments were subjected to expression analysis. Additionally, nerve morphology analysis revealed the morphological correlates.

MATERIAL AND METHODS

Ethical standards

All procedures were approved by the Bioethics Committee of the University of Torino, by the Institutional Animal Care and Use Committee of the University of Torino, and by the Italian Ministry of Health, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Surgery

In this study, a total of 64 adult female Wistar rats (Harlan Laboratories), weighing approximately 200 g, were used. Animals were housed in plastic cages with free access to food and water; their room was maintained at constant temperature and humidity under 12–12 h light/dark cycles.

For mRNA and protein analysis, a total of 52 rats were randomly divided into four experimental groups according to the nerve injury/repair: (i) in the crush injury group (axonotmesis), median nerves were bilaterally crushed at the mid humerus level with a non-serrated clamp, as previously described (Ronchi et al., 2009); (ii) for end-to-end repair (neurotmesis), median nerves were bilaterally transected at the same position as in (i) and the proximal and distal nerve ends were immediately reconnected with two epineurial sutures (9/0); (iii) in the degenerating nerve group, median nerves were bilaterally transected at the same location as in (i) and (ii) and left unrepaired (to prevent regeneration, the proximal stumps were sutured to the major pectoralis muscle); (iv) finally, in the control group (n=7), animals were left uninjured.

For electron microscopy analysis (nerve morphology), five additional rats were subjected to condition (i) and five rats to condition (iii). Finally, for *in vitro* nerve culture, two rats were used.

During surgery, rats were deeply anesthetized with Tiletamine and Zolazepam (Zoletil) i.m. (3 mg/kg). Adequate measures were taken to minimize pain and discomfort during the post-operative period.

Rats were sacrificed by lethal i.m. injection of Tiletamine and Zolazepam at different time-points after injury/repair/degeneration. For mRNA and protein analysis, n=3 animals per time-point were analysed. For morphological analysis, n=1 animal per time-point was sacrificed. Time points analysed were: 1, 3, 7, 14 and 28 days. Nerve segments (approximately 10mm) distally to the lesion site were collected. Control nerves were collected from healthy animals.

***In vitro* nerve culture**

For *in vitro* nerve degeneration, each median nerve was divided into two 5-mm segments and cultured for 1 day in high-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% heat-inactivated foetal bovine serum (FBS, Invitrogen). Nerves were grown at 37°C in a 5% CO₂ atmosphere saturated with H₂O.

RNA isolation, cDNA preparation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. 0.75 µg total RNA was subjected to a reverse transcriptase (RT) reaction in a 20 µl reaction volume containing: 1x RT-Buffer (Invitrogen), 0.1 µg/µl bovine serum albumin (BSA), 0.5 µM dNTPs; 7.5 µM random decamers (Invitrogen); 40 U RIBOlock (Fermentas) and 200 U SuperScript III Reverse Transcriptase (Invitrogen). The reaction was performed at 25°C 10 min, at 50°C 90 min, at 70°C 15 min. qRT-PCR was performed using an ABI Prism 7300 (Applied Biosystems, Life Technologies Europe BV, Monza, Italy) detection system. cDNA was diluted 12.5 fold in nuclease-free water and 5 µl (corresponding to 15ng starting RNA) were analysed in a 20 µl reaction volume, containing 1 x iTaq Universal SYBR Green Supermix (BioRad) and 300 nM forward and reverse primers. Dissociation curves were routinely performed to check for the presence of a single peak corresponding to the required amplicon. Analysis was performed in technical and biological triplicate. The data from the RT-PCR experiments were analysed using the $\Delta\Delta C_t$ method for the relative quantification. The threshold cycle number (C_t) values of both the calibrator and the samples of interest were normalized to the geometric average of two endogenous housekeeping genes: ANKRD27 (Ankyrin repeat domain 27) and RICTOR (RPTOR Independent Companion Of MTOR, Complex 2) (Gambarotta *et al.*, 2014a). As calibrator, the average of uninjured nerves was used. Primers were designed using Annhyb software (<http://www.bioinformatics.org/annhyb/>) and synthesized by Invitrogen (Life Technologies Europe BV, Monza, Italy). Primers sequences are reported in Table 1. All normalized relative quantitative data are depicted in the results section as $-\Delta\Delta C_T$ instead of $2^{-\Delta\Delta C_T}$, because in this way it is possible to appreciate both up-regulation (ranging between 0 and $+\infty$) and down-regulation (ranging between 0 and $-\infty$) while, when data are expressed as the more commonly used $2^{-\Delta\Delta C_t}$, the down-regulation is graphically compressed (ranging between 0 and +1).

CHAPTER 2

	Accession number	Primer name	Sequence 5'-3'	Amplicon size	Amplification efficiency
NRG1 type I/II	AF194993	rNRG1 I/II FW rNRG1 I/II REV	GGCGCAAACACTTCTTCATCCAC AAGTTTTCTCCTTCTCCGCGCAC	82	103%
NRG1 type III	AF194439	mrNRG1 III FW rNRG1 I/II/III REV	CCCTGAGGTGAGAACACCCAAGTC AAGTTTTCTCCTTCTCCGCGCAC	140	92%
NRG1 type alpha	AF194439	rNRG1-alfa/beta FW mrNRG1-alfa REV	TGCGGAGAAGGAGAAAACCTTC TTGCTCCAGTGAATCCAGGTTG	113	109%
NRG1 type beta	U02322 AY973245	rNRG1-alfa/beta FW mrNRG1-beta REV	TGCGGAGAAGGAGAAAACCTTC AACGATCACCAGTAAACTCATTGG	119	103%
NRG1 type a	U02322	mrNRG1 exon C3 FW rNRG1 exon a REV	CCCCTGACTCCTACAGAGACTCTCC TAGGGGAGCTTGCGTGTGG	107	100%
NRG1 type b	AY973245	mrNRG1 exon C3 FW rNRG1 exon b REV	CCCCTGACTCCTACAGAGACTCTCC AGGGTCTAAGATGAGTTGCTGACAGC	126	101%
NRG1 type c	U02324	mrNRG1 exon C3 FW rNRG1 exon c REV	CCCCTGACTCCTACAGAGACTCTCC CCGCAGGTGCTCATGGGATTC	108	104%
ErbB1	NM_031507	rErbB1 FW rErbB1 REV	CACCACGTACCAGATGGATG CGTAGTTTCTGGGGCATTC	83	103%
ErbB2	NM_017003	rErbB2 FW rErbB2 REV	TGACAAGCGCTGTCTGCCG CTTGAGTGGGCGCAGGCTG	106	100%
ErbB3	NM_017218	mrErbB3 FW mrErbB3 REV	CGAGATGGGCAACTCTCAGGC AGGTTACCCATGACCACCTCACAC	129	93%
MBP	NM_001025291	mrMBP FW rMBP REV	GGACCCAAGATGAAAACCCAGTAGTCC CCTTTCCTTGGGATGGAGGGGG	81	98%
ANKRD27	NM_001271264	rAnkrd27 FW rAnkrd27 REV	CCAGGATCCGAGAGGTGCTGTC CAGAGCCATATGGACTTCAGGGGG	95	99%
RICTOR	XM_001055633	rRICTOR FW rRICTOR REV	GAGGTGGAGAGGACACAAGCCC GGCCACAGAAGCTCGGAAACAAGG	81	106%

Table 1: Primers used for qRT-PCR analysis. The name of the amplified gene or isoform is indicated in the first column, followed by the accession number to reference sequences. The primer name indicates if the primers anneals only to Rattus norvegicus sequences (r) or also to Mus musculus (m). In the last column the primer amplification efficiency is indicated.

Total protein extraction and western blot analysis

Total proteins were extracted using the TRIzol Reagent (Invitrogen) after RNA extraction, according to the manufacturer's instructions. In the final passage, the protein pellet was re-suspended in boiling Laemli buffer [2.5% sodium dodecyl sulphate (SDS), 0.125M TrisHCl pH6.8]. Protein concentration was determined using the Bicinchoninic Acid assay kit (Sigma) on 1:4 diluted proteins to avoid detergent interference. Proteins (50 µg/sample) were routinely resolved by 8% SDS-polyacrylamide gel electrophoresis; for the analysis of NRG1 isoforms 4-15% precast gels were used (BioRad). Western blot analysis was carried out as previously described (Gambiarotta *et al.*, 2004). Primary antibodies used were: anti-ErbB1 (#sc-03), anti-ErbB2 (#sc-284), anti-ErbB3 (#sc-285), anti-NRG1 type "a" C-terminal (#sc-

348; 1:1000; all Santa Cruz, USA); anti-NRG1 type III N-terminal (#AB5551, 1:1000; Chemicon International, USA); anti-p-AKT (#4051), anti-AKT (#9272), anti-p-ERK (#9106), anti-ERK (#9102; 1:1000; all Cell Signaling Antibodies, MerckMillipore Europe); a pool of anti-MBP was used (#SMI 94 and SMI 99; 1:4000; Covance, Princeton, NJ, USA); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #4300; 1:20000; Ambion, ThermoFischer Scientific Inc, Europe); anti- β -actin (#A5316; 1:4000; Sigma, Germany); secondary antibodies used were horseradish peroxidase-linked anti-rabbit (#NA934) and anti-mouse (#NA931; both 1:40000; GE Healthcare Life Sciences, Europe).

Two housekeeping genes, actin and GAPDH, were shown in the Western blot panels because, as discussed in a previous paper focused on housekeeping gene stability at mRNA level (Gambarotta *et al.*, 2014a), it is difficult to find a gene whose expression is not influenced by the injury. Actin and GAPDH are not stably expressed and cannot be used for quantitative normalization. p-ERK and p-AKT bands were quantified using Quantity One Software (BioRad, California, USA), and their intensity was normalized to the total amount of ERK and AKT. Proteins previously extracted from primary cultured SC (Gnavi *et al.*, 2015) were used as a control for NRG1 expression.

Resin embedding and electron microscopy analysis

Nerve samples were fixed by immediate immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4-6 h at 4° C. Samples were then post-fixed in 2% osmium tetroxide for 2 h and dehydrated in passages in ethanol from 30% to 100%. After two passages of 7 min each in propylene oxide and O/N in a 1:1 mixture of propylene oxide and Glauerts' mixture of resins, specimens were embedded in Glauerts' mixture of resins (made of equal parts of Araldite M and the Araldite Harter, HY 964). In the resin mixture, 0.5% of the plasticizer dibutylphthalate was added. For the final step, 2% of accelerator 964 was added to the resin in order to promote the polymerization of the embedding mixture. Ultra-thin sections (70nm thick) were cut with an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) starting from the distal end and stained with saturated aqueous solution of uranyl acetate and lead citrate. Sections were analysed using a JEM-1010 transmission

electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany) for the computerized acquisition of the images.

Statistical methods

Gene expression analysis was carried out in technical triplicates of biological triplicates. Data statistical analysis (one-way ANOVA with Bonferroni's *post hoc*) was performed using SPSS software (New York, USA). Data discussed in the paper refer to the comparison between the gene expression at different time-points and the gene expression in the healthy nerve. Statistical analysis shown in the figures refers to the comparison among the three experimental models at the different analysed time-points, as indicated in the figure legends. For *in vitro* analysis, Student's t-test was used to assess statistical significance.

RESULTS

At the different time-points after injury (1, 3, 7, 14, and 28 days), mRNA and protein expression levels of ErbB receptors and different NRG1 isoforms were examined by qRT-PCR and Western blot analysis in median nerve segments obtained from healthy animals or distal nerve segments obtained from the three experimental groups: i) crush injury; ii) end-to-end repair; iii) degenerating nerve.

ErbB mRNAs are differentially regulated between regenerating and degenerating conditions

The expression of the NRG1 receptor ErbB3 and ErbB4, and their co-receptors ErbB1 and ErbB2, was assessed.

ErbB1 mRNA expression does not change significantly in the crush and degenerating groups following injury, whereas in the end-to-end group an up-regulation (1.73 ± 0.11 fold) is detectable at day-14 ($p=0.008$). At day-28 mRNA levels are comparable to control condition in the three experimental models (crush: 1.28 ± 0.21 fold; end-to-

end: 1.45 ± 0.24 fold; degenerating nerve: 0.99 ± 0.16). Statistical analysis revealed significant effects related to the type of injury (Figure 1).

ErbB2 mRNA expression level demonstrates a strong down-regulation from day 1 after injury under regenerating conditions (crush: 0.29 ± 0.06 fold, $p < 0.001$; end-to-end: 0.19 ± 0.03 fold, $p < 0.001$) that lasts until day 28 (crush: 0.17 ± 0.04 fold, $p = 0.034$; end-to-end: 0.51 ± 0.10 fold, $p < 0.001$). Under degenerating conditions ErbB2 expression does not change significantly. Significant differences among groups are detectable (Figure 1).

ErbB3 mRNA expression is strongly decreased 1 day after injury (crush: 0.42 ± 0.12 , $p = 0.009$; end-to-end: 0.35 ± 0.09 , $p = 0.005$; degenerating nerve: 0.54 ± 0.14 , $p = 0.053$), followed by an increase until day 7 in all experimental groups.

Under regenerating conditions, ErbB3 expression returns to control levels at day 28 (crush: 1.14 ± 0.19 fold; end-to-end repair: 1.61 ± 0.33 fold). Under degenerating conditions the up-regulation remains almost stable until day 28 after injury (2.84 ± 0.82 , $p = 0.001$). Statistical analysis showed significant differences related to the type of injury (Figure 1).

ErbB4 mRNA expression was also analysed, but in the peripheral nerve it is barely detectable (data not shown).

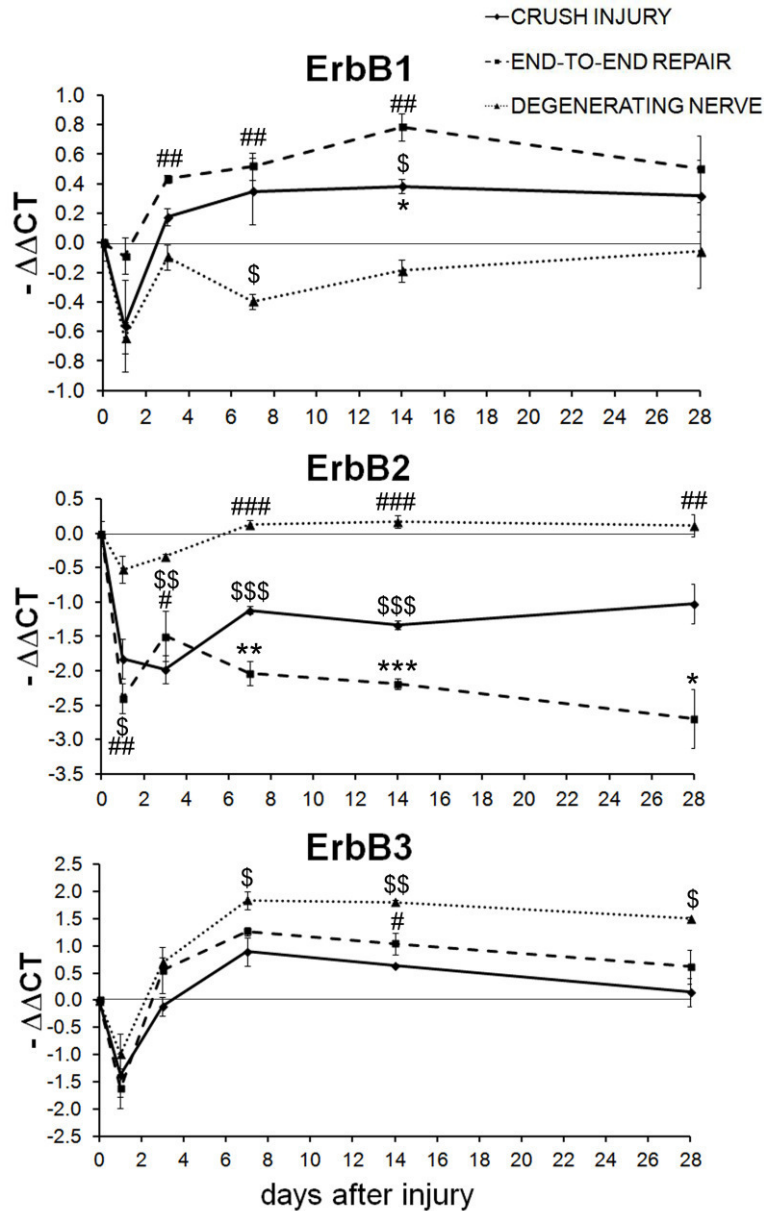


Fig. 1: ErbB mRNA is highly regulated after nerve injury. The relative quantification ($-\Delta\Delta C_t$) of ErbB1, ErbB2 and ErbB3 receptor was obtained by qRT-PCR: data were normalized to the geometric mean of two endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphics are expressed as mean $\pm$ SEM. Statistical analysis (one-way ANOVA with Bonferroni's post hoc) was carried out to compare the differences among the three injury models at each time-point. Asterisks (*) denote statistically significant differences between crush and end-to-end repair conditions; dollars (\$) between crush and degenerating nerves conditions; hashes (#) between end-to-end repair and degenerating nerve conditions (*\$/#: $p \leq 0.05$, **/\$\$/###: $p \leq 0.01$ and ***/\$\$\$/####: $p \leq 0.001$). Statistical analysis of the comparison of each time-point with control condition is discussed in the text.

mRNA expression levels of NRG1 soluble isoforms are strongly up-regulated immediately after nerve injury

The mRNA expression of soluble isoforms of NRG1 (NRG1 type I/II, mostly expressed by SC), shows a similar time course pattern in all experimental models: expression is highly increased ($p < 0.001$) immediately after injury, peaking at day 1 after injury (crush: 24.12 ± 6.02 fold; end-to-end: 26.75 ± 0.33 fold; degenerating nerve: 33.03 ± 6.03 fold). Then, the expression level decreases and returns to control values at day 28 after the injury. Intriguingly, in the degenerating group NRG1 type I/II expression is still higher at day 3 compared with control conditions (8.21 ± 2.10 fold, $p = 0.002$) Statistical analysis revealed no significant effects among injury types (Figure 2).

Soluble and transmembrane NRG1 can be either the isoform alpha or beta, according to the exon present in the C-terminal region of the EGF-like domain. The expression of mRNA encoding for NRG1 alpha and NRG1 beta demonstrated a different regulation pattern.

Expression of NRG1 alpha is strongly up-regulated immediately after nerve injury in all experimental groups ($p < 0.001$) displaying a peak at day 1 after injury (crush: 34.76 ± 12.45 fold; end-to-end: 36.80 ± 2.82 fold; degenerating nerve: 15.24 ± 3.73 fold). Under regenerating conditions, the expression level decreases towards control level which is reached and stabilized from day 14 to day 28 after injury. Under degenerating conditions, a decrease of expression reaching control values at day 7 after injury is detectable, remaining not significantly different until day 28. Significant differences among the groups are not detectable (Figure 2).

Expression of NRG1 beta mRNA is also strongly up-regulated ($p < 0.001$) peaking at day 1 after injury (crush: 12.18 ± 3.35 fold; end-to-end: 16.47 ± 0.37 fold; degenerating nerve: 13.55 ± 2.66 fold). In the following, the mRNA expression in the crush group reaches control expression levels already at day 3; in the end-to-end group mRNA expression decreases below control levels at day 7 (0.30 ± 0.12 fold, $p = 0.046$) and day 14 (0.13 ± 0.00 , $p = 0.002$); finally, under degenerating conditions NRG1 beta expression is still up-regulated at day 3 (3.74 ± 1.06 , $p = 0.043$) and down-regulated at day 7 (0.17 ± 0.07 , $p = 0.001$). Statistical analysis showed no significant differences related to the type of injury (Figure 2).

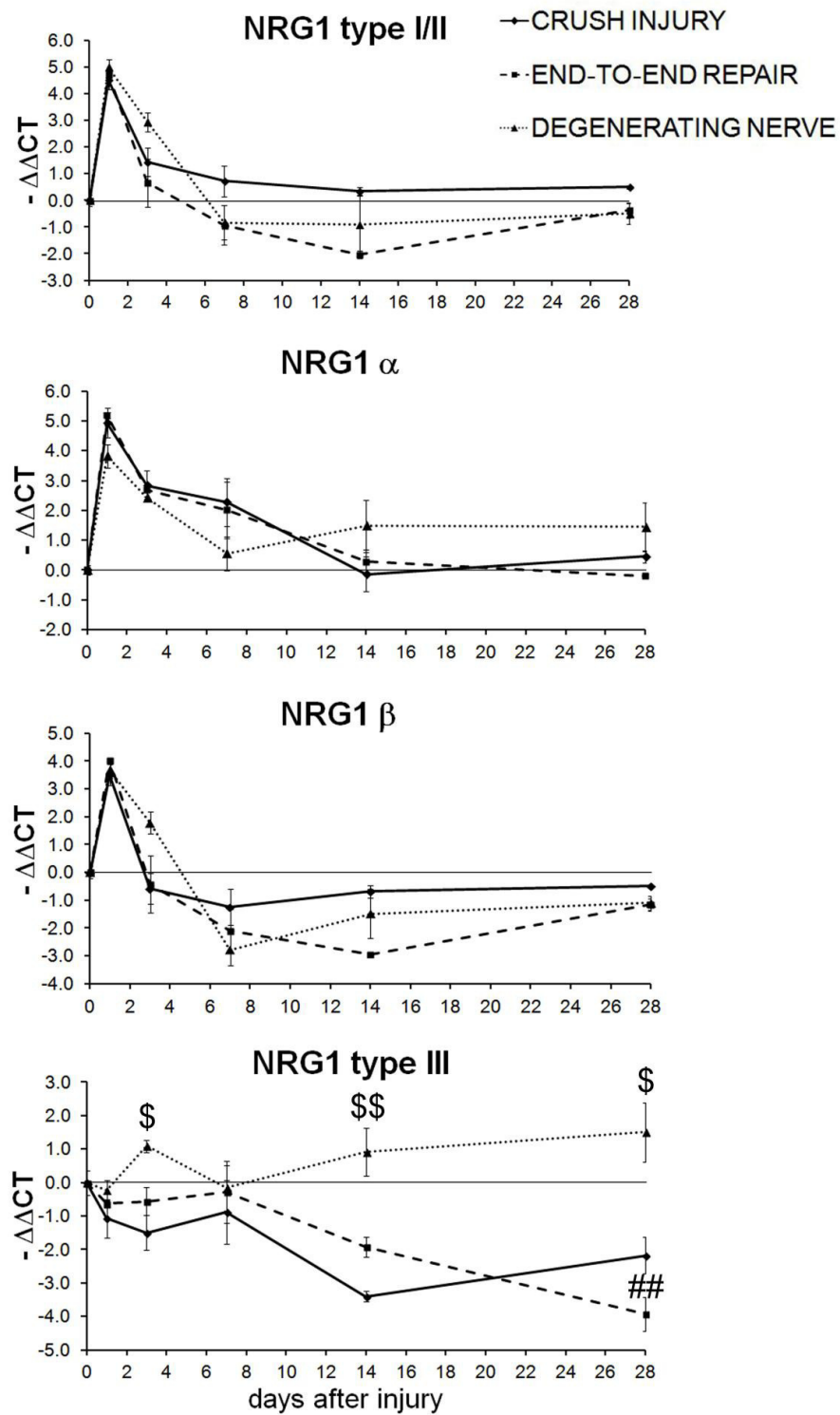


Fig. 2: NRG1 soluble isoforms are strongly up-regulated at mRNA level immediately after nerve injury. The relative quantification ($-\Delta\Delta C_t$) of NRG1 type I/II, NRG1 type III, NRG1 α and

NRG1 β was obtained by qRT-PCR: data were normalized to the geometric mean of two endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphics are expressed as mean $\pm$ SEM. Statistical analysis (one-way ANOVA with Bonferroni's post hoc) was carried out to compare the differences among the three injury models at each time-point. Asterisks () denote statistically significant differences between crush and end-to-end repair conditions; dollars (\$) between crush and degenerating nerves conditions; hashes (#) between end-to-end repair and degenerating nerve conditions (\$: $p \leq 0.05$, \$\$/###: $p \leq 0.01$). Statistical analysis of the comparison of each time-point with control condition is discussed in the text.*

mRNA expression level of NRG1 transmembrane isoforms is regulated under regenerating conditions

Transmembrane NRG1 is known to be mainly expressed by axons, whose cell bodies are in the dorsal root ganglia and in the ventral horn of the spinal cord where mRNA transcription occurs. For this reason, in the healthy and injured nerves NRG1 type III transcript level is low, with high expression variability. Expression levels in the degenerating nerve do not change significantly, whereas they decrease under regenerating conditions at day 14 in the crush group (0.10 ± 0.01 fold, $p=0.001$) and at day 28 in the end-to-end group (0.07 ± 0.02 fold, $p<0.001$). Statistical analysis revealed significant effects related to the type of injury (Figure 2).

mRNA expression level of NRG1 type a, type b and type c isoforms is deeply regulated following nerve injury

Finally, all NRG1 isoforms, both soluble and transmembrane, can be classified as type a, b, or c isoforms, according to the exon present in the C-terminal part of the protein (Falls, 2003, Mei and Xiong, 2008).

A strong expression of the type a isoform is detectable at day 1 after injury ($p<0.001$) in all experimental models (crush: 7.92 ± 0.35 fold; end-to-end: 11.32 ± 0.47 fold; degenerating nerve: 13.85 ± 3.12 fold). From day 3 the expression returns to control values in the three experimental models, although under degenerating conditions this decrease is slower (at day3: 1.76 ± 0.33 fold, $p=0.070$) (Figure 3).

The expression pattern of the type b NRG1 isoform is similar to type a, with a peak at day 1 from the injury ($p<0.001$) in three injury models (crush: 7.76 ± 1.83 fold; end-to-end: 11.33 ± 3.20 fold; degenerating nerve: 15.55 ± 3.09 fold). Under regenerating conditions the expression returns to control values from day-3 onward, whereas under degenerating conditions the expression is still high at day 3 (2.38 ± 0.31 , $p=0.011$; Figure 3).

Finally, the mRNA expression of the type c NRG1 isoform is strongly down-regulated after injury, remaining low until the end of the observation period (crush: 0.23 ± 0.12 fold, $p=0.016$; end-to-end: 0.05 ± 0.01 fold, $p<0.001$; degenerating nerve: 0.25 ± 0.02 fold, $p=0.050$; Figure 3). Statistical analysis revealed significant effects related to the type of injury for NRG1 type a, type b and type c, as shown in Figure 3.

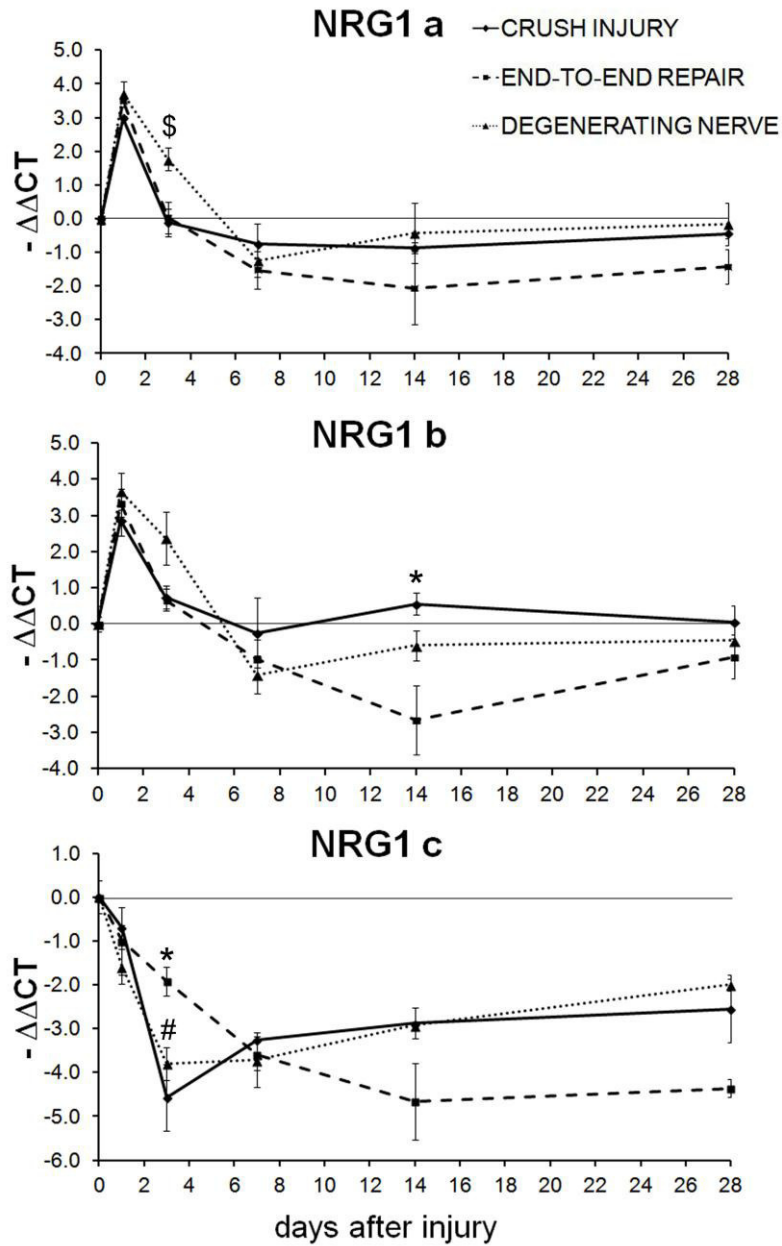


Fig. 3: NRG1 type a and type b isoforms are up-regulated at mRNA level after nerve injury. The relative quantification ($-\Delta\Delta Ct$) of NRG1 type a, type b and type c was obtained by qRT-PCR: data were normalized to the geometric mean of two endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphics are expressed as mean $\pm$ SEM. Statistical analysis (one-way ANOVA with Bonferroni's post hoc) was carried out to compare the differences among the three injury models at each time-point. Asterisks (*) denote statistically significant differences between crush and end-to-end repair conditions; dollars (\$) between crush and degenerating nerves conditions; hashes (#) between end-to-end repair

and degenerating nerve conditions (*\$/#: $p \leq 0.05$). Statistical analysis of the comparison of each time-point with control condition is discussed in the text.

NRG1/ErbB mRNA expression patterns depend on the expression by cells resident to the nerve

To investigate whether the deep changes observed at day 1 after injury are dependent on cells resident to the nerve or on cells invading it after injury, such as macrophages, healthy median nerve samples were put into degeneration *in vitro* for 1 day, and the mRNA expression level was analysed and compared to fresh healthy control levels. As depicted in Figure 4, all analysed genes demonstrated an expression pattern similar to the expression pattern detected *in vivo* under degenerating conditions.

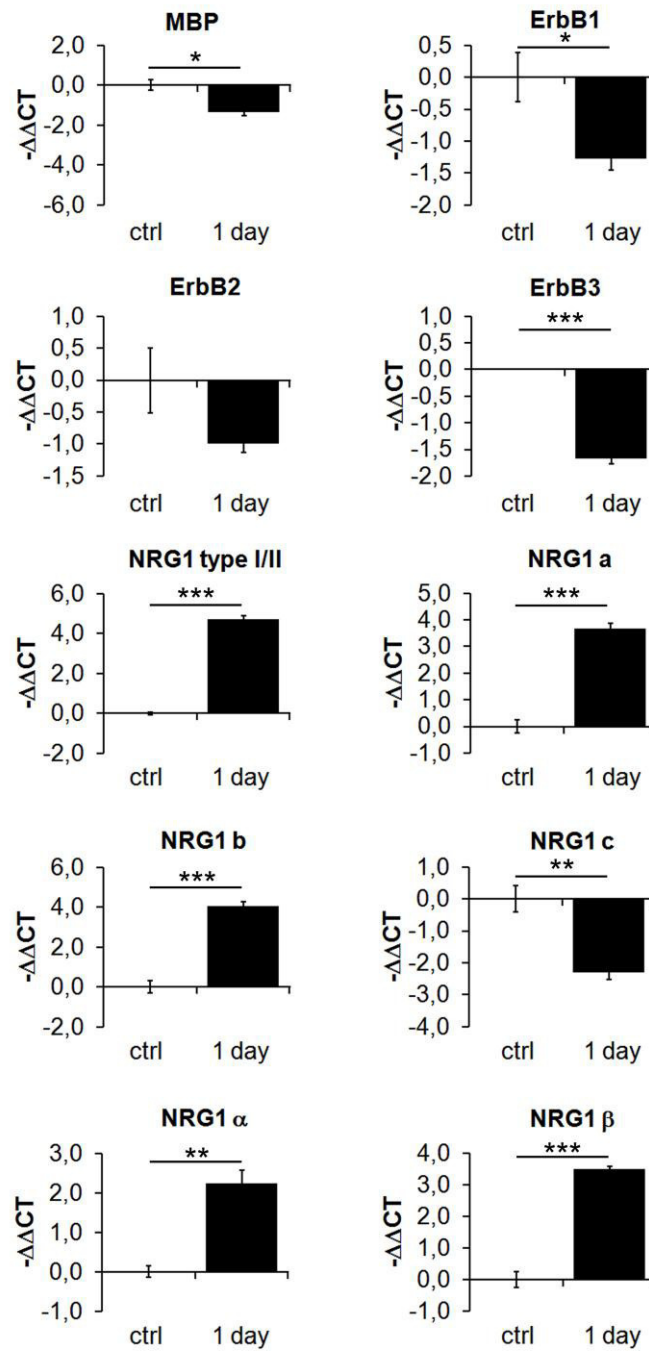


Fig. 4: NRG1/ErbB expression in in vitro degenerating nerves. Nerves were cultured for 1 day. This in vitro experimental procedure mimics in vivo nerve degeneration. The relative quantification ($-\Delta\Delta C_t$) of NRG1/ErbB was obtained by qRT-PCR: data were normalized to the geometric mean of two endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphics are expressed as mean + SEM. Student t-test was used for statistical analysis (*: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$).

ErbB receptor protein expression is strongly up-regulated at day 7 after injury

The protein expression level of ErbB1, ErbB2 and ErbB3 receptors and of some NRG1 isoforms following nerve injury was assessed by Western blot analysis of proteins obtained from the same samples analysed for mRNA expression levels.

ErbB1 protein (Figure 5) is expressed in the healthy control nerve and, following injury, its expression initially decreases. At day 3 after injury, however, protein expression is already increased and remains almost stably up-regulated until day 28 under regenerating conditions. Under degeneration conditions, ErbB1 expression level demonstrated a constantly increasing up-regulation until the end of the observation period. Protein expression levels do not reflect the changes detected for mRNA expression levels (Figure 1). This is most evident under the degenerating conditions.

ErbB2 shows a protein expression pattern (Figure 5) that is again strongly different from the mRNA expression pattern (Figure 1): ErbB2 protein is barely detectable in healthy control nerve samples or until day 3 after injury, while its expression is strongly up-regulated at day 7 to day 14, followed by a slight decrease until day 28. ErbB2 protein expression levels are similar under regenerating and degenerating conditions, while mRNA expression is differentially regulated and never displaying an up-regulation.

ErbB3 protein (Figure 5) is expressed in healthy control nerve samples and strongly down-regulated until it is almost undetectable at day 3 after injury. At day 7, however, similar to ErbB2 protein, ErbB3 protein expression levels are strongly up-regulated and remain high 14 and 28 days after injury. The ErbB3 protein expression levels reflect the changes detected for ErbB3 mRNA expression (Figure 1).

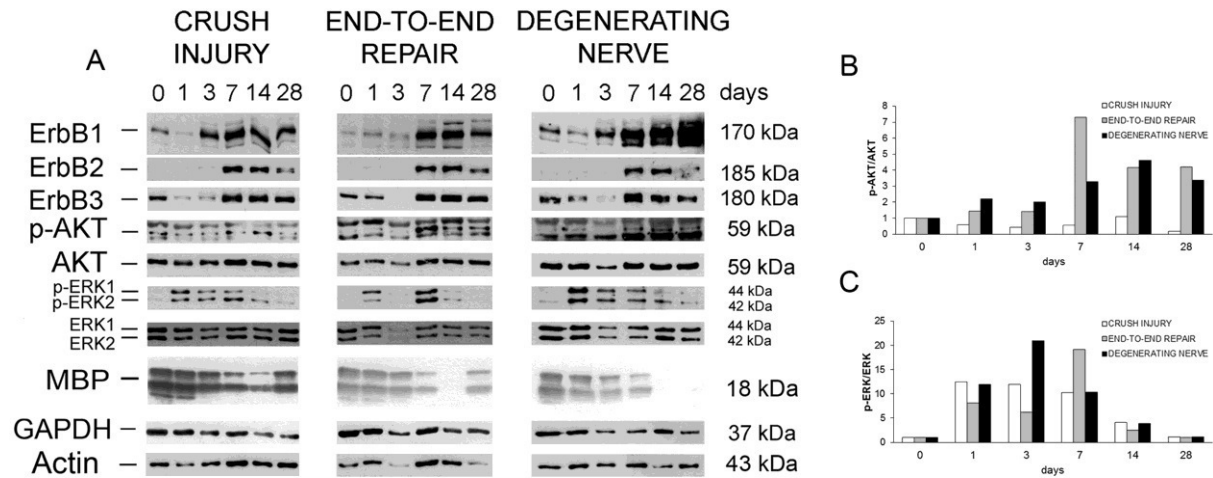


Fig. 5: ErbB receptor proteins are selectively regulated in the three experimental injury models. (A) Western blot analysis of proteins extracted from healthy nerves (0) and distal segments of injured nerves at different time-points, separated on 8% gels (50 µg/ lane) and probed with antibodies for ErbB1, ErbB2, ErbB3, p-AKT, total-AKT, p-ERK, total-ERK, MBP, GAPDH, β-actin. The same samples were run on three independent gels to analyse the expression of different proteins characterized by the same molecular weight. The positions of the detected proteins is indicated on the left, size markers on the right. The band corresponding to the phosphorylated AKT is detectable between two unspecific bands. Its identification was possible by comparison with the band corresponding to the total AKT protein. (B) p-AKT bands were quantified and normalized to the total of AKT. (C) p-ERK bands were quantified and normalized to the total amount of ERK.

The phosphatidylinositol-3 kinase (PI3K) and the ERK MAP kinase pathways are activated following nerve injury

Two signal transduction pathways were analysed under regenerating and degenerating conditions: the PI3K and the ERK MAP kinase pathways.

A basal expression level of phosphorylated AKT phosphorylation is detectable in healthy nerve samples, and an increased expression can be appreciated 7 days after injury in the end-to-end repaired and degenerating distal nerve samples (Figure 5B). In Figure 5A, the band corresponding to the phosphorylated AKT is detectable

between two unspecific bands. Its identification was possible by comparison with the band corresponding to the total AKT protein.

Phosphorylated ERK 1/2 protein (Figure 5A and C) is barely detectable in healthy nerve samples, and is strongly up-regulated from day 1 after injury where it remains high until day 7 prior to its slow decrease until day 28 after injury. At day 3 in the end-to-end group, and at day 3 and day 7 in the degenerating nerve ERK seems less phosphorylated, but this is due to a lower amount of protein extract loaded for these samples, as can be verified from total ERK analysis.

Protein levels of different NRG1 isoforms are differentially regulated after injury

The analysis of NRG1 expression is a very difficult aim, both at mRNA and protein level, because several isoforms exist, which are characterized by the combination of exons shared in part with other isoforms (Figure 6). NRG1 expression was analysed using two specific antibodies: AB5551, directed to the cytoplasmic N-terminus shared by all transmembrane type III isoforms; and sc-348, directed to the type “a” cytoplasmic C-terminus. The “a” exon is one of the three possible known C-termini (which can be a, b, or c) downstream the transmembrane and the cytoplasmic domain common to all transmembrane NRG1 isoforms (soluble type I/II pro-proteins, transmembrane type III, alpha, beta isoforms) except the $\beta 3$ isoforms, which lack this region and are produced as “ready-to-use” proteins (Falls, 2003, Mei and Xiong, 2008).

In the upper panel of Fig. 6A, the Western blot obtained with AB5551 antibody for NRG1 proteins is shown. This antibody recognizes the full-length (~100-130 kDa) and the N-terminal fragment (NTF; ~75 kDa) of all NRG1 Type III isoforms.

The 100-kDa band, which seems to be expressed not only by axons but also by SC, appears between day 3 and day 7 after injury, and its expression is switched off 28 days after injury under regenerating conditions, while it remains highly expressed under degenerating conditions. This band seems to reflect the expression of a full length type III NRG1, isoform b or c; it is likely not reflecting expression of full length type III NRG1 isoform type a, because it is not detectable with the antibody sc-348 (Figure 6B).

The NTF (~75kDa) is selectively regulated after injury. Indeed, the molecular weight of this band increases slightly after injury, suggesting that this small increase could be due to a switch between a disintegrin and metalloprotease (ADAM)17/tumour necrosis factor- α -converting enzyme (TACE) and β -site of amyloid precursor protein-cleaving enzyme 1 (BACE1) proteolytic cleavage of the detected type III NRG1 (Fleck *et al.*, 2013). As illustrated in Figure 6C, when analysing type III NRG1 protein expression with an antibody recognizing the N-terminus of the protein, the product after cleavage by the α -secretase ADAM17/TACE (the α -N-terminal fragment / α -NTF), has a lower molecular weight than the product cleaved off by the β -secretase BACE1 cleavage, the β -NTF. On the contrary, when the expression of the type III NRG1 protein is analysed with an antibody recognizing the C-terminus of the protein, the product of the ADAM17/TACE cleavage (the α -C-terminal fragment / α -CTF) has a higher molecular weight than the product of the BACE1 cleavage (β -CTF), as previously elegantly shown by others (Fleck *et al.*, 2013).

Regarding the expression levels of the NRG1 α -NTF and β -NTF, the healthy nerve expresses only the α -NTF (Figure 6A). Under regenerating conditions after mild injury (crush) both α -NTF and β -NTF are weakly expressed at day1 and exclusively β -NTF at day 3. At day 7 and day 14 after injury α -NTF is strongly and β -NTF weakly expressed with the expression of the latter being eliminated at day28 (resembling the status of a healthy nerve segment).

Under regenerating conditions after a more severe injury (end-to-end repair), α -NTF is still expressed in the distal nerve segment at day 1 after injury, then completely disappears until the end of the observation period (day 28), when it is expressed again (resembling the expression pattern of a healthy nerve segment). At day 3 and day 7 after end-to-end repair, the β -NTF band is only barely detectable in regenerating distal nerve segments, and its expression increases at day 14.

Under degenerating conditions, however, α -NTF expression disappears at day 1 after injury, while β -NTF is observed over the complete observation period, during which no regeneration occurs.

The same nerve specimen, as subjected to AB5551 Western blot analysis, were subjected to analysis with an antibody (sc-348) recognizing a 75k Da band corresponding to the CTF of type a NRG1 isoforms (Figure 6A, lower panel). An

expression increase of CTF was detectable after injury. This was followed by a decrease in protein levels under regenerating conditions (crush: at day 7; end-to-end: at day 28), while in the degenerating nerve the expression level remained stably elevated.

The CTF band detectable with sc-348 antibody does not show alpha and beta fragments like the NTF band, thus suggesting that this CTF derives mainly from the cleavage of type I/II NRG1 isoforms and not from type III NRG1 isoforms.

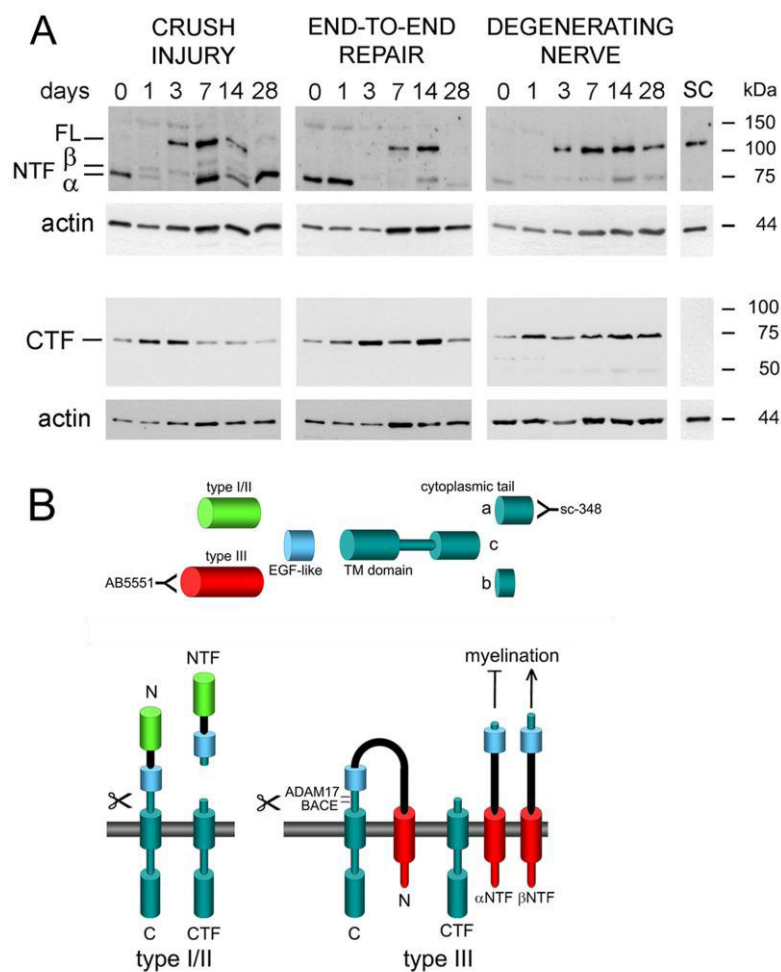


Fig. 6: NRG1 isoforms are selectively regulated at protein level in the three experimental injury models. Western blot analysis of proteins extracted from healthy nerves (0) and from the distal segment of injured nerves at different time-points. Proteins (50 μ g/lane) were separated on 4-15% gels. In the upper part of (A), proteins were probed with an antibody (AB5551) directed to the N-terminus of all type III NRG1 isoforms. This antibody recognizes:

a ~100 kDa band corresponding to the full length (FL) NRG1 (detected also in adult primary SC) whose expression switch on after injury and off after regeneration, but remains on in the degenerating nerve; a pair of bands ~75 kDa corresponding to the N-terminus fragments (NTF) resulting from α (ADAM17/TACE) or β (BACE) secretase cleavage of type III NRG1 (α -NTF, β -NTF). In the lower part of (A), the same samples were probed with an antibody (sc348) directed to the type a C-terminus common to many NRG1 isoforms. This antibody recognizes only a ~75 kDa band that corresponds to the C-terminus fragment (CTF) resulting from the cleavage of type a NRG1 isoforms (both soluble and transmembrane). The positions of the detected proteins are indicated on the left, size markers are indicated on the right. (B) A very simplified structure of type I/II soluble isoforms and type III transmembrane isoforms of NRG1 is schematized, together with antibody epitope. This scheme illustrates that type I/II and type III isoforms differ for the N-terminus regions, that the CTF can derive from both type I/II and type III NRG1 isoform cleavage, and that CTF can be recognized by sc-348 only if its C-terminal end is type a. ADAM17/TACE and BACE1 cleavage sites on type III NRG1 are shown, to illustrate how the NTF fragment produced by the BACE1 cleavage (β -NTF) has a molecular weight higher than the NTF fragment produced by the ADAM17/TACE (α -NTF) cleavage.

Distal nerve morphology displays different phases under regenerating and degenerating conditions

The ultrastructure of distal nerve segments was investigated by electron microscopy analysis (Figure 7). In particular, regenerating (crush) and degenerating nerves were focus on, assuming that end-to-end-repaired distal nerve morphology will resemble that of the crushed nerve just with a delay.

One day after injury (Figure 7A and F), the general organization of the median nerve morphology is preserved under both regenerating and degenerating conditions and similar to that of a healthy control nerve, just some signs of fibre degeneration are already detectable, like (for example) partially decompacted myelin sheaths surrounding collapsed axons.

Three days after injury (Figure 7B and G), first signs of Wallerian degeneration are clearly detectable in both the regenerating and the degenerating condition. Nerve fascicles display dispersed fibres with an increased amount of connective tissue

between them; some fibres show detachment and vacuolization of myelin lamellae, causing axolemma exposure. However, many fibre profiles still present a normal ultrastructure in both crushed and degenerating nerves.

Seven days after injury (Figure 7C and H), almost all nerve fibres display degeneration in both experimental groups. The nerve samples contain mainly collapsed axons with disintegrated myelin sheaths. Dedifferentiated SC with phagocytic properties are frequently detected by the numerous vacuoles in their cytoplasm.

Fourteen days after injury (Figure 7D and I), first differences between the distal nerve segments under regenerating and degenerating conditions are evident. In the crushed nerve segments, first small diameter and thin myelinated axon profiles are detectable and indicate that the regeneration phase has been initiated between day 7 and 14 post-injury. In contrast, distal segments under degenerating conditions display almost no more degenerating axons profiles, only a few onion bulb-like structures of degenerated myelinated axons are left. But also no regenerating axon profiles like in the crushed nerve are detectable.

Upon final analysis, 28 days after injury, (Figure 7E and J), differences between the regenerating and degenerating nerve segments are preserved. In the crushed nerve not only the number of regenerating axon profiles is increased, but also the diameter and myelin sheath thickness of the regenerating axons. This is indicative for the phase of fibre maturation. In contrast, under degenerating condition, the phase of chronic denervation is reflected by the completed clearance of myelin and the presence of dedifferentiated SC with no more phagocytic properties.

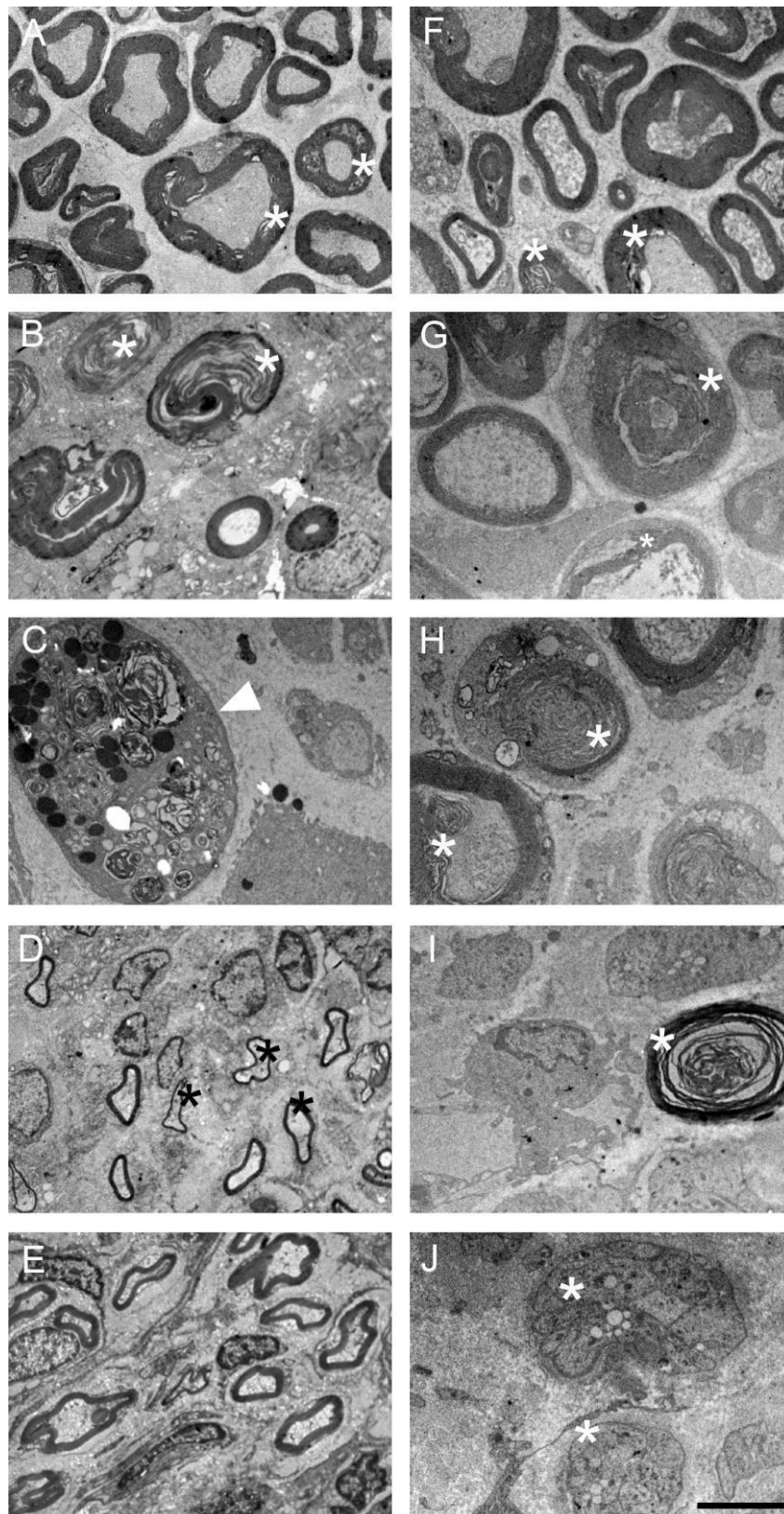


Fig. 7: Representative electron micrographs of transverse median nerve sections of crushed nerve (A-E) and degenerating nerve (F-J) after 1 day (A and F), 3 days (B and G), 7 days (C and H), 14 days (D and I) and 28 days (E and J) from the injury. One day, 3 days and 7 days after injury, nerve morphology appears similar in both experimental models: 1 day after injury (A and F), partially decompacted myelin sheaths (white asterisks) are visible; after 3 days (B and G), an increasing amount of connective tissue can be appreciated, as well as detached myelin lamellae (white asterisks). Seven days after injury (C and H), almost all nerve fibres display degeneration: collapsed axon with disintegrated myelin sheath (white asterisks) and the dedifferentiated SC with phagocytotic properties (white arrow head) are detected. Fourteen days after injury, differences between regenerating and degenerating nerves are evident - in the crushed nerve (D), small diameter regenerating axons with thin myelin (some fibres are indicated with black asterisks in the figure) are detected, whereas under degenerating condition (I) onion bulbs can be still seen (white asterisks). Finally, after 28 days, regenerating axon profiles with increased diameter and myelin sheaths are detected in crush injury group (E), while under degenerating conditions, dedifferentiated SC without vacuoles are present in the nerve distal segment (white asterisks, J). Scale bar: 5 μ m.

MBP protein expression reflects different nerve regeneration stages

The analysis of the MBP expression (Figure 8), both at mRNA and protein level, was utilized as a measure of myelination in distal nerve segments under regenerating and degenerating conditions.

MBP mRNA level (Figure 8A) strongly decreases ($p < 0.001$) after injury demonstrating lowest expression levels from day 3 to day 7. This reflects the phase of Wallerian degeneration also detectable on a morphological level (Figure 7B, C, G and H). In the following, MBP mRNA levels are up-regulated towards healthy control nerve levels under regenerating conditions. Reflecting the phases of axonal regrowth and maturation which are again also detectable on a morphological level (Figure 7D), healthy control values are already almost reached at day 14 after injury in distal segments of crushed nerves, while this is not detected before day 28 after end-to-end repair. The latter finding indicates that axonal regrowth and maturation as well as remyelination are delayed after end-to-end repair in comparison to crush injuries. Under degenerating conditions, however, MBP mRNA levels remain strongly down-

regulated, reflecting the entry into the morphologically detectable phase of chronic denervation (Figure 7I and J).

MBP protein expression level (Figure 8B) again reflects the morphologically evident phases although its decrease demonstrates a lower kinetic. Under regenerating conditions at day 14, MBP protein is weakly expressed in distal segments of crushed nerves while the protein completely disappeared after end-to-end repair. In the following, the MBP protein expression level starts to recover towards healthy control nerve levels, displaying a delay after end-to-end repair compared to crush injury. Under degenerating conditions, MBP protein expression continuously disappeared from day 14 onward.

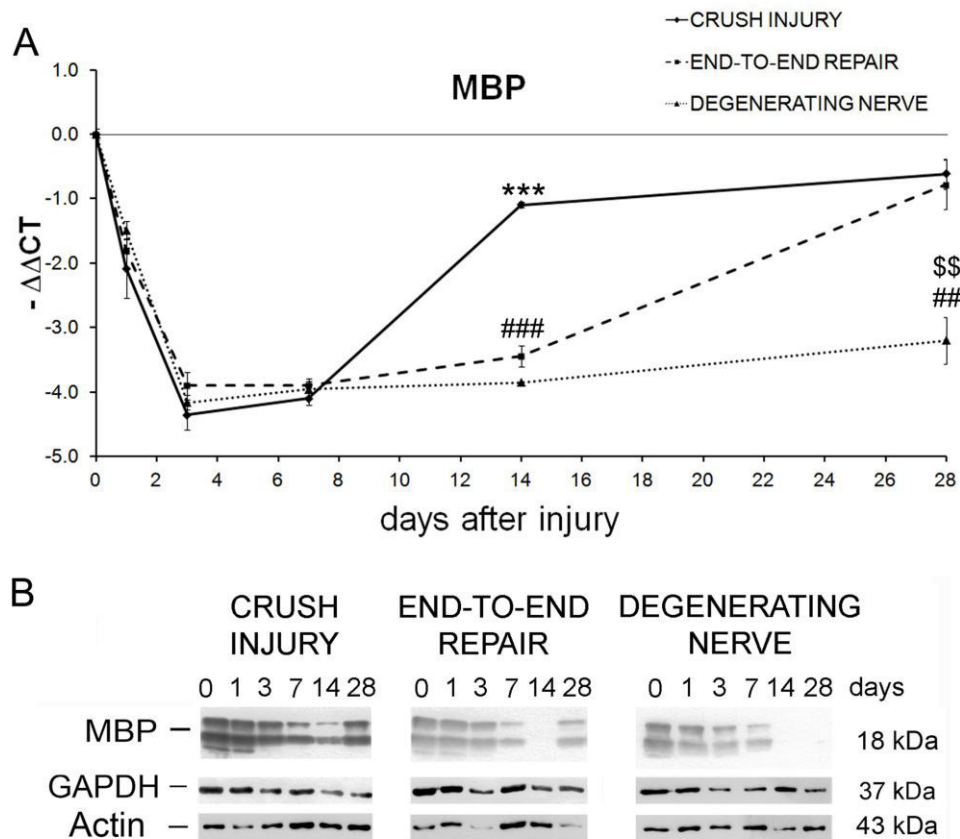


Fig. 8: Myelin basic protein (MBP) expression reflects the nerves-regenerating and -degenerating conditions. (A) The relative quantification ($-\Delta\Delta Ct$) of MBP was obtained by qRT-PCR - data were normalized to the geometric mean of two endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphics are expressed as mean $\pm$ SEM. One-way ANOVA was used for statistical analysis in order to compare the differences among the three model of injury at each time-point. Asterisks () denote statistically significant*

differences between crush and end-to-end repair conditions; dollars (\$) between crush and degenerating nerve conditions; hashes (#) between end-to-end repair and degenerating nerve conditions (\$\$/###: $p \leq 0.01$ and ***/####: $p \leq 0.001$). Statistical analysis of the comparison of each time-point with control condition is discussed in the text. (B) Western blot analysis showing MBP expression in the three injury models. β -actin and GAPDH were used as a loading control.

DISCUSSION

As summarized in Figure 9, the components of the NRG1/ErbB system are differentially regulated during the time course of peripheral nerve regeneration and degeneration. In the current study, three experimental conditions have been investigated, with crush injury and end-to-end repair reflecting two regenerating conditions that demonstrate all phases from Wallerian degeneration over axonal regrowth to axonal maturation and remyelination following a different time course. In contrast, the unrepaired nerve reflected the degeneration condition represented by complete removal of peripheral myelin on morphological and protein level and no signs of axonal regrowth or remyelination in the chronic denervation phase. Differential regulation of the NRG1/ErbB system components on mRNA and protein levels can be specifically linked to the different phases of biomolecular and morphological changes the distal nerve segments undergo under regenerating and degenerating conditions.

The NRG1/ErbB system is deeply involved in peripheral nerve myelination and remyelination processes, and several previous studies by others and us focused on the role of soluble and transmembrane NRG1 isoforms in these late phases of nerve regeneration (Fricker and Bennett, 2011, Gambarotta et al., 2013). In particular, it has been shown that transmembrane type III NRG1 plays a key role both in myelination and remyelination (Michailov et al., 2004, Taveggia et al., 2005, Fricker et al., 2011, Stassart et al., 2013), while it has been suggested that soluble type I/II NRG1 is mainly involved in SC survival, redifferentiation and, therefore, in remyelination (Stassart et al., 2013).

To the authors knowledge, this is the first time that mRNA expression levels also in the early phases after peripheral nerve injury were discriminated between not only soluble and transmembrane isoforms (which are involved in autocrine/paracrine and juxtacrine interactions) but also between type alpha and type beta isoforms (which are characterized by different activity) and between type a, type b and type c isoforms (which differ for the cytoplasmic C-terminal domain involved in NRG1 back signalling).

It has been demonstrated here that NRG1 isoforms are differentially regulated after nerve injury, where a strong increase of mRNA of the NRG1 type I/II, alpha and beta, as well as type a and type b at day1 were detected. Therefore, we hypothesize that the observed NRG1 expression peak corresponds to different combinations of NRG1 type I/II, alpha and beta, a and b. A strong increase of soluble NRG1 expression at day 1 after nerve injury was already described (Stassart *et al.*, 2013) as well as the up-regulation of NRG1 alpha by others and the authors (Carroll *et al.*, 1997, Nicolino *et al.*, 2003).

Alpha and beta NRG1 isoforms are known to differ in their activity, with alpha isoforms being about 10-fold less active than beta isoforms (Pinkas-Kramarski *et al.*, 1996). However, the difference between alpha and beta isoforms seems to be not only quantitative, but also qualitative. Indeed, it has been shown that NRG1 alpha and beta differentially affect the migration and invasion of malignant peripheral nerve sheath tumor cells (Eckert *et al.*, 2009) and that NRG1 alpha is able to regulate genes involved in phosphorylation, acetylation and alternative splicing in lymphoblastoid cells (Ghahramani-Seno *et al.*, 2013). The concomitant up-regulation of both the NRG1 alpha (up to 37-fold) and beta (up to 16-fold) isoforms demonstrated in this study indicates that both play a regulatory role following peripheral nerve injury.

The C-terminal domain of NRG1 (which can be type a, type b or type c) is a domain common to all soluble and transmembrane NRG1 isoforms, except NRG1 $\beta 3$, and it is involved in the back signalling mediated by transmembrane NRG1 isoforms following interaction with ErbB receptors. Indeed, it has been demonstrated that, following NRG1/ErbB interaction, a γ -secretase cleavage releases a NRG1 intracellular domain (ICD) that translocates into the nucleus and, according to the cell

model analysed, represses the expression of genes regulating apoptosis (Bao *et al.*, 2003), up-regulates the expression of post-synaptic density protein-95 (PSD-95) (Bao *et al.*, 2004) and the expression of lipocalin-type prostaglandin D2 synthase (L-PGDS) which, together with the G-protein-coupled receptor Gpr44, is involved in peripheral nerve myelination (Trimarco *et al.*, 2014). A transient up-regulation of NRG1 isoforms containing type a and type b C-terminal ends was observed here, whereas the type c isoforms stably decrease after nerve injury. Further studies are necessary to investigate the role of type a, b, and c NRG1 isoforms both during development of the PNS and after nerve injury.

At the protein level, NRG1 expression was analysed using two antibodies, one directed to the cytoplasmic N-terminus common to all transmembrane type III NRG1, and one directed to the type a NRG1 C-terminus that can be present both in transmembrane type III NRG1 and in the transmembrane precursors of soluble type I/II NRG1. The most interesting data were obtained with the first antibody.

After peripheral nerve injury, a “switch on” of a ~100 kDa band, expressed also by SC, and a “switch-off” of the same band 28 days after injury under regenerating conditions was observed, while it remained to be highly expressed in the degenerating condition, as previously described (Carroll *et al.*, 1997). The molecular weight of the band suggests that it is a full-length type III NRG1, isoform b or c (not a, because the antibody directed to the type a domain did not detect it). The protein expression pattern suggests that this isoform plays a crucial role for nerve regeneration.

Transmembrane NRG1 is usually considered to be expressed by axons and it has been demonstrated in this study that this expression is related to the phase of axonal regrowth. Nevertheless, its strong and stable expression observed also in the degenerating nerve and in SC suggests that this transmembrane isoform is of glial origin or potentially translated in the axon from previously transcribed mRNA. The latter hypothesis can be concluded from the NRG1 type III mRNA analysis that is not changed under regenerating conditions, while slightly increased (with a high standard error) in the degenerating nerve.

Intriguingly, the presence of a band, corresponding to the NTF of type III NRG1, with a molecular weight of ~75 kDa was observed, whose weight slightly increases after

injury, and decreases after regeneration. It has hypothesized that this small increase could be due to a switch between ADAM17/TACE to BACE1 proteolytic cleavage of type III NRG1.

It has been previously shown by others that products resulting from ADAM17/TACE cleavage of type III NRG1 inhibit remyelination (La Marca *et al.*, 2011), while products resulting from BACE1 cleavage promote myelination (Willem *et al.*, 2006). On the other hand, it has recently been shown that products resulting from BACE1 cleavage are not essential for the activation of myelination probably because additional proteases can also process NRG1 type III (Velanac *et al.*, 2012).

To the authors' knowledge, the data in this study evidence, for the first time, a switch between the two cleavage activities of ADAM17/TACE and BACE1 following peripheral nerve injury *in vivo*. The current data show that the healthy nerve expresses only the α -NTF of NRG1, which is the product of ADAM17/TACE cleavage, while regenerating nerves after end-to-end repair express only β -NTF, the product of the BACE1 cleavage that promotes remyelination. In contrast, regenerating nerves after a milder injury (crush) express both α -NTF and β -NTF of NRG1, suggesting that in this model axonal regrowth and remyelination phases are partially over-lapping. Finally, 4 weeks after injury regenerating nerves express only α -NTF, indicating that remyelination is completed at that time after a crush injury as well as after end-to-end repair. Under degenerating conditions β -NTF is still expressed 28 days after injury, indicating that the machinery is kept on "stand-by" for eventually still regrowing axons.

As after axonotmesis (crush) and neurotmesis (transection and repair or non-repaired), the continuity between distal axons and the corresponding cell bodies is disrupted, it is very likely that the NRG1 β -NTF band observed after nerve injury is locally synthesized in the analysed nerve segments. This hypothesis is strengthened by data demonstrating that peripheral nerves contain mRNA, ribosomal proteins and Golgi-like structures, and that local translation can occur in the injured nerve (Yoo *et al.*, 2010). Intriguingly, intercellular transport of ribosomes (Court *et al.*, 2011) and mRNA (Giuditta *et al.*, 2002, Sotelo *et al.*, 2014) from SC to axons has been demonstrated before. The latter reinforces the idea that SC may support local axonal protein synthesis.

In order to assign the 75 kDa CTF band to specific NRG1 isoforms, the following conclusions can be drawn from the current data. NRG1 protein expression levels were analysed with an antibody directed against the cytoplasmic NRG1 C-terminus that is shared by all isoforms containing the exon defining type a isoforms. Therefore, the 75 kDa CTF can derive either from the proteolytic cleavage of the transmembrane type III NRG1 isoforms or the proteolytic cleavage of the pro-protein type I/II isoforms (which are transmembrane, but release an extracellular soluble fragment, leaving a membrane anchored C-terminus).

However, because two different molecular weights for the NTF of NRG1 with the AB5551 antibody directed to the type III NRG1 (α -NTF and β -NTF), and only a single band with the sc-348 antibody directed to the CTF of NRG1 (α -CTF and β -CTF) were observed, it was hypothesized that this band corresponds to the CTF derived from the proteolytic cleavage of the precursor of type I/II NRG1 type a isoform. The two bands of type III NRG1 isoforms observed with the AB5551 antibody must then represent type b or type c isoforms.

It can be concluded that different NRG1 isoforms seem to be directly linked to the experimental paradigms; indeed, they follow a different pattern of expression and post-translational modifications depending on the severity of the lesion.

With regard to the NRG1 signalling after peripheral nerve injury, the expression of ErbB receptors, both at mRNA and protein level, was also analysed.

Under regenerating conditions, ErbB2 and ErbB3 mRNA levels show a down-regulation at day 1 after injury, followed by a strong up-regulation of ErbB3, while ErbB2 mRNA level decreases to constant low levels. At the protein level, the regenerating condition is reflected by an initial decrease of ErbB1 and ErbB3 protein followed by a strong up-regulation of ErbB1-3. Until the end of the observation period, ErbB1 and ErbB2 proteins are down-regulated again in the regenerating nerve samples at day 28 after injury, although their expression is still higher than control.

Under degenerating conditions, ErbB1 and ErbB2 mRNA expression is constantly comparable to the control during all time-points analysed, while the initially down-regulated ErbB3 mRNA level increases at day 7 after injury and remains highly up-regulated until day 28. At the protein level, ErbB1 expression is constantly increased

under degenerating conditions, thus suggesting that it is directly linked to the severity of the injury. On the contrary, the protein expression pattern of ErbB2 and ErbB3 are similar under regenerating and degenerating conditions, thus indicating that ErbB2 and ErbB3 receptor expression is not directly related to the different kinds of injury.

The current data obtained in the degenerating nerve are consistent with a previous study of ErbB and NRG1 expression during Wallerian degeneration after sciatic nerve transection injury (Carroll *et al.*, 1997).

The strong up-regulation of ErbB2 protein in the early phase after injury is independent of the repair conditions provided and does not correspond to an mRNA expression level increase. It can be confirmed, however, that the ErbB2 transcript level is stably high in the analysis of the threshold cycle by RT-PCR. Therefore, it is very likely that the strong protein up-regulation after nerve injury is the consequence of post-transcriptional regulation occurring through different signal transduction pathways. Indeed, different research groups analysed already the expression of microRNAs and their role in post-transcriptional regulation following nerve injury (Viader *et al.*, 2011, Wu *et al.*, 2011, Chang *et al.*, 2013, Nagata *et al.*, 2014, Zhou *et al.*, 2014), and, the injury-induced down-regulation of microRNAs having ErbB2 and ErbB3 as target genes could contribute to ErbB2 and ErbB3 protein up-regulation (Scott *et al.*, 2007). Moreover, post-transcriptional regulation could be the consequence of protein kinase A (PKA) activation occurring after nerve injury. Related to this hypothesis, it has been demonstrated *in vitro* that NRG1 and forskolin synergize, and that forskolin increases ErbB2-ErbB3 protein expression in human SC without increasing levels of mRNA encoding these receptors (Fregien *et al.*, 2005). Forskolin activates the enzyme adenylyl cyclase, thus increasing intracellular levels of cyclic adenosine monophosphate (cAMP), an important second messenger that acts by activating cAMP-sensitive pathways such as PKA. Therefore, the activation of cAMP signalling, which has also been shown to synergize with NRG1 in the promotion of myelination (Arthur-Farraj *et al.*, 2011), could contribute to ErbB2 protein up-regulation.

The regulation of NRG1 and ErbB mRNA expression observed in the degenerating nerve *in vivo* was confirmed also *in vitro*, suggesting that the corresponding genes are expressed by cells resident inside the nerve.

Finally, AKT and ERK phosphorylation were analysed to investigate the existence of possible differences in the signal transduction pathways activated in response to the regenerating or degenerating conditions after peripheral nerve injury. AKT is more phosphorylated in the end-to-end repaired and degenerating samples, suggesting that it is involved in the response to more severe injuries. ERK signalling was already described before to be rapidly and strongly activated following nerve injury (Sheu et al., 2000, Harrisingh et al., 2004, Napoli et al., 2012). The data obtained from the current study, however, do not display any differences among the regenerating and degenerating conditions during the observation period. With high levels of phosphorylated ERK from day 1 after injury and its disappearance at day 28, it is likely that the role of ERK is more linked to the general response of the nerve after injury than to regeneration or degeneration.

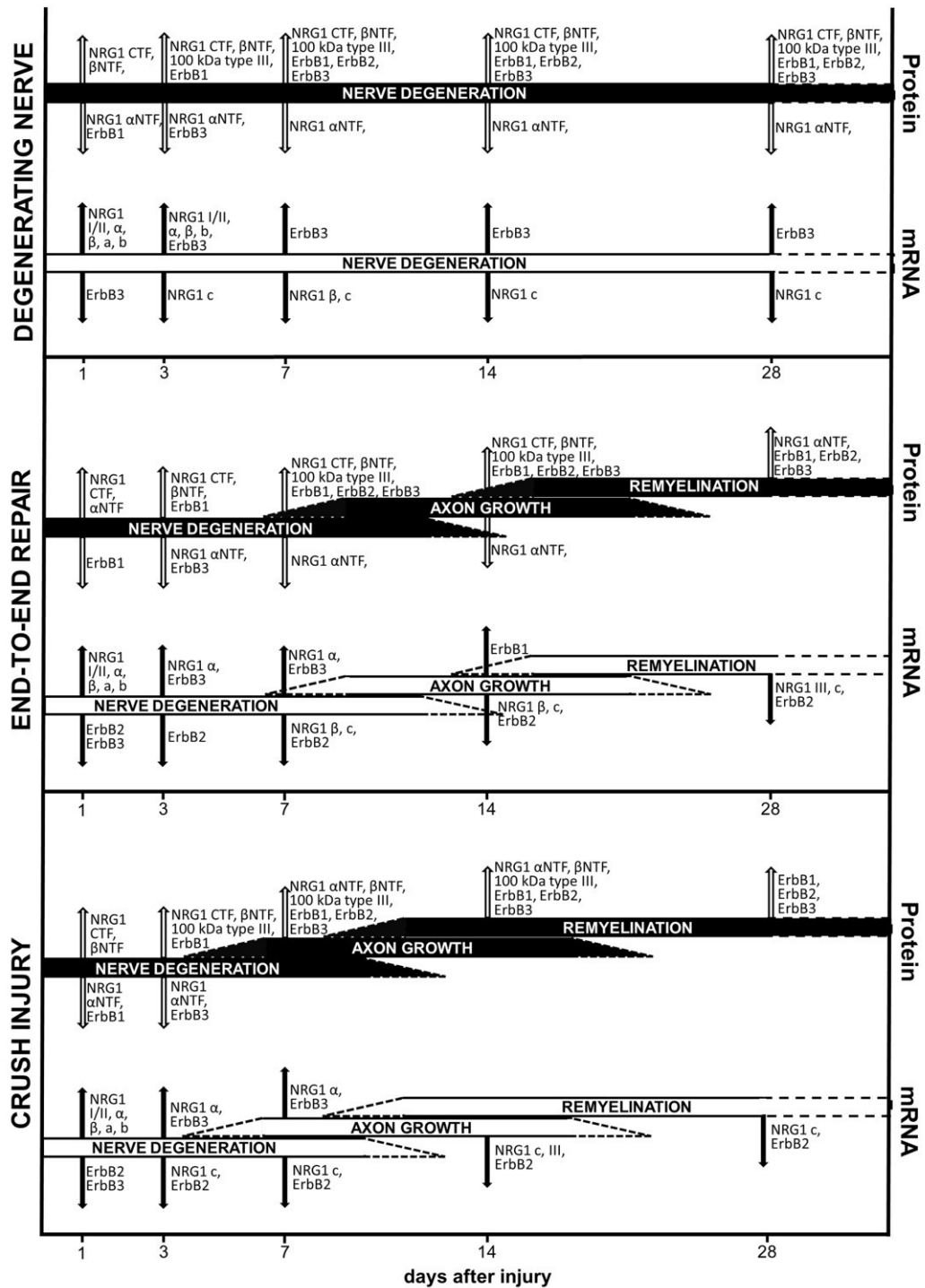


Figure 9: Schematic representation of all mRNA and protein data shown in this paper. Upward and downward arrows represent up-regulation and down-regulation, respectively. This graphical representation is not intended to exactly indicate the beginning and the ending of the three phases occurring after nerve injury (nerve degeneration, axon growth and remyelination), but rather the period when each process mainly occurs.

CONCLUSION

It has been demonstrated here that the components of the NRG1/ErbB system are differently regulated in the different phases a peripheral nerve undergoes after injury with the aim to regenerate. The immediate response to nerve injury is represented by ERK phosphorylation and by the up-regulation of soluble NRG1 (type alpha and beta, type a and b), which suggests that soluble NRG1 isoforms are involved in the response to nerve injury, stimulating SC survival and promoting axon regrowth. One week after injury, expression of ErbB2/ErbB3 co-receptor also is strongly up-regulated.

Additionally, a highly expressed type III NRG1 ~100 kDa full-length isoform was identified, the expression of which is only switched off after axon regeneration.

Furthermore, the current data suggest the existence of a reversible switch between ADAM17/TACE and BACE1 cleavage activity of type III NRG1 following nerve injury, which is very likely needed for the regulation of the remyelination process.

Moreover, for the first time the involvement of type a, b and c isoforms in peripheral nerve regeneration has been demonstrated here. Further studies will be necessary to better understand their role in the back signalling mediated by NRG1.

The precise regulation of the components of the NRG1/ErbB system indicates that each molecule that undergoes changes on the mRNA and protein level is crucially involved in successful peripheral nerve regeneration and could be a target for pre-clinical evaluation of regeneration-promoting factors.

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MY CONTRIBUTION TO THE MANUSCRIPT

I analysed and interpreted data obtained from *in vitro* experiments, I performed the biomolecular analysis of *in vivo* data and all the statistical analyses.

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CHAPTER 3

Chitosan tubes enriched with fresh skeletal muscle fibres for primary nerve repair

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ABSTRACT

Muscle-in-vein combined conduit is successfully employed for repairing nerve injuries: the vein prevents muscle fibers dispersion and scar tissue invasion, while the muscle prevents the vein to collapse and creates a favorable environment for Schwann cell migration and axon regrowth. However, the muscle-in-vein graft is technically challenged and requires microsurgical skills.

In this study we show a simple strategy aimed to improve the performance of a hollow tube made of chitosan by the introduction of fresh longitudinal skeletal muscle fibers. The hypothesis is (i) to overcome the technical issue of the muscle-in-vein preparation, and (ii) to take advantage of fiber muscle proprieties to create a surgically easy and effective conduit for nerve regeneration.

Rat median nerve gaps were repaired with (i) chitosan tubes filled with longitudinal skeletal muscle fibers (muscle-in-tube), (ii) hollow chitosan tubes or (iii) autologous nerve grafts (surgical gold standard). Regenerating nerve samples were harvested at early (up to 28 days) and late (12 weeks) time points for analyses (functional, morphological, morphometrical and biomolecular).

Our results demonstrate that nerve regeneration promoted by the muscle-in-tube was comparable to that obtained with the hollow chitosan tube and the autograft in terms of functional recovery, number and size of regenerating fibers. Yet, the fresh skeletal muscle inside the chitosan conduit is an endogenous source of soluble Neuregulin1, a key factor for Schwann cell survival and de-differentiation, absent in the hollow tube during the early phase of regeneration.

The muscle-in-tube technique is surgically easy to perform and we suggest that it might be a promising strategy to repair injuries when hollow conduits are not effective.

KEYWORDS

Chitosan; nerve repair; Neuregulin1; tubulization; skeletal muscle; tissue engineering

INTRODUCTION

Currently, the gold standard technique used to repair large peripheral nerve defects is the autologous nerve graft. However, this procedure has some well-known disadvantages: the need of an additional surgery to harvest the donor nerve, sensory deficits at the donor site, the possibility of neuroma-in-continuity formation with consequent neuropathic pain, and the limited availability of donor nerves in terms of number and diameter (Battiston et al., 2005, Konofaos and Ver Halen, 2013, Faroni et al., 2015). These limitations inspired researchers to develop alternative techniques for repairing large nerve defects.

The use of conduits made by non-nervous materials (tubulization technique) to bridge nerve gaps has been widely investigated and has shown promising results both experimentally and clinically (Battiston et al., 2005, Konofaos and Ver Halen, 2013). Tubular conduits have proven to be an excellent alternative to autologous nerve grafts because they act as physical guidance for the regenerating axons and provide a protective environment for axonal growth by limiting surrounding tissue invasion and by reducing neuroma and scar tissue formation (Pfister et al., 2007). Moreover, they facilitate the accumulation and the concentration of neurotrophic and neurotropic factors produced by the injured nerve stumps (Meek et al., 2004, Daly et al., 2012).

Several materials, both of biological or synthetic origin, have been used to build tubular conduits. Among these, muscle-in-vein combined conduits have been extensively and effectively employed to repair injured nerves. The success of this technique is due to the presence of the vein that prevents muscle fiber dispersion and scar tissue invasion, and muscle fibers which prevent the vein to collapse and create a favorable environment for Schwann cell migration and axon regrowth. However, the muscle-in-vein graft is technically challenged and requires microsurgical skills.

Chitosan is a natural polysaccharide that has been demonstrated to be a good biomaterial with a wide range of biomedical and tissue engineering applications (Zheng and Cui, 2010, Haastert-Talini et al., 2013) due to its biocompatibility, biodegradability, low toxicity and bioactivity. In recent studies its regenerative properties have been demonstrated in both standard and critical length nerve

reconstruction (Haastert-Talini et al., 2013, Gonzalez-Perez et al., 2015, Meyer et al., 2016, Shapira et al., 2016). These promising pre-clinical results confirmed that chitosan is a suitable biomaterial for peripheral nerve regeneration and in 2014 chitosan tubes have been accepted for clinical use (Reaxon® Nerve Guide, Medovent GmbH, Mainz, Germany).

Nevertheless, when more complex lesions occur, such as long gaps, hollow tubes not always lead to good results and conduit enrichment might create a favorable environment for nerve regrowth.

In this study we combined the use of a successful conduit (the chitosan tube) with the promising and simple intraluminal structure (fresh longitudinal skeletal muscle fibers) to evaluate peripheral nerve regeneration after rat median nerve reconstruction. The hypothesis is to (i) overcome the technical issue of the muscle-in-vein preparation by the use of a successful conduit, and to (ii) take advantage of fiber muscle properties to create a surgically easy and effective conduit for nerve regeneration.

Rat median nerve gaps were repaired with (i) chitosan tubes filled with skeletal muscle fibers (muscle-in-tube), (ii) hollow chitosan tubes or (iii) autologous nerve grafts (surgical gold standard). Samples were harvested at both early (1, 7, 14 and 28 days after nerve repair) and late (3 months) time points, and functional, morphological, morphometrical and biomolecular analyses were performed.

MATERIALS AND METHODS

Conditioned medium for cell stimulation and ELISA

Longitudinal pieces of rat *pectoralis major* muscle about 1 cm long were put in culture immediately after harvesting in a 24-multiwell using 0.5 ml (1, 3 days) or 1 ml (7, 14 days) 2% FBS DMEM/piece. Supernatant was collected, spun at 3000 rpm at room temperature for 5 minutes, and immediately frozen in liquid nitrogen for the following ELISA analysis. To quantify soluble Neuregulin1 (NRG1) in the muscle conditioned medium, ELISA analyses (#900-M316, PeproTech) were performed following manufacturer's instructions. 2% FBS DMEM was used as background negative

control. To investigate ELISA specificity, recombinant NRG1 α and NRG1 β 1 purchased from R&D (#296-HR-050 and #396-HB-050) were also analyzed.

At the same time points, degenerating muscles were collected and frozen for RNA extraction and quantitative real-time PCR (qRT-PCR) analyses. As control for RNA analysis, fresh muscle fibers were also collected. Experiments were carried out in biological triplicate.

RNA isolation, cDNA preparation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated according to the manufacturer's instructions using TRIzol Reagent (Invitrogen). Reverse transcription and quantitative real time PCR were performed and analysed as previously described (Ronchi et al., 2017). 0.75 μ g of total RNA were retrotranscribed,(Ronchi et al., 2017) the cDNA was diluted 10 fold and 5 μ l (corresponding to 15ng of the starting RNA) were analyzed for each PCR reaction. The presence of a single peak corresponding to the required amplicon was verified and dissociation curves were routinely performed. Technical and biological triplicate were performed.

As calibrator for relative quantification, the average of uninjured nerve Δ Ct was used for nerve regeneration *in vivo* experiments, the average of fresh muscle Δ Ct was used for muscle degradation *in vitro* analysis.

As housekeeping gene to normalize data, ANKRD27 (Ankyrin repeat domain 27) was used for *in vivo* nerve analysis (Gambarotta et al., 2014); TBP (TATA Binding protein) was used for *in vitro* muscle analysis. Primer sequences for ErbB1, ErbB2, ErbB3, NRG1 type I/II, type III, α , β , type a, type b, type c, ANKRD27 were previously published (Ronchi et al., 2015). New unpublished primer sequences are: ErbB4 forward: 5' AAGTTCTGGATGCGGAAGATGCC-3', ErbB4 reverse: 5'-TTGTTTCAGCACACACAGTCCTGG-3'; NRG1 α and NRG1 β forward: 5'-TGCGGAGAAGGAGAAAACCTTTC-3'.

Surgery

For *in vivo* study 68 female adult Wistar rats were used (Harlan, weight: 200-250g). Animals were housed in a room with controlled temperature and humidity, with 12h of

light and 12h of dark, free access to food and water. Every attempt was made to reduce animal suffering. All procedures were approved by the Bioethical Committee of the University of Torino and by the Italian Ministry of Health. Moreover, these procedures agree with the National Institutes of Health guidelines, the Italian Law for Care and Use of Experimental Animals (DL26/14), and the European Communities Council Directive (2010/63/EU).

During surgical procedures, animals were placed under general anaesthesia induced by i.m. injection of Tiletamine + Zolazepam (Zoletil, 3mg/kg) and were positioned in supine position. Using an incision from the nipple to the elbow, the median nerve was isolated to establish a defect in the middle of the exposed part, immediately followed by nerve repair according to the experimental group:

- *Chitosan-based hollow tube*: a nerve segment was removed and a 10mm-long chitosan tube was used to bridge the nerve defect by inserting 1mm of the two nerve ends inside the conduit. The nerve guide was sutured at each end.
- *Chitosan-based tube filled with fresh skeletal muscle – “muscle-in-tube”*: a nerve segment was removed and a 10mm-long chitosan tube enriched with a longitudinal piece of the *pectoralis major* muscle (withdrawn from the same animal) was used to bridge the nerve defect by inserting 1mm of the two nerve ends inside the conduit. The nerve guide was sutured at each end, as shown in Figure 1.
- *Autograft*: a median nerve segment was cut out, reversed (distal-proximal) and sutured to the nerve ends of the same animal. Chitosan-based tubes (Reaxon® Nerve Guide) were supplied by Medovent GmbH, Germany.

Animals were sacrificed by anaesthetic overdose at different times points: 1 day (only autograft and muscle-in-tube), 7, 14 and 28 days for early time-point analysis; 1 day hollow chitosan tube graft was not analyzed, because it is colonized only by fluid and it was not possible to withdraw it. For late time point analysis (only hollow tube and muscle-in-tube), animals were sacrificed 12 weeks after the surgery. Data about autograft at 12 weeks post-surgery are already present in literature (Ronchi et al., 2014, Gambarotta et al., 2015). For each time point (1, 7, 14, 28 days) n=4 samples for each experimental group were harvested for biomolecular analysis and n=1 for qualitative morphological analysis (7, 14, 28 days). For morphometrical analysis, 12 weeks regenerated distal stumps were harvested (n=5).

Healthy median nerve segments and healthy *pectoralis major* muscles from other rats were also harvested as control in the qRT-PCR analysis.

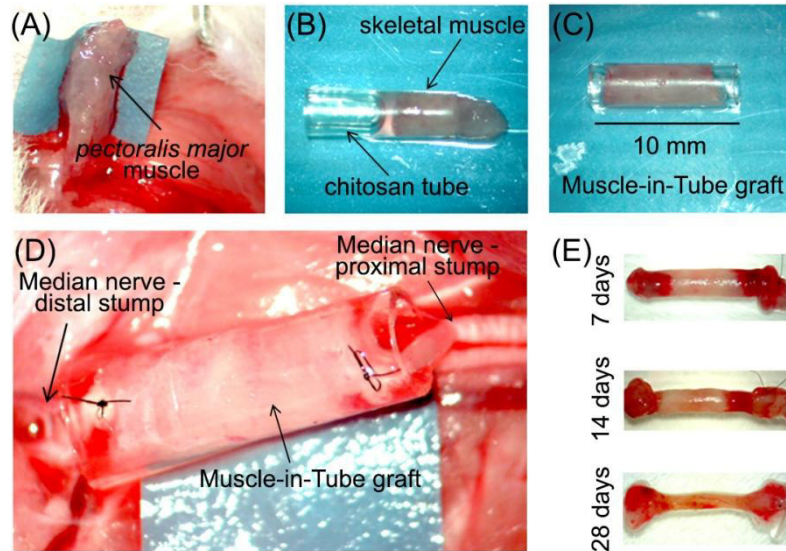


Fig. 1: Preparation of the muscle-in-tube graft. A longitudinal piece of the pectoralis major muscle was removed (A) and used to fill the chitosan conduit (B), to obtain a 10mm-long muscle-in-tube graft (C). The graft was then used to repair a median nerve defect (D). Pictures showing the regenerated nerves at different time points post-surgery (E). The conduit has been removed and a suture was used to mark the proximal stump (on the right in the pictures).

Grasping test

The grasping test was performed to estimate the functional recovery after nerve reconstruction. The analysis was carried out before animal sacrifice (12 weeks after nerve repair) following the same procedure previously described (Papalia et al., 2003, Ronchi et al., 2009). Rats were acclimatized before the testing. Each animal was tested three times and the average value was recorded.

Resin embedding, high resolution light microscopy and electron microscopy analysis

Samples corresponding to the grafted region (the autologous nerve and the filler of the chitosan tubes) harvested 7, 14 and 28 days after repair, and the distal portion of regenerated median nerve, harvested 12 weeks after repair, were processed for resin embedding as previously described and semi-thin and ultra-thin sections were cut for light and electron microscopy analysis (Ronchi et al., 2014).

Morphometrical analysis

The quantification of myelinated nerve fibers was performed on electron microscopy micrographs. Briefly, on one randomly selected ultra-thin section, 15-20 fields were chosen using a systematic random sampling protocol, as earlier described (Geuna, 2000, Geuna et al., 2000), with a magnification of 4000X. In each sampling field, a two dimensional (2-D) dissector procedure was used (Geuna et al., 2000). Mean fiber density, total fiber number, fiber and axon diameter, myelin thickness and *g-ratio* were then estimated. The total cross-sectional area of the whole nerve was measured on the toluidine blue-stained semi-thin section.

Statistical methods

Statistical analysis was performed using IBM SPSS Statistics 22.0 software. All data were expressed as mean $\pm$ standard error (SEM). Statistical analyses between two groups were performed using the two-tailed Student's t-test. Data sets containing more than two groups were tested by applying one-way analysis of variance (ANOVA) with Bonferroni's post hoc.

RESULTS

***In vitro* analysis: soluble NRG1 is expressed and released by *in vitro* degenerating muscles**

To evaluate the expression level of the different Neuregulin1 (NRG1) isoforms in degenerating muscles, longitudinal pieces of the *pectoralis major* muscle were put in culture in 0.5 ml 2% FBS medium immediately after harvesting. At different time points (1, 3, 7, 14 days) total RNA was extracted from *in vitro* degenerating muscles and from control health muscles and the expression level of different NRG1 isoforms was analyzed by quantitative real time PCR, discriminating among soluble and transmembrane, α and β , type a, type b and type c isoforms. Data analysis showed that during *in vitro* degradation, soluble isoforms were strongly and significantly up-regulated, and that soluble up-regulated isoforms belonged to α and β , type a, and type b isoforms; transmembrane and type c isoforms were also up-regulated, although with a high threshold cycle (Ct) indicative of a low expression level (Figure 2A).

Conditioned medium was collected and analyzed by ELISA to quantify soluble NRG1 release. ELISA was performed on 50 μ l muscle conditioned medium collected after 1, 3, 7, 14 days of *in vitro* culture. To investigate if ELISA antibodies were able to recognize both NRG1 α and NRG1 β , additional standard curves were performed using recombinant proteins; nevertheless, only NRG1 β isoforms were recognized (data not shown). ELISA data analysis showed that muscle was able to release soluble NRG1 β in the medium (1.721 ng/ml $\pm$ 0.231 after 24 hours, Figure 2B).

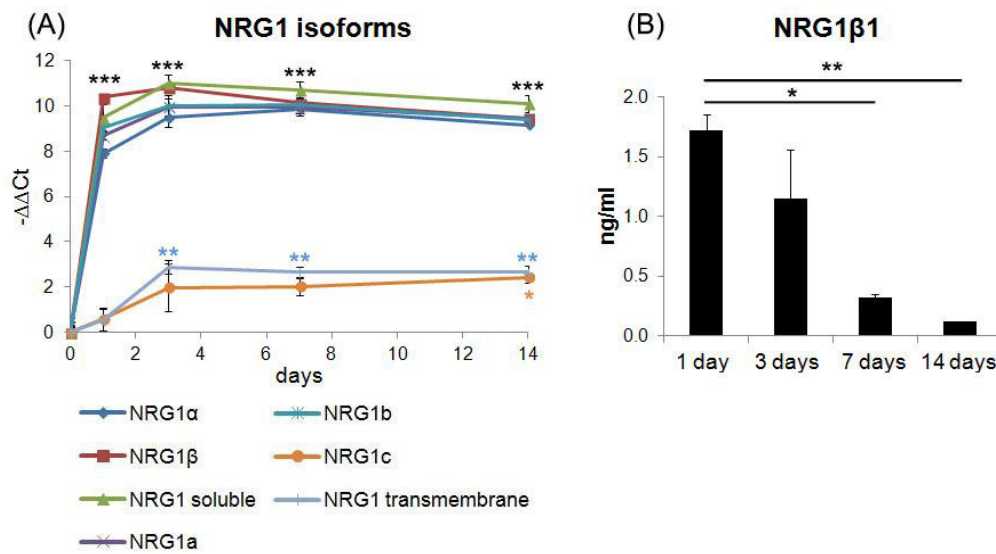


Figure 2: Degenerating muscle expresses NRG1. (A) qRT-PCR showing the relative quantification ($-\Delta\Delta Ct = \log_2 \text{fold change}$) of the different NRG1 isoforms in *in vitro* degenerating muscle. TBP was used as housekeeping gene to normalize data. (B) ELISA data showing NRG1 β 1 release in the medium of degenerating muscles. Data are expressed as mean $\pm$ SEM. Asterisks denote significant differences between degenerating muscles and the healthy muscle (A) or between the released NRG1 amounts at the different time points (B), * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

In vivo analysis

To verify the effectiveness of the muscle-in-tube to repair rat median nerve gaps, we compared this method with hollow chitosan tubes and autologous nerve grafts.

Short-term analysis of regenerated nerve: qualitative morphology

To observe the qualitative morphology of the nerve in the early stages of regeneration, high resolution light micrographs of toluidine-blue stained semithin sections were taken at short-term time points post-surgery (7, 14 and 28 days). Images were gathered inside the different grafts, both proximally and distally, to follow nerve regeneration alongside the conduits (Figure 3).

Nerves repaired with the autograft (positive control) showed an early regeneration, as expected (Figure 3A). 7 days after implantation, the nerve fibers inside the graft underwent Wallerian degeneration and the graft was filled with axons and myelin debris. After 14 days regenerated fibers were already present in the proximal part of the graft, while in the distal part endoneurial tubes were ready to be colonized by re-growing axons. Finally, after 28 days the whole graft was colonized by regenerated fibers.

Regeneration inside the hollow chitosan tube is slower when compared to the autograft (Figure 3B). Few days after implantation, extracellular matrix and fluid containing inflammatory cells filled the conduit. After 14 days, several cell types colonize the conduit, and only after 28 days myelinated fibers were detectable inside the conduit. Regenerated fibers were compacted in the central part of the tube (Figure 3B).

In the muscle-in-tube, longitudinal muscle fibers were inserted inside the chitosan conduit to provide a physical and trophic scaffold for axon and cell growth (Figure 3C). 7 days after nerve repair, big muscle fibers were clearly detectable inside the graft, together with different cell types. After 14 days, muscle fibers appeared smaller, showing that they were undergoing progressive degeneration over time. Few myelinated fibers organized in small fascicles were detectable. Finally, 28 days after implantation, the conduit was colonized by a high number of myelinated fibers organized in fascicles. Among nerve fascicles, small degenerating muscle fibers were still visible.

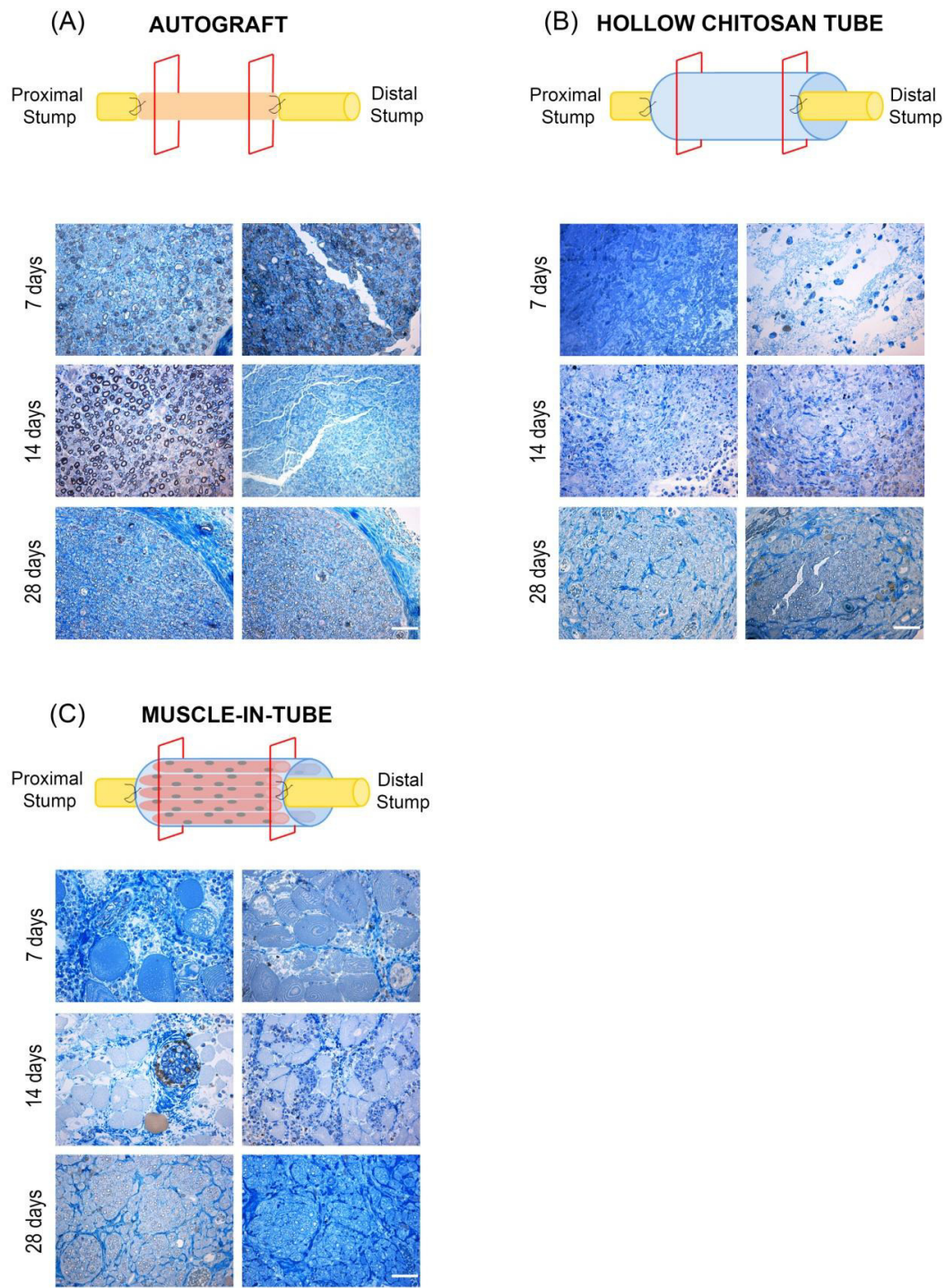


Fig. 3: Representative high resolution light images of toluidine-blue stained semi-thin transverse sections of regenerated median nerve repaired with autograft (A), hollow chitosan tube (B) or muscle-in-tube (C). Images were taken inside the grafts, both proximally (left columns) and distally (right columns), at different time points (7, 14, 28 days post repair). Bar: 40 μ m.

Short-term analysis of regenerated nerve: biomolecular evaluation

At different time-points after nerve reconstruction (1, 7, 14 and 28 days), ErbB receptors and different NRG1 isoforms were examined by qRT-PCR in the grafts of the three experimental groups (Figure 4). For the hollow tube, we analyzed the samples starting from day-7 post repair, because 1 day after repair the tube was colonized only by fluid material and was not possible to withdraw and analyze it.

ErbB1 expression level in the muscle-in-tube samples was down-regulated after injury and repair and was lower than in the hollow tube and autograft samples, where the expression was similar (Figure 4A).

ErbB2 expression in the muscle-in-tube samples was strongly and stably up-regulated at 7 and 14 days after injury relatively both to the healthy nerve and the muscle and to the autograft and the hollow chitosan group. At day 14 the autograft showed an expression level higher than the empty chitosan group (Figure 4B).

ErbB3 expression in the chitosan tube was lower than in autograft samples and even lower in the muscle-in-tube samples. 28 days after injury, ErbB3 expression in muscle-in-tube samples was significantly increased and autograft and hollow chitosan samples showed an expression level similar to the healthy nerve (Figure 4C).

ErbB4 expression strongly decreased in the muscle-in-tube samples - relatively to both healthy muscle and nerve - already 1 day after injury and remained really low until 28 days. In the hollow chitosan samples ErbB4 expression was similar to the muscle-in-tube samples, while in the autograft ErbB4 expression decreased after injury, but was higher than the muscle-in-tube and hollow chitosan samples. 28 days after injury only autograft samples showed an expression level similar to the control (Figure 4D).

NRG1 expression analysis is really complex, because each primer pair amplifies a single isoform, but soluble and transmembrane isoforms can be type α or type β , and type a, b or c. Transmembrane NRG1 expression was also analyzed but not shown, because its expression level is barely detectable, being this isoform expressed mainly by the axon and not by Schwann cells.

Autograft showed a strong early up-regulation (1 day) of the different NRG1 isoforms (except for NRG1c) followed by a return to values similar to control nerve. On the contrary, NRG1c was down-regulated from the 14th day (Figure 4 E-J).

In the first days after implantation, the hollow chitosan tube is filled by cells which do not express NRG1 or that express it at low level, indeed, at day 7 mRNA expression of most NRG1 isoforms was similar or lower to control nerve values. At day 14 NRG1 α started to be over-expressed and was still up-regulated after 28 days (Figure 4 E-J).

Finally, muscle-in-tube showed an expression pattern very similar to that of the autograft: all soluble NRG1 isoforms showed a strong peak one day after implantation (except for NRG1c) and then the up-regulation of most of them was still high at 7 and 14 days, returning to control values after 28 days (except for NRG1 α , that was still up-regulated also after 28 days) (Figure 4 E-J).

Overall, the up-regulation peak of NRG1 isoforms in the hollow chitosan tube started only 14 days after injury and is lower than muscle-in-tube. Intriguingly, the up-regulation at day-1 of soluble NRG1 (α and c isoforms) in muscle-in-tube samples was stronger than in autograft samples.

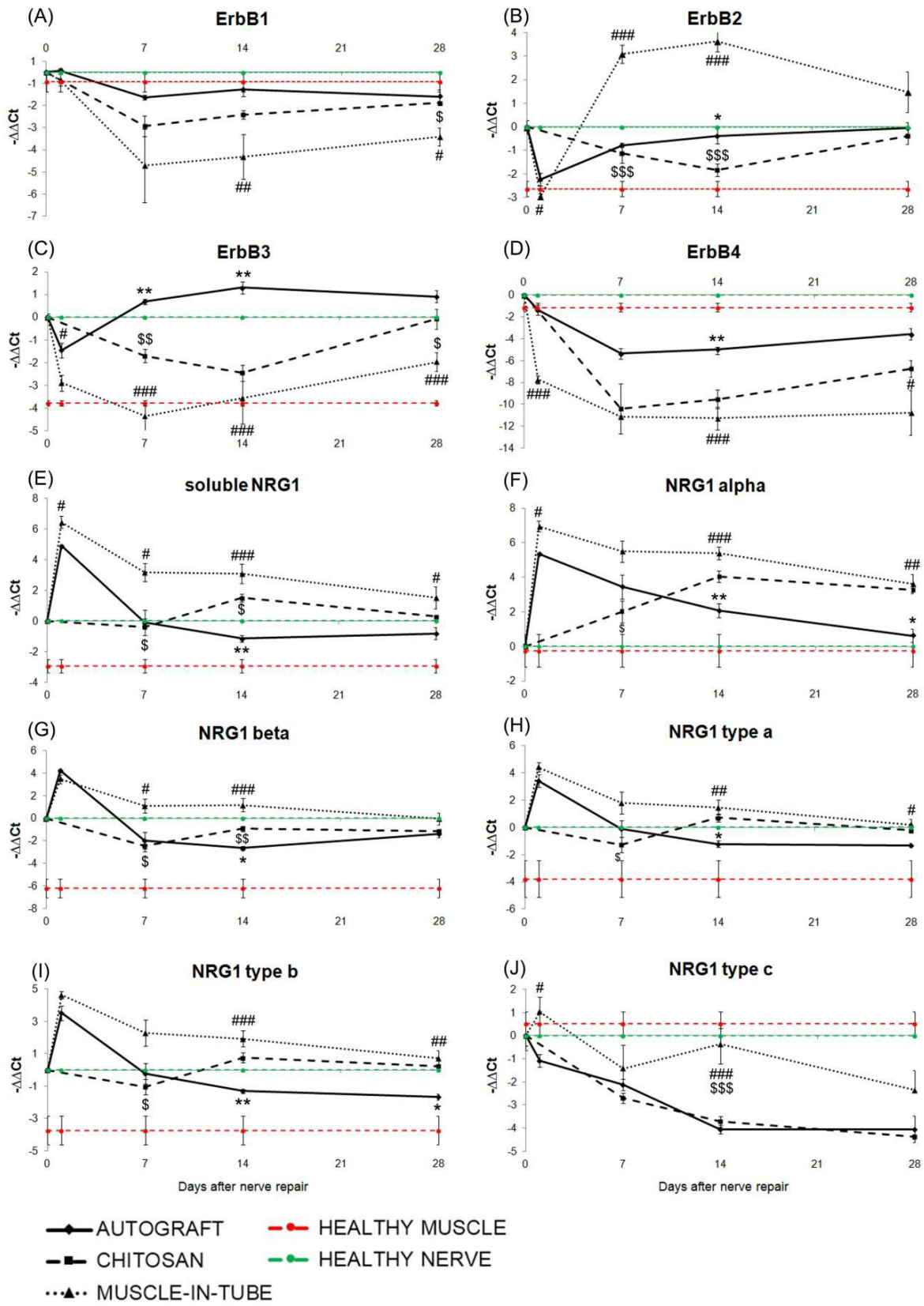


Fig. 4: Quantitative analysis of the NRG1/ErbB system expression. qRT-PCR showing the relative quantification ($-\Delta\Delta C_t = \log_2 \text{fold change}$) of ErbB receptors (A-D) and the different NRG1 isoforms (E-J). ANKRD27 was used as housekeeping gene to normalize data. Values in the graphics are expressed as mean $\pm$ SEM. Asterisks () denote statistically significant differences between autograft and hollow chitosan tube groups; hashes (#) between autograft and muscle-in-tube groups; dollars (\$) between hollow chitosan tube and muscle-in-tube. $^{*/#/\$}p \leq 0.05$, $^{**/##/\\\$}p \leq 0.01$ and $^{***/###/\\\$\\\$}p \leq 0.001$. Statistical analysis of the comparison of each time point with the healthy nerve is discussed in the text.*

Long-term analysis of regenerated nerve: functional, morphological and morphometrical analyses

To determine whether the presence of fresh muscle fibers inside the chitosan tube influences nerve regeneration, we performed a functional test (grasping test) and morphometrical analysis on the nerve segment distal to the graft 12 weeks after nerve reconstruction (Figure 5). Analyses were performed at electron microscopy level to enable accurate identification of all myelinated nerve fibers.

The functional test performed at week 12 post repair did not show statistical differences between the hollow chitosan tube and the muscle-in-tube groups (Figure 5A).

Results of the quantitative analysis performed on regenerated nerve fibers show no significant differences in all the analyzed parameters between the two experimental groups (Figure 5 B-F), as showed also by representative images (Figure 5G). The frequency distribution of nerve fiber diameters showed a shift of the muscle-in-tube histograms towards bigger size (Figure 5H). Finally, scatter plots displaying g-ratios of individual fibers in relation to respective axon diameter showed no differences between groups (Figure 5I).

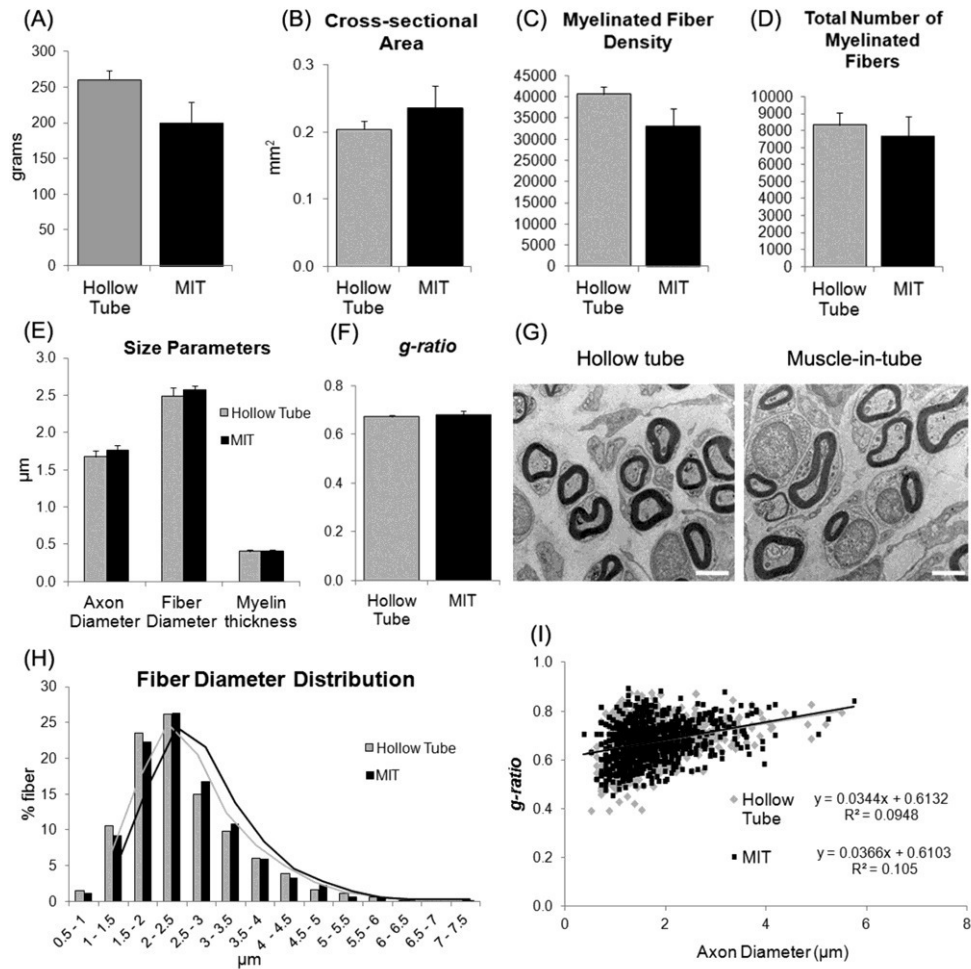


Fig. 5: Functional, morphological and morphometrical analyses of long-term regenerated nerves after hollow tube or muscle-in-tube (MIT) primary repair. (A) Histograms showing the result of the grasping test, performed 12 weeks after nerve repair. (B-F) Histograms of morphometrical analysis of the regenerated myelinated fibers: (B) cross-sectional area of the whole nerve section, (C) myelinated fiber density, (D) total number of myelinated fibers, (E) size parameters (axon and fiber diameter, myelin thickness) and (F) g-ratio. Values in the graphics are expressed as mean + SEM. (G) Representative electron microscopy images of the regenerated distal part of the two experimental groups. Bar: 2 μm . (H) Frequency distribution histograms of myelinated fiber diameters. The two lines are the fitting lines of the distribution. (I) Scatter plots showing g-ratio of individual myelinated axons as a function of axon diameter. Regression lines, their equations and R^2 are also shown. Both H and I data were obtained by pooling all the values from all animals for each experimental group; hollow tube: $n=830$; muscle-in-tube: $n=777$.

DISCUSSION

The use of hollow conduits to repair nerve defects is a valid alternative technique to autograft because of their well-demonstrated advantages, and in the last years several kinds of conduits have been proposed for their use in clinic.

However, the efficiency of hollow conduits is still insufficient, especially for large nerve gaps, probably due to an inadequate formation of the extracellular matrix, thus limiting cell migration and axonal regrowth (Daly et al., 2012). A number of intraluminal fillers of both biological and synthetic nature have been proposed to enrich hollow conduits. Skeletal muscle fibers have been demonstrated to be a suitable intraluminal filler for several reasons. Firstly, the three-dimensional (3-D) environment provided by the muscle basal lamina acts as a useful scaffold for growing axons and for migrating cells (Schwann cells, fibroblasts, and endothelial cells) (Meek et al., 2004). Second, the muscle tissue is readily available at the nerve injury site and can be easily translated into clinical practice.

In 1993, Brunelli et al. (Brunelli et al., 1993) described an innovative biological conduit made by a vein segment filled with fresh skeletal muscle fibers (muscle-in-vein combined conduit). This technique resulted experimentally successful and was therefore translated also to the clinic to treat certain cases of peripheral nerve injuries (Battiston et al., 2005, Marcoccio and Vigasio, 2010, Tos et al., 2012) (Schiefer et al., 2015). Nevertheless, the muscle-in-vein graft is not easy to perform and only few surgeons possess the technical skills necessary to build it.

To overcome this technical issue, the muscle tissue can be introduced inside a tubular conduit. However, so far, the placement of denatured skeletal muscle tissue (Meek et al., 1996, 1999, Meek et al., 2001, Mligiliche et al., 2003, Alluin et al., 2009, Dornseifer et al., 2011) or fresh skeletal muscle fibers (Varejao et al., 2003a, Varejao et al., 2003b) inside different kinds of tubular conduits (collagen, poly L-lactic acid and ϵ -caprolactone–PLAC, or DL-lactide and ϵ -caprolactone [p(DLLA–CL)]) have shown contrasting results in terms of nerve regeneration and functional recovery.

In this study we used a conduit made of chitosan (Reaxon® Nerve Guide) - that has been shown to promote successful nerve regeneration - combined with fresh longitudinal skeletal muscle fibers. The hypothesis is (i) to overcome the technical

issue of the muscle-in-vein preparation, and (ii) to take advantage of fiber muscle proprieties to create a surgically easy and effective conduit for nerve regeneration.

We used the fresh muscle tissue because it has been demonstrated that muscle cells produce and release factors that contribute to the survival of motoneurons *in vitro* (Montoya et al., 2009). Among these factors there are brain-derived neurotrophic factor, bone morphogenetic protein 6, cardiotrophin-1, glial cell-derived neurotrophic factor, heparan sulfate, hepatocyte growth factor, insulin-like growth factors, neurotrophin-3, neurotrophin-4 and vascular endothelial growth factor (Montoya et al., 2009).

Here we focused our attention on Neuregulin1 (NRG1), because it is known to be one of the most important factors regulating Schwann cell activity (survival, proliferation, de-differentiation, and migration) and, therefore, it is a key factor for peripheral nerve regeneration. *In vitro* experiments performed in this study demonstrated that (i) skeletal muscle fibers up-regulate the expression of soluble NRG1 mRNA while they are degenerating and that (ii) the soluble NRG1 protein produced by the muscle is released in the medium. Our results are in accordance with previous studies, showing a significant up-regulation of soluble NRG1 after skeletal muscle denervation (Nicolino et al., 2009), and an increase in NRG1 mRNA in the muscle-in-vein combined grafts at early regeneration stages – while muscle is degenerating (Geuna et al., 2003, Nicolino et al., 2003). However, as far as we know, this is the first study showing the release of soluble NRG1 protein in the environment. Taken together, these results show that the fresh skeletal muscle can be an endogenous source of the gliotrophic factor NRG1. For this reason, we moved to *in vivo* experiments using the rat median nerve experimental model for a primary repair of short gaps. The nerve gap has been repaired with the chitosan nerve guide (Reaxon® Nerve Guide), filled with fresh skeletal muscle fibers (muscle-in-tube) and compared with the hollow one.

Our *in vivo* experiments aimed to (i) evaluate the different behavior of regenerating fibers inside the conduits (muscle-in-tube versus hollow tube) in terms of gene expression and axonal regrowth in the early stages of nerve regeneration (up to 28 days), and to (ii) investigate whether the conduit filled with fresh skeletal muscle

fibers could improve nerve regeneration at later time points in terms of functional recovery and regeneration degree (12 weeks).

Our results indicate that muscle-in-tube promotes nerve regeneration. With respect to functional recovery and quantitative morphometry, no significant differences are observable between the two experimental groups at 12 weeks post-surgery, suggesting that both conduits are effective for repairing peripheral nerve defects in this experimental model (short gap primary repair). The comparison of morphometrical data obtained in this study with our previous data obtained using autograft (Ronchi et al., 2014), shows that the regenerated fiber number is slightly lower in the two experimental groups compared to autograft group, but the size parameters are very similar. Also, functional recovery reached similar values between the two chitosan experimental groups and the autograft (Gambiarotta et al., 2015), confirming the efficiency of the muscle-in-tube in promoting nerve regeneration.

The mRNA analysis of these samples is really complex. Indeed, muscle-in-tube samples at early time points (1 day and 7 days after injury and repair) are in fact mostly muscles and for this reason we have to compare them also with the muscle, not only with the nerve. Then, step by step, the muscle mRNA percentage decreases, while the nerve mRNA percentage increases. Consequently, although healthy nerves were used as a common calibrator for the three experimental groups, both healthy nerves and healthy muscle fibers were analyzed and showed in the figures (green and red lines respectively). Also hollow chitosan tubes represent a complex model, although in a different way: they need to be filled by migrating cell populations and for this reason the possible gene down-regulation in the first time point (7 days) is only apparent, because at time zero they are just empty tubes. Therefore, 7 days should be considered the expression starting point for these samples.

mRNA analysis, performed inside the conduits at shorter time points, differed greatly between the two groups in terms of gene expression: different isoforms of soluble NRG1 are highly expressed in the muscle-in-tube early after nerve repair, whereas no NRG1 expression is seen in the hollow chitosan tube. The NRG1 expression pattern observed in muscle-in-tube samples is very similar to that observed in

autograft samples, in which NRG1 is up-regulated by Schwann cells that colonize the graft. A similar expression pattern has also been described in other experimental models, such as crush injury and end-to-end repair, demonstrating that soluble NRG1 isoforms are involved in the response to nerve injury, stimulating Schwann cell survival and promoting axon regrowth (Ronchi et al., 2016).

CONCLUSIONS

Our results show that the muscle-in-tube promotes nerve regeneration as efficiently as the hollow chitosan tube, and that the fresh skeletal muscle inserted inside the chitosan conduit is an endogenous source of soluble NRG1, a source that is absent in the hollow tube. We recently showed that after prolonged degeneration of the median nerve distal stump, Schwann cells undergo atrophy and down-regulate the expression of soluble NRG1. Even after the cross-suture with the freshly axotomized ulnar nerve proximal stump, NRG1 remains at very low expression level, and nerve regeneration results impaired (Ronchi et al., 2017). Therefore, we suggest that soluble NRG1 supplied by fresh skeletal muscle-enriched conduit might be more useful to improve nerve regeneration in advanced experimental models, such as a longer gap or delayed (secondary) nerve repair.

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MY CONTRIBUTION TO THE MANUSCRIPT

I collaborated to the design and conception of all experiments and to write the manuscript; indeed, I shared the first authorship of the manuscript. Moreover, I have dealt with all *in vitro* experiments and with *in vivo* biomolecular analyses, and I supervised surgeries and behavioural tests.

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CHAPTER 4

The influence of electrospun fibre size on Schwann cell behaviour and axonal outgrowth

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ABSTRACT

Fibrous substrates functioning as temporary extracellular matrices can be prepared easily by electrospinning, yielding fibrous matrices suitable as internal fillers for nerve guidance channels. In this study, gelatin micro- or nano-fibres were prepared by electrospinning by tuning the gelatin concentration and solution flow rate. The effect of gelatin fibre diameter on cell adhesion and proliferation was tested in vitro using explant cultures of Schwann cells (SC) and dorsal root ganglia (DRG). Cell adhesion was assessed by quantifying the cell spreading area, actin cytoskeleton organization and focal adhesion complex formation. Nano-fibres promoted cell spreading and actin cytoskeleton organization, increasing cellular adhesion and the proliferation rate. However, both migration rate and motility, quantified by transwell and time lapse assays respectively, were greater in cells cultured on micro-fibres. Finally, there was more DRG axon outgrowth on micro-fibres. These data suggest that the topography of electrospun gelatin fibres can be adjusted to modulate SC and axon organization and that both nano- and micro-fibres are promising fillers for the design of devices for peripheral nerve repair.

KEYWORDS

electrospun fibres, extracellular matrix, cell adhesion, cell spreading, axon outgrowth, nerve tissue engineering

INTRODUCTION

In recent years, a considerable body of research has focused on the development of implantable polymeric scaffolds to be used in the repair of nerve gaps as alternative to autograft (Ichihara et al., 2008, Siemionow and Brzezicki, 2009, Biazar et al., 2010, Jiang et al., 2010, Gu et al., 2011). The need of artificial scaffolds for nerve tissue engineering arises from the limited autologous nerve availability and the requirement of secondary surgery that can result in donor sensory loss and/or pain from nerve autografts-based technique, and from the immune-rejection problems common to allografts- and xenografts-based techniques (Geuna et al., 2007, Campbell, 2008, Deumens et al., 2010, Geuna et al., 2013). Biomimetic materials for peripheral nerve reconstruction should: be biodegradable and biocompatible, display a large surface area and high porosity and provide adequate mechanical support for re-growing axons. To achieve these goals, a scaffold for peripheral nerve repair can be functionalized by acting on the properties of the inner filler (de Ruiter et al., 2009, Deumens et al., 2010, Gu et al., 2011, Steed et al., 2011) in order to mimic the native structure of the nerve and to enhance nerve repair processes (Agarwal et al., 2008, Bacakova et al., 2011, Harvey et al., 2013). It is necessary to take account of (i) the three-dimensional architecture (topography) of the internal filler and (ii) its biochemical composition in developing innovative implantable nerve scaffolds, since these features deeply influence cell behaviour and organization of the regeneration tissue. In particular, the internal filler of the conduit (i.e. fibre composition, diameter, three-dimensional structure) can be diversified in order to modulate Schwann cell (SC) and axon behaviour (cell proliferation rate, number of migrated cells, number of re-growing axons, etc.). Regarding the three-dimensional architecture of the internal filler, the basal lamina and the extracellular matrix (ECM) structures have to be considered. The tissue basal lamina membrane has unique nano-fibrous characteristics, suggesting the importance of substrate topography (Birk and Trelstad, 1984, Agarwal et al., 2008, Bacakova et al., 2011, Harvey et al., 2013). A number of studies have demonstrated that micro-to-nano scale topography is important for controlling the adhesion, proliferation and survival of different cell types such as human cord blood derived hemapoietic stem progenitor cells (Chua et al., 2006, Hu et al., 2008), bone marrow derived osteoprogenitors (Dalby et al., 2006,

Hart et al., 2007), mesenchymal stem cells (Dalby et al., 2007), MC3T3-E1 preosteoblast cells (Hu et al., 2008), hippocampal progenitor cells (Recknor et al., 2006), human keratinocytes (Rho et al., 2006), neural stem cells (Christopherson et al., 2009), SC (Gupta et al., 2009, Wang et al., 2009) and neurons (Xie et al., 2009, Huang et al., 2011). Surface modification of the biomaterial, roughness, porosity, and topographical cues such as grooves and ridges or pits and pillars influence protein absorption, cell interaction and host response to the material (Harvey et al., 2013, Rahmany and Van Dyke, 2013). Topographical modifications of implantable devices influence cell adhesion, growth and migration by affecting the size, shape, and distribution of focal adhesion plaque, actin cytoskeleton reorganization and/or *lamellipodium* and *filopodia* formation through integrin receptor signalling (Defilippi et al., 1999, Mattila and Lappalainen, 2008, Bacakova et al., 2011, Harvey et al., 2013, Rahmany and Van Dyke, 2013). Ideally, a candidate biomimetic scaffold should mimic native ECM structure and function, defining the optimal three-dimensional tissue organization for maintaining cell organization, viability, and invasion, proliferation and differentiation behaviour (Agarwal et al., 2008, Bacakova et al., 2011, Harvey et al., 2013).

Electrospinning is a fascinating and widely-used technique for producing fibres, mimicking the native ECM, from polymer solutions of both natural and synthetic origins. The resulting fibres are characterized by high surface to volume ratios, high porosity and enhanced physico-mechanical properties that can be adjusted by manipulating the polymer solution and process parameters to yield fibres with desired morphology and mechanical strength (Bhardwaj and Kundu, 2010). Electrospun fibres, mimicking the ECM structure have been used in a variety of applications such as wound healing, drug delivery, enzymes immobilization, filtration and scaffolds tissue engineering in nerve, dura mater, tendon/ligament and tendon-to-bone insertion repair (Venugopal and Ramakrishna, 2005, Rho et al., 2006, Agarwal et al., 2008, Prabhakaran et al., 2008, Venugopal et al., 2008, Bhardwaj and Kundu, 2010, Liu et al., 2012). Regarding biochemical composition, natural polymers are often used because of their high biocompatibility and presence of bio-functional cues, improving overall scaffold cytocompatibility (Liang et al., 2007, Jiang et al., 2010, Cunha et al., 2011). Collagen, alginate, silk protein, hyaluronic acid, chitosan and

others biomaterial have been used for preparing electrospun fibres (Liang et al., 2007, Bhardwaj and Kundu, 2010, Jiang et al., 2010, Cunha et al., 2011). However, ECM-based biomaterials have limitations such as poor mechanical properties and immunogenicity risk (Ciardelli and Chiono, 2006, Rahmany and Van Dyke, 2013).

In this work gelatin, a natural polymer obtained from the thermal denaturation of collagen, was chosen to produce random electrospun fibres owing to its advantageous features. In comparison to collagen, an animal protein, gelatin is biocompatible, biodegradable and does not induce immune-rejection problems, maintaining molecular cues that are key regulator of cell behavior (Ciardelli and Chiono, 2006). Despite these properties, the difficulty of dissolving gelatin in water at mild temperature (e.g. 37°C) limits its use in biomedical applications (Ciardelli and Chiono, 2006, Zhang et al., 2006, Zhang et al., 2009, Tonda-Turo et al., 2013). In a previous study, the authors successfully established a protocol for producing electrospun gelatin fibres. Gelatin was dissolved in distilled water, avoiding the use of potentially cytotoxic solvents and acidic solutions (Tonda-Turo et al., 2013). Furthermore, to increase gelatin stability in a physiological environment γ -glycidoxypolytrimethoxysilane (GPTMS) was used as cross-linker. GPTMS cross-linking has been reported not to alter the morphology of fibre matrices but it decreases the gelatin degradation rate in physiological solution (from few hours to more than seven days for gelatin and cross-linked gelatin nano-fibres, respectively). In that study, the authors identified the process and solution parameters to be applied in fabricating homogenous nano-fibres. In details, by tuning the gelatin concentration over the range 8 to 15 % w v⁻¹, the solution flow rate over the range 8 to 15 ml min⁻¹ and the nozzle collector distance in over the range 7 to 19 cm, electrospun fibres with a diameter size ranging from 200 to 700 nm were obtained. The biocompatibility of the nano-fibrous substrates was tested by culturing Neonatal Olfactory Bulb Ensheating Cells (NOBEC) on 300 nm fibres (Tonda-Turo et al., 2013). On the basis of these previous results (Tonda-Turo et al., 2013), we developed nanofibrous matrices characterized by different diameters in order to analyse the influence of fibres size on nerve tissue behaviour. Gelatin-GPTMS cross-linked electrospun fibres with diameters ranging from 300 nm to 1300 nm were prepared and *in vitro* and *ex vivo* primary SC culture and dorsal root ganglia (DRG) explants were established to

investigate whether the topography of micro-to-nano-scale fibres can modulate SC adhesion, proliferation and migration and axonal outgrowth.

MATERIALS AND METHODS

Fibre preparation

Gelatin solutions were prepared as previously described (Tonda-Turo et al., 2013) . Briefly, gelatin (type A from porcine skin) and GPTMS were supplied by Sigma Aldrich. Gelatin was dissolved in demineralised water at 50°C to obtain the desired concentration (15 or 20% w v⁻¹) and 137 µl of GPTMS was added to the solution and mixed for 1 h before spinning. The electrospinning system used for fibre preparation consists of an isothermal chamber equipped with: a high voltage generator (PS/EL30R01.5-22 Glassman High Voltage) providing a voltage of 0 to 30 kV and a current of 0 to 1.5 mA with reversible polarity; a volumetric pump (KDS210, KD Scientific); an electrode; a mobile syringe support; a syringe and a 1.5 mm thick flat aluminium collector. The gelatin solutions (containing 15% or 20% gelatin) were spun at 50 °C, 30 kV, flow rate 10 µl min⁻¹ or 15 µl min⁻¹ and a nozzle-collector distance of 15 cm as previously reported (Tonda-Turo et al., 2013). Briefly, 15% gelatin solution was spun at 10 or 15 µl/min flow rate in order to yield fibres of 300 and 600 nm in diameter respectively. Twenty percent gelatin solution was spun at 12.5 or 15 µl/min to yield fibres of 1000 and 1300 nm in diameter respectively (Figure 1 A). Electrospun scaffolds were prepared using a vertical electrospinning set-up to generate randomly-oriented nano- or micro-fibres on glass coverslips.

Scanning electron microscope analysis

The morphology of the electrospun matrices was examined using a scanning electron microscope (STANDARD DEVIATION Philips 525 M) at an acceleration voltage of 15 kV. The fibre samples were coated with gold using a sputter coater. The STANDARD DEVIATION magnification (6000×) was selected for 50 µm squared fields, allowing fibres distribution to be described. Pore and fibre diameters were quantified by analysing SEM micrographs through *ImageJ* software as previously

reported (Christopherson et al., 2009, Tonda-Turo et al., 2013). For each fibre type, three images from three different samples were examined and the diameters were recorded as average value $\pm$ standard deviation.

Cell culture

The RT4-D6P2T SC line and primary SC were grown on monolayers at 37 °C in a humidified atmosphere of 5% CO₂. The RT4-D6P2T were purchased from ATCC (American Type Culture Collection, 10801 University Blvd, Manassas, VA 20110-2209) and grown in complete high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) according to the designated ATCC protocol. SC for primary culture were isolated from sciatic nerve harvested from adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing approximately 190-220g. All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/ EEC). Adequate measures were taken to minimize pain and discomfort taking human endpoints for animal suffering and distress into account. The sciatic nerves were isolated, cut into 3 mm section and incubated at 37°C in 5% CO₂ in complete medium consisting of low glucose DMEM (Gibco) supplemented with 100 units ml⁻¹ of penicillin, 0.1 mg ml⁻¹ of streptomycin, 1 mM of sodium pyruvate, 2 mM of L-glutamine, 10% of heat-inactivated fetal bovine serum (FBS, Invitrogen), 63 ng/ml of glial growth factor (GGF, R&D System) and 10µM of forskolin (Sigma). The medium was changed every three days. After two weeks, 2 ml of digestion solution consisting of 0.6 mg/ml of collagenase type IV (Sigma) and 0.5 mg/ml of dispase (Invitrogen) diluted in low glucose complete medium was added. After 24 h the incubated nerve segments were transferred in a 50 ml tube and suspended in 5 ml low glucose complete medium using a glass Pasteur pipette. The cell suspension was filtrated through a 70 µm cell strainer (Falcon), centrifuged at 900 rpm for 5 min, re-suspended in 10 ml of SC complete medium and seeded on a poly-L-lysine (PLL, Sigma) coated plate. In order to remove contaminating fibroblasts, the confluent SCs were immunodepleted. Briefly, the confluent SC were harvested by trypsinization, re-suspended in 500 µl low glucose complete medium containing mouse anti-rat Thy1.1 antibody diluted 1:500 (Serotec, MCA04G) and incubated for 10 min at 37°C. Fresh

rabbit complement (250 μ l, Cederlane Labs) was added and incubated for 30 min at 37°C. The reaction was blocked by adding 10 ml of low glucose complete medium and 5 min of centrifugation at 900 rpm. The pellet was resuspended in low glucose complete medium and seeded on PLL-coated plate. Confluent cells were harvested twice a week by trypsinization and seeded at the desired dilution on a new plate. Before cell seeding, fibres samples were sterilised by overnight (O/N) exposure to UV irradiation (UV lamp Technoscientific Co., wavelength 254nm) and then incubated O/N with complete DMEM.

Adhesion assay

RT4-D6P2T and primary SC were seeded in complete DMEM at a density of 4000 or 8000 cells/cm² on both PLL- (control condition) and gelatin-fibre coated coverslips. After 3, 6 or 24 h, the culture medium was removed and the substrates with attached cells were rinsed with PBS containing Ca²⁺ and Mg²⁺ and fixed by incubation with 4% paraformaldehyde solution (PFA, Sigma-Aldrich). After 20 minutes, the PFA solution was removed and samples were rinsed with PBS containing Ca²⁺ and Mg²⁺. The fixed cells were permeabilized with 0.1% Triton X-100 diluted in PBS for 10 min at room temperature and blocking solution (normal goat serum, NGS, diluted 1:100 in PBS DAKO X0907) was applied for 1 hour at room temperature. The cells were stained by O/N incubation with anti-S-100 β rabbit polyclonal antibody (diluted 1:600 in PBS, Sigma) followed by 1 hour incubation with goat-anti-rabbit IgG (H+L) Cy3 secondary antibody at room temperature (diluted 1:200 in PBS, Jackson) or goat-anti rabbit IgG (H+L) AlexaFluor488 (diluted 1:200 in PBS, Invitrogen). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:1000 in PBS. The cells were photographed under an inverted optical video-confocal microscope (ViCo, Nikon Eclipse 80i) equipped with a Nikon ECLIPSE 80i camera using Image-Pro Plus 6.0 (Media Cybernetics USA). Adherent cells were counted with *ImageJ*. The experiments were repeated three times independently and included five sets of samples. Each set included three fibre matrix- and three control PLL-coated coverslips. For each sample, 30 images were taken at 20X magnification. The cell number and spreading area were calculated using *ImageJ* software. Cell numbers were averaged and expressed as the number of adherent cells/mm² $\pm$ standard

deviation. Spreading area was measured by calculating the pixel area occupied by cells, and the spreading areas were divided by the number of cells quantified for each field, and expressed as pixel area occupied by single cells $\pm$ standard deviation.

Proliferation assay

RT4-D6P2T and primary SC were seeded in complete DMEM at a density of 1000 or 2000 cells/cm² on both PLL- (control condition) and gelatine fibre-coated coverslips. After 1, 3, 5, and 7 days the cells were fixed, stained and photographed and cells were counted as described in the adhesion assay in Section 2.4. The experiments were repeated three times independently and included five sets of samples. For each time interval, three fibre matrix and three control PLL-coated coverslips were used. The numbers of cells counted for each assays were averaged and expressed as number of cells $\pm$ standard deviation.

Assessment of actin cytoskeleton organization

Cell adhesion and morphology were measured using immunocytochemistry. RT4-D6P2T and primary SC were seeded in complete DMEM at 4000 or 8000 cells/cm² on both PLL- (control condition) and gelatine fibre-coated coverslips. After 3, 6 or 24 h, the culture medium was removed and samples were fixed as described in the adhesion assay paragraph (Section 2.4). The fixed cells were permeabilized with 0.1% Triton X-100 in 1X PBS for 10 min at room temperature and NGS blocking solution was applied for 1 h at room temperature as described in the Section 2.4. F-actin was detected using FITC-conjugated phalloidin (diluted 1:1000 in blocking solution, Millipore) by 1 h incubation at room temperature followed by washing (3 x 5 min). Vinculin was detected by O/N incubation with anti-vinculin mouse monoclonal antibody (diluted 1:100 in PBS, Millipore) followed by 1 h incubation with goat-anti-mouse IgG (H+L) AlexaFlour488 secondary antibody (Invitrogen, diluted 1:200 in PBS). Fluorescent images were acquired using an inverted optical video-confocal microscope (ViCo, Nikon Eclipse 80i) equipped with a Nikon ECLIPSE 80i camera using Image-Pro Plus 6.0 (Media Cybernetics USA). The experiments were repeated three times independently and included five sets of samples. Each set included three fibre matrix- and three control PLL-coated coverslips. The entire procedure was carried out in a blind manner. For each sample 100 cells were examined making a

total of 300 cells for each experimental condition. An arbitrary score was given to each cell and the data were expressed as the percentage of cells displaying a low, medium or high actin cytoskeleton organization or presence of focal adhesion points $\pm$ standard deviation.

Lamellipodium and filopodia

Lamellipodia and *filopodia* were identified by direct microscopy of cells stained with anti-S-100 β rabbit polyclonal antibody. The cells were stained as described in Section 2.4. All images were captured with an inverted optical video-confocal microscope (ViCo, Nikon Eclipse 80i) equipped with a Nikon ECLIPSE 80i camera using Image-Pro Plus 6.0 (Media Cybernetics USA). The experiments were repeated three times independently and included five sets of samples. Each set included three fibre matrix and three control PLL-coated coverslips. The entire procedure was carried out in a blind manner. For each sample, 100 cells were examined making a total of 300 cells for each experimental condition. The data were expressed as percentage of cells with *lamellipodia* or *filopodia* $\pm$ standard deviation.

Bi-dimensional migration: time lapse

RT4-D6P2T and primary SC were seeded at 4×10^3 cells/cm² under fibre matrix and control conditions and allowed to adhere for 24 h. Fibres samples were inserted into 24-multiwell plates using CellCrowns (Sigma) to avoid floating of the samples.

For time-lapse video recording the cells were kept at 37°C in 5% CO₂ in an incubator chamber (Okolab). Cell movements were monitored with an inverted microscope (Eclipse Ti, Nikon) using Plan Fluor10 $\times$ /0.25 NA objectives (Nikon). Images were collected with CCD video cameras (Roper Scientific) at 30 minute intervals up to 48 h, digitized and stored as image stacks using MetaMorph 7.6.1.0 software (Universal Imaging Corp.). The experiments were repeated three times independently and included five sets of samples. Each set included three fibre matrix- and three control PLL-coated coverslips. Ten cells were analysed for each sample. Image stacks were analysed with *ImageJ* software using *MtrackJ* plugin. Cell motility was expressed as $\mu\text{m/s} \pm$ standard deviation.

Three-dimensional migration: transwell assay

Cells (10^5) suspended in 200 μ l complete DMEM containing 2% FBS were seeded in the upper chamber of a transwell (cell culture insert, BD Biosciences) on a porous transparent polyethylene terephthalate membrane (8.0 μ m pore size, 1×10^5 pores/cm²) with either no fibre matrices (control), or different fibre matrices inserted using CellCrowns (Scaffdex) to avoid floating of the samples. The lower chamber was filled with complete DMEM medium containing 10% FBS. The 24-well plates containing cell culture inserts were incubated at 37°C in a 5% CO₂/air atmosphere. After 6 h of incubation, cells attached to the upper side of the membrane were mechanically removed using a cotton-tipped applicator. Cells that had migrated cells to the lower side of the membrane were rinsed with PBS, fixed with 2% glutaraldehyde diluted in PBS for 20 min at room temperature, washed five times with water, stained with 0.1% crystal violet in 20% methanol for 20 min at room temperature, washed five times with water, air-dried, and photographed using an Nikon ECLIPSE TS100 inverted microscope equipped with a Nikon Digital Sight DSL1 camera; the images were analysed with *ImageJ* software. The experiments were repeated three times independently and include five sets of samples. Each set includes three control condition transwells and three transwells filled with the different fibre matrices. Cells counts were expressed as percentage of migrated cells/total number of cells $\pm$ standard deviation.

DRG explants

DRG explants were harvested from adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing approximately 190-220g. They were cultured on coverslips coated with the different fibre matrices or 100 μ l matrigel® in which culture medium was added, either without growth factors (negative control) or containing 50 ng NGF/ml (positive control). A total of ten ganglia were mounted per coverslips. After 30 min of incubation at 37°C, to allow the matrigel® to polymerize, complete F12-BME medium (2 ml) was added. Complete F12-BME medium comprises F12 and BME media (Gibco) in a 50:50 ratio containing 1% bovine serum albumin (BSA, Sigma), 0.5 g/ml D-glucose (Sigma), 100U/ml penicillin and streptomycin (Sigma), 1mg/100ml putrescine (Sigma), 2 mM L-glutamine (Sigma), 2 ml of 100X insulin-

selenium-transferrin (Gibco) and 0.125 ng/ml vitamin C. After 48h of incubation, the explants were fixed with 4% PFA for 20 min at RT. Axons were stained immunocytochemically as described in Section 2.4 using β -tubulin mouse mAb (diluted 1:100, in PBS, Sigma) and goat-anti-mouse IgG (H+L) AlexaFluor488 (diluted 1:200 in PBS, Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (diluted 1:1000 in PBS, Sigma). The experiments were repeated three times independently and included five sets of samples. Each set included three fibre matrix- and three control matrigel®-coated coverslips. For quantification, the whole explants were acquired through an inverted optical video-confocal microscope (ViCo, Nikon Eclipse 80i) equipped with a Nikon ECLIPSE 80i camera using Image-Pro Plus 6.0 (Media Cybernetics USA). Axon density, sprouting area and axon lengths were measured using *ImageJ* software as described elsewhere (Zamburlin et al., 2006, Gilardino et al., 2009). Axon density was expressed as pixel occupied by axons $\pm$ standard deviation. Sprouting area was expressed as DRG axon occupied area/DRG body area ratio $\pm$ standard deviation. Axon length was expressed in μm $\pm$ standard deviation.

Confocal microscopy

Samples were observed with a Nikon Eclipse E800 epifluorescence microscope under appropriate filters and a Leica TCS SP5 confocal laser scanning microscope (Leica, Mannheim, Germany) using a 40 $\times$ Plan-NEOFLUAR objective (numerical aperture(NA) = 0.50). High resolution images were acquired (1024 $\times$ 1024 pixels) at 100 Hz.

Statistics

The experiments were repeated three times independently and include five sets of samples. Each set included three control samples and three fibre matrix samples. The data were expressed as mean $\pm$ standard deviation. GraphPad Prism® software was used for single-factor analysis of variance (ANOVA). Values of * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ were considered as statistically significant.

RESULTS

Increasing gelatin concentration and flow rate resulted in production of fibres with larger diameter and greater porosity

Figure 1A summarizes fibre diameter and porosity. Increasing gelatin concentration and flow rate resulted in increased fibre diameter and porosity: a gelatin concentration of 15% and flow rate of 10 $\mu\text{l}/\text{min}$ allowed 340 ± 14 nm fibres to be produced, while a gelatin concentration of 15% and flow rate of 15 $\mu\text{l}/\text{min}$ allowed 594 ± 48 nm fibres to be produced, a gelatin concentration of 20% and flow rate of 12,5 $\mu\text{l}/\text{min}$ allowed 1005 ± 20 nm fibres to be produced and a gelatin concentration of 20% and flow rate of 15 $\mu\text{l}/\text{min}$ allowed 1306 ± 34 nm fibres to be produced. These will be denoted respectively as 300 nm, 600 nm, 1000 nm, and 1300 nm fibres. Figure 1(B-E) showed representative SEM micrographs of the samples prepared using the different process and solution parameters. The pore size of 300 nm fibres was significantly lower than the pore sizes of 600, 1000 nm and 1300 nm fibres (Figure 1A).

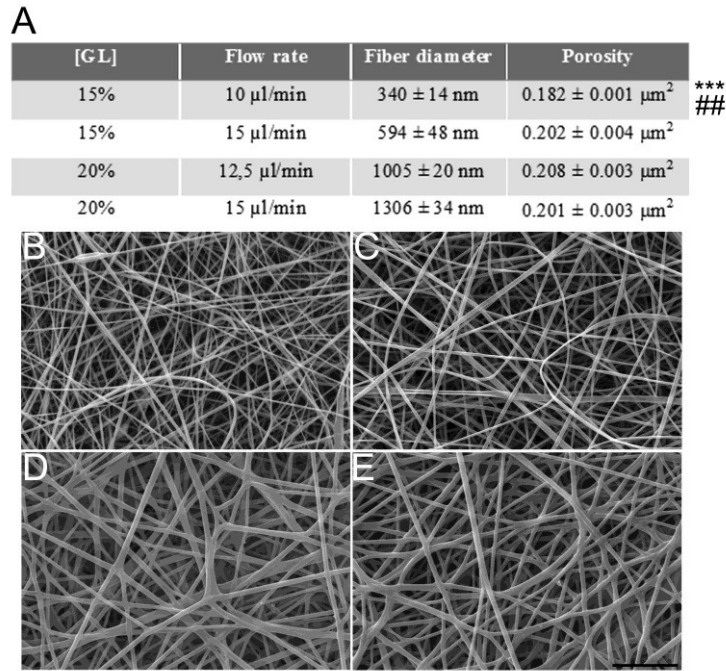


Fig. 1: The table summarizes the different spinning parameters used for gelatin fibre preparation, fibre diameter (mean nm $\pm$ standard deviation) and porosity (mean $\mu\text{m}^2 \pm$ standard deviation). One-way ANOVA was used for statistical analysis. Asterisks denote the statistical difference between the 300nm and the 600 nm and 1000 nm fibre matrices ($***p \leq 0.001$). Hashes denote the statistical difference in porosity between 300 nm and 1300 nm fibre matrices ($##p \leq 0.01$) (A). SEM micrographs of gelatin fibres obtained by varying the concentration and flow rate are presented. Gelatin fibres of 300 nm in diameter (B). Gelatin fibres of 600 nm in diameter (C). Gelatin fibres of 1000 nm in diameter (D). Gelatin fibres of 1300 nm in diameter (E). Scale bar: 10 μm .

Increasing fibre diameter did not affect the number of adherent cells but decreased the cell spreading area

Figure 2 shows the effect of fibre diameter on cell adhesion and the morphology of RT4-D6P2T and primary SC cultures. After 3, 6 and 24 h the adherent cells were counted and their morphology examined. Increasing the fibre diameter did not affect the number of adherent cells for either RT4-D6P2T (Figure 2P) or primary SC cultures (Figure 2R). However, the cell spreading area was decreased and the cells became more elongated, as evidenced by spreading area quantification (Figure 2 Q and S) and fluorescent microscopy images (Figure 2 A-O). This trend was maintained in all the time points tested (3, 6 and 24 h). For cells seeded under control conditions, 300 and 600 nm fibres induced a spread morphology while 1000 and 300 nm fibres induced a more elongated morphology (Figure 2 A-O). Fibre substrates appear green owing to auto-fluorescence.

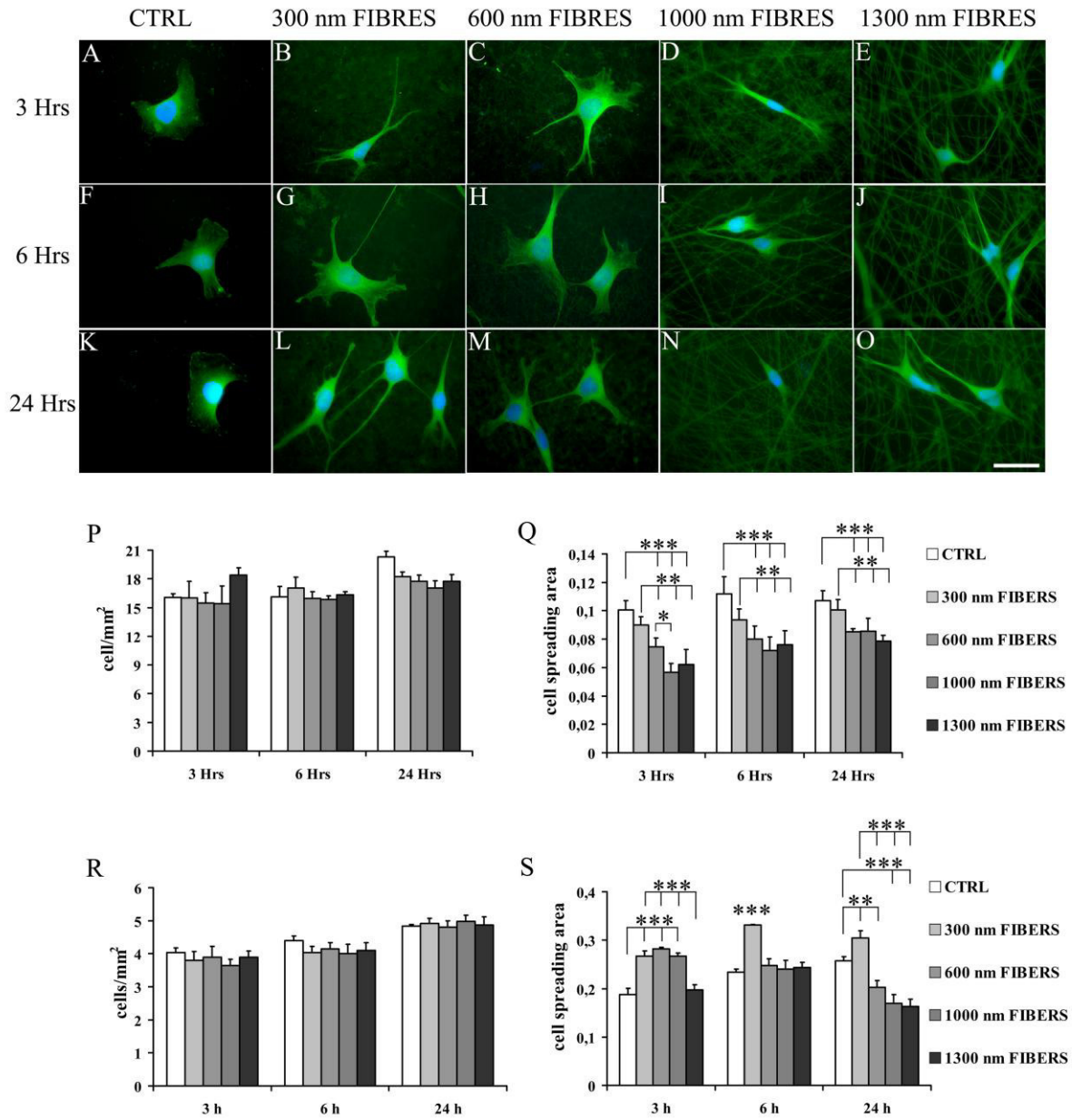


Fig. 2: Adhesion assay. Fluorescence images after S100 β (green) and DAPI (blue) staining of RT4-D6P2T on poly-L-lysine coated coverslips (control condition, A, F and K), 300 nm fibres (B, G and L), 600 nm fibres (C, H and M), 1000 nm fibres (D, I and N), and 1300 nm fibres (E, J and O) 3 (A-E), 6 (F-J) and 24 (K-O) h after seeding. Scale bar: 100 μ m. Numbers of adhering RT4-D6P2T (P) and primary SC cells (R) are reported. Cell number is expressed as cells/mm² $\pm$ standard deviation. Spreading area of RT4-D6P2T (Q) and primary SC (S) 3, 6 and 24 h after seeding. Spreading area is expressed as pixel area occupied by single cell $\pm$ standard deviation. One-way ANOVA was used for statistical analysis. Asterisks denote statistically significant differences with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Increasing fibre diameter resulted in lower actin cytoskeleton organization and fewer focal adhesion points

Both RT4-D6P2T (Figure 3) and primary SC (Figure 4) cultured under control conditions and on 300 nm fibres displayed high actin cytoskeleton organization and many focal adhesion points (Figure 3 A, B, F, G, K and L; Figure 4 A, B, F, G, K and L). Cells seeded on 600, 1000 and 1300 nm fibres exhibited lower actin cytoskeleton and fewer focal adhesion points and had a more elongated morphology (Figure 3 C-E, H-J, and M-O; Figure 4 C-E, H-J, and M-O). This trend was maintained at all the time points tested (3, 6 and 24 h). Finally, actin cytoskeleton staining showed that cells cultured under control conditions or 300 and 600 nm fibres displayed a spread morphology while those cultured on 1000 and 1300 nm fibres had an elongated morphology. These data accord with the adhesion assay results.

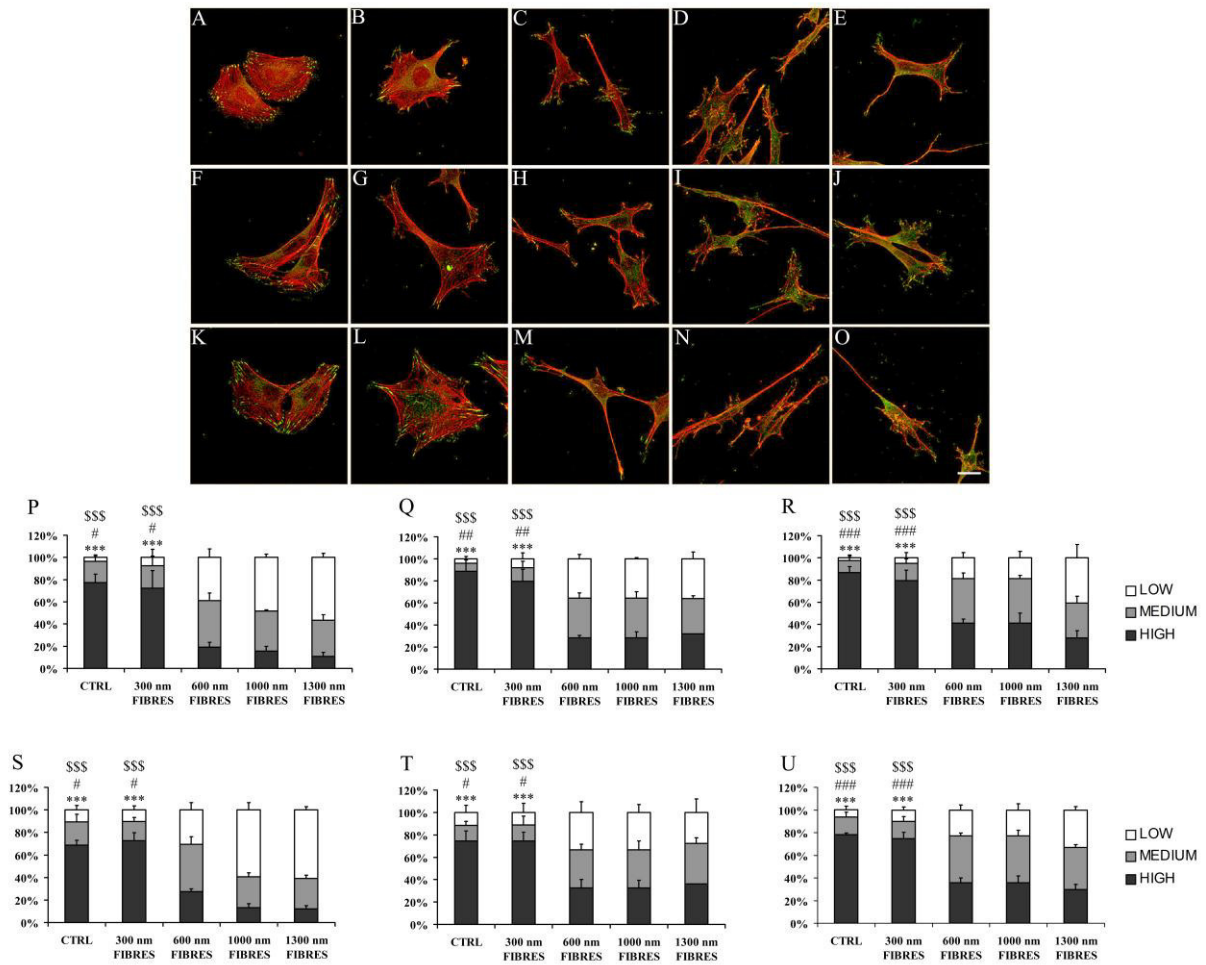


Fig. 3: Actin cytoskeleton organization and focal adhesion points. Fluorescence images following phalloidin-actin staining (red), vinculin (green) and DAPI (blue) staining of RT4-D6P2T on poly-L-lysine coated coverslips (control condition, A, F and K), 300 nm fibres (B, G and L), 600 nm fibres (C, H and M), 1000 nm fibres (D, I and N), and 1300 nm fibres (E, J and O) 3 (A-E), 6 (F-J) and 24 (K-O) h after seeding. Scale bar: 20 μm. Percentages of cells with low, medium or high actin cytoskeleton organization ± standard deviation 3 (P), 6 (Q) and 24 (R) h after seeding are reported. Percentages of cells with low, medium or focal adhesion points ± standard deviation 3 (S), 6 (T) and 24 (U) h after seeding are also reported. One-way ANOVA was used for statistical analysis with */#/\$ $p \leq 0.05$, **/##/\$\$ $p \leq 0.01$ and ***/###/\$\$\$ $p \leq 0.001$. Asterisks denote statistically significant differences between low actin cytoskeleton organization and focal adhesion point conditions. Hashes denote statistically significant differences between medium actin cytoskeleton organization and focal adhesion point conditions. Dollars denote statistically significant differences between high actin cytoskeleton organization and focal adhesion point conditions.

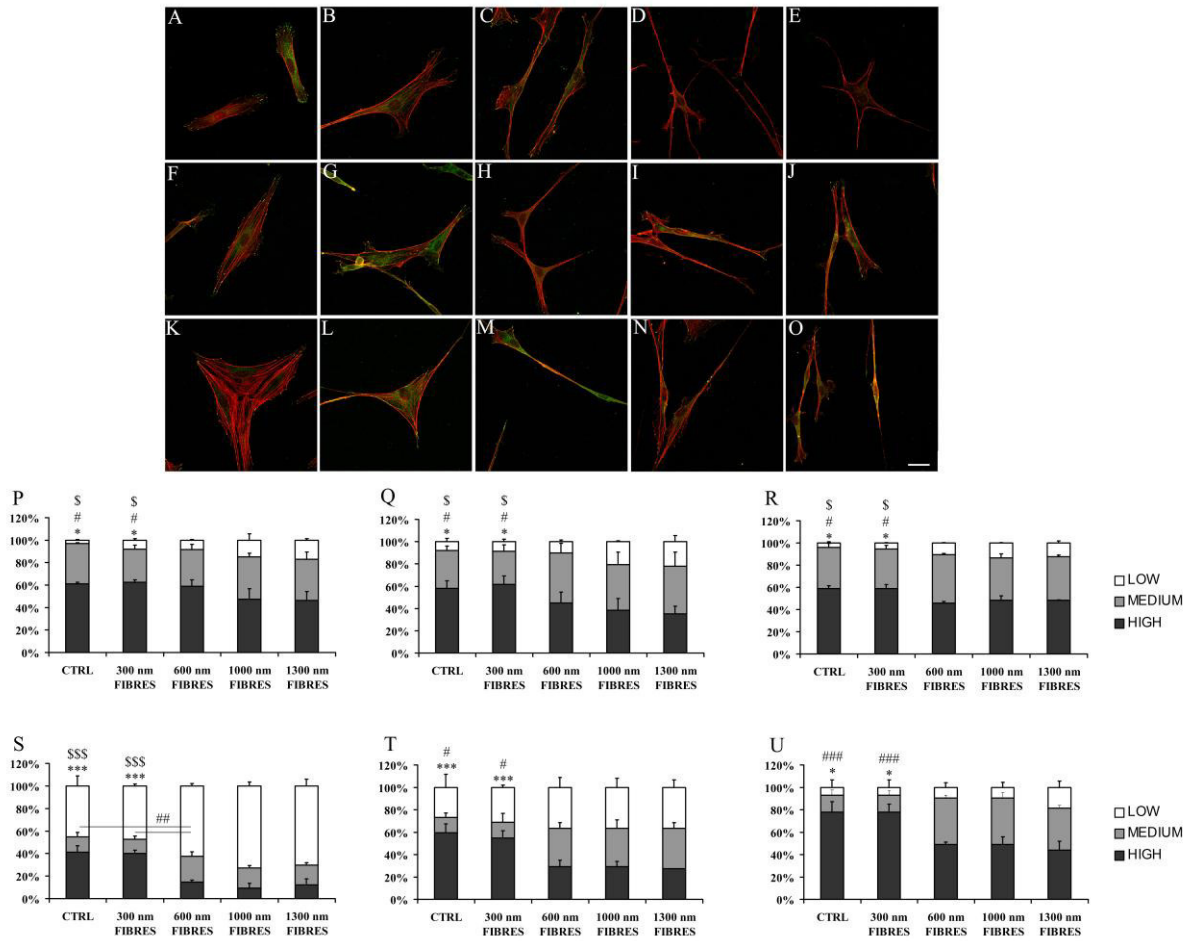


Fig. 4: Actin cytoskeleton organization and presence of focal adhesion points. Fluorescence images following phalloidin-actin staining (red), vinculin (green) and DAPI (blue) staining of SC primary culture on poly-L-lysine coated coverslips (control condition, A, F and K), 300 nm fibres (B, G and L), 600 nm fibres (C, H and M), 1000 nm fibres (D, I and N), and 1300 nm fibres (E, J and O) 3 (A-E), 6 (F-J) and 24 (K-O) h after seeding. Scale bar: 20 μ m. Percentages of cells with low, medium or high actin cytoskeleton organization $\pm$ standard deviation 3 (P), 6 (Q) and 24 (R) h after seeding are reported. Percentages of cells with low, medium or focal adhesion points presence $\pm$ standard deviation 3 (S), 6 (T) and 24 (U) h after seeding are also reported. One-way ANOVA was used for statistical analysis with */#/\$ $p \leq 0.05$, **/##/\$\$ $p \leq 0.01$ and ***/###/\$\$\$ $p \leq 0.001$. Asterisks denote statistically significant differences between low actin cytoskeleton organization and focal adhesion point conditions. Hashes denote statistically significant differences between medium actin cytoskeleton organization and focal adhesion point conditions. Dollars denote statistically significant differences between high actin cytoskeleton organization and focal adhesion point conditions.

Increasing fibre diameter enhances filopodia formation

Lamellipodia are motile organelles defined as a thin sheet of cytoplasm filled with a branched network of actin filaments arranged in a criss-cross pattern (Figure 5A). The width of a *lamellipodium* (commonly 0.1-0.2 μm) seldom exceeds 10 μm and the actin filament density decreases from the front to the back. *Filopodia* are rod-shaped cell protrusions filled with linear actin filaments and their formation often coincides with the presence of *lamellipodia*. *Filopodia* can reach 50 μm length (Figure 5B). Increasing fibre diameter led to more cells displaying *filopodia* in both RT4-D6P2T (Figure 5 C-E) and primary SC (Figure 5 F-H).

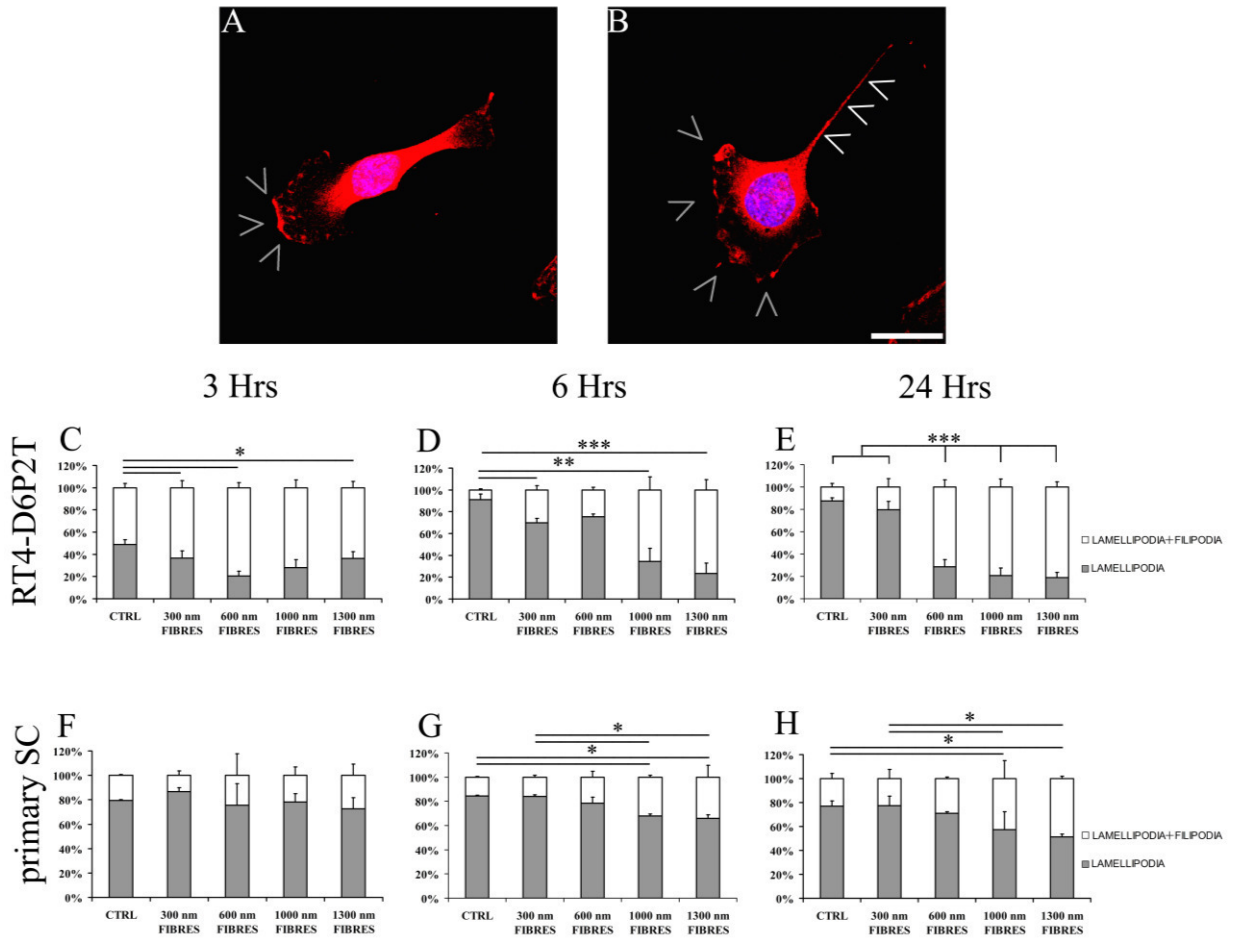


*Fig. 5: Lamellipodia and filopodia. Fluorescence images after S-100 β staining (red) and DAPI (blue) staining of RT4-D6P2T. Grey arrows indicate lamellipodium protrusion (A) whereas white arrows indicate filopodia protrusion (A and B). Scale bar: 20 μ m. Percentage of RT4-D6P2T cells characterized by lamellipodium and filopodia protrusions 3 (C), 6 (D) and 24 (E) h after seeding are expressed as mean $\pm$ standard deviation. Percentage of primary SC characterized by lamellipodium and filopodia protrusion 3 (F), 6 (G) and 24 (H) h after seeding are expressed as mean $\pm$ standard deviation. One-way ANOVA was used for statistical analysis. Asterisks denote statistically significant differences with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.*

Increasing fibre diameter increased cell motility and migration

Time lapse results showed no difference in cell motility between the control condition and 300 and 600 nm fibres for either RT4-D6P2T (Figure 6A) or primary SC (Figure 6B). In contrast, cells seeded on 1000 and 1300 nm fibres were more motile than those seeded on to 300 and 600 nm fibres (Figure 6). RT4-D6P2T cells migrated more extensively when seeded on 1300 nm fibres in comparison to 300 and 600 nm fibres (Figure 7A). Primary SC seeded on 1000 and 1300 nm fibres also migrated more extensively than those seeded on 300 nm fibres (Figure 7B).

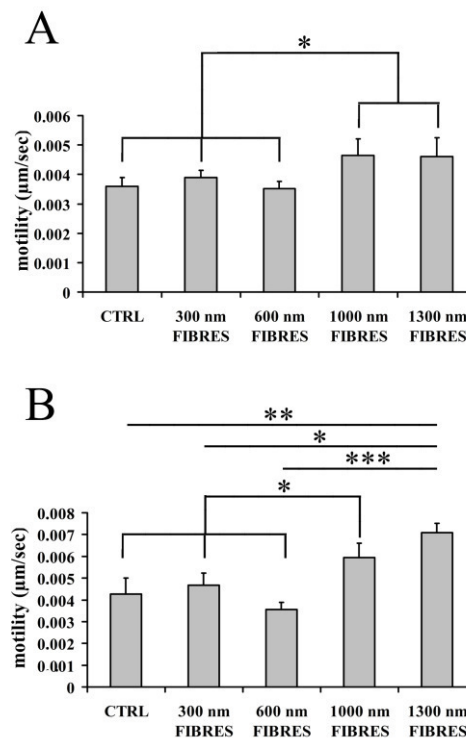


Fig. 6: Bi-dimensional motility: time lapse assay. RT4-D6P2T (A) and primary SC (B) motility is expressed as $\mu\text{m}/\text{sec} \pm$ standard deviation. One-way ANOVA was used for statistical analysis. Asterisks denote statistically significant differences with $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

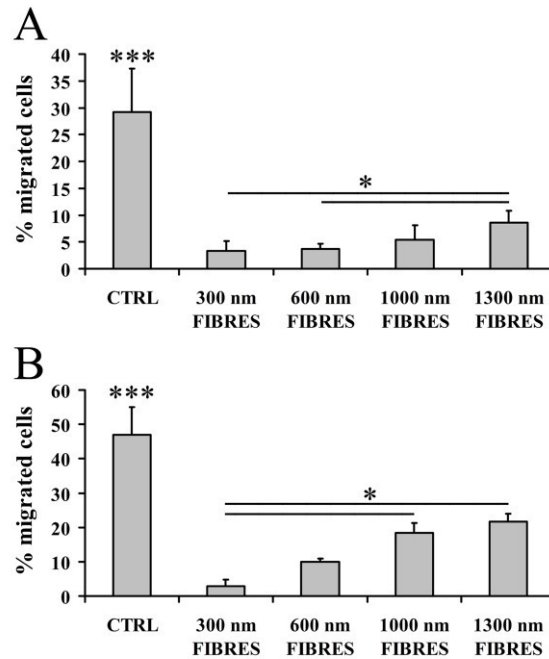


Fig. 7: Three-dimensional migration: transwell assay. RT4-D6P2T (A) and primary SC (B) migration are expressed as percentage of migrated cells $\pm$ standard deviation. One-way ANOVA was used for statistical analysis. Asterisks denote statistically significant differences with $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

Increasing fibre diameter reduced the cell proliferation rate

After 5 days, RT4-D6P2T cells had lower proliferation rates when seeded on 1300 nm fibres than under control conditions or seeded on 300, 600 and 1000 nm fibres (Figure 8U). After 7 days, primary SC had lower proliferation rates when seeded under control conditions and on 600, 1000 and 1300 nm fibres than those seeded on 300 nm fibres. Both RT4-D6P2T and primary SC cells reached confluence under in all the tested condition. Primary SC staining against S100 β revealed normal spread morphology at all time points tested, consistent with the adhesion assay (Figure 8 A-T). Similar results were obtained using RT4-D6P2T cells (data not shown).

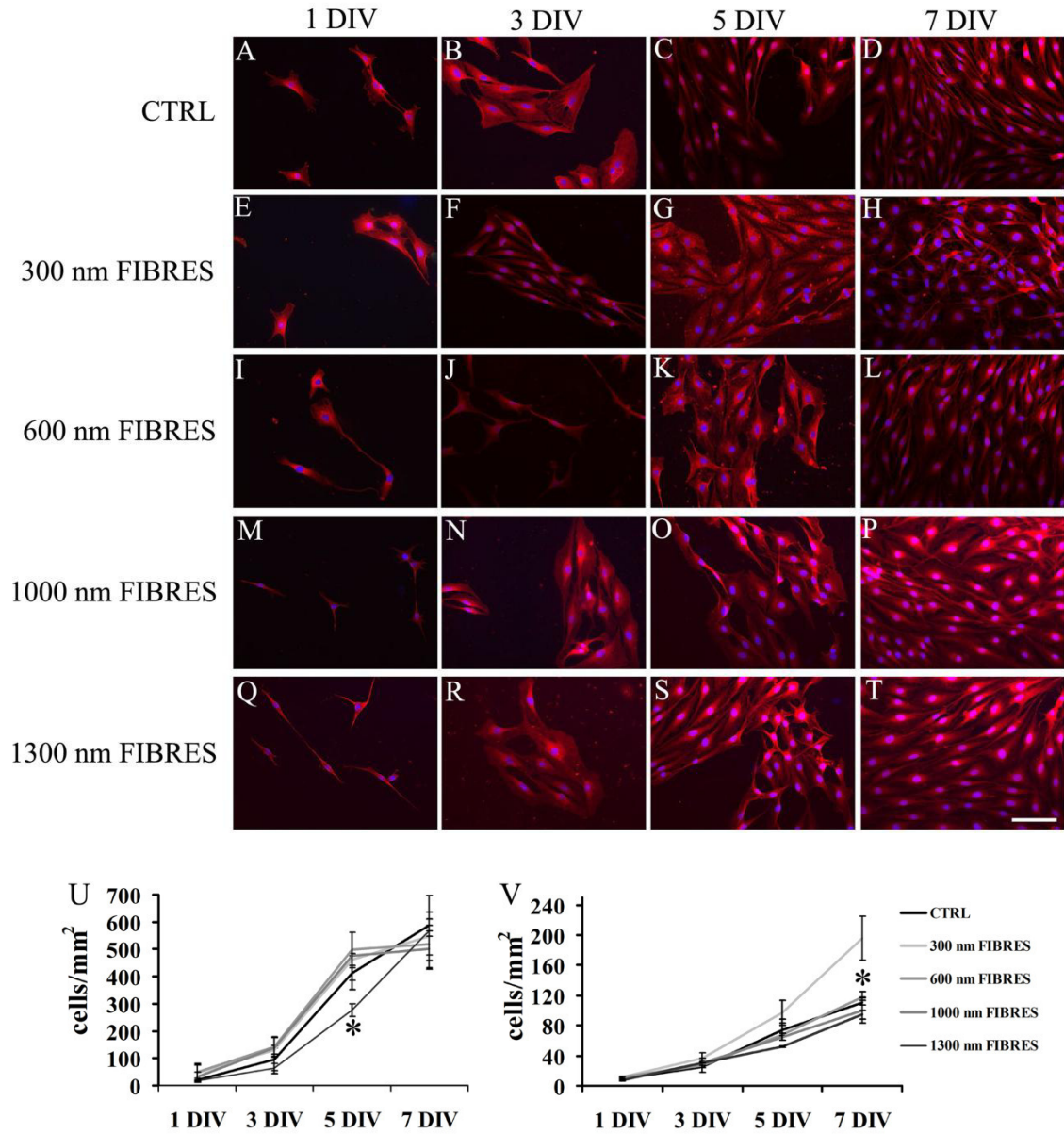


Fig. 8: Proliferation assay. Fluorescence images following S100 β (red) and DAPI (blue) staining of primary SC on poly-L-lysine coated coverslips (control condition, A-D), 300 nm fibres (E-H), 600 nm fibres (I-L), 1000 nm fibres (M-P), and 1300 nm fibres (Q-T) after 1 (A, E, I, M and Q), 3 (B, F, J, N and R), 5 (C, G, K, O and S) and 7 (D, H, L, P and T) days in vitro culture (DIV). Scale bar: 50 μ m. Proliferation rates of RT4-D6P2T (U) and primary SC cells (V) are reported. Cell number is expressed as cells/mm² $\pm$ standard deviation. One-way ANOVA was used for statistical analysis. Asterisks denote statistically significant differences with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Increasing fibre diameter enhanced axon outgrowth

No axon sprouted on DRG explants cultured on matrigel® or fibre matrices if no NGF was added to the culture medium (Figure 9 A and G). When NGF was added, axons sprouted under all conditions tested (Figure 9 B-F and H-L). Axon density was higher when DRG were cultured on 1000 and 1300 nm fibres than on 300 nm fibres, and there was no difference from the positive control condition (Figure 9M). DRGs cultured on 1300 nm fibres displayed higher spreading area than those cultured on 300 nm fibres. These data are consistent with axon density measurements (Figure 9N). Axon were shorten in DRG explants on 300 nm fibres than on 600, 1000 and 1300 nm fibres (Figure 9O).

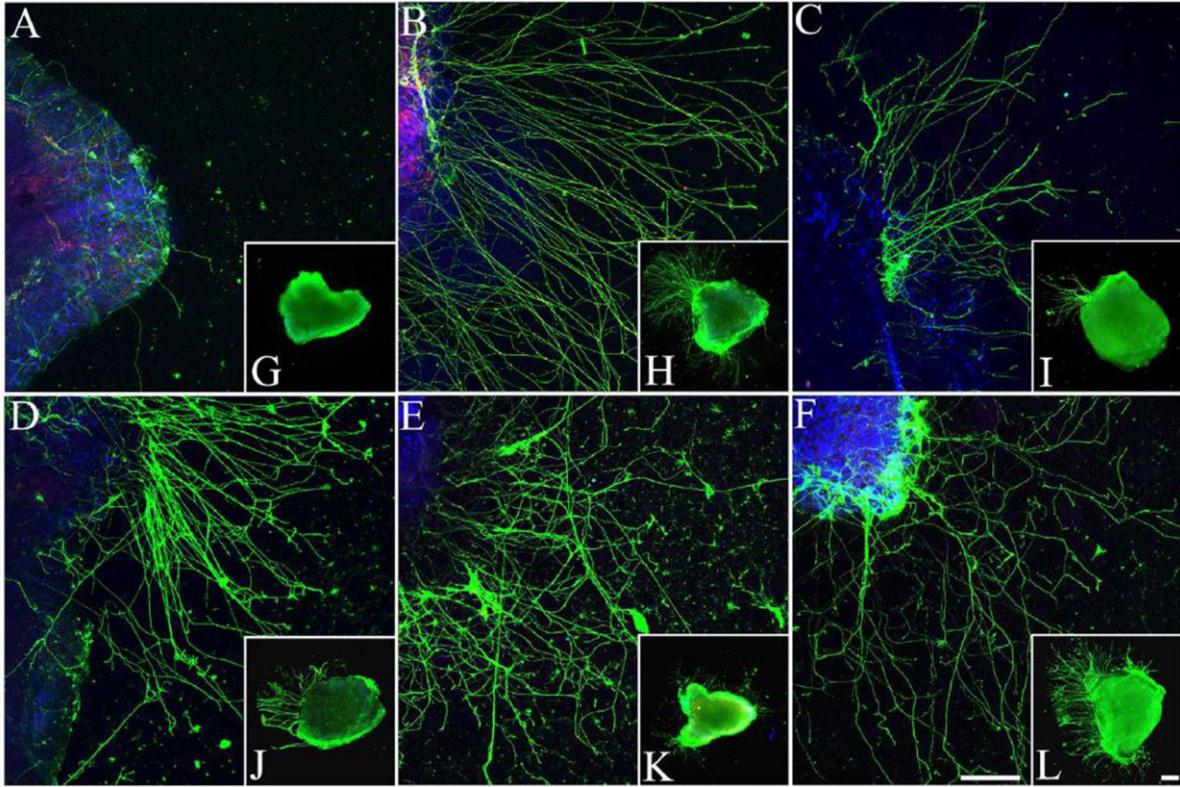


Fig. 9: DRG axon outgrowth. Fluorescence images after β -tubulin (green) and DAPI (blue) staining of DRG explants cultured on matrigel® coated coverslips without NGF (negative control conditions, A and G), matrigel® coated coverslips with 50 ng of NGF/ml in medium (positive control conditions, B and H), 300 nm fibres (C and I), 600 nm fibres (D and J), 1000 nm fibres (E-K), and 1300 nm fibres (F-L). Detailed confocal images (A-F) and fluorescence microscope images of the whole explants are presented (G-L). Scale bar: 20 μ m. Axon density expressed as $Kpixel \pm$ standard deviation (M), sprouting area expressed as DRG axon area/DRG axon area ratio $\pm$ standard deviation (N) and axon length as $\mu m \pm$ standard deviation (O) are reported. One-way ANOVA was used for statistical analysis. Asterisks denote statistically significant differences with $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

DISCUSSION

The distribution and growth of cells on a scaffold are key requirements for tissue engineering applications. Several studies have demonstrated that micro-to-nano-scale topography is important for controlling cell adhesion, survival, proliferation and differentiation (Chua et al., 2006, Dalby et al., 2006, Dalby et al., 2007, Bacakova et al., 2011, Harvey et al., 2013). The development of an artificial biomaterial scaffold mimicking native structure is important for improving peripheral nerve regeneration (Deumens et al., 2010, Harvey et al., 2013, Rahmany and Van Dyke, 2013). In particular, the regeneration phase needs an appropriate basal lamina to mediate the contact between migratory SC and regenerating axon (Letourneau et al., 1994, Ide, 1996, Ichihara et al., 2008). A major problem in generating an artificial scaffold mimicking the ECM and basal lamina is the use of native adhesion substrates, since they are animal derived materials and can cause immune rejection (Ciardelli and Chiono, 2006, Chiono et al., 2009, Siemionow and Brzezicki, 2009). Gelatin, a natural polymer obtained from thermal denaturation of collagen, is becoming widely used in biomimetic tissue engineering scaffold preparations because of its low immunogenicity (Ciardelli and Chiono, 2006, Chiono et al., 2009). Adequate fibrous substrates, functioning as an ECM, can be prepared easily by electrospinning, which allows random or aligned fibrous matrices suitable as internal filler for nerve guidance channels to be produced (Bhardwaj and Kundu, 2010). In a previous study (Tonda-Turo et al., 2013), the authors reported that electrospun gelatin fibres can be prepared by dissolving gelatin in water and using GPTMS as gelatin cross-linker. GPTMS- cross-linked gelatin nano-fibres yield substrates with high biocompatibility towards neural tissue and suitable biodegradation rates (Tonda-Turo et al., 2013).

In this work, different kinds of nano- and micro-fibres were successfully prepared by electrospinning by adjusting gelatin concentration and flow rate. The resulting nano-fibres had diameters ranging from 300 nm to 600 nm, whereas the micro-fibre diameters ranged from 1.0 to 1.3 μm . The nano-fibres (300 nm) had a lower porosity and a more intricate network (Mattila and Lappalainen, 2008, Parsons et al., 2010, Rottner and Stradal, 2011, Mullins and Hansen, 2013). In agreement with our previous study (Tonda-Turo et al., 2013), we provide further evidence on the biocompatibility of electrospun gelatin, in particular with respect to SCs, the key

element in peripheral nerve regeneration (Geuna et al., 2009). In addition, the results of this study provide new insights into the influence of fibre diameter on neural tissue organization and morphology. The different fibre diameters yield diverse three-dimensional scaffold architectures and porosities (distance between fibres), influencing cell organization by providing different substrate mechanical properties (stiffness and elasticity) and different topography and focal adhesions (FA) patterns (Engler et al., 2006, Saha et al., 2008, Parsons et al., 2010). It has been reported that soft hydrogels favour neurons survival whereas harder gels promote glial cultures. Soft gels also inhibit cell spreading, self-renewal, and differentiation (Saha et al., 2008, Leipzig and Shoichet, 2009). Another study reported that mesenchymal stem cells are sensitive to tissue elasticity and in particular that soft matrices mimicking the brain are neurogenic whereas stiffer matrices mimicking muscles are myogenic, suggesting that physical properties of the matrix play a key role in cell lineage commitment (Engler et al., 2006).

In addition to mechanical properties, substrate topography is important for controlling the adhesion, proliferation and survival of adult and the embryonic stem cells (Christopherson et al., 2009). Understanding the influence of micro-to-nano-scale topography on cellular behaviour is crucial for the design of functional scaffolds for neural tissue engineering. The strong correlation between fibre diameter and cell shape/morphology suggests that different ECM topographies modulate different cellular processes. The interactions of cells and their cytoskeletons with ECM proteins activate a variety of biological processes. A complex interplay between focal adhesions and actin cytoskeleton leads to the generation of membrane protrusions (*lamellipodia* and *filopodia*) and traction forces regulating cell shape, proliferation, motility and migration (Mitra et al., 2005, Mattila and Lappalainen, 2008, Fabry et al., 2011, Rottner and Stradal, 2011). Focal adhesion kinase (FAK) is central to the formation of FA points by interacting with integrins, paxillin, α -actinin and other proteins linking FAs to the actin cytoskeleton. It regulates several cytoskeletal and other FA proteins influencing cell motility, migration, morphology and contractility (Mitra et al., 2005, Parsons et al., 2010, Fabry et al., 2011, Mullins and Hansen, 2013). FAK can be activated (tyrosine-phosphorylated) by ECM components (through integrins). FAK phosphorylation causes the activation of several pathways such as:

Ras/Raf/MEK/Erk1-2, which regulate cell proliferation; Paxilin/Crk/Rac which regulates cytoskeleton reorganization, cell motility and migration; and PI3K pathway, which regulates cell survival (Defilippi et al., 1999, Mitra et al., 2005, Fabry et al., 2011). The results of this study demonstrated that SCs cultured on gelatin nano-fibres displayed a spread morphology whereas those cultured on micro-fibres were more elongated, in accordance to literature data (Qu, 2013). These findings are also consistent with reports that aminated nano-fibre meshes support cell adhesion better than aminated films (Chua et al., 2006). In addition, SC cultured on micro-fibres displayed a stretched shape with elongated actin filaments and fewer FA points organized at the cell extremities. The different SC spread morphology on nano-fibres may be due to the intricate network between fibres, which provides more FA points than micro-fibres (Agarwal et al., 2008). The assessment of actin filament organization, i.e. the so called stress fibres (Mattila and Lappalainen, 2008, Parsons et al., 2010), provides a possible explanation for the observed difference in cell morphology by showing that stress fibres are differently organized depending on gelatin fibres size, with a criss-cross arrangement on nano-fibres. This can be attributed to the higher fibre matrix density of nano-fibres (Agarwal et al., 2008, Venugopal et al., 2008) being a suitable basis for their spread morphology (Agarwal et al., 2008, Bacakova et al., 2011). These data are consistent with another study reporting that FAK-deficient cells display a rounded morphology with a smaller spreading area, a pronounced cortical distribution of the actin cytoskeleton and loss of stress fibres (Ren et al., 2000, Chen et al., 2002, Fabry et al., 2011). On the other hand, FAK rescue results in a larger spreading area and stress fibres reformation, probably attributable to formation of the correct FA pattern (Chen et al., 2002, Fabry et al., 2011). The formation of *lamellipodia* is associated with major actin filament polymerization and stress fibre formation (Mitra et al., 2005, Mattila and Lappalainen, 2008, Mullins and Hansen, 2013). Since we observed a difference in FA pattern and actin organization between cells seeded on nano-fibres and those seeded on micro-fibres we focused our attention on *lamellipodia* and *filopodia* formation. These structures are involved in SC migration and axon outgrowth (de Ruiter et al., 2009, Deumens et al., 2010, Xie et al., 2010, Rottner and Stradal, 2011) as well as myelination (Chan, 2007, Jin et al., 2011). *Filipodia* often originate from

lamellipodium protrusion and are important for cell migration and axon outgrowth (Chan, 2007, Mattila and Lappalainen, 2008, Xie et al., 2009, Jin et al., 2011, Rottner and Stradal, 2011). Our results showed that more SC develop *filipodia* if they are cultured on micro-fibres rather than nano-fibres. The constant remodelling of the actin cytoskeleton into *filopodia*, *lamellipodia*, and stress fibres is essential for cell migration (Mitra et al., 2005, Mattila and Lappalainen, 2008, Mullins and Hansen, 2013). Altogether the previously-discussed data suggest that micro-fibre substrates lead to higher motility and more migration. The pro-migratory effect of micro-fibres was confirmed by bi-dimensional and three-dimensional migration assays, which showed that micro-fibres promote greater cell motility and migration than nano-fibres. These results are in agreement with a previous *in vitro* study showing that a combination of micro-and nano-fibres produces better cell migration than nano-fibres alone (Shalumon et al., 2012). Finally, actin reorganization and stress fibre remodelling participate in cell division process (cell proliferation) (Mattila and Lappalainen, 2008, Mullins and Hansen, 2013). Our results showed that micro-fibres resulted in lower SC proliferation rate than nano-fibre substrates. This could be attributed to the different FA pattern and lower stress fibre organization presented by SC cultured on micro-fibers substrate and to the mechanical properties of the fibres as per the literature data discussed earlier (Engler et al., 2006, Saha et al., 2008, Leipzig and Shoichet, 2009). Our data are consistent with a study in which neural stem/progenitor cells displayed more rapid proliferation, greater cell spreading and less cell aggregation as the fibre diameter decreased (Christopherson et al., 2009). Altogether these results suggest that the mechanical properties and the topographical organization of the electrospun gelatin fibres influence SC morphology, organization, migration and proliferation by acting on the FA pattern and stress fibre polymerization in accordance with the previously discussed literature data (Ren et al., 2000, Chen et al., 2002, Chua et al., 2006, Engler et al., 2006, Saha et al., 2008, Christopherson et al., 2009, Leipzig and Shoichet, 2009, Parsons et al., 2010, Fabry et al., 2011). Finally, since both SC and re-growing axons are essential for nerve regeneration (Geuna et al., 2009), axon outgrowth on the different substrates was tested by culturing and stimulating DRG explants. More axons sprouted on the DRG explants if they were cultured on micro-fibre rather than nano-fibres substrates, and

this could have a negative effect on *in vivo* regeneration since excessive sprouting can cause aberrant regeneration (Eggers et al., 2013, Shakhbazau et al., 2013). Contrary to our results, other studies have demonstrated that neuronal differentiation, axonal outgrowth and cell viability are promoted significantly better by align nano-fibres than align micro-fibres (Xie et al., 2009, Wang, 2012, Qu, 2013). Moreover, DRG explants cultured on both align nano- and micro-fibres displayed longer and denser axons than those cultured on random fibres (Wang, 2012, Qu, 2013). However, these studies used a different fibre diameter range (400-800 nm) and different cell models such as human embryonic stem cell, central nervous system neurons and astrocytes, which could behave differently *in vitro* from peripheral nervous system cells. On the other hand, another study showed that axonal sprouting and maturation following rat sciatic nerve repair with a conduit containing align or align/ random nano-fibres was closed to isograft condition (Wang et al., 2009). On the basis of our *in vitro* and *ex vivo* results, it would be interesting to test *in vivo* the effect of a nerve guidance conduit filled with align fibres in combination with random fibres with different diameters. For peripheral nerve regeneration, an ideal biomimetic material should promote cell proliferation and adhesion as well as migration and axon outgrowth (de Ruiter et al., 2009, Geuna et al., 2009, Deumens et al., 2010). Recent studies have reported that nano-fibres improved functional recovery after rat sciatic nerve repair (Wang et al., 2009, Jiang et al., 2012) suggesting, that proliferation- and adhesion-promoting substrates are better for promoting peripheral nerve regeneration *in vivo*.

CONCLUSION

This study highlights the possibility of producing random GPTMS-cross-linked gelatine fibres of different diameters by electrospinning. Fibres with different topographies can be used *in vitro* and *ex vivo* to modulate Schwann cells and axons behaviour. In accordance with the previous study we confirmed that the prepared electrospun random fibres ranging from nanometre to micrometre size display biocompatible and biomimetic properties in relation to the main cellular components of the peripheral nerve, Schwann cells and axons. In this study we showed that random fibre size affects both Schwann cells and axon behaviour. In particular, electrospun micro-fibres promote Schwann cell migration and axonal outgrowth whereas electrospun nano-fibres promote Schwann cell proliferation and adhesion. Further *in vivo* investigations will be performed to determine the influence of the random fibre size on peripheral nerve regeneration. The probable conduit design will consist of an outer porous structure made of a 60/40 wt/wt blend poly(ϵ -caprolactone) and polyethylene oxide, which should ensure nutrient permeation and avoid fibroblast infiltration into the tube. In order to promote Schwann cell migration and differentiation and directional axon ingrowth, the conduit core will consist of an outer layer composed random fibres and an inner layer of aligned gelatine fibres. The inner layer of aligned fibres should influence Schwann cells migration and oriented organization and promote axonal growth parallel to the aligned fibres. The different fibre topography (size composition) of the outer conduit layer should inhibit scar tissue formation and influence Schwann cell differentiation and axon growth density. Thus, the use of topographically different fibres in different combination could help to modulate Schwann cell and axonal behaviour *in vivo* and thus promote better peripheral nerve regeneration.

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MY CONTRIBUTION TO THE MANUSCRIPT

I prepared electrospun gelatin fibres at the Department of Chemistry (University of Torino), and I performed and analysed most of the *in vitro* experiments. I participated to data interpretation and, to figure and manuscript preparation.

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CHAPTER 5

The effect of electrospun gelatin fibres alignment on Schwann cell and axon behavior and organization in the perspective of artificial nerve design

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ABSTRACT

Electrospun fibrous substrates mimicking extracellular matrices can be prepared by electrospinning, yielding aligned fibrous matrices as internal fillers to manufacture artificial nerves. Gelatin aligned nano-fibres were prepared by electrospinning after tuning the collector rotation speed. The effect of alignment on cell adhesion and proliferation was tested *in vitro* using primary cultures, the Schwann cell line, RT4-D6P2T, and the sensory neuron-like cell line, 50B11. Cell adhesion and proliferation was assessed by quantifying at several time-points. Aligned nano-fibres reduced adhesion and proliferation rate compared with random fibres. Schwann cell morphology and organization were investigated by immunostaining of the cytoskeleton. Cells were elongated with their longitudinal body parallel to the aligned fibres. B5011 neuron-like cells were aligned and had parallel axon growth when cultured on the aligned gelatin fibres. The data show that the alignment of electrospun gelatin fibres can modulate Schwann cells and axon organization *in vitro*, suggesting that this substrate shows promise as an internal filler for the design of artificial nerves for peripheral nerve reconstruction.

KEYWORDS

peripheral nerve injury, artificial nerve organs, gelatin nano-fibres, electrospinning, aligned fibres

INTRODUCTION

Peripheral nerve injury following trauma may lead to a substantial loss of nerve tissue, and long defect formation between the proximal and the distal nerve stump, rendering surgical intervention necessary. Currently, the gold standard for nerve repair involves bridging the nerve gap by using an autologous nerve graft taken from another part of the body. Because autograft techniques have several disadvantages, the use of artificial nerve organs composed of biomaterial may be the ideal choice among other options (Battiston et al., 2009, de Ruiter et al., 2009, Biazar et al., 2010, Deumens et al., 2010b, Geuna et al., 2013). Numerous substances, both of synthetic or natural origin, have been used to fabricate artificial nerve conduits (Ciardelli and Chiono, 2006, Willerth and Sakiyama-Elbert, 2007, Chiono et al., 2009, Tabesh et al., 2009, Deumens et al., 2010b). Synthetic non-degradable polymers, such as silicone, and synthetic biodegradable polymers, such as poly(lactic-co-glycolic acid) (PLGA), poly- ϵ -caprolactone (PCL), poly-L-lactic acid (PLLA) and conductive polymers (polypyrrole, polyaniline), have been used for nerve injury repair (Dahlin et al., 2001, Lundborg et al., 2004, Pfister et al., 2007, Jiang et al., 2010, Cunha et al., 2011). More recently, several natural biodegradable polymers, such as collagen, chitosan, alginate, elastin, silk, fibrins and gelatin have been used in tissue engineering due to their bioactivity, biocompatibility, low toxicity, tunable mechanical properties and degradation kinetics (Ciardelli and Chiono, 2006, Tabesh et al., 2009, Deumens et al., 2010b, Cunha et al., 2011, Xu et al., 2011). Natural biomaterials have been manipulated to obtain biomimetic materials, particularly the topography and the 3-D conformation being adjusted, to simulate extracellular matrix (ECM) (de Ruiter et al., 2009, Bacakova et al., 2011, Harvey et al., 2013, Rahmany and Van Dyke, 2013). Conduits that are Food and Drug Administration (FDA) and European Commission approved consist of biodegradable materials, among them NeurotubeTM, NeuraGenTM and Neurolac tubes made of poly(glycolide) (PGA), collagen and poly(dl-lactide- ϵ -caprolactone) respectively (de Ruiter et al., 2009, Deumens et al., 2010a, Deumens et al., 2010b, Kehoe et al., 2012). Collagen is the major connective tissue protein that is widely dispersed in the ECM of the peripheral nervous system (PNS) (Longo et al., 1984, Li et al., 2006). Over the past decades, collagen-based biomaterials have been widely used in tissue engineering (Parenteau-Bareil R.

Gauvin, 2010). Collagen is a biodegradable, biocompatible, highly versatile and a readily available polymer. Despite these advantages, it is hard to sterilize without altering its native structure (Chevallay and Herbage, 2000, Parenteau-Bareil R. Gauvin, 2010). Moreover, the use of collagen in the construction of artificial scaffolds might cause adverse immune responses (Parenteau-Bareil R. Gauvin, 2010). Gelatin may be used as an alternative to collagen in artificial organ preparation (Chiono et al., 2008, Santoro et al., 2014). Gelatin is produced by thermal denaturation or physical and chemical degradation of collagen. In comparison to collagen, gelatin has many advantages: it is biocompatible, biodegradable and does not induce immune rejection problems, maintaining molecular cues that may regulate cell behaviour (Chiono et al., 2008, Santoro et al., 2014). Cross-linking may also be used to modulate the mechanical, chemical and topographic properties of gelatin (Chiono et al., 2008, Tonda-Turo et al., 2013, Gnani et al., 2015). Both collagen and gelatin have been used in the preparation of different internal filler in artificial nerve conduit manufacturing, among them hydrogels and fibres (de Ruiter et al., 2009, Zhang et al., 2009, Gu et al., 2011, Tonda-Turo et al., 2013, Gnani et al., 2014, Gnani et al., 2015). Collagen is included as a scaffold in polycaprolactone tubular prostheses, resulting in stimulation of nerve regeneration in the rat sciatic nerve model (Maturana et al., 2013).

A thermosensitive collagen hydrogel mimicking ECM has been used as filler of poly-L-lactic acid scaffolds involving bone marrow mesenchymal stem cells (Huang et al., 2014). Collagen has also been used in an aligned collagen-glycosaminoglycan matrix preparation, mimicking Schwann cells (SCs) basal lamina that permits SC migration and repopulation (Shakhbazov et al., 2014). Oriented collagen fibres are included as fillers of collagen tubes, providing a guide for regenerating axons in an orientated manner to the distal nerve segment in rat sciatic nerve transaction model (Ma et al., 2014). Finally, the use of Revolnerv® collagen tubes for palmar digital nerve repair in human patients it has been reported; a 6-month follow-up showed that this did not improve regeneration in comparison to an uncoated direct suture (Arnaout et al., 2014).

Gelatin is now being used to prepare electrospun fibres (Zhang et al., 2009, Tonda-Turo et al., 2013, Gnani et al., 2015) and hydrogel (Hato et al., 2012, Li et al., 2013,

Gnavi et al., 2014) that have high biocompatibility towards SCs and axons. Cross-linked gelatin combined with ECM components and neurotrophins is a promising scaffold for SC and Dorsal Root Ganglia (DRG) (Gamez Sazo et al., 2012). Gelatin can also increase the biocompatibility of scaffolds used in neural tissue engineering (Gupta et al., 2009).

Fibres for internal fillers in artificial nerve conduits can be prepared through an electrospinning technique (Venugopal et al., 2008, Bhardwaj and Kundu, 2010, Xie et al., 2010). Several parameters can be adjusted to produce random or aligned fibres, these being classified into solution parameters (*i.e.* concentration, molecular weight, solvent and polymer type), solution properties (viscosity, surface tension and conductivity), process parameters (*i.e.* applied voltage, flow rate, collectors type and tip to collector distance), and ambient parameters (Bhardwaj and Kundu, 2010). Fibre alignment is mainly determined by the type of collector (flat or cylindrical) and its rotation speed (Venugopal et al., 2008, Bhardwaj and Kundu, 2010). The electrospun fibres produced by these methods may affect cell adhesion, morphology, proliferation and differentiation (Bacakova et al., 2011, Harvey et al., 2013, Rahmany and Van Dyke, 2013). Corrugated round fibre topography has given better adhesion and proliferation of C2C12 cells in comparison to solid round fibres (Bettahalli et al., 2013).

Human embryonic stem cell-derived neural precursors seeded on polycaprolactone (PCL) aligned fibres showed an elongated morphology with their longitudinal axis parallel to the direction of PCL fibres (Mahairaki et al., 2011). DRG cultured on PCL fibres extend their neurites without specific directionality when cultured on random fibres. On the other hand, the neurites grew along the long axis of the fibres cultured on aligned parallel fibres matrix (Xie et al., 2009). Aligned and random PCL/gelatine nano-fibres promote Schwann cell adhesion and growth in comparison to PCL nano-fibres (Gupta et al., 2009); both PCL/gelatin and PCL aligned fibres oriented cells along the longitudinal direction of the fibres (Gupta et al., 2009). PCL aligned nano-fibres coated with GRGDS and YIGSR peptide act guides, increasing actin filament alignment in Schwann cells (Zheng et al., 2015). Finally, poly(3-hydroxybutyrate) aligned fibres have been used to differentiate PC12 cells; cultured on aligned fibres

these cells had highly aligned and longer neurites in comparison to those on randomly oriented fibres (Genchi et al., 2015).

We chose gelatin to produce random and aligned electrospun nano-fibres by the electrospinning technique. The influence of the different electrospun nano-fibres topography and 3D-structure of several *in vitro* cell models has been studied. Since bands of Büngner formation by SCs and axons regrowth are the key elements in nerve regeneration, the RT4-D6P2T SC line, primary SC cultures and the neuron-like 50B11 cell line were used. Particularly, RT4-D6P2T line is an immortalized Schwann cell line derived from *N*-ethyl-*N*-nitrosourea (ENU) induced rat peripheral neurotumor. Primary SC cultures were obtained from rat sciatic nerves. The 50B11 cell line is an immortalized rat DRG sensory neuron cell line that can be induced to differentiate *in vitro*.

MATERIALS AND METHODS

Preparation of gelatin solution and nano-fibres

Gelatin (GL) solutions were prepared as previously described (Tonda-Turo et al., 2013). Briefly, gelatin (type A from porcine skin), (3-Glycidoxypropyl)methyldiethoxysilane (GPTMS) and polyethylene oxide (PEO, Mw 900 Kda) were supplied by Sigma Aldrich (Saint, MO, USA). Gelatin was dissolved in demineralised water at 50°C to the desired concentration (15% w/v); 137 µl GPTMS were added to the solution and mixed for 1 h before spinning (GL_GPTMS). GPTMS was selected as GL cross-linker to increase the water stability of nano-fibres (from few hours to up to 14 days) (Tonda-Turo et al., 2013). Compared to other GL cross-linkers, GPTMS was selected in this study since its cross-linking mechanism does not required a further step after fibres formation that could alter the fibre morphology, due to fibre swelling and partial or complete dissolution. A small amount of PEO was added to stabilize the polymer jet and to enhance aligned fibre formation. PEO was dissolved in distilled water to give a 15% w/v solution. GL/PEO blend with 90/10 volume ratio was prepared for aligned nano-fibre fabrication (GL/PEO_GPTMS).

Electrospinning of randomly oriented and aligned gelatin based nano-fibres

The electrospinning system consists of an isothermal chamber equipped with: a high voltage generator (PS/EL30R01.5-22 Glassman High Voltage, Inc., Hampshire, UK) giving a voltage up to 30 kV and a current up to 1.5 mA with reversible polarity; a volumetric pump (KDS210, KD Scientific, Hollistone, MA, USA); an electrode; a mobile syringe support; a syringe and different collectors. Electrospun scaffolds were prepared using a vertical electrospinning set-up and two different collectors were used: a 1.5 mm-thick flat aluminium collector for random fibres preparation, and a cylindrical rotating drum with an 8 cm diameter and a controllable rotating speed up to 3000 rpm for randomly oriented and aligned fibres deposition, respectively. Randomly oriented nano-fibres were obtained using a GL_GPTMS solution. The solution was spun at 50°C, 30 kV, flow rate 10 $\mu\text{l min}^{-1}$ and a nozzle-collector distance of 15 cm to yield fibres of 300 nm, as previously reported (Tonda-Turo et al., 2013). Aligned nano-fibres were collected on rotating mandrel. GL/PEO_GPTMS were spun at 50°C, 30 kV and the flow rate was increased to 17.5 $\mu\text{l min}^{-1}$ to obtain homogeneous nano-fibres. The influence of mandrel rotating velocity on fibre alignment was measured.

Scanning electron microscopy

The morphology of the electrospun matrices was examined through a scanning electron microscope (Standard Deviation Philips 525 M, SEMTech Solutions, Amsterdam, The Netherlands) at an acceleration voltage of 15 kV. The fibre samples were sputter-coated with gold. A magnification of 6000 was selected for 50 μm square fields, allowing fibre distribution to be recorded.

Pore and fibre diameters were quantified by analysing SEM micrographs with *ImageJ* software (National Institutes of Health, Bethesda, MD, USA), as previously reported (Christopherson et al., 2009, Tonda-Turo et al., 2013). For each fibre type, three images from three different samples were examined, and the diameters recorded as means $\pm$ standard error of the mean.

Nano-fibre orientation under different conditions was examined with a 2D Fast Fourier Transform (FFT) *ImageJ* processing tool. The applied processing tool shows

graphical peaks indicating predominant fiber orientation angles (Wu et al., 2010, Jha et al., 2011).

Cell culture

RT4-D6P2T cells and primary SC were grown on monolayers at 37°C in a humidified air atmosphere with 5% CO₂. RT4-D6P2T cells purchased from ATCC (American Type Culture Collection, Manassas, VA 20110-2209) were grown in complete high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Waltham, MA, USA) as per the ATCC protocol. SC for primary culture were isolated from the sciatic nerves of adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing 190-220g. All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Adequate measures were taken to minimize pain and discomfort, taking human endpoints for animal suffering and distress into account. The sciatic nerves were isolated, cut into 3 mm section and incubated at 37°C in air plus 5% CO₂ in a complete medium consisting of low glucose DMEM (Gibco, Waltham, MA, USA) supplemented with 100 units ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% heat-inactivated foetal bovine serum (FBS, Invitrogen) 63 ng/ml glial growth factor (GGF, R&D Systems, Minneapolis, MN, USA) and 10 µM forskolin (Sigma, Saint Louis, MO, USA). The medium was changed every 3 days. After 2 weeks, 2 ml digestion solution, consisting of 0.6 mg/ml collagenase type IV (Sigma) and 0.5 mg/ml dispase (Invitrogen) diluted in low glucose complete medium, was added. After 24 h the incubated nerve segments were transferred to a 50 ml tube and suspended in 5 ml low glucose complete medium. The cell suspension was filtered through a 70 µm strainer (BD Biosciences, San Jose, CA, USA), centrifuged at 900 rpm for 5 min, resuspended in 10 ml of complete SC medium and seeded on poly-L-lysine (PLL, Sigma) coated plates. To remove contaminating fibroblasts, the confluent SC were immunodepleted. Briefly, the confluent SCs were trypsinized and resuspended in 500 µl low glucose complete medium containing mouse anti-rat Thy1.1 antibody diluted 1:500 (Serotec, MCA04G) and incubated for 10 min at 37°C. Fresh rabbit complement (250 µl, Cederlane Labs, Burlington, ON, Canada) was added and incubated for 30 min at 37°C. The reaction

was blocked by adding 10 ml low glucose complete medium and the mixture spun for 5 min at 900 rpm. The pellet was resuspended in low glucose complete medium and seeded on PLL-coated plates. Confluent cells were harvested twice a week by trypsinization and seeded at the desired dilution on new plates.

50B11 cells, a gift from Dr. Ahmet Hoke (Chen et al., 2007), were grown on monolayers at 37°C in a humidified air atmosphere with 5% CO₂ in Neurobasal medium (Life Technologies, Gibco) supplemented with 10% FBS (Sigma-Aldrich), 2% B27 (Life Technologies), 0.22% glucose (Sigma) and 0.2mM glutamine (Sigma-Aldrich). Before cell seeding, fibre samples were sterilised overnight (O/N) by exposure to UV irradiation (wavelength 254 nm, UV lamp Technoscientific Co., Tokyo, Japan) and incubated in complete DMEM.

Adhesion assay

RT4-D6P2T and primary SC were seeded in complete DMEM at 4.000 or 8.000 cells/cm² on both PLL- (control condition) and gelatine fibre coated coverslips. After 3, 6 or 24 h, the culture medium was removed and the substrates with attached cells were rinsed with PBS containing Ca²⁺ and Mg²⁺ before being fixed by incubation with 4% paraformaldehyde (PFA, Sigma-Aldrich). After 20 min, the PFA solution was removed and the samples rinsed with PBS containing Ca²⁺ and Mg²⁺. The cells were permeabilized with 0.1% Triton X-100 diluted in PBS for 10 min and blocking solution (normal goat serum, NGS, diluted 1:100 in PBS DAKO X0907) was added for 1 h at room temperature. The cells were stained by O/N incubation with anti-vinculin rabbit polyclonal antibody (diluted 1:600 in PBS, Sigma), followed by 1 h incubation with FITC-conjugated phalloidin (diluted 1:1,000 in blocking solution, Millipore) at room temperature (diluted 1:1,000 in PBS, Millipore) and goat-anti rabbit IgG (H+L) AlexaFluor488 (diluted 1:200 in PBS, Invitrogen). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:1,000 in PBS.

The cells were photographed under an inverted fluorescence microscope Nikon Eclipse 80i equipped with a Nikon ECLIPSE 80i camera using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). Cell numbers were calculated using *ImageJ* software, averaged and expressed as the number of adherent cells/mm² + standard error of the mean (SEM).

Proliferation

RT4-D6P2T and primary SC were seeded in complete DMEM at 1000 or 2000 cells/cm² on both PLL- (control) and gelatin fibre coated coverslips. After 1, 3, 5, and 7 days, the cells were fixed, stained, photographed and counted as described above (Section 4.5). The number of cells counted for each assay was averaged and expressed as cells/mm² + standard error of the mean (SEM).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay

Potential biomaterial cytotoxicity was evaluated by the MTT assay. RT4-D6P2T and primary SC were plated in 0.2 ml of DMEM containing 10% FBS on both PLL- (control) and gelatine random or aligned nano-fibres (experimental group) coated 96-well tissue culture plate. After a 1, 3, 5 and 7 day incubation, 10 µl MTT substrate (Sigma, 5 mg/ml in phosphate buffered saline) was added, and the cells incubated at 37 °C for 4 h. The MTT solution was removed and cells washed twice with 0.1 ml of PBS. 0.1 ml of dimethyl sulfoxide (DMSO; Sigma) was added to each well to dissolve the formazan. Spectrophotometric absorbance was measured at 570 nm wavelength, using DMSO as the blank. Each assay was performed in triplicate.

50B11 differentiation

Cells were plated at low densities optimal for visualizing individual neurite growth on both PLL- (control) and gelatin fibre coated coverslips. Twenty-four hours after plating, the cells were differentiated by adding forskolin (Sigma–Aldrich, 75 µM) to the culture medium. Based on previous observations (Chen et al., 2007, Bhattacharjee et al., 2014), neuronal phenotype was most stable between 20 and 36 h post-forskolin treatment. Twenty-four hours after differentiation, cells were fixed, stained and photographed as described in (Section 4.5), using β-tubulin mouse mAb (diluted 1:100, in PBS, Sigma) and goat-anti-mouse IgG (H+L) AlexaFluor488 (diluted 1:200 in PBS, Invitrogen). Nuclei were stained with DAPI (diluted 1:1000 in PBS, Sigma).

Cell number and axon lengths were measured using *ImageJ* software as described elsewhere (Zamburlin et al., 2006, Gilardino et al., 2009). The cells counted for each

assay were averaged and expressed as cells/mm² + standard error of the mean (SEM). Axon length was expressed in μ m + standard error of the mean (SEM).

Confocal microscopy

Samples were observed with a Nikon Eclipse E800 epifluorescence microscope under appropriate filters and a Leica TCS SP5 confocal laser scanning microscope (Leica, Mannheim, Germany) using a 40 $\times$ Plan-NEOFLUAR objective (numerical aperture (NA) = 1.25) or 63 $\times$ Plan-NEOFLUAR objective (numerical aperture (NA) = 1.40). High resolution images were acquired (1024 $\times$ 1024 pixels) at 100 Hz.

Statistics

The experiments were repeated three times independently and included three sets of samples. Each set included three random nano-fibre matrix-, three align nano-fibre matrix- and three control PLL coated-coverslips. The data are expressed as mean $\pm$ standard error of the mean (SEM). GraphPad Prism® software was used for single-factor analysis of variance (ANOVA). Values of * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ were considered statistically significant.

RESULTS

Influence of mandrel collector speed on the alignment of the nano-fibres

Randomly oriented fibres with an average fibre dimension of 300 nm were fabricated as previously described (Tonda-Turo et al., 2013). Aligned GL/PEO_GPTMS (gelatin/polyethylene-oxide/(3-glycidoxypopyl)methyldiethoxysilane) nano-fibres were made using a rotating mandrel collector, and its rotation was varied from 0 to 2400 rpm to analyze the influence of this parameter on fibre alignment. FFT (2D Fast Fourier Transform) analysis of the SEM (Scanning Electron Microscopy) images was used to quantitatively analyze the degree of the GL based nano-fibres alignment. In FFT analysis, a graphical plot of frequency distribution was generated by summing the pixel intensities along the radius of the FFT output image obtained from the original SEM image. For a rotation of 2400 rpm, two sharp peaks were

observed at a distance of $\sim 180^\circ$, confirming the morphological data that showed a large number of fibres aligned in a preferential direction (Figure 1C). On the other hand, rotating at ~ 300 rpm produced no fibre orientation, confirmed by SEM image and FTT analysis (Figure 1A). Nano-fibres size was measured at different rotating mandrel speeds showing a slight reduction in the fibre average diameters when the speed is increased. The measured diameters 204 ± 48 nm for aligned nano-fibres using a rotating speed of 2400 rpm, 238.9 ± 74 nm for 1200 rpm and 254.7 ± 68.5 nm for 300 rpm. No significant differences in fibre size were observed from fibres collected on plane or rotating collector. Nano-fibres obtained using a rotating speed of 2400 rpm were used for cell test, as they showed a high degree of alignment on a preferential direction (as confirmed by FFT and SEM analysis).

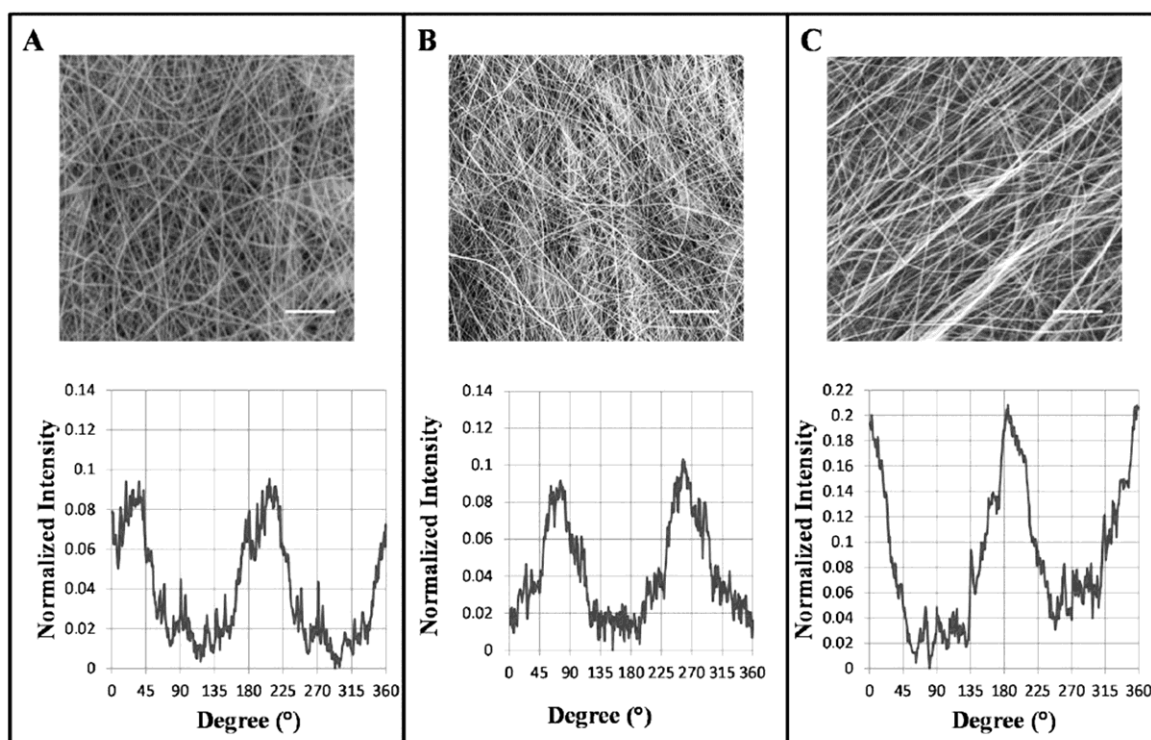


Fig. 1: Scanning electron microscopy (SEM) micrographs and 2D fast fourier transform (FTT) analysis of nano-fibres collected using rotating mandrel rates of 300 (A), 1200 (B) and 2400 (C) rpm.

Aligning gelatin nano-fibres decreased the number of adherent Schwann cells

RT4-D6P2T and primary SC cultures were seeded on control condition (poly-L-lysine coated coverslips), gelatin random fibres and aligned fibres. After 3 hour, the adherent cells were counted and their morphology examined. Figure 2 shows the effect of fibre alignment on cell adhesion and morphology. The alignment of gelatin electrospun fibres affected the number of adherent cells for RT4-D6P2T ($p<0.05$) (Figure 2C) and primary SC ($p<0.001$) (Figure 2D) cultures. When seeded on aligned fibres, there was less adhesion than under control conditions or random fibres. Both RT4-D6P2T and primary SC had high actin cytoskeleton organization and many focal adhesion points under all tested conditions. Its staining showed that both RT4-D6P2T and primary SC cultured on aligned fibres had elongated actin fibres compared to the control condition and random fibres (Figure 2A). Finally, cells had an elongated morphology with their longitudinal axis parallel to the direction of the aligned gelatin nano-fibres (Figure 2A).

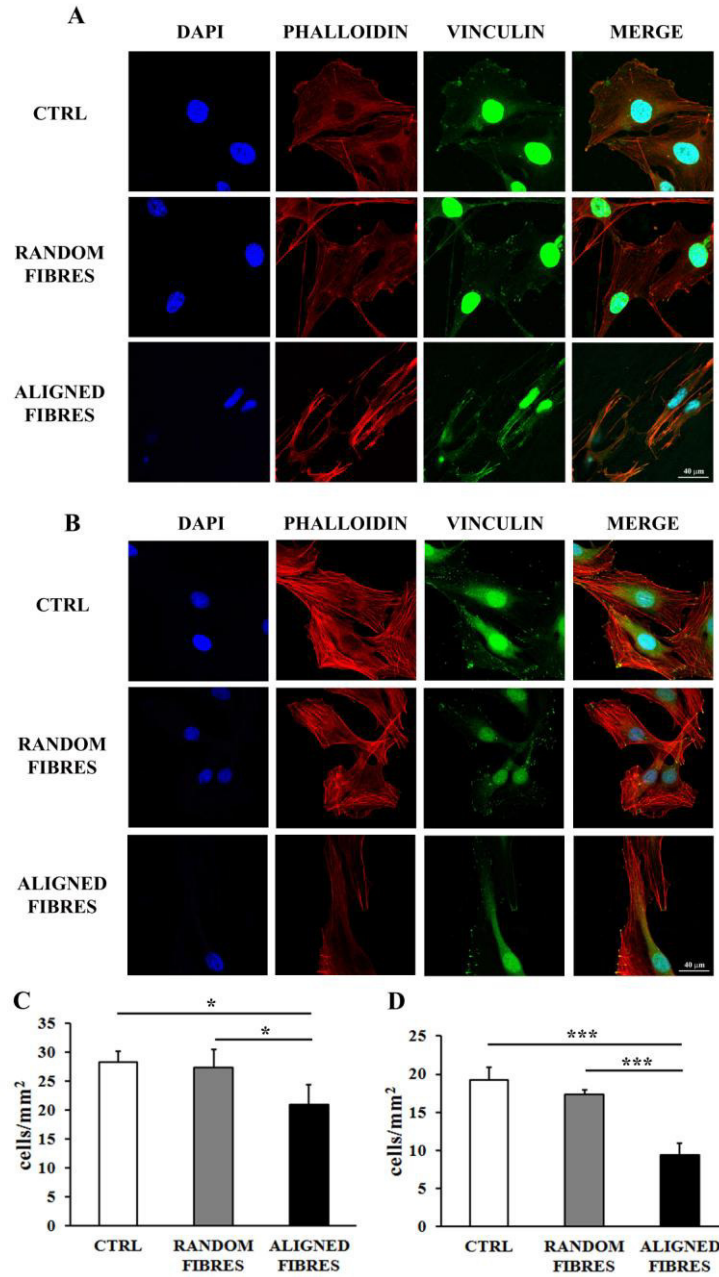


Fig. 2: Adhesion assay. Confocal images (63X magnification) after DAPI (blue), tetramethylrhodamine (TRITC)-conjugated phalloidin (red) and vinculin (green) staining of RT4-D6P2T (A) and primary SC (B) on poly-L-lysine coated coverslips (control condition), random fibres and aligned fibres 3 h after seeding. Scale bar: 40 μ m. RT4-D6P2T (C) and primary SC (D) cell numbers were expressed as cells/mm² $\pm$ standard error of the mean (SEM). Statistical analysis was carried out using one-way ANOVA. Asterisks refer to significant statistical differences with * $p \leq 0.05$ and *** $p \leq 0.001$.

Aligning gelatin nano-fibres reduced Schwann cell proliferation rate

After three ($p<0.001$), five ($p<0.001$) and seven ($p<0.001$) days, RT4-D6P2T cells proliferated more slowly when seeded on aligned fibres than under control conditions or random fibres (Figure 3C). After five ($p<0.01$) and seven ($p<0.01$) days, primary SC also had slower proliferation rates on aligned fibres than under control conditions or random fibres (Figure 3D). Both RT4-D6P2T and primary SC cells reached confluence under all tested conditions. Primary SC staining of actin showed normal spread morphology at all time-points, consistent with the adhesion assay data (Figure 3B), similar results being obtained with RT4-D6P2T cells (Figure 3A). Both RT4-D6P2T and primary SC cultured on aligned fibres had elongated actin fibres in comparison to control condition and random fibres at all time-points. Cells are organized in aligned bands with their longitudinal axis parallel to the direction of the aligned gelatin nano-fibres (Figure 3A-B). Finally, aligned gelatin fibres reduced Schwann cell proliferation rate after one, three, five and seven days (Figure 4). The MTT assay confirmed that Schwann cell were highly viable good vitality on both random and aligned fibres, indicating good biocompatibility of gelatin electrospun fibres.

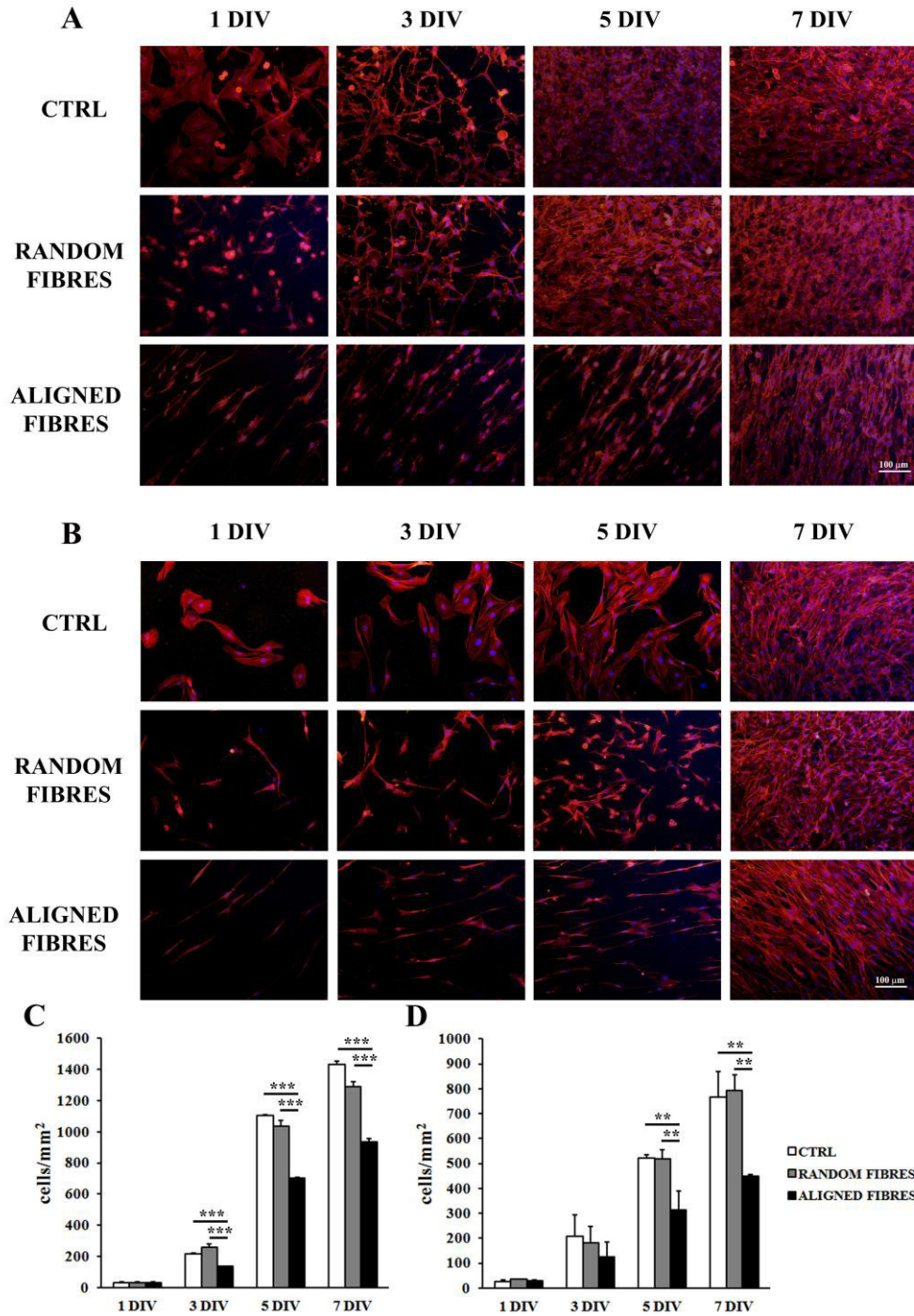


Fig. 3: Proliferation assay. Fluorescent images (20X magnification) after DAPI (blue) and phalloidin (red) staining of RT4-D6P2T (A) and primary SC (B) on poly-L-lysine coated coverslips (control condition), random fibres and aligned fibres 1, 3, 5, and 7 DIV (days in vitro) after seeding. Scale bar: 100 μm . RT4-D6P2T (C) and primary SC (D) cell number is expressed as $\text{cells}/\text{mm}^2 \pm$ standard error of the mean (SEM). Asterisks refer to significant statistical differences with $**p \leq 0.01$ and $***p \leq 0.001$.

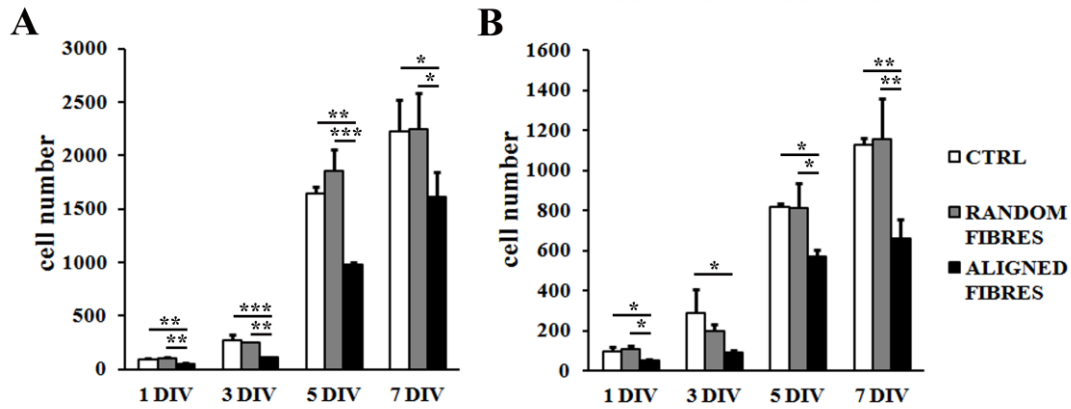


Fig. 4: MTT assay. RT4-D6P2T (A) and primary SC (B) were seeded on poly-L-lysine coated coverslips (control condition), random fibres and aligned fibres. 1, 3, 5, and 7 DIV (days in vitro) after seeding, cell viability was quantified. Asterisks refer to significant statistical differences with $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

Aligning gelatin nano-fibres resulted in neurites alignment

50B11 were seeded under control condition, and also on aligned and random fibres. After 24h the adherent cells were counted and their morphology examined by β -tubulin and DAPI staining. Addition of 75 μ M forskolin resulted in 50B11 differentiation under all tested conditions (Figure 5). The alignment of gelatin electrospun fibres did not affect the number of adherent 50B11 cells (Figure 5B). After forskolin, 50B11 cells stopped proliferating and started differentiating, resulting in a reduced cell number ($p < 0.001$) compared with non-treated conditions (Figure 5B). Immunostaining of β -tubulin showed that 50B11 cell maintain their ability to differentiate on gelatin electrospun fibres. Confocal images showed that aligned fibres made neurites align in parallel to the direction of the fibres. Neurites growth on aligned fibres was similar to random fibres and control condition (Figure 5A). There were no differences in 50B11 neurites length under all tested conditions (Figure 5C).

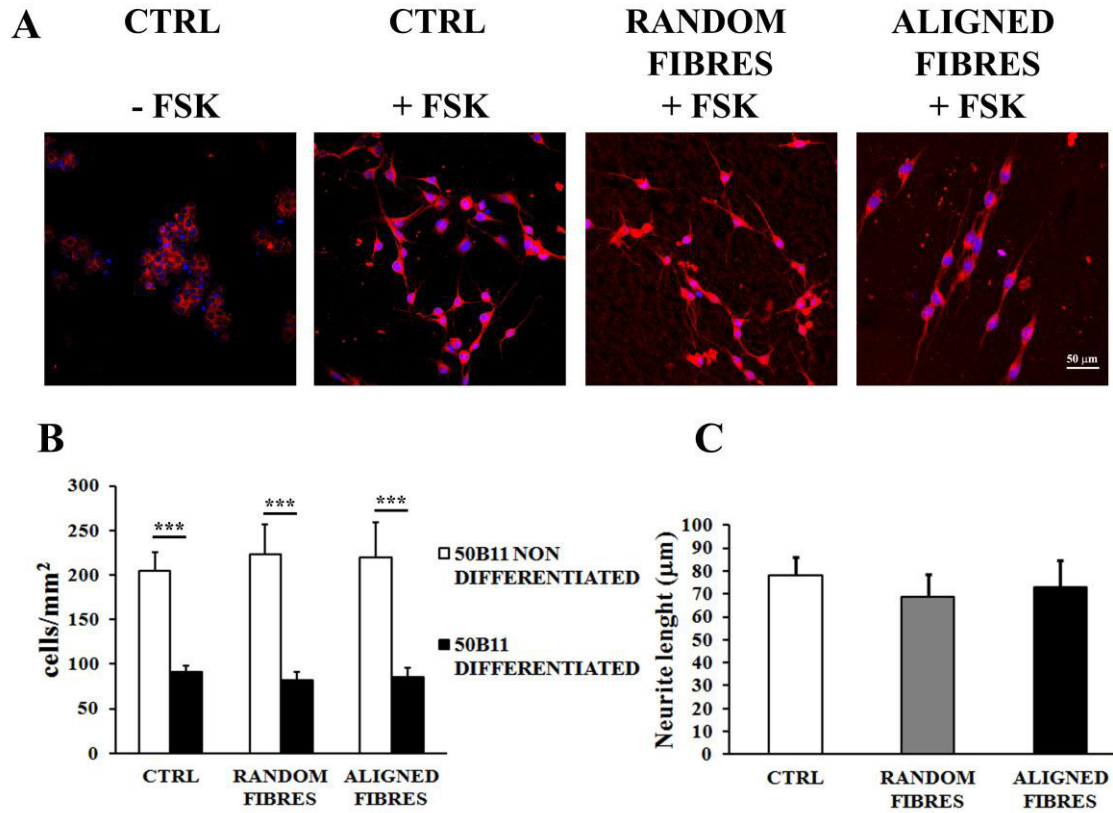


Fig. 5: 50B11 differentiation. Confocal images (40X magnification) after DAPI (blue) and β -tubulin (red) staining of 50B11 seeded on poly-L-lysine coated coverslips (control condition), random fibres and aligned fibres 24 h after forskolin treatment. Scale bar: 50 μ m. (A) 50B11 cell number is expressed as cells/mm² $\pm$ standard error of the mean (SEM) (B); neurite length is expressed in μ m (C). Asterisks refer to significant statistical differences with *** $p \leq 0.001$.

DISCUSSION

Interest in developing artificial nerve conduits composed of internal filler mimicking the extracellular tissue matrix has been increasing in recent years (de Ruiter et al., 2009, Nectow et al., 2012, Geuna et al., 2013).

Many synthetic and natural materials have been used in the fabrication of artificial nerve conduits (Ciardelli and Chiono, 2006, Willerth and Sakiyama-Elbert, 2007, Chiono et al., 2009, Tabesh et al., 2009, Deumens et al., 2010b). Initially synthetic non-degradable polymers, such as silicone, have been used to enhance nerve regeneration, even though they had several disadvantages for long-term recovery (Dahlin et al., 2001, Lundborg et al., 2004, Pfister et al., 2007, Jiang et al., 2010, Cunha et al., 2011). More recently, several natural bio-degradable-polymers, such as collagen (Chevallay and Herbage, 2000, Ciardelli and Chiono, 2006, Shakhbazau et al., 2014), chitosan (Xu et al., 2011, Gnavi et al., 2013) and gelatin (Chiono et al., 2008, Zhang et al., 2009, Gnavi et al., 2014, Santoro et al., 2014) have been used in tissue engineering applications due to their biocompatibility, mechanical and degradation properties (Ciardelli and Chiono, 2006, Tabesh et al., 2009, Deumens et al., 2010b, Cunha et al., 2011, Xu et al., 2011). These natural biomaterials have been used to obtain biomimetic materials displaying topography and the three-dimensional conformation similar to the ECM (de Ruiter et al., 2009, Bacakova et al., 2011, Harvey et al., 2013, Rahmany and Van Dyke, 2013).

Since collagen, the main component of ECM, has some disadvantages such as adverse immune response induction (Longo et al., 1984, Chevallay and Herbage, 2000, Li et al., 2006, Parenteau-Bareil R. Gauvin, 2010), gelatin may be a suitable alternative to be used in the construction of artificial nerve for tissue engineering applications (Ciardelli and Chiono, 2006, Chiono et al., 2008, Chiono et al., 2009, Gupta et al., 2009, Zhang et al., 2009, Tonda-Turo et al., 2013, Gnavi et al., 2014, Gnavi et al., 2015). The aim of this study was understand: (i) the effect of gelatin fibres on SC and neuron viability; and (ii) the influence of electrospun nano-fibre alignment on SC adhesion, morphology, proliferation and axonal growth. Since SC and neurons play an important role in peripheral nerve regeneration processes (de Ruiter et al., 2009, Deumens et al., 2010b, Gu et al., 2011) we used RT4-D6P2T SC line, primary SC culture and neuron-like 50B11 cell line to perform *in vitro* tests. In

order to understand if the alignment of gelatin electrospun align nano-fibres modulates SC organization and axonal growth PLL coated-coverslip and random nano-fibres have been used as control condition.

Increasing mandrel collector speed rotation resulted in fibres alignment

We were previously successful in using an electrospinning technique to spin water gelatin solution to prepare random nano- and micro-fibres (Tonda-Turo et al., 2013, Gnani et al., 2015). The use of aqueous solution for fibers preparation is known to reduce the risk of gelatin denaturation and nano-fibre cytotoxicity compared to organic solvents and acidic solutions. Homogenous nano-fibres with diameters of 200-300 nm were made and successfully aligned, using a rotating collector at 2400 rpm speed.

Fibres alignment reduced Schwann cells adhesion and proliferation but enhanced the alignment of Schwann cell actin filaments

In regard to cell adhesion, the reduced level in RT4-D6P2T and primary SC seeded on aligned nano-fibres may be due to the different fibre topography with which fewer focal adhesion points compared to random fibres (Agarwal, 2008, Bacakova et al., 2011). Yet SCs had higher actin cytoskeleton organization under all tested conditions, in accordance with our previous data (Gnani et al., 2015). In particular, the actin cytoskeleton of SCs was elongated fibres when cultured on aligned fibres. Moreover, cells were elongated with their longitudinal axis parallel to the direction of the aligned gelatin nano-fibres, in agreement with previous data (Gupta et al., 2009, Ma et al., 2014, Shakhbazov et al., 2014, Zheng et al., 2015). SC seeded on PCL/gelatin random fibres showed cell morphology similar to that on poly-lysine coated coverslips, whereas SC cultured on aligned nano-fibres resulted in aligned morphology, reaching confluence after 9/12 days of culture (Gupta et al., 2009). Ma et al. showed that oriented collagen fibre guide regenerating axons in an oriented way to the distal degenerating nerve stump, maximizing target reinnervation (Ma et al., 2014). Finally, aligned nano-fibres act as a potential guidance cue by enhancing the alignment of actin filaments, suggesting that these scaffolds would be useful in directing SCs for peripheral nerve regeneration (Zheng et al., 2015). The data suggest that the organization of SCs in aligned bands mimics the band of Bungers

formation and thus leads to improved nerve regeneration and functional recovery *in vivo* (de Ruiter et al., 2009, Gupta et al., 2009, Deumens et al., 2010b, Gu et al., 2011).

According to adhesion assay data, actin staining of SC showed normal spread morphology at all time-points. SCs proliferated more slowly when seeded on aligned fibres than under control conditions or random fibres. When seeded on aligned fibres, SCs are characterized by elongated actin fibre morphology (Zheng et al., 2015), maintaining an organization in aligned bands with their longitudinal axis parallel to the direction of the aligned gelatin nano-fibres.

MTT assay confirmed that Schwann cell were viable on both random and aligned fibres, demonstrating the biocompatibility of gelatin electrospun fibres (Tonda-Turo et al., 2013, Gnani et al., 2015). It has been reported that blending of gelatin with PCL results in better mechanical properties and hydrophilicity, enhancing nano-fibre biocompatibility towards SCs (Gupta et al., 2009).

Alignment of gelatin electrospun fibres did not affect neurite length but induced neurites alignment

50B11 sensory neurons-like cells have been used to study the influence of aligned nano-fibres on axonal growth. Alignment of gelatin electrospun fibres did not affect the 50B11 adhesion and proliferation rate. Moreover, 50B11 cells maintained their ability to differentiate on gelatin electrospun fibres; on aligned nano-fibre, this resulted in neurites alignment in parallel to the fibre direction. DRG neurites cultured on aligned PCL fibres grow parallel on the fibres, increasing their average length on aligned fibres in comparison with the random fibres (Xie et al., 2009). We found that different fibre orientation affects 50B11 neurite orientation, but does not affect neurite length. Neurite organization on aligned and random nano-fibres is in accordance with previous reports (Christopherson et al., 2009, Gupta et al., 2009, Xie et al., 2009, Shakhbazov et al., 2014, Zheng et al., 2015).

CONCLUSIONS

The results of the present study show that electrospinning can be used to prepare aligned nano-fibres. The aligned nano-fibres induces both primary SC and RT4-D6P2T SC line adhesion and growth in aligned bands with their longitudinal axis parallel to the direction of the fibres compared to control condition and random nano-fibre substrates. Moreover, SCs proliferate more slowly when seeded on aligned fibres than on random fibres. Finally, neurite outgrowth of 50B11 neuron-like differentiated cells runs parallel to the aligned fibres. Our data suggest that: (i) gelatin is a good biomaterial for SCs (both primary Sc and RT4-D6P2T SC line) and neurons viability; (ii) the topography of electrospun gelatin fibres can be adjusted to modulate SC organization and axon growth; (iii) aligned nano-fibres may be promising fillers for the design of artificial organs for peripheral nerve repair. Thus gelatin fibres may be used to induce a parallel orientation and growth of axons and SCs *in vivo* to stimulate bands of Büngner formation resulting in an enhancement of peripheral nerve regeneration.

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MY CONTRIBUTION TO THE MANUSCRIPT

I prepared electrospun random fibres, while aligned ones were prepared by our collaborators from the Politecnico di Torino. I participated to the interpretation of data, I performed the statistical analyses, I prepared the figures for the publication and I revised the manuscript.

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CHAPTER 6

In vitro evaluation of gelatin and chitosan electrospun fibres as artificial guide in peripheral nerve repair: a comparative study

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ABSTRACT

Random and aligned gelatin and nano-fibres have been prepared by electrospinning tuning the collector rotation speed. The effect of fibre alignment on cell adhesion and proliferation was assessed *in vitro* by using different Schwann cell and neuronal models. Moreover, actin cytoskeleton organization, lamellipodia and filipodia formation, and axon outgrowth were evaluated. Gelatin and chitosan fibres induced similar adhesion and proliferation rate. Gelatin and chitosan random fibres promoted higher adhesion and proliferation rates induction in comparison to the aligned ones, although gelatin and chitosan fibres alignment resulted in Schwann cell and axon oriented growth. Filipodia formation was higher on aligned fibres, suggesting that these substrates can promote higher cell migration in comparison to random ones. 50B11 (neuronal cell line) differentiation was higher on gelatin fibres, whereas no differences were observed in dorsal root ganglia explants model. These data suggest that both gelatin and chitosan fibres can be promising substrates to be used in peripheral nerve reconstruction.

KEYWORDS

Chitosan, gelatin, electrospun fibres, peripheral nerve repair, biomimetic materials, aligned fibres, random fibres

INTRODUCTION

In recent years, the research for effective biomimetic materials for peripheral nerve reconstruction has generated interest in the use of engineered electrospun fibres as alternatives to autografts (Ichihara et al., 2008, Siemionow and Brzezicki, 2009, Biazar et al., 2010, Gu et al., 2011). Electrospun fibre can be used as internal filler for hollow nerve guide. Electrospun random or aligned fibres can be easily prepared by electrospinning technique (Bhardwaj and Kundu, 2010). In the electrospinning technique, a polymer solution is forced into a capillary tube and subjected to an electric field. When the applied electric field reaches a critical value, the repulsive electrical forces overcome the surface tension forces and a charged jet of the solution is ejected from the capillary tip (Yarin et al., 2001, Adomaviciute and Milasius, 2007, Christopherson et al., 2009). Then, an unstable jet occurs in the gap between the Taylor cone and the collector, which leads to solvent and polymer fibre formation (Yarin et al., 2001, Adomaviciute and Milasius, 2007, Christopherson et al., 2009). Particularly, random fibres are collected on a fixed collector, whereas the orientation of nano-fibres along a preferred direction can be obtained by using a rapidly rotating cylindrical collector (Yarin et al., 2001, Adomaviciute and Milasius, 2007, Christopherson et al., 2009). Moreover, the diameter of fibres can range from nanometre to micrometre size depending on different parameters, such as polymer concentration and solution flow rate (Yarin et al., 2001, Adomaviciute and Milasius, 2007, Christopherson et al., 2009). The alignment of fibres is of interest for tissue engineering purposes in order to give to the cells a preferred growth direction. Several materials, both synthetic and natural, have been used in the preparation of electrospun fibres.

In the past years, many synthetic biodegradable polymers, polyglycolic acid (PGA) and polylactic acid (PLA) or polyhydroxybutyrate, and synthetic non-absorbable materials, such as silicone have been used as artificial nerve guides (Dahlin et al., 2001, Biazar et al., 2010). The Food and Drug Administration (FDA) and European Commission (EC) approved a number of synthetic (biodegradable and not degradable) and natural materials, among them Neurotube™, Neura-Gen™ and Neurolac tubes made of PGA, collagen and poly(DL-lactide- ϵ -caprolactone), respectively. These materials resulted in poor nerve repair and functional recovery in

comparison to autograft (Daly et al., 2012). Silicone nerve conduits have been clinically used providing mechanical support for peripheral nerve regeneration, although these conduits are not degradable and cannot be absorbed *in vivo* (de Ruiter et al., 2009, Carriel et al., 2014). Nerve conduits made of polyesters, such as PLA, poly- ϵ -caprolactone (PCL) and PGA have been used in nerve gaps repair (Deumens et al., 2010, Carriel et al., 2014). PCL nerve conduits have been widely used, although there are few clinical reports about their use in nerve reconstruction probably because of physical damage caused by this kind of conduit. PGA-based nerve conduits have excellent mechanical properties, however, they progressively lose their strength. Moreover, PGA hydrolysis decreases the pH that cannot be neutralized by the cellular microenvironment resulting in tissue inflammation and necrosis (Carriel et al., 2014). On the other hand, natural materials such as collagen, fibrin, glycoproteins and proteoglycans are highly biocompatible and biodegradable (Siemionow and Brzezicki, 2009, Deumens et al., 2010, Rahmany and Van Dyke, 2013, Carriel et al., 2014).

During the last years, in order to overcome the disadvantages of artificial materials, gelatine (GL) and chitosan (CS) scaffolds have been used as alternatives to synthetic polymers because of their biomimetic properties (Gnavi et al., 2013).

Biomimetic materials to be used in nerve reconstruction should reduce immunogenicity and inflammatory reactions, be biodegradable, support surgical manipulation, prevent collapse, display a certain degree of porosity to promote efficient exchange of nutrients and waste, and provide topological signalling to cells in order to facilitate reorganization of the inner configuration of the nerve (Ciardelli and Chiono, 2006, Chiono et al., 2009, Zhang et al., 2014). In recent years, a number of studies demonstrated that GL and CS are promising biomimetic materials (Ciardelli and Chiono, 2006, Gnavi et al., 2013). GL is a natural polymer obtained from the thermal denaturation of collagen. In comparison to collagen, GL is biocompatible, biodegradable, does not provoke immune-rejection reactions, and maintains molecular cues that regulate cell behaviour (Ciardelli and Chiono, 2006). However, GL can be dissolved in water, and the rapid degradation rate of GL can be the limiting factor of using GL in wound healing. Nevertheless, the degradation can be reduced by using cross-linking agents such as γ -glycidoxypolytrimethoxysilane

(GPTMS) or genipin, thus avoiding the use of acidic solutions and potentially cytotoxic solvents (Zhang et al., 2009, Tonda-Turo et al., 2013, Gnani et al., 2015a). CS is derived from chitin by performing alkaline hydrolysis of acetamide groups. In order to obtain CS a combination of high temperature (100°C) with strong aqueous alkali treatments are used to deacetylate chitin (Khor and Lim, 2003, Gnani et al., 2013). The major limitations in the employment of CS in biomedical applications are its low solubility at neutral pH and high viscosity (Khor and Lim, 2003, Gnani et al., 2013). Despite these disadvantages, CS has positive properties such as biocompatibility, biodegradability, and no toxicity that make this polymer suitable in the biomedical field (Khor and Lim, 2003, Gnani et al., 2013). Several CS products have been approved by the FDA. Moreover, CS has been used to produce laser-activated film surgical adhesive (SurgiLux) that can be used as an alternative to microsurgery for peripheral nerve repair (Foster and Karsten, 2012).

As previously mentioned, despite biocompatibility and biochemical composition, other parameters must be taken in account in innovative inner filler development. Particularly, the topography (three-dimensional architecture) of the inner filler influences cell organization of the regenerating tissue (Agarwal, 2008, Bacakova et al., 2011, Harvey et al., 2013). The properties of an internal filler such as fibre composition, diameter and orientation can be diversified to modulate Schwann cell (SC) and axon growth (Agarwal, 2008, Bacakova et al., 2011, Harvey et al., 2013). A number of studies have reported that topography plays an important role in controlling adhesion, proliferation, survival and migration of different cell types by affecting formation and distribution of focal adhesion plaques, actin cytoskeleton reorganization and/or lamellipodium and filopodia formation (Agarwal, 2008, Bacakova et al., 2011, Gnani et al., 2013, Harvey et al., 2013, Tonda-Turo et al., 2013, Gnani et al., 2015a).

In this study, we developed nano-fibrous matrices, made up of GL or CS, characterized by different fibres orientation (random or aligned). RT4-D6P2T schwannoma cell line, primary SC and dorsal root ganglia (DRG) neuronal 50B11 cell line cultures and DRG explants have been set up to perform the experiments. GL and CS fibres of 300 nm size were prepared, and *in vitro* and *ex vivo* studies were

performed to investigate whether the topography and polymer composition modulate SC adhesion, survival and proliferation, and neurite outgrowth.

MATERIAL AND METHODS

Gelatin and chitosan fibres preparation

Medical grade CS (Mw 200–400 kDa, deacetylation degree ≥ 92.6 %) was purchased by Kraeber GmbH, GL (type A from porcine skin), GPTMS and polyethylene oxide (PEO, Mw 900.000 Da), dibasic sodium phosphate (DSP), dimethyl sulfoxide (DMSO) and solvents were supplied from Sigma-Aldrich.

Cross-linked GL nano-fibres were prepared as previously described (Tonda-Turo et al., 2013, Gnani et al., 2015a, Gnani et al., 2015b). Briefly, for random fibres, a 15% w/v GL solution was obtained in demineralized water at 50 °C, and GPTMS was used as a GL cross-linker adding 137 μ L GPTMS for each 1.5 g of GL and mixing for 1 h before spinning. For aligned fibres, a small amount of PEO (90/10 volume ratio) was added to stabilize the polymer jet and to enhance aligned fibres formation.

CS 5 % (w/v) and 3% (w/v) PEO solutions were prepared separately by dissolving CS or PEO in 0.5 M acetic acid solution at room temperature by continuous stirring. After complete solubilization of each component, a 50/50 (v/v) CS/PEO solution was prepared by mixing equal volumes of CS and PEO solutions to obtain the mixtures with weight ratios of CS to PEO of 62/38; the resultant mixtures were kept under stirring about 2 h. A 5% (v/v) of DMSO was added to the CS/PEO solution as a co-solvent to relax CS chain entanglements and to increase the fibre yields, and consequently improve the spinnability of the CS-based solution (Bhattacharai et al., 2005). To increase water stability of nano-fibres, a 1 M solution of DSP was added (CS/PEO_DSP) with a concentration of 7.5 % v/v with respect to the natural polymer solution volume, as previously described (Ruini et al., 2015). Uncrosslinked solution was prepared as control. The final solution was coded as CS. CS nano-fibres were prepared through the electrospinning system using a voltage of 30kV, a temperature of 37 °C, a nozzle-collector distance of 12 cm and a flow rate of 30 μ L/min. Randomly

oriented nano-fibres were collected on a plane aluminum foil, while for aligned nano-fibres a rotating mandrel with a speed of 2400 rpm was used.

Both GL and CS nano-fibres were collected on glass coverslips and then sterilized by overnight (O/N) exposure to UV irradiation (UV lamp Technoscientific Co., wavelength 254 nm), and then incubated O/N with complete Dulbecco's modified Eagle's medium (DMEM; composition specified in "Cell cultures"), before proceeding with cell seeding.

Scanning electron microscopy and morphology evaluation of fibres

The morphology of fibres was investigated using Scansion Electronic Microscopy (SEM, LEO 1450VP). Prior to analysis, samples were sputtered using gold under vacuum for 100s by Agar Auto Sputter Coater. Then micrographs were analyzed through ImageJ software to measure the diameter of fibres. The fibre mats wettability was measured using a KSV instrument equipped with CAM 200 software for data acquisition at room temperature. A 5 μ l double-distilled water droplet and the sessile drop method was applied. For each sample, at least five measurements on different surface areas were acquired, and results were expressed as an average value $\pm$ standard deviation. The chemical composition of the fibres was detected through Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR). The FTIR-ATR spectra were recorded using a Perkin Elmer Spectrometer in the wavenumber range of 4000–600 cm^{-1} at a resolution of 4 cm^{-1} . Complete characterization of GL and CS nano-fibres has been described in previous work (Tonda-Turo et al., 2013).

Cell cultures

RT4-D6P2T SC line and primary SCs were grown on monolayers at 37 °C in a humidified atmosphere of 5% CO₂ as previously described (Tonda-Turo et al., 2013, Gnani et al., 2015a). RT4-D6P2T cell line was purchased from ATCC (American Type Culture Collection, 10801 University Blvd, Manassas, VA 20110-2209) and grown, according to the ATCC protocol, in complete medium consisting of high-glucose DMEM (Invitrogen) supplemented with 100units ml^{-1} penicillin, 0.1mg ml^{-1} streptomycin, 1mM sodium pyruvate, 2mM L-glutamine, 10%heat-inactivated fetal

bovine serum (FBS; Invitrogen). SCs for primary culture were isolated from 3 cm of both sciatic nerves harvested from three adult female Wistar rat (Charles River Laboratories, Milan, Italy), weighing approximately 190-220g. All procedures were in accordance with the European Communities Council Directive (2010/63/EU), with the National Institutes of Health guidelines, and with the Italian Law for Care and Use of Experimental Animals (DL26/14). Conformed measures were taken to minimize pain and discomfort, taking human endpoints for animal suffering and distress into account. The sciatic nerves were harvested, purified and cultured as previously described (Gnavi et al., 2015a, Gnavi et al., 2015b). Briefly, the sciatic nerves were isolated, cut into 3-mm section and incubated at 37°C in 5% CO₂ in complete medium consisting of low-glucose DMEM (Gibco) supplemented with 100units ml⁻¹ penicillin, 0.1mg ml⁻¹ streptomycin, 1mM sodium pyruvate, 2mM L-glutamine, 10%heat-inactivated FBS (Invitrogen), 63 ng/ml glial growth factor (GGF, R&D Systems) and 10μM forskolin (Sigma). The medium was changed every 3 days. After two weeks, 2 ml of digestion solution consisting of 0.6 mg/ml collagenase type IV (Sigma) and 0.5mg/ml dispase (Invitrogen) diluted in low-glucose complete medium were added. After 24 h, the incubated nerve segments were transferred to a 50-ml tube and suspended in 5ml low-glucose complete medium using a glass Pasteur pipette. The cell suspension was filtered through a 70 μm strainer (Falcon), centrifuged at 900 rpm for 5 minutes, re-suspended in 10 ml of complete DMEM medium and seeded on a poly-L-lysine (PLL; Sigma)-coated plate. In order to remove contaminating fibroblasts, the confluent SCs were immunodepleted. Briefly, the confluent SCs were harvested by trypsinization, re-suspended in 0.5 ml of low-glucose complete medium containing mouse anti-rat Thy1.1 antibody diluted 1:500 (Serotec, MCA04G), and incubated for 10 min at 37°C. Fresh rabbit complement (0.25 ml; Cederlane Labs) was added and incubated for 30 min at 37°C. The reaction was blocked by adding 10 ml low-glucose complete medium and 5 min centrifugation at 900 rpm. The pellet was re-suspended in low-glucose complete medium and seeded on PLL-coated plates. Confluent cells were harvested twice a week by trypsinization and seeded at the desired dilution on a new plate.

Adhesion and proliferation assay: 4,6-diamidino-2-phenylindole (DAPI) immunocytochemical detection of proliferating cells

RT4-D6P2T and primary SCs for adhesion assays were seeded in complete DMEM at 4000 and 8000 cells/cm², respectively. RT4-D6P2T and primary SCs for proliferation assays (DAPI immunocytochemical detection of proliferating cells) were seeded in complete DMEM at 1000 and 2000 cells/cm², respectively. Adhesion and proliferation assays were performed on both PLL- (control condition), random or aligned GL-fibre coated coverslips and random or aligned CS-fibre coated coverslips. After 3 h (adhesion assay), 1, 3, 5, and 7 days (proliferation assay), cells were fixed, stained, photographed and counted. Briefly, culture medium was removed and the substrates with attached cells were rinsed with phosphate-buffered saline (PBS) before being fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) as described before (Tonda-Turo et al., 2013, Carriel et al., 2015, Gnani et al., 2015a, Gnani et al., 2015b). After 20 min, the PFA solution was removed and the samples rinsed with PBS. The cells were permeabilized for 10 min with 0.1% Triton X-100 diluted in PBS, and blocking solution (normal goat serum diluted 1:100 in PBS DAKO X0907) was added for 1 h at room temperature. The cells were stained by O/N incubation with anti-vinculin rabbit polyclonal antibody (diluted 1:600 in PBS; Sigma), followed by 1 h incubation with FITC-conjugated phalloidin at room temperature (diluted 1:1000 in PBS, Millipore) and goat-anti rabbit IgG (H + L) AlexaFluor488 (diluted 1:200 in PBS; Invitrogen). Nuclei were stained with DAPI (Sigma) diluted 1:1000 in PBS. Cells were photographed under an inverted fluorescence microscope Nikon Eclipse 80i equipped with a Nikon ECLIPSE 80i camera using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). Cell numbers were calculated using ImageJ software, averaged and expressed as cells/mm² ± standard error of the mean (SEM). The experiments were repeated three times independently and included five sets of samples for each time-point. Each set included three control PLL-, three random GL fibre matrix-, three align GL fibre matrix-, three random CS fibre matrix-, and three align CS fibre matrix-coated coverslips.

MTT assay

RT4-D6P2T and primary SCs were plated in 0.2 ml of DMEM containing 10% FBS on both PLL- (control) and CS or GL random or aligned fibres (experimental group) as previously described (Tonda-Turo et al., 2013, Gnani et al., 2015a). In order to quantify the cell number, serial dilutions were performed by plating 1×10^3 , 2×10^3 , 4×10^3 , 8×10^3 , 1.6×10^4 , 3.2×10^4 , and 6.4×10^4 per well. After a 1, 3, 5 and 7 day incubation, 10 μ l MTT substrate (Sigma; 5 mg/ml in PBS) was added, and the cells incubated at 37 °C for 4 h. The MTT solution was removed and cells washed twice with 0.1 ml PBS. To dissolve formazan, 0.1 ml of DMSO (Sigma) was added to each well. Spectrophotometric absorbance was measured at 570 nm wavelength, using DMSO as the blank.

Evaluation of actin cytoskeleton organization

Cell adhesion and morphology were measured using immunocytochemistry staining against anti-vinculin and phalloidin. RT4-D6P2T and primary SCs were seeded, fixed and stained as described in “Adhesion and proliferation assay: DAPI immunocytochemical detection of proliferating cells”. Three, 6 or 24 hour time-points conditions have been performed and described elsewhere (Gnani et al., 2015a). For each sample, 100 cells were examined making a total of 300 cells for each experimental condition. An arbitrary score was given to each cell, and data were expressed as the percentage of cells displaying low, medium or high actin cytoskeleton organization or presence of focal adhesion points $\pm$ SEM. The entire procedure was carried out in a blind manner, as previously described (Gnani et al., 2015a).

Estimation of lamellipodia and filopodia formation

Lamellipodia and filopodia formation was evaluated by direct microscopy of cells stained with anti-S-100 β rabbit polyclonal antibody. The cells were seeded, fixed and stained as described in “Adhesion and proliferation assay: DAPI immunocytochemical detection of proliferating cells”. Three, 6 or 24 h time-point conditions have been performed as described before (Gnani et al., 2015a). For each sample, 100 cells were examined, making a total of 300 cells for each experimental

condition. The data were expressed as percentages of cells displaying only lamellipodia or lamellipodia and filopodia $\pm$ SEM. The entire procedure was carried out in a blind manner, as previously described (Gnavi et al., 2015a).

50B11 culture and differentiation

To visualize individual neurite growth, cells were plated at low densities on both PLL- (control), random or aligned GL-fibre coated coverslips and random or aligned CS-fibre coated coverslips. Twenty-four hours after plating, cells were differentiated by adding forskolin (Sigma-Aldrich; 75 μ M) to the culture medium as previously described (Gnavi et al., 2015b). Twenty-four hours after differentiation, cells were fixed, stained and as described in “Adhesion and proliferation assay: DAPI immunocytochemical detection of proliferating cells”, using β -tubulin mouse mAb (diluted 1:100, in PBS; Sigma-Aldrich) and goat-anti-mouse IgG (H + L) Cy3 (diluted 1:200 in PBS; Invitrogen). Nuclei were stained with DAPI (diluted 1:1000 in PBS; Sigma-Aldrich). Cell number and axon lengths were measured using ImageJ software. Cell number and percentages of differentiated or undifferentiated cells were evaluated in both differentiated and undifferentiated conditions. Counted cells for each assay were averaged and expressed as cells/mm² $\pm$ SEM. Axon length was expressed in μ m $\pm$ SEM.

DRG explants

Dorsal root ganglia explants were harvested from adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing approximately 190–220g. All procedures were in accordance with the European Communities Council Directive (2010/63/EU), with the National Institutes of Health guidelines, and with the Italian Law for Care and Use of Experimental Animals (DL26/14). Conformed measures were taken to minimize pain and discomfort, taking human endpoints for animal suffering and distress into account. Explants were cultured on coverslips coated with the different fibre matrices. Matrigel-coated coverslips were used as negative control. In order to avoid the detachment of the explants 100 μ l Matrigel® was added to the top of the cultures. After 1 h of incubation at 37 °C to allow the Matrigel® to polymerize, complete F12-BME medium (2 ml) was added. Complete F12–BME medium is

composed of F12 and BME media (Gibco) in a 50:50 ratio containing 1% bovine serum albumin (BSA, Sigma-Aldrich), 0.5 g/ml D-glucose (Sigma-Aldrich), 100U/ml penicillin and streptomycin (Sigma-Aldrich), 1mg/100 ml putrescine (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 2 ml of 100× insulin–selenium–transferrin (Gibco) and 0.125 ng/ml vitamin C. Culture medium was added, either without growth factors (negative control) or containing 50ng nerve growth factor (NGF)/ml (positive control, Millipore).

After 48 h of incubation, the explants were fixed with 4% PFA, for 20 min at room temperature. Axons were stained immunocytochemically as described in “Adhesion and proliferation assay: DAPI immunocytochemical detection of proliferating cells”, using β -tubulin mouse mAb (diluted 1:100, in PBS; Sigma-Aldrich) and goat-anti-mouse IgG (H + L) AlexaFluor488 (diluted 1:200 in PBS; Invitrogen). Nuclei were stained with DAPI (diluted 1:1000 in PBS; Sigma-Aldrich). For quantification, whole explants were acquired through an inverted optical video-confocal microscope (ViCo, Nikon Eclipse 80i) equipped with a Nikon ECLIPSE 80i camera using Image-Pro Plus 6.0 (Media Cybernetics USA). Axon density, sprouting area and axon lengths were measured using by ImageJ software as described elsewhere (Zamburlin et al., 2006, Gilardino et al., 2009). Axon density was expressed as pixels occupied by axons $\pm$ SEM. Sprouting area was expressed as the ratio between DRG axon occupied area and DRG body area $\pm$ SEM. Axon length was expressed in $\mu\text{m} \pm$ SEM.

Confocal microscopy

Samples were observed with a Nikon Eclipse E800 epifluorescence microscope under appropriate filters and a Leica TCS SP5 confocal laser-scanning microscope (Leica, Mannheim, Germany) using a 40× Plan-NEOFLUAR objective [numerical aperture (NA) = 1.25] or 63× Plan-NEOFLUAR objective [numerical aperture (NA) = 1.40]. High-resolution images were acquired (1024 × 1024 pixels) at 100 Hz.

Statistics

The experiments were repeated three times independently (biological triplicate) and included five sets of samples. Each set (technical triplicate) included three control PLL-, three random GL fibre matrix-, three align GL fibre matrix-, three random CS

fibre matrix-, and three align CS fibre matrix-coated coverslips for each time-point or growth factor condition. The data are expressed as mean $\pm$ SEM. GraphPad Prism® software was used for one- or two-way analysis of variance (ANOVA). Values of * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were considered statistically significant.

RESULTS AND DISCUSSION

Gelatin and chitosan polymers can be easily used to prepare electrospun nano-fibres

Gelatin and CS nano-fibres were obtained with no defects on the fibre surface (Figure 1). Despite the differences in materials and parameters used, the average diameter of the different fibres was similar and did not show significant differences (Table 1).

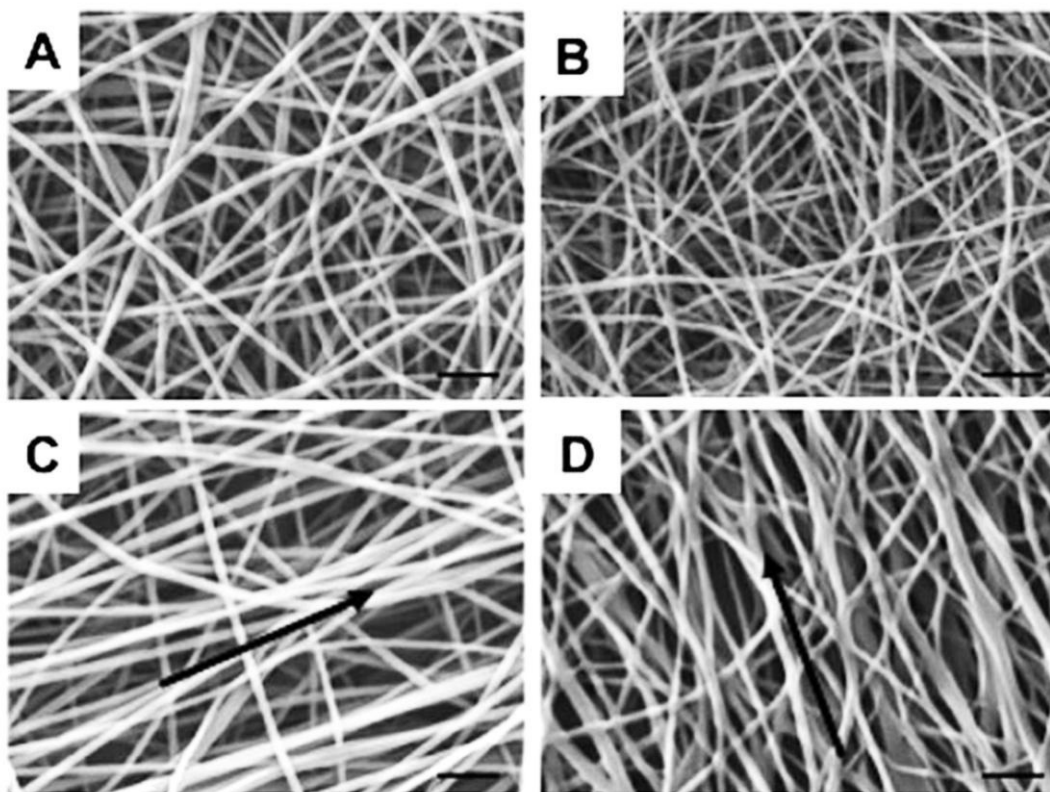


Fig. 1: Scanning electron micrographs of randomly oriented GL (A) and CS (B) nano-fibres and of aligned GL (C) and (D) CS nano-fibres. Arrows indicated aligned fibres orientation. Scale bars: 2 μm .

	GL random fibres	GL aligned fibres	CS random fibres	CS aligned fibres
Fibre size (nm)	242± 35	204 ± 48	167 ± 34	159± 41
Contact angle (°)	37±5	44±3	49±7	56±4

Table 1. Average size and contact angle of GL and CS nano-fibres.

A slight reduction of CS wettability compared to GL was detected due to the lower hydrophilicity of CS compared with GL. The FTIR-ATR spectra (Figure 2) showed the characteristic peaks of the constituent materials as reported in Tables 2 and 3. No differences were observed for randomly oriented and aligned fibres and data were reported only for randomly oriented samples.

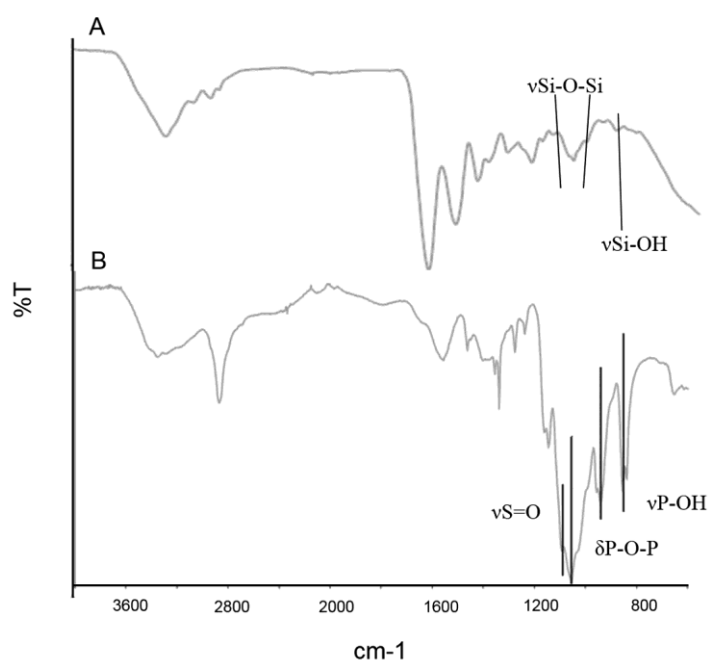


Fig. 2: Fourier transform infrared (FTIR) spectra.

GL nano-fibres

Bond Vibration		Wave Number (cm ⁻¹)
GL	ν O-H	3285.24
GL	Amide B	2937.99
GL - PEO	τ C-H	2870.23
GL	ν C=O	1635.79
GL	δ N-H	1534.83
PEO	δ CH ₂	1470.2
PEO	ω CH ₂	1360.31
PEO	τ CH ₂	1279.41 – 1241.56
GL - PEO	ν C-N e δ N-H	1241.76
GPTMS	Si-O-Si	1031.36 – 1160.51
PEO	ρ CH ₂	969.47 – 951.3
GPTMS	ν Si-OH	916.12
GPTMS	Cyclic epoxide	871.21

Table 2. FTIR peaks related to GL nano-fibres. GL, gelatin; GPTMS, γ -glicidoxypropyltrimethoxysilane; PEO, polyethylene oxide.

CS nano-fibres

Bond Vibration		Wave Number (cm ⁻¹)
CS	vO-H	3221.59
	vN-H	
CS/PEO	vC-H	2882.98
CS	vC=O	1633.56
CS	δN-H	1546.59
PEO	δCH ₂	1466.39
CS	vC-N	1409.64
PEO	ωCH ₂	1359.79
PEO	τCH ₂	1279.5 – 1241.07
CS - PEO	vC-O-C	1148.45 – 1095.02 – 1060.08
PEO	ρCH ₂	959.44 – 947.03
PEO	δC-O-C	841.87
DSP	vPO ₃	1059.3
DSP	δO-P-O	944.65

Table 3. FTIR peaks related to CS nano-fibres. CS, chitosan; DSP, dibasic sodium phosphate; PEO, polyethylene oxide.

Gelatin and chitosan nano-fibres promote similar sc adhesion rate

RT4-D6P2T cells and primary SCs were cultured on PLL-coated coverslips (control condition), GL and CS random and aligned fibres. After 3 h, the adherent cells were fixed, stained and counted. The alignment of GL and CS electrospun fibres decreased the number of adherent cells for RT4-D6P2T and primary SC cultures (Figure 3). RT4-D6P2T cells seeded on aligned GL fibres displayed lower adhesion rate than under control conditions ($p < 0.05$), GL random fibres ($p < 0.01$), and CS random fibres ($p < 0.05$) (Figure 3). RT4-D6P2T cells seeded on aligned CS fibres displayed lower adhesion rates than under control conditions ($p < 0.01$), GL random fibres ($p < 0.01$), and CS random fibres ($p < 0.05$) (Figure 3).

Primary SCs seeded on aligned GL fibres displayed lower adhesion rates than under control conditions ($p < 0.001$), GL random fibres ($p < 0.001$), and CS random fibres ($p < 0.01$) (Figure 3). Primary SCs seeded on aligned CS fibres displayed lower adhesion rate than under control conditions ($p < 0.001$), GL random fibres ($p < 0.001$), and CS random fibres ($p < 0.001$) (Figure 3). Moreover, primary SCs seeded on CS aligned fibres displayed lower adhesion rates in comparison to GL aligned fibres ($p < 0.05$) (Figure 3). All together, these results showed that GL and CS random fibres promote SC adhesion, reaching adhesion rate levels similar to cells cultured on control condition. Aligned fibres still promote SC adhesion even if adhesion rates are lower than those reached by cells cultured on control condition and random fibres. This can be due to the different topography of the aligned fibres in comparison to the random ones (Bacakova et al., 2011, Rahmany and Van Dyke, 2013). Particularly, the alignment of fibres provides less contact points to the cells, thus resulting in lower adhesion rates (Bacakova et al., 2011, Rahmany and Van Dyke, 2013, Gnavi et al., 2015a). On the other hand, the distribution of random fibres results in a complex network formation between fibres providing many contact points to the adhering cells (Bacakova et al., 2011, Harvey et al., 2013, Rahmany and Van Dyke, 2013, Gnavi et al., 2015a). Concerning cell morphology, fibre alignment results in adherent cells aligning their body to the direction of the fibre, whereas cells seeded on random fibres display a spread morphology in accordance with previous study (Cirillo et al., 2014, Gnavi et al., 2015a, Gnavi et al., 2015b).

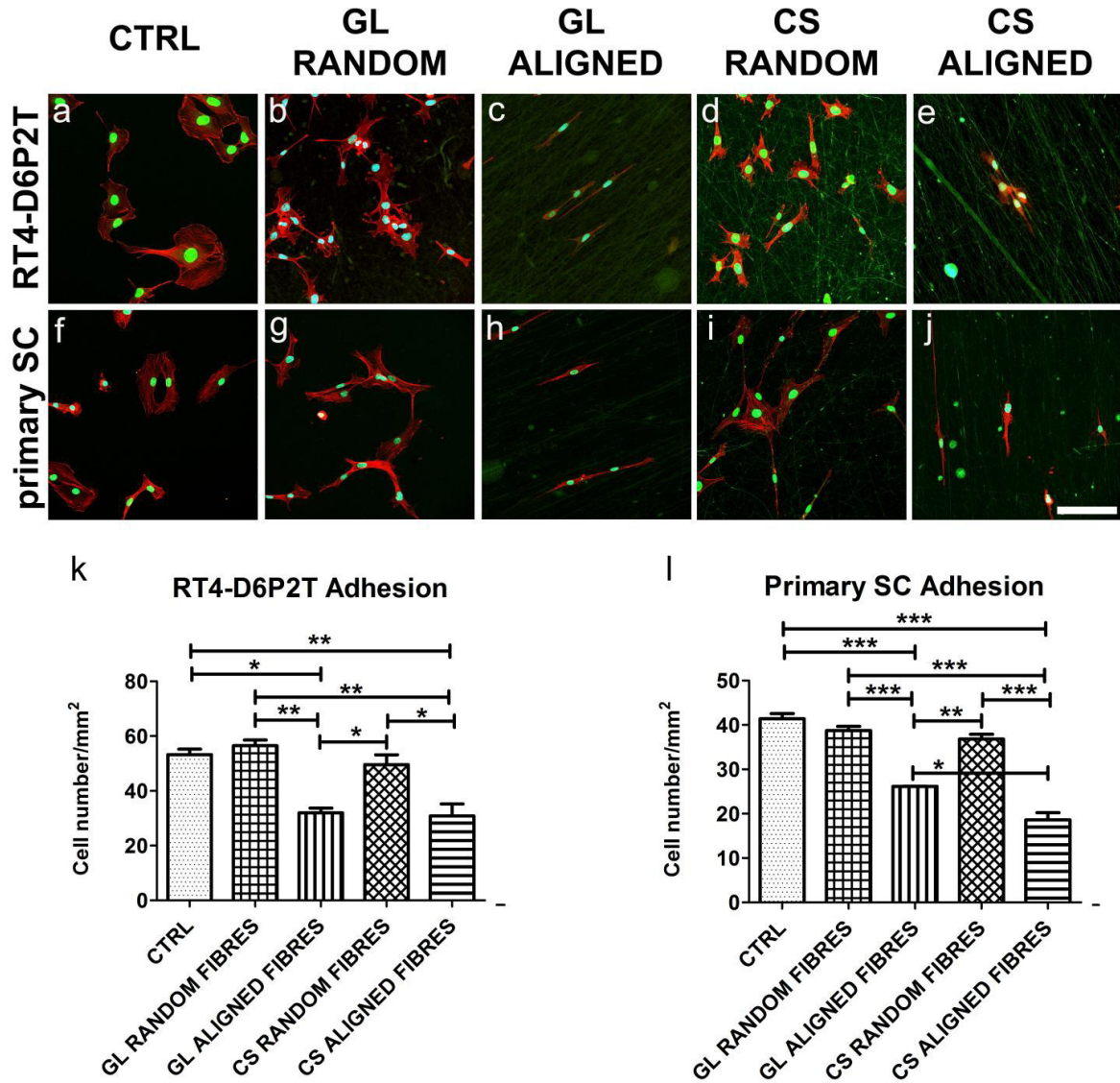


Fig. 3: Adhesion assay. Fluorescence images after vinculin (green), phalloidin (red) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of RT4-D6P2T (a-e) and primary Schwann cell (SC) culture (f-j) on poly-L-lysine (PLL)-coated coverslips (control condition, a and f), gelatin (GL) random fibres (b and g), GL aligned fibres (c and h), chitosan (CS) random fibres (d and i), and CS aligned fibres (e and j). Fibres substrates appear green due to material autofluorescence. Images were acquired at 40X magnification. Scale bar: 100 μm . Number of adhering RT4-D6P2T (k) and primary SCs (l) are reported. Cell number is expressed as cells/ $\text{mm}^2 \pm$ standard error of the mean (SEM). One-way ANOVA was used for statistical analysis. Asterisks denote significant differences with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Chitosan nano-fibres resulted in lower sc proliferation rates in comparison with gelatin nano-fibres

RT4-D6P2T cells and primary SCs were cultured on PLL-coated coverslips (control condition), GL and CS random fibres and aligned fibres. After 1, 3, 5 and 7 *days in vitro* (DIV) the proliferating cells were fixed, stained and counted (Figures 4 and 5). The alignment of GL and CS electrospun fibres decreased the number of proliferating cells for RT4-D6P2T and primary SC cultures (Figures 4 and 5, respectively). Moreover, RT4-D6P2T and primary SCs displayed lower proliferation rates when cultured on CS fibres, both random and aligned in comparison to control condition and random GL fibres. Two-way ANOVA analysis reported in Table S1 showed that the different fibre substrate ($p < 0.001$) and the time ($p < 0.001$) affect RT4-D6P2T cell proliferation. Interaction between the different fibre substrates and time accounts for 13.04% of the total variance, which significantly affects cell proliferation ($p < 0.001$).

Particularly, RT4-D6P2T cells seeded on random CS fibres, aligned GL fibres, or aligned CS fibres displayed lower proliferation rates than under control condition and GL random fibres both at 5 and 7 DIV ($p < 0.001$), and CS random fibres ($p < 0.05$; two-way ANOVA analysis is reported in supplementary Table S1). RT4-D6P2T cells seeded on aligned CS fibres displayed lower proliferation rate than under GL aligned fibres at 5 ($p < 0.05$) and 7 ($p < 0.001$) DIV (Two-way ANOVA analysis is reported in Table S1). Finally, RT4-D6P2T cells seeded on aligned CS fibres displayed lower proliferation rates than under CS random fibres at 5 ($p < 0.01$) and 7 ($p < 0.001$) DIV (Two-way ANOVA analysis is reported in Table S1). Similar results have been obtained by using primary SC cultures (Figure 5; two-way ANOVA analysis is reported in Table S2).

In order to evaluate cell vitality, MTT assay has been performed on both RT4-D6P2T cells and primary SCs cultured on the different substrates (Figures 4v and 5v). MTT results are in agreement with proliferation assay data for both RT4-D6P2T cells and primary SCs (MTT assay, two-way ANOVA results for RT4-D6P2T and primary SCs are reported in Table S3 and S4, respectively). All together, proliferation and MTT assay results demonstrate that the different substrates allow SC proliferation and vitality, in agreement with previous data (Gnavi et al., 2015a, Gnavi et al., 2015b), although, random GL fibres seems to be the better substrate for SC proliferation and

vitality, followed by GL aligned fibres, CS random fibres and CS aligned fibres. The higher cell proliferation on random fibres can be due to the fibres topography that, as already discussed, provides more contact points to the proliferating cells (Bacakova et al., 2011, Harvey et al., 2013, Rahmany and Van Dyke, 2013). Even if random fibres induce higher proliferation rate, a number of study reported that the alignment of fibres promotes better nerve regeneration (Neal et al., 2012, Gnavi et al., 2013, Cirillo et al., 2014, Huang et al., 2015). Particularly, fluorescent images show that SCs cultured on aligned fibres arrange themselves in parallel bands with elongate cell bodies parallel to the direction of the fibres. Thus, aligned fibres can be use to promote band of Bungner formation *in vivo* enhancing nerve regeneration processes (de Ruiter et al., 2009, Deumens et al., 2010).

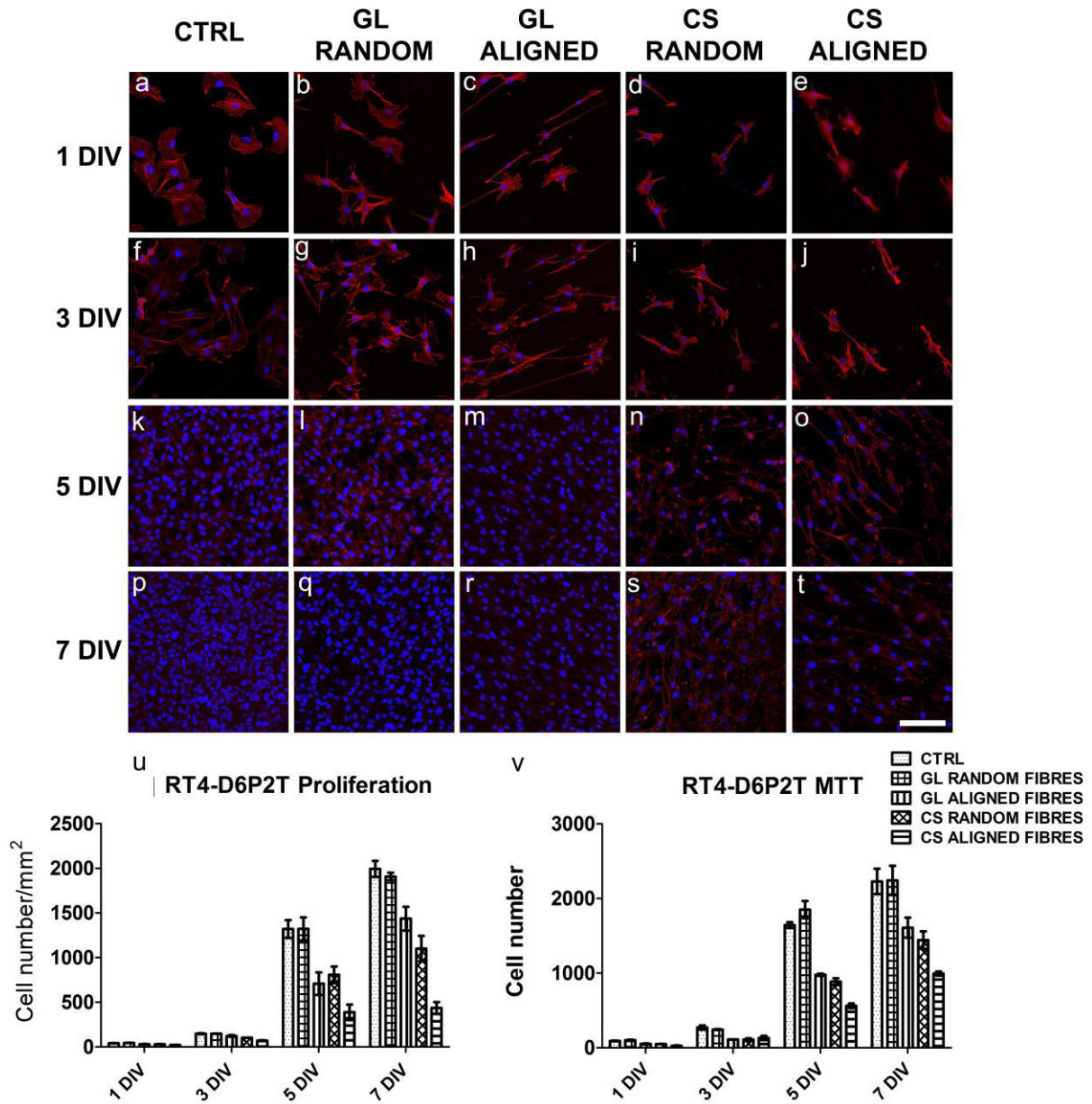


Fig. 4: RT4-D6P2T proliferation and MTT assay. Fluorescence images after phalloidin (red) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of RT4-D6P2T (a-t), on poly-L-lysine (PLL)-coated coverslips (control condition, a, f, k, and p), gelatin (GL) random fibres (b, g, l, and q), GL aligned fibres (c, h, m, and r), chitosan (CS) random fibres (d, i, n, and s), and CS aligned fibres (e, j, o, and t) after 1 (a-e), 3 (f-j), 5 (k-o) and 7 (p-t) days in vitro (DIV). Images were acquired at 40X magnification. Scale bar: 100 μm . Number of proliferating RT4-D6P2T is reported as cells/ $\text{mm}^2 \pm$ standard error of the mean (SEM) (u). Cell vitality was quantified through MTT assay and reported as cell number $\pm$ SEM (v). Two-way ANOVA was used for statistical analysis $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ (Table S1 and S3).

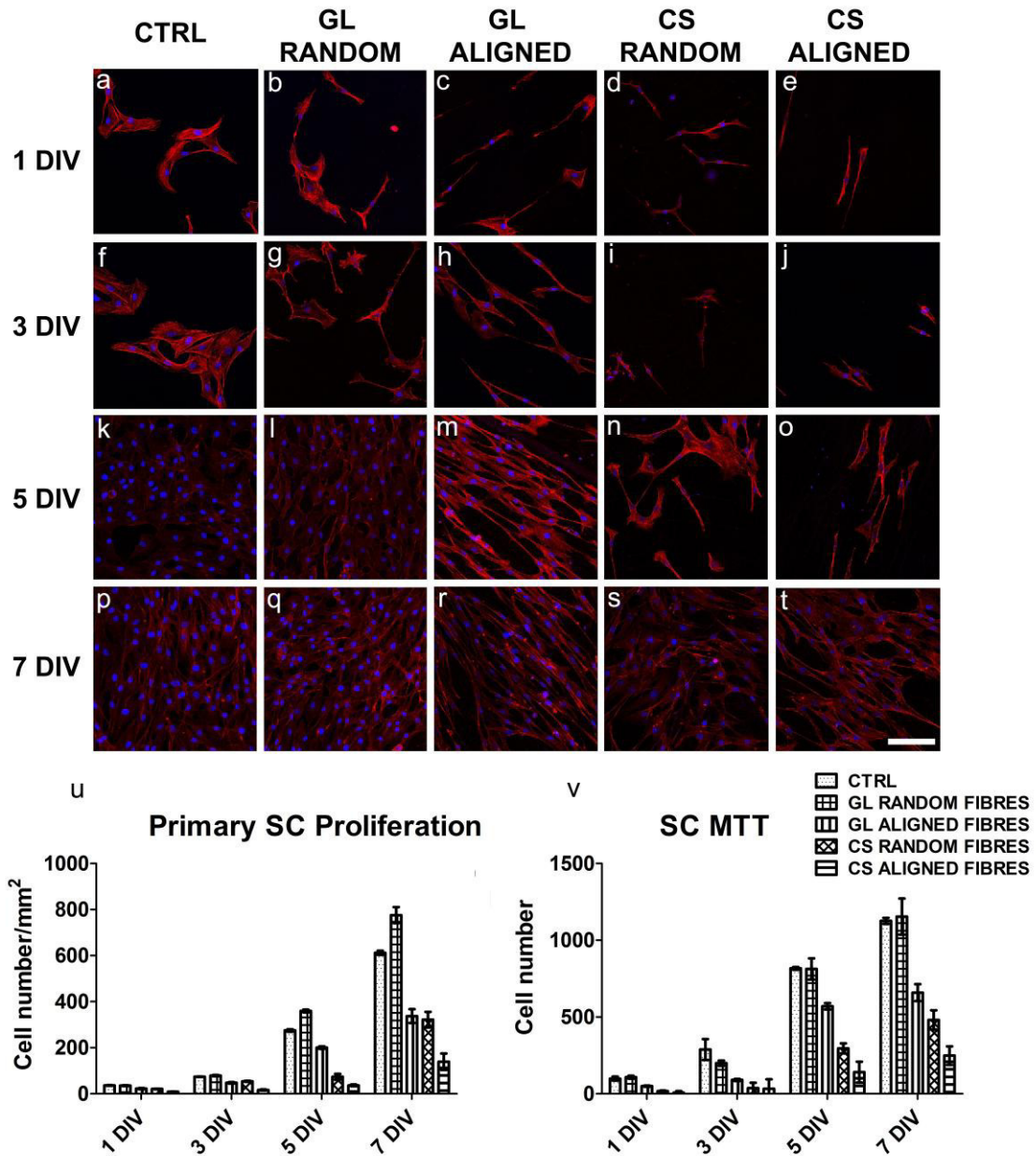


Fig. 5: Primary SC proliferation and MTT assay. Fluorescence images after phalloidin (red) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of SCs (a-t), on poly-L-lysine (PLL)-coated coverslips (control condition, a, f, k, and p), gelatin (GL) random fibres (b, g, l, and q), GL aligned fibres (c, h, m, and r), chitosan (CS) random fibres (d, i, n, and s), and CS aligned fibres (e, j, o, and t) after 1 (a-e), 3 (f-j), 5 (k-o) and 7 (p-t) days in vitro (DIV). Images were acquired at 40X magnification. Scale bar: 100 μm . Number of proliferating RT4-D6P2T is reported as cells/ $\text{mm}^2 \pm$ standard error of the mean (SEM) (u). Cell vitality was quantified through MTT assay and reported as cell number $\pm$ SEM (v). Two-way ANOVA was used for statistical analysis * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (Table S2 and S4).

Gelatin and chitosan nano-fibres alignment resulted in lower actin cytoskeleton organization and fewer focal adhesion points in comparison with gl nano-fibres

Both RT4-D6P2T cells (Figure 6) and primary SCs (Figure 7) were cultured under control conditions and on aligned and random GL and CS fibres. Cells seeded on control conditions and random GL fibres displayed higher actin cytoskeleton organization and many focal adhesion points compared with those cultured on GL aligned fibres and CS fibres both random and aligned. This trend was maintained at all the time-points tested (3, 6 and 24 h). Two-way ANOVA analysis is reported in Table S5-S10 for RT4-D6P2T cells, and in Table S11-16 for primary SCs. Finally, actin cytoskeleton staining showed that cells cultured under control conditions (Figure 5 and 6 a, f, k and p) and on GL random fibres (Figure 5 and 6 b, g, l and q) displayed a spread morphology, whereas those cultured on aligned and CS fibres had an elongated morphology. These data agreed with the adhesion assay results, and with previous studies (Cirillo et al., 2014, Gnani et al., 2015a, Gnani et al., 2015b). Several authors reported that actin cytoskeleton organization plays an important role in lamellipodia and filopodia formation (Parsons et al., 2010, Mullins and Hansen, 2013). Because a different actin cytoskeleton organization on SCs cultured on the different substrates has been observed, immunofluorescence assays to investigate if there were differences on lamellipodia and filopodia formation on the different substrates have been performed.

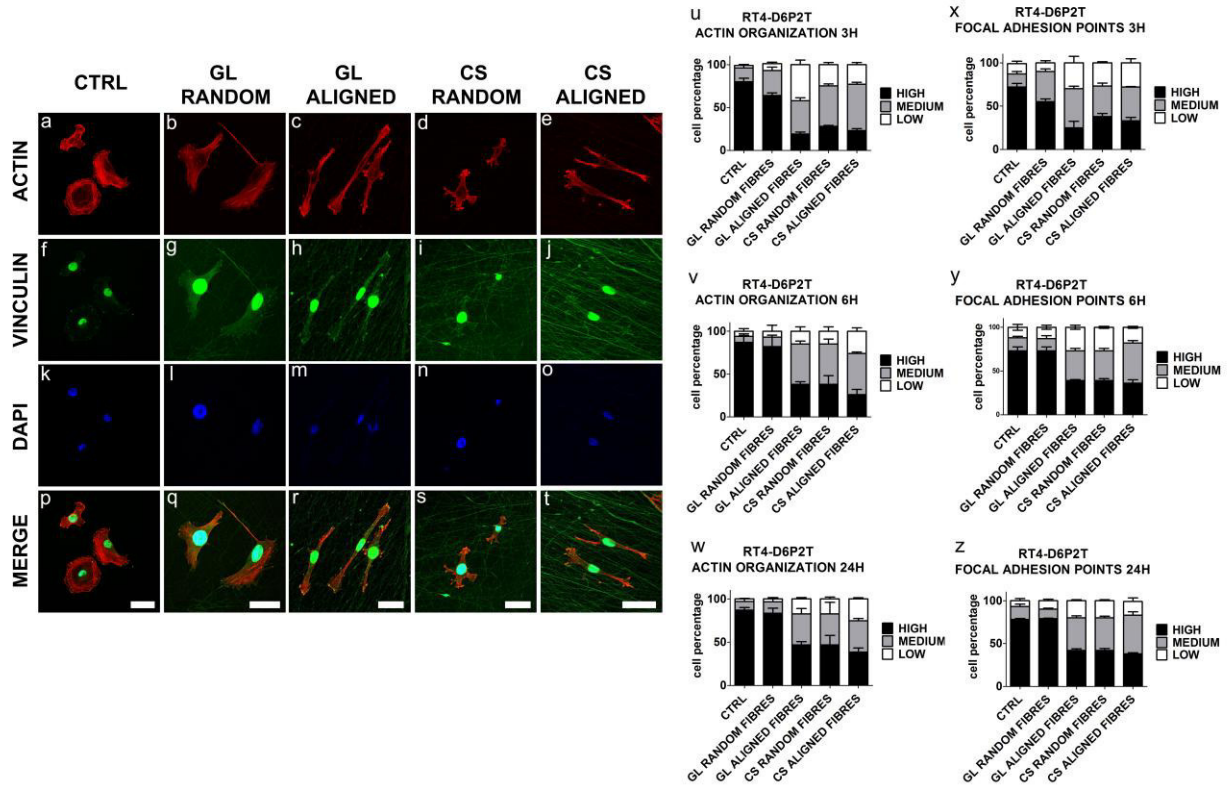


Fig. 6: RT4-D6P2T actin cytoskeleton and focal adhesion points organization. Fluorescence images following phalloidin-actin (red), vinculin (green) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of RT4-D6P2T on poly-L-lysine (PLL)-coated coverslips (control condition, a, f, k and p), gelatin (GL) random fibres (b, g, l and q), GL aligned fibres (c, h, m and r), chitosan (CS) random fibres (d, i, n and s), and CS aligned fibres (e, j, o and t). Fibres substrates appear green due to material autofluorescence. Images were acquired at 60X magnification with different zoom. Scale bar: 40 μ m. Percentages of cells with low, medium or high actin cytoskeleton organization $\pm$ standard error of the mean (SEM) 3 (u), 6 (v) and 24 (w) h after seeding are reported. Percentages of cells with low, medium or high numbers of focal adhesion points $\pm$ SEM 3 (x), 6 (y) and 24 (z) h after seeding are also reported. One-way ANOVA was used for statistical analysis. Asterisks denote significant differences with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (Tables S5-S10).

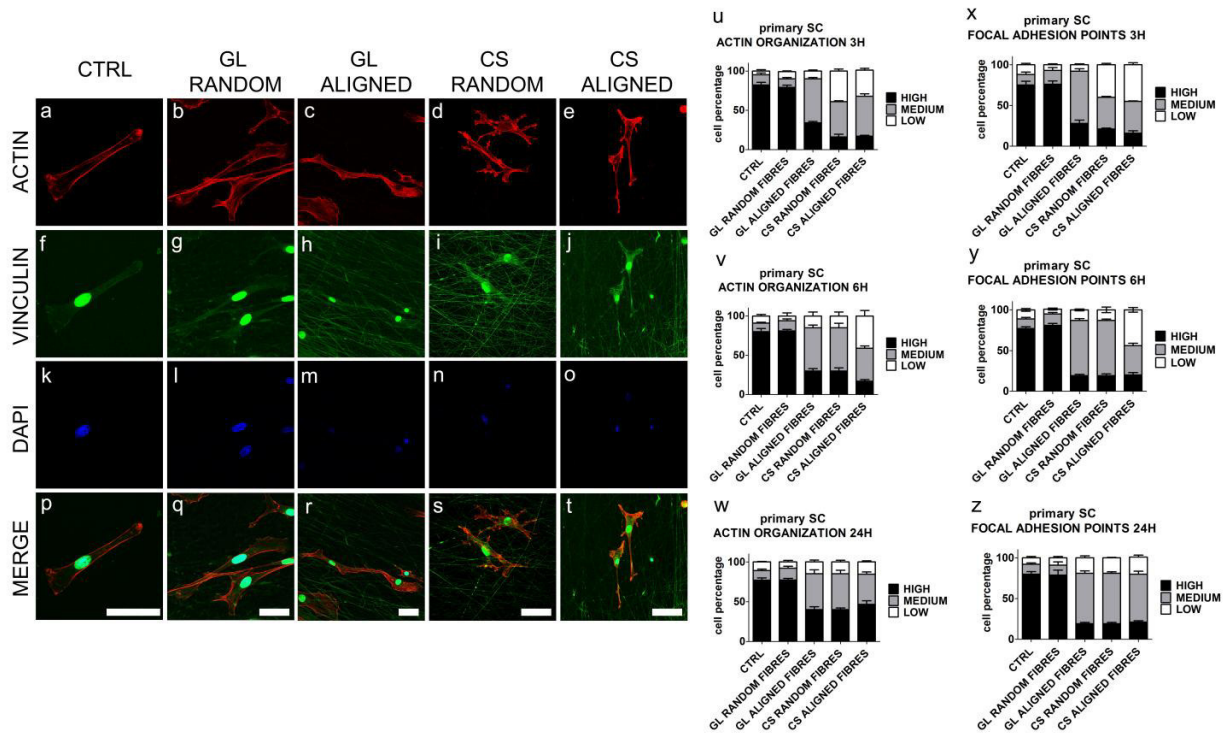


Fig. 7: Primary Schwann cell (SC) actin cytoskeleton and focal adhesion points organization. Fluorescence images following phalloidin-actin (red), vinculin (green) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of SCs on poly-L-lysine (PLL)-coated coverslips (control condition, a, f, k and p), gelatin (GL) random fibres (b, g, l and q), GL aligned fibres (c, h, m and r), chitosan (CS) random fibres (d, i, n and s), and CS aligned fibres (e, j, o and t). Fibres substrates appear green due to material autofluorescence. Images were acquired at 60X magnification with different zoom. Scale bar: 40 μ m. Percentages of cells with low, medium or high actin cytoskeleton organization $\pm$ standard error of the mean (SEM) 3 (u), 6 (v) and 24 (w) h after seeding are reported. Percentages of cells with low, medium or high numbers of focal adhesion points $\pm$ SEM 3 (x), 6 (y) and 24 (z) h after seeding are also reported. One-way ANOVA was used for statistical analysis. Asterisks denote significant differences with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (Tables S11-S16).

GL and CS nano-fibres alignment enhances filipodia formation

Lamellipodia and filipodia formation has been evaluated by culturing both RT4-D6P2T cell line and primary SCs on the different substrates. Results showed that the alignment of fibre led to more cells displaying filipodia in both RT4-D6P2T cells (Figure 8) and primary SCs (Figure 9). This trend was maintained at all the time-points tested (3, 6 and 24 h; Figure 8-9 f, g and h, respectively). Two-way ANOVA analysis is reported Tables S17-S19 and S20-S22 for RT4-D6P2T cells and primary SCs, respectively). Lamellipodia are motile structures formed by a thin sheet of cytoplasm filled with a criss-cross arranged network of actin filaments. Filipodia consist of rod-shaped cell projections full of linear actin filaments and their formation often is associated with lamellipodia formation. Several studied reported that filipodia formation is associated to cell motility and migration (Qu, 2013); thus, our data suggest that fibre alignment may induce SC migration.

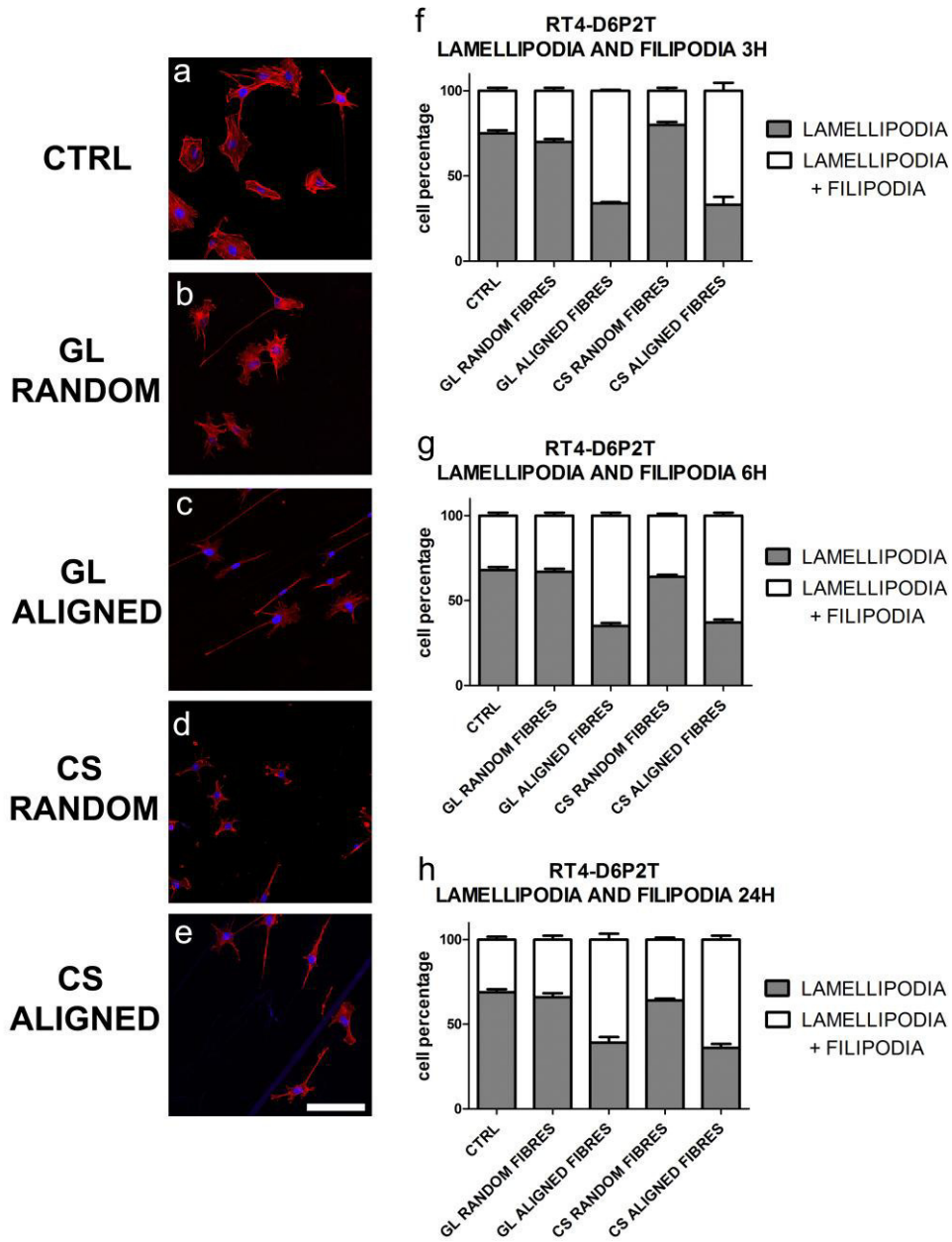


Fig. 8: Lamellipodia and filipodia organization in RT4-D6P2T cells. Fluorescence images phalloidin (red) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of RT4-D6P2T cells seeded on poly-L-lysine (PLL)-coated coverslips (control condition, a), gelatin (GL) random fibres (b), GL aligned fibres (c), chitosan (CS) random fibres (d), and CS aligned fibres (e) 6 h after seeding. Images were acquired at 40X magnification. Scale bar: 100 μ m. Percentage of RT4-D6P2T cells characterized by lamellipodium and filipodia protrusions 3 (f), 6 (g) and 24 (h) h after seeding are expressed as mean $\pm$ standard error of the mean (SEM). One-way ANOVA was used for statistical analysis. Asterisks denote significant differences with $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ (Tables S17-S19).

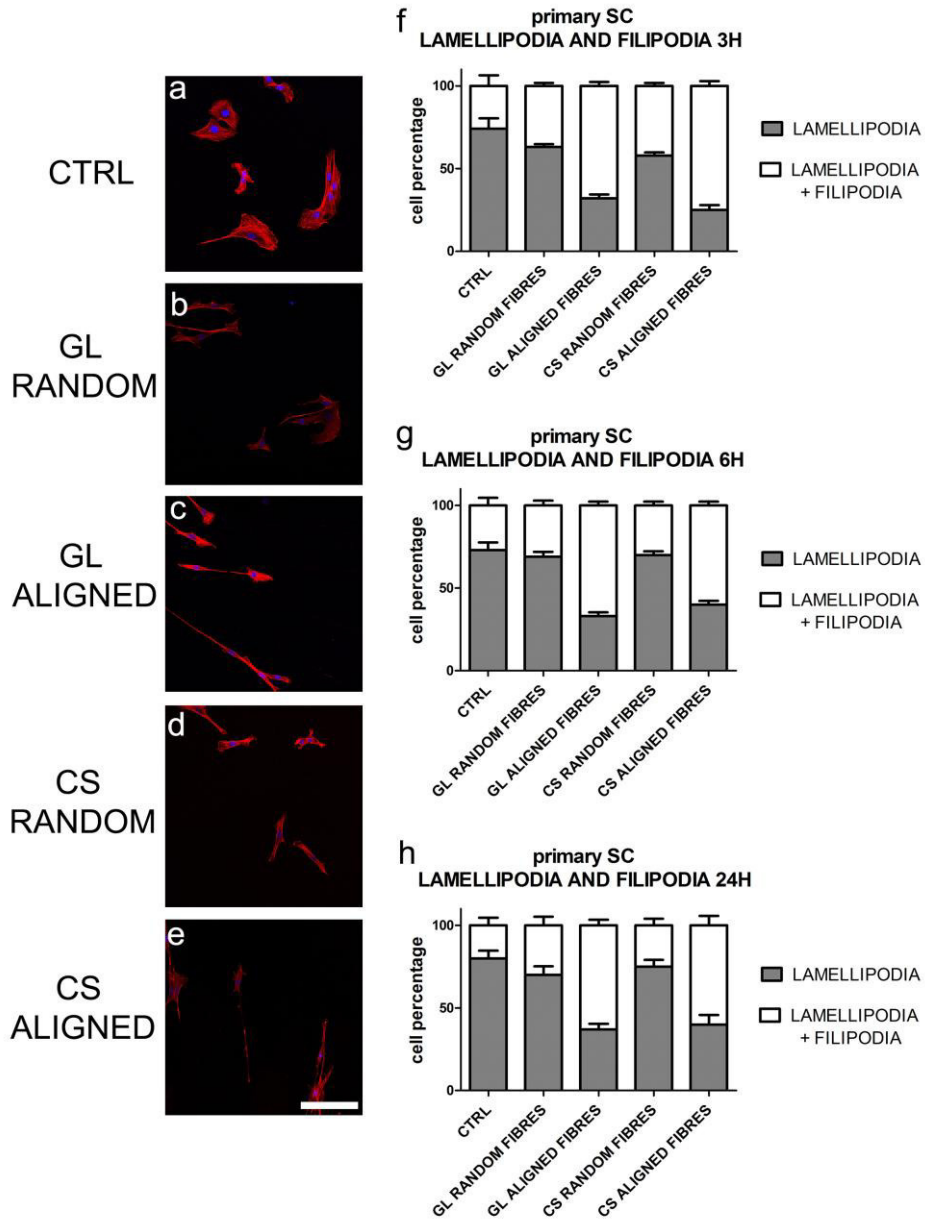


Fig. 9: Lamellipodia and filipodia organization in primary Schwann cells (SCs). Fluorescence images phalloidin (red) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of SCs seeded on poly-L-lysine (PLL)-coated coverslips (control condition, a), gelatin (GL) random fibres (b), GL aligned fibres (c), chitosan (CS) random fibres (d), and CS aligned fibres (e) 6 h after seeding. Images were acquired at 40X magnification. Scale bar: 100 μ m. Percentage of SCs characterized by lamellipodium and filipodia protrusions 3 (f), 6 (g) and 24 (h) h after seeding are expressed as mean $\pm$ standard error of the mean (SEM). One-way ANOVA was used for statistical analysis. Asterisks denote significant differences with $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ (Tables S20-S22).

Gelatin nano-fibres resulted in higher 50B11 cell differentiation and neurites length in comparison with chitosan nano-fibres

50B11 cells were seeded under control conditions, and on aligned and random GL and CS fibres. After 24 h, 50B11 cells displayed high adhesion rates under the different substrates without any statistical difference between groups (Table S23). The alignment of GL and CS fibres did not affect the number of adherent 50B11 cells (Figure 10p). The addition of forskolin resulted in 50B11 differentiation under all tested conditions (Figure 10p) without any statistical differences (Table S23). After forskolin addition, 50B11 cells stopped proliferating and started to differentiate, resulting in a reduction of cell number compared with non-treated conditions (one-way ANOVA statistical analysis is reported in Figure 10). The number of differentiated 50B11 cells is slightly decreased when 50B11 cells are cultured on GL aligned fibres in comparison to control conditions ($p < 0.05$). 50B11 neurite length was higher in control conditions, GL random fibres and GL aligned fibres in comparison with CS random and aligned fibres (Figure 10 q). According to these results, confocal images showed that aligned fibres made neurites parallel to the direction of the fibres (Figure 10 c, h and m for GL fibres; and Figure 10 e, j and o for CS fibres). In order to understand if fibres themselves can induce cell differentiation, the number of differentiated cells was evaluated in the presence or absence of forskolin in culture medium. Results, expressed as percentage of differentiated and undifferentiated cells, show that with forskolin addition (Figure 10 r) there are high percentages of differentiated cells without any statistical differences between the different substrates (Table S24). When 50B11 cells are cultured without forskolin, there are higher percentages of undifferentiated cells (Figure 10 s), although GL aligned fibres seems to induce 50B11 differentiation in comparison with control conditions, GL random fibres and CS random and aligned fibres ($p < 0.001$) (Table S25). These results suggest that the topography of GL random and aligned fibres may induce neuron differentiation and neurite alignment in agreement with several studies (Xie et al., 2009, Mahairaki et al., 2011, Cirillo et al., 2014, Gnani et al., 2015b, Huang et al., 2015). Particularly, longitudinally aligned nano-fibres promote ordered neurites outgrowth (Huang et al., 2015), PC12 and DRGs alignment and parallel axonal growth, and hMSC and PC-12 cells neurogenic differentiation (Cirillo et al., 2014).

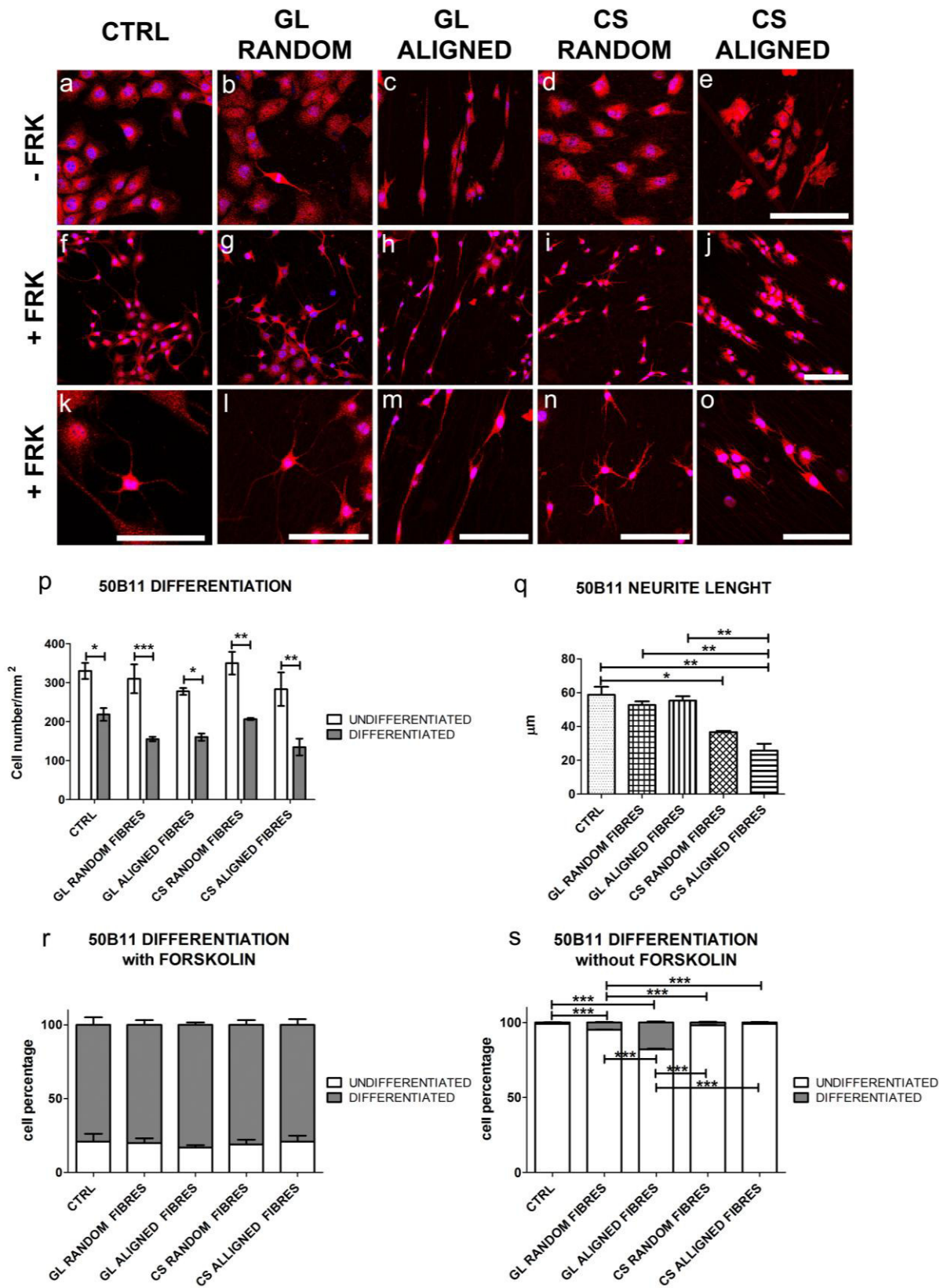


*Fig. 10: 50B11 cells differentiation. Confocal images after β -tubulin (red) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of B5011 cells seeded on poly-L-lysine (PLL)-coated coverslips (control condition, a, f, and k), random gelatin (GL) fibres (b, g, and l), aligned GL fibres (c, h and m), random chitosan (CS) fibres (d, i and n) and aligned CS fibres (e, j and o). 50B11 cells were cultured without (a-e) or with forskolin (f-o) addition to the culture medium. Images were acquired at 40X or 60X magnification with different zoom. Scale bar: 100 μm . 50B11 cell were counted and cell number is expressed as $\text{cells}/\text{mm}^2 \pm$ standard error of the mean (SEM) (p); Neurite length is expressed as $\mu\text{m} \pm$ SEM (q); 50B11 differentiated and undifferentiated cells in presence (r) or absence (s) of forskolin treatment are expressed as percentage of cells $\pm$ SEM. One way ANOVA was used for statistical analysis. Asterisks reported in the figure refer to significant statistical difference with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Two-way ANOVA was also used for statistical analysis. Asterisks refer to significant differences with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (Table S23-S25).*

Gelatin and chitosan nano-fibres resulted in similar DRG neurites growth and alignment

Dorsal root ganglia explants were cultured on PLL-coated coverslips (control conditions), GL and CS random fibres and aligned fibres with or without NGF in culture medium. After 48 h, the explants were fixed, stained and neurite length, neurite occupied area and neurite area ratio were quantified. Neurite length, neurite occupied area and neurite area ratio were higher when DRG explants were cultured in presence of NGF (one-way ANOVA statistical analysis is reported in Figure 11). When DRG explants were cultured in the presence of NGF, neurite length was higher on GL and CS aligned fibres in comparison with all the other tested conditions (Figure 11f; Table S26). There are no differences between neurite length of DRG explants cultured on GL and CS aligned fibres in the presence of NGF (Table S26). Moreover, when DRG explants were cultured without NGF, neurite length was higher on GL aligned fibres, CS random and aligned fibres in comparison to control conditions and GL random fibres (Table S26).

When DRG explants were cultured in the presence of NGF, the neurite occupied area was higher in control condition and random fibres in comparison to aligned fibres (Figure 11 g; Table S27). When DRG explants were cultured without NGF, the neurite occupied area was lower in control condition and aligned fibres in comparison with the other tested substrate (Table S27). Similar results have been obtained by evaluating the ratio between the area occupied by the neurites and the area occupied by the DRG bodies (Figure 11 h; Table S28). Fluorescence images (Figure 11 c and e) show that aligned fibres result in parallel neurites outgrowth in agreement with literature data (Xie et al., 2009, Cirillo et al., 2014).

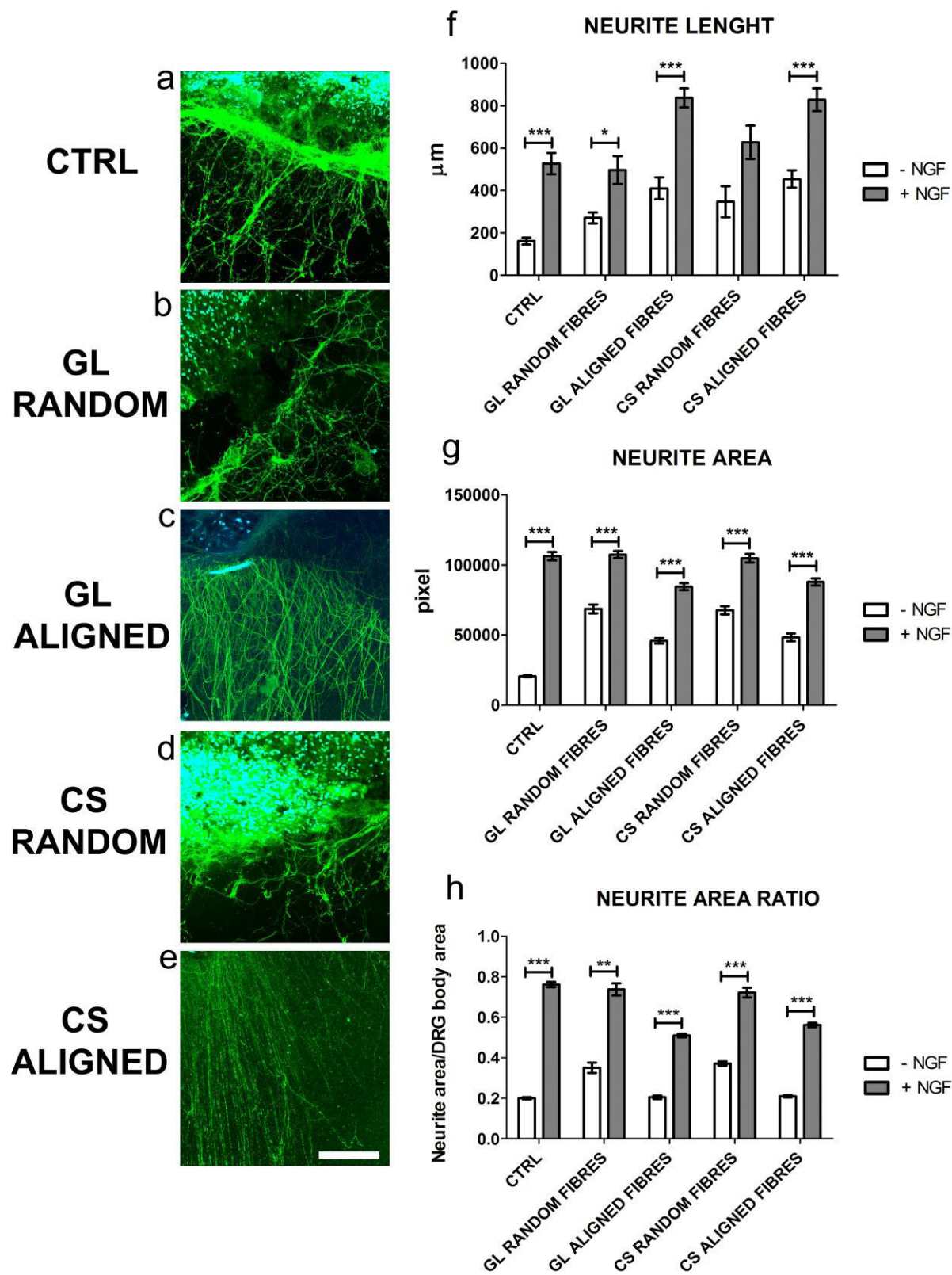


*Fig.11: Fluorescence images after β -tubulin (green) and 4,6-diamino-2-phenylindole (DAPI; blue) staining of DRG explants cultured on control condition (poly-L-lysine-coated coverslips, a), random gelatin (GL) fibres (b), aligned GL fibres (c), random chitosan (CS) fibres (d) and aligned CS fibres (e) without (not shown) or with NGF (a-e). Images were acquired at 40X magnification. Scale bar: 100 μ m. Axon length is expressed as μ m $\pm$ standard error of the mean (SEM) (f), density as pixel $\pm$ SEM (g), sprouting area as DRG axon area/DRG axon area ratio $\pm$ SEM (h). One way ANOVA was used for statistical analysis. Asterisks reported in figure refer to significant difference with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Two-way ANOVA was also used for statistical analysis. Asterisks refer to significant difference with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (Tables S26-S28).*

CONCLUSION

Reported data show that both GL and CS fibres can be promising filler for the development of peripheral nerve conduits. Particularly, aligned fibres induce SCs and axon oriented growth with cell bodies parallel to the direction of the fibres. DRG neurite outgrowth was similar on the different tested substrates, although, GL fibres resulted in higher 50B11 neuronal differentiation in comparison to CS fibres. Concerning CS fibres, we observed an oriented SCs and neurite growth that can be due to both the polymer biomimetic and biochemical intrinsic properties of the material (Gnavi et al., 2013, Meyer et al., 2016). Both GL and CS have been widely used for peripheral nerve repair displaying high biocompatibility (Ciardelli and Chiono, 2006, Chiono et al., 2009, Foster and Karsten, 2012, Gnavi et al., 2013, Huang et al., 2015, Meyer et al., 2016). In this work, GL and CS fibres have been tested, showing that depending on their morphology and biochemical composition they can induce and promote different regenerative events (i.e. SC adhesion, SC proliferation, neuronal differentiation and growth). In conclusion, although *in vitro* positive results are not always predictors of similar *in vivo* performances (Gomes et al., 2015), our data support that all the tested substrates are potential candidates for nerve reconstruction.

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MY CONTRIBUTION TO THE MANUSCRIPT

I performed the experiments on dorsal root ganglia explants, I supervised *in vitro* experiments and revised all the statistical analyses. I participated to the interpretation of data and to the draft of the manuscript.

SUPPLEMENTARY MATERIALS

Because the supplementary materials of this chapter are 35 pages long, they were not inserted in the thesis and are available at this link:

<https://www.dropbox.com/s/64d1rh7ekxxkf8v/SUPPLEMENTARY%20MATERIALS%20CHAPTER%206%20FORNASARI%20PhD%20THESIS.pdf?dl=0>

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CHAPTER 7

Irreversible changes occurring in long-term denervated Schwann cells affect delayed nerve repair

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ABSTRACT

Objective Multiple factors may affect functional recovery after peripheral nerve injury, among them the lesion site and the interval between the injury and the surgical repair. When the nerve segment distal to the lesion site undergoes chronic degeneration, the ensuing regeneration (when allowed) is often poor.

The aims of the current study were as follows: 1) to examine the expression changes of the Neuregulin1/ErbB system during long-term nerve degeneration; and 2) to investigate whether a chronically denervated distal nerve stump can sustain nerve regeneration of freshly axotomized axons.

Methods This study used a rat surgical model of delayed nerve repair consisting of a cross suture between the chronically degenerated median nerve distal stump and the freshly axotomized ulnar proximal stump. Before the suture, a segment of long-term degenerated median nerve stump was harvested for analysis. Functional, morphological, morphometric, and biomolecular analyses were performed.

Results The results showed that Neuregulin1 is highly down-regulated after chronic degeneration, as well as some Schwann cell markers, demonstrating that these cells undergo atrophy, which was also confirmed by ultrastructural analysis. After delayed nerve repair, it was observed that chronic degeneration of the distal nerve stump compromises nerve regeneration in terms of functional recovery, as well as number and size of regenerated myelinated fibres. Moreover, Neuregulin1 is still down-regulated after delayed regeneration.

Conclusions The poor outcome after delayed nerve regeneration might be explained by Schwann cells impairment and the consequent ineffective support for nerve regeneration.

Understanding the molecular and biological changes occurring both in the chronically degenerating nerve and in the delayed repaired nerve may be useful to the development of new strategies to promote nerve regeneration. The results suggest that Neuregulin1 has an important role in Schwann cell activity after denervation, indicating that its manipulation might be a good strategy for improving outcome after delayed nerve repair.

KEYWORDS

delayed nerve repair; NRG1/ErbB system; neuregulin1; nerve degeneration; nerve regeneration; stereology; peripheral nerve

INTRODUCTION

Despite advances in microsurgical techniques, patient outcome after severe peripheral nerve injury is still often poor. Functional recovery depends on many factors, including the lesion site and the interval of time between the injury and the surgical repair (Hoke, 2011).

Concerning the lesion site, the more proximal the injury is, the more time is required for nerve regeneration and target reinnervation, because axons have to regenerate over longer distances (Sulaiman and Gordon, 2013). The rate of axonal regrowth is estimated to be approximately 1 mm per day. As a consequence, when the nerve lesion is more distal (e.g. median or ulnar nerve injuries close to the wrist), the functional recovery of the hand muscles is expected to be reached within a few months. On the contrary, when the nerve lesion is more proximal (e.g. brachial plexus injuries close to the spinal cord), axons require 2-3 years to regrow before reaching target muscles (Hoke, 2011, Sulaiman and Gordon, 2013, Grinsell and Keating, 2014).

The timing of surgical intervention depends on the nerve injury type. In cases of sharp nerve transection, when the wound is clean and there are no other major injuries, primary nerve repair is usually the best choice (within 72 hours up to 7 days) (Dvali and Mackinnon, 2003, Siemionow and Brzezicki, 2009). When the nerve injury is more severe (e.g. injury caused by a gunshot) and an immediate repair is not possible due to other concomitant and complicating injuries, the nerve reconstruction is delayed in time until all other tissues are accurately restored (Siemionow and Brzezicki, 2009). In both cases (proximal injury or delayed nerve repair), a full functional recovery rarely occurs. This poor outcome can be attributed to many factors, which include the following: 1) the inability of denervated muscle to accept reinnervation and to recover from muscle atrophy; 2) the reduced ability of injured

axons to regenerate after a long-term axotomy; and 3) the loss of the Schwann cell capability to support regeneration (Grinsell and Keating, 2014).

This issue has been widely studied over the last several years (Fu and Gordon, 1995, Sulaiman and Gordon, 2000, Saito and Dahlin, 2008, Saito et al., 2009, Gordon et al., 2011, Jonsson et al., 2013, Sulaiman and Gordon, 2013). It has been shown that a decrease in the number of regenerating motoneurons and myelinated axons is accompanied by a decline in Schwann cells marker expression and an increase of fibrosis in the sciatic distal nerve stump following delayed nerve grafting (Jonsson et al., 2013). Also, previous studies, in which after a delay the tibial nerve was cross-sutured to the common peroneal nerve with a denervated nerve autograft, showed a decrease in the number of regenerated motoneurons, demonstrating that chronically denervated Schwann cells have an inhibitory effect on nerve regeneration (Gordon et al., 2011).

Moreover, recent studies that have focused on the expression of different molecules in the chronically axotomized distal nerve stump as well as in the delayed repaired nerves, have provided a neurobiological explanation for the poor functional outcome. A progressive down-regulation of some regeneration associated genes - namely α 1-tubulin, actin and GAP-43 - has been recently shown in chronically axotomized motoneurons (Gordon and Tetzlaff, 2015), even after the transient increase observed after a refreshment axotomy. Brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) mRNA are significantly up-regulated in transected distal tibial nerve following denervation (up to 6 months), whereas neurotrophin-3 (NT-3) and nerve growth factor (NGF) mRNA levels are comparable to control levels (Michalski et al., 2008). After delayed repair, Schwann cells in the distal stump significantly overexpress cleaved caspase 3 and down-regulate ATF3 (Saito and Dahlin, 2008, Saito et al., 2009).

Previous data from our laboratory and others demonstrated that Neuregulin1 (NRG1)/ErbB system is strongly and selectively regulated after acute peripheral nerve injury and during the early phases of regeneration (Stassart et al., 2013, Ronchi et al., 2015), but little is known about expression changes of this system during chronic denervation and delayed repair.

NRG1 is one of the most important factors regulating the activity of Schwann cells, both during their development (Schwann cell survival, proliferation, differentiation and migration) and during nerve regeneration after injury in adulthood (Schwann cell survival, axon guidance and remyelination) (Pereira et al., 2003, Taveggia et al., 2010, Fricker and Bennett, 2011, Salzer, 2012, Haastert-Talini et al., 2013, Heermann and Schwab, 2013, Gambarotta et al., 2014b, Ronchi et al., 2015). The different NRG1 isoforms, generated through the use of different promoters and alternative exon splicing (Adlkofer and Lai, 2000), can be soluble or transmembrane. Type I/II isoforms are produced either as soluble peptides ready to signal in a paracrine or autocrine manner or as transmembrane precursors needing a proteolytic cleavage to release a soluble fragment into the extracellular environment. Type III isoforms are produced as transmembrane proteins that are ready to signal in a juxtacrine manner or that need proteolytic cleavage to be active (Falls, 2003).

The aims of the current study were therefore as following: 1) to study the expression of the NRG1/ErbB system during long-term degeneration (up to 9 months); and 2) to investigate - at functional, morphological and biomolecular levels - whether chronically denervated distal nerve stump can still sustain nerve regeneration of freshly axotomized axons.

MATERIALS AND METHODS

Surgical procedure

A total of 36 adult female Wistar rats, weighing approximately 200g each, were used in this study. All animals were housed in a room with controlled temperature and humidity, with light/dark cycles of 12:12 hours, and had access to food and water ad libitum. All procedures were approved by the Bioethics Committee of the University of Torino, the Institutional Animal Care and Use Committee of the University of Torino, and by the Italian Ministry of Health, in accordance with the European Communities Council (Directive 2010/63/EU).

Animals were operated under general anaesthesia induced by intramuscular injection of 3mg/kg tiletamine plus zolazepam (Zoletil). Surgical procedures were performed

with rats placed supine with the legs wide apart. Using a linear incision from the nipple to the elbow crease, the left median nerve was exposed, isolated, and transected approximately in the middle of the exposed part; the proximal nerve stump was buried in the pectoral muscle to prevent regeneration; the distal stump was sutured to adjacent innervated muscles and allowed to degenerate for 3 or 6 months. Three or 6 months after median nerve axotomy, cross-suture of the proximal stump of freshly axotomized ulnar nerve and the distal stump of the degenerated median nerve was performed with 2 Ethilon 9/0 stitches (3-months delayed repair group, n=7, and 6-months delayed repair group, n=7). Before the suture between the ulnar and median nerves, the first 5 mm of the distal degenerated median nerve stump was harvested in each rat. In others 7 animals, immediate cross-suture between the median and the ulnar nerves was performed (immediate repair group) (Figure 1). To prevent interference during the grasping test, the right median nerve was transected at the middle one-third of the brachium, and its proximal stump was sutured to the *pectoralis major* muscle to avoid spontaneous reinnervation. All animals were euthanized 6 months after the surgery by anaesthetic overdose, and the regenerated nerves were harvested. Healthy median nerve segments (n=5), 9-months degenerated nerves (n=5) and 3-months regenerated end-to-end repaired median nerves (n=5) from other animals were also harvested.

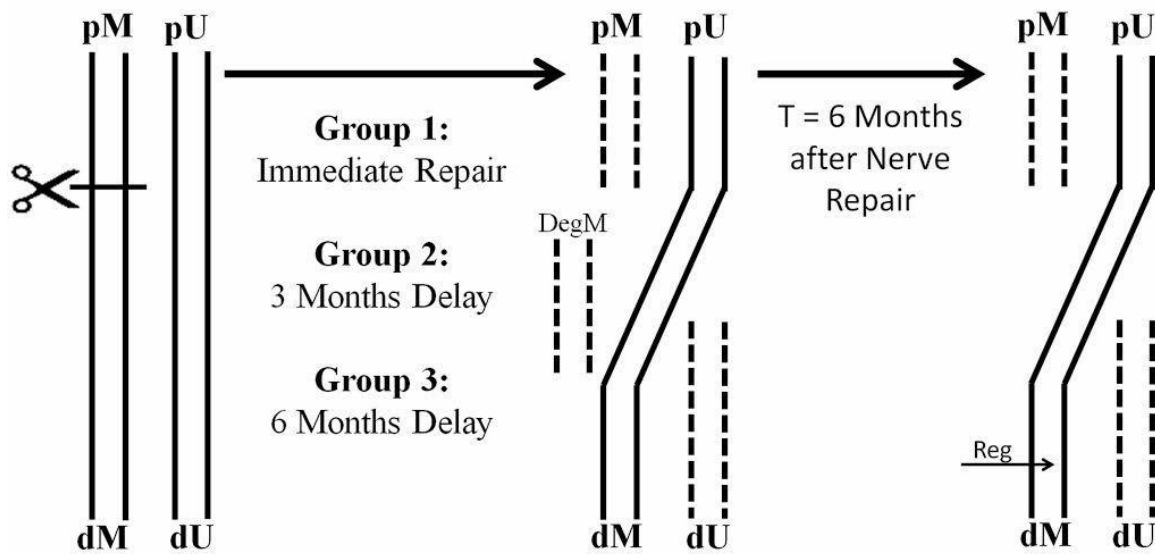


Figure 1: Schematic diagram showing the surgical model used in this study and a description of the specimens analysed. The median nerve was cut and its distal part was allowed to degenerate. Immediately after median nerve was cut (Group 1: immediate repair group), 3 months (Group 2: 3-months delay group), or 6 months later (Group 3: 6-months delay group), the ulnar nerve was cut and its proximal stump was sutured to the median nerve distal stump. Before suturing of the two nerves, a segment of the degenerating median nerve was collected and analysed. Six months after nerve repair, the regenerated nerve was harvested and analysed. DegM = degenerating median nerve; dM = median nerve distal stump; dU = ulnar nerve distal stump; pM = median nerve proximal stump; pU = ulnar nerve proximal stump; Reg = regenerated nerve; T = time.

Grasping test

The strengths of the *flexor digitorum sublimis* muscle and the *flexor digitorum profundus* muscle, which are both innervated by the median nerve in the rat, were assessed by the grasping test. This test was done every 3 weeks, starting, from 3 weeks post-surgery to 24 weeks (6 months). The grasping test was performed according to the same procedure previously described (Papalia et al., 2003, Ronchi et al., 2009) using the BS-GRIP Grip Meter device (2Biological Instruments). Each animal was tested three times and the average value was recorded.

Isolation of RNA, preparation of cDNA, and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription (RT) of 0.4µg total RNA was performed in a 25 µl reaction volume containing 1x RT-Buffer, 0.1µg/µl bovine serum albumin (BSA), 0.05% Triton, 1mM deoxynucleoside triphosphate (dNTPs); 7.5µM Random Hexamer Primers; 40U RIBOlock and 200U RevertAid® Reverse Transcriptase (all RT ingredients were provided by Thermo Scientific). The reaction was performed at 25°C 10 min, at 42°C 90 min, at 70°C 10 min. Quantitative real-time PCR was performed through an ABI Prism 7300 (Applied Biosystems, Life Technologies Europe BV) detection system. The cDNA was diluted 10 fold in nuclease-free water, and 5µl (corresponding to 15ng starting RNA) were analyzed in a 20µl reaction volume containing 1 x iTaq Universal SYBR Green supermix (BioRad) and 300nM forward and reverse primers. Dissociation curves were routinely performed to verify the presence of a single peak corresponding to the required amplicon. Analyses were performed in technical and biological triplicate. Degenerating nerve data from the real-time PCR experiments were analyzed using the $-\Delta\Delta C_t$ method for the relative quantification to appreciate both up-regulation (ranging between 0 to $+\infty$) and down-regulation (ranging between 0 to $-\infty$). Meanwhile, relative quantitative data of regenerating nerve were analyzed as $2^{-\Delta\Delta C_t}$ (ranging between 0 to +1).

The threshold cycle number (C_t) values of calibrator and samples of interest were normalized to the geometric average of two endogenous housekeeping genes: ANKRD27 (Ankyrin repeat domain 27) and RICTOR (RPTOR Independent Companion of MTOR, Complex 2) (Gambarotta et al., 2014a). The average of uninjured nerves was used as calibrator. Primers were designed using Annhyb software (<http://www.bioinformatics.org/annhyb/>) and synthesized by BMR Genomics. Primer sequences for ErbB2, ErbB3, NRG1 type I/II and MBP were previously published (Ronchi et al., 2015). Primer sequences for S100, low-affinity nerve growth factor receptor (p75) and glial fibrillary acidic protein (GFAP) are as follows:

S100: forward 5'-GGGTGACAAGCACAAGCTGAAGAA-3' reverse 5'-TTGTCCACCACTTCCTGCTCTTTG-3' ;
 p75: forward 5'-AGCAGACCCATACGCAGACTG-3', reverse 5'-TCTCTACCTCCTCACGCTTGG-3';
 GFAP: forward 5'-GAGGCAGTGGCCACCAGTAACATG-3', reverse 5'-GGAAGCAACGTCTGTGAGGTCTGC-3'.

Total protein extraction and western blot analysis

Total proteins were extracted using the TRIzol Reagent (Invitrogen) after RNA extraction, according to the manufacturer's instructions. In the final passage, the protein pellet was resuspended in Laemli buffer [2.5% sodium dodecyl sulphate (SDS), 0.125M TrisHCl pH6.8] at 100°C. Protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Sigma-Aldrich) on 1:4 diluted proteins to avoid detergent interference. Proteins (50µg/sample) were resolved by 8% SDS-polyacrylamide gel electrophoresis; for the analysis of NRG1 isoforms, 4-15% precast gels were used (BioRad). Western blot analysis was performed as previously described (Gambarotta et al., 2004). Primary antibodies used included anti-total HER2/ErbB2 (#sc-284) and anti-total HER3/ErbB3 (#sc-285) (diluted 1:1000, both purchased from Santa Cruz); anti-NRG1 type III N terminal (#AB5551) from Chemicon International, diluted 1:1000; glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) (#AM4300) diluted 1:20000 from Ambion. Secondary antibodies used are horseradish peroxidase-linked anti-rabbit (#NA934) and anti-mouse (#NA931) diluted 1:40000, (GE Health).

Resin embedding and electron microscopy analysis

Degenerated and regenerated nerve samples were fixed by immediate immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 5-6 hours at 4° C. Samples were then post-fixed in 2% osmium tetroxide for 2 hours and dehydrated in passages in ethanol from 30% to 100% (5 minutes each passage). After two passages of 7 min in propylene oxide and 1 hour in a 1:1 mixture of propylene oxide and Glauerts' mixture of resins, samples were embedded in Glauerts' mixture of resins (comprising equal parts of Araldite M and the Araldite Harter, HY 964). In the

resin mixture, 0.5% of the plasticizer dibutyl phthalate was added. For the final step, 2% of accelerator 964 was added to the resin to promote polymerization of the embedding mixture at 60°C.

Semithin sections (2.5µm thick) were cut using an Ultracut UCT ultramicrotome (Leica Microsystems) and stained with 1% toluidine blue for high-resolution light microscopy examination and design-based stereology. A DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems) were used for section analysis. With the same ultramicrotome, ultrathin sections (70nm thick) were cut and stained with saturated aqueous solution of uranyl acetate and lead citrate. Sections were analysed using a JEM-1010 transmission electron microscope (JEOL) equipped with a MegaView III digital camera and a Soft Imaging System (SIS) for the computerized acquisition of the images.

Stereological and morphometric analysis

To quantify myelinated nerve fibres with high-resolution light microscopy, one toluidine blue stained semithin section was selected and the total cross-sectional area of the whole nerve was measured. Thirteen to 15 sampling fields were selected using a systematic random sampling protocol, as previously described (Geuna, 2000, Geuna et al., 2000). In each sampling field, a 2D dissector procedure was adopted (Geuna et al., 2000). Total fibre numbers were estimated and fibre and axon diameter, myelin thickness and *g-ratio* were measured. For Schwann cell nuclei quantification, electron microscopy micrographs were used. Briefly, on one randomly selected ultrathin section, 15-20 fields were selected using a systematic random sampling protocol, with a magnification of 5000X. The number of Schwann cell nuclei was estimated using the same protocol described for the fibre quantification.

Statistical methods

Statistical analysis was performed using SPSS software. All data (stereological analysis and gene expression analysis) were statistically analyzed using the t-test or one-way analysis of variance (ANOVA), and *post hoc* analysis was done with the Bonferroni test. Regression lines were analyzed by the Student t-test using the Prism Software Package (GraphPad).

RESULTS

Degenerating nerve

We first focused on studying the degenerating median nerve from a morphological and biomolecular point of view, to understand its characteristics before repairing the chronically degenerated nerve with a freshly axotomized nerve.

The chronically degenerated median nerves (3, 6 and 9 months after axotomy) were analyzed by transmission electron microscopy. Three months after the axotomy, neither myelinated nor unmyelinated fibres were detected, and axonal and myelin debris had been cleared. Dedifferentiated Schwann cells, as well as fibroblasts, colonized the distal part of the degenerating nerve (Figure 2A). Some inflammatory cells (mast cells) are also still detected (Figure 2A'). After 6 months, atrophic Schwann cells were observed (Fig. 2B), as well as endoneurial tubes (the basal laminae of Schwann cells) with some debris inside (Figure 2B'). Similar morphological features were also detected after 9 months of nerve degeneration (Figure 2C-C').

Quantitative real-time PCR analysis was performed to evaluate mRNA expression during long-term median nerve degeneration of Schwann cell-specific markers: myelin basic protein (MBP), S100, p75 and GFAP.

As expected, the MBP mRNA level (Figure 2D) strongly decreased 3 months after injury, and its level remains strongly down-regulated also 6 and 9 months later, reflecting the chronic degeneration phase detected at morphological level (Figure 2 A-C, A'-C'). The expression pattern of S100 is similar to that of MBP; it strongly decreased and remained down-regulated for all of the analyzed time points (Figure

2E). On the contrary, p75 was intensely up-regulated during long-term degeneration (Figure 2F). Finally, GFAP was down-regulated after 3 and 9 months after the injury (Figure 2G).

Next, we focused on the NRG1/ErbB system, which is known to play an important role during nerve degeneration/regeneration. In particular, mRNA and protein expression levels were examined by quantitative real-time PCR and Western blot analysis in long-term degenerating median nerves (Figure 3).

Although ErbB2 and ErbB3 mRNA expression did not change after axotomy (Figure 3A and 3B), ErbB2 and ErbB3 proteins were still present at 3 and 6 months. Three months after axotomy, mRNA expression of soluble NRG1 (type I/II) was down-regulated, and this down-regulation was even more accentuated after 6 and 9 months (Figure 3C). After 9 months, ErbB2 and ErbB3 proteins were no longer detectable (Figure 3D)

For NRG1 protein expression, an antibody directed to the cytoplasmic N-terminus common to all transmembrane type III isoforms was used (Ronchi et al., 2015). As previously described (Ronchi et al., 2015), this antibody recognizes different bands; among these, a band can be detected at approximately 75 kDa, which can be the product of NRG1 type III cleavage by the α -secretase ADAM17/TACE [the α -N-terminal fragment (NTF), with a lower molecular weight] or the product after cleavage by the β -secretase BACE1 (β -NTF, with an higher molecular weight). In the control nerve, α -NTF is expressed. During chronic degeneration, no bands recognized by this antibody (α -NTF nor β -NTF) were detected.

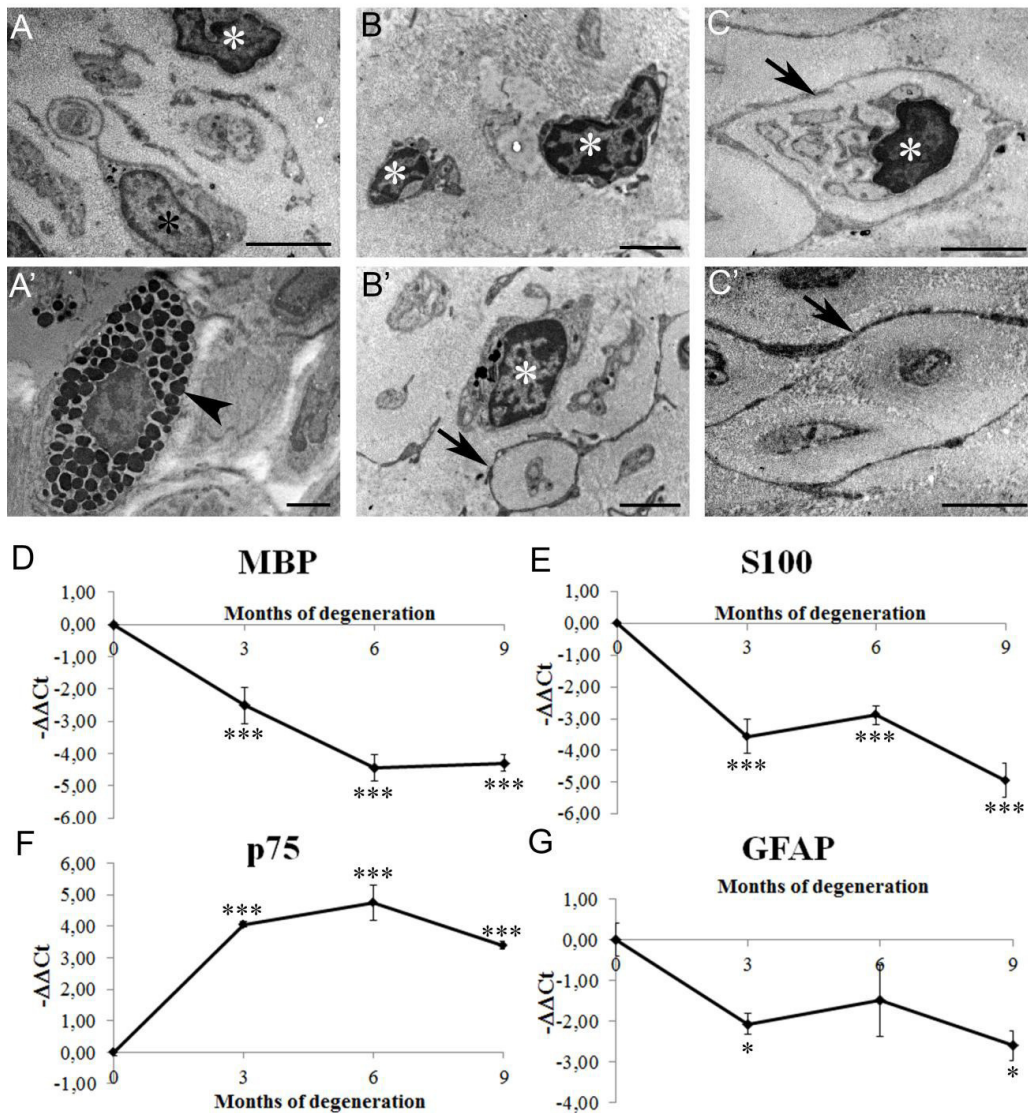


*Fig. 2: Representative transmission electron micrographs of degenerating median nerve (corresponding to degM, Figure 1) 3 months (A and A'), 6 months (B and B') and 9 months (C and C') after axotomy. Bar = 2 μ m. Neither myelinated nor unmyelinated fibres were detectable at the 3 time-points of degeneration. At 3 months, the degenerated nerve showed atrophic Schwann cells (white asterisk), fibroblastic-like cells (black asterisk), and mast cells (arrowhead). After 6 and 9 months, atrophic Schwann cells (white asterisks) and endoneurial tubes (arrows) with debris inside were detectable. D-G: Quantitative real-time PCR results of MBP, S100, p75 and GFAP mRNA expression during median nerve degeneration (performed on degM, Figure 1). Data were normalized to the geometrical mean of 2 endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphics are expressed as the mean $\pm$ SEM. *: $p \leq 0.05$, ***: $p \leq 0.001$). Schwann cell markers were strongly regulated in the long-term degenerating nerve.*

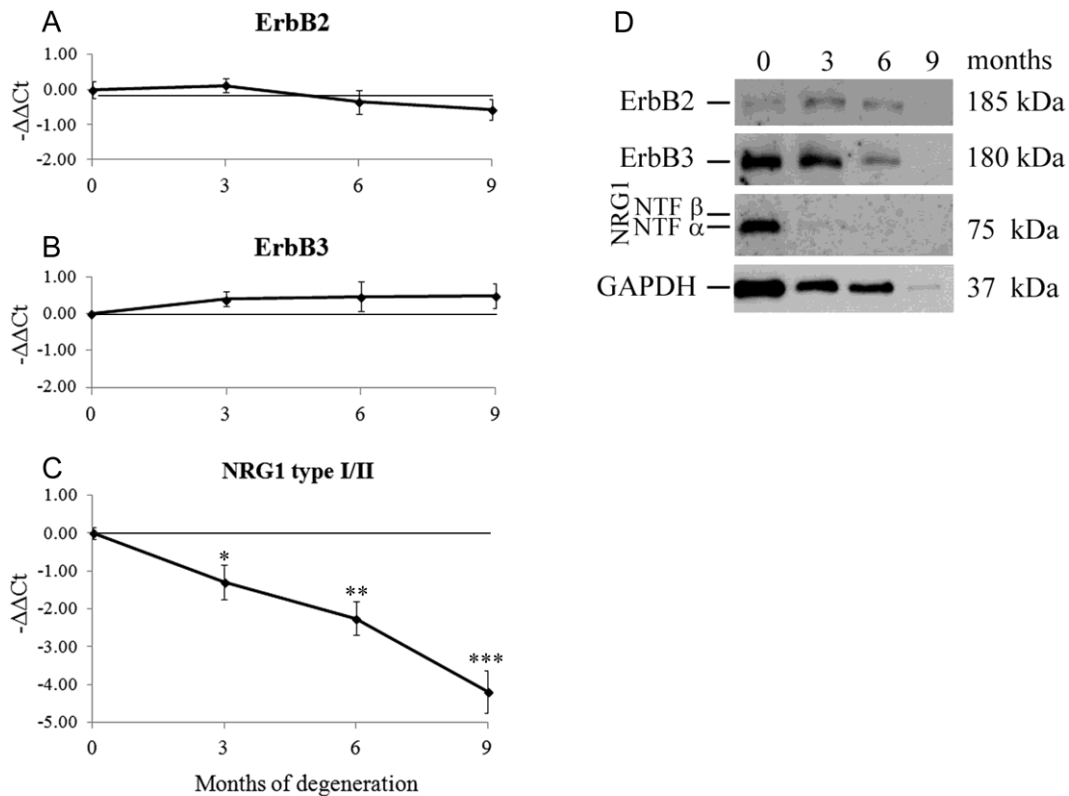


Fig. 3: A-C: Quantitative real-time PCR results of ErbB2 and ErbB3 receptors and soluble type I/II NRG1 isoform mRNA expression in degenerated median nerves (performed on DegM, Figure 1). Data were normalized to the geometrical mean of 2 endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphs are expressed as the mean $\pm$ SEM (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$). Soluble NRG1 is strongly down-regulated. D: Western blot analysis of proteins extracted from healthy control nerves (0) and long-term degenerating nerves (3, 6 and 9 months, corresponding to DegM, Figure 1) and probed with antibodies for ErbB2, ErbB3, and NRG1 (AB5551 antibody, which can recognize 2 bands of approximately 75 kDa [α -NTF, β -NTF]); GAPDH was used as a loading control. The positions of the detected proteins are indicated on the left; size markers are indicated on the right. The NRG1 is not detectable after 3, 6 and 9 months of degeneration. The ErbB expression decreases over time, becoming undetectable at 9 months.

Regenerating nerve

Immediately after median nerve axotomy (immediate repair group), 3 months after the axotomy (3-months delayed repair group), or 6 months after the axotomy (6-months delayed repair group), the distal median nerve stump was sutured to the freshly axotomized ulnar nerve and axons were allowed to regenerate into the median nerve distal stump for 6 months (as displayed in Figure 1).

During the postoperative period, animals were tested every 3 weeks for *flexor digitorum* muscle function by means of the grasping test. One rat in the immediate repair group had already started to recover 6 weeks after nerve repair. Starting from week 12, 3 animals began to recover, and by the end of the postoperative period (24 weeks), 4 of 7 animals showed a functional recovery (Figure 4A). However, the grasping force, expressed in grams (Figure 4B), was lower compared with healthy control values, where is approximately 450 g (data not shown). No animals in the 2 delayed repair groups (3- and 6-months delays) showed functional recovery over time.

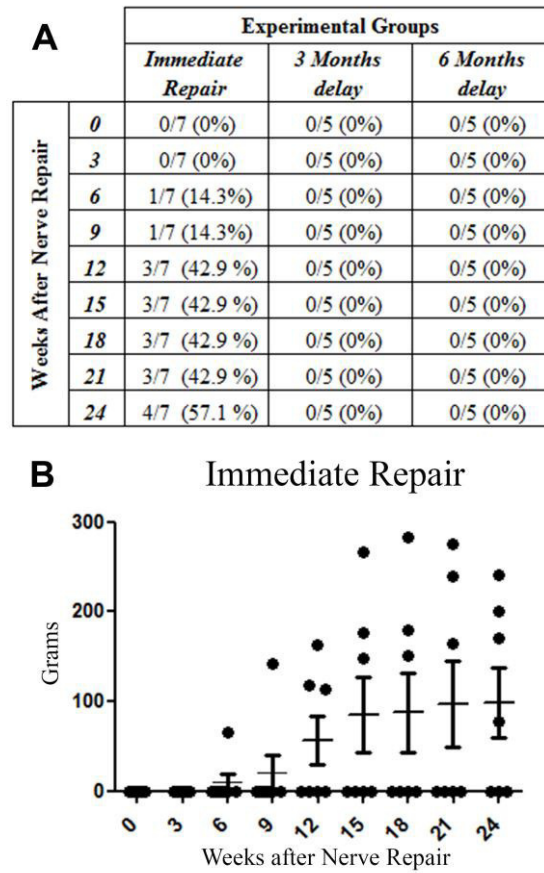


Fig. 4: A: Overview of the number and percentage of animals in the experimental groups that recover motor function over time. Few animals in the immediate repair group and no animals in the two delayed repair groups showed functional recovery. B: Scatterplots showing individual animal values with integrated mean and variance values of the quantitative analysis of motor recovery. Only data of the immediate repair group are presented, because only animals in this group showed partial functional recovery.

The structure of the nerve was evaluated, and the total number of myelinated fibres was estimated. All samples were quantitatively analyzed, as well as nerves withdrawn from animals that did not show functional recovery. Morphological evaluation on toluidine blue-stained semithin sections reveals that 6 months after nerve repair [compared with uninjured healthy control nerves (Figure 5A)], regenerated nerves from the 3 experimental groups [immediate repair (Figure 5B), 3-months delayed repair (Figure 5C) and 6-months delayed repair (Figure 5D)] exhibited regrowth of many myelinated fibres organized in microfascicles with a well-defined axoplasm and well-organized myelin sheaths. As expected, from a qualitative point of view, they were smaller compared with uninjured healthy control nerves. Moreover, the amount of connective tissue is greater in the experimental groups compared with the healthy control nerves.

Quantitative analysis on the semithin sections showed that after immediate repair, the cross-sectional area (mm^2) of the regenerated nerve is larger than in the healthy control group (Figure 5E). On the contrary, no difference was observed in the cross-sectional area for the 2 delayed repair groups compared with both the healthy control group and the immediate repair group. The total number of myelinated fibres, obtained by stereological methods, was higher in the immediate repair group compared with the healthy control group. The 2 delayed repair groups showed few fibres than the immediate repaired group, but there were no discernible differences in the total number of myelinated fibres between these 2 groups and the healthy control group (Figure 5F).

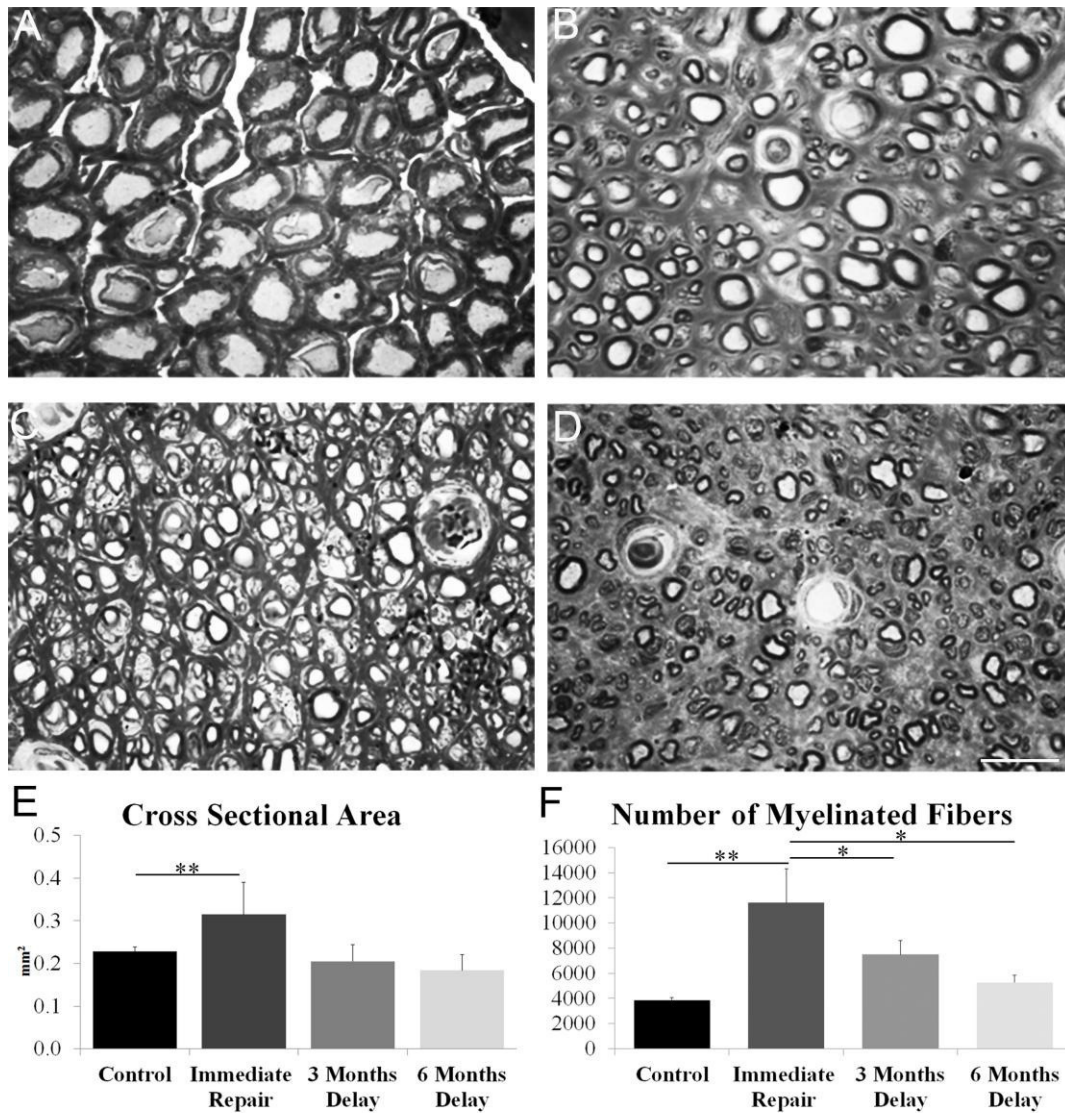


Fig. 5: Representative light micrographs of toluidine blue-stained semi-thin cross sections of a control nerve (A) and of regenerated nerves after immediate repair (B), 3-months delay (C), and 6-months delay (D) (corresponding to Reg, Figure 1). Bar = 10 μ m. After 6 months of regeneration, all experimental groups showed many re-growing myelinated fibers. Histograms show the results of stereological evaluations of nerve regeneration 6 months after nerve repair (Reg, Figure 1); the cross-sectional area of the nerve (E) and the total number of myelinated fibers (F) are represented. Values in the graphics are expressed as mean + SEM (*: $p < 0.05$; **: $p < 0.01$). The immediate repair group showed a bigger cross-sectional area and a higher number of myelinated fibers compared with the healthy control group. No differences were detected between the 2 delayed repair groups and the healthy control nerve.

With respect to axon and fibre size, a difference was detectable between the healthy control group and the 3 experimental groups. Moreover, the two delayed repair groups showed smaller axon and fibre diameters when compared with the immediate repaired group (Figure 6A). As expected, the mean myelin thickness was decreased in the three experimental groups compared with the healthy control group (Figure 6B). No differences were seen among the three experimental groups. Finally, the *g-ratio* (axon diameter/fibre diameter) did not differ among the 4 groups.

In Figure 6C-F, the frequency distributions of fibre diameters are shown. Healthy control nerve fibre diameters show a bimodal distribution with peaks at 5-6 μm and 9-11 μm , whereas all three experimental groups showed a unimodal distribution of the fibre diameters. In particular, all of these groups showed a peak at 2-3 μm , but the two delayed repair groups showed a higher frequency of distribution of very small fibres (1-2 μm) compared with the immediate repair group.

Finally, we analyzed the *g-ratio*/axon diameter correlation of individual fibres by means of scatterplots, evaluating the differences in linear regression (Figure 6 G-J). As shown in the figure, the linear regression line for the healthy control nerve displays a flatter slope compared with the regenerated nerves.

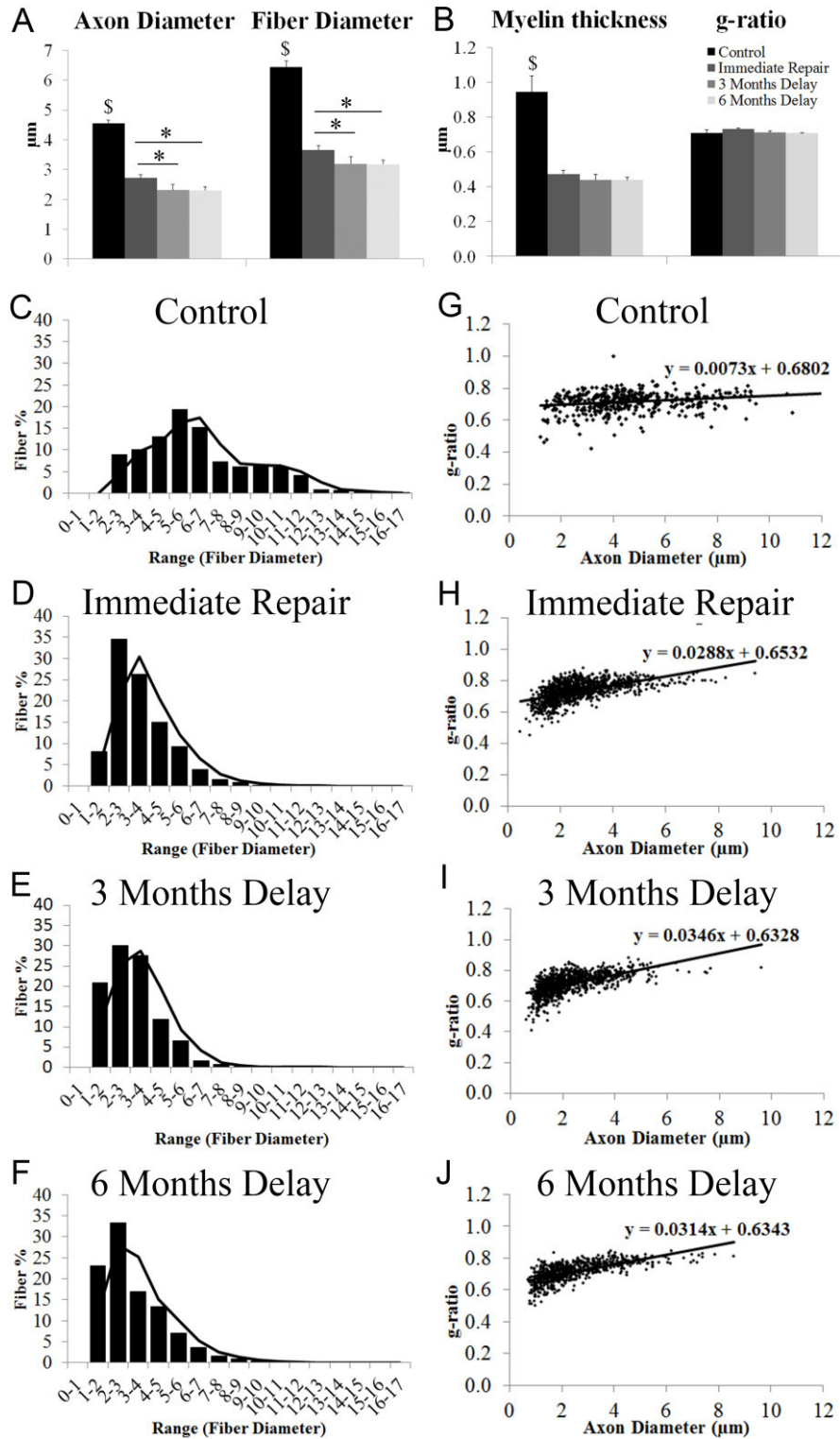


*Fig. 6: Histograms show the results of histomorphometric evaluation (performed on Reg samples, Figure 1). Axon diameter and fiber diameter (A) and myelin thickness and g-ratio (axon diameter/fiber diameter) (B) are represented. Values in the graphs are expressed as the mean + SEM (\$: $p \leq 0.001$ vs all the experimental groups, *: $p \leq 0.05$). The 2 delayed repair groups showed smaller axon and fiber diameters when compared with both immediate repaired group and healthy nerve control. Histograms show the percentage of the nerve fiber diameter distribution of control nerves ($n=432$) (C) and of the regenerated nerves after immediate repair ($n=1117$) (D), 3-month delayed repair ($n=1004$) (E) and 6-month delayed repair ($n=735$) (F). The 2 delayed repair groups have a higher percentage of very small fibers (1-2 μm) compared with the immediate-repaired group. Scatter plot displaying the g-ratio of individual myelinated axons as a function of the respective axon diameter in the control nerve (G) and in the regenerated nerves after immediate repair (H), 3-month delayed repair (I) and 6-month delayed repair (J). Linear regression lines and the relative equation are also represented, showing that the healthy control nerve displays a significantly flatter slope compared with the regenerated nerves.*

Quantitative assessment of Schwann cell nuclei was performed by electron microscopy analysis on the distal regenerated nerve stumps. In particular, we quantified only myelinating Schwann cells (that is, Schwann cells for which the nucleus was associated with an axon). Figure 7 shows the results of the quantification, with representative electron micrographs. Data are expressed as the ratio between the total number of myelinated fibres and the total number of myelinating Schwann cell nuclei. In the healthy control group, this ratio is 13.65 ± 1.26 , meaning that for each counted Schwann cell nucleus, there were 13.65 myelinated fibres. In the immediate repair group, the ratio is 11.61 ± 0.62 , whereas in the 3 and 6-months delayed repair groups, the ratios were 7.00 ± 0.28 and 5.53 ± 0.91 , respectively. These results demonstrate that, in proportion to the total number of regenerated myelinated fibres, there are more Schwann cells in the two delayed repair groups compared with both the healthy control and immediate repair groups.

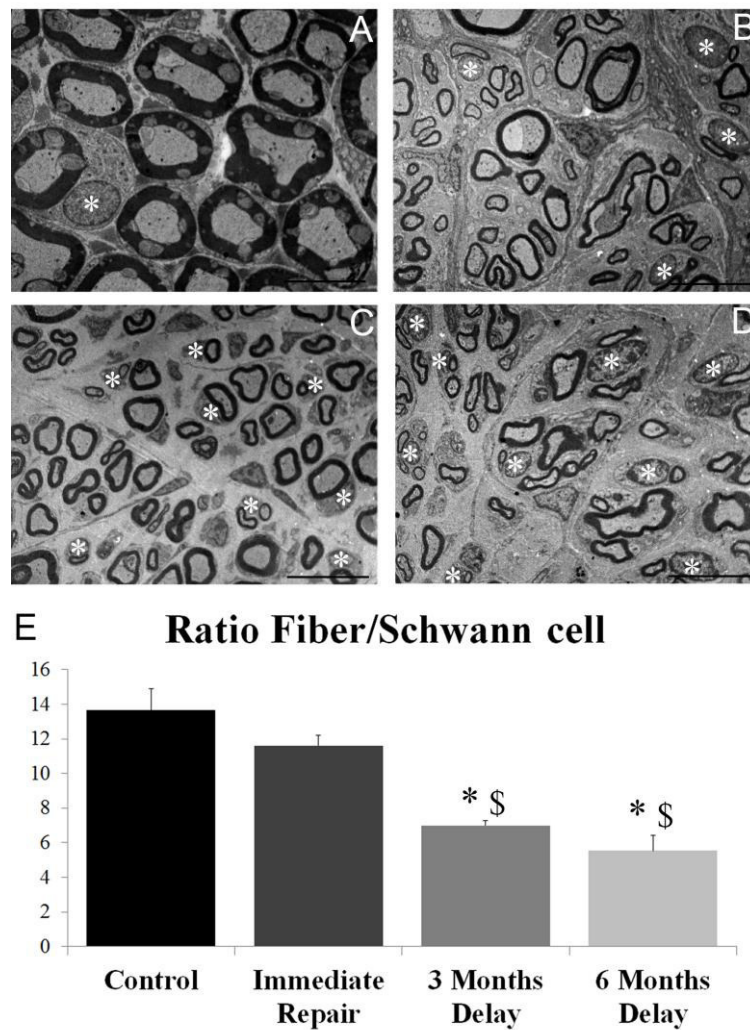


*Fig. 7: Representative transmission electron micrographs of ultra-thin cross-sections of a control nerve (A) and of regenerated nerves in the immediate repair (B), 3-month delayed repair (C) and 6-month delayed repair (D) groups (corresponding to Reg samples, Figure 1). Bar = 10 μ m. White asterisks indicate myelinating Schwann cell nuclei. Histograms show the ratio between myelinated fibers and myelinating Schwann cell nuclei (E). Values in the graphs are expressed as the mean + SEM (*: $p < 0.01$ vs control group; \$: $p < 0.01$ vs immediate repair group). The 2 delayed repair groups show a fiber/Schwann cells ratio lower than both healthy control and immediate repair groups, corresponding to a higher density of Schwann cells.*

To understand whether the expression level of Schwann cell-specific markers (MBP, S100, p75 and GFAP) changed after delayed nerve regeneration, we performed quantitative real-time PCR analysis on the regenerated distal nerve stumps. We focused on the worst case, which was the 6-months delayed repair group. We compared these real-time PCR results with the results obtained after 6 months of degeneration (as a negative control) and with healthy control nerves (as a positive control). These last two samples are the same as those discussed in Figure 2. Moreover, as additional positive control for regeneration, we used a 3-months regenerated distal segment of an end-to-end repaired median nerve. As expected, the MBP mRNA level (Figure 8A) increased after delayed nerve regeneration compared with the 6-months degenerating nerve; however, its expression was still different compared with both the healthy control values and the end-to-end repaired median nerve.

Intriguingly, S100 (Figure 8B) mRNA expression in the 6-months delayed repair group remained down-regulated compared with both the healthy control nerve and the end-to-end repaired median nerve; however, its expression was higher than the 6-months degenerating nerve. Expression of p75 (Figure 8C) strongly increased after nerve degeneration, and it was still up-regulated after delayed nerve regeneration when compared with the healthy control group. Expression of GFAP (Figure 8D) was lower than in the two positive control groups.

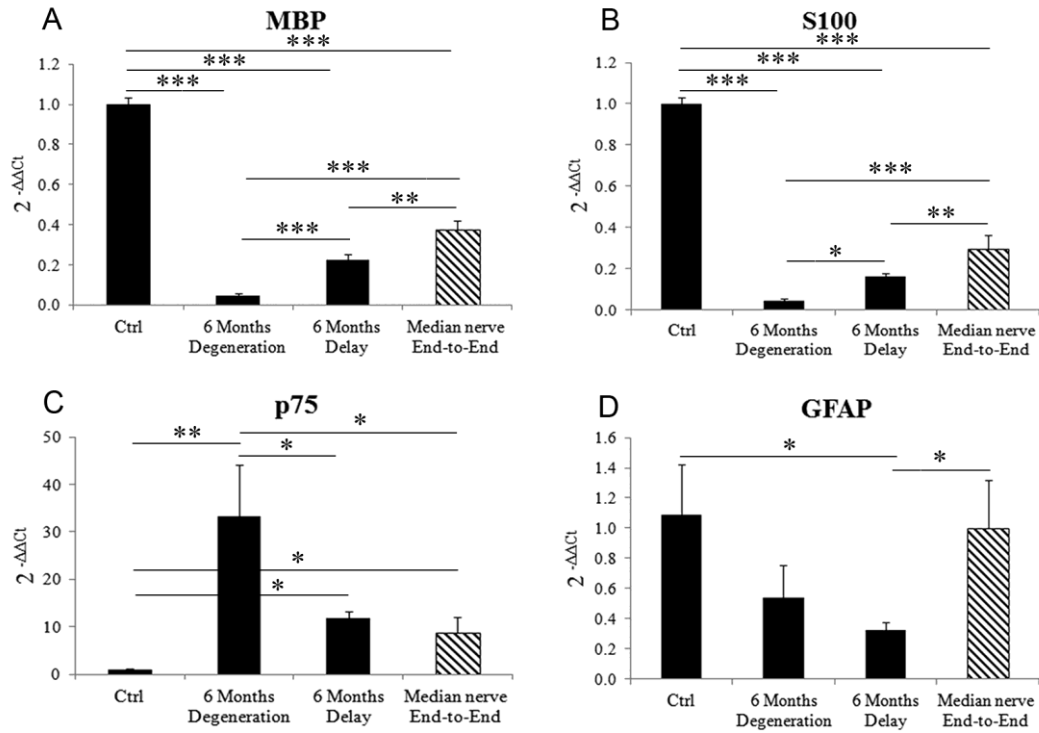


Fig. 8: Quantitative real-time PCR results of MBP (A), S100 (B), p75 (C) and GFAP (D) mRNA expression in the 6-month delayed repair group, after 6 months of regeneration (Reg, Figure 1). This result was compared with the expression level obtained in the 6-month degenerating nerve (DegM, Figure 1) and in the control nerves (already shown in Figure 2, but now expressed as $2^{-\Delta\Delta C_t}$ instead of $-\Delta\Delta C_t$). An additional positive control for regeneration (end-to-end repaired median nerve, 3 months after regeneration) is shown. Data were normalized to the geometrical mean of 2 endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphs are expressed as the mean + SEM (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). After 6 months of delayed regeneration, Schwann cell markers were regulated differently compared with the healthy control nerve and the end-to-end-repaired median nerve.

Finally, we evaluated the regulation of the NRG1/ErbB system after delayed nerve repair. We observed that ErbB2 and ErbB3 mRNA expression was slightly up-regulated after delayed regeneration compared with the 6-months degeneration and the end-to-end repaired median nerve (for ErbB2; Figure 9A) or the healthy control group (for ErbB3; Figure 9B). Intriguingly, mRNA expression of soluble NRG1 (type I/II), for which expression strongly decreased after nerve degeneration, was still strongly down-regulated after delayed regeneration. Indeed, its level was comparable to the 6-months degeneration expression level and differed from the level in the healthy control and to end-to-end repaired median nerve (Figure 9C).

For the protein analysis (Figure 9D), we observed a very low ErbB2 expression in all experimental groups. The ErbB3 protein seemed to be slightly up-regulated during delayed regeneration compared with the degenerating nerve, but it was still lower than the two positive controls. With respect to NRG1 protein expression, we did not detect any band after delayed regeneration, whereas in the end-to-end repaired median nerve, a barely detectable band was present.

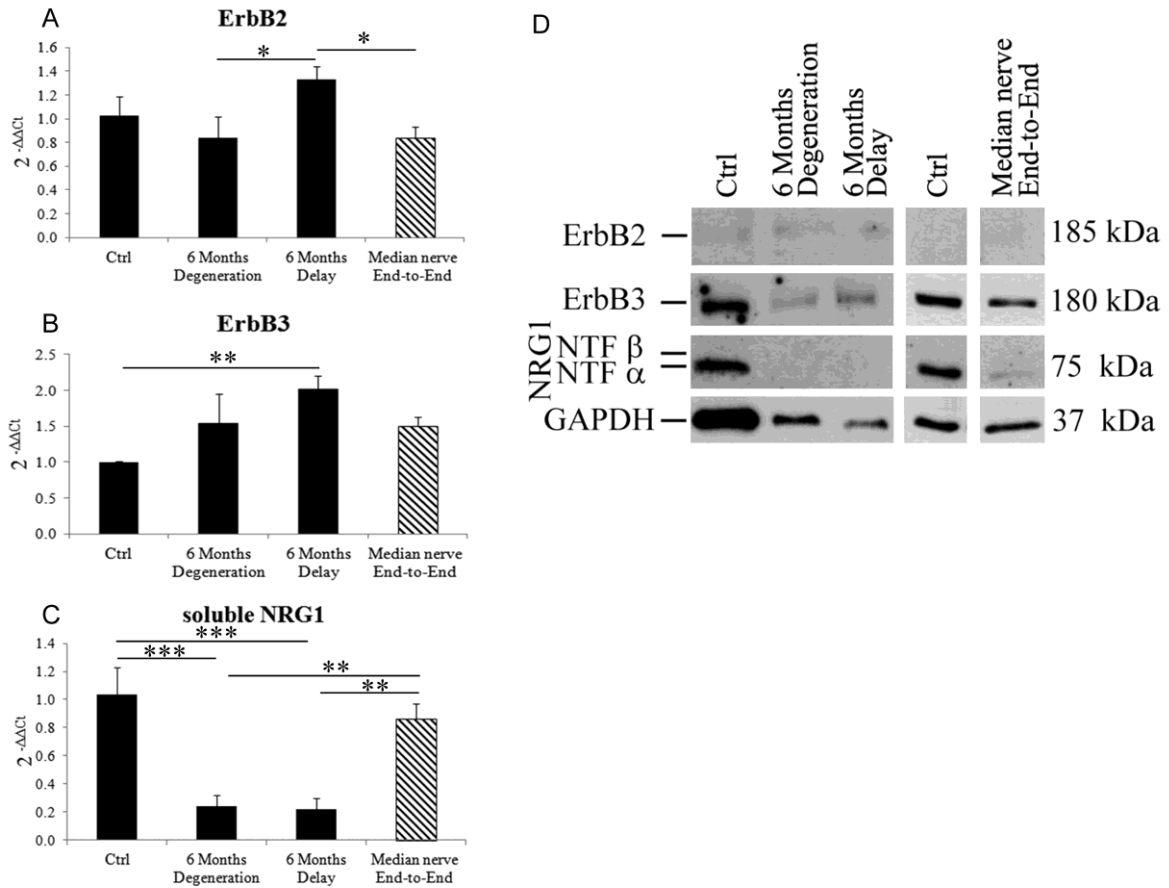


Fig.9: Quantitative real-time PCR results of ErbB2 (A) and ErbB3 (B) receptors and soluble type I/II NRG1 isoform (C) expression in the 6-month delayed repair group, after 6 months of regeneration (Reg, Figure 1). This result was compared with the expression obtained in the 6-month degenerating nerve (DegM, Figure 1) and in the control nerves (already shown in Figure 3, but now expressed as $2^{-\Delta\Delta C_t}$ instead of $-\Delta\Delta C_t$). An additional positive control for regeneration (end-to-end-repaired median nerve, 3-months after regeneration) is shown. Data were normalized to the geometrical mean of 2 endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphs are expressed as the mean + SEM (*: $p \leq 0.05$; **: $p \leq 0.01$; * $p \leq 0.001$). Soluble NRG1 (type I/II) was still strongly down-regulated after 6 months of delayed nerve regeneration. D: Western blot analysis of proteins extracted from the 6-month delayed repair group and probed with antibodies for ErbB2, ErbB3, and NRG1 (AB5551 antibody, which can recognize 2 bands of approximately 75 kDa [α -NTF, β -NTF]); GAPDH was used as a loading control. The positions of the detected proteins are indicated on the left, size markers are indicated on the right. NRG1 was not detectable after delayed regeneration.**

DISCUSSION

In this study, focused on 1) the molecular changes in the chronically degenerated nerve, with specific attention paid to the NRG1/ErbB gliotrophic system; and 2) the ability of the chronically degenerated nerve to sustain regeneration of freshly axotomized axons. This study demonstrated that chronic degeneration of the distal nerve stump compromises nerve regeneration in terms of functional recovery, as well as the number and size of regenerated myelinated fibres. The inability of the distal nerve stump to sustain regeneration could be due to impaired Schwann cells that are not able to completely recover from atrophy. Indeed, we hypothesized that the down-regulation of NRG1 still observed after delayed regeneration negatively affects Schwann cell activities and, consequently, nerve regeneration.

Surgical paradigm choice

We used the surgical paradigm of the cross-suture between the chronically denervated median nerve distal stump and the freshly axotomized ulnar nerve proximal stump. A similar paradigm was used in studies by Sulaiman & Gordon and by Sulaiman et al., where they cross-sutured the denervated common peroneal nerve with the freshly axotomized tibial nerve (Sulaiman and Gordon, 2000, Sulaiman et al., 2002). We used a similar surgical procedure (but shifting from the hindlimb to the forelimb) for three main reasons. First, we wanted to mimic the clinical surgical procedure, in which the proximal nerve stump is usually refreshed before repairing a nerve injury (Gordon and Tetzlaff, 2015) (in our experiments, the ulnar nerve axotomy mimics this event). Second, before the cross-suture we withdrawn degenerating median nerve segments from the same animals, to reduce the number of used animals. Indeed, “Reduction” (together with “Replacement” and “Refinement”) represents one of the 3 R’s principle. “Reduction” means to minimize the number of animals used in the study, by obtaining more information from the same number of animals. Indeed, we were able to study the long-term degenerating process and the regeneration after delayed repair from the same animals. Third, this technique needs a single suture, while the grafting technique requires two sutures to repair the nerve. In this way, it is possible to limit the variability due to the surgery

(indeed, a double suture means more variability in terms of regeneration, since re-growing axons must cross two consecutive sutures).

Nevertheless, it is important to remember that the translation from animal models to humans is sometimes unreliable for nerve regeneration, due to the difference between rat and human. These differences are reflected in a different outcome after nerve repair: depending on the surgical paradigm, peripheral nerves regenerate faster and better in rat compared to human, and rat may completely recover, unlike the human. However, this is a general issue, not unique to nerve regeneration studies, as the most of *in vivo* researches are carried out using animal models (Kaplan et al., 2015).

Long-term degenerating nerve strongly down-regulates soluble NRG1

In the first part of our study, we analyzed the chronically denervated median nerve distal stumps. In particular, we evaluated the expression of Schwann cell markers and of the NRG1/ErbB gliotrophic system, in order to understand which molecular changes occur after long-term degeneration before repairing the median nerve with a freshly axotomized ulnar nerve proximal stump.

The analysis of the myelin basic protein (MBP) expression was used as an internal control of degeneration, as well as the ultrastructural analysis performed by electron microscopy. As expected, MBP mRNA level strongly decreases during degeneration, reflecting the absence of myelinated fibres, also not more detected on a morphological level.

Since the differentiation status of Schwann cells has been demonstrated to change after injury (i.e. when the Schwann cell – axonal contact is lost), we analyzed the expression of the most common Schwann cell markers (S100, p75 and GFAP). S100 is known to be expressed by both immature and mature myelinating and non-myelinating Schwann cells (Conrad et al., 2009). By immunofluorescence analysis, it has been shown a progressive decrease in S100-positive cells in long term denervated (4 or 6 months) distal stumps (Li et al., 1997, You et al., 1997). Our results, obtained by quantitative real time PCR mRNA analysis, are consistent with these data and we hypothesize that S100 down-regulation is due to Schwann cell atrophy.

p75 receptor is a member of the tumor necrosis factor receptor family and binds neurotrophins with low affinity. Neurotrophins and their receptors are known to be involved in peripheral nerve regeneration. In particular, p75 is expressed in axotomized motoneurons after injury (Funakoshi et al., 1993), is up-regulated in Schwann cells after nerve injury, and this up-regulation has been associated with Schwann cell migration (Anton et al., 1994) and apoptosis (Ferri and Bisby, 1999, Soilu-Hanninen et al., 1999). The subsequent down-regulation is generally associated with target reinnervation (You et al., 1997). Here we observed a strong increase of p75 mRNA during long-term degeneration. Since no axons are present in the chronically degenerated distal nerve stump (axons are indeed already degenerated within 3 months and are not more visible by electron microscopy), and no regeneration occurs, p75 is more likely up-regulated by Schwann cells that progressively undergo atrophy.

Finally, it has been demonstrated that Glial Fibrillary Acidic Protein (GFAP), a Schwann-cell-specific cytoskeleton constituent expressed by non-myelinating Schwann cells, is upregulated after damage (3-5 days after injury) (Jessen et al., 1990, Triolo et al., 2006). After long-term denervation (up to 9 months), we observed a down-regulation of GFAP, which follows a similar pattern described for S100 and MBP. The down-regulation, also in this case, is likely due to Schwann cells that are undergoing atrophy after chronic denervation.

Previous studies demonstrated that soluble NRG1 (type I/II), expressed by Schwann cells, is strongly up-regulated in rat median nerve in response to acute injury (Stassart et al., 2013, Ronchi et al., 2015). However, this up-regulation is transient and soluble NRG1 mRNA expression returns to control values within 28 days. In this study, we demonstrated that soluble NRG1 mRNA expression strongly decreases during chronic degeneration, starting from 3 months from nerve axotomy. We also analyzed axonal transmembrane NRG1 at protein level, by using an antibody directed to the cytoplasmic N-terminus common to all transmembrane type III NRG1. This antibody recognizes different bands (Ronchi et al., 2015) which can be the product of NRG1 type III cleavage by the α -secretase ADAM17/TACE, or by the β -secretase BACE1. In a previous work we hypothesized a “switch” between ADAM17/TACE to BACE1 proteolytic cleavage of type III NRG1 under regenerative

conditions (Ronchi et al., 2015). From a functional point of view, this change means a “switch” between the inhibition of the myelination (mediated by ADAM17/TACE cleavage of type III NRG1) (La Marca et al., 2011) and the promotion of the myelination (mediated by BACE1 cleavage of type III NRG1) (Willem et al., 2006). Here we observed that during chronic degeneration (3, 6 and 9 months), transmembrane NRG1 is totally missing, while in the control healthy nerves only the NRG1 fragment produced by the ADAM17/TACE cleavage is detected. These results suggest that both transmembrane type III NRG1 (mainly expressed by axons) and soluble type I/II NRG1 (mainly expressed by Schwann cells) are no more expressed during long-term nerve degeneration, probably because axons are no more present in the chronically denervated distal nerve stump and Schwann cells are atrophic.

Then we focused our attention on the heterodimer ErbB2-ErbB3 that is the NRG1 receptor expressed by Schwann cells. ErbB2 and ErbB3 mRNA did not change in the chronically denervated nerves (they remain similar to control healthy nerve values). However, ErbB2 and ErbB3 proteins follow a different expression pattern compared to mRNA since a decrease is detected after 9 months of degeneration. A discrepancy between mRNA and protein expression has already been described in a previous study (Ronchi et al., 2015) and can be explained with a post-transcriptional regulation microRNA-mediated. Previous studies reported that ErbB2 mRNA is still expressed in Schwann cells that had been denervated for 1 month (Li et al., 1997, Ronchi et al., 2015), whereas it is not more expressed after long-term denervation (Li et al., 1997), in accordance with our data. We suggest that this down-regulation at protein level could be due to Schwann cell atrophy.

Nerve Regeneration is impaired after delayed repair

As already demonstrated, healthy Schwann cells are indispensable for nerve regeneration (Fu and Gordon, 1997, Arthur-Farraj et al., 2012). In the last years, different studies tried to understand whether and how chronic denervation of Schwann cells affects axonal regeneration. It has been demonstrated that, after delayed repair, Schwann cells in the distal stump of a long-term degenerated nerve lose their ability to support nerve regeneration, suggesting that they need to be reinnervated in a timely manner (Sulaiman and Gordon, 2000, Hoke, 2011). Indeed,

these cells progressively undergo atrophy, down-regulate the expression of factors that sustain nerve regeneration (Li et al., 1997, Hoke et al., 2002, Hoke, 2011) and up-regulate the expression of molecules that inhibit axon regeneration (Zuo et al., 1998, Heine et al., 2004, Hoke, 2011).

In our study we also demonstrate that long-term chronic denervation dramatically reduces nerve regeneration from different points of view. First, we did not observe any functional recovery in the two experimental groups where nerve repair was delayed in time (both 3 and 6 months delayed repair groups). Nevertheless, we observed only a partial functional recovery of the immediate repair group: at the end of the observation time (24 weeks), only 4 animals out of 7 showed a functional recovery (but no one of the animals reached control values, that is approximately 450 grams) (Ronchi et al., 2009). Moreover, this recovery is also lower compared to the end-to-end repaired median nerve, where animals reach approximately 335 grams (Lee et al., 2007). This incomplete recovery can be explained with the complexity of the surgical model (cross suture between ulnar and median nerves), which requires a reorganization of projections from the central nervous system. Indeed, it has already been widely demonstrated that following a peripheral injury there is a reorganization of cortical representation, as well as plastic changes in subcortical structures such as the thalamus, brainstem or spinal cord (Chen et al., 2002, Wall et al., 2002). Moreover, in our surgical paradigm, axons belonging to the ulnar nerve, that in the rat innervates the intrinsic muscles of the paw (Papalia et al., 2006), after nerve repair regenerate into the median nerve distal stump that, in the rat, innervates extrinsic flexor muscles of the fingers (Papalia et al., 2006). This means that regenerated axons (from the ulnar nerve to the median nerve) have to innervate different muscles and the time required to learn this new skill is longer compared to a direct suture.

Despite this negative functional recovery, we observe many myelinated fibres in all experimental groups (not only in the immediate repair group where there is a partial functional recovery, but also in the two delayed repair groups). However, by performing stereological and morphometrical analysis, we observe a significantly lower number of myelinated fibres in the delayed repair groups compared to the immediate repair group. On the other hand, only the latter shows a significant higher fibre number compared to healthy nerves. However, this is a typical condition of

peripheral nerve regeneration, where axons sprout from a single regenerating fibre. Indeed, also in the end-to-end repaired median nerve, the number of myelinated fibres increases compared to the healthy nerve (Lee et al., 2007). Size parameters reveal that myelinated fibres (as well as axons) of the delayed repair groups are smaller when compared to the immediate repair group. These data are even more clear when we observe the fibre diameter distribution: despite the distribution pattern is similar among the three experimental groups (they show an unimodal distribution, whereas in the control healthy nerve a typical bimodal distribution can be observed), in the two delayed repair groups, the percentage of very small fibres (1-2 μm) is higher when compared to the immediate repair group. Finally, we calculated the g-ratio (inner axonal diameter divided by the outer axonal diameter), a common tool to evaluate myelination: lower g-ratio indicate thicker myelin and *vice versa*. The mean g-ratio did not change among groups, but when we plot the g-ratio of individual fibres in relation to respective axon diameter, we observe that the slope of the linear regression is higher in the two delayed repair groups compared to both immediate repair and control healthy groups. The slope of the linear regression is an indirect parameter of nerve fibre maturation: the higher the slope, the higher the ratio between the g-ratio and the axon diameter and the lower the fibre maturation (Gambarotta et al., 2015).

Taken together, these results confirm that when the repair is delayed in time (both 3 and 6 months), myelinated fibres which regenerated within the degenerated distal stump are less numerous, smaller and less mature compared to fibres which are allowed to regenerate immediately after axotomy, demonstrating that a poor regeneration occurred.

The poor outcome after delayed repair is due to impaired Schwann cells

To explain the reduced regeneration, we focused our attention on Schwann cells. The ratio between myelinated fibres and Schwann cells showed that, in proportion to the total number of regenerating fibres, Schwann cells are more numerous in the two delayed repair groups compared to both control healthy nerve and immediate repair groups. We hypothesize that a greater number of Schwann cells reflects shorter internodal length. Indeed, it has already been demonstrated that internodes are

shorter after regeneration (Court et al., 2004, Wu et al., 2012). In demyelinating pathologies the repair of demyelinating regions is accompanied by proliferation of supernumerary Schwann cells and the formation of new shorter internodes (Mathey and Armati, 2007). Also during nerve regeneration, an higher number of Schwann cells has been described: there is an overproduction of Schwann cells which envelop the regrowing axons followed by a decrease after 10 months from the nerve repair (Schroder, 1968, Hirata et al., 1998). It is well known that internodal distance affects the velocity of nerve impulse conduction (Brill et al., 1977, Wu et al., 2012); shorter internodes can reflect slower conduction velocity that might explain the negative function recovery in the two delayed repair groups.

We then analyzed Schwann cells from a biomolecular point of view. To identify the molecular changes between degeneration and regeneration conditions we focused our attention on the worst case (6 months delay) and we compared expression profiles with its negative control (6 months of degeneration). Moreover, end-to-end repaired median nerve was used as positive control of regeneration. We observed an expected increase of MBP mRNA expression after delayed nerve regeneration (of course its expression is still different compared to the healthy control and to the end-to-end repaired median nerve, due to the presence of smaller fibres with thinner myelin thickness). p75 is still up-regulated while S100 mRNA expression is still strongly down-regulated compared to control healthy nerves. These data confirm a previous work where a decrease in S100 level has been shown in the distal stump of 3 and 6 months delayed repaired nerves (Jonsson et al., 2013). It is interesting to notice that the down-regulation of S100 does not reflect a decrease of Schwann cell relative number. Therefore, we hypothesize that, after chronic degeneration, Schwann cells are impaired in their activities and characteristics, even after 6 months of regeneration. This hypothesis is also supported by the analysis of the NRG1/ErbB system: the mRNA expression of the soluble NRG1 isoform (type I/II) is still strongly down-regulated after nerve regeneration, even if its receptors are slightly up-regulated probably to compensate the ligand lack. On the contrary, in the end-to-end repaired median nerve soluble NRG1 expression level is similar to the healthy nerve. At the protein level, the axonal transmembrane NRG1 type III is still missing after delayed regeneration, even whether axons partially regenerated, as showed by

morphological and stereological analysis. Indeed, it has been already demonstrated that axonal transmembrane NRG1 type III is a rate limiting for nerve remyelination in the early phases after injury, while at later stages other signaling pathways appear to compensate, therefore suggesting that axonal NRG1 is not necessary for long term nerve remyelination (Fricker et al., 2013).

CONCLUSION

This study demonstrates that the chronic degeneration of the distal nerve stump compromises nerve regeneration: motor functional recovery, number and size of regenerated myelinated fibres are reduced. This poor outcome might be explained with impaired Schwann cells (NRG1 as well as other Schwann cell markers are deregulated after delayed regeneration) that are not able to support nerve regeneration properly.

All the results support the view that soluble NRG1 could be a good candidate to improve peripheral nerve regeneration. Manipulating its expression (i.e. overexpression of NRG1 in denervated Schwann cells) might possibly lead to better results. Future studies are therefore necessary to elucidate if this strategy could be promising to improve the outcome after delayed nerve repair.

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MY CONTRIBUTION TO THE MANUSCRIPT

I mainly contributed to biomolecular analyses and interpretation of *in vivo* data. Moreover, I prepared the pictures and I revised the manuscript.

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CHAPTER 8

Soluble Neuregulin1 is strongly up-regulated in the rat model of Charcot-Marie-Tooth 1A disease

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ABSTRACT

Neuregulin1 (NRG1) is a growth factor playing a pivotal role in peripheral nerve development through the activation of the transmembrane co-receptors ErbB2-ErbB3. Soluble NRG1 isoforms, mainly secreted by Schwann cells, are strongly and transiently up-regulated after acute peripheral nerve injury, thus suggesting that they play a crucial role also in the response to nerve damage. Here we show that in the rat experimental model of the peripheral demyelinating neuropathy Charcot-Marie-Tooth 1A (CMT1A) the expression of the different NRG1 isoforms (soluble, type α and β , type a and b) is strongly up-regulated, as well as the expression of NRG1 co-receptors ErbB2-ErbB3, thus showing that CMT1A nerves have a gene expression pattern highly reminiscent of injured nerves. Because it has been shown that high concentrations of soluble NRG1 negatively affect myelination, we suggest that soluble NRG1 over-expression might play a negative role in the pathogenesis of CMT1A disease, and that a therapeutic approach, aimed to interfere with NRG1 activity, might be beneficial for CMT1A patients. Further studies will be necessary to test this hypothesis in animal models and to evaluate NRG1 expression in human patients.

KEYWORDS

Charcot-Marie-Tooth 1A (CMT1A); Neuregulin1 (NRG1); ErbB; peripheral nerve; Schwann cells; neuropathy

INTRODUCTION

Charcot-Marie-Tooth (CMT) is one of the most frequent inherited neurological diseases, affecting both motor and sensory nerves; it is clinically characterized by weakness and muscle wasting of distal limb, skeletal foot deformities, distal sensory loss and problems with hand function (Schenone et al., 2011). CMT is caused by mutations in several genes encoding proteins involved in the physiological function of the peripheral nerve axon or in the myelin sheath formation (Baets et al., 2014). CMT1A, the most common form of CMT, is an autosomal dominant disease that derives from peripheral myelin protein 22 (PMP22) gene duplication (Patel et al., 1992).

Schwann cell activity is regulated by several factors, including soluble and transmembrane isoforms of the ligand NRG1, which play different roles both in myelination during development and in remyelination following nerve injury (Gambarotta et al., 2013). Soluble NRG1 isoforms, mainly secreted by Schwann cells, play their role following nerve injury: their absence or over-expression do not affect myelination, while their down-regulation negatively affects re-myelination. Transmembrane NRG1 isoforms, mainly expressed by axons, are involved in both development and nerve repair: their down-regulation impairs - and their over-expression promotes - both myelination and re-myelination. NRG1 activates tyrosine-kinase receptors belonging to the ErbB family (Mei and Xiong, 2008). ErbB2-ErbB3 is the NRG1 heterodimer receptor expressed by Schwann cells.

To the best of our knowledge, no data about NRG1 isoform expression in CMT1A nerves are available. In this study the expression of different NRG1 isoforms and receptors was analysed during different stages of the disease in an animal model of CMT1A represented by a transgenic rat over-expressing Pmp22 (Sereda et al., 1996).

MATERIALS AND METHODS

Sciatic nerves were obtained from both females and males P3, P16, P28, P56 transgenic Sprague-Dawley rats over-expressing Pmp22 (CMT1A rats) (Sereda et al., 1996). Animals were bred at the IRCCS-AOU San Martino IST Animal Facility of Genova, Italy. Heterozygous transgenic animals (CMT1A) from three independent litters were compared to their age-matched non-transgenic (WT) littermates following Italian Health Ministry guidelines. Total RNA and proteins were extracted by TRIzol Reagent (Invitrogen) following manufacturer's instructions, and were analysed by quantitative real time PCR (qRT-PCR) and Western blot analysis as previously described (Ronchi et al., 2016). Densitometric analysis was performed by Quantity One Software (Bio-Rad).

RESULTS

mRNA and protein expression level of different NRG1 isoforms and ErbB2-ErbB3 receptors was evaluated in sciatic nerves obtained from CMT1A or WT rats at different development stages.

NRG1 isoforms can be classified as transmembrane or soluble according to their structure, and further subdivided into α and β isoforms according to the epidermal growth factor (EGF)-like domain, and into type a, b and c isoforms, according to the C-terminal domain (Falls, 2003). Different primer pairs (Ronchi et al., 2016) allowed us to discriminate among the different isoforms.

Data show (Figure 1A) that soluble NRG1 mRNA is strongly up-regulated in CMT1A nerves from P3. A significant up-regulation is appreciable also for NRG1 isoforms α and β and for type a and b isoforms from P16. Statistical analysis with two-way ANOVA using phenotype and age as factors revealed significant effects and significant interaction between factors for soluble, α , β , type a, and type b NRG1 isoforms, while NRG1 type c shows significant effects only for age.

At the protein level (Figure 1B), soluble NRG1 in CMT1A nerves shows an up-regulation of the 75-kDa band, corresponding to the C-terminus fragment of NRG1 deriving from precursor protein proteolytic cleavage, a necessary step for soluble ligand release (Mei and Xiong, 2008, Fleck et al., 2016). Moreover, a 100-kDa band,

corresponding to the precursor of the soluble NRG1, is strongly expressed starting from P16, suggesting that NRG1 over-expression saturates the protease machinery responsible of protein cleavage and soluble ligand release.

The axonal transmembrane NRG1 precursor (150-kDa) is expressed at similar levels in WT and CMT1A animals until P16, while at P28 its level slightly decreases in transgenic rats. A 75-kDa band corresponding to the N-terminus fragment of transmembrane NRG1 following proteolytic cleavage (Mei and Xiong, 2008), seems to be slightly more highly expressed in WT nerves from P28, coherently with CMT1A demyelinating phenotype.

At mRNA level (Figure 1A), ErbB2 and ErbB3 show a small decrease during development both in WT and in transgenic animals; at P56 a higher expression of both ErbB2 and ErbB3 is detected in CMT1A animals in comparison to WT. Statistical analysis with two-way ANOVA using phenotype and age as factors, revealed a significant effect of both for ErbB2 and ErbB3. At the protein level (Figure 1B), both ErbB2 and ErbB3 are more highly expressed in transgenic animals starting from P28.

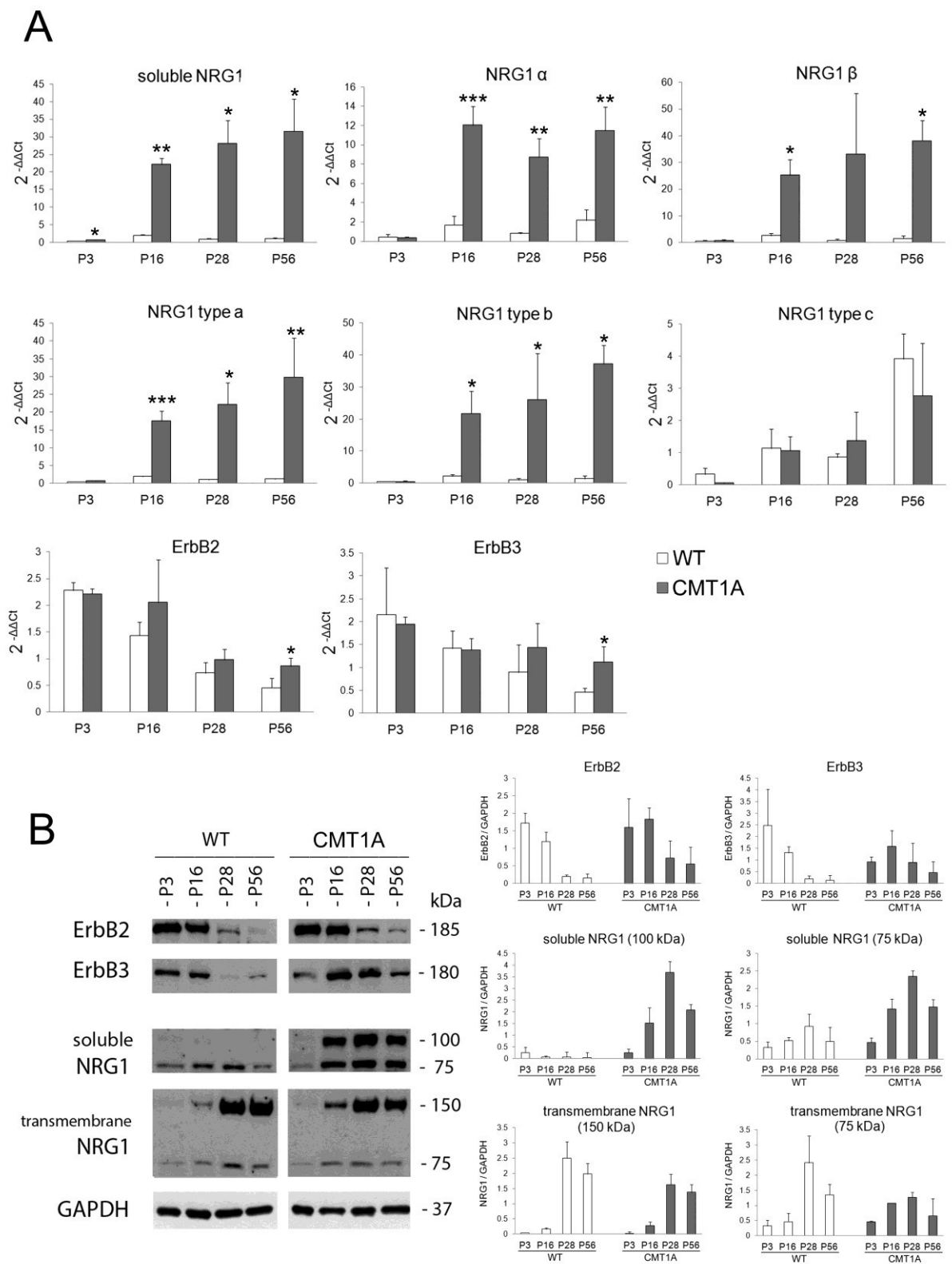


Fig. 1: Soluble NRG1 is strongly up-regulated in CMT1A nerves. Panel A - The relative quantification ($2^{-\Delta\Delta C_t}$) of different isoforms of NRG1, ErbB2 and ErbB3 receptors was obtained by qRT-PCR carried out on RNA obtained from sciatic nerves withdrawn at different time points from WT and CMT1A rats (n=3 for each time point). For each gene, the ΔC_t average of P3 WT nerves was used as calibrator. Data were normalized to the geometric mean of two endogenous housekeeping genes (ANKRD27 and RICTOR) (Gambarotta et al., 2014). Values in the graphics are expressed as mean + standard deviation (SD). Statistical analysis (Student's t-test) was performed to compare the differences between WT (white bars) and CMT1A (black bars) animals at the different time points ($p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Panel B - Western blot analysis of proteins extracted from WT and CMT1A sciatic nerves probed with antibodies for ErbB2 (#sc-284, Santa Cruz), ErbB3 (#sc-285, Santa Cruz), soluble NRG1 type a (#sc-348, Santa Cruz), transmembrane NRG1 (#AB5551, Chemicon), GAPDH (#4300, Ambion). A representative image of two analyses carried out on the biological duplicate and the corresponding densitometric analyses are shown.*

DISCUSSION

CMT1A nerves are characterized by Schwann cell de-differentiation and immaturity marker up-regulation (Fledrich et al., 2014). Most of these genes are up-regulated also following acute nerve injury, thus suggesting that CMT1A nerves show a gene expression pattern highly reminiscent of Wallerian degeneration (Martini et al., 2013). Indeed, we and others previously demonstrated that soluble NRG1 isoforms are strongly and transiently up-regulated after acute nerve injury (Stassart et al., 2013, Ronchi et al., 2016) and here we show that soluble NRG1 isoforms are strongly up-regulated in CMT1A nerves from P3. ErbB receptors are also up-regulated, as shown in human samples too (Massa et al., 2006), and this up-regulation may be the result of a positive feedback loop or dysregulated ErbB trafficking (Lee et al., 2016).

The strong NRG1 over-expression found in CMT1A nerves raises the question on the function of NRG1 in chronic nerve injuries: does NRG1 counteract this disease, attenuating its symptoms and promoting nerve repair, or does it worsen it?

Recent results (Fledrich et al., 2014) demonstrated that the crossbred of CMT1A mice with transgenic mice over-expressing soluble NRG1 led to a phenotype rescue. Furthermore, systemic injection from P6 to P18 of recombinant soluble NRG1 in CMT1A rat pups lead to similar results, thus demonstrating that, when provided in early development stages, soluble NRG1 ameliorates CMT1A pathogenesis, while no improvements were detected when the treatment started later.

Nevertheless, it has been also demonstrated by others that soluble NRG1 has bifunctional concentration-dependent effects on myelination: low concentration promotes, high concentration inhibits myelination (Zanazzi et al., 2001, Syed et al., 2010, Eshed-Eisenbach et al., 2016). Therefore, we hypothesize that, although early acute treatment with recombinant NRG1 seemed to play a positive role (Fledrich et al., 2014) (maybe due to the low expression of endogenous NRG1 protein at early development stages), over-expression of endogenous NRG1 in the following stages might play a negative role, worsening CMT1A pathogenesis. If our hypothesis is true, a possible therapeutic approach might consist in inactivation of the signal transduction pathways activated by NRG1, through the use of drugs targeting ErbB receptors (Awada et al., 2016).

In support to this therapeutic proposal, it has been shown that treatment with ErbB2 inhibitor, Herceptin, promotes axonal outgrowth after acute nerve transection and repair (Placheta et al., 2014, Hendry et al., 2016). Herceptin treatment might promote regeneration by limiting the excess of soluble NRG1 activity in CMT1A chronic injury. This therapy might bypass the limits of the early phase NRG1 treatment (Fledrich et al., 2014) - that might have a neoplastic potential (Martini, 2014) - and might have a broader use, because it would not be restricted to those patients who receive an early diagnosis of the disease.

Soluble NRG1 over-expression observed in CMT1A animal model needs to be confirmed also in human patients, but this analysis requires accurate human sample collection and further studies. If soluble NRG1 is over-expressed also in human

CMT1A nerves, a therapeutic approach aimed to inhibit the signal transduction pathways driven by NRG1 might be carefully developed.

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MY CONTRIBUTION TO THE MANUSCRIPT

I extracted and analysed RNA and proteins, I interpreted the obtained data and I analysed them statistically. Moreover, I prepared the figures and collaborated to write the manuscript.

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CHAPTER 9

Neuregulin1 alpha activates migration of neuronal progenitors expressing ErbB4

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ABSTRACT

Deficits in neuronal migration during development in the central nervous system may contribute to psychiatric diseases. The ligand neuregulin1 (NRG1) and its receptor ErbB4 are genes conferring susceptibility to schizophrenia, playing a key role in the control of neuronal migration both during development and adulthood. Several NRG1 and ErbB4 isoforms were identified, which deeply differ in their characteristics. Here we focused on the four ErbB4 isoforms and the two NRG1 isoforms differing in their EGF-like domain, namely α and β . We hypothesized that these isoforms, which are differently regulated in schizophrenic patients, could play different roles in neuronal migration. Our hypothesis was strengthened by the observation that both NRG1 α and NRG1 β and the four ErbB4 isoforms are expressed in the medial and lateral ganglionic eminences and in the cortex during development in rat. We analysed in vitro the signal transduction pathways activated by the different ErbB4 isoforms following the treatment with soluble recombinant NRG1 α or NRG1 β and the ability to stimulate migration. Our data show that two ErbB4 isoforms, namely JMa-cyt2 and JMb-cyt1, following NRG1 α and NRG1 β treatment, strongly activate AKT phosphorylation, conferring high migratory activity to neuronal progenitors, thus demonstrating that both NRG1 α and NRG1 β can play a role in neuronal migration.

KEYWORDS

alternative splicing, neuronal migration, signal transduction, ErbB4, neuregulin1

INTRODUCTION

Neuronal migration represents a critical step in the development of the central nervous system, where neuronal progenitors migrate from their birth site to their final destination (Ghashghaei et al., 2007, Marin et al., 2010).

The tyrosine kinase receptor ErbB4 and one of its ligands, neuregulin1 (NRG1), are involved in the migration of neuronal progenitors from the medial ganglionic eminence (MGE) to the cortex during development (Flames et al., 2004, Lopez-Bendito et al., 2006, Marin et al., 2010, Marin, 2013, Villar-Cervino et al., 2015). Moreover, they also control the migration of subventricular zone (SVZ)-derived neuroblasts toward the olfactory bulb (OB), a process that continues throughout the whole life of the animal (Anton et al., 2004, Ghashghaei et al., 2007, Birchmeier, 2009).

Since NRG1 and its receptors ErbB3 and ErbB4 are genes conferring susceptibility to schizophrenia (Hahn et al., 2006, Iwakura and Nawa, 2013), deeper studies on the NRG-ErbB system could contribute to a better understanding of the role played by this system in both physiological and pathological conditions (Mei and Nave, 2014).

ErbB4 is a tyrosine kinase receptor belonging to the ErbB family. Alternative splicing determines the expression of four different ErbB4 isoforms: JMa-cyt1, JMa-cyt2, JMb-cyt1 and JMb-cyt2 (Mei and Xiong, 2008). The mutually exclusive insertion of exon 15 or 16 determines the expression of JMa (juxtamembrane a) or JMb (juxtamembrane b) isoforms (Mei and Xiong, 2008). Isoforms containing the exon JMa are sensitive to proteolytic cleavage elicited by the metalloprotease ADAM17/TACE (A disintegrin and metalloprotease 17/tumour necrosis factor- α -converting enzyme) which causes the shedding of the extracellular domain, followed by a second cleavage carried out by a presenilin-dependent γ -secretase enzyme (Lee et al., 2002), which releases an intracellular fragment that can be translocated into the nucleus, thus influencing gene transcription (Sardi et al., 2006). Isoforms containing the exon JMb are uncleavable. Isoforms containing exon 26 are named cyt1, while those missing it are called cyt2. The presence of exon 26 in the intracellular domain of the cyt1 isoform confers to the receptor the ability to bind PI3K (phosphatidylinositol-3-kinase) and to activate the corresponding downstream pathway (Junttila et al., 2000); isoforms containing this exon are also bounded by the

E3 ubiquitin ligase and are degraded faster than ErbB4 cyt2 isoforms (Sundvall et al., 2008). The major differences displayed by the four ErbB4 isoforms suggest that they could play different roles by transducing different signals.

ErbB4 can form homodimers or heterodimers with other members of the ErbB family (ErbB1, ErbB2, ErbB3), therefore the signal transduction pathways activated downstream ErbB4 can be deeply influenced by the isoform expressed and by the co-receptor interacting with it (Roskoski, 2013).

In this paper, we investigated the ability of the four ErbB4 isoforms to confer a different migratory activity to neuronal progenitors and to activate the downstream pathways following the stimulation with two NRG1 isoforms. NRG1 gene gives rise to more than 30 different isoforms. The alternative splicing of exons located at the N-terminus allows to divide the NRG1 in 6 different protein types (I-VI): some are synthesized as transmembrane pro-proteins releasing a soluble fragment, others as soluble proteins or transmembrane proteins. NRG1 type I, II, IV, V, VI generate a soluble fragment, while NRG1 type III is a transmembrane isoform (Mei and Xiong, 2008). Alternative splicing of exons located in the C-terminus of the EGF-like domain gives rise to NRG1 α and NRG1 β isoforms (Wen et al., 1994, Falls, 2003, Edwards and Bottenstein, 2006). In many tissues NRG1 β has demonstrated a higher bioactivity compared to NRG1 α (Wen et al., 1994, Eckert et al., 2009).

Intriguingly, NRG1 β is significantly increased while NRG1 α is decreased in the prefrontal cortex of schizophrenic patients (Bernstein and Bogerts, 2013, Bernstein et al., 2013), thus suggesting that the different NRG1 isoforms could play different roles. It has been demonstrated that ErbB4 expressing neuronal progenitors migrate toward their target, attracted by soluble NRG1, interacting with a permissive cell corridor expressing transmembrane NRG1 (Anton et al., 2004, Flames et al., 2004). However, in these previous studies, the expression of the different ErbB4 isoforms, as well as the expression of NRG1 α and NRG1 β isoforms and their role in neuronal migration, were not investigated.

We previously analysed *in vitro* the role played by the different ErbB4 isoforms in NRG1 β -induced migration (Gambarotta et al., 2004), the expression of the different ErbB4 isoforms in the OB and their ability, *in vitro*, to elicit substrate preference (Fregnan et al., 2014). In this study, we further characterized the expression of the

different ErbB4 and NRG1 isoforms in the embryonic ganglionic eminences and in the cerebral cortex and we studied the role played by NRG1 α and NRG1 β in neuronal migration and signal transduction.

MATERIALS AND METHODS

Cell culture

The ST14A cell line was derived from primary cells dissociated from rat striatal primordia at embryonic day 14 and conditionally immortalized by retroviral transduction of the temperature-sensitive variant of the SV40 large T antigen, as previously described (Cattaneo and Conti, 1998). In this work, we used four previously obtained ST14A stable clones (Gambarotta et al., 2004) each expressing one of the four ErbB4 isoforms: JMa-cyt1 (clone A1.1), JMa-cyt2 (clone A2.1), JMb-cyt1 (clone B1.15) and JMb-cyt2 (clone B2.16). Cells stably transfected with the empty expression vector (pIRESpuro2 vector, Clontech) were used as mock samples. ST14A cells endogenously express ErbB1, ErbB2 and ErbB3. ErbB4-transfected cells were grown as previously described (Gambarotta et al., 2004) at 33°C in medium containing 5 μ g/ml puromycin.

Recombinant peptides corresponding to the EGF-like domain of NRG1 α (#296-HR) and NRG1 β (#396-HB) were purchased from R&D systems.

Ethical standards

Tissue samples (cortex and MGE/LGE, medial/lateral ganglionic eminences) were dissected under the stereomicroscope from four E15 Wistar rats (Harlan). Embryos were obtained by caesarean section from a rat dam deeply anaesthetised by intraperitoneal injection of ketamine (100 mg/kg; Ketavet, Bayer) supplemented by xylazine (5 mg/kg; Rompun, Bayer), and immediately sacrificed by decapitation. All procedures were in accordance with the Council Directive of the European Communities (2010/63/EU), the National Institutes of Health guidelines, and the Italian Law for Care and Use of Experimental Animals (DL26/14) and were approved by the Italian Ministry of Health and the Bioethical Committee of the University of

Torino. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

RNA isolation, cDNA preparation and quantitative real-time PCR (qRT-PCR)

RNA was extracted from MGE/LGE and cortex from four E15 Wistar rats. Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Retrotranscription (RT) of 0.75 µg total RNA was carried out in a 25 µl reaction volume containing: 1x RT-Buffer, 0.1 µg/µl bovine serum albumin (BSA), 0.05% Triton, 1 mM dNTPs, 7.5 µM Random Hexamer Primers, 40 U RIBOlock and 200 U RevertAid® Reverse Transcriptase (all RT ingredients were provided by Thermo Scientific). The reaction was performed 10 min at 25°C, 90 min at 42°C, 15 min at 70°C. Quantitative real-time PCR (q-RT-PCR) was carried out using an ABI Prism 7300 (Applied Biosystems) detection system. cDNA was diluted tenfold in nuclease-free water and 5 µl (corresponding to 15ng starting RNA) were analysed in a 20 µl reaction volume, containing 1 x iTaq Universal SYBR Green Supermix (BioRad) and 300 nM forward and reverse primers. Analyses were performed in technical and biological triplicate.

The data from the real-time PCR experiments were analysed using the $-\Delta\Delta C_t$ method for the relative quantification. The threshold cycle number (C_t) values were normalized to an endogenous housekeeping gene: TBP (TATA box Binding Protein). As calibrator the C_t average of all samples was used. All normalized relative quantitative data are shown as $2^{-\Delta\Delta C_t}$.

Primers were designed using Annhyb software (<http://www.bioinformatics.org/annhyb/>) and synthesized by Invitrogen. Primer sequences are reported in Table 1. Predicted amplicon size was validated through capillary electrophoresis analysis of the amplification products (Fragment Analyzer™, Advanced Analytical Technologies).

GENE	Accession Number	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')	Amplicon size
ErbB1	NM_031507.1	CACCACGTACCAGATGGATG	CGTAGTTTCTGGGGCATTTC	83
ErbB2	NM_017003	TGACAAGCGCTGTCTGCCG	CTGTAGTGGGCGCAGGCTG	106
ErbB3	NM_017218.2	CGAGATGGGCAACTCTCAGGC	AGGTTACCCATGACCACCTCACAC	129
ErbB4 JMa	AY375306.1	GGACGGGCCATTCCACTTTACC	CCATGATGACCAGGATGAAGAGCC	90
ErbB4 JMb	AY375308.1	CGGCCTGATGGATAGAACTCCACTG	CCATGATGACCAGGATGAAGAGCC	71
ErbB4 cyt1	AY375306.1	GGAATATTTGGTCCCCCAGGCTTTC	GAGGAGGGCTGTGTCCAATTCAC	101
ErbB4 cyt2	AY375307.1	GGAATATTTGGTCCCCCAGGCTTTC	GTACACAACTGATTCTATTGGAGTCAATTC	91
NRG1 type α	AF194439	CGACTGGGACCAGCCATCTCATAAAG	TTGCTCCAGTGAATCCAGGTTG	141
NRG1 type β	AF194438	CGACTGGGACCAGCCATCTCATAAAG	AACGATCACCAGTAACTCATTTGG	144
soluble NRG1 (type I-II-IV-V)	AF194993	GGCGCAAACACTTCTTCATCCAC	AAGTTTTCTCCTTCTCCGCGCAC	82
transmembrane NRG1 (type III)	AF194439	CCCTGAGGTGAGAACACCCAAGTC	AAGTTTTCTCCTTCTCCGCGCAC	140
TBP	NM_001004198.1	TAAGGCTGGAAGGCCTTGTG	TCCAGGAAATAATTCTGGCTCATAG	68
Dlx2	NM_001191746.1	CAACGAGCCCGACAAGGAAGAC	GAAACTGGAGTAGATGGTGCCTGG	97
Ascl1	NM_022384.1	GAGGGATCCTACGACCCCTTAGTC	CTCCTGCCATCCTGCTTCCAAAG	117
Pax6	NM_013001.2	CATCGGGTTCATGTTGGGCC	GGACTGGGGTTGCATAGGC	114

Table 1. Primers for quantitative real time PCR analysis. The name of the amplified gene or isoform is indicated in the first column, followed by the accession number to the reference sequence. Primer sequences (forward and reverse) are shown in “forward primer” and “reverse primer” columns. In the last column the size (bp) of the amplification product is indicated. A NRG1 isoform diagram in the supplementary information (Fig. S1) shows where the specific primers were designed.

Transwell assays

Transwell assays were carried out using cell culture inserts (Cat. #353097, BD Falcon) as previously described (Pregno et al., 2011). Briefly, 10^5 cells resuspended in 200 μ l 2%FBS DMEM were seeded in the upper chamber of the cell culture insert, on a porous transparent polyethylene terephthalate membrane (8.0 μ m pore size, 1×10^5 pores/cm²). The lower chambers contained 800 μ l 2%FBS DMEM as control condition, or 2%FBS medium added with NRG1 α (200ng/ml, 28.5nM) or NRG1 β (50ng/ml, 6.24nM). Cells were allowed to migrate 18 hours at 33°C. At the end of the incubation time, inserts were washed with PBS containing calcium and magnesium, and the top side of the membrane was wiped clean with cotton tipped applicators to eliminate unmigrated cells. Migrated cells, present on the lower side of the filter, were fixed in 2% glutaraldehyde for 20 minutes at room temperature, washed with water

and then stained with 0.1% crystal violet in 20% methanol. Transwells were photographed (8-bit images, 4x magnification) using a Nikon ECLIPSE TS100 inverted microscope equipped with a Nikon Digital Sight DSL1 camera. All conditions were performed in technical and biological triplicate. Four images were analysed for each transwell using the *ImageJ* software and the amount of migrated cells was expressed as the total area of migrated cells (pixel²). Cells were discriminated by the pores of the transwell membrane by applying a threshold of 300 pixel². To take baseline migration into account, values from random migration (in 2% FBS, baseline) were subtracted from chemotactic migration (in 2% FBS + NRG1 α or NRG1 β) to generate signals of net chemoattraction (Limame et al., 2012).

Protein extraction and western blot analysis

Confluent cells grown on 6-cm diameter dishes were serum-starved for 48 hours and then stimulated with NRG1 α (200ng/ml, corresponding to 28.5nM), or NRG1 β (50ng/ml, corresponding to 6.24nM) in serum free medium. Cells were washed once with warm PBS (33°C) and proteins were extracted by solubilizing cells in 200 μ l boiling Laemmli buffer (LB, 2.5%SDS, 125mM Tris-HCl, pH6.8). Lysates were collected with a cell scraper, denatured at 100°C for 3 minutes, syringed and spun for 20 minutes at 12000 rpm to discard cell debris. Protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Sigma-Aldrich) and equal amounts of proteins (30 μ g) were loaded into each lane. Proteins were resolved by 8% SDS-PAGE, transferred to a supported nitrocellulose membrane (Biorad #162-0093) and blocked 1 hour at 37°C in 1X TBST containing 5% nonfat milk. Primary antibodies used are: anti-phospho-Erk (1:2000, #9106), anti-phospho-Akt Ser 473 (#4051), anti-phospho-Akt Thr 308 (#4056), anti-total Akt (#9272), anti-total Erk (#9102), anti-phospho-EGF Receptor (#4404), anti-phospho-HER2/ErbB2 (#2247), anti-phospho-HER3/ErbB3 (#4561), anti-phospho-HER4/ErbB4 (#4757) (diluted 1:1000, unless differently specified, and all purchased from Cell Signalling Antibodies); anti-total EGF Receptor (#sc-03), anti-total HER2/ErbB2 (#sc-284), anti-total HER3/ErbB3 (#sc-285), anti-total HER4/ErbB4 (#sc-283) (diluted 1:1000, all purchased from Santa Cruz); anti- β -actin (#A5316, diluted 1:4000, purchased from Sigma); GAPDH (#AM4300, diluted 1:20000, purchased from Ambion). Secondary

antibodies used are horseradish peroxidase linked anti-rabbit (#NA934) and anti-mouse (#NA931) diluted 1:40000, purchased from GE Health. All primary antibodies were diluted in 1X TBST containing 5% BSA and 0,02% sodium azide; all secondary antibodies were diluted in 1X TBST containing 1% BSA. Bands were quantified through *Quantity One* software (Biorad).

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics 22.0 software. All data were expressed as mean + standard error (SEM). Statistical differences between two groups were determined using the two-tailed Student's t-test. Data sets containing more than two groups were tested by applying one-way analysis of variance (ANOVA) with Bonferroni's *post hoc*.

RESULTS

Both NRG1 α and NRG1 β are expressed in the embryonic medial/lateral ganglionic eminences and cortex

To investigate the expression of the different NRG1 and ErbB4 isoforms in embryonic (E15) rat MGE/LGE and cortex, a quantitative real time PCR (qRT-PCR) analysis was performed (Figure 1). To validate the accuracy of the dissection, the expression of marker genes for MGE/LGE (*Dlx2* e *Ascl1*) and for the cortex (*Pax6*) was evaluated (Figure S2, panel A).

Data show that soluble NRG1 is more highly expressed in the cortex than in the MGE/LGE (Figure 1A; $p=0.0008$); conversely, transmembrane NRG1 shows higher expression in the MGE/LGE than in the cortex (Fig. 1B; $p=0.0005$), in agreement with previous data published by others (Flames et al., 2004). NRG1 α expression is similar in the two tissues (Figure 1C), while NRG1 β is more highly expressed in the MGE/LGE region (Figure 1D; $p=0.0005$).

The expression of the NRG1 receptor ErbB4 was also analysed; four isoforms of ErbB4 can be produced by alternative splicing: JMa-cyt1, JMa-cyt2, JMb-cyt1 and JMb-cyt2. By qRT-PCR it is not possible to quantify the relative expression of the

different exon combinations, because the amplification product would be too long (more than 1200 bp) for quantitative analysis. Therefore, it was only possible to carry out the expression analysis of the single exons (JMa, JMb, cyt1, and cyt2).

Data show that all the analysed ErbB4 exons are more highly expressed in the MGE/LGE than in the cortex (Figure 1 D-G; JMa $p=4.6724E-09$, JMb $p=7.5843E-05$, cyt1 $p=1.2795E-07$, cyt2 $p=1.2244E-07$). In these samples we also analysed the expression of ErbB4 co-receptors ErbB1, ErbB2 and ErbB3 at mRNA and protein level: ErbB2 and ErbB3 proteins are detectable in the MGE/LGE and cortex, while ErbB1 is barely detectable only in the MGE/LGE (Figure S2, panel B).

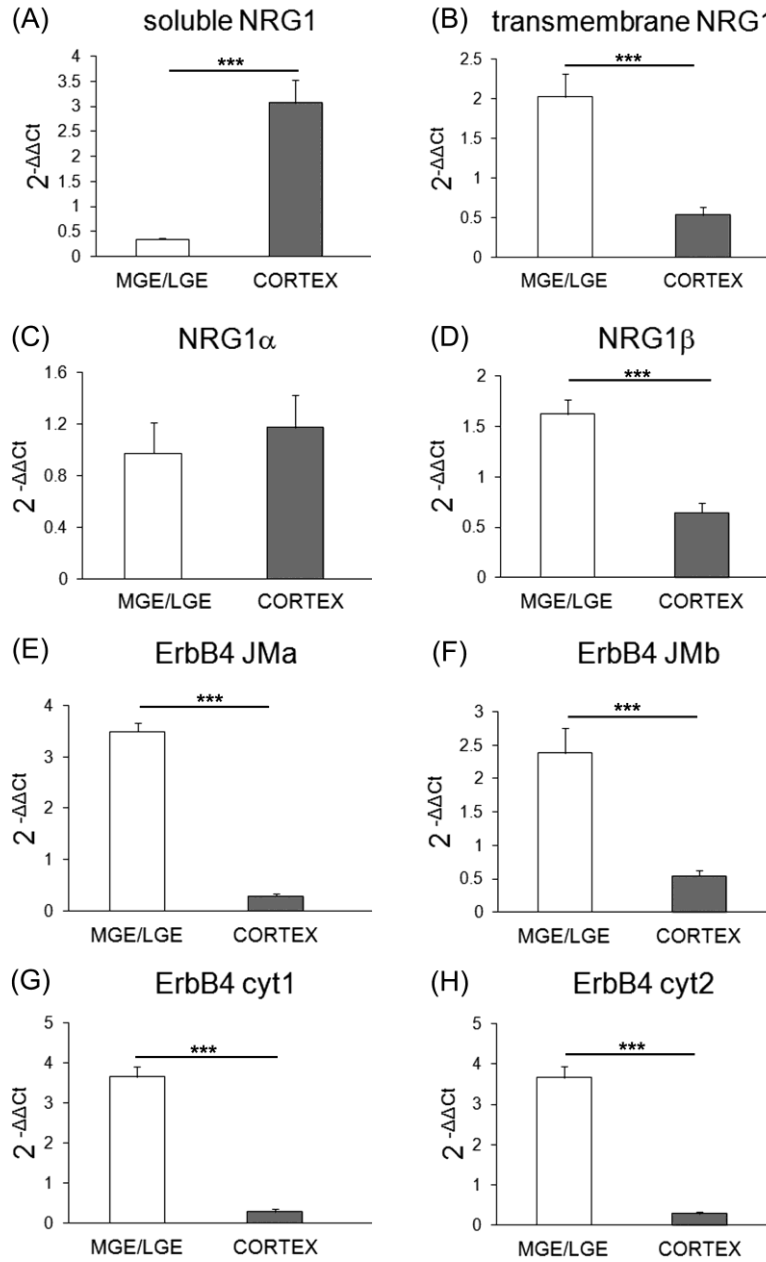


*Fig. 1. Expression analysis of NRG1 and ErbB4 isoforms in embryonic (E15) MGE/LGE and cortex. Analysis of RNA samples obtained from MGE/LGE and cortex of rat embryos at E15. The relative quantification ($-2^{\Delta\Delta C_t}$) of soluble NRG1 (A), transmembrane NRG1 (type III) (B), NRG1 α (C), NRG1 β (D), ErbB4-JMa (E), ErbB4-JMb (F), ErbB4-cyt1 (G) and ErbB4-cyt2 (H) was obtained by quantitative real-time PCR: data were normalized to an endogenous housekeeping gene (TBP). In the absence of a reference calibrator sample, quantitative data were expressed relatively to the average of the two tissue samples. Statistical analysis was carried out using Student's t-Test. Asterisks refer to significant statistical differences, with *** $p \leq 0.001$.*

NRG1 α is able to stimulate the migration of neuronal progenitors stably expressing JMa-cyt2 or JMb-cyt1 ErbB4 isoforms

As both NRG1 α and NRG1 β and the four ErbB4 isoforms are expressed in the MGE/LGE/cortex, migration assays were carried out to test whether NRG1 α is able to stimulate the migration of neuronal progenitors expressing different ErbB4 isoforms.

The migratory activity of the stable clones derived from the neuronal progenitor cells (ST14A) (Cattaneo and Conti, 1998), each expressing one of the ErbB4 isoform (JMa-cyt1, JMa-cyt2, JMb-cyt1 or JMb-cy2) (Gambarotta et al., 2004) was evaluated through transwell assays following the stimulation with either NRG1 α or NRG1 β recombinant peptides, corresponding to the EGF-like domain- α and the EGF-like domain- β .

For NRG1 β we chose 50ng/ml, which is the concentration used in the previously published experiments; since NRG1 α is less bioactive than NRG1 β , different ligand concentrations were tested in preliminary assays to identify the best NRG1 α concentration generating a detectable ErbB3 phosphorylation (data not shown). Preliminary tests of NRG1 stimulation on ErbB4 phosphorylation were not conducted, as the aim of the experiment was to investigate the response of the different ErbB4 isoforms to NRG1 α and it was impossible to know in advance if the different ErbB4 isoforms were going to have the same response. So, 200ng/ml NRG1 α was the chosen concentration for performing the following experiments. Although the stimulation with 200ng/ml NRG1 α induced a lower migration and a lower phosphorylation response compared to the stimulation with NRG1 β , we chose to use a NRG1 α concentration that is not too high, so as to be as close as possible to the physiological conditions; moreover, our aim was to investigate the response of the different ErbB4 isoforms to NRG1 α or NRG1 β stimulation, and not to compare the stimulation strength of the two NRG1 isoforms.

Accordingly, the migratory activity of the different stable clones following the treatment with NRG1 α (or with NRG1 β) was compared to the migratory activity of the corresponding untreated samples (df1=7, df2=16).

Data analysis shows that following the stimulation with NRG1 β , all stable clones expressing the different ErbB4 isoforms migrate more highly than control samples

(Fig. 2, JMa-cyt1 $p=0.0481$, JMa-cyt2 $p=7.3804E-05$, JMb-cyt1 $p=0.0002$, JMb-cyt2 $p=0.0032$). On the contrary, following the stimulation with NRG1 α , only clones expressing JMa-cyt2 and JMb-cyt1 ErbB4 isoforms show increased migratory activity compared to control samples (JMa-cyt2 $p=5.0731E-06$, JMb-cyt1 $p=8.6344E-06$).

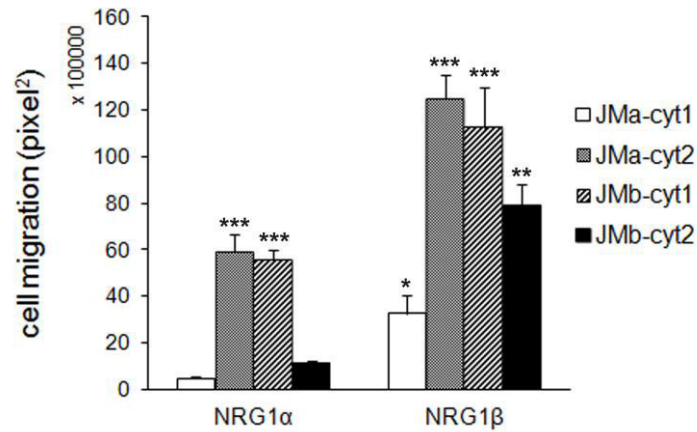


Fig. 2: NRG1 α stimulates migration of neuronal progenitors stably expressing JMa-cyt2 or JMb-cyt1 ErbB4 isoforms.

*Neuronal progenitors stably expressing different ErbB4 isoforms (JMa-cyt1, JMa-cyt2, JMb-cyt1, JMb-cyt2) were stimulated with NRG1 α or NRG1 β . The migratory activity was assessed by transwell assay analysis. Migration is expressed in terms of total area of migrating cells (pixel²). Baseline migration values were subtracted from the corresponding chemotactic migration to show the net chemoattraction. Biological triplicate experiments were carried out in technical triplicate. Asterisks refer to one-way ANOVA significant differences between the treated (NRG1 α or NRG1 β) and the corresponding untreated samples, with * $p\leq 0.05$, ** $p\leq 0.01$ and *** $p\leq 0.001$. No statistical differences between samples treated with NRG1 α or NRG1 β were highlighted, because the concentration of the two ligands is different.*

Stimulation of the different ErbB4 isoforms with NRG1 α and NRG1 β

To study the signal transduction pathways activated by NRG1 α or NRG1 β , neuronal progenitors stably expressing one ErbB4 isoform (JMa-cyt1, JMa-cyt2, JMb-cyt1 or JMb-cyt2) (Gambarotta et al., 2004) were starved in serum free medium for 48 hours and then were stimulated for 15 minutes with 200ng/ml NRG1 α or 50ng/ml NRG1 β . Cells stably transfected with the empty vector were used as mock control.

Experiments were carried out in biological triplicate. Total proteins were extracted, analysed by Western blot and quantified, as shown in Figure 3, where a representative western blot is shown. One-way ANOVA statistical analysis was carried out to compare the protein phosphorylation following the treatment with NRG1 α (or with NRG1 β) with the corresponding untreated sample (df1=9, df2=20).

In the absence of ligand stimulation, ErbB4 is not phosphorylated. Statistical analysis shows that, after stimulation with NRG1 β , ErbB4 is phosphorylated in all stable clones (Fig. 3, JMa-cyt1 $p=7.1520E-07$, JMa-cyt2 $p=4.2316E-08$, JMb-cyt1 $p=0.0248$, JMb-cyt2 $p=1.2633E-06$), while after stimulation with NRG1 α is only phosphorylated in clones expressing JMa-cyt2 and JMb-cyt1 ErbB4 isoforms (JMa-cyt2 $p=0.0468$, JMb-cyt1 $p=0.0355$).

Western blot shows that the expression level of total ErbB4 is not exactly the same in all stable clones. Nevertheless, total ErbB4 quantification (data not shown) shows that there is no correlation between ErbB4 level and phosphorylation following NRG1 stimulation. Indeed, JMb-cyt1 is less expressed, but highly phosphorylated, while JMb-cyt2 is more expressed, but less phosphorylated; JMa-cyt1 and JMb-cyt1, with comparable expression levels, are differently phosphorylated.

ST14A cell line, stably transfected with different ErbB4 isoforms, endogenously express other members of the ErbB family, namely ErbB1, ErbB2 and ErbB3, which could heterodimerize with ErbB4 following ligand stimulation, thus activating different signal transduction pathways. Therefore, the phosphorylation of ErbB1, ErbB2 and ErbB3 following the stimulation with NRG1 α or NRG1 β was analysed in the neuronal progenitor cells stably expressing different ErbB4 isoforms.

Statistical analysis shows that ErbB3 is phosphorylated after NRG1 β stimulation in stable clones expressing JMb-cyt1 and JMb-cyt2 ErbB4 isoforms (Figure 3, JMb-cyt1 $p=0.0294$, JMb-cyt2 $p=0.0083$) and it is not phosphorylated following stimulation with

NRG1 α . ErbB1 is not phosphorylated, ErbB2 phosphorylation is barely detectable after a very long exposure (data not shown).

To investigate the possible differences among the four ErbB4 isoforms in the signal transduction pathways activated following the stimulation with NRG1 α or NRG1 β , the phosphorylation of AKT (at Threonine-308 or Serine-473 sites) and of ERK1 and ERK2 were assayed.

Statistical analysis shows that AKT is phosphorylated at Thr³⁰⁸ site after the stimulation with NRG1 β in all stable clones expressing ErbB4 (Figure 3, JMa-cyt1 $p=0.0020$, JMa-cyt2 $p=0.0010$, JMb-cyt1 $p=9.0674E-06$, JMb-cyt2 $p=0.0037$) and after stimulation with NRG1 α in stable clones expressing JMa-cyt2 or JMb-cyt1 ErbB4 isoforms (JMa-cyt2 $p=0.0193$, JMb-cyt1 $p=0.0001$).

As concerns AKT-Ser⁴⁷³, statistical analysis shows that AKT is phosphorylated at Ser⁴⁷³ site after the stimulation with NRG1 β in all stable clones expressing ErbB4 except the clone expressing JMb-cyt2 isoform (Figure 3, JMa-cyt1 $p=0.0263$, JMa-cyt2 $p=0.0008$, JMb-cyt1 $p=3.3558E-08$), and after stimulation with NRG1 α in stable clones expressing JMa-cyt2 or JMb-cyt1 ErbB4 isoforms (JMa-cyt2 $p=0.0200$, JMb-cyt1 $p=0.0005$).

ERK1 and ERK2 were quantified separately, because their regulation could be different. Nevertheless, because their phosphorylation pattern is the same, the graph in Figure 3 shows the sum of ERK1 and ERK2 phosphorylation. Statistical analysis shows that ERK1/2 is phosphorylated in clones stably expressing JMa-cyt1 and JMa-cyt2 ErbB4 isoforms after the stimulation with NRG1 β (Figure 3, JMa-cyt1 $p=0.0039$, JMa-cyt2 $p=0.0462$).

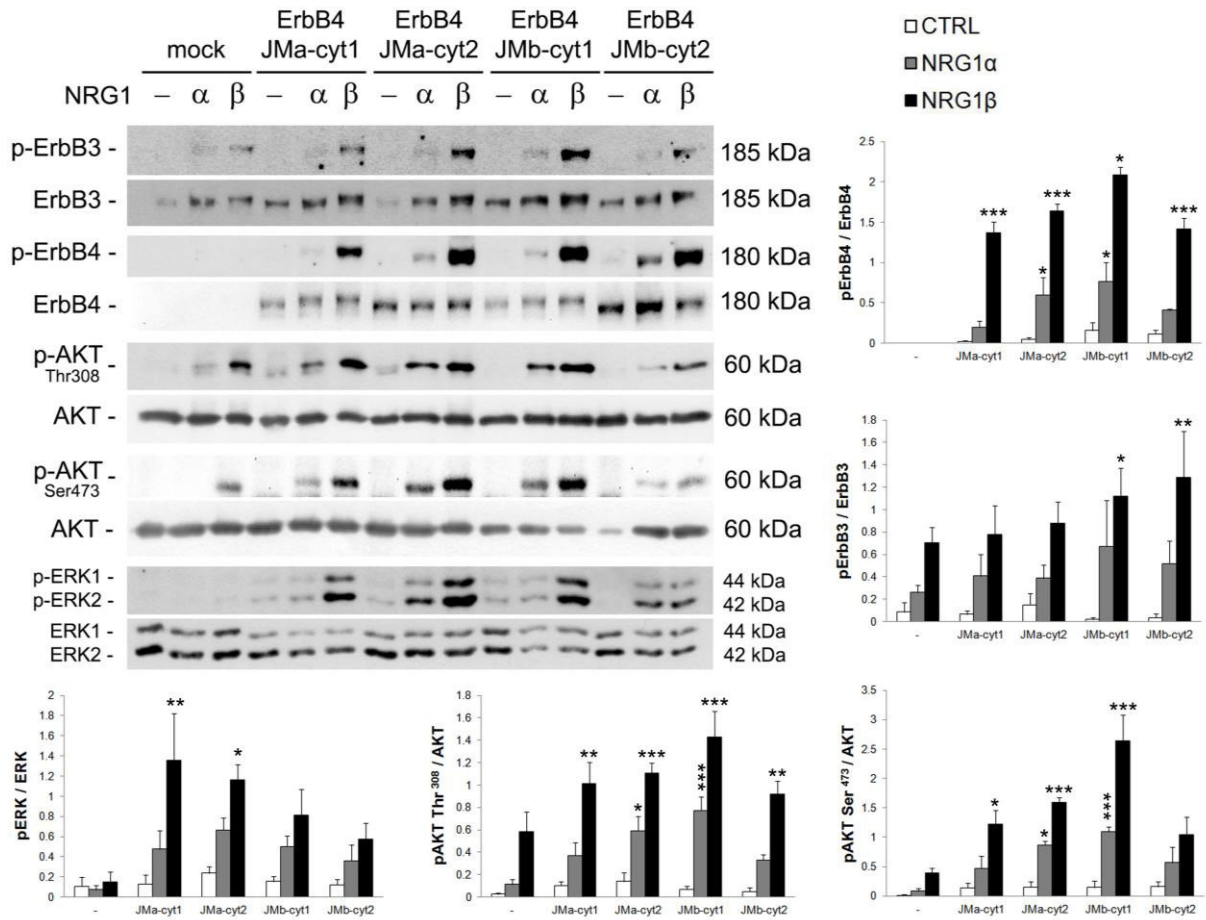


Fig. 3: ErbB3, ErbB4, PI3K and MAPK pathway activation following NRG1 α and NRG1 β stimulation. Western blot panels showing phosphorylation of ErbB3, ErbB4, AKT at Thr³⁰⁸ or Ser⁴⁷³ residues, ERK1 and ERK2 following 15 minutes of stimulation with NRG1 α or NRG1 β . Experiments were carried out in biological triplicate; the panel shows a western blot representative of the three independent experiments. Molecular mass standards are expressed in kilodaltons (kDa). Protein phosphorylation was normalized to the total amount of the corresponding protein (for each sample the ratio between the phosphorylated band and the total protein was calculated) and the results are shown in the graphics. One-way ANOVA statistical analysis shows significant differences between samples treated with NRG1 α or NRG1 β and untreated samples (CTRL) (* p<0.05; ** p<0.01; *** p<0.001). No statistical differences between samples treated with NRG1 α or NRG1 β were highlighted, because the concentration of the two ligands is different.

DISCUSSION

Deficits in the NRG1/ErbB4 system affect neuronal migration and could account for developmental diseases, such as schizophrenia, for which they are considered susceptibility conferring genes (Harrison and Law, 2006, Law et al., 2007, Chong et al., 2008, Shamir et al., 2012).

When inhibitory interneurons migrate toward the developing cortex, soluble NRG1 isoforms act as chemotactic factors that are expressed by the cortex to attract interneurons expressing ErbB4 (Flames et al., 2004, Ghashghaei et al., 2007). Cortical interneurons are generated from the MGE of the subpallium and migrate through a permissive corridor represented by the LGE, where membrane-bound NRG1 isoforms are expressed (Birchmeier, 2009).

Although many authors described NRG1 as a chemoattractant both in the MGE/LGE/cortex system (Yau et al., 2003, Flames et al., 2004) and in the SVZ/OB system (Anton et al., 2004), it has been recently proposed that NRG1 acts as repellent that funnels interneurons as they migrate from the MGE to cortical destinations (Li et al., 2012). The reasons underlying the discrepancy between the two models are currently unknown. Nevertheless, our previous and current results are consistent with the attractive role of the NRG1 signalling: we previously observed *in vitro* that ErbB4 expression confers to neuronal progenitor cells the ability to migrate toward a soluble NRG1 source (Gambarotta et al., 2004) and to adhere preferentially to a substrate expressing transmembrane NRG1 (Fregnan et al., 2014). The mRNA expression analysis confirms that soluble NRG1 is more highly expressed in the embryonic cortex, while transmembrane NRG1 is more highly expressed in the MGE/LGE region. NRG1 isoforms can also differ in the C terminus of the EGF-like domain, which can be α or β . Ligand displacement analyses indicated that the structural difference between α and β significantly affects the affinity of NRG1 to its receptors: the β isoforms bind to mammary cells (expressing NRG1 receptors) with an affinity that is approximately 10-fold higher than that of the α isoforms (Wen et al., 1994, Eckert et al., 2009).

Nevertheless, NRG1 α has a wide localization in organs and tissues: it has been found in neuronal cells during development in mouse (Meyer and Birchmeier, 1994) and it is expressed in rat, monkey and human brain (Bernstein et al., 2006). An

interesting aspect of NRG1 α and NRG1 β isoforms relatively to the neuropsychiatric disorders is that their expression pattern changes in opposite directions in the prefrontal cortex of schizophrenic patients: NRG1 β expression is increased while NRG1 α expression is decreased (Bernstein et al., 2006, Bernstein and Bogerts, 2013).

These data prompted us to further investigate the role of NRG1 α : we studied *in vivo* the expression of NRG1 α and NRG1 β isoforms and we analysed *in vitro* the activity of these isoforms in terms of migration and signal transduction. Our data show that NRG1 α is expressed at a comparable level in both MGE/LGE and cortex, while NRG1 β is more highly expressed in the MGE/LGE region, thus suggesting that also NRG1 α could play a role in the migration from the MGE/LGE to the cortex.

ErbB4 is the tyrosine kinase receptor mediating the NRG1 induced neuronal migration, as demonstrated by the analysis of conditional knockout mice (Gerecke et al., 2001, Anton et al., 2004, Flames et al., 2004). However, the expression and the role of the single ErbB4 isoforms were not investigated in these regions. For this reason, we quantified the expression of JMa, JMb, cyt1 and cyt2 in MGE/LGE and cortex. Our data demonstrate that all the ErbB4 isoforms are expressed in the analysed areas and confirm that ErbB4 is more highly expressed in the MGE/LGE region than in the cortex. Indeed, migrating interneurons express ErbB4 and the differential expression of the ErbB4 isoforms between MGE/LGE and cortex is likely due to the fact that migrating interneurons at E15 are not yet invading the cortex (Yau et al., 2003). The observation that different members of the NRG1/ErbB4 system are expressed in regions where neuronal migration occurs, induced us to study *in vitro* the migratory behaviour and the activated signal transduction pathways of neuronal progenitors expressing the different ErbB4 isoforms, following the stimulation with two different NRG1 isoforms.

In the MGE/LGE, we observed the expression of ErbB1, ErbB2 and ErbB3, together with ErbB4. The expression of ErbB co-receptors in the MGE/LGE region disagrees with other published data, where it is shown that murine GAD67⁽⁺⁾ migrating cortical interneurons express only ErbB4 (Rakic et al., 2015). It is noteworthy that ErbB expression was analysed using end-point PCR on FACS purified murine samples, while our analysis was performed using quantitative RT-PCR, which is a more

sensitive technique, on the whole rat MGE/LGE region, and was confirmed also by western blot analysis.

Neuronal progenitor cells (ST14A) stably expressing different ErbB4 isoforms (Gambarotta et al., 2004) were used as *in vitro* model. ST14A cells endogenously express ErbB co-receptors and therefore are a good model to investigate the migratory activity and the signal transduction pathways activated by different ErbB4 isoforms following the stimulation with NRG1. To analyse the activity of NRG1 α and NRG1 β , recombinant peptides corresponding to the EGF-like domain were used, as recombinant proteins corresponding to the entire extracellular domain of NRG1 α were not commercially available.

The migratory activity of stable clones expressing different ErbB4 isoforms was assessed by transwell assay. All stable clones migrate following the stimulation with NRG1 β , while only clones expressing JMa-cyt2 and JMb-cyt1 isoforms respond to the stimulation with NRG1 α .

In malignant peripheral nerve sheath tumour cells it had been previously shown that NRG1 α had no effect on migration (Eckert et al., 2009). Using a different experimental paradigm, we demonstrated that NRG1 α is able to stimulate neuronal progenitor migration.

To investigate the signal transduction pathways involved in the migratory activity, stable clones expressing the different ErbB4 isoforms were stimulated with NRG1 α or NRG1 β and the phosphorylation of ErbB receptors and the activation of PI3K/AKT and MAPK/ERK signal transduction pathways were analysed. In some cases, phosphorylation can be appreciated in the western blot, but the quantitative analysis of the biological triplicate did not give significant results. This discrepancy could be due to the technical limitations of western blot analysis, which is strongly influenced by different factors such as the exposure time, which affects the ratio between the phosphorylated form and the corresponding total protein.

The statistical analysis of ErbB4 phosphorylation shows that all ErbB4 isoforms are significantly phosphorylated following the stimulation with NRG1 β , while only JMa-cyt2 and JMb-cyt1 isoforms are phosphorylated following the stimulation with NRG1 α . These data show a correlation between NRG1 induced ErbB4 phosphorylation and neuronal progenitor migration.

Although the expression level of total ErbB4 is not exactly the same in all the stable clones, it was found that it does not affect neither ErbB4 phosphorylation nor migration, which seem to be affected only by the expressed ErbB4 isoform type.

Quantitative analysis of ErbB3 phosphorylation exhibits a very high variability. Statistical analysis shows that ErbB3 is significantly phosphorylated in clones expressing JMb isoforms following the stimulation with NRG1 β , thus suggesting that ErbB3 might participate in the signal transduction pathways activated by NRG1 treatment. Nevertheless, ErbB3 in the absence of ErbB4 is unable to stimulate cell migration (data not shown).

ErbB1 and ErbB2 are not phosphorylated either in the presence or absence of ErbB4, thus suggesting that they do not heterodimerize with ErbB3 or ErbB4 in this neuronal model.

It has been shown in zebrafish embryos that ERK1 and ERK2 control genes involved in cell migration (Krens et al., 2008a, Krens et al., 2008b). ERK1 and ERK2 in the absence of ErbB4 are not phosphorylated. Statistical analysis shows ERK1 and ERK2 are phosphorylated after the stimulation with NRG1 β in clones expressing JMa ErbB4 isoforms.

AKT is an essential mediator of the PI3K signal transduction pathway; it is a serine/threonine kinase playing a critical role in cell growth, differentiation, survival and migration and its activity is tightly regulated through threonine 308 and serine 473 phosphorylation (Hart and Vogt, 2011). Phosphorylation of Thr³⁰⁸ is essential for the catalytic activity of AKT and for signalling; this phosphorylation allows the organization of a network of interactions essential for the functioning of the catalytic domain and the formation of the active site (Yang et al., 2002). Phosphorylation of Ser⁴⁷³ is required for maximal activation of AKT. Indeed, this phosphorylation leads to further stabilization of the kinase, pushing the equilibrium toward the active conformation and increasing AKT catalytic activity by approximately 10 fold (Alessi et al., 1996).

Our analysis shows that the phosphorylation pattern of AKT-Thr³⁰⁸ and AKT-Ser⁴⁷³ is the same for all stable clones except for JMb-cyt2.

Data show a strong correlation between ErbB4, AKT-Thr³⁰⁸ and AKT-Ser⁴⁷³ phosphorylation level and the migratory activity of the clones stably expressing ErbB4.

Indeed, following the stimulation with NRG1 α and NRG1 β , the two ErbB4 isoforms (JMa-cyt2 and JMb-cyt1) conferring the highest migratory activity show high AKT-Thr³⁰⁸ and high AKT-Ser⁴⁷³ phosphorylation. In mock cells, which do not migrate following NRG1 stimulation, AKT-Thr³⁰⁸ and AKT-Ser⁴⁷³ are not phosphorylated. Accordingly, we previously showed that, in the presence of PI3K inhibitors, the migratory activity mediated by ErbB4 following the stimulation with NRG1 β is inhibited (Gambarotta et al., 2004).

The ErbB4 isoforms JMa-cyt2 and JMb-cyt1, conferring the highest migratory activity, are deeply different: JMa is susceptible to proteolytic cleavage and can release a cytoplasmic fragment migrating to the nucleus, JMb is resistant to cleavage; cyt1 isoforms contain a domain that mediates the activation of PI3K/AKT pathway (Elenius et al., 1999) and the interaction with E3 ubiquitin ligase (Elenius et al., 1999), cyt2 isoforms lack this domain; both cyt1 and cyt2 isoforms can stimulate MAPK pathway (Sundvall et al., 2008). Therefore, we expect that they activate AKT phosphorylation and cell migration through different ways: cyt1 isoforms can activate PI3K pathway directly, cyt2 isoforms need to heterodimerize with ErbB3, which contains six binding sites for PI3K (Vijapurkar et al., 2003, Gambarotta et al., 2004, Roskoski, 2013). AKT phosphorylation observed following the stimulation with NRG1 could be mediated either by ErbB4 homodimerization (in the case of cyt1 isoforms), or ErbB4-ErbB3 heterodimerization (in the case of cyt1 and cyt2 isoforms).

CONCLUSION

We showed *in vivo* that different ErbB4 and NRG1 isoforms are expressed in regions where neuronal migration occurs during development. We demonstrated *in vitro* that different ErbB4 and NRG1 isoforms confer to neuronal progenitor cells distinct characteristics and behaviours and that both NRG1 α and NRG1 β can play a role in signal transduction activation and neuronal migration.

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MY CONTRIBUTION TO THE MANUSCRIPT

I collaborated to the design and conception of all experiments. I supervised *in vivo* explants and I participated to their analysis through qRT-PCR and Western blot. Moreover, I performed all *in vitro* experiments on ST14A cell line and I interpreted the data obtained by the different experiments. I wrote most of the manuscript.

SUPPLEMENTARY MATERIALS

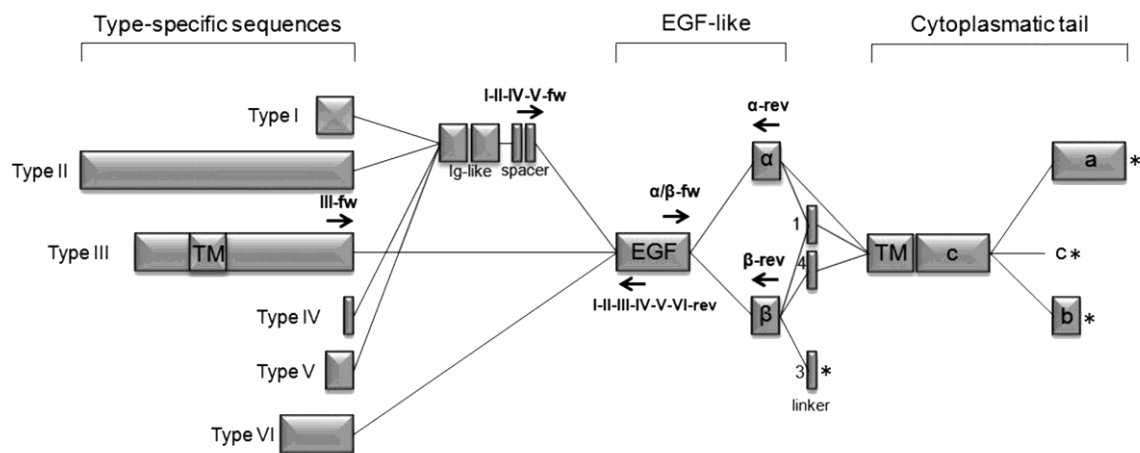


Fig S1: NRG1 isoform diagram showing where the specific primers were designed. Soluble NRG1 isoforms were amplified with the primers “I-II-IV-V-fw (forward) and “I-II-III-IV-V-VI-rev” (reverse). As can be deduced from the diagram, PCR primers for NRG1 soluble isoforms detect all (I, II, IV and V) soluble isoforms except type VI. Transmembrane NRG1 isoforms were amplified with the primer “III-fw” and the primer “I-II-III-IV-V-VI-rev”. To distinguish between NRG1 α and NRG1 β , a common forward primer “ α/β -fw” and specific reverse primers “ α -rev” and “ β -rev” were used. The scheme was modified from Mei et. al 2008 (Mei and Xiong, 2008).

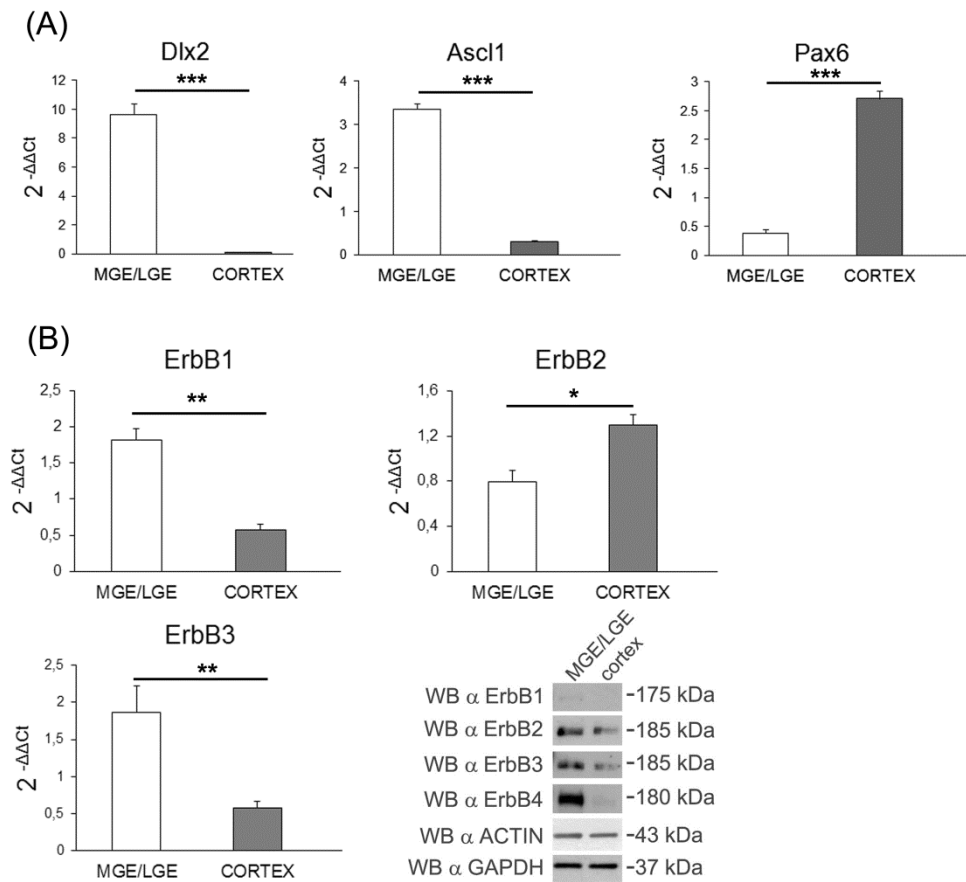


Fig. S2: RNA and protein expression of ErbB4 co-receptors in MGE/LGE and cortex at E15. The relative quantification ($-2^{\Delta\Delta C_t}$) of Dlx2, Ascl1, Pax6 (Panel A), ErbB1, ErbB2 and ErbB3 (Panel B) was obtained by quantitative real-time PCR: data were normalized to an endogenous housekeeping gene (TBP). In the absence of a reference calibrator sample, quantitative data were expressed relatively to the average of the two tissue samples. Statistical analysis was carried out using Student's t-Test. Asterisks refer to significant statistical differences with $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. Western blot panel shows ErbB1, ErbB2, ErbB3, ErbB4 protein expression in MGE/LGE and cortex regions. Housekeeping proteins actin and GAPDH were used as a loading control. Molecular mass standards are expressed in kilodaltons (kDa).

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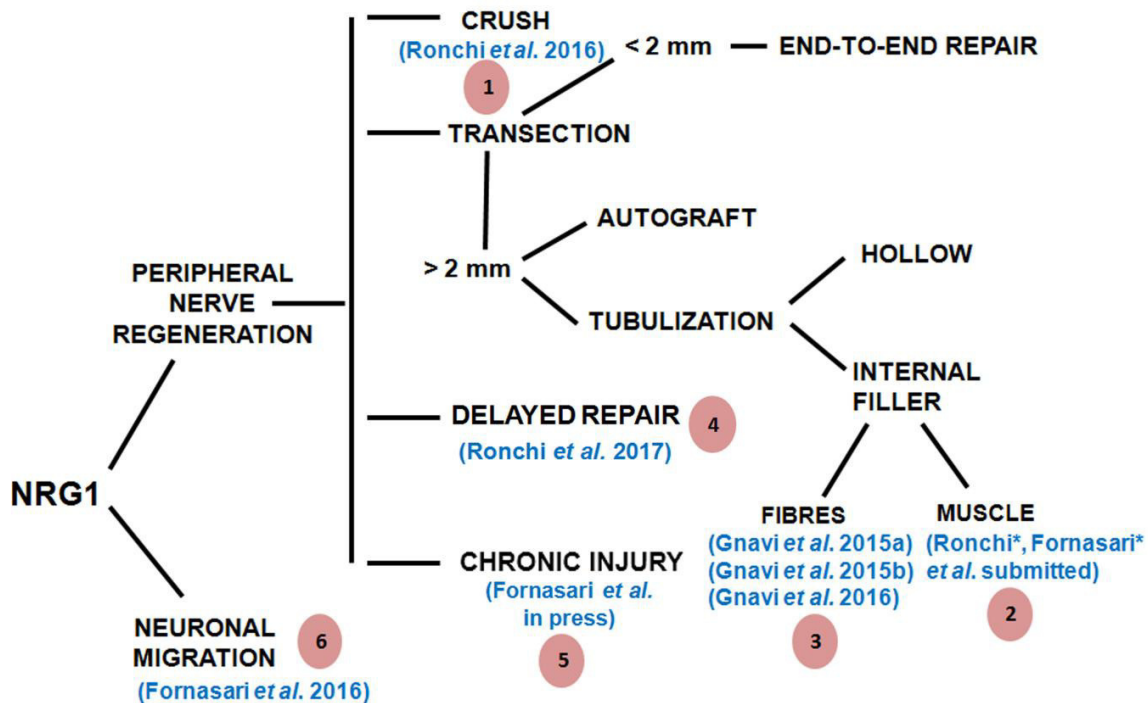
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CHAPTER 10

General discussion

My PhD thesis is focused on NRG1 involvement in two different biological events: the peripheral nerve regeneration and neuronal migration in the central nervous system.



Scheme of the different aspects investigated in this thesis with the related publications; the numbers correspond to the order followed in the discussion, that reflects the logical order followed in the thesis.

For what concerns **peripheral nerve regeneration**, I investigated the expression of different NRG1 isoforms after peripheral nerve injury followed by immediate or delayed repair and in chronic injuries represented by the inherited peripheral neuropathy Charcot-Marie-Tooth1A.

Moreover, when severe peripheral nerve injuries occur, tubulization can be used, as alternative to autograft, to fill the gap between the two nerve stumps. I investigated two possible internal fillers for hollow conduits: muscle fibres, which provide physical support for regrowing axons and release soluble NRG1, and electrospun fibres of different biomaterials, different diameter and orientation.

For what concerns the central nervous system, I demonstrated that NRG1 α , an underestimated NRG1 isoform, can stimulate *in vitro* the **migration of neuronal progenitors** expressing different isoforms of NRG1 receptor ErbB4.

1. NRG1 expression after nerve injury in case of nerve degeneration and regeneration (Ronchi *et al.*, 2016)

To improve and ensure peripheral nerve regeneration, tissue engineering is a valid aid, but is not enough. Indeed, a thorough knowledge of the molecular mechanisms underlying nerve degeneration and regeneration and the involved molecules, is required. Among these molecules, we focused our attention on NRG1 and its isoform expression and regulation during the phases of peripheral nerve degeneration and regeneration, assessed in three experimental models. A crush injury (first experimental model) and a net transection followed by an end-to-end repair (second experimental model) are the two conditions (with increased level of injury severity) reflecting all phases of Wallerian degeneration over to axonal outgrowth and remyelination (regenerating conditions), while the unrepaired nerve injury (third experimental model) reflected the degenerating condition, with the signs of chronic denervation. The expression of different NRG1 isoforms and of their ErbB receptors was evaluated in the early phases after nerve injury, at mRNA and protein level, in the distal stump of rat median nerve samples in the different experimental models. Our results confirmed that one day after the injury there is a strong increase of soluble NRG1 type I/II, as already demonstrated by others (Stassart *et al.*, 2013). Nevertheless, we observed, for the first time, that the expression peak one day after the injury corresponds to different combinations of soluble NRG1 isoforms type α and β , a and b, while NRG1 type c was not up-regulated and decreased after nerve injury.

At the protein level, the analysis is really complex, because a few suitable antibodies are commercially available and the several isoforms and proteolytic cleavage make the data interpretation really complex (Fleck *et al.*, 2013). We observed that full length transmembrane NRG1 expression is switched on after nerve injury and it remains expressed under degenerating conditions, while it is turned off when the regeneration is completed. This band was also recognized by Carroll and colleagues with an antibody that recognizes all NRG1 isoforms; our data show that this band corresponds to the transmembrane isoform (Carroll *et al.*, 1997). The same antibody, that targets the N-terminus of all type III NRG1 (transmembrane isoforms),

recognizes a couple of bands (~75kDa) corresponding to the N-terminus fragment (NTF) that results from the cleavage of NRG1 (Fleck et al., 2013). The different molecular weights of the NTF depend on which α (ADAM/TACE) or β (BACE1) secretase makes the cleavage, giving rise to α NTF or β NTF (Fleck et al., 2013). The different NTFs have an opposite role, α NTF inhibits myelination (La Marca et al., 2011), while β NTF promotes myelination (Willem et al., 2006). Here, for the first time (Ronchi et al., 2016), we demonstrated *in vivo* the switch between the α NTF and β NTF of transmembrane NRG1 after nerve injury, highlighting that when the nerve is under regenerating condition transmembrane NRG1 is cleaved by BACE1, while when the nerve repair and the myelination are completed there is a switch to the cleavage by ADAM/TACE that generates the NRG1 fragment inhibiting myelination, that is not more necessary. Under degenerating conditions (until 28 days), transmembrane NRG1 is still cleaved by BACE1, because the regenerative machinery is still active and ready to restart. In condition of chronic nerve degeneration (up to 3 months), we demonstrated that transmembrane NRG1 is no longer expressed, because axons are totally degenerated (Ronchi et al., 2017).

2. Enrichment of chitosan hollow tubes with muscle fibres (Ronchi, Fornasari *et al.*, submitted)

When a severe injury with loss of substance between the two stumps of the nerve occurs, tubular conduits have proven to be an excellent alternative to autologous nerve grafts because they act as physical guidance for the regenerating axons and provide a protective environment for axonal growth by limiting surrounding tissue invasion and by reducing neuroma and scar tissue formation. Despite this, hollow tubes not always lead to good outcome, due to an inadequate formation of extracellular matrix or to a decay of growth factors and the conduit enrichment might better support nerve regrowth, by creating a favourable environment. Here we observed that *in vitro* degenerating skeletal muscle fibres over-expressed soluble NRG1 isoforms type α , β , a and b, the same isoforms that we observed up-regulated one day after the injury (Ronchi et al., 2016). Since NRG1 is important in the early stages of peripheral nerve regeneration (Ronchi et al., 2016), this up-regulation lead us to the introduction of muscle fibres inside a chitosan hollow conduit (Reaxon®

Nerve Guide), whose effectiveness in peripheral nerve repair was already proved (Haastert-Talini et al., 2013, Gonzalez-Perez et al., 2015, Shapira et al., 2016). The degenerating muscle not only provides physical support to regrowing axons, through its basal lamina (Meek et al., 2004), but also releases different growth factors (Montoya et al., 2009). Among the growth factors, we demonstrated that the fresh muscle also releases soluble NRG1 in the surrounding environment. Moreover, our data are supported by another previous study, showing a significant up-regulation of soluble NRG1 after skeletal muscle denervation (Nicolino et al., 2009). Starting from the *in vitro* results and the previous studies of others about the introduction of muscle inside conduits (Varejao et al., 2003a, Varejao et al., 2003b) or inside a vein, in the experimental paradigm of the *muscle-in-vein* (Marcoccio and Vigasio, 2010, Tos et al., 2012, Manoli et al., 2014), we tested the expression of NRG1 isoforms and its effectiveness inside chitosan tube *in vivo*. We used an experimental model of short gap primary repair of rat median nerve of rat: 8mm nerve gap has been repaired with the chitosan conduit (Reaxon® Nerve Guide) filled with skeletal muscle fibres and compared with the hollow one.

At mRNA level, the introduction of fresh muscle inside the hollow tubes restores the peak of soluble NRG1 isoforms (type α , β , a and b) expression one day after the injury, whose expression has not been observed in the hollow tube. This NRG1 expression pattern is similar to that observed in autograft samples and in our previous data, one day after the injury (Ronchi et al., 2016). Moreover, an increase of NRG1 mRNA was also observed in the *muscle-in-vein* at the early stages of regeneration in other studies (Geuna et al., 2003, Nicolino et al., 2003). These data demonstrated that the soluble NRG1 isoforms are involved in the early response to injury and that the fresh skeletal muscle can be an endogenous source of soluble NRG1 inside a hollow tube, in which NRG1 expression peak is shifted to 14 days instead of one day after the injury and it is lower. Regarding the effectiveness of the introduction of the muscle inside the tube, the quantitative morphology and the functional recovery tests highlight no differences with the hollow chitosan tubes, suggesting that both conduits are effective for the primary repair of short gaps, but the introduction of muscle fibres might be an useful tool to repair more complex injury (such as longer gap or in the delayed repair).

3. Influence of electrospun fibres of different biomaterials, size and orientation on Schwann cell behaviour and axonal outgrowth

The distribution and growth of cells on a scaffold are key requirements. A number of studies demonstrates that micro-to-nano-scale topography is important in the control of cell adhesion, proliferation and survival (Harvey et al., 2013). The topography of the surface affects the cytoskeleton organization; moreover, changing in cell morphology and shape can lead to different functional behaviours (Hoffman-Kim et al., 2010). Appropriate fibrous substrates, functioning as a temporary extracellular matrix, can be easily prepared by electrospinning, a technique which allows the obtainment of fibrous matrices with different diameter and orientation, suitable as internal fillers for nerve guide (Bhardwaj and Kundu, 2010).

3.1 Influence of the diameter size of gelatin electrospun fibres on Schwann cell behaviour and axonal outgrowth (Gnavi *et al.*, 2015a)

Gelatin fibres with different diameters can be prepared by varying different parameters, such as the solution concentration and the flow rate, starting from a water solution cross-linked with γ -glycidoxypolytrimethoxysilane (GPTMS). In this thesis, we investigated cell behaviour and axonal outgrowth on random gelatin fibres with different diameters. A SC cell line and primary SCs were selected because the distinct ability of peripheral nerves to grow back to their targets is due to SCs, while DRG explants were chosen as a model to evaluate axonal re-growth, since successful nerve regeneration depends on the proper reinnervation of sprouting axons from the proximal stump, towards the injury site. Through *in vitro* assays, we demonstrated that nano-fibres, mimicking the extracellular matrix, promoted cell spreading and cytoskeleton organization resulting in higher cellular adhesion and proliferation rate in comparison to micro-fibers, while cells cultured on micro-fibres displayed higher motility and migration in comparison to cells in contact with nano-fibres. Finally, DRG explants cultured on micro-fibres resulted in higher neurite outgrowth; this might be due to the dimension similarity between micro-fibre diameter and bands of Büngner size. Taken together these data confirm that these substrates display high biocompatibility and that fibre diameter size affects SC behaviour and axonal outgrowth, promoting different processes. Despite random micro-fibres better

promote SC migration and neurites outgrowth, also cell adhesion and proliferation are required for nerve regeneration.

3.2 Effect of random or aligned gelatin electrospun fibres on Schwann cell behaviour and axonal outgrowth (Gnavi *et al.*, 2015b)

Although Cardwell and colleagues in tendon/ligament lineage demonstrated that fibre diameter affects cellular behaviour more significantly than fibre alignment, the orientation of fibres is an important factor to consider for the design of internal frameworks for nerve guides (Cardwell *et al.*, 2014). Indeed, the fibre size is not the only parameter that can be set during fibre production: depending on the type of collector and its rotation speed, fibres with a different orientation can be prepared. Flat fixed collectors give rise to random fibres, while cylindrical rotating ones collect aligned fibres. The rotation speed influences fibre alignment and size, indeed, faster collector rotation leads to a great number of aligned fibres with fewer diameter in comparison to fibres produced by a slower rotation, as also demonstrated by Xie and colleagues (Xie *et al.*, 2010). Therefore, the obtainment of aligned fibres with a large diameter is hard, especially stating from an aqueous solution of gelatin. Moreover, other studies demonstrated that cell viability, neuritis outgrowth and neuronal differentiation are better promoted on aligned nano-fibres in comparison to aligned micro-fibres (Xie *et al.*, 2009, Wang *et al.*, 2012, Qu *et al.*, 2013). For these reasons, we analyzed the effect of nano-fibres alignment on SC behaviour and on axonal outgrowth. The fibre alignment reduced SC proliferation and the number adherent SCs in comparison to cells seeded on random fibres or on control condition (poly-L-lysine-coverslips). Despite the fact that all tested conditions showed actin filaments and focal adhesion points on aligned nano-fibres, SCs displayed elongated actin filaments that were also parallel to the direction of the nano-fibres and present fewer focal adhesion points that are consistent with the reduction of SC proliferation. This reduction of SC proliferation on aligned nano-fibres is probably due to a reduced number of adhesion points for cell anchoring on fibres (Bacakova *et al.*, 2011, Harvey *et al.*, 2013, Rahmany and Van Dyke, 2013), as also demonstrated by Gupta and colleagues on PCL/gelatin nano-fibres (Gupta *et al.*, 2009). Although SC proliferated less on aligned nano-fibres, all tested conditions reached the confluence, confirming

that gelatin nano-fibres are biocompatible. Biocompatibility was also evaluated by MTT assays. Our results are in accordance with previous studies on SC behaviour on aligned and random nano-fibres (Gupta et al., 2009, Xie et al., 2009, Shakhbazau et al., 2014, Zheng et al., 2015). To evaluate neuritis outgrowth, instead of DRG that required rat sacrifices, we used an immortalized rat DRG neuron cell line, that can be induced to differentiate *in vitro* (Chen et al., 2007). Although the alignment of nano-fibres guides the direction of the neuritis of differentiated cells parallel to the direction of fibres, no differences in neuritis length were observed between the tested conditions. Therefore, orientated fibres are a potential tool to maximize target reinnervation, by guiding regenerating axons to the distal stump (Ma et al., 2014, Zheng et al., 2015).

3.3 Comparison between gelatin and chitosan electrospun fibres with different orientation (Gnavi *et al.*, 2016)

Besides fibre size and orientation, also the solution used to prepare fibres can be different. Fibres deriving from biomaterials of different origins can lead to different results; indeed, various biomaterials have been proposed and tested to build up scaffolds to promote nerve repair. Among them, gelatin and chitosan have been raising more and more interest for basic and clinical scientists, because of their characteristics, as alternative to synthetic polymers (Ciardelli and Chiono, 2006, Gnavi et al., 2013). Both biomaterials are of natural origins and are highly biocompatible as confirmed by our studies.

Chitosan derives from the deacetylation of chitin, obtained by the combination of high temperature and strong aqueous alkali treatments (Khor and Lim, 2003). Chitosan has a low solubility at neutral pH; indeed, the solvent used to prepare chitosan fibres is acetic acid with the addition of supplementary components like, dimethyl sulfoxide (DMSO) as co-solvent to increase the fibre yield and dibasic sodium phosphate (DSP) to increase water stability (Ruini et al., 2015). Otherwise, gelatin derives from the thermal denaturation of collagen, is highly soluble in water and fibres can be prepared using water as solvent. Given its features, gelatin fibres degrade faster and possess weak mechanical strength, but the addition of a cross-linker as GPTMS increases fibre stability, thus avoiding the use of other acidic solutions and potentially

cytotoxic solvents (Tonda-Turo et al., 2013). To investigate whether the fibre composition in combination with their topography modulates SC behaviour and neurite outgrowth, random or aligned nano-fibres of gelatin or chitosan were prepared.

In vitro experiments confirmed that fibre alignment reduced SC adhesion and proliferation. It is remarkably of notice that, although alignment reduced proliferation, SC seeded on aligned gelatin fibres showed a proliferation rate similar to random chitosan fibres and proliferated more than those seeded on chitosan aligned nano-fibres. Moreover, on gelatin aligned nano-fibres DRG neuron cells differentiate, in the absence of forskolin (that is their inductor of differentiation *in vitro*) and their neurite length is similar to those seeded on control condition and on random fibres, while neurite length is reduced on aligned chitosan fibres. On aligned nano-fibres of both compositions, the number of filipodia is higher than on random fibres, suggesting that fibre alignment may induce SC migration, independently from the composition of fibres. For what concerns the actin cytoskeleton organization and the presence of focal adhesion points, we highlight differences between random gelatin and random chitosan fibres; indeed, on gelatin fibres we individuate cells with an actin cytoskeleton more organized and many focal adhesion points in comparison to cells seeded on random chitosan fibres, thus suggesting that gelatin solubilised in water is a good choice for fibre preparation. The use of acidic solution can change the properties of the materials.

Taken together these data suggest that both gelatin and chitosan are biocompatible, ensuring the physiological behaviour of SCs and axonal outgrowth, but the composition of fibres in combination with their orientation must be taken into account. Indeed, even if nano-fibres alignment reduced SC proliferation, overall gelatin aligned nano-fibres resulted as a potential internal filler for nerve guide thanks to gelatin properties and orientation properties that seem to induce neuron differentiation and neurite alignment in agreement with other studies, reporting that fibre alignment promotes better nerve regeneration (Neal et al., 2012, Cirillo et al., 2014, Huang et al., 2015).

4. Molecular and morphological changes occurring during long-term nerve degeneration and after delayed nerve repair (Ronchi *et al.*, 2017)

Often the immediate repair (primary repair) of nerve injury is not possible and the repair must be delayed in time (secondary repair) to allow the resolution of concomitant more severe trauma (Siemionow and Brzezicki, 2009). A lag in nerve repair may impair nerve regeneration and functional recovery. This poor outcome can be ascribed to the chronic denervation of the distal stump of the nerve. The chronic axotomy reduces the ability of the injured axon to regenerate and a progressive down-regulation of some regeneration-associated genes in chronically axotomized motoneurons was demonstrated (Grinsell and Keating, 2014, Gordon and Tetzlaff, 2015). The degeneration of the distal stump leads to the loss of SC capability to support nerve regeneration (Gordon *et al.*, 2011, Sulaiman and Gordon, 2013) and to fibrosis, as confirmed by an increased expression level of transforming growth factor beta, that is involved in scar formation (Jonsson *et al.*, 2013). Moreover, after chronic nerve degeneration, the muscle reinnervation is limited by the deterioration of intramuscular nerve sheaths (Fu and Gordon, 1995) and by the progressive muscle atrophy (Jonsson *et al.*, 2013). Indeed, the chronic denervation of the distal stump in combination with the chronic muscle denervation impairs the functional recovery (Gordon *et al.*, 2011).

After injury SCs dedifferentiate and change their phenotype and expression pattern; this phenomenon occurs also after a chronic denervation. Indeed, we focused our attention on the molecular changes occurring in the chronic degenerating distal stump of the nerve, by evaluating the expression of common SC markers after 3, 6 and 9 months of degeneration. The expression pattern of these markers is in accordance with previous studies (Li *et al.*, 1997, You *et al.*, 1997) and demonstrated that SCs progressively undergo atrophy, as also demonstrated by the morphological analysis. More interesting is the strong down-regulation of the soluble NRG1 in long-term degenerating nerves at both RNA and protein level, that can be one of the factors limiting nerve regeneration after a chronic denervation. Indeed, Li and colleagues demonstrated that the addition of the glial growth factor-2 (GGF2), corresponding to NRG1 type II, on chronically denervated SCs *in vitro*, promoted SC

proliferation and migration (Li et al., 1998), supporting the idea that atrophic SCs retain the ability to respond and that NRG1 expression is important for SC responsiveness to regrowing axons signals, also after chronic denervation. The inability of the distal stump to sustain nerve regeneration after a delayed repair could be due to atrophic SCs that are not able to completely recover from their impairment. Moreover, we observed the total lack of transmembrane at protein level, index of the absence of axons in the degenerated nerve stumps.

Jonsson and colleagues demonstrated that 3 months of denervation seem to be the time-point beyond which the secondary repair outcome become ineffective, showing that the number of regenerating motoneurons and myelinated axons decline starting from one month of denervation, accompanied by reduced muscle reinnervation and muscle atrophy (Jonsson et al., 2013). Our results confirmed that when the repair is delayed in time, up to 3 months, in the distal stump, myelinated fibres are fewer, smaller and less mature in comparison to those deriving from an immediate repair, demonstrating that with a secondary repair a poor regeneration occurred. Moreover, SCs in the distal stump of the delayed-repaired nerve showed a SC marker pattern of expression reminiscent of those of atrophic SCs in the chronic degenerating nerve (down-regulation of S100 and glial fibrillary acidic protein and up-regulation of p75 in comparison to the healthy nerves), supporting the idea that SCs lost their ability to support nerve regeneration (Gordon et al., 2011).

This hypothesis is also supported by our evaluation of the NRG1/ErbB system, where soluble NRG1 expression is still down-regulated after the delayed repair. These data, supported also by those of Li and colleagues, suggest that soluble NRG1 provided in the case of a delayed nerve repair could improve nerve regeneration outcome by stimulating atrophic SCs (Li et al., 1998).

5. Expression of NRG1 isoforms in chronic injuries represented by Charcot-Marie-Tooth1A disorder (Fornasari, Ronchi *et al.*, in press)

A transient SC dedifferentiation, after nerve injury, is part of an efficient regenerative process, while a persistent SC dedifferentiation negatively affects nerve regeneration and is detrimental for axonal and nerve integrity. Several studies demonstrated that the activation of the Raf/MEK/ERK signalling pathway is sufficient to induce SC dedifferentiation both *in vitro* (Harrisingh et al., 2004) and *in vivo* in transgenic mice (Napoli et al., 2012). Interestingly, a persistent ERK activation was also observed in models of a hereditary peripheral nerve neuropathies, among which Charcot-Marie-Tooth1A (CMT1A) (Fledrich et al., 2014), a demyelinating form of CMT, caused by the duplication of *Pmp22* gene (Patel et al., 1992). Moreover, we (Ronchi et al., 2016) and others (Sheu et al., 2000, Harrisingh et al., 2004) observed a strong and transient activation of ERK pathway, early after nerve injury, thus confirming that this signalling pathway mediates SC dedifferentiation in Wallerian degeneration (Napoli et al., 2012). These data, together with a similar gene expression pattern of other genes (such as cell surface molecules), show that CMT1A nerves share many similarities with nerves undergoing Wallerian degeneration (Martini et al., 2013); in CMT nerves ERK activation starts before axonal damage, while in injured nerves it is induced after the damage. Indeed, the activation of this signalling pathway also leads to an increase of monocyte chemoattractant protein-1 that is strongly involved in macrophage recruitment and macrophage-related demyelination (Martini et al., 2008), mechanisms that seem crucially implicated in CMT1A pathogenesis and are a response to injury related to the Wallerian degeneration.

The loss of balance between PI3K/Akt and MEK/ERK pathways contributes to the dedifferentiation of SCs that characterizes CMT1A. Indeed, Fledrich and colleagues demonstrated a strong activation of MEK/ERK pathway and a decreased activation of PI3K/Akt pathway in the peripheral nerves of a rat model of CMT1A, that over-expressed *Pmp22* (Fledrich et al., 2014). Moreover, they demonstrated that the administration of MEK-ERK inhibitors to SCs *in vitro* or to sciatic nerves of CMT1A rats, reduced the expression of SC dedifferentiation markers (Fledrich et al., 2014). These data are also supported by the evidence that in CMT1A-derived SC primary cultures, the administration of a pharmacological activator of PI3K/Akt is sufficient to

down-regulate genes associated to the dedifferentiation of SCs (Fledrich et al., 2014).

They also observed that from the cross-breeding between CMT1A model mice over-expressing PMP22 with transgenic mice over-expressing soluble NRG1, they obtained mice with an increased PI3K-Akt activation and undamaged axons in double-transgenic mice suggesting the idea that soluble NRG1 can be a therapeutic tool to promote SC differentiation. This therapeutic approach has been shown to offer beneficial effects only if administered in a precise time-window (from P6 to P18). Indeed, from our results we demonstrated that in the nerves of the same CMT1A rat model (Sereda et al., 1996) early during development, the expression of endogenous soluble NRG1 is lower, so its administration may be useful to ameliorate the pathology symptoms, while from P16 soluble NRG1 type α , β , a and b isoforms result to be over-expressed in comparison to wild-type animals. Indeed, NRG1 is a physiological activator of both PI3K/Akt and MEK/ERK pathways, with a bi-functional concentration-dependent effect on myelination: low NRG1 concentration activates PI3K/Akt pathway which promotes myelination, while high NRG1 concentration activates MEK/ERK pathway which is important for SC dedifferentiation and inhibits myelination (Zanazzi et al., 2001, Syed et al., 2010, Eshed-Eisenbach et al., 2016). Since the soluble NRG1 is over-expressed in CMT1A nerves, its administration can be useless and harmful. Moreover, these data of soluble NRG1 isoform over-expression confirmed the similarity between injured nerves undergoing Wallerian degeneration and CMT1A nerves (Martini et al., 2013).

6. NRG1 α stimulates the migration of neuronal progenitors expressing ErbB4 (Fornasari *et al.*, 2016)

Besides its role during peripheral nerve development and regeneration, NRG1/ErbB system is involved in many other biological processes, such as development and progression of different kinds of cancer (Roskoski, 2014), cardiac development and angiogenesis (Rupert and Coulombe, 2015), neuromuscular junction formation (Wang et al., 2017), synapse formation, oligodendrocyte development and neuronal migration in the central nervous system (Mei and Xiong, 2008).

The correct functioning of the central nervous system requires the proper balance between inhibitory and excitatory neurons, that correctly migrate to their target sites. Alteration of this equilibrium may lead to the development of neurological disorders, such as schizophrenia (Birchmeier, 2009). It has been demonstrated that GABAergic interneurons require NRG1 to reach the cortex from the medial ganglionic eminence (MGE) of the *subpallium* during development. During the migration, inhibitory interneurons express ErbB4 and are attracted by the soluble NRG1 expressed by the cortex and migrate along the lateral ganglionic eminence (LGE) that expresses transmembrane NRG1 (Yau et al., 2003, Flames et al., 2004). Our results support the findings about an attractive role for NRG1, indeed it has been previously demonstrated that neuronal progenitor cells expressing ErbB4 acquire the ability to migrate toward a source of soluble NRG1 (Gambarella et al., 2004) and prefer to adhere on substrates expressing transmembrane NRG1 (Fregnan et al., 2014). We analysed *in vivo* the expression of α and β NRG1 isoforms in the MGE/LGE and in the cortex, in order to investigate the role played by these two isoforms in neuronal migration. The isoforms α and β derived from the alternative splicing of the exons located downstream the EGF-like domain (Falls, 2003, Edwards and Bottenstein, 2006) and NRG1 β in many tissues has demonstrated to be more bioactive than NRG1 α (Wen et al., 1994, Eckert et al., 2009).

Moreover, NRG1 and ErbB4 are considered genes that confer susceptibility to schizophrenia (Harrison and Law, 2006, Law et al., 2007, Shamir et al., 2012) and it has been demonstrated in several studies that in the prefrontal cortex of schizophrenic patients NRG1 β expression is increased while, on the contrary, NRG1 α expression is decreased (Bernstein et al., 2006, Bertram et al., 2007, Bernstein and Bogerts, 2013). Our data demonstrated that during development, NRG1 α is expressed at the same level in both MGE/LGE and in the embryonic cortex, while NRG1 β is more highly expressed in the MGE/LGE. Since schizophrenia may be due to an impaired neuronal migration and NRG1 α is decreased in the patients, we suggest that NRG1 α could play a role in the migration of inhibitory interneurons expressing ErbB4 from the MGE to the cortex. Indeed, we investigated which ErbB4 isoform confers to neuronal progenitors the ability to migrate following stimulation with NRG1 α . We demonstrated that NRG1 α stimulates the migration of

stable clones expressing the ErbB4 isoforms JMa-cyt2 and JMb-cyt1 and that their migration is strongly correlated with the phosphorylation of ErbB4 and with the downstream activation of the PI3K/Akt signalling pathways. Indeed, PI3K/Akt pathways is involved in migration (Vogt and Hart, 2011) and it has been previously demonstrated that with an inhibitor of the PI3K, the migration mediated by ErbB4, following the stimulation with NRG1 β , is inhibited (Gambarotta et al., 2004). Thus, in this study we demonstrated *in vitro* that NRG1 α can play a role in neuronal migration and that the different ErbB4 isoforms can confer to the neuronal progenitors a different ability to migrate in response to different isoforms of NRG1.

CONCLUSIONS

In this thesis I focused my attention on different aspects of peripheral nerve injury and repair, paying attention to the development of tools to improve nerve regeneration, together with a deep attention to the NRG1/ErbB system, in order to understand how these molecules might be used to improve nerve regeneration.

From all these observations emerges that NRG1 is important in the first phases of regeneration and that its down-regulation after chronic denervation may affect nerve regeneration. Indeed, after delayed nerve repair NRG1 is still down-regulated. For these reasons the release of soluble NRG1 through skeletal muscle fibres, inside nerve conduits, might improve the outcome when the repair is delayed in time or in case of long gaps. Besides muscle fibres, different substrates can be used as internal filler, among them electrospun fibres. Electrospun fibres can own different diameter, different orientation and can be made of different biomaterials such as chitosan or gelatin. Among the different kinds of tested fibres, we observed that aligned gelatin nano-fibres give the better results as internal filler. These fibres might be functionalized with NRG1 and introduced inside hollow chitosan tubes as internal framework, to support peripheral nerve regeneration thanks to the topography of the substrate in combination with NRG1 release. NRG1 supplement is important in the early regeneration phase, but its expression needs to be finely tuned. Indeed, when soluble NRG1 is chronically over-expressed by the nerves, such as in CMT1A, its

expression may be harmful and the treatment with drugs targeting ErbB receptors, such as Herceptin, may limit the excess of NRG1 (Awada et al., 2016).

Finally, since NRG1 and ErbB4 are genes that confer susceptibility to schizophrenia, understanding their role in neuronal migration may contribute to better understand the etiopathogenesis of this disease.

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