Potential of genetically modified ensheathing cells for regeneration after spinal cord injury

Potencial de la glía envolvente genéticamente modificada para la regeneración después de lesión medular

Sara Nocentini

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FACULTAT DE BIOLOGIA

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POTENTIAL OF GENETICALLY MODIFIED ENSHEATHING CELLS FOR REGENERATION AFTER SPINAL CORD INJURY

(Potencial de la glía envolvente genéticamente modificada para la regeneración después de lesión medular)

Memoria presentada por la licenciada en Biología

SARA NOCENTINI

para optar al grado de Doctor por la Universidad de Barcelona

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Barcelona, 2014

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ibec Institute for bioengineering of Catalonia

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ABBREVIATIONS:

aa	aminoacid
AC	astrocyte
ADAM22	a disintegrin and metalloprotease 22
ADF	actin depolarizing factor
AMIGO3	amphoterine-induced gene and open reading frame 3
BBB	Barson, Beattie and Bresnahan
BMSC	bone marrow stromal cell
C or COOH	carboxy
CNS	central nervous system
CRMP-2	collapsing response mediator protein-2
CSPG	chondroitin sulfate proteoglycans
CST	corticospinal tract
DA	differential cell adhesion
d	days
DRG	dorsal root ganglia
E	embryonic day
ECM	extracellular matrix
eGFP	enhanced GFP
EGFR	epidermal growth factor receptor
E-NCAM	embryonic N-CAM
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
FA	focal adhesion
FAK	FA kinase
Fig	Figure
GDNF	glial-cell line derived neutrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
GTP	guanosine-5'-triphosphate
h	hours
IB	immunomagnetic beads
Ig	immunoglobulin
JNK	c-Jun N-terminal kinases
LBD	ligand-binding domain
LGI1	leucine-rich glioma inactivated 1
LIMK	LIM kinase
LINGO1	leucine rich repeat and Ig domain containing 1
LOTUS	lateral olfactory tract usher substance
LPA	lysophosphatidic acid
LRR	leucine-rich repeats
LacZ	bacterial enzyme β-galactosidase gene
MAG	myelin associated glycoprotein
MAIs	myelin associated inhibitors
MAPK	mitogen-activated protein kinase

MEK	MAPK/extracellular signal regulated kinase
MLC	myosin light-chain
MLCK	MLC kinase
MNPs	magnetic nanoparticles
MRI	magnetic resonance imaging
MSC	mesenchymal stem cells
N or NH_2	amino
NC	neural crest
N-CAM	neural cell adhesion molecule
NEP1-40	Nogo extracellular peptide, residues 1-40
NG2	neuron-glial antigen 2
NgR	Nogo receptor
NgR(Ecto)	Nogo receptor 1(310) ectodomain
NPY	Neuropeptide Y
NT	neurotrophin
NYU	New York University
OB	olfactory bulb
OE	olfactory epithelium
OECs	olfactory ensheathing cells
OMgp	oligodendrocyte-myelin glycoprotein
ONF	olfactory nerve fibroblasts
ONL	olfactory nerve layer
ORN	olfactory receptor neuron
PAA	polyacrylamide
PDMS	polydimethylsiloxane
PI	post-injury
PI-3K	phosphatidylinositol 3-kinase
PirB	paired-immunoglobulin-like receptor B
PNS	peripheral nervous system
PKC	protein kinase C
ROCK	Rho-associated serine/threonine kinase
SCs	Schwann cells
SCI	spinal cord injury
SHP	scr homology protein tyrosine phosphatase
TFM	traction force microscopy
TNF-α	tumor necrosis factor α
Tx	transection
w	week

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INTRODUCTION

1. Spinal cord injury

Spinal cord injury (SCI) may be defined as an injury resulting from an insult inflicted on the spinal cord that compromises its major functions (motor, sensory, autonomic, and reflex) (Dumont et al., 2001; Furlan et al., 2013). SCI remains an important cause of morbidity, being a crucial clinical problem in modern society. In fact, although numerous therapeutic interventions have been attempted they have very limited success (Thuret et al., 2006; Varma et al., 2013). Due to the complexity of the nervous system and SCI pathophysiology it is likely that effective therapy will encompass multiple approaches and targets. In fact, although in peripheral nervous system (PNS) axon shows relevant properties of regeneration and functional recovery; central nervous system (CNS) neurons lack of these properties, but more relevantly CNS as a whole impairs actively the regenerative process. Enhancing regeneration of surviving neurons to reestablish neuronal connectivity and function is an area of great interest and proven to be extremely challenging.

1.1 Inhibition of regeneration after SCI

The CNS has a very little regenerative capacity after injury or disease. In fact, axons undergo a process of abortive restoration that ends in the degeneration of the injured area. This is in contradiction with what happens in the PNS where axons can spontaneously re-establish functional connection after a lesion.

The first author to observe that axons fail to regenerate in the CNS was Santiago Ramón y Cajal in 1928 (Ramón y Cajal, 1928). He described that the end of the lesioned axons become swollen into "dystrofic endballs" that were no longer capable for regeneration (Ramón y Cajal, 1928). However, some years later, Aguayo and colleagues demonstrated that damaged CNS axons retain a limited capacity of regeneration and, if provided with a permissive substrate, such as a peripheral nerve graft (a source of reactive Schwann cells (SCs)), can extend over long distances (David et al., 1981). Moreover it was observed that neurons from the dorsal root ganglia (DRG) that have extensions in both CNS and PNS can only regenerate their peripheral

processes (Tuttle and Matthew, 1995).Some studies revealed that the dystrophic growth cones observed after an injury are not quiescent but are highly active (e.g., (Tom et al., 2004)) and that macrophage could induce their retraction but only in a hostile environment, such as the one present after a lesion (Horn et al., 2008). All these data suggested that the regeneration potential of the lesioned axons directly depends on the interaction with the environment and that the presence of inhibitory molecules and/or receptors/ligands is responsible for the lack of regeneration observed. Indeed, after an injury at the spinal cord level, inflammation in the form of invading macrophages and microglia can produce a large amount of secondary damage to the nerve, affecting even uncut axons (Rowland et al., 2008). Without proper growth factor support cut or crushed axons retract their growth cones and a large percentage die (Mey and Thanos, 1993; Berkelaar et al., 1994), while cell bodies of those that are spared often atrophy to the point of being unrecognizable as neurons (Villegas-Perez et al., 1988). At the same time, a fluid-filled cyst forms at the site of injury, and invading reactive astrocytes together with oligodendrocyte precursors and meningeal cells proliferate and secrete extracellular matrix (ECM) molecules, forming the glial scar (Yiu and He, 2006). This scar creates a physical barrier to the entry of regenerating axons, but also is a biochemical barrier since it contains a large number of chondroitin sulfate proteoglycans (CSPGs) that have been shown to impede the growth of CNS axonal processes (Yiu and He, 2006; Sharma et al., 2012). Axons of surviving neurons that manage to avoid the glial scar must navigate through myelin debris (Yiu and He, 2006; Yang et al., 2011) that persists months to years after the initial insult and that contain a number of inhibitory proteins (Fawcett et al., 2012).

1.2 Myelin associated inhibitors

Myelin, was first postulated as an environmental factor inhibiting axonal regrowth as it was observed that CNS neurons could grow extensively only on gray matter portions over sections of the whole brain (Carbonetto et al., 1987; Crutcher, 1989; Savio and Schwab, 1989). Although this difference could have resulted from a lack of positive substrates in the white matter, additional studies have shown that the myelin itself is inhibitory to neurite outgrowth. Indeed, DRG neurons were unable to extend axons when cultured on differentiated oligodendrocytes (the myelinating cells of the CNS) or purified myelin extracted from the brain (Schwab and Caroni, 1988). Since

then, a number of studies have demonstrated the importance of CNS myelin to failure axon regeneration. For example, axons of newborn rats exhibit enhanced regeneration after demyelination induced by killing oligodendrocyte precursor cells with lethal doses of x-irradiation (Savio and Schwab, 1989; Weibel et al., 1994). Immunization with antibodies recognizing CNS myelin, have also been shown to promote CNS regeneration in the spinal cord and the optic nerve (Huang et al., 1999; Ellezam et al., 2003).

Three molecules isolated from CNS myelin, designed as myelin associates inhibitors (MAIs) have been shown to highly inhibit axonal growth *in vitro*: NogoA, myelin associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (Figure 1).

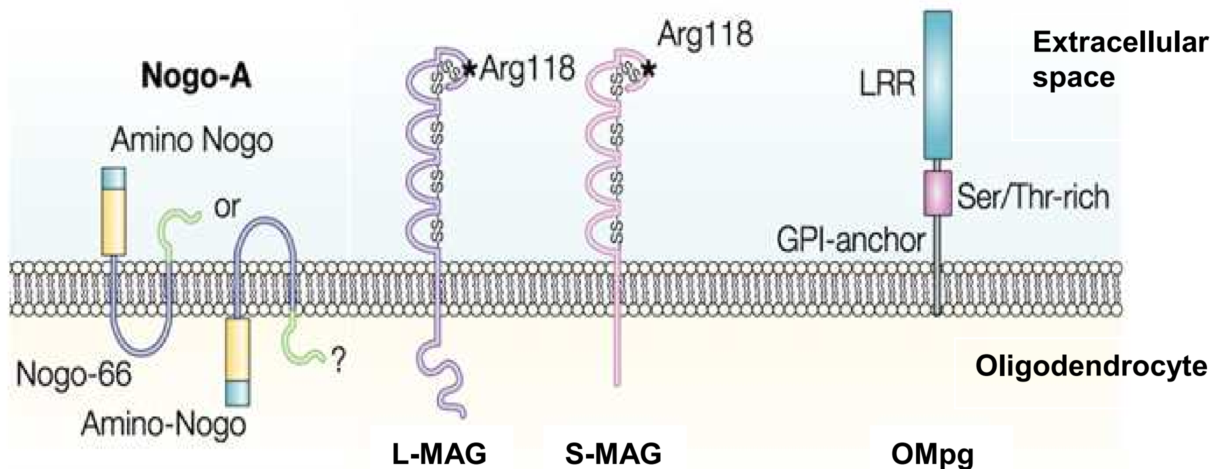

Figure 1: Schematic structures of MAG, NogoA and OMgp. The NogoA structure highlights its inhibitory domains: Nogo-66, the 66 aminoacid loop between two the transmembrane domains and amino-nogo (in yellow) near the N-terminal domain. MAG exists as two isoforms, small MAG (S-MAG) and large MAG (L-MAG). The extracellular domain consists of 5 Ig-like domains. The sialic acid binding site (*) is present in the first Ig domain.. OMgp is a GPI-linked protein consisting of a leucine-rich repeat and a serine/threonine rich domain. (Modified from Filbin et al., 2003).

1.2.1 NogoA

The inhibitory activity of NogoA (previously identified as NI250/80) was discovered by Schwab and colleagues over 20 years ago (Caroni and Schwab, 1988a; Caroni and Schwab, 1988b). From 2000, we know that NogoA is a member of the

Reticulon family, also known as Reticulon 4-A (Oertle et al., 2003). It is a membrane protein with two hydrophobic transmembrane domains separated by a loop containing 66 aminoacid, termed Nogo66. Nogo-A is thought to have at least two different membrane topologies, one in which the amino (N)-terminal domain and Nogo-66 domain face the extracellular space and a second in which the N-terminal domain and Nogo-A-specific part are exposed to the cytoplasmic side of the membrane (Filbin, 2003).Nogo66 has a potent axon growth inhibitory effect *in vitro* and binds to multiple receptors, including Nogo66 receptor (NgR1). The N-terminal domain of NogoA contains a unique sequence (amino-nogo) that also inhibits axon outgrowth and the adhesion of non-neuronal cells; however the receptor mediating these actions has yet to be discovered (Fournier et al., 2002b; Filbin, 2003; Mingorance et al., 2006). Amino-Nogo may associate, is not known whether directly or indirectly, to certain integrins to alter their function and finally block their growth-promoting signling (Hu and Strittmatter, 2008). Antibodies against the N-terminal of NogoA and Nogo66 show that both regions are localized on the cell surface of oligodendrocytes on the inner loop of myelin, where they can make contact with the axon (Fournier et al., 2002b). A small peptide, called Nogo extracellular peptide 1-40 (NEP1-40), was developed to interfere with Nogo66's inhibitory activity and when applied to the injured spinal cord, promoted CNS axon regeneration and sprouting (GrandPre et al., 2002). NogoA is highly expressed on oligodendrocytes and only minimally on PNS SCs (Fournier et al., 2002b; Niederost et al., 2002). This data is consistent with a role in mediating axonal growth inhibition specifically in the CNS. Within the carboxy (C)-terminal, NogoA exhibits a dilysine endoplasmic reticulum (ER) retention signal (Oertle et al., 2003). Indeed NogoA can be localized in the ER (Oertle et al., 2003) and from here may play many diverse roles in oligodendrocytes and neurons that I will not discuss in this thesis.

1.2.2 Myelin associated glycoprotein

Myelin associated glycoprotein (MAG) was identified through purification and separation of molecules present in myelin (McKerracher et al., 1994). Is expressed by both CNS and PNS glial cells and has been shown to participate in the formation and maintenance of the myelin sheath (Filbin, 2003). MAG is a sialic acid-binding protein and member of the immunoglobulin (Ig) super family and its extracellular domain contains five Ig domains. MAG has a single transmembrane domain and a cytoplasmic

domain (Wang et al., 2002a). To exert axon-inhibitory activity on axonal regrowth, MAG, interacts with the NgR1, but it has been also shown to interact also with Nogo receptor 2 (NgR2) (McKerracher et al., 1994; Filbin, 2003). When neurons were grown over MAG-immunodepleted CNS myelin extracts (McKerracher et al., 1994) or denaturized MAG (Li et al., 1996) partial recovery from neurite growth inhibition and collapse was observed. Anyway, CNS myelin derived from mice carrying a null mutation of the MAG gene, inhibited neurite outgrowth to a similar degree to the wild-type CNS myelin (Li et al., 1996; Ng et al., 1996). These results suggest that although MAG contributes significantly to axon growth inhibition associated with myelin, its lack in MAG-deficient mice may be masked by other non-MAG inhibitors.

1.2.3 Oligodendrocyte myelin glycoprotein

Oligodendrocyte myelin glycoprotein (OMgp), is a glycosylphosphatidylinositol (GPI)-linked protein that is expressed on oligodendrocytes, peripheral neurons, and in high levels on several types of CNS neurons (Habib et al., 1998; Wang et al., 2002b). OMgp has five tandem leucine-rich repeats (LRR) followed by a C-terminal with serine/threonine repeats. Similar to NogoA and MAG, OMgp expression induces growth cone collapse and inhibits neurite outgrowth (Wang et al., 2002b). The NgR1 has been shown to bind OMgp to initiate signal transduction to inhibit axonal outgrowth (Kottis et al., 2002; Atwal et al., 2008).

1.3 Other inhibitory proteins in the CNS

In addition to MAIs, there are other molecules present in the CNS that mediate growth-inhibitory effects that will be summarized briefly hereafter. Myelin is constituted for 70-75% by lipids in weight and it is likely that they may contribute to the inhibitory properties of myelin. Indeed, recently it has been observed that sulfatide, that comprises 4-7% of CNS myelin lipid (Norton and Autilio, 1966) contributes to axon regenerative failure *in vitro* and *in vivo* (Winzeler et al., 2011). CSPGs are released by astrocytes in the CNS injury sites and are potent inhibitors of axon growth (McKeon et al., 1995; McKeon et al., 1999). Canonical axon guidance molecules such as the semaphorin, netrin, and ephrin families are expressed through development and in the adult CNS. There is evidence that these proteins contribute to the inhibitory environment and upon injury the expression levels of these proteins often change

(Benson et al., 2005; Kaneko et al., 2006). For example semaphorin 6A was found to be upregulated at lesions in the CNS, suggesting an inhibitory role in regeneration (Shim et al., 2012).

1.4 The Nogo Receptor complex

Fournier et al., in 2001 identified the NgR1 as a receptor for the 66-amino acid inhibitory peptide, Nogo66, of the NogoA (Fournier et al., 2001). Surprisingly this receptor resulted to bind also to MAG and OMgp (Domeniconi et al., 2002; Wang et al., 2002a), suggesting that NgR1 is an important point of convergence for inhibitory signaling pathways.

NgR1 consists of a 288 residue N-terminal LRR domain, responsible for binding MAIs, followed by a 137 residue C-terminal domain necessary, but not sufficient for signal transduction, anchored to the cellular membrane with a GPI-ancor. To mediate intracellular signaling NgR1 must associate with co-receptors. The low affinity neurotrophin receptor p75 that, interacts with NgR1 through its extracellular domain (Wang et al., 2002a), and the LRR and Ig domain containing Nogo Receptor interacting protein (LINGO1) have been identified as a component of the NgR-complex (Mi et al., 2004). TAJ/TROY, an orphan tumor necrosis factor receptor family member can substitute for p75 as an alternative co-receptor for NgR1/LINGO1 (Park et al., 2005; Shao et al., 2005).

1.4.1 p75

p75 has a role in transducing the growth inhibitory signals initiated by several MAIs (Wang et al., 2002a). In fact, a robust outgrowth of p75 null sympathetic neurons was observed in myelinated portions of the cerebellum in comparison to wild-type neurons (Walsh et al., 1999). Following axotomy or lesions, p75 is upregulated (Montazeri et al., 2013), suggesting that although it is not normally expressed at high levels in many adult neurons, p75 expression could be regulated following injury to mediate outgrowth inhibition.

p75 acts activating the small guanosine-5'-triphosphate (GTP)ase, RhoA, inhibiting axon outgrowth (Fig. 2). Indeed, neurons treated with MAG, NogoA, OMgp, or myelin extracts showed increased activation of RhoA, whereas p75 mutant neurons did not exhibit increased RhoA activity in response to MAG (Wang et al., 2002a;

Yamashita et al., 2002). Moreover MAG can increase cytoplasmic Ca^{2+} levels interacting with the NgR but only in presence of p75 (Sivasankaran et al., 2004).This increase in calcium leads to the activation of the protein kinase C (PKCs). PKC in turn may be directly or indirectly involved in RhoA activation and in executing specific cytoskeleton rearrangements. In fact, it is known that a few prominent cytoskeleton regulators, such as GAP-43, CAP-23 and MARCKS, are major PKC substrates in axons, suggesting a potential role for PKC in directly influencing axonal behavior (Sivasankaran et al., 2004).

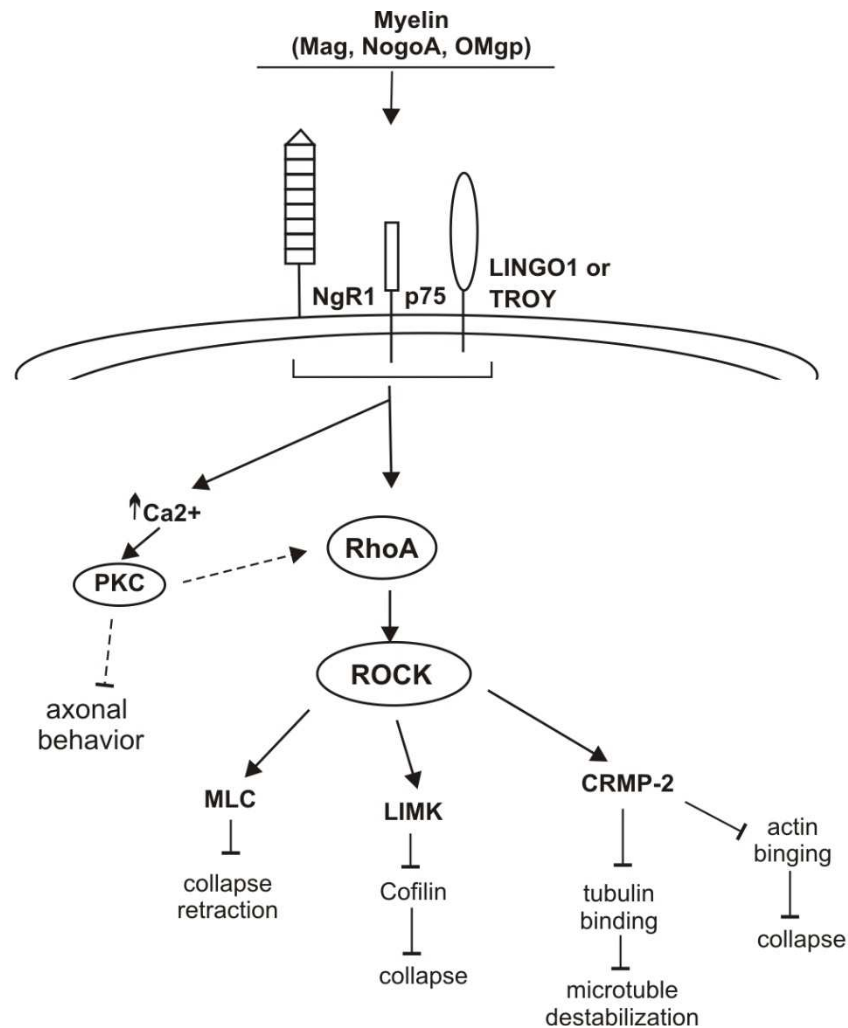

Figure 2: **Inhibitory signaling achieved by MAIs**. Myelin inhibitors signal via receptor complexes NgR/LINGO1/p75 or NgR/LINGO1/TROY to induce collapse and retraction. MAIs signal through Ca^{2+} dependent activation of PKC and RhoA. PKC may directly or indirectly induce RhoA activation and execute cytoskeleton rearrangements. RhoA mediates inhibition through activation of ROCK, which in turn acts on multiple cytoskeletal regulatory proteins. Target of ROCK phosphorylation are the myosin light chain (MLC) which in turn acts on myosin II finally enhancing cell contractility (Amano et al., 1998). Additionally ROCK can regulate actin dynamics and microtubule destabilization through the activation of LIM kinase (LIMK) and collapsing response mediator protein-2 (CRMP-2), a neuronal protein involved in growth cone collapse (Arimura et al., 2000).

1.4.2 LINGO1

Like NgR1, LINGO1 is a member of the LRR superfamily with an extracellular domain containing LRRs and an Ig domain. It also has a transmembrane domain and an intracellular domain that may play a role in signaling events downstream of MAIs (Mi et al., 2004). To date the function of LINGO1 is not well known, but its presence in the NgR-complex is necessary for inhibition of neurite outgrowth and the regulation of Rho GTPases by several MAIs (Mi et al., 2004). Indeed, when COS-7 cells were transfected with combinations of *NgR1*, *p75* and *LINGO-1*. Triply transfected cells responded to OMgp by activating RhoA, but no activation occurred when binary pairs of *NgR1/p75*, *LINGO-1/p75* or *NgR1/LINGO-1* were tested (although it cannot be excluded that such binary complexes have no roles *in vivo*) (Mi et al., 2004).

1.4.3 TROY

TROY was identified as a co-receptor for NgR1 that can functionally substitute for p75 in neurite outgrowth inhibition signaling (Park et al., 2005; Shao et al., 2005) and mediates activation of RhoA by MAIs, when expressed in CHO or COS-7 cells. Overexpression of a mutant TROY lacking its intracellular domain reversed outgrowth inhibition by Nogo66, OMgp, or myelin (Park et al., 2005; Shao et al., 2005). TROY is more widely expressed in mature neurons than p75 (Park et al., 2005) and may be important for mediating MAI signaling in neurons lacking p75.

1.5 Endogenous antagonist for NgR-complex

In 2010, it was identified the leucine-rich glioma inactivated 1 (LGI1) as the first endogenous NgR1 antagonist. LGI1 is a secreted protein that shares homology with members of the SLIT family (Thomas et al., 2010). LGI1 has been shown to antagonize myelin-induced growth cone collapse and neurite outgrowth inhibition by binding directly to NgR1 and competing with Nogo66 for the occupancy of the receptor (Thomas et al., 2010). It seems that LGI1 may also facilitate neurite extension over myelin and Nogo66. NgR1 may forms a complex with a disintegrine and metalloprotease 22 (ADAM22) and facilitates LGI1 binding to this receptor, finally exerting a positive effect on neurite extension (Thomas et al., 2010). More recently, a molecule denominated lateral olfactory tract usher substance (LOTUS) has been

described as a ligand of the NgR1 (Sato et al., 2011). It has been observed that LOTUS suppressed Nogo-NgR1 binding and Nogo-induced growth cone collapse. *In situ* hybridization revealed that LOTUS mRNA is expressed in several specific brain areas, including the spinal cord in mouse embryos. Overexpression of LOTUS attenuated Nogo-66 growth cone collapse in cultured DRG neurons from E13 chick embryos, preventing Nogo66 (or NogoA)–NgR (Sato et al., 2011). Thus, LGI1 and LOTUS may exert an antagonistic action on Nogo66-induced repulsive signaling and therefore may contribute to future therapeutic approaches for SCI. Moreover, these findings raise the possibility that NgR1 activation *in vivo* may reflect relative occupancy by endogenous agonists and antagonists.

1.6 Other receptors for MAIs

Since LINGO-1 protein expression levels do not rise in the spinal cord until 14 days after SCI (Mi et al., 2004), other NgR1 co-receptors mediating axon growth inhibition are likely to be expressed and function during SCI acute stages. Recently, it was observed that levels of the amphoterin-induced gene and open reading frame 3 (AMIGO3) are significantly higher than those of LINGO-1 acutely post-dorsal column lesions (Ahmed et al., 2013). AMIGO3 interacted functionally with NgR1-p75/TROY in non-neuronal cells and in brain lysates, mediating RhoA activation in response to CNS myelin (Ahmed et al., 2013). Knockdown of AMIGO3 in myelin-inhibited adult primary DRG and retinal cultures promoted disinhibited neurite growth when cells were stimulated with appropriate neurotrophic factors. These findings demonstrate that AMIGO3 may be a substitute for LINGO-1 in the NgR1-p75/TROY inhibitory signaling complex and suggests that the NgR1-p75/TROY-AMIGO3 receptor complex mediates myelin-induced inhibition of axon growth acutely in the CNS (Ahmed et al., 2013). Thus, antagonizing AMIGO3 rather than LINGO-1 immediately after SCI is likely to be a more effective therapeutic strategy for promoting CNS axon regeneration.

When NgR-complex function is abolished, there is still some residual neurite outgrowth inhibition exerted by MAIs, suggesting that additional receptors are involved in this process. Among others, gangliosides have been proposed as receptors for MAG, although their exact contribution remains largely controversial (McKerracher, 2002; Schnaar and Lopez, 2009; Cao et al., 2010). Two of the most recently identified

receptors for MAIs are the paired-immunoglobulin-like receptor B (PirB) (Atwal et al., 2008) and β1-integrin (Goh et al., 2008). To date, the mechanism by which PirB inhibits neurite outgrowth remains unknown. However, PirB is able to form complexes with src homology 2-containing protein tyrosine phosphatases (SHP-1 and SHP-2) in the brain (Syken et al., 2006) and it has been observed that SHP-2 could act as a "stop" signal for neurons approaching their targets (Chen et al., 2002). β1-integrin acts as a direct receptor to mediate MAG-induced growth cone response of CNS neurons, through the phosphorilation of the focal adhesion kinase (FAK) (Goh et al., 2008).This signaling is important for both MAG induced repulsion and attraction of neurons during different developmental stages or different cellular status (Goh et al., 2008).

2. Olfactory ensheathing cells

Olfactory ensheathing cells (OECs) are unique glia found in the primary olfactory system that retain exceptional plasticity, and play a key role in the stimulation and maintenance of olfactory neuron turnover. These cells possess the unique ability to promote neurogenesis and targeting across the PNS/CNS boundary. Even though, the understanding of OEC intrinsic properties is limited they have become a prime candidate for cell-mediated repair following SCI (Richter and Roskams, 2008).

2.1 The primary olfactory system

The primary olfactory system of vertebrates is composed by a peripheral part, the olfactory mucosa, located in the nasal cavity and its target tissue, the olfactory bulb (OBs), that reside inside the CNS (Barber, 1982; Murray and Calof, 1999). The olfactory mucosa consists of an olfactory epithelium (OE) and an underlying lamina propria separated by a basal lamina. In the OE we can find the somas of the olfactory receptor neurons (ORNs). ORNs send their dendritic processes to the apical surface of the epithelium whereas their axons project into the lamina propria together with other ORNs axons form the olfactory fascicles that pass through the cribriform plate to enter the OB located in the anterior cranium of mammals (Murray and Calof, 1999). Here the axons of the ORNs converge into numerous spherical structures called glomeruli where they synapse with second order neurons (mitral and tufted cells) (Huart et al., 2013). Surrounding the glomeruli there are periglomerular interneurons that also receive inputs from ORNs (Huart et al., 2013) (Figure 3 and 4). Although ORNs that express a specific individual odorant receptor are widespread throughout the olfactory epithelium, their axons converge to one or few glomeruli in the OB (Mombaerts et al., 1996; Mori and Sakano, 2011). In this way, each glomerulus is tuned to detect a specific set of odors forming a sensory odorant map (Mori and Sakano, 2011) (Figure 4).

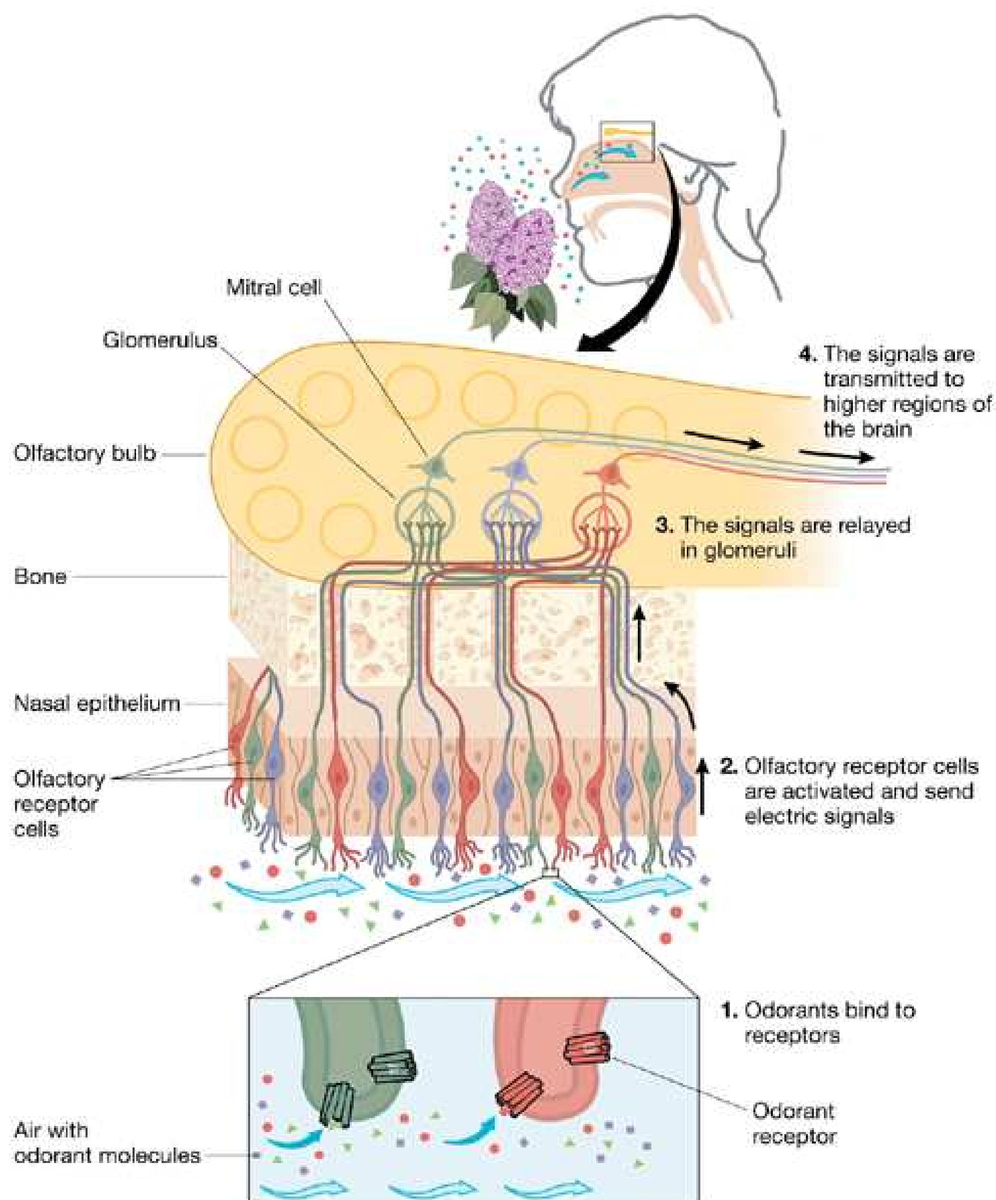

Figure 3: Scheme of the primary olfactory system in humans. The odorant receptors are localized on ORN, which occupy a small area in the upper part of the nasal epithelium. Every olfactory receptor cell expresses only one odorant receptor. On activation, signals from olfactory receptor cells are relayed in the glomeruli-well defined micro-regions in the olfactory bulb. Receptor cells of the same type are randomly distributed in the nasal mucosa but converge on the same glomerulus. In the glomerulus, the receptor nerve endings excite mitral cells that forward the signal to higher regions of the brain (from (Rinaldi, 2007)).

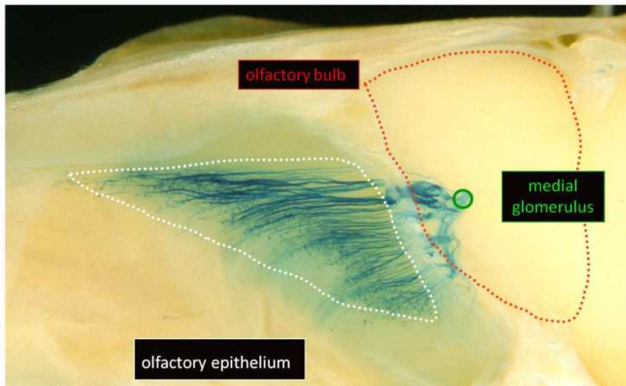

Figure 4: Receptor cells of the same type converge on the same glomerulus. An example of a midline whole mount in which both the olfactory epithelium and olfactory bulb can be seen, showing the convergence of the axons into a single glomerulus in the olfactory bulb. (Modified from http://www.yalescientific.org/2011/05/the-neural-basis-of-olfaction)

Neurogenesis in the olfactory system takes place both in the olfactory mucosa (Schwob, 2002; Huart et al., 2013) and in the OB throughout life (Doetsch et al., 1999; Lotsch et al., 2013). The globose basal cells in the olfactory mucosa are able to give rise to new ORNs that live approximately 70 days in humans. The rate of neurogenesis is normally regulated by environmental factors. In fact, after contact with any damaging factor (for example toxin, virus, physical injury) proliferation of neuronal progenitors increase to replace ORN that have been lost (Murray and Calof, 1999). In the OB neuronal progenitors cell derive from the supraventricular zone or directly from the OB (Pagano et al., 2000; Gritti et al., 2002) and they give rise to the interneurons of OB (Doetsch et al., 1999). Neurogenesis, in this case, depends on the animal age, thus it has been demonstrated that neurogenesis decrease over time. Anyway, in the last years, it has been described that other factors may regulate regeneration in the OB although further research is needed to clarify the exact role and regulation of these mechanisms (Gritti et al., 2002; Gudziol et al., 2009).

2.2 OECs and olfactory axons

The ability of regeneration of the olfactory system throughout life has been partially associated with the presence of the OECs that create a permissive environment for axonal elongation (Doucette et al., 1983; Doucette, 1984). This particular glial cell type is found both in the PNS and CNS regions of the olfactory system. OECs are capable to ensheath unmyelinated axons (0.1-0.7 um en diameter in mammals) of ORNs that exit from the OE and accompany them through the lamina propria, the cribriform plate to reach their final target in the OB (Boyd et al., 2005). Axons arising from the OE group in olfactory nerves fascicles. Located within these fascicles are the OECs that

extend sheet-like cytoplasmatic processes around hundreds of packed axons (Nedelec et al., 2005) to form a sort of tunnel-like structure that ease ORN axons elongation and transition from PNS to CNS (Fig. 5). Moreover, OECs help the axons to form an appropriate synaptic connection with mitral, periglomerular and tufted cells in the OB (Valverde et al., 1992). In fact, once the nerve fascicle gets to the olfactory nerve layer (ONL) of the OB, the ensheathing cylinders open to allow individual ORN axons to synapse with second-order neurons (Nedelec et al., 2005). At this level, OECs interact with CNS astrocytes to direct regenerating axons to the correct synaptic target (Doucette et al., 1983; Doucette, 1996; Li et al., 2005). Furthermore, OECs are able to produce a variety of molecules known to be involved in cell adhesion and that promote neurite initiation, axonal elongation and growth cone attachment as, among others, laminin (Doucette, 1996), L1 (Miragall et al., 1988), neural cell adhesion molecule (N-CAM) (Chuah and Au, 1993) and the polysialic acid embryonic N-CAM (E-NCAM) (Sonigra et al., 1999). Finally, a huge number of neurite growth promoting factors have, also, been found to be expressed in OECs *in vitro* including neurotrophins (NT)-4/5 (Vincent et al., 2003), glial-cell line derived neutrophic factor (GDNF) (Lipson et al., 2003), nerve growth factor (Vincent et al., 2003) and brain derived neutofic factor (Boruch et al., 2001).

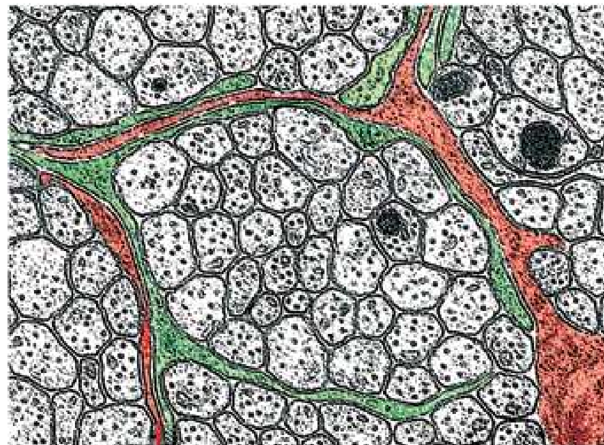

Figure 5: OECs processes enclosing olfactory axons. Electron micrograph of a cross section through a bundle of over 30 olfactory axons (small circular profiles) ensheathed in a channel formed by the interweaving of the sheet-like OECs processes (red and green) arising from adjacent OECs bodies (out of the picture) (from (Raisman and Li, 2007)).

2.3 OECs origin and role in development

OECs were thought to derive from the olfactory placode (Couly and Le Douarin, 1985) but, recently, it has been demonstrated that OECs precursors arise from the neural crest (NC) by fate-mapping techniques in chick embryos (Barraud et al., 2010). In mice,

at embryonic day 10 (E10) the olfactory placodes invaginate to form the olfactory pits giving rise to the primary olfactory epithelium (Hinds, 1972). Axons from pioneer sensory neurons leave the nasal pit and extend dorsally to the telencephalon. These axons are accompanied by a heterogeneous population of cells including OECs (Doucette, 1989). OECs, in contrast to SCs which migrate along already defined axonal pathways (Jessen and Mirsky, 2005), always extend processes ahead of the axons they ensheath, so that the growth of the olfactory axons seems to depend on their presence (Valverde et al., 1992; Chehrehasa et al., 2010; Ekberg et al., 2012). At this stage, OECs proliferate rapidly and develop elaborated processes to maintain the ensheathment of the increasing number of axons (Valverde et al., 1992). At E13, the olfactory sensory axons together with OECs arrive at the telencephalon and stimulate the formation of the OB (Doucette, 1989). The axons remain in this region for 2-3 days while they defasciculate and extend towards their target (Royal and Key, 1999). In the outer ONL of the OB, OECs are thought to contribute to the defasciculation of the mixed bundles of axons, whereas, within the inner ONL, OECs are thought to facilitate the sorting and refasciculation of axons of the same odorant receptor (Au et al., 2002).

2.4 Are OECs a homogenous population of cells?

The existence of antigenically different populations of OECs *in vivo* was suggested by the variable expression of the glial fibrillary acidic protein (GFAP) in peripheral nerves (Barber and Lindsay, 1982). Through the years, individual subpopulation of OECs with distinct anatomical localization, different behavior and expression pattern of certain molecules have been observed. For example OECs from the peripheral olfactory nerve express among others Notch-3 and chondroitin sulfate proteoglycan NG2. But, this two markers are not expressed by OECs from the ONL (Au and Roskams, 2003). Even molecules considered markers for OECs are not universally expressed. In fact, all OECs in ONL express S100ß and weak GFAP, but only the cells from the outer part express p75 and E-NCAM (Au et al., 2002) while only OECs from the inner ONL express neuropeptide Y (NPY) (Ubink and Hokfelt, 2000). Indeed, it was thought that two populations of OECs exist in the adult ONL. However the exact definition of these populations is questionable due to the variability among studies (strains, age of animals used) and difficulties in identifying the structure labeled as molecules can be expressed by both OECs and axons such as p75 or E-NCAM (Vincent

et al., 2005).

Despite all this uncertainty, all OECs derive from the neural crest and can be distinguished from other glial type by their electron-dense cytoplasm (Barnett et al., 1993), scattered intermediate filaments (Doucette, 1984) and irregular nuclei (Boyd et al., 2005). The possible existence of subpopulations of OECs *in vivo* is reflected *in vitro* (Ramon-Cueto et al., 1993). Indeed, in culture, two subpopulation with different morphology have been described, one resembling SC (SC-OECs) and one resembling astrocyte (AC) (AC-OEC) (Pixley, 1992; Huang et al., 2008). SC-OECs present a long fusiform bipolar structure with two processes, express the low affinity neurotrophin receptor p75 and show diffuse expression of GFAP (Pixley, 1992). AC-OECs resemble type 1 astrocytes and display a flat, multipolar, stellated structure, intense GFAP staining, E-NCAM expression and may lack expression of p75 (Franceschini and Barnett, 1996; Raisman, 2001; Huang et al., 2008). Although some researchers have suggested that the SC- and AC-like phenotypes represent separate subpopulations of OECs or different developmental stages of OECs (Huang et al., 2008), others postulate that OECs may change their morphologies *in vitro*, depending on the cell culture environment and the source of OECs (Barnett and Roskams, 2008). Recent studies demonstrated the plasticity of these cell types and suggested that each OECs cell subtype may switch between morphologies (van den Pol and Santarelli, 2003). Further evidence for this hypothesis has been provided by time-lapse imaging of single isolated cells that demonstrated that OECs subpopulation transformed into each other spontaneously (Huang et al., 2008). This last result supports the notion that OECs are a single cell type with a highly malleable functional phenotype. Indeed SC-like OECs have been shown to migrate three times faster than AC-like cells (Huang et al., 2008) so that the morphology could be reflecting a different functional state.

3. Strategies for regeneration after SCI

Over the past years, tremendous progress has been made in understanding the inhibitory components of CNS myelin, the axonal receptors that respond to these cues and the intracellular signaling mediating the impairment of axon outgrowth derived from myelin signaling. Several approaches designed to antagonize the molecular mediators of axon inhibition have been tested. Reversing the inhibitory properties of MAIs has been done by blocking the inhibitory molecules with the use of antibodies/antagonists, eliminating these molecules or their receptors by using animal knockouts or blocking the signaling interfering with the receptor via receptor bodies (Table 1). These studies validated the role of MAIs in impairing axonal regeneration. However approaches such as the generation of knockout mice for MAIs have raised new questions. For example, administration of IN-1 antibody which recognize, but is not specific for NogoA (Caroni and Schwab, 1988b) promoted axonal regeneration of the corticospinal tract (CST) (Schnell and Schwab, 1990; Bregman et al., 1995) and functional recovery after SCI (Z'Graggen et al., 1998). More specific antibodies against NogoA yielded less regenerative profiles (Liebscher et al., 2005; Maier et al., 2009) but results were consistent with NogoA’s role in axon regeneration inhibition. When using genetically modified NogoA knockout mice three different outcomes were observed ranging from robust (Simonen et al., 2003), suggestive (Dimou et al., 2006) to no regeneration (Lee et al., 2009). This observation led to major controversy regarding NogoA‘s role in axon regeneration. MAG knockout failed to show enhanced regeneration (Bartsch et al., 1995) while OMgp knockout results are controversial (Ji et al., 2008). All these results raised the question whether any of the three MAIs by themselves is a significant inhibitor of axon regeneration. Maybe all the three molecules need to be blocked simultaneously in order to promote regeneration. To test this hypothesis a triple knockout was generated (Cafferty et al., 2010). Cafferty and colleagues reported significant enhancement of CST axon regeneration and locomotor improvement after SCI in the triple mutant in comparison to wildtype or single NogoA knockouts (Cafferty et al., 2010). This result indicated a possible synergic effect of the

three MAIs. Anyway Lee and colleagues didn't find any significant enhancement after a similar hemisection of the spinal cord in the same model (Lee et al., 2010).

Intervention method	Target	Injury model	Result	Reference
Antibodies				
IN-1	NogoA	Spinal cord hemisection (rat)	CST regeneration	Bregman et al., 1995 Schnell et al., 1990
		Pyramidotomy (rat)	Functional recovery	Z'Graggen et al., 1998
11C7, 7B12	NogoA	T-shaped lesion (mouse)	CST regeneration and functional recovery	Liebscher et al., 2005
11C7	NogoA	T-shaped lesion (mouse)	CST regeneration and functional recovery	Maier et al., 2009
Knockout models				
Knockout for:	NogoA	Spinal cord hemisection (mouse)	CST regeneration and improved functional recovery	Simonen et al., 2003
	NogoA	Spinal cord hemisection (mouse)	Slight CST regeneration	Dimou et al., 2006
	Nogo	Spinal cord hemisection (mouse)	No CST regeneration	Lee et al., 2009
	MAG	Spinal cord hemisection (mouse)	No CST regeneration	Bartsch et al., 1995
	OMgp	Spinal complete transection (mouse)	Axonal regeneration	Ji et al., 2008
	OMgp	Spinal cord hemisection (mouse)	No axonal regeneration neither funcional recovery	Ji et al., 2008
	NgR1	Spinal cord transection or dorsal hemisection (mouse)	Raphespinal and rubro-spinal fibers regeneration, improved motor function recovery but no CST regeneration	Kim et al., 2004
	NogoA/MAG/OMgp	Spinal cord hemisection (mouse)	CST regeneration and functional recovery	Cafferty et al., 2010
	NogoA/MAG/OMgp	Spinal cord hemisection or complete transection (mouse)	No CST regeneration neither funcional recovery	Lee et al., 2010
Receptor Bodies				
NEP1-40	NgR1	Spinal cord hemisection (rat)	CST regeneration and improved functional recovery	GrandPre et al., 2002
NEP1-40	NgR1	Spinal cord hemisection (mouse)	Slight CST regeneration, but no funcional recovery	Steward et al., 2008
NgR1(310) Ectodomain	NgR1	Spinal cord hemisection (rat)	CST and raphespinal regeneration and improved functional recovery	Li et al., 2004
NgR1(310) Ectodomain	NgR1	Spinal cord contusion (rat)	Axonal regeneration and functional recovery	Wang et al., 2006

Table 1: Improving regeneration after SCI targeting MAIs. A table with a summary of studies cited in the introduction of methods used to improve regeneration after SCI in animal models.

An alternative strategy to block MAIs simultaneously is to target their common receptor. Administration of the peptide NEP1-40, consisting in the first 40 residues of the Nogo-66 loop of Nogo-A (the domain interacting with NgR), prevents Nogo-66 binding acting as a competitive antagonist of NgR1 (GrandPre et al., 2002). NEP1-40 promoted CST axons in one study (GrandPre et al., 2002), however the robust effect could not be reproduced (Steward et al., 2008). As the ligand-binding domain (LBD) of

NgR1 contains overlapping, but distinct, binding pockets for NogoA, OMgp and MAG (Lauren et al., 2007) a soluble form of the NgR1, NgR1(310)-ectodomain (from now on NgR(Ecto)) was generated. NgR(Ecto) consists of residues 1 through 310 and includes the entire LBD, but lacks both the C-terminal region that is required for NgR signaling and the GPI linkage region of the receptor (Fournier et al., 2002a) (Fig. 6). This ectodomain exerts its action binding on the MAIs and preventing their binding to the NgR1 on the neuronal cell surface, and also interacting with full-length NgR1 to prevent oligomerization or interaction with the other components of the NgR-complex, blocking their intracellular signaling. NgR(Ecto) showed CNS myelin inhibitor antagonistic properties in vitro (Fournier et al., 2002a; He et al., 2003). Indeed, following SCI, NgR(Ecto) promoted some sprouting and regenerative growth of severed CST and raphespinal fibers and functional recovery (Li et al., 2004; Wang, X. et al., 2006). Anyway, the use of a knockout mouse for the NgR1 receptor showed no CST regeneration (Kim et al., 2004). This indicates that targeting only NgR1 is not sufficient to promote robust regeneration. Therefore other approaches have been studied during the years. In particular, cell transplantation for SCI may promote regeneration and rescue impaired neuronal function as grafted cells may secrete permissive neutrofic factors at lesion site to create an environment more conducive to regrowth and remyelination. Moreover, other types of cells could help filling in the lesion and provide a scaffolding to support regeneration.

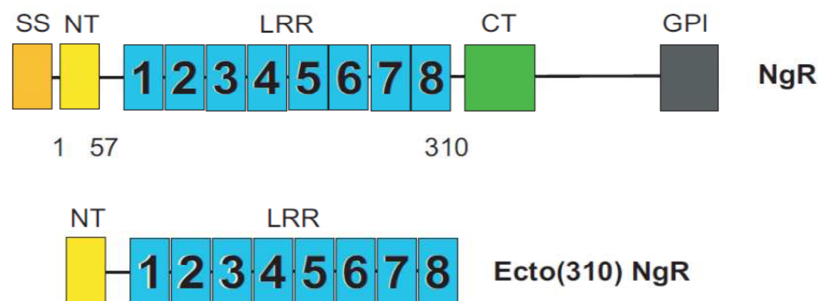

Figure 6: Schematic representation of the wild type NgR and the NgR(Ecto). The NgR(Ecto) lacks of the C-terminal (CT) region that is required for NgR signaling and the GPI linkage region of the receptor. SS, signal sequence; NT, N-terminal region.

3.1 Transplantation of OECs after SCI

Many types of cells have been implanted in the spinal cord, among them, oligodendroglial lineage cells (Almad et al., 2011; Sun et al., 2013), SCs (Zhou et al., 2012; Hu et al., 2013), OECs (Kocsis et al., 2009; Tabakow et al., 2013) and different types of stem cells (Karimi-Abdolrezaee and Eftekharpour, 2012; Ozdemir et al., 2012; Wang et al., 2013). We will concentrate on the use of OECs as they are the focus of our research (Table 2). Given their intrinsic properties OECs have been implanted into the damaged spinal cord in hopes of promoting axonal regeneration. Ramon-Cueto *et al.*, were the first to demonstrate the beneficial effects of implanting OECs after SCI (Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto et al., 1998). Three weeks after OECs implantation from the OB into the dorsal root entry zone of T9 transected spinal cords of adult rats, an enhancement in the regeneration of dorsal root axons was observed (Ramon-Cueto et al., 1998). Since then, OECs have been used as a therapeutic strategy for enhancing axonal repair in various SCI animal models. Research lead to controversial results because of methodological differences, such as the source of OECs (bulbar and/or mucosal), the SCI model (crushed, transected, X-irradiation; acute or chronic), the axon tracts involved and the length of the recovery period. For example, OECs from olfactory mucosa implanted into a completely transected T10 SCI promoted functional recovery, and enhanced the regeneration of serotonergic axonal tracts (Lu et al., 2002). Axonal elongation was demonstrated, also, after implanting embryonic bulbar cells in a similar study (Lopez-Vales et al., 2006). Positive results have been shown following implantations of OECs in a contusion model in some studies (Deng et al., 2008; Gorrie et al., 2010); and a hemisection lesion model (Deumens et al., 2006b). In contrast to these results, a lack of axonal regeneration was observed following implantation of bulbar OECs into chronical SCI (Deumens et al., 2006a) or of mucosal OECs after a full C4 transection. (Lu et al., 2006). Similar failures have been shown by others (Takami et al., 2002; Boyd et al., 2004; Collazos-Castro et al., 2005; Lopez-Vales et al., 2006).

Main graft	SCI model	Transplantation	Outcome	Reference
OECs from rat **OB**	T10 **complete Tx** (rat)	Injection (0 hr PI) ACUTE	Numerous regenerating dorsal root axons were observed reentering the spinal cord.	Ramon-Cueto and Nieto-Sampedro, 1994
OECs from rat **OB**	T9 **complete Tx** (4mm of spinal cord removed, rat)	Injection rostral and caudal to the lesion site (0 hr PI) ACUTE	OECs integrated and migrated up to 1,5 mm from injection site. Labeled spinal neurons seen caudally to the graft (0,5-3mm) and ascending propriospinal axons observed in rostral spinal cord, up to 2,5 cm.	Ramon-Cueto et al., 1998
OECs from rat **OB**	T11 demyelinated (rat) spinal cord lesion	Injection rostral and caudal (3+3) to the lesion site (3 d PI) SUBACUTE	Remyelinated axons near and remote from the cell injection site, indicating extensive migration of OECs within the lesion	Imiazumi et al., 1999
OECs from rat **OB**	L3-L6 lumbar rhizotomy (rat)	Injection at site of lesion (0 hr PI) ACUTE	Transplanted ensheathing cells promoted central regeneration and functional reconnection of regenerating sensory afferents	Navarro et al., 1999
OECs from rat **lamina propria**	T10 **complete Tx** (rat 1-2mm gap)	Pieces of lamina propria rostral and caudal to lesion epicenter (0hr PI) ACUTE	Nerve fiber passed through transaction site, serotoninergic fiber and descending pathway regeneration.	Lu et al., 2002
OECs from rat **OB**	T9 **contusion** NYU impactor (rat)	OECs injected at injury center with SCs or alone (7d PI) SUBACUTE	Less cavitation and more sparing, higher number of retrograde traced propriospinal and brainstem axons in the SCs-OEC group (not in OEC alone) CST axons terminated closer to lesion epicenter. No improvement in BBB in OEC group.	Takami et al., 2002
OECs from rat **OB**	T8 lesion **obtained by illumination of Rose Bengal** (rat)	Injection at lesion site (30 min PI) ACUTE	OECs promoted functional and morphological preservation of the spinal cord.	Verdu et al., 2003
OECs from rat **OB**	T9 **contusion** NYU impactor (rat)	OECs injected at injury center (0 h PI) ACUTE	OECs were seen several mm from the injection site. No functional recovery enhancement.	Resnick et al., 2003
OECs from rat **OB**	T10 **compression** injury (rat)	OECs injected at injury center (7d PI) SUBACUTE	A robust growth of CNS axons was observed but OEC did not myelinate axons, and didn't migrate from the lesion site.	Boyd et al., 2004
OECs from rat **OB**	C-7 weight drop **mild contusion** (rat)	5 injection near the site of lesion (0 hr PI) ACUTE	No evidence of functional repair was observed 3 months after lesion	Collazos-Castro et al., 2005
OECs from rat **OB**	T8 **complete Tx** (rat)	2 injection 1mm rostral and caudal to lesion epicenter (0hr, 30 min or 7 d PI) ACUTE and SUBACUTE	Long axonal regeneration, enhancement of locomotory recovery.	Lopez-Vales et al., 2006
OEC from **immortalized cell lines**	C3 bilateral dorsal column lesion with **forceps** (rat)	TEG3 from OECs of the olfactory bulb of rats (0 h PI) ACUTE	TEG3 survived at least 10 weeks, sensory projection axon grow into lesion site robust sprouting/ axonal growth of the CST into and beyond lesion. Enhanced Locomotor recovery	Moreno-Flores, 2006
OECs from rat **OB**	T11-T12 **dorsal hemisection** (rat)	Mixed OECs and fibroblasts from the OB implanted into the lesion site and 1 mm rostral or caudal (4weeks PI) CHRONIC	Enhanced presence of CST axons rostral to the lesion, no behavioral recovery.	Deumens et al., 2006b
OECs from rat **lamina propria**	T10 **complete Tx** (rat 1-2mm gap)	Pieces of lamina propria rostral and caudal to lesion epicenter (30 d PI) CHRONIC	Slight enhancement of axonal regeneration. No effect on functional recovery.	Steward et al., 2006
OECs from rat **lamina propria**	C4 **complete Tx** (rat)	2 injection 1mm rostral and caudal to lesion epicenter (0hr PI) ACUTE	OECs do not appear to exhibit significant migratory properties. They don't support bridging of CST axons through the lesion.	Lu et al., 2006
OECs from rat **OB**	**Dorsal hemisection** injury at T11/T12 (rat)	Aligned OEC/ONF-biomatrix complexes injected 1 mm rostal and caudal to the lesion (4w PI) CHRONIC	Injured axons did not penetrate the OEC/ONF biomatrix. No functional recovery enhancement.	Deumens et al., 2006a
OECs from rat **OB**	T8 **complete Tx** (rat)	4 injections: rostral, caudal and 2 lateral (45d PI) CHRONIC	No significant CST and serotoninergic axonal regeneration.	Lopez-Vales et al., 2007
OECs from **human OB**	T10 weight drop **contusion** (rat)	Human OECs injected at injury center and 2mm rostral and caudal to the lesion with BMSCs or alone (0 h PI) ACUTE	Significantly more axons were seen in the lesion site. Enhanced functional recovery When implanted with BMSCs the regeneration was enhanced.	Deng et al., 2008
OECs from **human OB**	T10 **contusion** (rat)	Human OECs injected at injury center (7 d PI) SUBACUTE	Reduction in volume of lesion and cavities, enhancement of locomotory recovery.	Gorrie et al., 2010

Table 2: OECs implantation after SCI. Summary of the studies where OECs are implanted after a spinal cord injury cited in the text. Tx: Transection; BBB: Barson, Beattie and Bresnahan; ONF: Olfactory nerve fibroblasts; BMSC: brown marrow stromal cell; PI: post injury; d: days; h: hours; w: weeks.

3.2 Problems with OECs transplantation after SCI

The properties, fate and migration capacity of OECs following implantation into the injured spinal cord has raised many issues. Some are related to the fact that these cells are phenotypically very similar to SCs. As commented before, OECs express p75, GFAP and S100β which are also expressed by the SCs that can be found in the rodent olfactory system (Boyd et al., 2005; Rizek and Kawaja, 2006). OECs grafts used in SCI studies could be contaminated by SCs as demonstrated recently (Rizek and Kawaja, 2006) and the presence of this cell type in the grafts could account, in part, to the different results observed after SCI implantation (as different percentage of SCs versus OECs could be present in the graft).

The source of OECs is another issue to consider when comparing studies. Rubio *et al*. (Rubio et al., 2008) stated that bulbar OECs are the best source of OECs as they are able to effectively migrate into the injured spinal cord and promote long distance regeneration (Ramon-Cueto et al., 1998; Verdu et al., 2003; Lopez-Vales et al., 2006), while mucosal OECs failed to exhibit these results (Lu et al., 2006; Steward et al., 2006). Nevertheless, there are numerous studies showing the negative effects of bulbar OECs and the beneficial effects of mucosal OECs on spinal cord repair. For example, Takami *et al*. (2002) demonstrated that OECs isolated from the olfactory bulb are ineffective at promoting spinal cord regeneration (Takami et al., 2002; Lopez-Vales et al., 2007).

Several studies reported that OECs are able to migrate for long distances from the injection in a SCI (Ramon-Cueto et al., 1998; Resnick et al., 2003). As few have adequately pre-labeled cells before transplantation there is a possibility of dye leakage or the absorbance of dead pre-labeled OECs by macrophages. However, further studies using viral vector-mediated expression of green fluorescent protein (eGFP) in OECs revealed a clear lower migratory capacity, and that OECs localization could be due to passive spreading rather than active migration (Boyd et al., 2004; Lu et al., 2006).

The general consensus is that OECs grafts facilitate neural repair, although the mechanisms through which this is accomplished are under debate (Ramon-Cueto et al., 1998; Boyd et al., 2005). Important issues that remain to be resolved include the optimal source of cells (mucosal versus bulbar), age of the animal for obtaining these cells (embryonic *versus* adult) and the graft strategy (for example, injection of a suspensions

or transfer within a cellular matrix). It will also be important to determine whether enriching cultures for specific phenotypes of OECs improves the structural and/or functional recovery, since SC-OECs have been demonstrated to migrate more and to be more effective in enhancing axonal regeneration in some studies in contrast to AC-OECs (Franklin et al., 1996; Imaizumi et al., 1998; Boyd et al., 2005; Kumar et al., 2005).

However, primary cultures of OECs, which have been used in the afore-mentioned studies, are not the best sources of transplantation material in SCI research. In fact, they often contain contaminating cells; usually survive only few weeks in culture (therefore their constant preparation from fresh tissue is needed); are limited in number by the efficacy of harvesting; their efficacy to support regeneration depends on the method of culture preparation; and require immune-suppression that may lead to changes in behavioral recovery. Moreover, to study the molecular and cellular characteristic responsible for their regeneration enhancing properties a homogenous population of OECs is needed. An immortalized cell line of OECs that maintain the regenerative properties of primary cultures would provide an unlimited source of OECs for use in culture and *in vivo* (Moreno-Flores et al., 2003). Indeed, one specific rat OECs clonal line (TEG3) proved to be as good as primary OECs in promoting axon regeneration in culture models and in an animal model of SCI, dorsal column crush (Moreno-Flores et al., 2003). TEG3 cells can acquire AC-like or SC-like morphology depending on the conditions under which they are cultured. Moreover in TEG3-transplanted animals cells survived for at least ten weeks after grafting; sensory projection axons grown into the lesion site and there was robust sprouting/axonal growth of the CST (Moreno-Flores et al., 2003). As regenerative properties of OEC seem to depend on their migration capacity, we aimed to explore the behavioral and migratory properties over MAIs using this cell line.

3.3 Methods to improve OECs efficacy after SCI

Combining transplantation of OECs with gene therapy to have a local and continuous delivery of factors enhancing axonal growth, with factors neutralizing the inhibitory environment signals or with biomaterials could be one powerful strategy to promote SCI repair.

Indeed, OECs have been genetically modified to express GDNF (Cao et al., 2004) and NT3 (Ruitenberg et al., 2005; Ma et al., 2010), two factors that when injected directly after SCI are able to enhance recovery.

GDNF is a factor that is able to exert a trophic effect on CST neurons and promote long-term survival after axotomy, and behavioral and anatomical neuroprotection following SCI (Blesch and Tuszynski, 2003; Hashimoto et al., 2005; Ansorena et al., 2013).Genetically modified OECs expressing GDNF, were capable to survive and express the foreign gene when implanted in SCI (Cao et al., 2004). GDNF-OECs could stimulate an increase in the regeneration of CST or rubrospinal axons trough the transection site in adult rats compared with normal OECs (Cao et al., 2004). The functional of locomotion recovery in the GDNF-OECs group showed statistically significant improvements compared with the normal OECs group (Cao et al., 2004).

NT3 is well known for its beneficial effects on survival of several neuronal cells, and neurite outgrowth. The the local delivery of this factor counteract pathological events and induce a regenerative response after SCI. NT3 expressing OECs using once implanted in the SCI stimulated increased amount of regenerating CST or rubrospinsal axons, in comparison with non-modified OECs. Moreover, the NT3-OECs rat group achieved statistically significant improvement of locomotor functions compared with the normal OECs group (Ruitenberg et al., 2005; Ma et al., 2010).

As myelin debris are present for long times after SCI in the lesion site, we think that a good candidate for OECs genetic modification is the NgR(Ecto). In fact, (as described before) this molecule binds to NogoA and prevents its binding to the NgR1 on the neuronal cell surface, and can also interact with full-length NgR1 to prevent oligomerization or interaction with other components of the NgR-complex, blocking the intracellular signaling (Fournier et al., 2002a; He et al., 2003). Indeed, following SCI, NgR(Ecto) promoted some sprouting and regenerative growth of severed CST and functional recovery (Li et al., 2004; Wang, X. et al., 2006).

Migratory properties of OECs after SCI lead to controversial results. A system capable of guiding OECs to the desired spot and controlling their migration after SCI could be useful to enhance regeneration results. Biomaterials like magnetic nanoparticles (MNPs) have been used to track labeled OECs by magnetic resonance imaging (MRI), *in vivo* (Lee et al., 2004). Superparamagnetic iron oxide MNPs permit the non-invasive monitoring of grafted cells *in vivo*. These non-toxic particles are

effectively endocytosed by cells and accumulate in their cytoplasmic endosomes (Bulte et al., 2001). In rats it was possible to determine, by this method, migration details of OECs in normal and injured spinal cord (Lee et al., 2004). Anyway, this technique can also be used to actively guide cells to a location (Dobson, 2008). In this study we want to observe if OECs labeled with MNPs can be guided by an external magnetic field *in vitro*. If so, this method could be used *in vivo* to control OECs delivery and migration towards specific zone in the spinal cord. Indeed, it has been demonstrated that mesenchymal stem cells (MSCs) labeled with MNP could be guided to the desired location by the application of a magnetic field, i*n vivo*. Specifically, when MNP-labeled MSCs were injected in the portal vein of rats, cells could be guided to the liver, with a consequent reduction of their migration to other organs (Vittorio et al., 2011). In another study, labeled MSC cells transplantation intrathecally via lumbar puncture was performed 1 week after SCI, in rats. MRI and histological analysis revealed significant differences in cell numbers and cell distribution close to the lesion site under magnetic field in comparison to control groups (Vanecek et al., 2012). Moreover it was observed that the targeting efficiency could be increased by using magnets that produce spatially modulated stray fields (Vanecek et al., 2012). These magnets with tunable geometric parameters, in the future, might provide the additional level of control needed to enhance the efficiency of cell delivery in SCI.

4. Cell migration

Embryogenesis, nerve growth, tissue repair and invasion are all biological processes that require cell attachment and migration. These processes require many temporally and spatially integrated components that must be precisely regulated. Despite the differences between of cell types, the major mechanisms and molecular basis of migration, whose component have been functionally conserved in evolution, occur in a similar way (Kurosaka and Kashina, 2008).

4.1 Migration mechanisms: an overview

Cell migration requires a series of repetitive integrated procedures to produce cell movements. The body must modify its shape and stiffness to interact with the surrounding tissue structures. First, the moving cell becomes polarized and elongates (Ridley et al., 2003). An active leading edge consisting of lamellipodia and filopodia is then formed (Yang et al., 2007). Following protrusion, small site of cell attachment to the extracellular matrix (ECM), known as focal contacts, are formed. These focal adhesion contacts link the ECM to the actin cytoskeleton through integrins and other molecular complexes (Kurosaka and Kashina, 2008). Subsequently, while cell contact at rear release, regions of the leading edge or the entire cell body contract generating a traction force that leads to the moving of the cell body (Kurosaka and Kashina, 2008) (Fig. 7).

4.2 Molecular basis of for migration

Initial propulsion and elongation of leading pseudopods are driven by actin polymerization and assembly to filaments. Polymerization is tightly regulated and actin is precisely assembled in a branched pattern to form lamellipodia. This process is controlled by actin-binding protein, among them actin-related protein 2/3 complex, actin depolarizing factor (ADF) and cofilin (Zebda et al., 2000). Phosphatases are really important in these processes (Larsen et al., 2003). In fact, to maintain polarized protrusion of a single lamellipodium for continuous migration ADF/cofilin family protein must be maintained in a non-phosphorylated active state (Larsen et al., 2003).

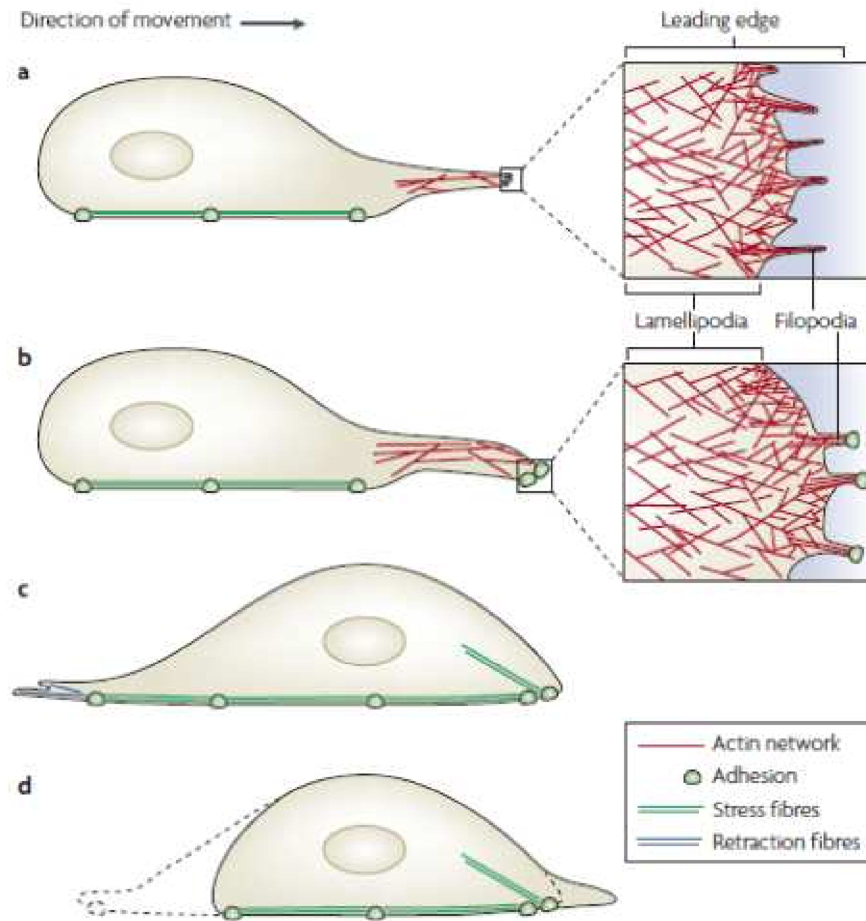

Figure 7: Cell migration is dependent on different actin filament structures. a) Motility is initiated by an actin-dependent protrusion of the leading edge, which is composed of lamellipodia and filopodia. These protrusive structures contain actin filaments, with elongating barbed ends orientated towards the plasma membrane. b) During cellular extension, new adhesions with the substratum are formed under the leading edge. c) Next, the nucleus and the cell body are translocated forward through actomyosin-based contraction forces that might be mediated by focal adhesion-linked stress fibers, which also mediate the attachment to the substratum. d) Then, retraction fibers pull the rear of the cell forward, adhesions at the rear of the cell disassemble and the trailing edge retracts. (from Mattila and Lappalainen, 2008)

Growing cell protrusions then touch the adjacent ECM and initiate binding via adhesion molecules, most notably transmembrane receptors of the integrin family. Integrins couple to the actin cytoskeleton via adaptor proteins, then become locally enriched, cluster and develop into an initial small focal complex that can grow and stabilize within minutes to form a focal contact (Kurosaka and Kashina, 2008).

Before and while focal contacts develop, actin filaments locally elongate and assemble, through the action of crosslinking proteins such as α-actinin, myosin II and others to give rise to branched actin networks below the inner leaflet of the plasma membrane termed cortical actin and cytoplasmic bundles designated as stress fibers (Larsen et al., 2003). These fibers assembly and contraction are predominantly induced by RhoA and its downstream effector, the Rho-associated serine/threonine kinase (ROCK). By contrast, the cortical actin network is regulated by the myosin light-chain kinase (MLCK) to allow the cell to separately control cortical actin dynamics from contractions in inner regions (Kurosaka and Kashina, 2008). Actomyosin contraction promotes the shortening of the cell's length axis and generates inward tension towards focal contacts that are located at outward edges. By several mechanisms, not completely understood, cell-substrate linkages resolve preferentially in the back of the cell, whereas the leading edge remains attached to the ECM and further elongates. Following focal contact disassembly in the rear, the cell body and nucleus slowly glide forward (Larsen et al., 2003; Kurosaka and Kashina, 2008).

The speed generated by the migration cycle is limited by the turnover rates of adhesion and de-adhesion events, yielding an inverse relationship between focal contact strength and migration rates. Stabilization of focal contacts increases attachment and impairs migration rates, whereas weakening of adhesion strength, to a certain degree, propels migration.

4.3 Cellular and molecular basis of OECs migration

Cell migration (described above) is attributed mainly to the activity of the leading edge exploring the environment, establishing adhesion, followed by movement of the cell body and retraction of the rear. It is described that, subpopulations of OECs present differences in their migratory properties (Huang et al., 2008). In fact, SC-OEC send out two processes during migration. One of them, the leading process, with a larger lamellipodia or higher dynamic filopodia, extends in the direction of cell migration. The other process, or tailing process, is found in the opposite direction and has smaller lamellipodia and less dynamic filopodia. As the leading process moves ahead the nucleus of OECs moves forward simultaneously, while the tailing process retracts (Huang et al., 2008). The AC-OEC show instead, as do not have leading and trailing

process, display one large lamellipodium extending forward with the soma following in the back. The cell in this case moves as a unit (Huang et al., 2008).

Surprisingly, it was observed that migrating OECs present also a highly motile lamellipodial protrusion along the cell body which increases the migration of peripheral OECs *in vitro* (Windus et al., 2007). These lamellipodial waves induce cell-cell adhesion and subsequently contact-stimulated migration. This is in accordance with the fact that OECs, *in vivo*, migrate in close contact with each other and olfactory axons. Therefore adhesion induced by lamellipodial waves may result in contact-stimulated migration (Windus et al., 2007). Specific inhibition of MAPK/extracellular signal-regulated kinase (MEK), which acts down-stream of GDNF as part of Ras/Raf/MEK/ERK pathway blocked peripheral waves activity while leaving the leading edge intact, suggesting that in OECs leading edge and peripheral lamellipodial waves are both involved in initiating cell-cell contact and promoting migration (Windus et al., 2007; Windus et al., 2010). The mechanisms that regulate the interaction and migration of different OECs subpopulations and how OECs morphology relates to cellular behavior still remain unknown.

At molecular level, only few factors have been described to functionally modulate OECs migration. The first one observed was GDNF (Cao et al., 2006). OECs express and secrete GDNF and also express the GDNF receptor. This factor acts through GFRα-1 and Ret receptor activating JNK and Src intracellular cascades, and at a cellular level stimulates the activity of peripheral lamellipodial waves resulting in an increased cell-cell contact and subsequently contact-dependent migration (Cao et al., 2006). Lysophosphatidic acid (LPA) is a lipid that influences diverse functions in the nervous system. When OECs were treated with LPA, their migration was enhanced and their actin cytoskeleton reorganized (Huang et al., 2008). This factor was shown to operate through the LPA receptor, leading to the inhibition of the Rho-GTPase and RhoA kinase activity and enhancing of phophorilation of ERK1/2 (Yamada et al., 2004; Huang et al., 2008). While contact between OECs stimulates migration, increased adhesion has the opposite effect. Nogo66 acts on the NgR-complex and has been demonstrated to increase adhesion of OECs to the extracellular matrix, significantly decreasing their migration rate (Su et al., 2007). Another factor capable of inhibiting OECs migration, via their Robo receptor, is the repulsive guidance cue Slit-2. Slit-2 also caused the collapse of leading edge via the activation of actin depolarizing cofilin in a

calcium dependent manner and reversal of migrating direction via activation of RhoA kinase activity (Huang et al., 2011). Recent *in vitro* experiments have revealed that tumor necrosis factor α (TNF-α) secreted by activated astrocytes stimulates OEC chemotaxis. Interestingly a linear expression gradient of TNF-α has been detected in the glial scar after spinal hemisection that could conceivably regulate the migration of transplanted OECs (Su et al., 2009). TNF-α level peaked at 7 days after injury and in studies that failed to show OEC migration, the timing or location of OEC transplantation may not have coincided with the TNF-α level required for a correct chemotaxis (Chuah et al., 2010).

4.4 Force involved in cellular migration

As described before, the first step of cell movement is protrusion of the leading edge. The basic active mechanism underlying this step is the polymerization of actin filaments towards the cell membrane. In fact, polymerizing actin filaments alone can generate significant propulsive force to move the cell leading edge (Fig. 8). While the leading edge of the cell protrudes, focal contacts are formed. The spatial distribution patterns of attachments and the adhesive force vary among cell types and are important factors that determine the protrusion rate and rate of translocation (Lee et al., 1994; Munevar et al., 2001). The adhesions near the cell leading edge are crucially required to convert some of the polymerization force into protrusion; the rest of it contributes to the flow of the actin network. This flow is modulated by retrograde rate, which can be explained by the existence of a molecular clutch (Mitchison and Kirschner, 1988; Jurado et al., 2005). The molecular clutch is thought to be composed of vinculin, talin and other adhesion complexes, and determines the extent to which the cytoskeleton and underlying substrate are linked and can interact (Ananthakrishnan and Ehrlicher, 2007).When the clutch is engaged, there is an increase in the rate of translocation and a decrease in the retrograde flow because the tight cytoskeleton-substrate linkage enables an effective transmission of the acto-myosin contractile forces to the substrate. This enables the cell to move forward by pushing against the substrate with traction forces (Ananthakrishnan and Ehrlicher, 2007). This hypothesis thus predicts that slow moving cells should have a high retrograde flow and generate less traction force, while in fast moving cells, a smaller retrograde flow and a larger traction force are expected. However, this is not completely true, as when the clutch is engaged and raking of the

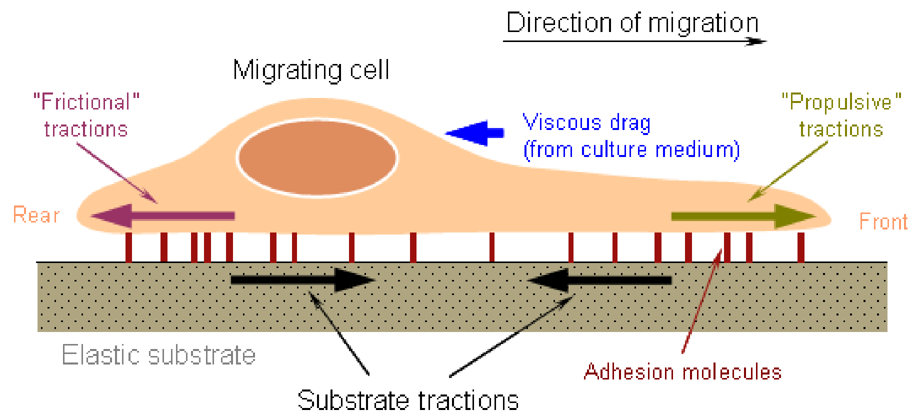

Figure 8: Scheme of a migrating cell and the tractions it generates on the substrate. The thick arrows are forces with which the cell acts upon the substrate (black) and the elastic substrate reaction forces that act upon the cell (green and purple). In case of the stuck cell presented here, the "propulsive" tractions act mainly on the cell front, while "frictional" tractions act at the cell rear. (from www.cellmigration.org)

cytoskeleton against the substrate occurs, the traction forces exerted on the substrate are large, but when the clutch is not engaged and retrograde flow is high, the traction forces are small. Since both phenomena (adhesion raking and clutch disengagement) occur simultaneously in the cell, their combined effects produce a non-linear relation between cell speed and adhesiveness or traction force, a result in agreement with experimental and computational studies on fibroblasts, neutrophils etc. (Zaman et al., 2005) . In conclusion, for rapid cell movement, the adhesion force needs to be optimum (which is different for each cell type) and retrograde flow minimum, while for slow movement, adhesion could be below or above optimum and retrograde flow should be high.

The final step of cell movement is translocation of the cell body and the rear of the cell. The “frictional” force required for this process is thought to be generated by the sliding of myosin motors on actin filaments in the cell body and rear. Since this actin filaments and bundles are connected to the cell membrane and the substrate, the force generated can be converted to a traction force that enables the cell to move forward (Ananthakrishnan and Ehrlicher, 2007).

A technique called traction force microscopy (TFM) has been developed to determine how much force is exerted by the cells onto an extracellular substrate. In TFM, cells are seeded on a flexible substrate and displacements of the substrate caused

by cell contraction are tracked. Using the physical characteristics appropriate to each substrate these displacements are converted to a traction stress field.

Some methods use continuous gels, comprised typically of either polyacrylamide (PAA) or polydimethylsiloxane (PDMS) embedded with fluorescent beads (0.2μm) to visualize the gel displacement field induced by cellular traction. The displacement of any bead on the substrate is coupled with the displacements of neighboring points. Thus, the force sensor is the entire surface of the substrate (Fig. 9 a,b). Computational routines are then utilized to convert the displacements of the gel surface to estimate the magnitude and direction of cell-induced traction stresses.

Other methods use substrates that consist of an array of discrete, isolated force sensors denominated nano-pillars (Fig. 9 c,d). These pillars are deflected by the cell and the magnitude of deflection, as well as the direction in which the cell moved can be recorded. Since adhesions are attached to discrete locations, the spatial resolution of the force measurement is well defined by the geometry of the sensor array. However, this geometry also puts geometrical constraints on cellular adhesions and introduces topographical cues that may alter native cellular physiology.

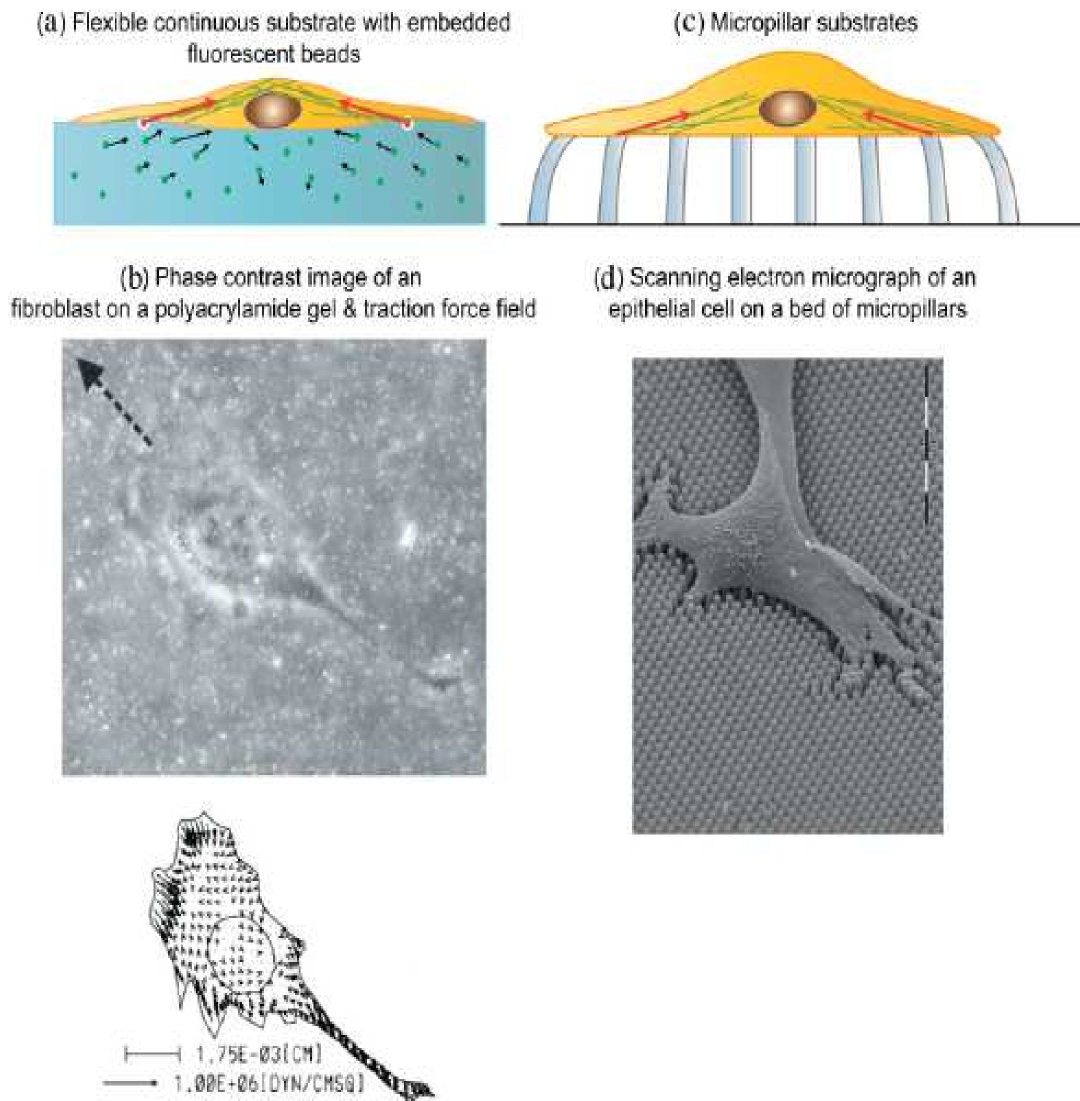

Figure 9: TFM techniques to measure cell-generated forces. (a), (b) Schematic representation of the TFM using fluorescent beads embedded into a PAA gel and an example of the substrate deformations and the resulting forces (b). (c), (d) Schematic representation of the TFM using the nano-pillar method and an example of a cell lying on a bed of nano-pillars.

OBJECTIVES

The failure of axonal regeneration after SCI is thought to be attributed at least in part to the overexpression of the inhibitory molecules such as MAIs (Schwab, 1990; Wang et al., 2002). Given their axon growth-promoting properties, natural or genetically modified OECs have been studied extensively and transplanted into the injured spinal cord to promote axonal regeneration after SCI (Ramon-Cueto and Nieto-Sampedro, 1994; Navarro et al., 1999; Tabakow et al., 2013). Several OEC subpopulations have been identified in the olfactory system on the basis of their topographical distribution, distinctive morphology, intracellular cytoskeletal distribution and antigenic or gene expression profiles (Au and Roskams, 2003; Vincent et al., 2005; Guerout et al., 2010; Windus et al., 2010). Indeed, two populations of OECs have been described *in vitro* and in some *in vivo* studies: SC-OECs, with fusiform bipolar form; and AC-OECs, with a more flatted structure (Franceschini and Barnett, 1996; Kumar et al., 2005). It has been suggested that SC-OECs have higher regenerative potential than AC-OECs (Franklin et al., 1996; Imaizumi et al., 1998). This property has been associated with the greater migratory potential of the former. However, different migratory properties have also been reported after transplantation in lesioned central CNS (Ramon-Cueto et al., 1998; Boyd et al., 2004). Hence, Lu et al. failed to find unique migratory properties of OECs when these cells were implanted into SCI (Lu et al., 2006). Moreover it has been reported that NogoA (one of the MAIs) enhances the adhesion of OECs affecting their migration (Su et al., 2007). Cell migration requires the spatial and temporal coordination of cell adhesion and protrusion with the generation of traction forces at the cell/ECM interface (Ananthakrishnan and Ehrlicher, 2007). These traction forces are mainly generated by the acto-myosin cytoskeleton and transmitted to the ECM through focal adhesions (FAs) (Ananthakrishnan and Ehrlicher, 2007).

Thus, in this thesis we wanted to **determine the migratory properties of OECs in presence of MAIs** using the cellular line TEG3 with the **final aim of improving their effectiveness after transplantation**. Therefore we designed some specific objectives:

Objective 1 Characterize the OEC cell line TEG3 and the expression of the molecular machinery required to transduce the inhibitory effects of MAIs.

Objective 2 Examine the effects of MAIs on TEG3s migration by using single cell tracking imaging on micropatterned substrate and on their traction forces

using TFM.

From the results of the previous objectives, in order to overcome the effects of MAIs over TEG3:

Objective 3 Investigate whether a particular type of MNP could be used to control the localization and migration of TEG3s.

Objective 4 Generate and characterize genetically modified TEG3s to express the NgR(Ecto) to overcome the inhibitory action of MAIs and therefore enhance the migratory properties of TEG3.

RESULTS

The results of the thesis will be presented as a compound of articles:

Chapter I: "**Myelin-associated proteins block the migration of olfactory ensheathing cells: an in vitro study using single-cell tracking and traction force microscopy**." Authors: **Nocentini S**[1], Reginensi D[1], Garcia S, Carulla P, Moreno-Flores MT, Wandosell F, Trepat X, Bribian A, del Río JA. Published in: *Cell Mol Life Sci. 2012 May;69(10):1689-703. doi: 10.1007/s00018-011-0893-1. Epub 2011 Dec 29. PMID: 22205212*

Chapter II: "**Generation of magnetized olfactory ensheathing cells for regenerative studies in the central and peripheral nervous tissue**." Authors: Riggio C[1], **Nocentini S**[1], Catalayud MP, Goya GF, Cuschieri A, Raffa V, Del Río JA. Published in: *Int J Mol Sci. 2013 May 24;14(6):10852-68. doi: 10.3390/*ijms140610852. *PMID: 23708092*

Chapter III:" **Enhanced migration of olfactory ensheathing cells genetically modified to express the NgR1(310) ectodomain over myelin**." Autors: Carulla P, **Nocentini S**, Reginensi D, Seira O, Torres-Espín A, Navarro X, Del Rio JA. in preparation for publication in *Cell Mol Life Sci.*

Chapter I

Myelin-associated proteins block the migration of olfactory ensheathing cells: an in vitro study using single-cell tracking and traction force microscopy.

Authors: **Nocentini Sara**[1], Reginensi Diego[1], Garcia Simon, Carulla Patricia, Moreno-Flores Maria Teresa, Wandosell Francisco, Trepat Xavier, Bribian Ana, del Río José Antonio. ([1]: Nocentini Sara and Reginensi Diego contributed equally to this study)

Published in: *Cellular and Molecular Life Sciences. 2012 May;69(10):1689-703.*

RESUMEN:

La implantación de OECs ha surgido como terapia prometedora para lesiones a la medula espinal. Pero las propiedades regenerativas de estas células parecen depender de la presencia de un sustrato permisivo para la migración celular (Gudino-Cabrera et al., 2000; Ramon-Cueto and Munoz-Quiles, 2011; Ekberg et al., 2012). Los datos de otros estudios demuestran que las OECs migran menores distancias una vez trasplantadas en la médula espinal lesionada respecto a medula espinal no lesionada (Gudino-Cabrera et al., 2000; Deng et al., 2006). Esto sugiere que los inhibidores asociados a mielina (IAMs), que se sobre-expresan después de una lesión (Schwab, 1990), podrían estar modulando la capacitad migración de las OECs. Por lo tanto, en nuestro estudio hemos examinado el efecto de los IAMs sobre la línea clonal de OECs de rata, TEG3. Hemos demostrado que estas células expresan todos los componentes del complejo receptor NgR y que este complejo es activo. De hecho, en respuesta a estímulos agudos de mielina se puede observar un aumento de activación de RhoA y de fosforilacion de ERK1-2 en las TEG3. En presencia de mielina como substrato la capacitad migratoria del las OECs es reducida. Esta diminución de capacitad migratoria córrela con una reducción en fuerza de tracción ejercida por la célula sobre el substrato, una reducción en número de contactos focales y con la redistribución del citoesqueleto de F-actina. Por último, observamos que la incubación de las TEG3s cultivadas sobre mielina, con el péptido NEP-40, que bloquea NgR1, puede restaurar parcialmente las propiedades enumeradas precedentemente.

Estos datos nos sugirieron que, para mejorar las propiedades migratorias del las OECs después de trasplante en lesión espinal, es necesaria una estrategia que tenga en cuenta los efectos inhibitorios de los IAMs sobre estas células.

ABSTRACT (English version):

OECs implantation has emerged as a promising therapy for SCI, but their regenerative properties seem to depend on the presence of a substrate permissive to cell migration (Gudino-Cabrera et al., 2000; Ramon-Cueto and Munoz-Quiles, 2011; Ekberg et al., 2012). But, studies demonstrate that when transplanted after SCI, the migratory properties of OECs are reduced compared to transplantation in not-injured spinal cords (Gudino-Cabrera et al., 2000; Deng et al., 2006). As after SCI, inhibitory molecules such as MAIs are overexpressed in the site of lesion (Schwab, 1990)we thought that this molecules could modulate OECs migration capacity.

In this study we used a rodent clonal OEC line, TEG3, and we demonstrated that these cells express all the components of the NgR-complex. This complex resulted to be active since TEG3s activated RhoA and increased ERK1-2 phosphorylation in response to acute stimuli of myelin. Indeed, myelin inhibited OECs migration over glass surfaces and also over linearly elastic PAA gels. Moreover using TFM, we quantitatively demonstrate that TEG3s largely decrease their traction stress over myelin. This decrease in traction force correlated with decreased focal contacts and redistribution the of F-actin cytoskeleton. In fact, OECs cultured on myelin largely reduced the number of protrusions, stress fibers and FAs. Finally we observed that the incubation of TEG3s with the NgR1 blocking peptide NEP1-40 partially overcame MAIs-mediated migratory inhibition, restoring the traction forces of these cells and their cytoskeletal organization.

Altogether these data suggested us that a cell-based strategy, using TEG3s that has the capacity to overcome the inhibitory action of MAIs, is required in other to enhance their migratory properties after SCI transplantation.

Complete reference: "**Myelin-associated proteins block the migration of olfactory ensheathing cells: an in vitro study using single-cell tracking and traction force microscopy**." Authors: **Nocentini S**[1], Reginensi D[1], Garcia S, Carulla P, Moreno-Flores MT, Wandosell F, Trepat X, Bribian A, del Río JA. Published in: *Cell Mol Life Sci. 2012 May;69(10):1689-703. doi: 10.1007/s00018-011-0893-1. Epub 2011 Dec 29. PMID: 22205212*

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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Myelin-associated proteins block the migration of olfactory ensheathing cells: an in vitro study using single-cell tracking and traction force microscopy

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Abstract Newly generated olfactory receptor axons grow from the peripheral to the central nervous system aided by olfactory ensheathing cells (OECs). Thus, OEC transplantation has emerged as a promising therapy for spinal cord injuries and for other neural diseases. However, these cells do not present a uniform population, but instead a functionally heterogeneous population that exhibits a variety of responses including adhesion, repulsion, and crossover during cell–cell and cell–matrix interactions. Some studies report that the migratory properties of OECs are compromised by inhibitory molecules and potentiated by chemical gradients. Here, we demonstrated that rodent OECs express all the components of the Nogo receptor complex and that their migration is blocked by myelin. Next, we used cell tracking and traction force microscopy to analyze OEC migration and its mechanical properties over myelin. Our data relate the decrease of traction force of OEC with lower migratory capacity over myelin, which correlates with changes in the F-actin cytoskeleton and focal adhesion distribution. Lastly, OEC traction force and migratory capacity is enhanced after cell incubation with the Nogo receptor inhibitor NEP1-40.

S. Nocentini and D. Reginensi contribute equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s00018-011-0893-1) contains supplementary material, which is available to authorized users.

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Introduction

The olfactory system in the adult nervous system is renewed throughout life with the help of olfactory ensheathing cells (OECs). The elongation of newly generated olfactory receptor axons between the peripheral and central nervous system may be largely attributed to the properties of OECs. They ensheath and guide the axons of olfactory neurons that extend from the olfactory epithelium to the olfactory bulb [1–3]. Given their axon growth-promoting properties, natural or genetically modified OECs have been studied extensively, and they have been transplanted into the injured spinal cord to promote axonal regeneration [4–12]. Thus, OEC transplantation has emerged as a promising therapy for spinal cord injuries and other neural diseases [13–17]. Some OEC subpopulations have been identified in the olfactory system on the basis of their topographical distribution, intracellular cytoskeletal distribution, regenerative properties, and antigenic or gene expression profiles [18–24]. In fact, from a morphological point of view, two populations of OECs have been described in vitro and in vivo: Schwann cell-like OECs (sOECs), with fusiform bipolar form; and astrocyte-like OECs (aOECs), with a more flattened structure [25, 26]. However, the morphology of cultured OECs is strongly dependent on the culture conditions [21, 27, 28]. They transform from a fusiform to a flat-sheet shape spontaneously [29], and both sOECs and aOECs are likely to represent different morphologies of this motile and mitotic cell (see [30] for example).

Although they are useful for spinal cord repair, different OECs migratory properties have been reported after transplantation in lesioned central nervous system (CNS) [31, 32]. Indeed, Lee et al. determined by magnetic resonance tracking that OECs showed limited migration in injured spinal cord [33]. This was also reported in other studies using different techniques (e.g., [12], [34–38] among others). These results suggest that migration of OECs is modulated by specific interactions with the inhibitory substrate [39]. Indeed, in the injured spinal cord, lesioned axons and transplanted cells are confronted with a changing environment with a huge variety of growth inhibitory molecules located in the meningo-glial scar and adjacent spinal cord regions [40, 41]. Among others, the role of myelin-associated inhibitors (MAIs): Nogo-A, the myelin-associated glycoprotein (MAG) and the oligodendrocyte-myelin glycoprotein (OMgp) have been extensively studied after spinal cord lesions [42, 43]. These molecules block axon regeneration by acting thorough a common receptor: the Nogo receptor complex. This membrane receptor is formed by the GPI-anchored protein NgR1 and three putative co-receptors (p75, TROY, and Lingo-1) [42, 43]. In addition, new ligands have recently been reported to bind to the Nogo receptor complex (e.g., leucine-rich, glioma-inactivated (LGI) gene product [44], the B lymphocyte stimulator (BLyS) [45], members of the fibroblast growth factor FGF1 and FGF2 [46] and sulfatide [47]). Moreover, new receptors for MAIs have also been described: gangliosides [48], integrins [49], an NgR1 isoform: the NgR2 [50], G protein-coupled receptor 50 (GPR50) [51] and PirB [52].

OEC migration in vitro is modulated by neurotrophins, such as the glial cell-derived neurotrophic factor (GDNF), or chemicals such as lysophosphatidic acid (LPA) [53–55]. In addition, other secreted molecules involved in cell and axon migration, such as Slit-2, negatively modulate OEC migration [56]. However, although further studies are needed, it seems that Slit-2 is not over-expressed after spinal cord lesion [57], in contrast to Slit-1 and Slit-3 [57], semaphorins [58], Netrin-1 [59], chondroitin sulphate proteoglycans [60] and MAIs [61]. OECs show changes in their intracellular signaling mechanisms (Cofilin phosphorylation, RhoK activity and changes in Ca^{2+} stores) after Slit-2 treatment [56] that are shown by other inhibitory molecules [41]. In fact, these observations raised the notion that OECs may respond to a wide range of molecules. For MAIs, it has been reported that Nogo-66 fusion proteins (GST-Nogo-66 and His-Nogo-66) and Nogo-A enhance the adhesion of OECs, thus decreasing their migration in vitro [62]. Moreover, OECs increased its migration after PI-PLC treatment (to remove GPI-anchored NgR1) and α-NgR antibody treatment in vitro; or when NgR1-mediated signaling in a spinal cord lesion model is neutralized by α-NgR antibody infusion [62]. Taken together, these results indicate that Nogo-A/NgR signaling impairs OECs migration in vitro as well as in vivo. However, as indicated above, lesioned regions of the spinal cord may contain myelin debris as well as other inhibitors for long periods (more than 2 months in rodents [63] and years in humans [64]). Thus, in the present study, we aimed to explore the behavior and the migratory properties of OECs in the presence of myelin extracts by using single-cell tracking imaging on micro-patterned substrates. On the other hand, directed cell migration requires the spatial and temporal coordination of cell adhesion and protrusion with the generation of traction forces at the cell/extra-cellular matrix (ECM) interface [65]. These traction forces are mainly generated by the acto-myosin cytoskeleton and transmitted to the ECM through focal adhesions (FAs) [66–68]. Thus, we

also examined the effects of myelin on rodent OEC migration by traction force microscopy (TFM). First, we found that OECs express all the molecular machinery (Nogo receptor complex) required to transduce the inhibitory effects of MAIs, and that the migratory properties of OECs were blocked by myelin. Moreover, using TFM, we quantitatively demonstrate that OECs strongly decrease their traction stress over myelin, a finding that correlates with decreased focal contacts and redistribution of the F-actin cytoskeleton. Finally, we show that incubation of OEC cultures with the Nogo receptor 1 (NgR1) blocking peptide NEP1-40 [69] partially overcomes myelin-mediated migratory inhibition, thereby restoring the traction forces of the cells that correlate with cytoskeletal re-organization and re-appearance of focal contacts.

Materials and methods

Antibodies and biochemical reagents

The following antibodies were used at a dilution of 1:1,000 for Western blotting and/or 1:500 for immunohistochemical staining, unless otherwise indicated. Lingo-1 and actin (dilution 1:10,000) were from Millipore (Billerica, MA, USA). S100β was purchased from Abcam (Cambridge, MA, USA) and TROY from R&D System (Minneapolis, MN, USA). GFAP was from DAKO (Glostrup, Denmark), p75 was from Promega (Madison, WI, USA). NgR1 was a gift from Prof. B.L. Tang (Singapore). Tubulin (1:5,000), vinculin (1:400), phalloidin-alexa594 and DAPI were purchased from Sigma (St. Louis, MO, USA). ERK phospho-threonine 202/phospho-tyrosine 204 (pERK1-2) was from Cell Signaling Technology (Beverly, MA, USA). Total ERK antibody was from Transduction Laboratories (Lexington, KY, USA). Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit immunoglobulins were purchased from Molecular Probes (Leiden, Netherlands). The goat anti-mouse horseradish peroxidase (HRP) and rabbit anti-goat-HRP secondary antibodies used in the Western blots were from DAKO. Goat anti-rabbit-HRP was supplied by Sigma. Green fluorescent FITC-beads (0.75 μm ∅) used to bind myelin were kindly provided by Dr. A. Homs and Prof. J. Samitier (IBEC, Barcelona). In addition, the Nogo-66 (1–40) antagonist peptide (NEP1-40) was purchased from Alpha Diagnostic International (San Antonio, TX, USA). Myelin was purified from adult Sprague–Dawley rat CNS, as described [70]. All animal procedures were performed in accordance with the guidelines established by the Spanish Ministry of Science and Technology and the European Community Council Directive 86/609 EEC.

TEG3 cultures

The immortalized clonal cell line TEG3, which contains the SV40 large T antigen stable transfectant of OEG primary cultures, was prepared from adult rat olfactory bulbs [71]. Cells were maintained in ME10: DMEM–F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% bovine calf serum (SAFC Biosciences, Lanexa, VA, USA), 20 μg/ml pituitary extract (Invitrogen), 2 μM forskolin (Sigma), 1% penicillin–streptomycin and 1% fungizone (Invitrogen). TEG3 cells between passages 4–8 were used for the experiments.

Western-blot techniques

Cultured TEG3 cells were collected with a scraper and homogenized on ice in a buffer containing 150 mM NaCl, 50 mM HEPES, 1 mM ethylene–glycol-tetraacetic acid, 10% glycerol, 1% Triton X-100, and 13 protease inhibitor cocktail (Roche, Basel, Switzerland). The lysate was clarified by centrifugation at 12,000 × g for 15 min, and the protein content of soluble fractions was determined using the Bio-Rad detergent-compatible assay (BCA) (Bio-Rad, Hercules, CA, USA). Cell extracts (20 μg) were boiled in Laemmli sample buffer at 100°C for 10 min, subjected to 8 or 15% SDS-PAGE, and electrotransferred to nitrocellulose membranes (Amersham Biosciences, United Kingdom). Extracts of mouse brain at postnatal day five were prepared and used as controls (see [70], for technical details). After transfer, membranes were incubated overnight with primary antibodies at 4°C. The following day, they were subsequently incubated with peroxidase-tagged secondary antibodies, and peroxidase activity was detected using the ECL-plus kit (Amersham Biosciences). Active RhoA was determined with a pull-down technique using the GST-Rhotekin-binding domain, as described previously [72], following the manufacturer's instructions (Rho Activation assay kit BK036, Tebu-Bio Barcelona, Spain).

In vitro experiments and immunocytochemical methods

Glass coverslips (12 mm ∅) were coated essentially as described [73]. Briefly, coverslips were precoated with poly-L-lysine 10 μg/ml dissolved in 0.1 M PBS (pH 7.3) and then washed. They were then incubated with myelin (0.01–0.02 μg/μl), heat-inactivated myelin (0.02 μg/μl heated at 96°C for 1 h) or 0.1 M PBS alone, and then allowed to dry. Next, the coverslips were washed and coated with laminin (2 μg/ml, dissolved in 0.1 M PBS) and washed again with 0.1 M PBS. TEG3 counted cells were seeded onto substrate-coated coverslips in ME10 medium. Some of the cells seeded on coverslips containing myelin were treated with 1 μM NEP1-40 by adding the peptide

directly to the culture media. Cells were cultured for 20 h and then the coverslips were fixed in 4% buffered paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1 M PBS, and blocked with 10% normal serum in 0.1 M PBS. Cells were sequentially incubated overnight with primary antibodies at 4°C and then with Alexa Fluor-tagged secondary antibodies for 1 h. Cells were rinsed in 0.1 M PBS, stained with 0.1 μM DAPI diluted in 0.1 M PBS for 10 min, rinsed in 0.1 M PBS, and mounted on Fluoromount™ (Vector Labs, Burlingame, CA, USA). Time course of RhoA activation and ERK1-2 phosphorylation in TEG3 cells after myelin treatment was carried out adding myelin (0.02 μg/μl) directly to the cellular media (ME10) at different times.

Adhesion stripe assays

Stripe assays were carried out as described previously [74–76]. Briefly, acid-washed coverslips were incubated overnight with poly-L-lysine (10 μg/ml; Sigma) at 37°C, and after were rinsed several times with water and then air-dried. They were then inverted onto a silicon matrix provided by Dr. J. Jung (Max Planck Institute, Tübingen, Germany). Myelin (0.02 μg/μl) or laminin (2 μg/ml; Sigma) were mixed in 0.1 M PBS and the stripes were performed. The first protein was injected into the matrix channels and incubated for 2 h at 37°C. A solution of fluorescent Alexa 594-conjugated bovine serum albumin (2% in laminin; Molecular Probes) was then injected into the channels. After 2 h at 37°C, the channels were rinsed four times by injecting 0.1 M PBS. TEG3 cells were placed on the stripes and cultured for 24 h as indicated above. Stripe-functionalized coverslips were then fixed with 4% buffered paraformaldehyde for 10 min before DAPI staining, mounted in mounted on Fluoromount™ and photo-documented in an Olympus BX61 fluorescence microscope.

Time-lapse analysis of TEG3 migration

Fluorodish cell culture dishes (World Precision Instruments, Sarasota, FL, USA) were coated with laminin or myelin as described above. We seeded 5×10^4 TEG3 cells on the coated dishes and 20–24 h later we performed the time-lapse analysis. To study cell migration, we transferred the culture dishes to an LCI system (Live Cell Instruments, Seoul, Korea) for 20 h. The multi-tracking analysis was performed with the ImageJ™ software using the plugin mTrackJ (Biomedical Imaging Group Rotterdam of the Erasmus MC-University Medical Center Rotterdam, Netherlands). Tracking was performed in an inverted Olympus microscope IX-71 (20× or 63× objectives) and the images (5 megapixels) were captured by an Olympus XC-50 camera (150 frames, one frame every 8 min). Cell tracking allows the analysis of the scrolling speed and position frame (Xt, Yt). The cell position for each frame (position pixel) was determined with Matlab™ (Math Works, Natick, MA, USA), which provides a 3D graphic (x–y–z, z = time) and the MSD. The same experiments were run over fluorodishes covered with ~70–100-μm-thick PAA gels prepared as previously described [77]. Briefly, to obtain a stiff gel of 12-kPa Young's modulus (PAA), 265 μl of an acrylamide/bis-acrylamide mixture (15% acrylamide and 6.5% bis-acrylamide, Bio-Rad) was dissolved in ultrapure water containing 0.4% of 0.2 μm diameter red fluorescent beads (Invitrogen), 0.5% ammonia persulfate and 0.05% TEMED (Bio-Rad). For multi-tracking, 3D plot, and MSD of the TEG3 migration, the mixture was added to the center of the dish, which was then coated and stored overnight at 4°C. Previous to this procedure, OEC adhesion analyses were performed using 12-, 1.4-, and 0.15-kPa PAA gels. The 12-kPa PAA gels were selected because OECs show greater adherence and migration in these gels.

Traction force measurement

Cell tractions were evaluated using constrained Fourier-transform traction microscopy (FTTM) [78]. Briefly, the displacement field was calculated by comparing fluorescent microbead images obtained during the experiment with a reference image taken at the end of the experiment after the trypsinization and the consequent detachment of OECs from the underlying substrate. The projected cell area was calculated with Matlab™, based on the manual tracing of the OEC contours determined by a phase contrast image obtained at the start of the experiment. A particle imaging velocimetry algorithm [79] was used to determine the deformation of the substrate caused by the traction forces.

Statistical analysis

Summary data are expressed as mean ± SEM (standard error of the mean) of at least three independent experiments. Means were compared by one-way ANOVA test. A value of $p \leq 0.05$ was considered statistically significant.

Results

TEG3 is a clonal OEC line that shows similar growth-promoting capacity to non-modified OECs [71]. In addition, this cell line shows all the types of morphology described for primary OECs in culture [11, 80]. In a first set of experiments, we characterized the TEG3 cell line in our culture conditions (Fig. 1). In these conditions, fusiform forms were predominant over flat-shaped ones (Fig. 1a–d).

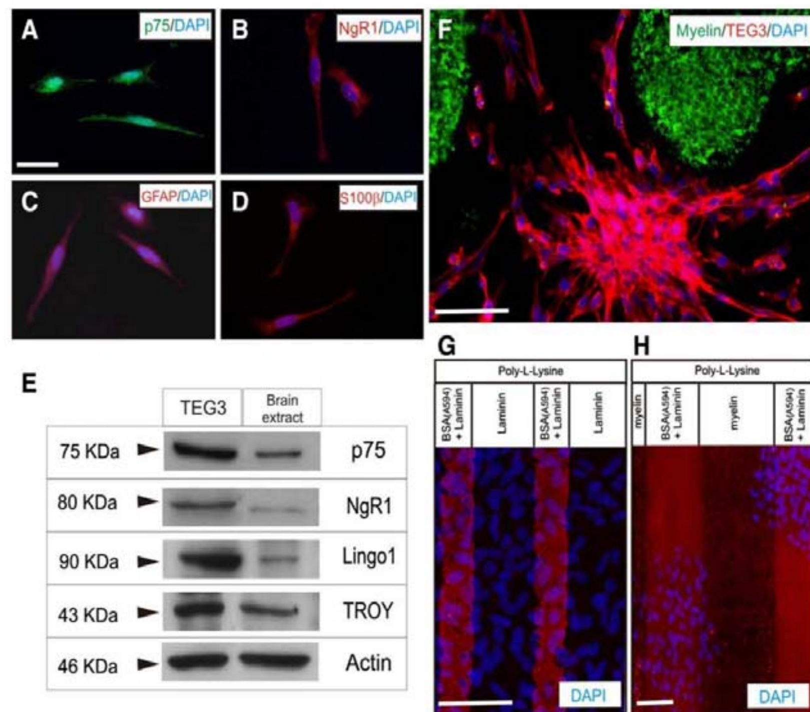
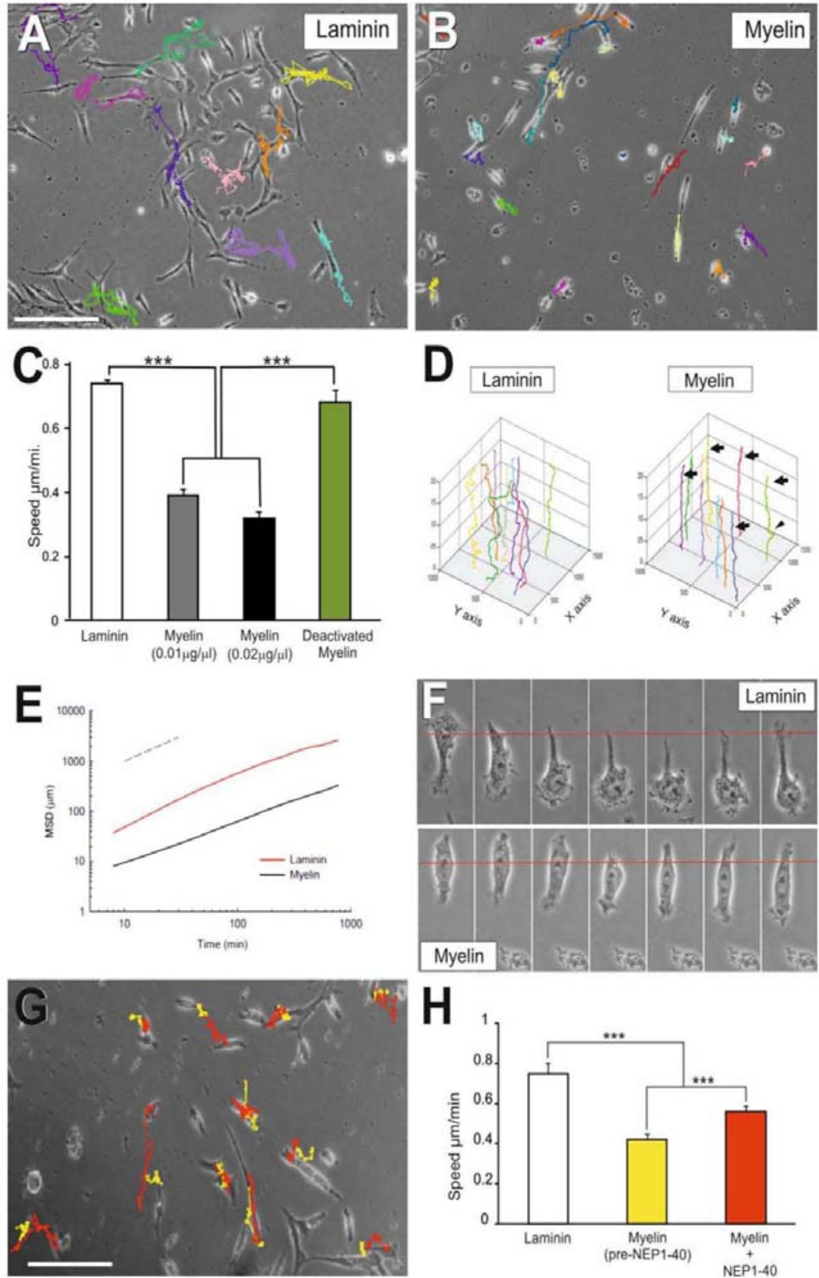


Fig. 1 Expression of Nogo receptor complex elements and effects of myelin in TEG3 cell adhesion. **a–d** Photomicrographs illustrating the expression of p75 (**a**), NgR1 (**b**), GFAP (**c**), and S100*β* (**d**) in cultured TEG3 cells. **e** Western blotting detected the expression of p75, NgR1, Lingo 1, and TROY in cultured TEG3 cells and brain extracts (see the section Materials and methods for details). Actin was blotted as internal control. **f** Photomicrographs illustrating an example of the distribution of TEG3 cells cultured on glass substrates containing spots of Alexa-tagged myelin. Note that TEG3 cells (Phalloidin-positive, *red*) are not localized on myelin containing regions (*green*). **g**, **h** Examples of stripe assays using TEG3 cells. In both cases, a coating of poly-L-lysine was uniformly distributed in all the culture area. Alternative stripes containing Alexa594-tagged BSA + laminin and laminin were generated (**g**) or Alexa594-tagged BSA + laminin and myelin (**h**). DAPI-stained TEG3 cells do not adhere in myelin-containing stripes (**h**) (see also Results). *Scale bars*: **a** = 25 μm pertains to **b–d**; **f** = 50 μm; **g** and **h** = 50 μm

Specific markers of OECs (p75, S100*β*, or GFAP) were present in our cultured cells (Fig. 1a–d). Next, using Western blotting, we examined whether TEG3 cells express members of the common receptor machinery for MAIs (Nogo receptor complex) [81–83]. Indeed, the revealed blots demonstrated that p75, NgR1, TROY, and Lingo1 were expressed by cultured TEG3 cells (Fig. 1e). This complex was functional since incubation with myelin increased activated RhoA as well as ERK1-2 phosphorylation (supplementary material Fig. S1). In a second set of experiments, we cultured these cells in 35-mm ∅ culture dishes patterned with dots of myelin tagged with 0.75 μm ∅ FITC beads. After Phalloidin-Alexa594 incubation and DAPI counterstaining, cells were exclusively located in regions not containing myelin-FITC (Fig. 1f). To avoid unspecific effects produced by FITC-tagged microbeads on OEC adhesion, we performed a stripe assay using brain myelin extract as substrate (Fig. 1f, h). DAPI-stained TEG3 cells were observed homogenously distributed in stripes containing poly-L-lysine and either laminin or BSA-Alexa594-laminin (Fig. 1g). In contrast, cells did not adhere to stripes containing poly-L-lysine/myelin compared to poly-L-lysine/BSA-Alexa594-laminin (Fig. 1h). Next, we performed a multiple cell migration analysis of TGE3 cells growing over poly-L-lysine + laminin- (Fig. 2a) or poly-L-lysine + myelin-coated substrates (Fig. 2b). Low-density TEG3 cultures were monitored for 20 h (20× objective; one frame every 8 min) in an inverted Olympus IX-71 microscope equipped with a cooled fluorescence camera and a cell culture incubation chamber (see the section Materials and methods for details). The migration speed of OECs grown on myelin-coated substrates



(0.01 µg/µl and 0.02 µg/µl) showed a decrease of 46.7% and 57.1%, respectively, compared to those on laminin-coated substrates (Fig. 2c) (0.77 ± 0.01 µm/min (laminin); 0.41 ± 0.01 µm/min (myelin; 0.01 µg/µl); 0.33 ± 0.01 µm/min (myelin; 0.02 µg/µl) (Fig. 2c). In contrast, cell migratory capacity was restored (up to 92.2%, 0.71 ± 0.04

◀**Fig. 2** Analysis of the migratory properties of TEG3 cells on coated glass substrates. **a**, **b** Examples of TEG3 cell migration on laminin (**a**) or myelin (**b**) coated glass substrates. Each cell trajectory is labeled with a different color line after the software analysis (ImageJ™). **c** Histogram showing the speed (*Y* axis) of cultured TEG3 cells on laminin, two different myelin concentrations and heat-denatured myelin. **d** Examples of three-axis representation of the trajectories of identified TEG3 cells in the focal plane (*x*,*y*) along time (*z*-axis, h). Note that identified TEG3 cells do not modify its (*x*,*y*) position over time (*arrows*) with few of them showing small displacements (*arrowhead*) compared to laminin. **e** Plot analysis of the MSD of TEG3 cells growing on laminin (*red*) or myelin (*black*). **f** Photomicrographs illustrating images of TEG3 cells behavior when cultured on laminin (*upper panels*) or myelin (*lower panels*). Each picture was obtained after 8 min. Note the small displacement and nuclear translocation of TEG3 cells on myelin compared to the clear nuclear translocation of parallel cultures on laminin. **g** Example of the NEP1-40 effects of individual TEG3 migration on myelin-coated glass substrate. The trajectory of selected TEG3 cells without treatment is labeled in *yellow*. *Red lines* show the changes observed after the incubation with NEP1-40 in the selected cells. **h** Histogram showing the results of the time lapse analysis and the effect of the NEP1-40 incubation. Data are represented by mean ± SEM of three different experiments and ten identified cells per experiment. *Scale bars*: **a** = 200 μm pertains to **b**; **g** = 200 μm. *Asterisks* in **c** and **h** indicate statistical differences ($p < 0.05$, ANOVA test)

of the laminin migration value) by culture cells in heat-denatured myelin-coated substrate (Fig. 2c, green bar). A three-axis plot [*x*/*y* position vs. time (h)] revealed higher motile persistence on laminin-coated than on myelin-coated substrate (Fig. 2d).

To systematically analyze cell migration, we computed the mean square displacement (MSD) of cell trajectories. We then fitted the MSD to a power law expression: $MSD = D \times \Delta t^{\beta}$, where D is a scaling parameter and β indicates the degree of persistence. When $\beta = 1$ the motion is random, when $\beta < 1$ the motion is subdiffusive (anti-persistent), and when $\beta > 1$ the motion is superdiffusive (persistent) [84]. For each time interval studied, cells on laminin-coated substrates migrated further than those on myelin-coated substrates (Fig. 2e). To assess the degree of persistence as a function of the time interval, we calculated the local value of β as: $\beta = \partial \log(MSD)/\partial \log(\Delta t)$ [84]. Cells growing on myelin-coated substrates exhibited a slightly subdiffuse behavior regardless of the time interval studied. In contrast, those on laminin-coated substrates moved in a slightly superdiffusive manner at short time intervals but their trajectories became progressively sub-diffusive with increasing time (Fig. 2e). Taken together, these data indicate that while cells seeded on laminin-coated substrates were more motile than those on myelin-coated, they oscillated around their initial position over long intervals (Fig. 2d).

High-power microscopic observation of cells growing on laminin and myelin showed the specific details of their migration (Fig. 2f). Cells growing on laminin-coated substrate migrated in a similar manner to a fibroblast-like cell with dynamic lamellipodia and further nuclear translocation to the cell leading edge, leaving a tail process (Fig. 2f, upper panels). In fact, the cells translocated the nuclei between caudal to rostral cell locations following the largest lamellipodia. However, cells growing on myelin displayed decreased lamellipodia dynamics and motile small lamellipodia were observed in several cell domains (Fig. 2f, lower panels). This finding contrasts with the behavior of cells growing on laminin, where two main lamellipodia were located on the opposite sides of the fusiform cell, with few transient lamellipodia that displaced laterally along the cell (see also Fig. 3d, e).

We next analyzed the migration distance of identified cells growing on myelin in the absence or presence of the NgR1 inhibitor peptide NEP1-40, to block MAIs effects (Fig. 2g, h). Non-treated cells migrated shorter distances, showing a decrease of 48% compared to those on laminin-coated substrates (0.39 ± 0.03 vs. 0.75 ± 0.02 μm/min, respectively). These distances increased by around 33% after the addition of the NgR1 blocking peptide (0.52 ± 0.03 μm/min) (Fig. 2h).

Cells generate traction forces against their substrate during adhesion and migration, and traction forces are used, in part, by cells to sense the substrate. Thus, using TFM, we aimed to determine the distribution of traction forces in OECs cultured on a well-characterized poly-acrylamide (PAA) gel, which is linearly elastic, optically clear, and amenable to protein conjugation [85] (Fig. 3). First we cultured OECs in PAA gels containing laminin (2 μg/ml) or myelin (0.02 μg/μl) and performed time-lapse analysis as above for 10 h (Fig. 3a, b). TEG3 cells growing on PAA coated with laminin or myelin displayed slightly higher migration speeds compared to those cultured on coated glass culture plates (Fig. 3c). Cultured cells over PAA containing laminin or myelin showed similar morphologies than those cultured on glass (Fig. 3d, e). MSD analysis indicates that cells on laminin-coated substrates (glass or PAA) showed similar motion properties being more motile than those on myelin-coated substrates (Fig. 3f, g).

Using TFM, we observed that TEG3 cells seeded on laminin-coated substrates transferred higher strain energy (0.037 ± 0.0061 pJ) to their underlying substrate than those seeded on myelin-coated substrates (0.0033 ± 0.0007 pJ) (Fig. 4). Treatment with NEP1-40 induced a significant increase (2.75-fold increase) in strain energy (0.0091 ± 0.0016 pJ) (Fig. 4b). These findings suggest that increased traction force generation provides OECs with greater migratory capacity. To study the mechanisms underlying changes in traction force generation in laminin versus myelin substrates as well as the effect of Nogo receptor complex blockage by NEP1-40, we examined the

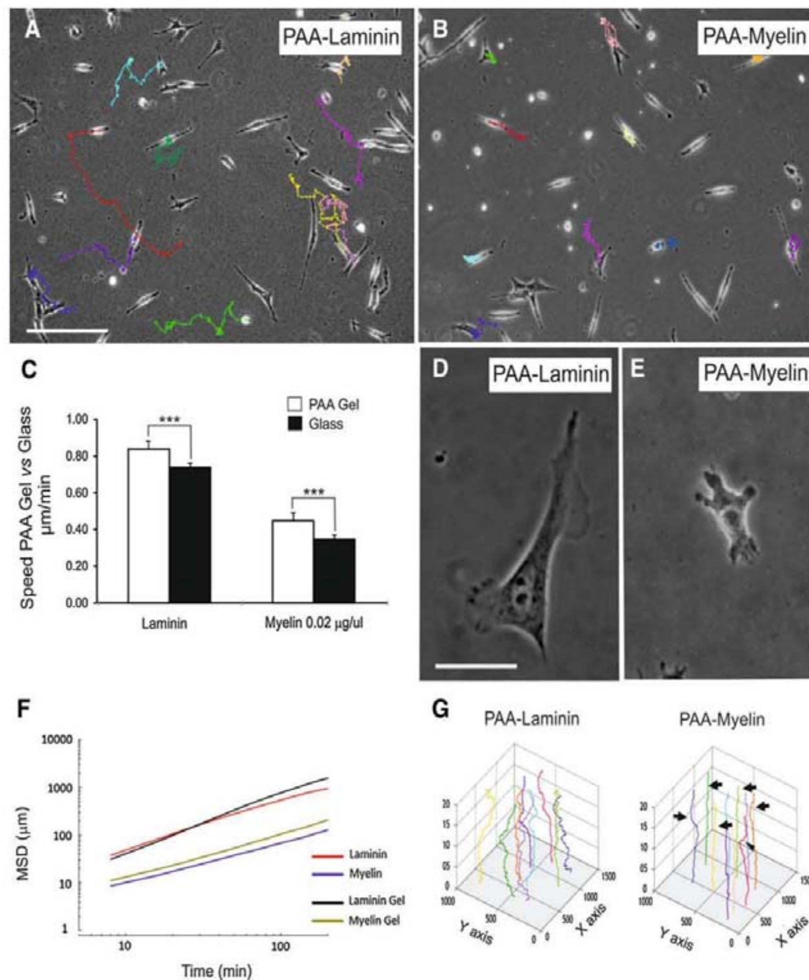


Fig. 3 Analysis of the migratory properties of TEG3 cells on coated PAA gels. **a**, **b** Examples of TEG3 cell migration on PAA-laminin (**a**) or PAA-Myelin (**b**) gels. Each cell trajectory is labeled with a different color line after the software analysis (ImageJ™). **c** Histograms illustrating the quantitative results of the migration assays. Notice that TEG3 cells, irrespective of the coating (laminin or myelin), showed increased speed on PAA gels than glass substrates. **d**, **e** High-power photomicrographs illustrating examples of TEG3 cells growing on PAA-laminin (**d**) and PAA-myelin (**e**). Notice the strong reduction of cell protrusions over PAA-myelin gels. **f** MSD plot of TEG3 cells growing on PAA (laminin and myelin gel, *black* and *green lines*) or in glass-coated substrates (laminin and myelin, *red* and *blue lines*). See Results for details. **g** Three-axis plots illustrating the migratory trajectories of cultured TEG3 cells in PAA-laminin and PAA-myelin. Notice the decreased number of trajectory changes of identified TEG3 cells on PAA-myelin (*arrows*). Plot scales are of Fig. 2. *Scale bars*: **a** = 200 µm; **d** = 20 µm. Data in (**c**) is represented as mean ± SEM of a three different experiments. *Asterisks* in **c** indicate statistical differences ($p < 0.05$, ANOVA test)

intracellular distribution of F-actin (Fig. 5a, b) and FAs (vinculin-positive) (Fig. 5c–f). Results indicate the negligible presence of Phalloidin-Alexa594-positive stress fibers (Fig. 5b) and FAs (Fig. 5e) in TEG3 cells cultured on myelin substrates in contrast to laminin (Fig. 5a (F-actin) and Fig. 5c, d (FAs)). In addition, the distribution of FAs in cells incubated with NEP1-40 was partially recovered as determined after vinculin labeling (Fig. 5f).

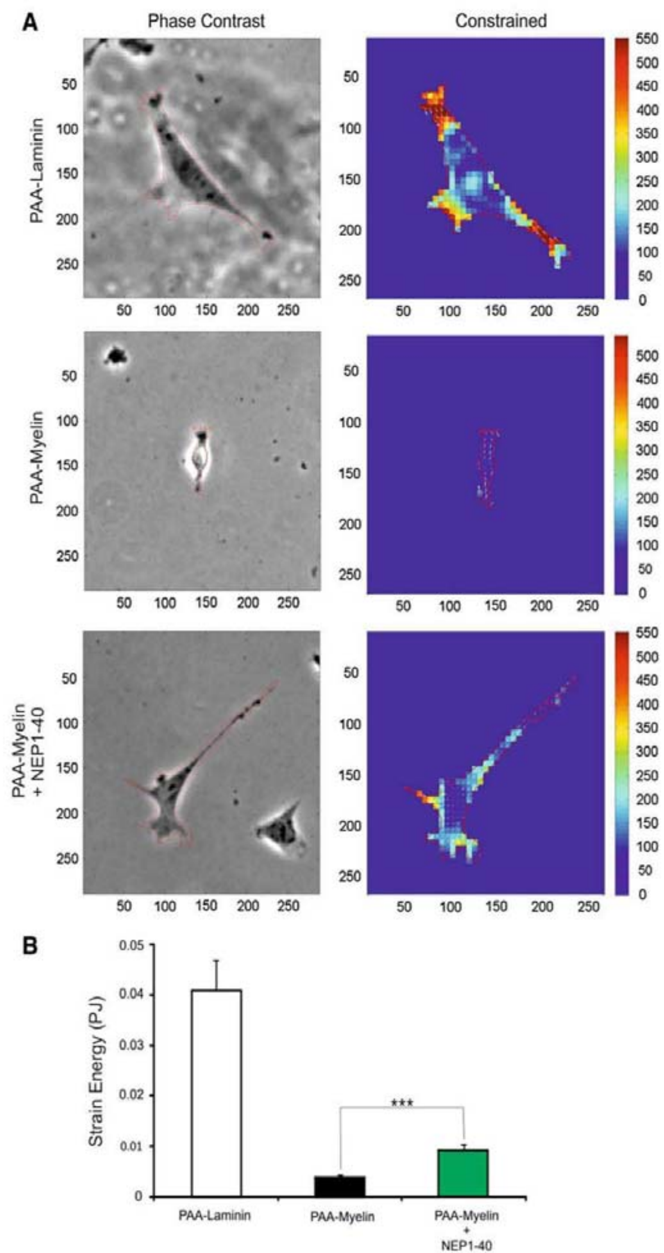


Fig. 4 TFM analysis of cultured TEG3 cells. **a** Quantitation of cellular traction forces of cultured TEG3 cells in PAA-laminin, PAA-myelin, and PAA-myelin + NEP1-40. A phase-contrast image of examples of cultured cells is shown on the *left side*. The scale in µm is also displayed in the lower and the *left side* of each image. In addition, the force map of cultured cells is shown on the *right* column. *Arrows* indicate the direction of the bead displacement. The color scale indicates the magnitude of the cellular traction forces (measured in Pa). **b** Histogram illustrating the quantitative results of the TFM analysis. Data in **b** is represented as mean ± SEM of the TFM analysis of 61 cells (PAA-laminin), 83 cells (PAA-myelin) and 62 cells (PAA-myelin + NEP1-40). *Asterisks* in **b** indicate statistical differences ($p < 0.05$, ANOVA test)

Discussion

As a result of their intrinsic properties, OECs and genetically modified OECs have been extensively transplanted into the injured spinal cord to promote axonal regeneration [4–11]. However, several studies failed to find unique migratory properties of OECs when implanted into damaged brain or spinal cord lesion (see Introduction for

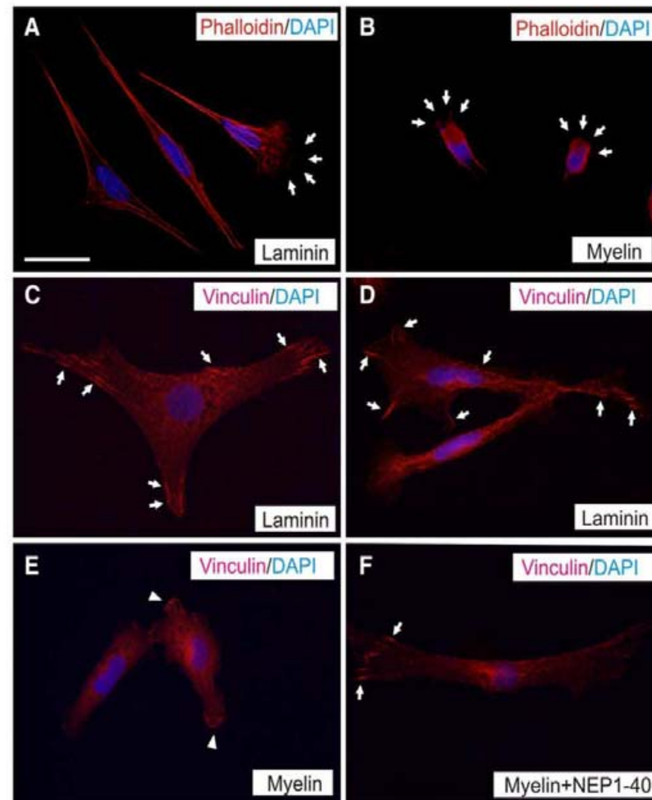


Fig. 5 Changes in stress fibers and FA distribution in cultured TEG3 cells. **a, b** Photomicrographs illustrating the distribution of F-actin in cultured TEG3 cells growing on laminin (**a**) or myelin (**b**). Stress fibers are clearly identified in **a** and poorly defined in **b**. *Arrows* in **a** and **b** point to cellular protrusions. **c–f** Distribution of FA (*arrows*) in cultured TEG3 cells on laminin (**c**, **d**), myelin (**e**), and myelin + NEP1-40. Notice the loss of FA and the diffuse vinculin-staining in TEG3 cells cultured over myelin (*arrowheads* in **e**) and the recovery of the FA (vinculin-positive) after incubation with NEP1-40 (*arrows* in **f**). *Scale bars*: **a** = 20 μm pertains to **b–f**

references). Although several authors examined OEC interaction with growing axons [86, 87], a number of parallel studies began to analyze the intrinsic migratory properties of OECs in order to improve their effectiveness after transplantation. Thus, research into OEC migration and proliferation has recently been performed on over glass surfaces with artificially generated gradients [53, 54, 56, 88], in collagen scaffolds [89, 90], in nanofibers [91], and in biomaterial-coated substrates [92, 93]. It has been demonstrated that TROY [94] and NgR1 are expressed in OECs [62, 95]. However, the present study is the first to report that cultured rodent OECs express all the elements of the Nogo receptor complex. In addition, we demonstrate that this receptor complex is active since OECs activate RhoK and increase ERK1-2 phosphorylation in response to myelin. Indeed, myelin inhibits OEC migration over glass surfaces but also in linearly elastic PAA gels.

From a biophysical point of view, our MSD data analysis indicates that cultured OECs migrate in an anti-persistent (subdiffusive) manner both over laminin and myelin on glass and PAA. In addition, our results may explain, to some extent, the findings of Gudiño-Cabrera and coworkers, who reported that transplanted OECs migrate preferentially in the opposite direction to the regenerating axon target and thus fail to promote axon regeneration [31]. Furthermore, OECs transplanted into the lesioned spinal cord must be placed on both sides of the lesion and, close to it, in order to be effective (e.g., [35]). On the basis of our MSD data, we propose that additional factors are required to generate a persistent and directed movement of OECs in vivo [96], as reported in vitro [29]. Indeed, a study by Su et al. indicates that TNF-α released by reactive astrocytes in the meningo-glial scar attracts OECs [97]. This result may explain why OECs invade the lesion cavity containing reactive glia when transplanted at shorter distances [35]. In fact, OECs interact and intermingle with astroglial cells in contrast to other cell types (e.g., Schwann cells) [98]. Moreover, OECs transplanted into the lesion cavity only migrate over short distances in adjacent regions [12]. Indeed, these surrounding areas are

rich in myelin debris as well as other inhibitory molecules (e.g., [41]).

OECs show lower migratory potential in lesioned spinal cord than controls [99]. In this regard, anti-NgR-treated OECs show greater migration in spinal cord in white matter tracts than controls [62]. In addition, in the same study, OECs cultured on Nogo-66- or Nogo-His-coated coverslips showed increased adhesion and reduced migration as a result of enhanced formation of paxillin-positive FAs and increased cell spreading, which was overcome by incubation with Y-27632 (a RhoK inhibitor) [62]. In our study, OECs cultured on myelin showed decreased spreading and fewer cell protrusions, stress fibers and FAs (vinculin-positive), and these effects were partially reversed by the NEP1-40 peptide. These data also paralleled those obtained from PAA gels. The differences in cell spreading between our data and those reported by Su and coworkers could be attributed to their use of a recombinant peptide or Nogo-A protein instead of complete myelin extract (containing several inhibitory proteins and lipids; see Introduction for details). The studies show different cell spreading of OECs growing over myelin or Nogo-66 peptides. Although the cell spreading of OECs cultured over Nogo-A is not illustrated by Su et al. we cannot completely rule out that the cell spreading mechanism in OECs could be different from that of other cell types. A study by Oertle et al. reported that Nogo-66, Nogo-C, and Nogo-66 peptide 4 (shown to be the inhibitory regions of Nogo-66 by GrandPre and coworkers [100]) are not inhibitory for fibroblasts spreading in culture (see Fig. 2 of [101]). In fact, two Nogo-A regions (59–172 aa and 544–725 aa) showed the increased inhibition of cell spreading in cultured fibroblasts. Thus, we conclude that specific Nogo-A domains may have a different effect on OECs spreading in a balanced way. On the other hand, in the same study, the authors report that higher concentrations of the cell-spreading inhibitory peptides became anti-adhesive. It has been reported that cell adhesion and migration showed a biphasic distribution and an optimal balance between adhesion and migration must be achieved by cells before they can migrate [102]. Indeed, if adhesion is lower or higher than an optimal level, migration may be decreased.

On the other hand, molecules other than Nogo-A may interact with Nogo receptor complex (e.g., OMgp, MAG, sulfatide) as well as with other undetermined MAIs receptors expressed by cultured OECs. Although further studies for OECs are needed, it has been described that the N-terminal domain inhibits COS-7 or HUVEC adhesion by acting through $\alpha V\beta 3$, $\alpha 5$ and $\alpha 4$ integrins, leading to decreased activity of focal adhesion kinase (FAK) in treated cells [49]. Our data showing a moderate recovery of the migratory properties and strain energy (see below) of TEG3 after NEP1-40 incubation suggest the presence of parallel inhibitory mechanisms.

Cell migration relies on the interplay between two cycles, one involving extension and contraction of its cytoskelekon, and the other involving formation and retraction of its adhesions. Traction forces come into play in the synchronization of both cycles by causing forced de-adhesion at the trailing edge and by driving the resulting forward motion of the cell body. Therefore, the balance between traction force and adhesion strength can be understood as a direct indicator of cell migration capacity. Using TFM and single-cell tracking, we showed that even if OECs cells on laminin exhibit increased concentration of focal adhesion proteins, the increased traction force is sufficient to tilt the mechanical balance toward a pro-migratory phenotype. The contribution of FAs to cell migration has been widely debated. Su et al. (2007) reported that OECs growing on Nogo-66 resembled flat-sheet cells. This observation could be attributed to a maintenance role of FA with little migration potential, as suggested for flat-shaped OECs. However, although we cannot rule out cell-specific factors for OECs, FA distribution and FA-mediated signaling in several cell types correlates with traction force parameters, nuclear translocation and cell migration (e.g., keratinocytes [103], invasive tumor cells [104], or fibroblasts [105, 106]).

In addition, our data show that OECs cultured on PAA substrates, the mechanical properties of which clearly differ from substrates coated on glass slides, increase their migratory potential. In this regard, recent data report increased proliferation and migration of OECs growing on several scaffolds (see above). Thus, it is feasible that a nanostructured scaffold would serve as a mechanical guide for OEC migration, thereby preventing the interaction of these cells with a putative inhibitory ECM. In this respect, migrating cells sense and respond to external stimuli. In fact, mechanical inputs can also be powerful regulators of cell behavior [103]. In physiological conditions, the migration of most cells occurs in an adhesion-dependent manner and involves the formation of FA at the membrane and the generation of mechanical forces via the actin-myosin network [103]. MAIs and receptors modulate the establishment of this network as well as microtubule dynamics [81, 107]. Indeed, MAI-induced arrest of CNS axon growth is either induced after MAI receptor, the Ca^{2+}-dependent activation of the epidermal growth factor receptor (EGFr), or induced by sequential RhoA/RhoK/LIM-kinase/cofilin phosphorylation, leading to actin depolymerization [108]. In parallel, microtubule stabilization is also compromised after exposure to MAIs [109]. Our results reinforce the notion that molecules present in myelin extracts act on Nogo receptor complex as well as on other receptors, and may modulate OEC cytoskeleton and

FAs distribution. In conclusion, our data suggest that a cell-based strategy using OECs to overcome the inhibitory action of myelin is required in order to enhance the migratory properties and the persistence of OECs in axon re-growth and their functional recovery in the lesioned CNS.

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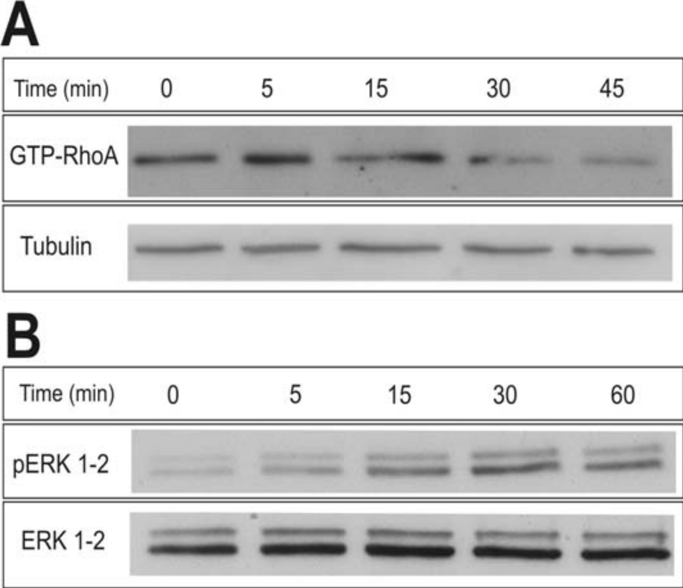

Suppl. Figure S1

Supplementary Figure S1: Myelin treatment activates RhoA and induces ERK1-2 phosphorylation in TEG3 cells. Time course of RhoA activation (**a**) and ERK1-2 phosphorylation (**b**) in TEG3 cells after myelin treatment. See the sections Materials and methods and Results for details.

Chapter II

Generation of magnetized olfactory ensheathing cells for regenerative studies in the central and peripheral nervous tissue.

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RESUMEN:

Dentro del marco de la búsqueda de posibles estrategias terapéuticas dirigidas al manejo de las lesiones de médula espinal, las OECs llamaron la atención de los científicos por su habilidad de envolver grupos de axones de neuronas sensoriales olfatorias y al servir como ayuda para la dirección de éstos en su trayecto desde el sistema nervioso periférico hacia el sistema nervioso central (Doucette et al., 1983). Después de trasplante en medulas lesionadas los resultados funcionales y anatómicos que han sido reportados demuestran una alta inconsistencia. Una de la causas de esta falta de coherencia es el hecho de que la OECs no representan una populación homogénea sino una populación funcionalmente heterogénea con células que exhiben diferente capacidad migratoria(Huang et al., 2008)

En este trabajo, hemos querido observar si las nanoparticulas magnéticas (NPMs) podrían ser utilizadas como un sistema para guiar y controlar las propiedades migratorias de las OECs. Pudimos observar que, *in vitro*, las TEG3s magnetizadas no exhiben respuestas celulares asociadas al estrés ni alteraciones a nivel de citoesqueleto. Además, su migración puede ser modulada por campos magnéticos. Para comprobar la posible capacitad de integrarse en un modelo más similar a una situación *in vivo*, implantamos estas células en un cultivo organotípico de médula espinal y nervio periférico. La células se integraron bien en este modelo y integrarse en el cultivo y algunas de ellas se encontraban en estrecha relación con axones en crecimiento.

En conjunto, estos dados indican que las OECs marcadas con NPMs podrían tener un potencial terapéutico para lesiones medulares.

ABSTRACT (English version):

OECs are a unique kind of glia found only in the olfactory system that supports olfactory neurogenesis and the re-targeting across the PNS:CNS boundary in the olfactory system (Doucette et al., 1983). Because they are also relatively accessible in an adult rodent or human, OECs have become a prime candidate for cell-mediated repair following SCI. Anyway high inconsistency in functional or anatomical outcome has been reported after OECs cells transplantation. One of the cause of this inconsistency is the fact that OECs do not represent a single homogeneous entity, but, instead, a functionally heterogeneous population that exhibits a variety of responses, including different migratory capacity.

In this paper, we wanted to observe if MNPs could be used as a system to guide OECs in their migration. We confirmed that, *in vitro,* magnetized TEG3s can survive well without exhibiting stress-associated cellular responses. Moreover, their migration can be modulated by magnetic fields. Their transplantation in organotypic culture of spinal cord and peripheral nerve, a model more similar to an *in vivo* situation, showed positive integration in the model.

Altogether, these findings indicate that OECs labeled with MNP could have a therapeutic potential for SCI.

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Article

Generation of Magnetized Olfactory Ensheathing Cells for Regenerative Studies in the Central and Peripheral Nervous Tissue

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Abstract: As olfactory receptor axons grow from the peripheral to the central nervous system (CNS) aided by olfactory ensheathing cells (OECs), the transplantation of OECs has been suggested as a plausible therapy for spinal cord lesions. The problem with this hypothesis is that OECs do not represent a single homogeneous entity, but, instead, a functionally heterogeneous population that exhibits a variety of responses, including adhesion and repulsion during cell-matrix interactions. Some studies report that the migratory properties of OECs are compromised by inhibitory molecules and potentiated by chemical gradients. In this paper, we report a system based on modified OECs carrying magnetic nanoparticles as a proof of concept experiment enabling specific studies aimed at

displacement of cells toward the more intensive magnetic fields was observed, and results suggested that cell displacement preferentially occurs during cell duplications [31,32]. In another study, mesenchymal stem cells (MSCs) were cultured in a CNT containing medium and, as a result of cell interaction with CNTs, the application of a magnetic field enabled shepherding of MSCs to the desired location *in vivo*. Specifically, when CNT labelled MSCs were injected in the portal vein of rats, we were able to significantly increase the localization of MSCs within the liver, with a consequent reduction of their migration to other organs [33]. More recently, we synthesized iron oxide MNPs with high saturation magnetization and tested them for cellular actuation of a neuroblastoma cell line through external magnetic forces. Results from scanning electron microscopy (SEM) and dual-beam SEM/focused ion beam (FIB) microscopy demonstrated the effective MNP internalization in large amounts. Since the magnetic force exerted on a single cell is proportional to the magnetic moment of the incorporated MNPs for a given magnetic field value (and its spatial derivative), this MNP internalization resulted in a high capability to induce cell migration under the effect of the magnetic source [34].

In an *ex vivo* model involving organotypic culture, the present study explored the utility of magnetizing OECs by labelling them with homemade MNPs, as this would enable precise shepherding of the transplanted cells to a nerve lesion. The results indicate that the labelling does not harm the OECs, and that migration of the magnetized OECs can be directed precisely by a magnetic field. Lastly, magnetized OECs can be transplanted in organotypic slices of spinal cord and peripheral nerve. In these organotypic co-cultures, magnetized OECs survive and are able to integrate within the tissue. Altogether, the method described represents a new approach for controlling the migration of the OECs *in vitro* and appears useful for future studies aimed at effective regeneration of spinal cord injuries.

2. Results

2.1. Magnetization of OECs via MNPs

OECs were cultured with MNPs (M-OECs) for 24 h and analyzed by light microscopy (Figure 1a,b) and electron microscopy (Figure 1c,d). Few particles could be detected on the cell surface, whereas clusters of particles were found inside cell cytoplasm. EDS (energy-dispersive X-ray spectroscopy) spectra clearly showed that these agglomerates are composed of iron, reflecting the intracellular presence of the MNPs (Figure 1e,f) and confirming the efficient labelling process of the OECs.

2.2. M-OECs Viability

Cell viability of M-OECs was tested in both a time- and dose-dependent manner by using propidium iodide (PI) dye exclusion assay. Flow cytometer analysis showed that the treatment induces a negligible toxicity after 72 h of treatment with all the concentrations of MNPs tested. In particular, at the highest concentration tested (25 μg/mL), the viability was found ~94.25% ± 3.12%, and not far from the control (~97.02% ± 5.05%) (Figure 2f). These results were confirmed by PI staining via fluorescent microscopy (Figure 2a–e); cells treated with MNPs exhibited permeability to PI similar to the control, except for 25 μg/mL, where few red spots are noticeable. Immunoblot analysis was performed to document any differences in the expression of proteins involved in cell replication (AKT,

exploring the potential of OECs in the treatment of spinal cord injuries. Our studies have confirmed that magnetized OECs (i) survive well without exhibiting stress-associated cellular responses; (ii) *in vitro*, their migration can be modulated by magnetic fields; and (iii) their transplantation in organotypic slices of spinal cord and peripheral nerve showed positive integration in the model. Altogether, these findings indicate the therapeutic potential of magnetized OECs for CNS injuries.

Keywords: nerve regeneration; olfactory ensheathing cell; magnetic nanoparticle; organotypic culture

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1. Introduction

The elongation of newly generated olfactory receptor axons in the adult from the olfactory mucosa towards the central nervous system (CNS) is attributed to the supporting properties of olfactory ensheating cells (OECs), which ensheath and guide these axons [1,2]. Given their axon growth-promoting properties, OECs (natural or modified) have been transplanted into the injured spinal cord to promote axonal regeneration and functional recovery [3–8]. Indeed, OEC transplantation has emerged as a promising therapy for spinal cord injuries [9,10]. It has been suggested that OECs provide a higher regenerative potential than Schwann cells [11–13]. This property has been attributed to the greater migratory potential of OECs [14]. However, different migratory properties have been reported after OEC transplantation in injured CNS [15,16]. For example, Lu *et al.* did not observe special migratory properties following their implantation in spinal cord lesion, and some of the transplanted cells migrated far from the lesion site [17]. In the injured spinal cord, damaged axons and transplanted cells are exposed to a changing environment with a large variety of growth inhibitory molecules located in the meningo-glial scar and adjacent spinal cord regions [18,19]. OEC migration is modulated by neurotrophins, such as the glial cell-derived neurotrophic factor (GDNF), or chemicals, such as lysophosphatidic acid (LPA) [20–22]. These observations raise the notion that OECs are able to respond to a large array of molecules, which affect their migration, including inhibitory molecules, like Nogo-A [23]. Hence, knowledge of the mechanisms involved in inhibiting OEC migration is useful in the development of appropriate therapeutic strategies. It has already been established in rats that OECs labelled with magnetic nanoparticles (MNPs) can be tracked *in vivo* by MRI to determine their migration details in normal and injured spinal cords, including the possibility that OECs can cross a complete spinal cord injury zone [24]. MNPs are largely employed in biomedicine. The use of MNP has been established for many clinical diagnostic/therapeutic uses, e.g., MRI contrast agents in magnetic resonance imaging [25], for cell tracking via MRI [26], magnetic hyperthermia [27], gene therapy [28], vectors for drug delivery [29], *etc.*

The idea to use MNPs for magnetic labeling of cells has been already proposed in the literature [30] and also proven by our group. When mammalian cells are cultured in a MNP modified medium, MNPs interact with the cells and, following an exposition to a magnetic field, they are able to move cells in the direction imposed by the external magnetic field. By incubating neuroblastoma cell lines with magnetic carbon nanotubes (CNTs), under the effect of a permanent dipole magnet, a progressive

displacement of cells toward the more intensive magnetic fields was observed, and results suggested that cell displacement preferentially occurs during cell duplications [31,32]. In another study, mesenchymal stem cells (MSCs) were cultured in a CNT containing medium and, as a result of cell interaction with CNTs, the application of a magnetic field enabled shepherding of MSCs to the desired location *in vivo*. Specifically, when CNT labelled MSCs were injected in the portal vein of rats, we were able to significantly increase the localization of MSCs within the liver, with a consequent reduction of their migration to other organs [33]. More recently, we synthesized iron oxide MNPs with high saturation magnetization and tested them for cellular actuation of a neuroblastoma cell line through external magnetic forces. Results from scanning electron microscopy (SEM) and dual-beam SEM/focused ion beam (FIB) microscopy demonstrated the effective MNP internalization in large amounts. Since the magnetic force exerted on a single cell is proportional to the magnetic moment of the incorporated MNPs for a given magnetic field value (and its spatial derivative), this MNP internalization resulted in a high capability to induce cell migration under the effect of the magnetic source [34].

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protein kinase B), apoptosis (P53) and cell death (ERK, extracellular-signal-regulated kinases) between the control and the cell treated with 10 µg/mL of MNPs over time. At any time tested, no differences were found in the protein expression and their phosphorylation level confirming the negligible toxicity induced on OECs by MNP labelling (Figure 3a).

Figure 1. (**a**,**b**) Light microscopy photomicrographs of olfactory ensheathing cells (OECs) treated with 10 µg/mL (**a**) or 25 µg/mL (**b**) of MNPs (M-OECs) (arrows in a and b); (**c**,**d**) dual beam SEM/FIB images of M-OECs treated with 10 µg/mL of MNPs. The internalization of the MNPs can be seen in the cross section of single OEC cells (light grey spots in **e**) and confirmed by the Fe-content from EDS analysis of these areas (spectral analysis in **f**).

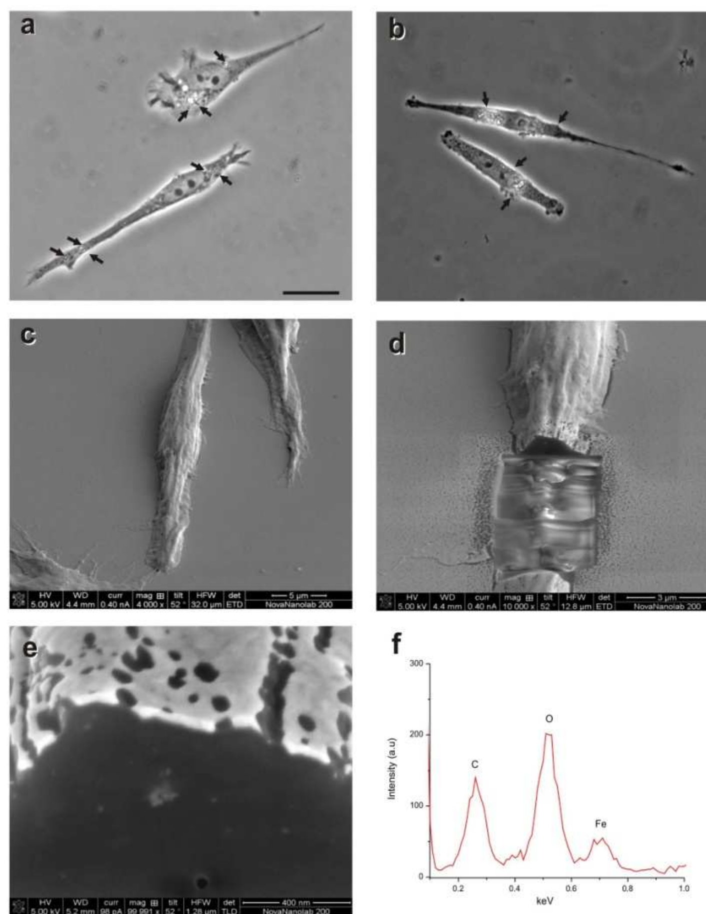
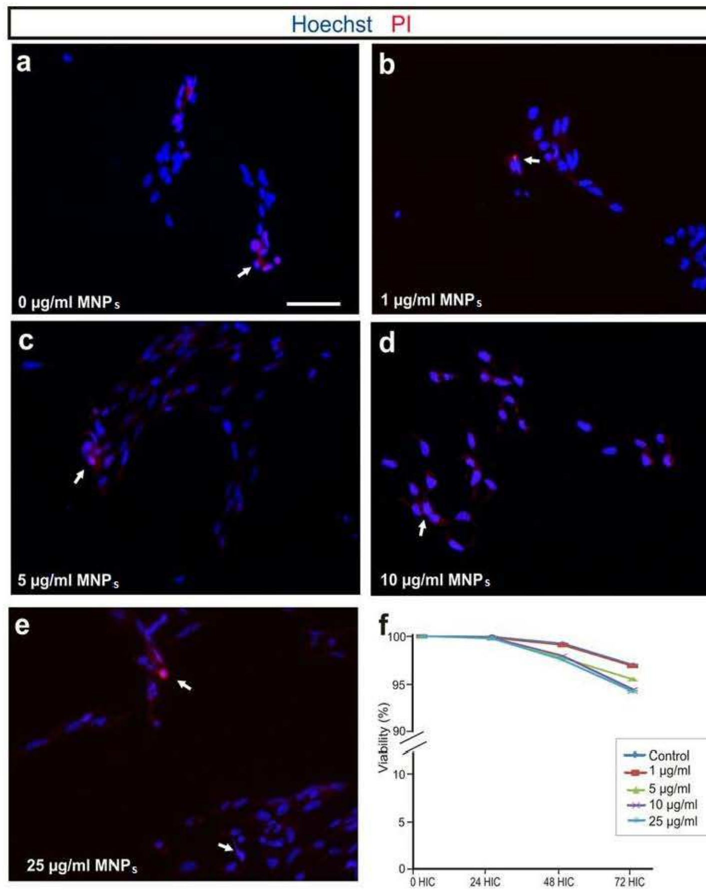
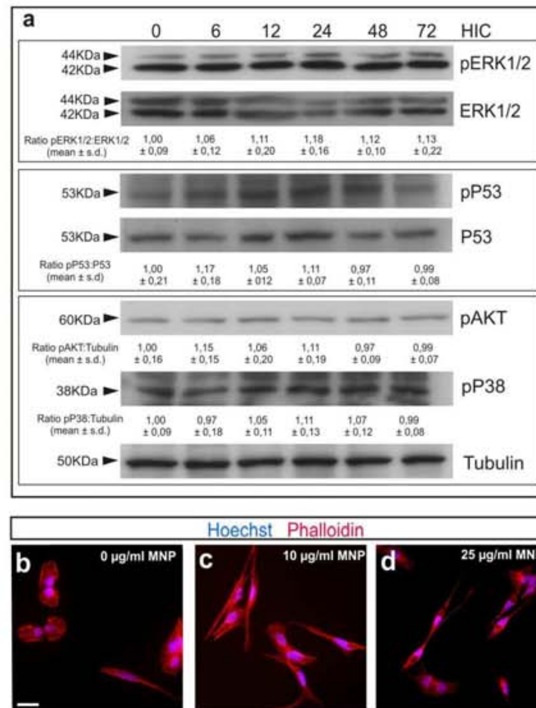


Figure 2. (**a**–**e**) Fluorescent microscopy of cells treated with MNPs 0, 1, 5, 10 and 25 μg/mL, respectively. Blue: Hoechst nuclear staining. Red: PI staining (white arrows show PI positive cells); (**f**) PI staining via flow cytometry of cells treated for 72 h with MNPs (1–25 μg/mL) ($p > 0.05$, ANOVA).



The cytoskeleton of OECs is the main target during the inhibition of cell migration [35]. Actin filaments staining confirmed that the labelling with MNPs did not alter cell morphology or induce cytoskeleton rearrangement (Figure 3b–d). Overall, these results confirm the negligible cytotoxic profile of MNP labelling.

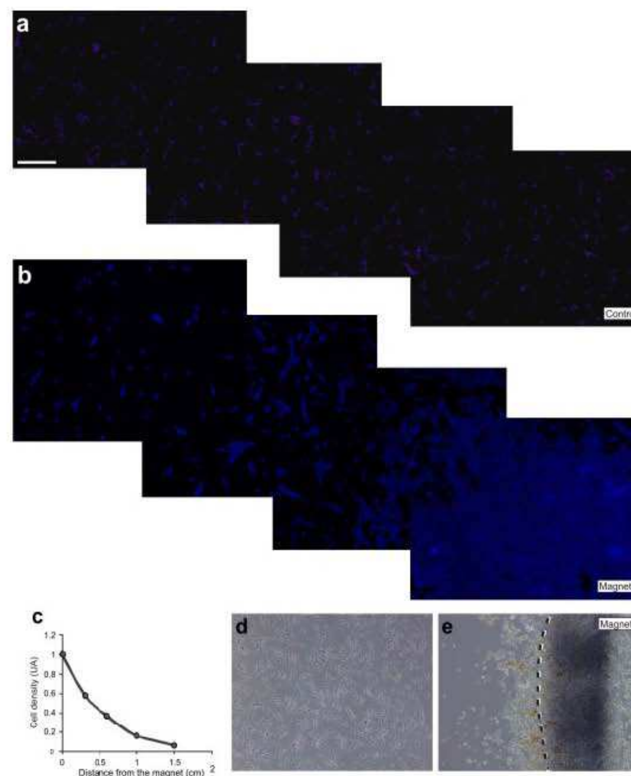
Figure 3. (**a**) Western blot analysis for M-OECs treated with MNPs (10 µg/mL) for 0, 6, 12, 24, 48 and 72 h in culture (HIC). Notice that activation levels of pERK1/2, pAKT, pP38 and pP53 are unchanged after MNP treatment. $p > 0.1$, ANOVA. (**b–d**) Phalloidin (red) and Hoechst (blue) staining for cells treated with 0, 10 and 25 µg/mL of MNPs for 24 h (b,c and d, respectively).



2.3. M-OECs Movement toward a Magnetic Source

When magnetically labelled cells are exposed to a non-homogeneous static magnetic field, the magnetic force exerted on the incorporated MNPs should result in a preferential growth and cell migration towards the magnetic source. Cell movement of M-OECs towards the magnetic source was monitored by fluorescent microscopy (nuclear staining). A magnet was attached to the cover of the Petri dish, and cell density was measured after 12 h as a function of the distance from the magnet (Figure 4). As expected, cell density increases exponentially with the decrease of the distance from the magnet, indicating that the magnetic field can actuate the labelled cells and pull them towards the magnetic poles, in agreement with our previous findings with different cellular models [33,36].

Figure 4. (**a**) Hoechst staining of M-OECs treated with MNPs (10 µg/mL); (**b**) Hoechst staining of M-OECs treated with MNPs (10 µg/mL); 8 h after the seeding, the magnet was placed inside the 3,5 mm ø petri dish for 12 h, when the images were acquired. (**c**) Quantification of cell density (*y*-axis) *vs*. distance from the magnet (*x*-axis) in the experiments. Note the increased number of M-OECs close to the magnet. (**d**,**e**) White field of a and b, respectively.

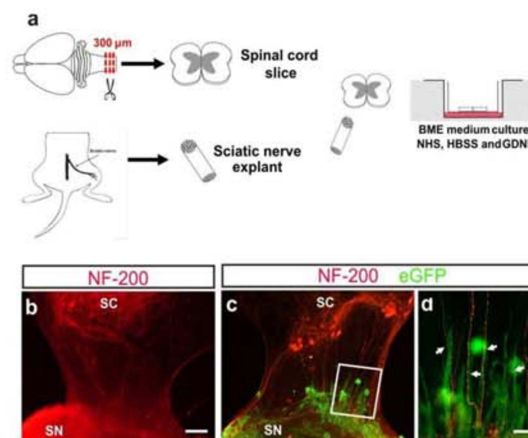


2.4. M-OECs Survive in an Organotypic Co-Culture of Peripheral Nerve and Spinal Cord

OECs have been used in numerous studies of axon regeneration (see introduction for details). Some of the studies determined both the poor survival of transplanted OECs and migratory deficits of these cells [9].

The olfactory ensheathing cell line used in 3D co-cultures experiments were obtained by lentiviral infection with eGFP-expressing lentivirus (see [37] for details). The model used consists of spinal cord slices co-cultured with a sciatic nerve graft for ventral root reconstruction (Figure 5a) (see also [38] for details).

Figure 5. (**a**) Representation of spinal cord and sciatic nerve co-culture (see material and methods for details). (**b**) Fluorescence photomicrograph illustrating an example of organotypic co-cultures of spinal cord (SC) and peripheral nerve (SN) in absence of M-OECs. After seven days *in vitro* (DIV), axons from motor neurons (red) reach and innervate the sciatic nerve explant. (**c**,**d**) Example of the morphology and behavior of M-OECs after transplantation in the organotypic slices. Numerous eGFP-positive cells can be seen, and some of them oriented parallel to the motor neuron axons (arrows in d). Scale bars: b, c = 150 μm, d = 50 μm.



Immunocytochemistry performed on organotypic co-cultures of this peripheral nerve regeneration model revealed that after seven days *in vitro*, motor neuron axons (NF-200 positive) were able to reach the sciatic nerve explants (Figure 5b). M-OECs were added to the model in order to assess the survival and the behavior of grafted cells. We observed the presence of M-OECs (eGFP-positive) after seven days of incubation in close distribution to NF-200 positive fibers (Figure 5c,d). In addition, numerous M-OECs were oriented parallel to NF-200 positive axons (Figure 5d). These data indicate that M-OECs were able to integrate into organotypic culture with some of them in close relationship with growing axons. Thus, the behavior of M-OECs is similar to those reported in other *in vitro* models using OECs [39].

3. Discussion

Cell transplantation has been used as therapy after CNS lesions. Since nerve regeneration can be promoted by supplying supportive ECM components and NFs and cell adhesion molecules, cells (e.g., Schwann cells) are effective and appropriate vehicles for supplying these factors [40–42]. Thus, normal or engineered cells have been largely used in several lesions in both central and peripheral nervous systems (e.g., [9,43,44]). However, as cell survival and migration may be compromised when cells are transplanted in an inhibitory environment [41,45], measures to enhance and direct transplanted cells are needed. In the present study, we propose a cell therapy strategy based on magnetized OECs [46,47]. Glial cells (*i.e.*, Schwann cells) and macrophages support regeneration by clearing

debris and secreting neurotrophic factors to aid axonal outgrowth [42]. In addition, OECs, a special type of glia cells that share phenotypic similarities to Schwann cells, are being extensively investigated as transplants to support nerve regeneration [48,49]. OECs support and guide the growth of olfactory axons and ensheath the bundles of olfactory nerves that extend from the olfactory epithelium to the olfactory bulb (e.g., [45]). Based on these features together with the capacity of OECs to express a number of neurotrophic factors [50], these cells are considered as candidate donor cells for axonal injuries and demyelinating diseases (e.g., [9,51]).

The migratory properties of the OECs are crucial for neural regeneration [35]. Several studies have reported that the migrating behavior of OECs varies and falls into two subpopulations: Schwann cell-like and the astrocyte-like morphology [45,52]. Irrespective of type, it is possible to magnetize OECs with MNPs and, thus, control their varied migratory behavior into "controlled shepherding" by magnetic fields. In order to confirm that MNP-labelled cells move towards a magnetic source, we performed cell migration experiments and demonstrated that magnetic labelling induces controlled and efficient cell migration under the influence of magnetic field gradients. These results are in agreement with our previous findings on MNP-labelled human neuroblastoma cells, showing that incorporation of MNPs into cells can render them suitable for magnetic actuation by external magnetic fields. Specifically, migration experiments under external magnetic fields confirmed that MNPs produced in our lab can effectively actuate the cells, inducing directional measurable migration towards predefined targets more effectively than commercial nanoparticles (e.g., fluidMAG-ARA supplied by Chemicell) [34].

In the present study, we demonstrated that the magnetization protocol we have developed does not alter biological features of OECs, which interacted strongly with MNPs with internalization of the MNPs demonstrated by electron microscopy. Western blot analysis revealed that MNP labelling does not induce activation (or phosphorylation) of proteins involved in cell replication, apoptosis and cell death (Figure 3a). The same results were obtained by both quantitative and qualitative PI dye exclusion assays, which confirmed cell viability exceeding 90% at all concentrations tested. Likewise, there was no cytoskeletal reorganization demonstrated with actin staining (Figure 3b–d).

In this study, we analyzed the survival of M-OECs in an organotypic model of spinal cord and peripheral nerve. Although *in vitro* transplantation of OECs and spinal cord has been reported [39], to our knowledge, this is the first report transplanting magnetized OECs in 3D co-cultures of spinal cord and peripheral nerve. Organotypic cultures are biochemically and physiologically similar to *in vivo* tissue and are very useful for studies on neural regeneration and drug delivery [53,54]. Several reports [55] have confirmed that nerve regeneration could be reproduced *in vitro* by using an organotypic model made of spinal cord slices and ulnar nerve [38]. In the present study, spinal cord slices and sciatic nerve explants from neonatal mice were cultured for seven days. Sciatic nerve was placed in front of ventral roots to allow motor neurons to innervate the sciatic nerve. After seven days of incubation, axons from motor neurons of the ventral horn of the spinal cord reached sciatic nerve. Hence, this system represents a good *in vitro* model for studying nerve regeneration of a peripheral nerve.

The behavior of M-OECs entrapped in Matrigel™ was tested by placement on the membrane used for organotypic co-cultures, with the regeneration process being monitored by staining motor neurons with NF-200 antibody (see Experimental section for details). This regeneration model showed that axons from the motor neurons of the ventral root of spinal cord re-innervated the sciatic nerve by grossing the 7 mm gap (site of lesion) between the two explants. The transplantation of M-OECs

(Figure 5) reported a good survival in these conditions (at least after seven days), with behavior resembling those reported by untreated OECs in similar models [39].

4. Experimental Section

4.1. MNP Synthesis

The synthesis protocol was based on the oxidative hydrolysis method, *i.e.*, the precipitation of an iron salt ($FeSO_4$) in basic media (NaOH) with a mild oxidant. In a typical synthesis, a mixture of 1.364 g of KNO_3 and 0.486 g of NaOH was dissolved in 135 mL of distilled water in a three-necked flask bubbled with N_2. Then, 15 mL of 0.01 M H_2SO_4 solution containing 0.308 g of $FeSO_4{\cdot}7H_2O$ (previously flowed with N_2 for 2 h) and polyethyleneimine (PEI) was added dropwise under constant stirring [56]. When the precipitation was completed, nitrogen was allowed to pass for another 5 min, and the suspension with black precipitate was held at 90 °C for 24 h under N_2. Afterward, the solution was cooled at room temperature with an ice bath, and the solid was separated by magnetic decantation and washed several times with distilled water. All reagents were commercially available and used as received without further purification. Iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), sodium hydroxide (NaOH), potassium nitrate (KNO_3), sulfuric acid (H_2SO_4) and polyethylenimine (PEI, MW = 25 kDa) were obtained from Sigma Aldrich.

4.2. OEC Cultures

The immortalized clonal OEC cell line, TEG3, was maintained in ME10: DMEM–F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% bovine calf serum (SAFC Biosciences, Lanexa, VA, USA), 20 μg/mL pituitary extract (Invitrogen, Carlsbad, CA, USA), 2 μM forskolin (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin-streptomycin and 1% Fungizone (Invitrogen, Carlsbad, CA, USA).

4.3. Spinal Cord (SC) Isolation and Sciatic Nerve(SN) Isolation

Mouse pups (P5-P6) were decapitated cleanly outside the sterile area by cutting with large scissors at the foramen magnum. The backbone was approached through a midline laparotomy; the bone cut with scissors and the lumbar region isolated, removed and put in cold PBS 0.1 M with 6.5 mg/mL glucose. Using a dissection microscope, the bone was carefully peeled off, taking care to remove also the meninges. The isolated lumbar spinal cord was put in the tissue chopper and cut into 350 μm slices, which were placed in incubation medium for 30 min and then seeded over a Millicell transwell. The incubation medium consisted of basal medium Eagle (BME) (50%), horse serum (25%), Hanks (2.5%), glucose (1%), glutamine (1%), GDNF (10 ng/mL) and water. After removing the excess of medium from the insert and placing the insert in the 6 well plates, slices were incubated at 37 °C. The sciatic nerve was harvested from an adult mouse after sacrifice. Again, the dissection microscope was used to expose the sciatic nerve, which was harvested and put in the incubation medium. The sciatic nerve was co-cultured with the spinal cord slice, by placing in front of the ventral root of the spinal cord, leaving a small gap in which M-OECs were injected in Matrigel™.

4.4. PI Dye Exclusion Assay through Fluorescence Microscopy

Cells (5×10^4) were seeded in 24 well plates. 24 h later, cells were incubated for 72 h in modified cell culture medium. PI was added at 2.5 µg/mL to each well and let to incubate for 5 min at room temperature (RT). Cells were washed two times with PBS and then fixed with paraformaldehyde 4% for 10 min at RT. Cells were washed again 2 times with PBS and then re-suspended in PBS. Hoechst 1 µM was added and incubated for 15 min at RT. Cells were washed three times with PBS and then analyzed in fluorescence.

4.5. PI Dye Exclusion Assay through Flow Cytometry

Cells (5×10^4) were seeded onto coverslips in 24 well plates in ME10 medium. Twenty-four hours later, cells were incubated for 72 h in the presence of different concentrations of nanoparticles (1, 5, 10, 25 µg/mL) in the medium. PI was added at 2.5 µg/mL to each well and incubated for 5 min at room temperature (RT). Cells were then fixed in 4% buffered paraformaldehyde, rinsed in 0.1 M PBS, stained with 0.1 µM Hoechst diluted in 0.1 M PBS for 10 min, rinsed in 0.1 M PBS and mounted on Fluoromount™ (Vector Labs, Burlingame, CA, USA). Alternatively, the percentage of nonviable cells was measured using a modification of the method described by [57]. Five thousand cells were plated into a 6-well plate with 2.5 mL of ME10 medium. Different concentrations of magnetic nanoparticles (0–50 µg/mL) were added to the cell medium. Baseline fluorescence F_1 was measured 1 h after addition of PI (30 µM) as an index of cell death not related to the treatment. Subsequently, fluorescence readings were taken at different times after the onset of the treatment (24, 48 and 72 h of incubation with MNPs). At the end of the experiment, the cells were permeabilized for 10 min with 500 µM digitonin at 37 °C to obtain the maximum fluorescence corresponding to 100% of cell death (F_{max}). PI fluorescence was measured in 24-well plates using a CytoFluor 2350 scanner (Millipore Corporation, Billerica, MA, USA) with 530 nm excitation (25 nm band pass) and 645 nm (40 nm band pass) emission filters. The percentage of cell death was calculated as follows:

$$\% \text{ cell death} = (F_n - F_1) \times 100/(F_{max} - F_1)$$

where F_n is the fluorescence at any given time.

4.6. Immunoblotting Analysis

This was performed to examine the expression level of specific proteins in cells treated with 10 µg/mL of MNPs for 0, 6, 12, 24, 48 and 72 h. The total cell lysate was prepared using a lysis buffer (50 mM Hepes, 1.5 mM $MgCl_2$, 150 mM NaCl, 1% Triton 100X, 10% glycerol 10%, 1 mM EGTA and 13 protease inhibitor cocktail (Roche, Basel, Switzerland)), followed by centrifugation and magnetic separation removal. The protein content was determined using the Bio-Rad detergent-compatible assay (BCA) (Bio-Rad, Hercules, CA, USA). Cell extracts (20 µg total protein) were boiled in Laemmli sample buffer at 100 °C for 10 min, subjected to 10% SDS-PAGE and electro-transferred to nitrocellulose membranes (Amersham Biosciences, Amersham, UK). After transfer, the membrane was blocked for 1 h with blocking buffer (5% milk in TBTS); the membrane was incubated overnight

at 4 °C with anti-tubulin (1:5000), anti-pERK1/2 and anti-ERK1-2, anti-pAKT, anti-pP38, anti-pP53 and anti-P53, all diluted to 1:1000 (*v*/*v*) in TBTS.

After washing (0.1% Tween 20 in PBS, pH 7.4), the membrane was incubated for 2 h at RT with secondary antibodies diluted to 1:1000 (*v*/*v*). The membranes were visualized using the ECL detecting solution (Millipore Corporation, Billerica, MA, USA) and analyzed using a Syngene imaging analysis system and software (Syngene, Cambridge, UK).

4.7. Phalloidin Staining

The cytoskeletal rearrangement of cells was studied by means of actin staining. Cells were seeded in a coverslip, at a concentration of $5 \cdot \times 10^4$ cells/well and incubated overnight in ME10 medium to allow cell adhesion. MNPs were added in the medium at a concentration of 10 µg/mL. Cells were cultured for 3 days before carrying out F-Actin staining. The culture medium was removed, and the cells were gently washed with PBS at 4 °C and, then, fixed with formaldehyde 4% for 15 min. After washing, cells were permeabilized with 0.1% Triton X-100 in 0.1 M PBS and blocked with 10% normal serum in 0.1 M PBS. Cells were sequentially incubated with Phalloidin-Rhodamine (R415, Invitrogen, Carlsbad, CA, USA) for 1 h, stained for Hoechst for 10 min, rinsed in 0.1 M PBS and mounted on Fluoromount™. The images were analyzed by fluorescent microscopy.

4.8. Immunocytochemistry of SC/SN Slice/OEC Co-Cultures

The SC/SN slice/OEC co-cultures were fixed in 4% PFA in PBS for 30 min at RT and washed with Triton 0.7% and PBS with gelatin 0.02% to permeabilize the cultures. Blocking was performed using Triton 0.7%, PBS with gelatin 0.02% and FBS 10% for 5 h 4 °C. Before proceeding with the incubation, the preparations were washed 4 times for 5 min with PBS. The co-cultures were then incubated with Triton 0.7%, PBS with gelatin 0.02% and FBS 5% with NF-200 (1:250) primary antibody for 48 h at 4 °C. After washing again for 5 min with PBS, the co-cultures were incubated with secondary antibody. Membranes with co-cultured SC/SN slice and OECs were cut off and mounted on glass slides with Fluoromount™ aqueous mounting medium and examined using an Olympus BX61 fluorescence microscope.

4.9. SEM/FIB Analysis

SEM/FIB cross sectioned cells were performed using SEM INSPECT F50, FEI Company, Hilsboro, OR, USA) and dual-beam FIB/SEM (Nova 200 NanoLab, FEI Company, Hilsboro, OR, USA). SEM images were taken at 5 and 30 kV, with a FEG column, and a combined Ga-based 30 kV (10 pA) ion beam was used to cross-section single cells. These investigations were completed by EDS for chemical analysis.

OECs were grown on coverslips and treated with MNPs (10 µg/mL). After 24 h of incubation, the cells were washed with PBS, fixed and dehydrated. After drying, the samples were sputtered with 10 nm of gold.

4.10. Cell Migration Assay

OECs were detached by trypsinization, collected by centrifugation and seed with fresh medium. After 24 h of incubation, 10 µg/mL of MNPs were added and then let to incubate for other 48 h. For the selection of the "magnetized" cell population, cells were trypsinized and subjected to magnetic separation. M-OECs were counted in a Burker chamber and seeded in 3.5 cm Petri dish. After 8 h of incubation, a neodymium cylindric magnet (N12, radius 0.3 length, height 0.8 mm) was attached to the cover of the Petri dish (Figure S1). Cell density was visualized by nuclear staining (Hoechst, Sigma, 33258) after 12 h.

4.11. Statistical Analysis

Values are reported as the mean ± standard error of the mean (S.E.M). Statistical significance was assessed by one way analysis of variance (ANOVA).

5. Conclusions

In this study we demonstrated that OeC labeled with magnetic nanoparticles can be manipulated by an external magnetic field and show a positive integration in organotypic slices of spinal cord and peripheral nerve.

These results pave the way for *in vivo* use of magnetized OECs and magnetic fields as a tool to localize the OEC-mediated production of therapeutic molecules at the lesion site. In such *in vivo* protocol, M-OECs would be injected at the site of the nerve injury site and magnetic fields (with the appropriate simulation and design of the magnetic field geometry) used to enhance homing and the localization of M-OECs to the injury site. In a previous study, we reported that magnetically labelled cells can be shepherded *in vivo* to a desired location following their intravenous administration and application of a proper magnetic field [33]. Further work is needed to demonstrate the capability to shepherd *in vivo* magnetically labelled OECs to the nerve injury site to promote regeneration by enhancing axon growth across the lesion and functional recovery.

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Conflict of Interest

The authors declare no conflict of interest.

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Chapter III

Enhanced migration of olfactory ensheathing cells genetically modified to express the NgR1(310) ectodomain over myelin.

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RESUMEN:

La implantación de las OECs para ayudar la regeneración axonal después de una lesión medular ha surgido como terapia prometedora, pero esta capacitad de las OECs parece depender de la presencia de un sustrato permisivo para su migración (Gudino-Cabrera et al., 2000; Ramon-Cueto and Munoz-Quiles, 2011; Ekberg et al., 2012). Después de una lesión se pueden observar restos de mielina en la médula espinal que afectan negativamente la regeneración axonal (Schwab, 1990). Además, resultados previos en uno de nuestros estudios demostraron que la mielina bloquea la migración de la OECs *in vitro* (Nocentini et al., 2012). El ectodominio NgR1(310) (desde ahora NgR(Ecto)), inhibe la señalización de la mielina y facilitando la regeneración axonal después de la lesión (Fournier et al., 2002a; He et al., 2003).

En este estudio hemos modificado genéticamente estas células para la expression de NgR(Ecto) usando un sistema lentiviral. Las OECs modificadas fueron capaces de expresar y secretar altos niveles del ectodominio *in vitro*. Mediante el análisis de su migración pudimos observar que estas células presentan una mayor motilidad que las células no modificadas sobre un substrato que presenta mielina. Para observar el comportamiento de las NgR(Ecto)-OECs *in vivo* nos planteamos un experimento preliminar e implantamos las células en médula espinal no lesionada de ratas adultas. La células modificadas migraron distancias más largas respecto laa células no modificadas, tanto en sentido caudal como rostral desde el punto de inyección.

Estos datos sugieren un potencial terapéutico de las NgR(Ecto)-OECs ya que una vez implantadas después de la lesión mostrarían una motilidad más alta y esto aumentaría su capacidad de regeneración. Por otra parte el ectodominio secretado podría

interferir con la mielina presente en el lugar de la lesión mejorando la regeneración axonal intrínseca.

ABSTRACT (English version):

Implantation of OECs to repair SCI has emerged as a promising therapy, but their regenerative capacity seems to depend on a substrate permissive for migration (Gudino-Cabrera et al., 2000; Ramon-Cueto and Munoz-Quiles, 2011; Ekberg et al., 2012). After SCI myelin debris are present for long times in lesioned spinal cord and previous results from our *in vitro* study demonstrated that myelin inhibits OECs migration (Nocentini et al., 2012). Moreover it is well known that myelin impairs CNS axonal regeneration (Schwab, 1990). The NgR1(310) ectodomain (NgR(Ecto)), containing the amino acids 1–310 of NgR1, blocks the signaling of myelin and previous studies demonstrated that its presence enhances axonal regeneration after SCI (Fournier et al., 2002a; He et al., 2003).

Therefore here we genetically modified OEC to express the NgR(Ecto)coupled with a GFP using a lentiviral based system in order to visualize them. Engineered OEC were able to express and secrete at high levels the ectodomain *in vitro*. Moreover, by time lapse analysis we could observe that these cells migrate longer distances than normal OEC over a myelin substrate. To observe the behavior of NgR(Ecto)-OECs *in vivo* we did a preliminary experiment where cells were implanted in a not-lesioned spinal cord of adult rats. The NgR(Ecto)-OECs migrated more distance both caudal and rostral from the site of injection in comparison with normal OEC.

These data suggest a therapeutic potential of NgR(Ecto)-OECs as once implanted after SCI they would migrate more and this would enhance their regeneration capacity. Moreover the secreted ectodomain would interfere with the myelin present at the lesion site improving intrinsic axonal regeneration.

Enhanced migration of olfactory ensheathing cells genetically modified to express the NgR1(310) ectodomain over myelin.

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Abstract: Implantation of olfactory ensheathing cell (OEC) to repair spinal cord injury (SCI) has emerged as a promising therapy. However, myelin debris are present for long times in lesioned spinal cord inhibiting OEC migration and CNS axonal regeneration. The NgR1 ectodomain (comprising the amino acids 1–310 of the NgR1) (NgR(Ecto)), blocks the signaling of myelin and previous studies demonstrated that its presence enhances axonal regeneration after SCI. Therefore we genetically modified TEG3, a clonal line for OEC, to express the NgR(Ecto) coupled with eGFP using a lentiviral based system. Engineered TEG3 were, then, tested for their ability to express and secrete at high levels the ectodomain *in vitro*. By time lapse analysis we could prove that these cells migrate more distances than normal TEG3 over a myelin substrate. Preliminary results show that, after implantation in a not-lesioned spinal cord of adult rats, NgR(Ecto) TEG3 migrate more than normal TEG3. This data suggest a therapeutic potential of NgR(Ecto) TEG3s as once implanted after SCI they could migrate more and this would enhance their regeneration capacity. Moreover the secreted ectodomain would shed the myelin present around the lesion site and therefore improve axonal regeneration.

Key words: Olfactory enshathing cells; Nogo Receptor ectodomain (1-310) (Ngr1(310)Ecto); Spinal cord injury.

Introduction:

Spinal cord injury (SCI) is one of the hardest forms of trauma experienced by humans. In fact, after a lesion to the spinal cord self-repair and regeneration of the damaged area of the central nervous system (CNS) is poor and often results in a permanent loss of function (Dumont et al., 2001; Furlan et al., 2013). This is mainly due to limited neuronal survival, lack of spontaneous axonal regeneration and the presence of an inhibitory environment (Yiu and He, 2006; Yang et al., 2011). The recent progress in cellular transplantation, gene therapy and molecular treatment has generated optimism about future cures of SCI (Li et al., 2005; Sun et al., 2013; Wang et al., 2013). Given their ability to support CNS axon regeneration, olfactory ensheathing cells (OECs) have attracted attention as potential therapeutic agent. Indeed, during mammalian lifespan OECs are capable to ensheath, accompany and guide newly unmyelinated growing axons of olfactory

sensory neurons from the peripheral nervous system (PNS) to their target in the CNS (Doucette et al., 1983; Nedelec et al., 2005). The implantation of these cells after SCI would generate a more favorable and supportive environment for axonal re-growth, guide axons through the lesioned area to their correct destination and promote functional recovery. Indeed, OECs have been successfully used in many transplantation experiments with encouraging outcomes (e.g., (Ramon-Cueto and Santos-Benito, 2001; Lu et al., 2002; Lopez-Vales et al., 2006)). The precise mechanisms accounting for the observed recovery are not well characterized but may include promotion of axonal regeneration, remyelination, neuroprotection, and induction of neovascularization. The migratory ability of OECs is important for these cells to exert their function. Indeed, *in vivo,* it was observed that OECs migrate ahead of newly formed axons to guide them and support their growth by secreting growth promoting factor (Ekberg et al., 2012). Studies on the migration capacity of OECs after transplantation in SCI models led to contradictory results (Ramon-Cueto et al., 1998; Resnick et al., 2003; Boyd et al., 2004; Lu et al., 2006). Recently, it was observed that in injured cord, in comparison to not-injured spinal cords, rat and human OECs migrate for shorter distances, in both rostral and caudal directions (Deng et al., 2006). Moreover, in a previous study we demonstrated that OECs migratory properties are impaired in the presence of myelin (Nocentini et al., 2012) and that this inhibition is mediated in major part by the NgR-complex (Nocentini et al., 2012). After SCI myelin associated inhibitors (MAIs) are overexpressed at the site of lesion and myelin debris persist over years in the spinal cords (Fawcett et al., 2012). This debris can inhibit OEC migration and CNS axonal regeneration. Moreover OECs also respond to chondroitin sulphate proteoglycans (Reginensi et al., unpublished results) Therefore we thought that modifying OECs to secrete de NgR1(310) ectodomain (NgR(Ecto)) could increase their migration capacity and in parallel increase intrinsic axonal regeneration potential after SCI. In fact, the NgR(Ecto) exerts his action by binding on NogoA (one of MAIs) and preventing its binding to the NgR-complex; and by interacting with full-length NgR1, finally blocking the intracellular signaling of myelin (Fournier et al., 2002a). *In vitro* studies showed that it can act as an antagonist for CNS myelin (Fournier et al., 2002a; He et al., 2003). Moreover, following SCI, intrathecal and i.p. injections of NgR(Ecto) improved regeneration of transected axonal tracts and functional recovery (Li et al., 2004; Wang, X. et al., 2006).

In the present study we examined the properties of a clonal line for OECs, TEG3, genetically modified to express the NgR(Ecto). The engineered TEG3 were generated using a lentiviral based system and subsequently were characterized by their ability to express and secrete the NgR1 ectodomain. By time lapse analysis we studied their migration capacity over myelin and observed that NgR(Ecto)-TEG3s migrated longer distances *in vitro* in comparison to normal TEG3s. In addition, as preliminary experiment we show that, after implantation in a not-lesioned spinal cord of adult rat, NgR(Ecto)-TEG3s migrate more than normal TEG3. This data suggest a therapeutic potential of NgR(Ecto)-TEG3s as once implanted after SCI they would migrate more and this would enhance their regeneration capacity. Moreover the secreted ectodomain would shed the myelin inhibitors and therefore improve intrinsic axonal regeneration.

Materials and methods:

Antibodies and biochemical reagents

The following antibodies were used at a dilution of 1:500 for immunohistochemical staining and/or western blotting, unless otherwise indicated. S100b was purchased from Abcam (Cambridge, MA, USA) GFAP and GFP were from DAKO (Glostrup, Denmark), p75 was from Promega (Madison, WI, USA). NgR1 was a gift from Prof. B.L. Tang (Singapore). Tubulin (1:5,000), phalloidin-alexa594 and DAPI were purchased from Sigma (St. Louis, MO, USA). Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit immunoglobulins were purchased from Molecular Probes (Leiden, Netherlands). The goat anti-mouse horseradish peroxidase (HRP) and rabbit anti-goat-HRP secondary antibodies used in western blots were from DAKO. Myelin was purified from adult Sprague-Dawley rat CNS, as described (Seira et al., 2010). All animal procedures were performed in accordance with the guidelines established by the Spanish Ministry of Science and Technology and the European Community Council Directive 86/609 EEC.

The NgR(Ecto) contruct

The NgR(Ecto) (Fig.1) construct was prepared as follows: the cDNA of the region was cloned by PCR from adult mouse brain with the primers: NgR(Ecto) Forward 5'- AAA GGA TCC ATG AAG AGG GCC TCC TCC GGA-3', and NgR(Ecto) Reverse 5'-AAT GGA TCC TTA TCA AGC ACA ACC CTC TAA GTC ACT-3'. The NgR(Ecto) PCR fragment was extracted (QIAquick, QIAGEN) and ligated to the pLenti6/V5-DEST vector (Invitgogen) into ApaI and BclI sites. This NgR(Ecto) vector was then sequenced to ensure correct nucleotide sequence. Subsequently lentiviral particle were produces by transient transfection of 293FT cells with Lipofectamine 2000 (Invitrogen), using the NgR(Ecto) vector, the second generation packaging construct psPAX (Tronolab, Lausanne, Switzerland) and the envelope plasmid pMD2G (Tronolab). 293FT were cultured in DMEM supplemented with 10% fetal calf serum and without antibiotics before transfection. Medium was changed and supplemented with antibiotics after 6 h. Vector supernatants containing viral particles were harvested 24 and 48 h later and concentrated by ultracentrifugation (2h at 2.6000 x g at 4°C).

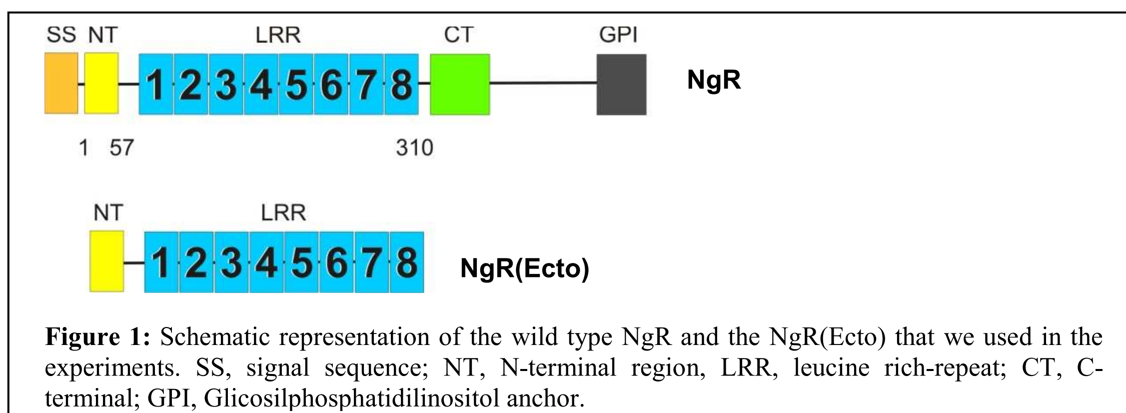

Figure 1: Schematic representation of the wild type NgR and the NgR(Ecto) that we used in the experiments. SS, signal sequence; NT, N-terminal region, LRR, leucine rich-repeat; CT, C-terminal; GPI, Glicosilphosphatidilinositol anchor.

TEG3 cultures and genetic modification

The immortalized clonal cell line TEG3, which contains the SV40 large T antigen stable transfectant of OEG primary cultures, was used. Cells were maintained in ME10: DMEM–F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% bovine calf serum (SAFC Biosciences, Lanexa, VA, USA), 20 µg/ml pituitary extract (Invitrogen), 2 µM forskolin (Sigma), 1% penicillin–streptomycin and 1% fungizone (Invitrogen). To obtain genetically modified TEG3, cells were transfected using one lentivirus carring the NgR(Ecto) construct and/or one lentivirus carring the eGFP construct (Morales et al., 2008) (Fig.2). Cells in passage 2 were plated at 2000 cells/cm^2 and incubated with one of the lentiviruses during 48h. Then, the medium was changed and the cells cultured as described above with the addition of blasticidin to the media (3µg/ml) in the case of the NgR(Ecto). For double tranfection cells were first incubated with the NgR(Ecto) lentivirus, and afterwards with the eGFP. Single clones to amplify were selected with cloning disks (Sigma) and expression of NgR(Ecto) was proven via Western blotting. The selected clone was grown in ME10 media and cells were expanded to produce enough number of TEG3 for the experiment.

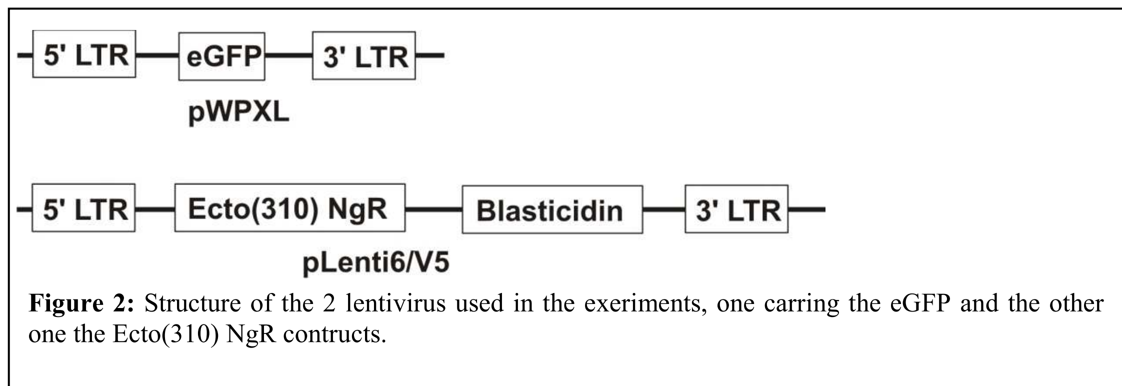

Figure 2: Structure of the 2 lentivirus used in the exeriments, one carring the eGFP and the other one the Ecto(310) NgR contructs.

Glass coverslips (12 mm diameter) were coated essentially as described (Fournier et al., 2002a). Briefly, coverslips were precoated with poly-L-lysine 10 µg/ml dissolved in 0.1 M PBS (pH 7.3) and then washed. They were coated with laminin (2 µg/ml, dissolved in 0.1 M PBS) and washed again with 0.1 M PBS. NgR(Ecto)-TEG3 counted cells were seeded onto substrate-coated coverslips in ME10 medium. Cells were cultured for 20 h and then the coverslips were fixed in 4% buffered paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1 M PBS, and blocked with 10% normal serum in 0.1 M PBS. Cells were sequentially incubated overnight with primary antibodies at 4°C and then with Alexa Fluor-tagged secondary antibodies for 1 h. Cells were rinsed in 0.1 M PBS, stained with 0.1 M DAPI diluted in 0.1 M PBS for 10 min, rinsed in 0.1 M PBS, and mounted on FluoromountTM (Vector Labs, Burlingame, CA, USA).

Time-lapse analysis of NgR(Ecto)-TEG3s migration

Fluorodish cell culture dishes (World Precision Instruments, Sarasota, FL, USA) were coated with laminin or myelin as described (Nocentini et al., 2012). We seeded $5*10^4$ cells on the coated dishes and 20–24 h later we performed the time-lapse analysis. To study cell migration, we transferred the culture dishes to an LCI system (Live Cell Instruments, Seoul, Korea) for 20 h. The multi-tracking analysis was performed with the ImageJ software using the plugin mTrackJ (Biomedical Imaging Group Rotterdam of the Erasmus MC-University Medical Center Rotterdam, Netherlands). Tracking was performed in an inverted Olympus microscope IX-71 (20X) and the images (5 megapixels) were captured by an Olympus XC-50 camera (150 frames, one frame every 8 min). Cell tracking allows the analysis of the scrolling speed and position frame (Xt, Yt).

Surgical procedure

Adult Sprague-Dawley female rats (9 weeks old; 250-300g) were used in the spinal cord experiments. The animals were housed with free access to food and water at a room temperature of 22 ± 2ºC under a 12:12h light-dark cycle. The experimental procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Directive 86/609/EEC. Under anesthesia with ketamine (90mg/kg) and xylacine (10mg/kg) and aseptic conditions, laminectomy was performed in T8-T9 vertebra. The cells for transplantation (eGFF-TEG3 or eGFP/NgR(Ecto)-TEG3) were suspended in L15 medium (Life Technologies) at 50,000 cells/µl and maintained in ice during the time of surgery. Using a glass needle (100 µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10ul Hamilton syringe (Hamilton #701 , Hamilton Co, Reno, NV, USA), 6µl of the corresponding cell suspension were intraspinally injected (1mm deep into the spinal cord, 2 injection of 3µl each, one at each side 0.5mm lateral to the cord line), with a total of 300,000 cells per injection. A perfusion speed of 2µl/min was controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip was maintained inside the tissue 3min after injection to avoid liquid reflux. The wound was sutured by planes and the animals allowed to recover in a warm environment. To prevent infection, amoxicillin (500 mg/l) was given in the drinking water for one week.

Tissue processing.

7 days post implantation, rats were deeply anesthetized (pentobarbital 60 mg/kg b.w. i.p.) and intracardially perfused with 4% paraformaldehyde in 0.1 M PBS. The spinal cord segment from 1.5 cm rostral to 1.5 cm caudal to the injection (± 3 cm total length) was harvested and post-fixed in the same fixative solution for 24h and cryopreserved in 30% sucrose. For GFP+ cell localization 30µm thick longitudinal sections of the spinal cord segment were obtained. Spinal cord sections of GFP+ cell transplanted rats were processed for immunohistochemistry against GFP and GFAP. Tissue sections were blocked with PBS-0.3% Triton-5% fetal bovine serum and incubated for 24h at 4ºC with the primary antibodies. After washes, sections were incubated for 2h at room temperature with secondary antibody. Slides were dehydrated and mounted with Citoseal 60 (Thermo Fisher Scientific, Madrid, Spain). For analysis,

images (10 megapixels) were obtained with a digital camera (Leica DFC 550) attached to the microscope (Leica AF700). Starting from the injections point 11 consecutive section of the length of approximately, 150μm were taken in account. GFP+ cells in each section were counted.

Statistical analysis

Summary data are expressed as mean ± SEM (standard error of the mean) of at least three independent experiments. Means were compared by one-way ANOVA test. A value of $p \leq 0.05$ was considered statistically significant.

Results

Characterization of NgR(Ecto)-TEG3

TEG3 is a clonal OEC line that shows similar growth promoting capacity to non-modified OECs (Moreno-Flores et al., 2006). In a fist set of experiments we selected the modified NgR(Ecto)-TEG3s clone that expressed high levels of the ectodomain. By Western blotting technique we could detect the ectodomain not only in cellular extract but even in concentrated media from cultured cells (Fig. 3a). Afterwards, the selected clone was trasduced for eGFP and the resulting colony was tested again for the production of the ectodomain by Western blotting (Fig. 3a). eGFP expression was detected by observing cells under a fluorescence microscope (Fig. 3b). eGFP was detected in almost all cells (>90%). eGFP/NgR(Ecto)-TEG3s could be maintained in culture and cells show all the types of morphology described for primary OECs and TEG3 cells (Nocentini et al., 2012). Indeed we could distinguish between Schwann cell like modified TEG3 with a fusiform morphology and Astrocyte cell like modified TEG3 with a flat shaped form as shown in detail by Phalloidin-Alexa594 staining (Fig.3c). In culture, The fusiform morphology was predominant. Specific markers of OECs (p75, S100b, or GFAP, NgR) were present in our cultured cells (Fig. 3c).

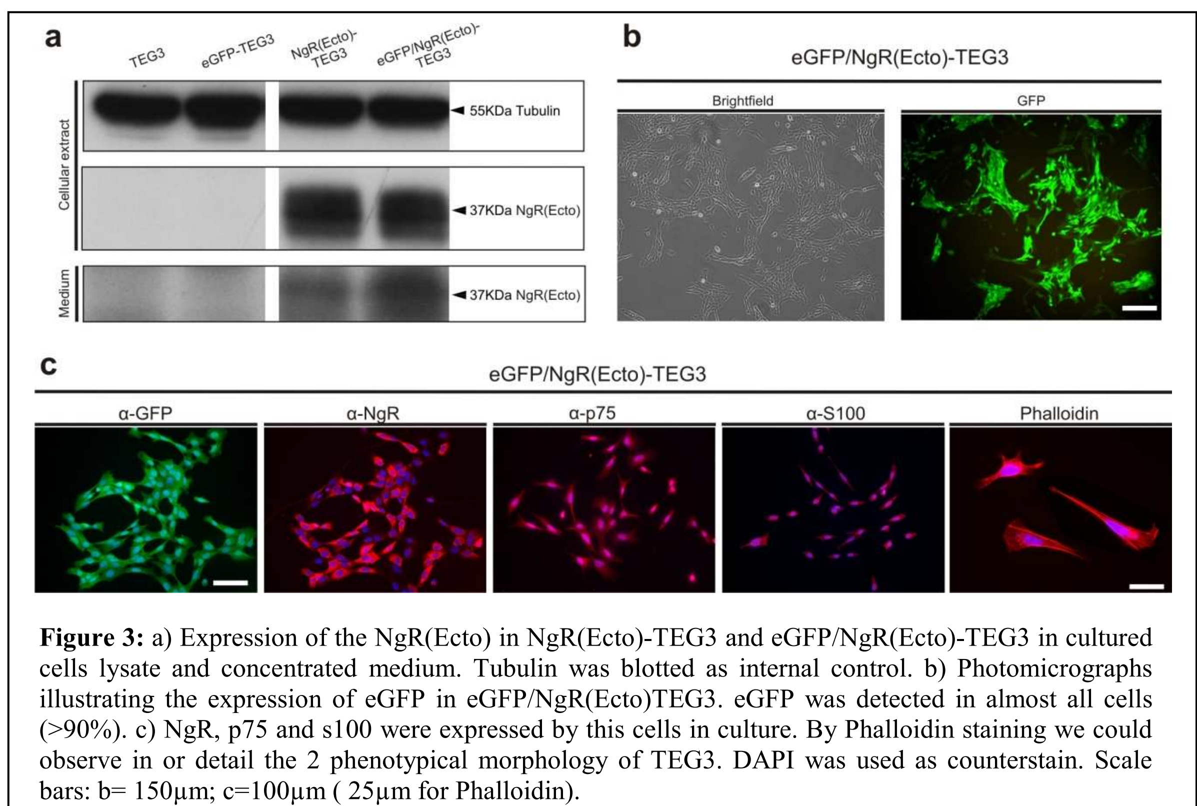

Figure 3: a) Expression of the NgR(Ecto) in NgR(Ecto)-TEG3 and eGFP/NgR(Ecto)-TEG3 in cultured cells lysate and concentrated medium. Tubulin was blotted as internal control. b) Photomicrographs illustrating the expression of eGFP in eGFP/NgR(Ecto)TEG3. eGFP was detected in almost all cells (>90%). c) NgR, p75 and s100 were expressed by this cells in culture. By Phalloidin staining we could observe in or detail the 2 phenotypical morphology of TEG3. DAPI was used as counterstain. Scale bars: b= 150μm; c=100μm (25μm for Phalloidin).

Migration properties of NgR(Ecto)-TEG3

Low density TEG3 cultures were monitored for 20 h (20X objective; one frame every 8 min) in an inverted Olympus IX-71 microscope equipped with a cooled fluorescence camera and a cell culture incubation chamber. The migration speed of normal TEG3s grown on myelin-coated substrates (0.02 µg/µl) showed a decrease of 56.0%, compared to those on laminin coated substrates (Fig. 4a,c) (0.75 ± 0.02 µm/min (laminin); 0.33 ± 0.05 µm/min (myelin)). NgR(Ecto)-TEG3s display the same migration speed then normal TEG3s over laminin (0.78 ± 0.06 µm/min)(Fig. 4c). Over myelin NgR(Ecto)-TEG3s cell migratory capacity was a 16.5% lower than over laminin (0.66 ± 0.05 µm/min) (Fig. 4b,c). Comparing normal TEG3s with NgR(Ecto)-TEG3s we can appreciate partial recovery of the migratory capacity of the second ones over myelin. The lack of complete recovery is probably due to the presence of inhibitors in the myelin that don't act via the NgR-complex and/or different receptors on OECs.

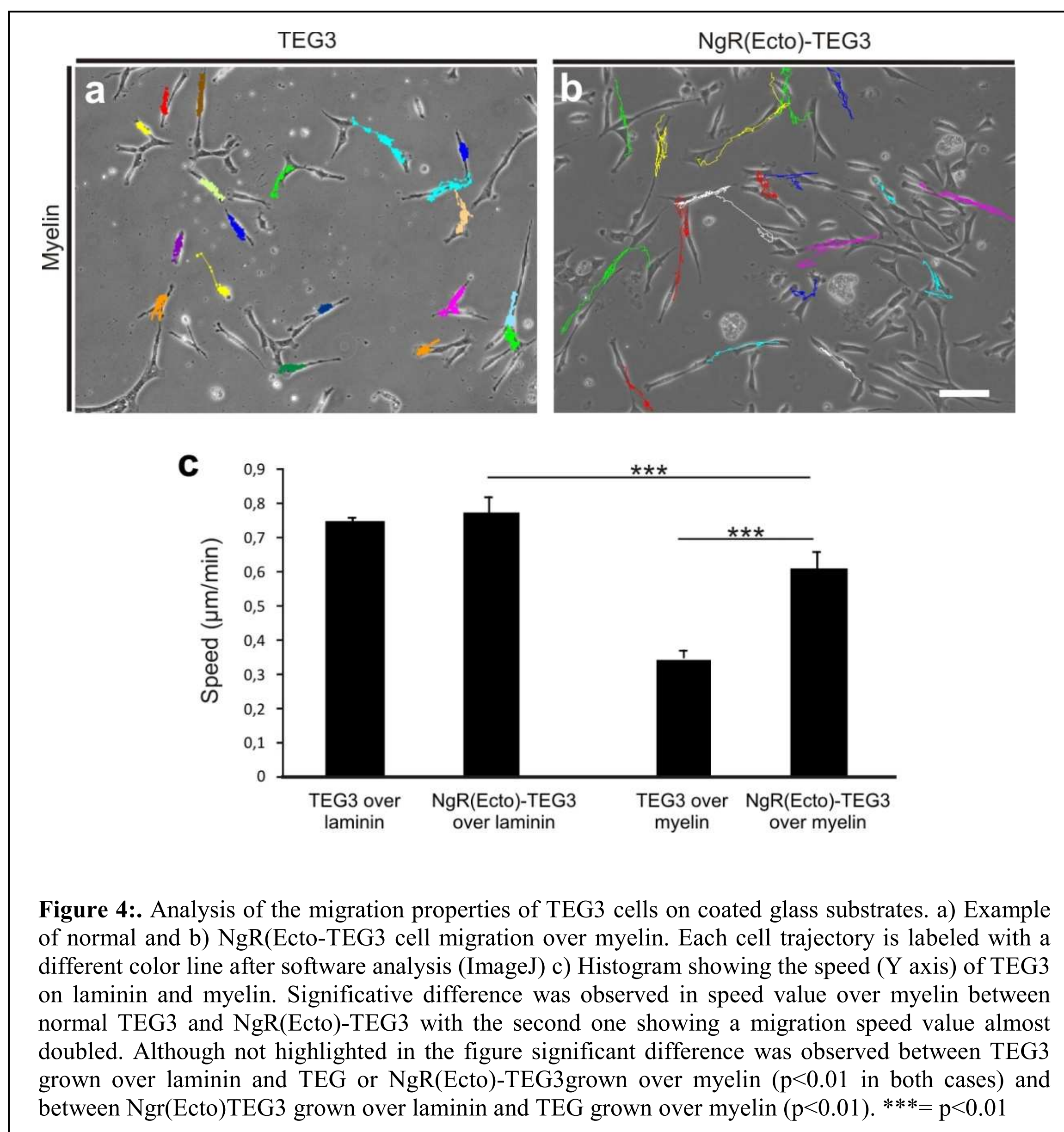

Figure 4:. Analysis of the migration properties of TEG3 cells on coated glass substrates. a) Example of normal and b) NgR(Ecto-TEG3 cell migration over myelin. Each cell trajectory is labeled with a different color line after software analysis (ImageJ) c) Histogram showing the speed (Y axis) of TEG3 on laminin and myelin. Significative difference was observed in speed value over myelin between normal TEG3 and NgR(Ecto)-TEG3 with the second one showing a migration speed value almost doubled. Although not highlighted in the figure significant difference was observed between TEG3 grown over laminin and TEG or NgR(Ecto)-TEG3grown over myelin (p<0.01 in both cases) and between Ngr(Ecto)TEG3 grown over laminin and TEG grown over myelin (p<0.01). ***= p<0.01

Behavior of NgR(Ecto)-TEG3 in not-lesioned spinal cords

Grafts of about 300.000 cells of eGFP- or eGFP/NgR(Ecto)-TEG3s were implanted in not injured spinal cords to analyze the capacity of this cell to integrate and migrate in the model. A total of 4 rats were used (2 rats received eGFP-TEG3s, another 2 eGFP/NgR(Ecto)-TEG3s). A week after implantation spinal cord where fixed and after staining (see material and method) the localization and distribution of TEG3 cells was observed. The slices with major number of cell were selected for analysis. The total number GFP positive cells in the case of eGFP-TEG3s (6000 ± 250 cells/slice) was slightly minor than the one of eGFP/NgR(Ecto)-TEG3s (7000 ± 200 cells/slice). Given the low number of animal used in the experiment we consider this difference not significant. To assess the distance migrated by TEG3 we counted the number of GFP positive cells, in areas of about 150μm in length starting from the injection point. 11 sections caudal or rostral to this point were taken in account, denominating section number 1 the closest to the injection site (Fig. 5).

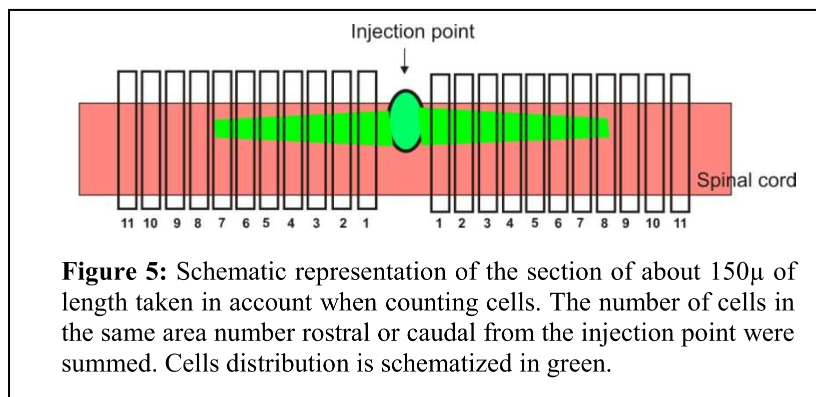

Figure 5: Schematic representation of the section of about 150μ of length taken in account when counting cells. The number of cells in the same area number rostral or caudal from the injection point were summed. Cells distribution is schematized in green.

The data obtained (Fig.6) revealed that eGFP-TEG3s (Fig. 6a,c) were distributed majorly near the injection point with the maximum cell percentage (25%) found in the section 300μm rostral or caudal to this point (Fig. 6c). Moreover no cell was seen more than 1200μm from injection point. eGFP/NgR(Ecto)-TEG3s, instead, were found in all the sections taken in account (Fig. 6b,c) with the major number of cells (16%) found in the section 600μm rostral or caudal to the injection point (Fig 6b,c). Given these results we can infer that eGFP/NgR(Ecto)-TEG3s integrate well in spinal cords and migrate more than eGFP-TEG3s. As cited before in the case of eGFP/NgR(Ecto)-TEG3s we observed a slightly higher number of cells. Although we did not consider this difference significant given the low number of animal used in the study, the effect of the ectodomain on TEG3 cellular survival or proliferation rate warrant further studies.

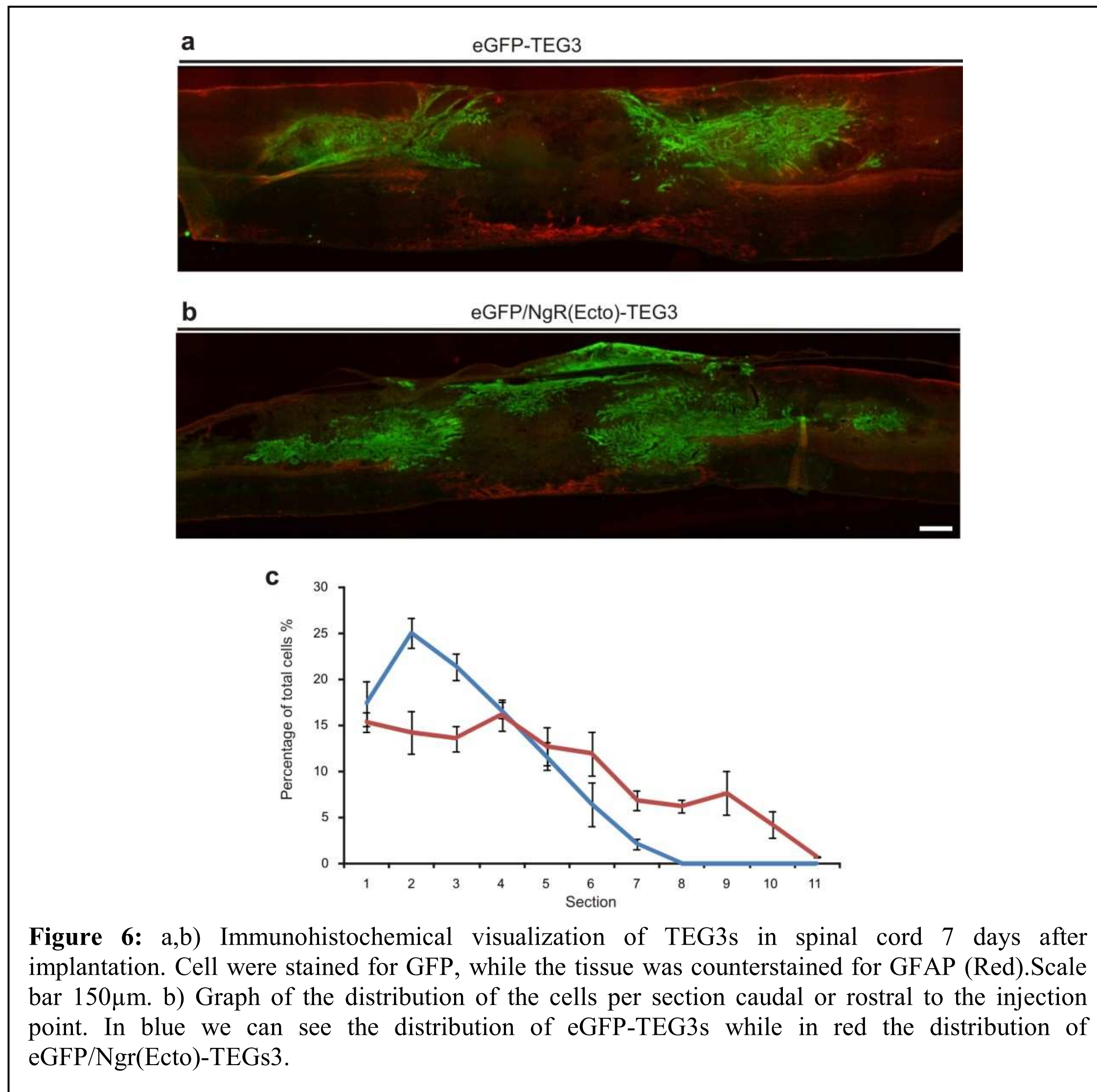

Figure 6: a,b) Immunohistochemical visualization of TEG3s in spinal cord 7 days after implantation. Cell were stained for GFP, while the tissue was counterstained for GFAP (Red).Scale bar 150µm. b) Graph of the distribution of the cells per section caudal or rostral to the injection point. In blue we can see the distribution of eGFP-TEG3s while in red the distribution of eGFP/Ngr(Ecto)-TEGs3.

Discussion

Combining transplantation and gene therapy is one of the most powerful strategies to promote regeneration after SCI. OECs implantation has been shown to promote regeneration and functional recovery in lesioned spinal cord. Thus there is interest in upgrading OECs capacities in order to enhance their regenerative potencial. Indeed, it has been reported that OECs with higher migration capacity show enhanced regenerative properties (Ramon-Cueto et al., 1998; Resnick et al., 2003; Boyd et al., 2004; Lu et al., 2006). In fact, it is supposed that OECs migrate ahead of growing axons and by secreting permissive factors aid and guide their elongation. In a previous study we demonstrated that OECs migration capacity is impaired by the presence of myelin (Nocentini et al., 2012). MAIs act through the common NgR-receptor complex. Therefore we genetically modified TEG3 to express a soluble form of this receptor known as NgR(Ecto) (Fournier et al., 2002a). This ectodomain has been show to interact with both MAIs and the complete NgR1 inhibiting their signalling. We observed that NgR(Ecto)-TEG3s express all the typical marker for OECs and both of the morphological phenotype

described in literature were present in the culture. By Western blotting analysis we could confirm the high expression of the NgR(Ecto) both at cellular and extracellular level. This data suggest that if implanted after SCI cells would secrete the ectodomain in the extracellular space. By time-lapse analysis of migrating cell we could demonstrate that although NgR(Ecto)-TEG3 and normal TEG3 show the same velocity over laminin substrate the first one, over myelin, show an enhanced migration. Giving this result we can infer that the interaction of the ectodomain with the myelin present in the substrate or the NgR-complex is causing this enhancement. The recovery in migration capacity observed in NgR(Ecto)-TEG3s is not complete, suggesting that other factors are involved in this process. Indeed, OECs could present MAIs receptor or/and myelin may contain other inhibitors. For example, recent studies reported that lipids such as sulfatide (Winzeler et al., 2011), could exert inhibitory action on axonal growth. Therefore this lipid could act even on OECs. NgR(Ecto)-TEG3 present random migration as described for normal TEG3 and this suggest that other factors may be needed in order to generate a persistent and directed migration (Huang et al., 2008; Nocentini et al., 2012). When implanted in not-lesioned spinal cords NgR(Ecto)-TEG3 migrated longer distances from the site of injection. Although a "real lesion" was not present to inject cells the tissue has to be disrupted therefore myelin debris and inflammation even though in minimal concentration may be present. This result is encouraging and highlights that the NgR(Ecto) exerts his action even in vivo situation and that modified TEG3 integrate well in lesion models. Although our data was not significant, we observed a slightly higher number of cells in presence of the ectodomain. This may suggest that the NgR(Ecto) could exert an action on OECs cellular survival or proliferation rate. Indeed, in a recent study it was observed that a soluble NgR fusion protein could enhance the proliferation of neural progenitor cells via the activation of Notch pathway *in vitro* (Li et al., 2011). Although this pathway may not control the proliferative capacity of OECs, it has been observed that its activation leads to an increase of production of nerve growth factor by OECs in culture (Fei et al., 2010).

Further studies are necessary to characterize the effect of the secreted NgR(Ecto) on OECs. But, in case of a lesion, if modified OECs were implanted, NgR(Ecto) would be secreted continuously. This ectodomain could interfere with myelin/NgR-complex impairing the inhibitory effect on both OECs migration and axonal regeneration

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RESUME OF RESULTS AND DISCUSSION

OECs implantation has emerged as a promising therapy for SCI, but their regenerative properties seem to depend on the presence of a substrate permissive for cell migration (Gudino-Cabrera et al., 2000; Moreno-Flores and Avila, 2006; Ramon-Cueto and Munoz-Quiles, 2011). Although several studies reported that OECs are able to migrate for long distances from the injection point in SCI (Ramon-Cueto et al., 1998; Resnick et al., 2003), few have adequately pre-labeled cells before transplantation. In fact, other studies where OECs were labeled by viral vector-mediated expression of GFP revealed that localization of OECs, after the lesion, could be due to passive spreading rather than active migration (Boyd et al., 2004; Lu et al., 2006). Only few factors have been described to functionally modulate OECs migration. Among them, the Nogo66 that significantly decrease their migration rate (Su et al., 2007). Nogo66 is a functional domain of the myelin-derived inhibitor NogoA. This suggested us that myelin could be able to modulate OEC migration. Indeed, after SCI myelin debris are present at the site of lesion and are in part responsible of the failure of axonal regeneration (Wang et al., 2002b). Therefore, in our study we examined the effect of MAIs on a rodent clonal OEC, TEG3. We demonstrated that these cells express all the components of the NgR-complex (the common receptor for MAIs) and that their migration is blocked by MAIs (Nocentini et al., 2012). Moreover we quantitatively demonstrate that TEG3s largely decrease their traction stress over myelin. This data correlated with decreased focal contacts and redistribution the of F-actin cytoskeleton. The incubation with the NgR1 blocking peptide, NEP1-40, partially overcome MAI-mediated migratory inhibition (Nocentini et al., 2012). Giving these results we explored the feasibility of two different strategies to enhance OECs migration. One was the possible use of MNPs to try to guide the TEG3 cells (Riggio et al., 2013). Although this technique would not increase the intrinsic migration speed of cells it would permit the forced driving and concentration of cells in a specific area after implantation (Vittorio et al., 2011). Indeed we could prove that MNPs-TEG3 can be guided by a magnetic field in culture and that cells integrate well in an organotypic model of spinal cord/sciatic nerve regeneration (Riggio et al., 2013). The second strategy was to genetically modify the TEG3 to express an ectodomain for NgR1 (Fournier et al., 2002a) to block the negative effects of MAIs. We could observe that modified cells migrated more *in vitro*

over a myelin substrate and *in vivo* after implantation in a not-lesioned spinal cord. The results obtained indicate a therapeutic potential of both of these strategies. A resume of the single results and their discussion is hereafter.

Why did we use a cellular line of OECs?

A stated before OECs have been used in many transplantation experiments with a variety of outcomes (see Introduction for details). To date the majority of OECs used in SCI regeneration studies derive from primary cultures. However, these primary cultures of OECs are not the best sources to obtain suitable transplantation material in SCI research as grafts often contain contaminating cells. In addition, their efficacy to support regeneration depends on the culture method and previous preparation that may differ from one laboratory to another. Moreover, as OECs primary culture usually survive only a few week in culture, their constant preparation from fresh tissue is needed and it is subject to batch-to-batch variability even in the same conditions and laboratory.

Isolation of OECs from the olfactory mucosa and olfactory bulb may yield cultures contaminated with SCs or fibroblasts. One reason for this has been the paucity of phenotypic markers that can provide unequivocal identification of OECs versus SCs *in vitro* (Rizek and Kawaja, 2006). These cells could be responsible for the remyelination observed after transplantation in SCI (Rizek and Kawaja, 2006). Indeed, in a study it was observed that OECs, but not SCs, express calponin, a muscle fiber actin-binding protein, and that many cells in OEC culture preparations are calponin-negative (Boyd et al., 2006). Anyway, Franklin et al, using a OECs line achieved remyelination providing a strong counterargument to the theory that SCs contamination accounts for remyelination by OECs (Franklin et al., 1996). A recent study demonstrated that calponin was present in the olfactory fibroblast meningeal cells but not in the adult OECs (Ibanez et al., 2007), strengthening the argument that OECs can form myelin. Fibroblasts contaminating the culture has been shown to promote the proliferation of OECs (Yui et al., 2011), therefore the presence of these cells could alter the behavior of the transplanted OECs.

Primary OECs cultures purified using method of differential cell adhesion (DA-OEC) or separated with immunomagnetic beads (IB-OEC) showed different efficacy in supporting regeneration. After SCI, short-term (3weeks) cultured DA-OECs supported

regrowth and attenuated retrograde degeneration, but IB-OECs failed to promote axonal regeneration (Novikova et al., 2011). In contrast, long-term cultured OEC neither enhanced axonal growth nor prevented retrograde cell death (Novikova et al., 2011). The results suggest that not only the method of cell purification but even the age of OEC in culture affect the efficacy of OEC to support neuronal survival and regeneration after SCI.

To study the molecular and cellular characteristic responsible for OECs regeneration enhancing properties and their motility under different conditions a homogenous population of cells is needed. One specific rat OECs clonal line, TEG3, proved to be as good as primary OECs in promoting axon regeneration in culture models and in an animal model of SCI (dorsal column crush; (Moreno-Flores and Avila, 2006). In transplanted animals, TEG3 cells grafts survived for at least 10 weeks after grafting (although our preliminary data indicate less survival time); sensory projection axons grow into the lesion site and there was robust sprouting/axonal growth of the CST (Moreno-Flores et al., 2003; Moreno-Flores et al., 2006). Therefore we choose to use this line to explore OECs migration properties in presence of one of the inhibitors of regeneration after SCI: myelin. Indeed, immortalized cell lines could lead to the formation of gliomas at late passages (round passage 25-30). Therefore we amplified cells at passage 2-3 and froze them in order to have a large stock of cells with which we could work. The first step of our study was to characterize this cell line. We observed that cells express all the typical markers of OECs: S100β; GFAP; p75. Moreover they can acquire AC-like or SC-like morphology, like primary cultured OECs. It had already been demonstrated that TROY and NgR1 were expressed in OECs (Su et al., 2007; Morikawa et al., 2008). However we reported for the first time that cultured OECs express all the element of the NgR-complex. In addition, we demonstrate that this receptor complex is active since OECs activate RhoA and increase ERK1-2 phosphorylation in response to myelin (Nocentini et al., 2012). The activation of the epidermal growth factor receptor (EGFR) due to the transactivation process, described in neurons, may account for this increase in phosphorylation (Koprivica et al., 2005). Indeed, in neurons, activation of NgR-complex leads to direct activation of RhoA, which activates ROCK and MLC in sequence, resulting in rearrangement of the cytoskeleton and inhibition of axonal growth (Fig. 2 page 9). Moreover, the rise in free intracellular calcium concentrations, induced by NgR activation leads to the

transactivation of the EGFR with the consequent activation of the ERK cascade (Koprivica et al., 2005; Chuderland and Seger, 2008)

OECs migration properties

Although OECs have been implanted extensively after SCI their migration properties are still not well known from the biophysical point of view. Regenerative capacity of OECs seems to depend on their migration properties. Indeed, in the olfactory system OECs migrate ahead of newly generated axons guiding and providing them a substrate permissive to growth (Ekberg et al., 2012). Therefore, in our study, using time-lapse imaging we examined the migratory properties of single OECs (Nocentini et al., 2012). We considered the whole population of OECs as single one and didn't differentiate between the 2 subpopulation (AC- or SC- TEG3) observed in culture. In fact, it was demonstrated that OECs can change from one morphology to the other spontaneously and this change is not due to environmental stimuli (Huang et al., 2008). We could observe the same behavior in our cultured TEG3s, supporting the idea that OECs are a single type of cell with highly malleable phenotypes.

Laminin was chosen as permissive substrate as it is spacio-temporally expressed in the developing olfactory nerve pathway. Moreover it had been demonstrated that, when OECs are grown on laminin substrate, they are more motile that on other substrates like collagenI or poly-L-lisine (Huang et al., 2008).

From a biophysical point of view we observed that cultured OECs migrate in an antipersistent manner both over laminin (permissive substrate) and myelin on glass and PAA gels. These results may partly explain why OECs have to be transplanted on both sides of a lesion and close to it to be effective (Gudino-Cabrera et al., 2000; Pearse et al., 2007). We propose that additional factors are required to generate a persistent and directed movement of OECs *in vivo*, as reported *in vitro* (Huang et al., 2008; Huang et al., 2011). Until now, only few factors have been described to modulate OECs migration (Fig. 10).

GDNF has been shown to promote OECs migration *in vitro* and *in vivo* (Cao et al., 2006). This factor operates through a receptor complex consisting of Ret and GFRα-1 to activate JNK and finally enhance OECs migration (Cao et al., 2006). Combining

OECs transplantation with GDNF delivery increased OECs migration properties in not-injured spinal cords, but no chemotactic effect was observed (Cao et al., 2006).

OECs appeared to have enhanced motility toward a gradient of LPA (Huang et al., 2008). When a LPA gradient was applied in culture, cells appeared to have enhanced motility compared with the control situation. Their leading processes became longer and the lamellipodia became larger with increased dynamics and finally, the cells were attracted to the source of LPA (Huang et al., 2008). LPA exerts this effect acting on LPA receptors and subsequently activating multiple intracellular signaling including RhoA activation,the stimulation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI-3K) (Yan et al., 2003). LPA has an important role in mediating inflammation and demyelination after SCI and it seems that can promote neuronal death (Goldshmit et al., 2012).These effects are achieved by an up-regulation of the LPA receptors in the cells after the injury (Goldshmit et al., 2012). To date, it is not clear whether LPA concentrations increase after SCI, therefore this factor could exert no effect on implanted OECs migratory capacity.

The repulsive guidance factor Slit-2 has been shown to guide OECs migration (Huang et al., 2011). Indeed, a gradient of Slit-2 in front of cultured OECs first caused the collapse of the leading front, then the reversal of cell migration. Frontal exposure of this gradient triggers an elevation intracellular Ca^{2+} at the leading front of the soma that leads to the activation of cofilin, whose activity is responsible for the disruption of F-actin and subsequent collapse of the leading front (Huang et al., 2011). Interestingly the reversal of the soma migration depended on the reversal of RhoA activity across the cell. Free migrating OECs exhibit a front-high and back-low polarity of active RhoA, that in presence of Slit-2 was reversed (Huang et al., 2011).The mechanism by which Slit-2 alters RhoA activity remains elusive, but a recent study observed that Slit-2 might inactivate Fyn, maybe acting through the CXCR4 receptor. Fyn is a kinase that forms complexes with Robo1 (Liu et al., 2012). Therefore it was suggested that Slit-2 deactivates Fyn and then activates RhoA through Robo1 signaling (Liu et al., 2012).Because Slit-2 is highly expressed in the OE, it is likely that it might help OECs and olfactory axons migrate out of the OE through chemorepulsion during early development. Slits expressing in the OB might also regulate the stop and scattering of OECs that have arrived at the surface of the OB (Huang et al., 2011). In one study, Slit-2 expression was not detected after SCI in or around the lesion point (Wehrle et al.,

2005). Anyway, another group observed that the Slit2 expression level increased at day 7 until day 14 after, and then returned to normal level at day 21 after injury (Liu et al., 2011). Therefore Slit-2 could play a role in modulating OECs migration after SCI.

Recently, a study by Su et al. indicated that TNF-α released by reactive astrocytes in the meningo-glial scar attracts OECs *in vitro* (Su et al., 2009) via binding to the receptor TNFR1 and subsequent activation of ERK1-2. A linear expression gradient of TNF-α has been detected in the glial scar after spinal hemisection and this could conceivably regulate the migration of transplanted OECs (Su et al., 2009). As TNF-α levels peak 7 days after SCI one of the reason that studies failed to show OECs migration towards the lesion, could be that the timing or location of OECs transplantation may not have coincided with the TNF-α levels required for correct chemotaxis (Chuah et al., 2011). This data may also explain why OECs invade the lesion cavity containing reactive glia when transplanted at short distances (Pearse et al., 2007; Chuah et al., 2011). Other reason why OECs transplanted into the lesion cavity only migrate over short distances in adjacent regions could be the presence of MAIs. Indeed, areas surrounding the lesion are rich in myelin debris as well as other inhibitory molecules (Yiu and He, 2006; Yang et al., 2011).

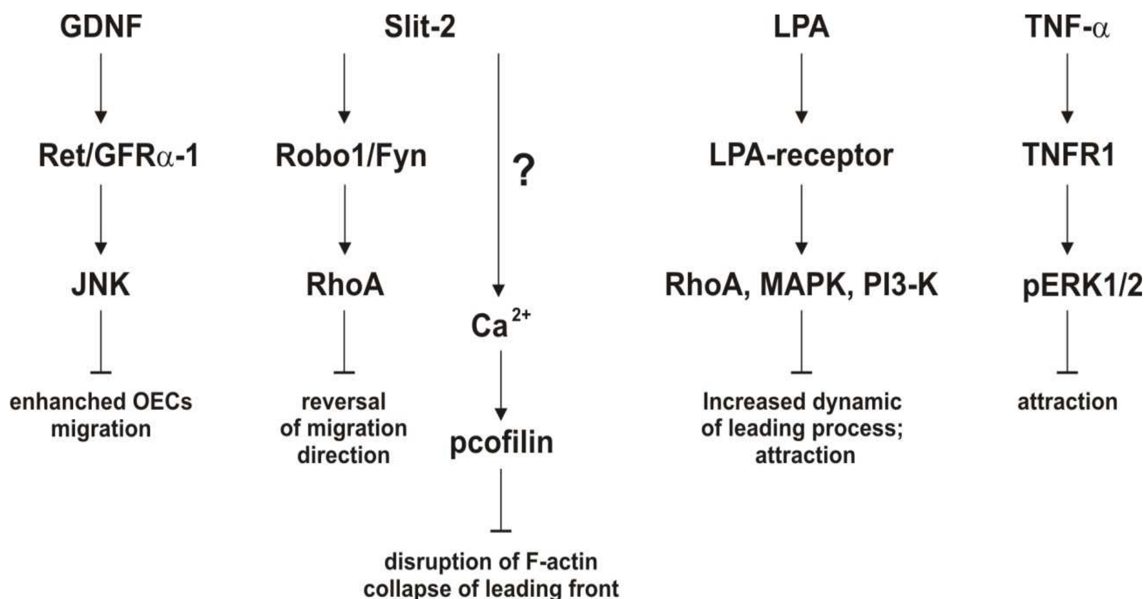

Figure 10: Factors able to modulate OECs migration. A resume of the factor that, to date, have been described to modulate OECs migration and the possible targets and signaling pathway activated by this factors.

OECs behavior over myelin

Data from other studies showed that both rat and human OECs migrated shorter distances in lesioned spinal cord than in not lesioned one (Gudino-Cabrera et al., 2000; Deng et al., 2006). Moreover it has been demonstrated that the migration of implanted OECs is facilitated by neutralizing NgR1 using anti-NgR1 antibodies (Su et al., 2007). However the specific mechanisms for the poor motility of OECs in the damaged CNS remain unknown.

In the present study we show that OECs cultured on myelin decreased their migration potential, spreading and presented fewer FAs (vinculin-positive) both over glass or PAA in comparison to cells grown over laminin (Nocentini et al., 2012). These data are in contrast with what was observed by Su and coworkers. In fact, although they observed a reduced migration of OECs cultured on Nogo66- or NogoA-His coated coverslips, cells showed enhanced formation of FAs (paxilin-positive) and increased spreading (Su et al., 2007). The differences between our data and those reported in this study could be attributed to their use of a recombinant peptide or NogoA protein instead of complete myelin extract (containing several inhibitory proteins and lipids). Indeed, the cell spreading mechanism in OECs could be similar to that of other cell types. A study on fibroblast spreading in culture reported that while Nogo66 and Nogo66 peptide 4 (the inhibitory regions of Nogo66 (GrandPre et al., 2002)) are not inhibitory for fibroblasts spreading in culture, two NogoA regions (59–172 aa and 544–725 aa) induced inhibition of cell spreading (Oertle et al., 2003). Therefore, specific NogoA domains may have a different effect on OECs spreading. Moreover, higher concentration of the cell spreading inhibitors peptides impaired cell adhesion (Oertle et al., 2003). For rapid cell movement, the adhesion force needs to be optimum (different for each cell type). When adhesion is below or above this optimum cells move slower (Ananthakrishnan and Ehrlicher, 2007). This could be the reason why, in our study, OECs over myelin showed a decrease in their migration capacity.

Cell migration is a complex mechanism that relies on the interplay between extension and contraction of the cell cytoskelekon, and the formation and retraction of its adhesions. Traction forces come into play in the synchronization of these mechanisms by causing forced de-adhesion at the trailing edge and by driving the resulting forward motion of the cell body. In our study OECs grown over laminin

exhibit high motility in parallel with increased concentration of FA proteins and a high traction force while on myelin cells show reduced migration capacity and decrease in FAs number and traction force (Nocentini et al., 2012). These data are in accordance with the observation that in migrating fibroblast near the leading edge, small nascent FAs transmit strong propulsive traction forces (Beningo et al., 2001). The subsequent maturation of FAs result in a shift of their function from transmitting force for strong propulsion to maintaining spread cell morphology as a passive anchorage apparatus (Beningo et al., 2001). This may in part explain the results obtained of Su et al. (2007) in which OECs presented reduced migration but an enhanced presence of FAs over Nogo66 substrate (Su et al., 2007). Indeed, cells, in this case, showed flat-sheet morphology, typical of static cells, suggesting a maintenance role of FAs.

Myelin modulates cytoskeletal organization of OECs

Although further studies are needed to describe the mechanisms by which MAIs exert their action on OECs adhesion, it has been described that the N-terminal domain of NogoA inhibits COS7 adhesions acting through integrins (Hu and Strittmatter, 2008). Integrins are cell surface glycoproteins that mediate cell–cell and cell–ECM interactions. Integrin engagement of ECM ligands results in the formation of adhesion complexes that provide a coupling to the actin cytoskeleton that is necessary for cell spreading and for the force generation during growth cone advance (Beningo et al., 2001). FAK becomes activated after integrin activation and its activity is required for the formation of growth cone point contacts to promote rapid neurite outgrowth by stabilizing lamellipodial protrusions (Robles and Gomez, 2006). In COS7 amino-Nogo binds to and selectively blocks signal transduction initiated by certain integrins leading to a decreased activity of the FAK and final reduction of focal contacts and cellular adhesion to the substrate. Thus, MAIs may act at this level to modulate cellular adhesion even in OECs (Fig. 12).

Recently it was shown that a gradient of MAG could stimulate NgR-complex dependent focal Ca^{2+} signals in growth cones of *Xenopus* spinal neurons that are necessary for chemorepulsion (Hines et al., 2010). These Ca^{2+} signals induce polarized endocytosis and concomitant asymmetric loss of β1-integrin and vinculin-containing adhesions on the repellent side during repulsive turning. Such redistribution allows the

growth cone to rapidly adjust adhesiveness across its axis and is an essential feature for initiating chemotactic turning (Hines et al., 2010). In our case this active endocytosis could explain why cells grown over myelin exhibit less number of FA-vinculin positive.

In addition to Ca^{2+} signal, NgR activation can stimulate the RhoA-ROCK pathway which is known to regulate the actin cytoskeleton and adhesion complexes (Yamashita et al., 2002). For a productive migration, RhoA activity has to be correctly spatial regulated. Indeed, RhoA must be actively inhibited at the leading edge to allow lamellipodial protrusions formation but activated at the rear of the cell to limit membrane activity in this region. Moreover the RhoA pathway activates downstream of repulsive guidance cues leading to growth cone collapse in neurons. In OECs MAIs interacting with NgR activate RhoA, as demonstrated by western blotting (Nocentini et al., 2012), and this in turn could lead to an incorrect spatial regulation of the pathway or activation of downstream of repulsive cues with the final result of reducing OECs migration capacity.

Integrin receptor or LINGO-1 (a component of the Ngr-complex) activation can modulate the activity of another small GTPase of the Rho family, Cdc42. Recently it was observed that Nogo66 could reduce the activity of Cdc42 in microglial cells strongly inhibiting their adhesion and migration capacity *in vitro* (Yan et al., 2012). Cdc42 is the main regulator of the formation and extension of filopodia and an upstream signal molecule for RhoA in the control of formation of protrusion and adhesive complex and therefore in the control of cell migration (Yan et al., 2012). A decrease in its activity may be responsible for the failure of polarization and protrusion formation in OECs, leading to a reduced migration capability in presence of myelin.

Another mechanism by which MAIs may modulate OECs migration is by compromising microtubule stabilization (Mimura et al., 2006; Llorens et al., 2011). Indeed, MAG and NogoA have been show to increase the phosphorylation of CRMP-2 (inactive), and in turn microtubule disassembly, downstream of RhoA/ROCK in postnatal cerebellar neurons (Mimura et al., 2006). Microtubules, in migrating cells, seem to be physically associated with FAs and appear to be essential in the control of their dynamics. Indeed, the stabilization of microtubules may facilitate the transport of integrins and participate in the growth of early FAs at the leading edge of migrating cells (Gu et al., 2011).

Molecules present in the myelin other than MAIs may interact with the NgR-complex and/or with other undetermined MAIs receptors expressed by cultured OECs (Fig. 10). Canonical axon guidance molecules such as the netrin, semaphorin and ephrin families are expressed through development and in the adult CNS. Both netrin-1 and its receptors are expressed in the intact and lesioned adult CNS. Netrin-1 is expressed by oligodendrocytes in similar location to that described for two MAIs, OMgp and MAG. The repulsion-mediating class of netrin UNC5 receptors is expressed by rubrospinal and corticospinal motor neurons, and by neurons in all laminas of spinal cord gray matter (Low et al., 2008). Its signaling downstream is relatively poorly understood. Anyway, neutralization of netrin signaling in extracts of adult spinal cord myelin enhances neurite outgrowth *in vitro*, and axonal growth is inhibited in a zone containing netrin-1 that is devoid of other MAIs in *in vivo* sites of SCI (Low et al., 2008). Thus, these results suggest that netrin-1 is an oligodendrocyte-associated inhibitor to regeneration in the adult spinal cord. In mice, ephrin-B3 functions during development as a midline repellant for axons of the corticospinal tract. In adult mice, it is expressed in myelinating oligodendrocytes and it was observed that could act on EphA4 receptor to activate RhoA to inhibite neurite outgrowth in culture (Benson et al., 2005). Sema4D is a membrane-bound semaphorin expressed by oligodendrocytes and myelin. It has been shown to induce the collapse of growth cones of CNS axons, operating through the plexinB1 receptor to activate RhoA (Moreau-Fauvarque et al., 2003).This molecule seems to be up-regulated upon SCI (Moreau-Fauvarque et al., 2003). These results do not exclude possible roles for these molecules in the modulation of OECs migration when implanted after SCI.

Recently it has been observed that sulfatide, that comprises 4-7% of CNS myelin lipid (Norton and Autilio, 1966) contributes to axon regenerative failure *in vitro* and *in vivo* (Winzeler et al., 2011). The mechanism involved in sulfatide-mediated inhibition is not well known but may share features with other known inhibitors, because the Rho activity inhibitor lessened the effects observed (Fig. 11).

Indeed, all these molecules present in the myelin may exert their function over OECs. In fact, we reported that the incubation of TEG3 with NEP1-40 peptide, blocking NgR function, leads to a moderate recovery of the migratory properties, traction forces and cytoskeletal organization.

Our results reinforce the notion that molecules present in myelin extracts act on NgR-complex as well as on other receptors, and in this way may modulate OECs cytoskeleton, FAs distribution and migration.

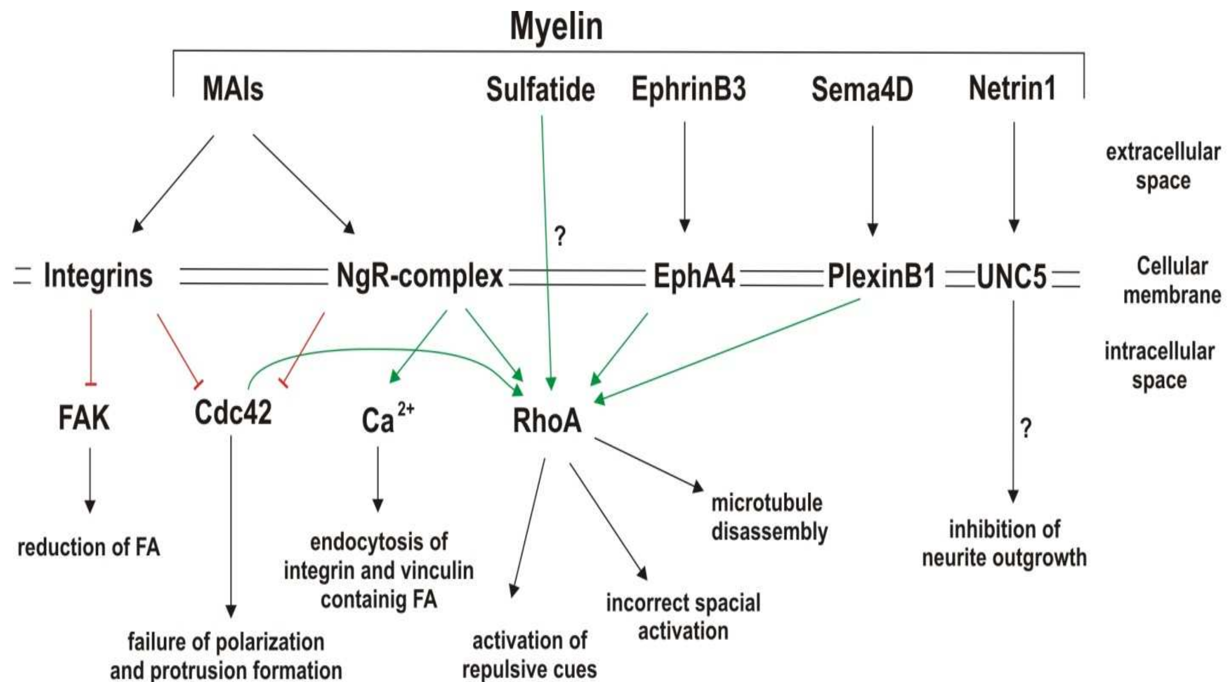

Figure 11: Mechanisms by which MAIs or other inhibitors may act on OECs. A schematic view of the mechanism by which myelin could act on OECs described in the chapter above in more detail. Although this pathways may contribute in the modulation of the migration of OECs further study are necessary to prove their importance in this cell type.

MNPs interaction with OEC *in vitro*

It has been observed that OECs once implanted intersperse amongst other cells in the host tissue (Ruitenberg et al., 2002). It could be possible that they do not maintain a high degree of cell-cell contact with each other and therefore do not form a continuous uniform mass. In an injury model *in vivo* it was observed that in the absence of axons, OECs rapidly form an extensive uniform mass and that the presentation of OECs in this format results in a subsequent higher axon growth (Chehrehasa et al., 2010). Therefore, it would be of interest to examine whether a delivery of OECs, which would encourage their migration as an uniform mass within spinal transplant models would result in an

improvement of the axonal growth. In order to control the location of the transplanted OECs, in our study we propose a strategy based on the use of MNPs. MNPs have been used for noninvasive imaging of transplanted OECs in SCI (Sandvig et al., 2012). Cultured OECs readily take up MNPs from a suspension resulting in a high level of cell labeling and this allowed labeled cells to be readily identified by MRI (Sandvig et al., 2012). Anyway with this method, it can be possible to control the varied migratory behavior of OECs subpopulations and concentrate cell at a specific location. The MNPs we used have a larger saturation magnetization compared to commercial ones (Riggio et al., 2012). As the magnetic force exerted on a single cell is proportional to the magnetic moment of the incorporated MNPs for a given magnetic field value we expected a high efficiency in controlling cell migration. First of all we demonstrated that the magnetization protocol we developed did not alter biological features of OECs. By electron microscopy we could observe that most of MNPs were found inside the cytoplasm in form of agglomerates. This tendency for particle agglomeration can be explained by the strong dipole-dipole interactions among magnetic particles. It has been reported that MNPs are normally ingested via endocytosis (Lee et al., 2004) and the products of MNP degradation could be responsible for the production of reactive oxygen species (Riggio et al., 2012). However, at the concentration of use the toxicity of MNP is negligible. Indeed, Western blot analysis revealed that MNP labeling does not induce activation (or phosphorylation) of proteins involved in cell replication, apoptosis and cell death. The same results were obtained by both quantitative and qualitative propodium iodide dye exclusion assays, which confirmed cell viability exceeding 90% at all concentrations tested. Likewise, there was no cytoskeletal reorganization, as demonstrated by actin staining (Riggio et al., 2013). Therefore these MNPs can be considerate safe for biological use at the concentrations tested. Moreover, OECs containing MNPs don’t show migration capacity impairment.

Magnetic fields can guide and control location of MNP-OECs.

The possibility of shepherding OECs migration toward a magnetic source is significant in terms of therapeutic use of these cells. We performed cell migration experiments to demonstrate that magnetic labeling induces controlled and efficient cell directed migration under the influence of magnetic field gradients. These results are in

agreement with previous *in vitro* finding where MNP-labeled human neuroblastoma cells where guided towards an external magnetic field (Riggio et al., 2012). Also, with *in vivo* experiments, where MSCs, were transplanted intrathecally via lumbar puncture and guided near the site of SCI under a magnetic field (Vanecek et al., 2012). Moreover it was observed that the targeting efficiency of MNPs-MSC could be increased by using magnets that produce spatially modulated stray fields (Vanecek et al., 2012). These magnets with tunable geometric parameters (Fig. 12), in the future, might provide the additional level of control needed to enhance the efficiency of cell delivery in SCI *in vivo*.

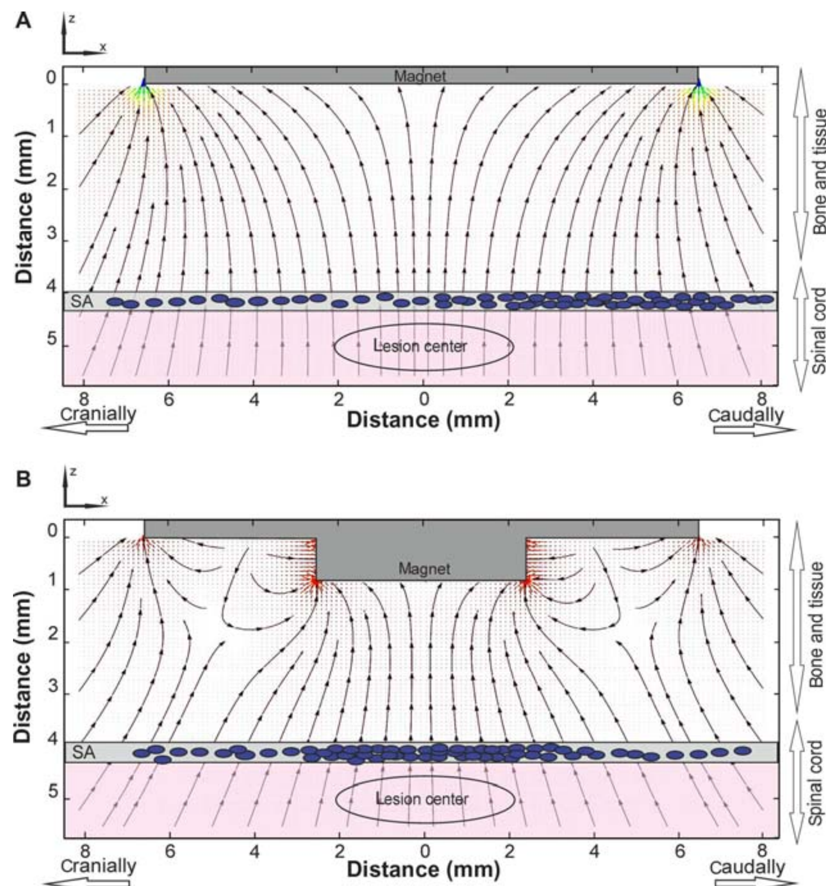

Figure 12: Magnetic force lines near the surfaces of flat (A) and stepped (B) magnets. The arrow lines show the directions of forces acting on a magnetically labeled cell. The corresponding distribution of transplanted cells in the subarachnoid space of the spinal cord under both types of magnet is schematically shown by the blue dots. The areas with the highest values of the magnetic force are progressively marked in green, orange, and red (Vanecek et al., 2012).

Integration of MNPs-OECs in an organotypic culture

We used an organotypic culture of spinal cord and sciatic nerve to analyze the survival and integration of MNP-OECs. Indeed, these cultures are very similar biochemically and physiologically to *in vivo* tissue and are also very useful for studies on neural regeneration and drug delivery (Dash et al., 2012). To our knowledge, this is the first report of transplantation of magnetized OECs in 3D co-cultures of spinal cord and peripheral nerve. Sciatic nerve from neonatal mice was placed in front of the ventral roots of the spinal cord slice to allow motor neurons to innervate the nerve (Riggio et al., 2013). After seven days of incubation, axons from motor neurons of the ventral horn of the spinal cord reached sciatic nerve explants. Hence, this system represents a good *in vitro* model for studying regeneration of a peripheral nerve. MNP-OECs were added to the model in order to assess the survival and the behavior of grafted cells. The results obtained indicate that MNP-OECs are able to integrate into the organotypic culture with some of them in close relationship with growing axons (Riggio et al., 2013). Thus, the behavior of MNP-OECs is similar to those reported in *in vivo* studies.

The NgR(Ecto) enhances OECs migration properties

As we demonstrate that myelin inhibits OECs migration a strategy to enhance OECs migration would be by interfering with the myelin signal. The NgR(Ecto) binds to NogoA (one of MAIs) and, thereby, prevents its binding to the NgR-complex. Moreover the ectodomain interacts with full-length NgR1, blocking the intracellular signaling. In vitro studies showed that NgR(Ecto) can act as an antagonistic for CNS myelin (Fournier et al., 2002a; He et al., 2003). Moreover, following SCI, NgR(Ecto) promoted regeneration and functional recovery (Li et al., 2004; Wang, X. et al., 2006). When TEG3 were genetically modified to express the ectodomain their migration properties over myelin were mainly restored. As commented before although the NgR seem to be the primary mediator of myelin action other receptor on OECs could be involved in the inhibitory signaling. As preliminary experiment we implanted modified OEC into not injured spinal cord. Although a “real lesion” was not present to inject cells the tissue needs to be disrupted, and therefore myelin debris and inflammation, even though in minimal concentration, may be present at the site of implantation. A week after implantation we could observe that NgR(Ecto)-TEG3

migrated longer distance in comparison to not-modified ones. This result is encouraging and points out that the NgR(Ecto) exerts its action even in an in vivo situation.

It has been observed that after axonal injury, NogoA expression is increased in dorsal root ganglion neurons which are unable to regenerate following injury (Peng et al., 2010), and that in vitro myelin enhances NogoA mRNA production (Peng et al., 2010). The incubation with a soluble form of NgR down regulated this over-expression and enhanced regeneration (Peng et al., 2010) indicating another mechanism by which the NgR(Ecto) could exert its action. Indeed in the case of a lesion, if modified OECs were implanted, NgR(Ecto) would be secreted continuously. Then the ectodomain could interfere with myelin/NgR-complex impairing the inhibitory effect and in parallel could modulate NogoA expression: In that case, the final result would be the enhancement of OECs migration and the proper facilitatition axonal outgrowth (Fig.13).

Although results from our study were not significant, due to the low number of animal used, we could observe a slightly higher number of eGFP/NgR(Ecto)-TEG3s in comparison to eGFP-TEG3s in spinal cords a week after implantation. This suggests that the ectodomain NgR(Ecto) could exert an action over TEG3 cell survival or proliferation in vivo. Recently it was observed that a soluble form of NgR

could induce neural progenitors cell proliferation activating the Notch pathway (Li et al., 2011). The effect of this pathway on OECs proliferation has not been studied yet, but it was observed that Notch activation can increase the synthesis of the nerve growth factor (NGF) in OECs *in vitro* (Fei et al., 2010). As OECs express the receptor for this factor, NGF could act both in a paracrine and autocrine manner (Fig. 13). Indeed, increased expression of NGF after SCI induces sprouting of primary nociceptive axons leading to autonomic dysfunction and pain. Anyway, from the results obtained in our study, we cannot infer that the levels of NGF expression in the modified TEG3 were different to the ones in normal TEG3 as this would need further studies.

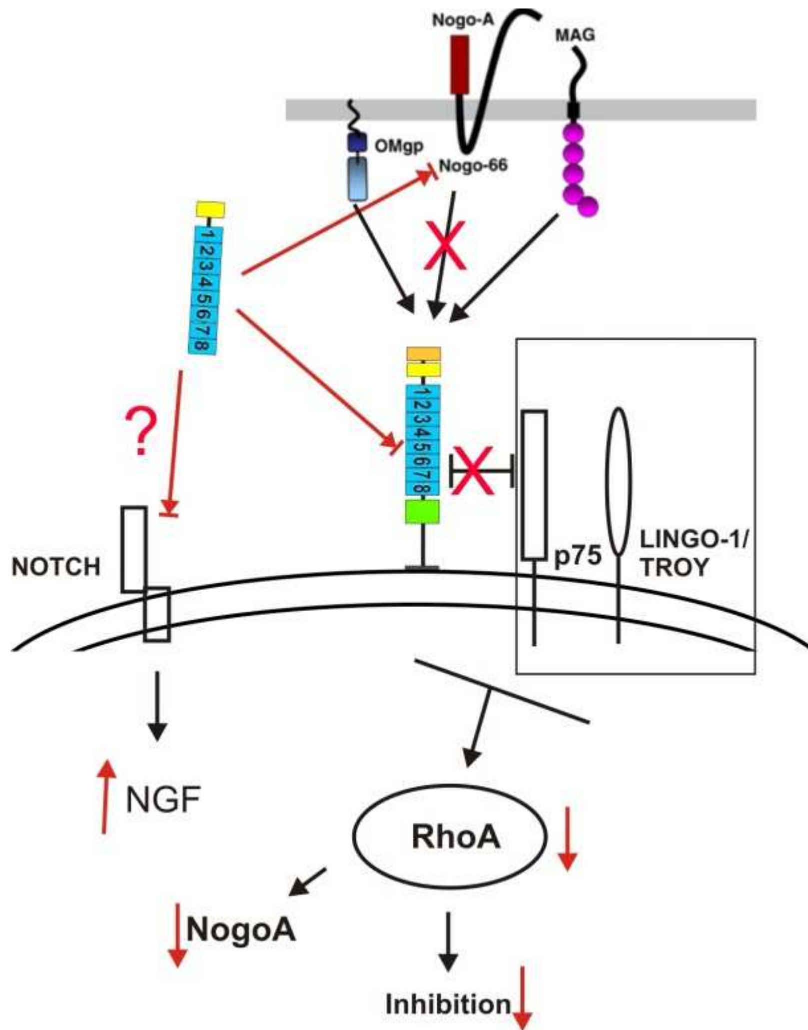

Figure 13: Possible way of action of the Ngr(Ecto) are schematized in this figure. The ectodomain interacts with NogoA inhibiting his interaction with th NgR-complex. Moreover it can bind to NgR1 inhibiting intracellular signaling that leads to inhibition of outgrowth and NogoA sintesis. It could even interact with the Notch receptor to increase expression of NGF.

OECs for spinal cord repair

Cell transplantation for SCI may promote regeneration and rescue impaired neuronal function as grafted cells may secrete permissive neutrofic factors at lesion site to create an environment more conducive to axonal regrowth and remyelination. Moreover implanted cells help filling in the lesion and provide a scaffolding to support regeneration. Given their properties OECs have been extensively implanted after SCI. However, high variable neuroanatomical and functional outcomes have been documented (as commented in the Introduction). This is due in part to the source of the

OECs and the purification methods used in the trials. Indeed, each laboratory optimizes his own dissection, purification and culturing protocols. The result is that there are almost as many different preparations of OECs as models of neural injury into which the OECs are transplanted. Although several authors examined how OECs interact with growing axons (Sorensen et al., 2008; Chuah et al., 2011) only recently advances have been made in determining how OECs respond to guidance cues in order to improve their effectiveness after transplantation. From the data of our study we can infer that OECs migration is inhibited by the presence of myelin and that this property is partly reversed by blocking NgR; that this cells move in antipersitent way and therefore other factors may be involved in generating a persistent and directed migration. These are factors that have to be taken in account for future research over OECs. We also observed that OECs cultured on PAA substrates, the mechanical properties of which clearly differ from coated glass slides, increase their migratory potential. In this regard, recent data report increased proliferation and migration of OECs growing on several scaffolds (Wang, B. et al., 2006b). Thus is possible that nanostructured scaffold could be used as a mechanical guide for OEC migration as they would not only increase OECs migration but could help preventing the interaction of these cells with putative inhibitory molecules in SCI models.

Future experiments with OECs

As described before we only implanted NgR(Ecto)-OECs in not lesioned spinal cord to prove if they would integrate and migrate in an *in vivo* system. Further studies are necessary to observe their behavior in a SCI model. Indeed we could combine the use of these modified cells with MNPs and/or the application of axonal growth factor. With the design of specific magnetic sources we could concentrate OECs near the lesion site and therefore increase their possible interaction with damaged axons. Moreover the secretion of NgR(Ecto) from OECs and application of axon growth promoting factor could enhance the sparing of these axons in part by shedding the myelin present in the lesion site and by enhancing axon's natural regenerative properties.

CONCLUSIONS

CONCLUSIONS

1. Cultured OECs express all the element of the NgR-complex. In addition, this receptor complex is active since OECs activate RhoA and increase ERK1-2 phosphorylation by EGFR transactivation in response to myelin.

2. OECs grown in presence of myelin show a decrease in migratory capacity. This capacity is mainly restored in presence of NEP1-40.

3. The decrease in speed of OECs parallels with a decrease in FAs number and traction force. Even in this case, the incubation with NEP1-40 brings to a recovery of the main cytoskeletal organization observed over myelin (e.g., focal adhesion).

4. The incomplete recovery of migration speed, traction force and cytoskeletal organization, over myelin substrate, in the presence of NEP1 -40, suggests that receptor, other than NgR-complex and/or different inhibitors in myelin may be exerting a role on OECs inhibition.

5. *In vitro,* magnetized OECs can survive well without exhibiting stress-associated cellular responses. Their migration can be modulated by magnetic fields; and their transplantation in organotypic cultures of spinal cord and sciatic nerve show a positive integration.

6. Engineered OEC to express the ectodomain for NgR1 were able to express and secrete, at high levels, the ectodomain *in vitro*.

7. NgR(Ecto)-TEG3 migrate longer distances over a myelin substrate. Anyway, recovery is not complete. This suggests that other receptors and/or inhibitors of myelin exert a role in modulating OECs migration.

8. Once implanted in a not-lesioned spinal cord NgR(Ecto)-TEG3s integrate into the tissue and migrate over longer distances both caudal or rostral to the site of injection in comparison with not-engineered cells.

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RESUMEN EN ESPAÑOL

Abreviaciones

aa	aminoácidos
AC	astrocita
AFs	adhesiones focales
CS	células de Schwann
Fig.	figura
FNT	factor de necrosis tumoral
GRD	ganglios de la raíz dorsal
IAMs	inhibidores asociados a la mielina
LINGO1	LRR and Ig domain-containing, Nogo receptor-interacting protein
MAG	glucoproteínas asociadas a mielina
MT	microscopia de tracción
NEP1-40	Nogo Etracellular Peptide 1-40
NgR1	Nogo Receptor
NgR(Ecto)	Ectodominio (aa 1-310) del Nogo Receptor 1
NPM	nanopartículas magnéticas
OECs	células de glía envolvente olfatoria
OMgp	glucoproteínas de la mielina de los oligodendrocitos
PAA	poli-acrilamida
Pag.	página
SNC	sistema nervioso central
SNP	sistema nervioso periférico
TCS	tracto cortico-espinal

Introducción

1. Lesiones de la médula espinal

Las lesiones de la médula espinal son muy frecuentes en nuestra sociedad y son el principal ejemplo de lesión del sistema nervioso central (SNC). Estas lesiones ocasionan consecuencias funcionales devastadoras en los pacientes que las sufren, con secuelas motoras, cognitivas y sensoriales (Dumont et al., 2001; Furlan et al., 2013). A pesar de las numerosas intervenciones terapéuticas que se han intentado se ha tenido un éxito muy limitado (Varma et al., 2013). Al contrario de lo que ocurre en el sistema nervioso periférico (SNP), los axones del SNC maduro son incapaces de regenerarse espontáneamente tras una lesión o enfermedad (Horner et al., 2000; David and Lacroix, 2003). El primero en observar que los axones no se regeneraban en el SNC fue Ramón y Cajal en 1928 (Ramón y Cajal, 1928). Sin embargo, algunos años más tarde, Aguayo y colaboradores demostraron que los axones del SNC dañado conservan una capacidad limitada de regeneración y que pueden extenderse por largas distancias si se les proporciona un sustrato permisivo (Aguayo et al., 1981; David and Aguayo, 1981). Además, las neuronas de los ganglios de la raíz dorsal (GRD) que tienen extensiones tanto en SNC como en el SNP, sólo pueden regenerar sus procesos periféricos (Tuttle and Matthew, 1995). Estos datos sugieren que el potencial de regeneración de los axones lesionados depende directamente de la interacción con el medio ambiente y que la presencia de moléculas inhibidoras y/o receptores es responsable de la falta de regeneración observada. De hecho, parte de la inhibición observada radica en la presencia de los inhibidores asociados a la mielina (IAMs) (Caroni et al., 1988; GrandPre et al., 2002; Wang et al., 2002b).

1.1 Los inhibidores asociados a la mielina y sus receptores

Inmediatamente después de un daño a la medula espinal, los fragmentos resultantes de mielina dejan expuestas moléculas inhibidoras del crecimiento axonal como las glucoproteínas asociadas a mielina (MAG) (McKerracher et al., 1994), NogoA (Caroni and Schwab, 1988a) y glucoproteínas de la mielina de los oligodendrocitos (OMgp) (Habib et al., 1998) (Fig.1;pag.5). Estas proteínas se localizan en las capas más internas de las envueltas de mielina en contacto directo con los axones (Fournier et al.,

2002a; Profyris et al., 2004) y son capaces de interaccionar con el mismo receptor neuronal (llamado Nogo-Receptor (NgR1)) para prevenir el crecimiento axonal (Fournier et al., 2002a). Para mediar en la señalización intracelular NgR1 se debe asociar con co-receptores: p75, que interactúa con NgR1 a través de su dominio extracelular (Wang et al., 2002a) y LINGO1 (LRR and Ig domain-containing, Nogo receptor-interacting protein) (Mi et al., 2004). TAJ/ TROY, un miembro de la familia de receptores huérfano del factor de necrosis tumoral, puede sustituir p75 como un co-receptor (Park et al., 2005). Una vez se produce la unión de las moléculas inhibidoras a los receptores, se desencadenan una serie de cascadas de señalización que consiguen, en último término, el colapso de los conos de crecimiento de los axones (Fig.2; pag.9). Además de estos receptores se han descripto receptores adicionales para los IAMs, que intervienen en la modulación de la respuesta neuronal (Venkatesh et al., 2005; Filbin, 2008). Asimismo, hay otras moléculas en la mielina que median efectos inhibidores del crecimiento. De hecho, recientemente se ha observado que la presencia de sulfátido, que representa el 4-7% de los lípidos de la mielina (Norton and Autilio, 1966), contribuye a la falta de regeneración axonal *in vitro* e *in vivo* (Winzeler et al., 2011).

1.2 Estrategias para promover la regeneración axonal después de lesión medular

En los últimos años, se ha progresado enormemente en la comprensión de las componentes inhibitorias de la mielina del SNC, de los receptores axonales que responden a estas moléculas y de la señalización intracelular que media la inhibición del crecimiento de axonal. Varias estrategias han sido diseñadas para antagonizar los efectos inhibitorios de los IAMs con el fin de permitir el crecimiento axonal como paso clave para la regeneración axonal. Estas estrategias han incluido el uso de anticuerpos o antagonistas para unirse las moléculas inhibitorias e impedir su interacción con el receptor, la eliminación de estas moléculas o de sus receptores mediante el uso de animales knockout o el bloqueo de la señalización, obtenido interfiriendo con el receptor (Tabla 1; pag.20).

El antiquerpo más utilizado para el bloqueo de los IAM ha sido el IN-1, que reconoce, pero no específicamente, el dominio central de NogoA (Caroni and Schwab,

1988b). Su uso ha dado éxito en diversos modelos de lesión *in* vivo (Schnell and Schwab, 1990; Bregman et al., 1995). Pero, con el uso de ratones knockout por NogoA se observaron tres resultados diferentes tras lesión que iban desde una regeneración robusta (Simonen et al., 2003), sugerente (Dimou et al., 2006) a ninguna (Lee et al., 2009). Esta observación dio lugar a una gran controversia sobre el papel de NogoA en la regeneración axonal. En los knockout por MAG no se pudo demostrar ninguna mejora en la regeneración (Bartsch et al., 1995) mientras que los resultados obtenidos con los knockout de OMgp son controvertidos (Ji et al., 2008). Tal vez las tres moléculas necesitan ser bloqueadas contemporáneamente con el fin de promover una regeneración robusta. Para comprobar esta hipótesis se generó un triple knockout. Pero, aunque Cafferty y colaboradores observaron una mejora significativa en la regeneración axonal después de lesión en comparación con animales wild-type o knockout simples (Cafferty et al., 2010); otro grupo no pudo replicar los resultados (Lee et al., 2010).

Una estrategia alternativa para bloquear los IAMs simultáneamente es actuar sobre su receptor común. La administración del péptido NEP1-40, que tiene la capacidad de unirse a NgR1 pero no de activarlo (GrandPre et al., 2002) promovió la regeneración de los axones del tracto cortico-espinal (TCS) en un estudio (GrandPre et al., 2002). Sin embargo los efectos observados no se pudieron reproducir (Steward et al., 2008). Recientemente fue generada una forma soluble del NgR1, el ectodominio (NgR1(310)) de ahora en adelante NgR(Ecto) (Fournier et al., 2002a). Este ectodominio contiene el dominio de unión al ligando de NgR1 para unirse a NogoA, MAG y OMgp (Lauren et al., 2007) pero carece de la región carboxi-terminal, que se requiere para la señalización, y de la región de ancoraje a la membrana (Fournier et al., 2002a) (Fig. 6; pag.21). NgR(Ecto) ejerce su acción uniéndose a los IAMs, inhibiendo así sus unión al NgR1 en la superficie de las células neuronales, o interactuando con el receptor completo para evitar la oligomerización o la interacción con los otros componentes del complejo de NgR, finalmente bloqueando la señalización intracelular. NgR(Ecto) mostró propiedades antagonistas a los IAMs *in vitro* (Fournier et al., 2002a; He et al., 2003). Además, el tratamiento con éste tras lesión medular promovió crecimiento regenerativo y recuperación funcional *in vivo* (Li et al., 2004; Wang, X. et al., 2006). De todos modos, el uso de un ratón knockout para el receptor NgR1 no mostró ninguna mejora en la regeneración de TCS después de lesión (Kim et al., 2004). Este dado indica

que una estrategia que focaliza sólo en el bloqueo de NgR1 o de los IAMs no es suficiente para promover una regeneración robusta después de lesión. Por lo tanto otros enfoques se han estudiado durante los años. En particular, se ha observado que el trasplante de células después de lesión medular puede promover la regeneración y rescatar la función neuronal. De hecho, las células implantadas pueden secretar factores neutróficos permisivos en el sitio de la lesión para crear un entorno más propicio para el nuevo crecimiento axonal. Además, ayudan a rellenar el sitio de lesión y proporcionan un andamiaje para apoyar la regeneración.

2. Células de la glía envolvente olfatoria

Con el fin de promover la supervivencia celular, la regeneración axonal, remielinizar axones, reemplazar las células pérdidas o restablecer la funcionalidad, tras una lesión medular, se han trasplantado diferentes tipos celulares. Nosotros nos concentraremos sobre el uso de las células de la glía envolvente olfatoria (OECs).

2.1 Sistema olfatorio y las OECs

El epitelio olfatorio es uno de los pocos lugares del SNC en el que se produce una continua neurogénesis y crecimiento axonal durante toda la vida de un organismo (Schwob, 2002; Huart et al., 2013). Los axones de las nuevas neuronas olfatorias tienen que crecer en la dirección correcta, y extenderse a través del epitelio olfatorio que es parte del SNP, atravesar la lámina cribosa, entrar en el bulbo olfatorio del SNC atravesando la glía limitante y hacer sinapsis con las dendritas de las segundas neuronas de la vía olfatoria (Huart et al., 2013) (Fig.3; pag.14). En todo esto proceso las OECs acompañan y envuelven a estos axones (Doucette et al., 1983; Doucette, 1984). Estas células extienden procesos citoplasmáticos alrededor fascículos de axones que salen desde el epitelio olfatorio formando una estructura similar a un túnel para facilitarles la elongación y la transición de PNS a CNS (Nedelec et al., 2005). Además, las OECs son capaces de producir una variedad de moléculas implicadas en la adhesión celular que promueven la iniciación de neuritas, elongación axonal y adhesión del cono de crecimiento como, entre otras, laminina (Doucette, 1996), L1 (Miragall et al., 1988), molécula de adhesión celular neural (Chuah and Au, 1993). Asimismo, las OECs *in vitro* expresan un gran número factores de promoción de crecimiento de neuritas, como neurotrofinas 4/5 (Vincent et al., 2003), factor neurotrófico derivado de células gliales

(Lipson et al., 2003), factor de crecimiento nervioso (Vincent et al., 2003) y factor neurotrófico derivado del cerebro (Boruch et al., 2001).

2.2 Trasplante de OECs después de lesión espinal

Debido a sus propiedades intrínsecas, las OECs se han implantado en modelos de lesión de médula espinal con la esperanza de promover la regeneración axonal (Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto et al., 1998; Boyd et al., 2004; Cao et al., 2004; Lee et al., 2004; Lu et al., 2006; Gorrie et al., 2010) (Table 2; pag.20). Sin embargo, los resultados obtenidos han sido altamente variables desde un punto de vista neuroanatómico y funcional. Esto se debe en parte a la fuente de la OECs y a los métodos de purificación utilizados en los ensayos. De hecho, cada laboratorio optimiza sus protocolos de disección, de purificación y de cultivo con el resultado final de que hay casi tantas preparaciones diferentes de la OECs como de modelos de lesión en el que se trasplantan. Además las OECs tienen un fenotipo funcional altamente maleable. De hecho *in vitro* se ha descrito la posible existencia de dos subpoblaciones de la OECs. Una con morfología similar a las células de Schwann (CS) con estructura bipolar y fusiforme; y la otra a astrocitas (AC) con morfología multipolar y estructura plana (Huang et al., 2008). Cada subtipo celular de OECs puede cambiar entre morfologías de forma espontánea (van den Pol and Santarelli, 2003; Huang et al., 2008), pero las CS-OECs pueden migrar tres veces más rápido que las AC-OECs (Huang et al., 2008) de manera que la morfología podría estar reflejando un estado funcional diferente.

Los cultivos primarios de OECs que se han utilizado en los estudios citados anteriormente, no son las mejores fuentes de material de trasplante en la investigación de lesión de la medula espinal. A menudo contienen células contaminantes, generalmente sobreviven sólo unas pocas semanas en cultivo y están limitadas en número por la eficacia de la extracción. Para estudiar los mecanismos moleculares y celulares responsables de la ayuda a la regeneración se necesita una población homogénea de la OECs. Por eso en nuestro estudio hemos usado una línea celular inmortalizada de OEC que mantiene las propiedades regenerativas de cultivos primarios y que nos proporciona una fuente ilimitada de OECs (Moreno-Flores et al., 2003; Moreno-Flores et al., 2006). Esta línea clonal de OECs (TEG3) resultó ser tan buena

como las OECs de cultivo primario en promover la regeneración axonal tanto *in vivo* como *in vitro* (Moreno-Flores et al., 2006). Además las TEG3s pueden adquirir la morfología de AC o SC.

Aunque varios autores han examinado cómo las OECs interactúan con axones en crecimiento (Sorensen et al., 2008; Chuah et al., 2011), sólo recientemente se han logrado avances en la determinación de cómo migran estas células (Huang et al., 2008; Ekberg et al., 2012). Se ha descrito que las propiedades regenerativas de las OEC parecen depender de su capacidad de migración (Gudino-Cabrera et al., 2000; Ramon-Cueto and Munoz-Quiles, 2011; Ekberg et al., 2012). Una vez implantadas en la medula espinal se puede observar que estas células migran menos en presencia de lesión que en una situación control (Deng et al., 2006). Además se ha descrito que en presencia de NogoA (uno de los IAMs) la velocidad de migración de las OECs disminuye significativamente (Su et al., 2007). Estos resultados nos sugieren que la mielina presente después de la lesión podría ejercer un papel sobre las propiedades de comportamiento y migratorias de las OECs.

3. Migración celular

La embriogénesis, el crecimiento nervioso, la reparación de tejidos y la invasión son todos los procesos biológicos que requieren la migración celular. A pesar de la diferencia de tipos celular, los principales mecanismos y base molecular de la migración se producen de una manera similar (Kurosaka and Kashina, 2008). La migración celular se produce mediante un ciclo biomecánico consistente en cuatro pasos. El primero de estos pasos es la extensión de una protrusión en el polo anterior de la célula (Yang et al., 2007). Seguidamente, esta protrusión se adhiere al sustrato mediante adhesiones focales (AFs). Una vez la protrusión está firmemente adherida al sustrato, la célula activa su maquinaria contráctil y genera una fuerza de tracción. Finalmente las adhesiones del polo posterior de la célula ceden a este incremento de fuerza, lo cual da lugar al avance del cuerpo celular (Kurosaka and Kashina, 2008) (Fig. 8; pag. 33). Estas fuerzas se pueden medir gracias al utilizo de la microscopia de tracción (MT). Esta técnica se basa en estudiar las células migrando sobre un sustrato elástico y transparente. Si el sustrato es suficientemente blando, la fuerza que ejercen las células es suficiente para deformarlo. Estas deformaciones se pueden medir introduciendo nanomarcadores

(beads) fluorescentes que se visualizan utilizando microscopia óptica y algoritmos avanzados de procesado de imágenes para obtener un mapa bidimensional de la deformación del sustrado (Fig.9; pag. 35). A través de modelos computacionales avanzados que tienen en cuenta la viscoelasticidad de los substratos se puede obtener localización y la magnitud de estas fuerzas.

Objetivos

En esta tesis hemos querido **determinar las propiedades migratorias de la OECs en presencia de los IAMs** utilizando la línea celular TEG3 con el objetivo final de mejorar su eficacia después de un trasplante en medula espinal. Por lo tanto hemos diseñado algunos objetivos específicos:

Objetivo 1: Caracterizar la línea celular TEG3 para estudiar si estas células expresan toda la maquinaria molecular necesaria para la transducción de los efectos inhibitorios de los IAMs.

Objetivo 2: Examinar los efectos de los IAMs sobre la migración de las TEG3 mediante seguimiento de células solas y sobre las fuerzas de tracción utilizando MT.

A partir de los resultados obtenidos y con el fin de **superar los efectos de los IAMs sobre las TEG3**, hemos elaborados los siguientes objectivos:

Objetivo 3: Investigar si un determinado tipo de nanopartículas magnéticas (NPM) podría ser utilizado para controlar la localización y migración de las TEG3s. Estas partículas se han utilizado para realizar seguimiento *in vivo* de las OECs por imágenes de resonancia magnética (Lee et al., 2004). Pero, las NPM se pueden utilizar también para guiar activamente las células a una ubicación (Dobson, 2008). De hecho, se ha demostrado que células mesenquimales etiquetadas con MNP pueden ser guiadas a la ubicación deseada gracias a la aplicación de un campo magnético, *in vitro* e *in vivo* (Vittorio et al., 2011; Vanecek et al., 2012).

Objetivo 4: Generar y caracterizar TEG3s modificadas genéticamente para expresar el NgR(Ecto) para superar la acción inhibitoria de los IAMs y por lo tanto mejorar las propiedades migratorias de las TEG3. De hecho, (como se ha descrito antes) esta molécula se une a NogoA (uno de los IAMs) y previene su unión con NgR1. Además puede interactuar con el NgR1 completo para evitar su interacción con los otros componentes del complejo NgR. De estas maneras bloquea la señalización intracelular inhibidora activada por los IAMs.

Resultados

La implantación de OECs ha surgido como una terapia prometedora para lesiones medulares, pero sus propiedades regenerativas parecen depender de la presencia de un sustrato permisivo para la migración celular (Gudino-Cabrera et al., 2000; Ramon-Cueto and Munoz-Quiles, 2011; Ekberg et al., 2012). Sólo unos pocos factores han sido descritos como capaces de modular funcionalmente la migración de las OECs. Entre ellos se encuentra NogoA, que disminuye significativamente sus tasa de migración (Su et al., 2007). Esto nos sugiere que los IAMs podrían ser capaces de modular la capacitad migración de las OECs. Por lo tanto, en nuestro estudio hemos examinado el efecto de los IAMs sobre la línea clonal de OECs de rata, TEG3. Hemos demostrado que estas células expresan todos los componentes del complejo NgR (el receptor común para los IAMs) y que este complejo es activo. De hecho, en respuestas a estímulos agudos con mielina se observa una activación de RhoA y un aumento de fosforilación de ERK1-2 (Nocentini et al., 2012). Además, la mielina inhibe la migración de las OECs sobre superficies de cristal o sobre geles linealmente elásticos de poli-acrilamida (PAA)(Nocentini et al., 2012). Con el uso de la MT, demostramos cuantitativamente que las TEG3s disminuyen en gran medida su fuerza de tracción sobre la mielina. Esta disminución se pudo correlacionar con una disminución de las AFs y la redistribución del citoesqueleto de F-actina (Nocentini et al., 2012). La incubación con el péptido NEP1-40, que bloquea NgR1, restableció parcialmente la capacitad migratoria, las fuerzas de tracción de estas células y su organización citoesqueletal (Nocentini et al., 2012). En seguida hemos explorado la viabilidad de dos estrategias diferentes para mejorar las propiedades migratorias de la OECs. Una de ellas era el posible uso de NPM para tratar de guiar a las células TEG3 (Riggio et al., 2013). Aunque esta técnica no aumenta la velocidad intrínseca de migración de las células, permite la concentración y la guía de las mismas hace a un área específica después de la implantación *in vivo* (Vittorio et al., 2011). Pudimos comprobar que las TEG3 marcada con NPM sobreviven bien, sin exhibir respuestas celulares asociadas al estrés, y pueden ser guiadas por un campo magnético *in vitro.* Además comprobamos que las células se integran bien después de su trasplante de en un modelo organotípico de regeneración de médula espinal/nervio ciático (Riggio et al., 2013). La segunda estrategia consistió en modificar genéticamente las TEG3 para que expresaran un ectodomínio por el NgR1

(Fournier et al., 2002a) para bloquear los efectos negativos de los IAMs sobre sus migración. Pudimos observar que las células modificadas migran más *in vitro* sobre un sustrato de mielinas comparadas con TEG3 normales. Además, una vez implantadas en una médula no lesionada, las células modificadas se integran bien en el modelo y migran más que células normal. Los resultados obtenidos indican un potencial terapéutico de ambas estrategias. A continuación se discuten en más detalle los resultados obtenidos.

Discusión

¿Por qué hemos usado una línea celular de OECs?

Como he descrito en la introducción, las OECs han sido usadas en muchos experimentos de trasplante con resultados variados. La mayoría de las OECs utilizadas en estudios derivan de cultivos primarios. El problema es que para estudiar las características moleculares y celulares responsables de la mejora de regeneración en presencia de OECs y sus motilidades bajo diferentes condiciones se necesita una población homogénea de células. La línea clonal TEG3 resultó ser tan buena como las OECs primarias en promover la regeneración axonal *in vitro* y en un modelo animal de la lesión medular (Moreno-Flores et al., 2006). Por lo tanto, optamos por usar esta línea para explorar las propiedades migratorias de las OECs en presencia de uno de los inhibidores de la regeneración después de la lesión medular: la mielina. El primer paso de nuestro estudio fue caracterizar esta línea celular. Observamos que las células expresan todos los marcadores típicos de la OECs: S100β; GFAP; p75. Además, pueden adquirir la morfología tipo AC o SC. Ya se había demostrado que TROY y NgR1 se expresan en la OECs(Su et al., 2007; Morikawa et al., 2008). Sin embargo hemos descrito por primera vez que las OECs expresan todos los elementos del complejo del receptor NgR. Además, este complejo receptor resultó ser activo, ya que en presencia de mielina se activa RhoA y aumenta la fosforilación de ERK1-2.

Propiedades migratorias de las OECs

Aunque las OECs se hayan implantado en la medula espinal, sus propiedades migratorias no son bien conocidas. Por lo tanto, en este estudio, hemos analizado estas propiedades *in vitro*. No hemos diferenciado entre las dos subpoblaciones de OECs (tipo AC o SC) observadas en cultivo. De hecho, recién se demostró que las OECs

pueden cambiar de una morfología a la otra de forma espontánea y que este cambio no es debido a estímulos ambientales (Huang et al., 2008). Este dado confirma que las OECs son un único tipo celular con alta variabilidad fenotípica. Elegimos la laminina como sustrato permisivo ya que es fisiológicamente expresada en el nervio olfatorio en desarrollo. Además, cuando las OECs se cultivan en sustrato de laminina son más móviles que sobre otros sustratos como colágeno I o poli-L-lisina (Huang et al., 2008). Desde un punto de vista biofísico pudimos observar que las OECs cultivadas migran de una manera antipersistente tanto sobre la laminina (sustrato permisivo) como sobre mielina (Nocentini et al., 2012). Este resultado puede explicar, en parte, porqué las OECs han de ser trasplantadas a ambos lados de una lesión, y cerca de ella, para ser eficaz (Gudino-Cabrera et al., 2000; Pearse et al., 2007). Factores adicionales se necesitan para generar un movimiento persistente y dirigido de OECs (Huang et al., 2008; Huang et al., 2011). Recientemente, un estudio realizado por Su y colaboradores indicó que el factor de necrosis tumoral (FNT)-α, expresado por los astrocitos reactivos en la cicatriz glial, atrae a las OECs *in vitro*(Su et al., 2009). Un gradiente lineal de expresión de FNT-α se ha detectado en la cicatriz glial después de hemisección de la médula y esto podría concebiblemente regular la migración de las OECs trasplantadas (Su et al., 2009). Como los niveles de FNT-α tienen un pico de expresión a los 7 días después de lesión, una de las razones por la cual en algunos estudios no se ha observado una migración de las OECs hacia a la zona de lesión, podría ser la falta de coincidencia con los niveles FNT-α necesarios para la quimiotaxis a la hora de la implantación (Chuah et al., 2011). Estos datos también pueden explicar porqué las OECs invaden la cavidad de la lesión que contiene glía reactiva cuando se trasplantan a distancias cortas (Pearse et al., 2007; Chuah et al., 2011). Otra razón por la cual las OECs implantadas después de una lesión medular solo migran distancias reducidas podría ser que las regiones adyacentes a la lesión son ricas en restos de mielina, así como de otras moléculas inhibitorias (Yiu and He, 2006; Yang et al., 2011).

Comportamiento de las OECs sobre mielina

Los datos de otros estudios demuestran que las OECs migran menores distancias una vez trasplantadas en la médula espinal lesionada respecto a medula espinal no lesionada (Gudino-Cabrera et al., 2000; Deng et al., 2006). Además se ha observado que la migración de las OECs es inhibida por la presencia de NogoA (Su et al., 2007). En el

presente estudio hemos demostrado que las OECs cultivadas en presencia de mielina disminuyen su potencial de migración, sus fuerzas de tracción, y presentan una menor extensión celular (cellular spreading) y un reducido número de fibras de estrés y AFs (vinculina-positivas), sobre vidrio o PAA en comparación con las células cultivadas sobre laminina (Nocentini et al., 2012). Estos datos están en contraste con lo observado por Su y colegas (Su et al., 2007). Aunque observaron una migración reducida de las OECs cultivada en presencia de NogoA, las células mostraban una mayor formación de AFs (paxilina-positivas) y un aumento de extensión celular (Su et al., 2007). Las diferencias entre nuestros datos y los reportados en este estudio podrían atribuirse al uso de un proteína recombinante (NogoA) en vez de extracto de mielina completa (que contiene varias proteínas (los IAMs) y lípidos inhibitorios). En efecto, las OECs podrían comportarse de forma similar a otros tipos celulares. Por ejemplo, el péptido Nogo66 (una región de NogoA) no ha resultado ser inhibitorio para la extensión celular de los fibroblasto en cultivo, pero otras dos regiones de NogoA (que contienen los aminoacidos 59-172 o 544-725) sí que han demostrado ser inhibitorios (Oertle et al., 2003). Por lo tanto, los dominios específicos de NogoA podrían tener un efecto diferente sobre la extensión de las OECs. En el mismo estudio, se observó que una mayor concentración de los péptidos inhibidores afectada la adhesión celular (Oertle et al., 2003). Para que las células puedan moverse rápido, la fuerza de adhesión debe ser óptima. Cuando la adherencia es inferior o superior a este punto óptimo, las células se mueven más lentamente (Ananthakrishnan and Ehrlicher, 2007). Esta podría ser la razón por la cual las OECs cultivadas en presencia de mielina muestran una disminución de la capacidad de migración en nuestro estudio. La incubación con el péptido NEP1-40 que bloquea la señalización de los IAMs través su interacción con el complejo del receptor NgR lleva a una recuperación moderada de las propiedades migratorias de las OECs. Esto sugiere la presencia otros mecanismos inhibitorios activados en presencia de mielina.

La mielina modula la fuerza de tracción y la organización del citoesqueleto de las OECs

La migración de una célula es un mecanismo complejo que se basa en la interacción entre la extensión y la contracción de su citoesqueleto, y la formación y retracción de sus adhesiones. Las fuerzas de tracción entran en juego en la

sincronización de estos mecanismos. En nuestro estudio las OECs cultivadas sobre laminina presentan una alta movilidad y una elevada concentración de AFs a la vez que una gran fuerza de tracción. En presencia de mielina, como se ha descrito antes, las células muestran una disminución en la capacidad de migración y una disminución en el número AFs a la vez que una significativa reducción de la fuerza de tracción (Nocentini et al., 2012). Datos sobre la migración de fibroblastos demuestran que las AFs nacientes en el polo anterior de la célula pueden transmitir grandes fuerzas de tracción de propulsión (Beningo et al., 2001). La posterior maduración de estas AFs resulta en un cambio de su función desde un aparado para la transmisión de las fuerzas de propulsión durante la migración a aparato de anclaje pasivo para el mantenimiento de la morfología celular en células estáticas (Beningo et al., 2001). Esto puede explicar los resultados obtenidos de Su y colaboradores. En presencia NogoA las OECs presentaban una migración reducida pero una mayor presencia de AFs (Su et al., 2007). Las células mostraban una morfología plana típica de una célula estática, lo que sugiere que, en este caso, las AFs jugaban un papel de mantenimiento de morfología. Aunque se necesitan más estudios para describir los mecanismos de acción de los IAMs sobre la adhesión de las OECs , se ha descrito que el dominio N -terminal de NogoA inhibe la adhesión de células COS7 actuando a través de las integrinas y la disminución final de la actividad de la AF cinasa (Hu and Strittmatter, 2008). Otras moléculas presentes en la mielina podrían estar actuando a este nivel para modular la adhesión celular, incluso en las OECs. Otro mecanismo por el cual los IAMs pueden modular la migración OECs podría ser la faltante estabilización de los microtúbulos (Mimura et al., 2006; Llorens et al., 2011). Los microtúbulos parecen estar asociados físicamente con las AFs y parecen ser esenciales en el control de sus dinámicas durante la migración celular. De hecho, la estabilización de los microtúbulos puede facilitar el transporte de las integrinas y participar en el crecimiento inicial de las AFs (Gu et al., 2011).

Nuestros resultados refuerzan la noción de que las moléculas presentes en los extractos de mielina actúan sobre el complejo-NgR, así como en otros receptores, y que de esta manera pueden modular citoesqueleto OEC y la distribución de las AFs. Sin embargo, la incubación de las OECs con el peptido NEP1-40, en presencia de mielina, conduce a una ligera recuperación de las fuerzas de tracción y de la organización del

citoesqueleto. Esto sugiere que otras moléculas presentes en la mielina u otros receptores pueden estar implicados en la modulación de estos procesos.

NPMs para guiar las OECs in vitro

Una vez implantadas en la medula las OECs se dispersan entre otras células del tejido (Ruitenberg et al., 2002). De esta manera las células no mantienen un alto grado de contacto entre célula y célula y por tanto no forman una masa uniforme. En un modelo de lesión *in vivo*, se observó que la presentación de la OECs en forma de masa uniforme resultó en un aumento de la regeneración axonal (Chehrehasa et al., 2010). Por tanto, sería interesante examinar si la implantación las OECs de una forma que favoreciese su migración como masa uniforme se traduciría en un aumento de regeneración en los modelos lesión medular. Con el fin de controlar la ubicación OECs en el presente estudio hemos utilizado una estrategia basada en el uso de NPMs. Estas partículas han sido utilizadas para la visualización no invasiva de las OECs después de trasplante en medulas. El cultivo de las OECs en presencia de NPMs en suspensión lleva a un alto nivel etiquetamiento celular y esto permitió identificar fácilmente las células marcadas por resonancia magnética (Sandvig et al., 2012). Este método podría ser utilizado para controlar la localización y el comportamiento migratorio variado de las subpoblación de OECs. Las NPMs empleadas en el estudio tienen una saturación de magnetización mayor que NPMs comerciales (Riggio et al., 2012). Dado que la fuerza magnética ejercida sobre una sola célula es proporcional al momento magnético de las NPMs incorporadas nos espetábamos una alta eficiencia en el control de la migración celular. En primer lugar demostramos que el protocolo de magnetización desarrollado no alteraba las características biológicas de las OECs. Gracias a imágenes de microscopía electrónica pudimos observar que la mayoría de las NPMs estaban localizadas en el citoplasma de las células en forma de aglomerados (Riggio et al., 2013). Esta tendencia a la aglomeración de partículas se puede explicar por las fuertes interacciones dipolo-dipolo entre ellas. Normalmente las NPMs son ingeridas por la célula a través de endocitosis (Lee et al., 2004) y los productos de la degradación de las partículas podrían ser responsables de la producción de especies reactivas de oxígeno (Riggio et al., 2012). Sin embargo, a la concentración de uso de la toxicidad de la NPMs no es significativa. De hecho, la análisis por Western blotting de proteínas implicadas en la replicación celular, la apoptosis y la muerte celular no reveló algún cambio en

activación (o fosforilación) de estas proteínas en las células marcadas con NPMs en respecto a células no marcadas (Riggio et al., 2013). Los mismos resultados fueron obtenidos con ensayos de exclusión de ioduro de propidio, que confirmaron una viabilidad celular superior al 90% en todas las concentraciones ensayadas. Además, no hemos podido apreciar ninguna reorganización en el citoesqueleto de actina (Riggio et al., 2013). Por lo tanto podemos concluir que las NPMs pueden ser consideradas seguras para el uso biológico a las concentraciones ensayadas. Aunque no se muestra en papel, la capacitad de migración de las OECs marcadas con NPMs es igual a la de las OECs no marcada.

Orientación de las OECs marcadas con NPM por campos magnéticos

La posibilidad de dirigir la migración OECs hacia a una fuente magnética es importante en términos de uso terapéutico de estas células. Gracias a experimentos de migración celular se pudo demostrar que un campo magnético induce la migración dirigida de las células marcadas (Riggio et al., 2013). Estos resultados sugieren que la misma técnica se podría usar *in vivo* para guiar y acumular las células en una determinada zona. De hecho, células mesenquimales implantadas por vía intratecal a través de la punción lumbar se han podido guiar gracias al uso de un campo magnético hacia la zona de lesión en la medula espinal en un estudio (Vanecek et al., 2012). Además con el uso de imanes con geometrías particulares se pudo aumentar la eficiencia en la localización en un área específica de las células (Vanecek et al., 2012). Estos imanes con parámetros geométricos sintonizables, en el futuro, podrían proporcionar el nivel adicional de control de localización necesario para mejorar la eficiencia de administración de las OECs en lesiones medular *in vivo*.

Integración de las NPM-OECs en un cultivo organotípico

Utilizamos un cultivo organotípico de médula espinal y nervio ciático para analizar la supervivencia e integración de las NPM-OECs en un modelo bioquímicamente y fisiológicamente más similar a una situación *in vivo*. De hecho, estos cultivos son muy útiles para los estudios sobre la regeneración neuronal (Dash et al., 2012). Parte del nervio ciático de ratones recién nacidos se colocó en frente de las raíces ventrales de una rebanada de médula espinal para permitir a las neuronas motoras de inervar el nervio ciático. Después de siete días en cultivo, los axones de las neuronas

motoras alcanzaron inervar el nervio ciático (Riggio et al., 2013). Por lo tanto, este sistema representa un buen modelo *in vitro* para estudios de regeneración. NPM-OECs se añadieron al modelo con el fin de evaluar su supervivencia y su comportamiento. Los resultados obtenidos indican que las NPM-OECs son capaces de integrarse en el cultivo y algunas de ellas se encontraban en estrecha relación con axones en crecimiento (Riggio et al., 2013). Por lo tanto, el comportamiento de los NPM-OECs es similar a los reportados en los estudios *in vivo* por la OECs normales.

El NgR(Ecto) mejora las propiedades migratorias de las OECs sobre mielina

Como demostramos que la mielina inhibe la migración de las OECs (Nocentini et al., 2012) una estrategia para mejorar la movilidad de estas células seria interfiriendo con la señalización de la mielina misma. El NgR(Ecto) ejerce su acción uniéndose a NogoA (uno de los IAMs) para prevenir su unión a la del complejo NgR y interactuando con el mismo receptor NgR1, para bloquear la señalización intracelular. Estudios *in vitro* demostraron que puede actuar como un antagonista para la mielina del SNC (Fournier et al., 2002a; He et al., 2003). Además, tras una lesión a la médula, NgR(Ecto) promovió la regeneración axonal y recuperación funcional (Li et al., 2004; Wang, X. et al., 2006). Nosotros modificamos genéticamente las TEG3 para que expresaran este ecodominio. Una vez cultivadas en presencia de mielina sus propiedades de migración fueron parcialmente restauradas. Como se ha comentado antes, aunque el complejo NgR parece ser el principal mediador de la acción de mielina otro receptor de las OECs podrían estar involucrados en la señalización inhibitoria. Como experimento preliminar implantamos las OECs modificadas en medula espinal no lesionada. Para inyectar las células se tiene que romper el tejido, por lo tanto restos de mielina e inflamación (aunque de forma mínima) puede estar presente en el sitio de implantación de la células. Una semana después del trasplante pudimos observar que los NgR(Ecto)-TEG3 habían migrado distancia más larga que las TEG3 normales. Este resultado nos sugiere que el NgR(Ecto) ejerce su acción in vivo. Después de lesión medular se ha observado que la expresión de NogoA incrementa en las neuronas incapaces de regenerarse (Peng et al., 2010), y que la mielina aumenta la expresión de NogoA *in vitro* (Peng et al., 2010). La incubación con el ecodominio de NgR regula a la baja esta sobre-expresión, finalmente mejorando la regeneración (Peng et al., 2010). En caso de una lesión, si se implantarían OECs modificadas para expresar el NgR(Ecto).

Este último podría interferir con la señalización de la mielina bajando su efecto inhibitorio y paralelamente podría modular la expresión intracelular de NogoA con el resultado final de mejorar la migración de las OECs y de facilitar la regeneración axonal.

OECs para la reparación de lesión de la médula espinal

El trasplante de las OECs puede promover la regeneración y rescatar la función neuronal deteriorada gracias a la secreción de factores neurotróficos permisivos en el sitio de la lesión para crear un entorno más propicio para la regeneración. Además las células ayudan a rellenar el sitio de lesión proporcionado un andamiaje sobre el cual los axones puedan crecer. Sin embargo, los resultados obtenidos en modelos de lesión son altamente variables. Esto se debe en parte a la fuente de la OECs y a los métodos de purificación utilizados en los ensayos. Aunque varios autores han examinado cómo las OECs interactúan con los axones en crecimiento (Sorensen et al., 2008; Chuah et al., 2011), sólo recientemente se han logrado avances en la determinación de cómo OECs migran y responden a señales extracelulares. A partir de los datos de nuestro estudio podemos inferir que la migración OECs es inhibida por la presencia de mielina después de lesión medular y que esta propiedad es sólo parcialmente revertidos mediante el bloqueo del complejo-NgR; que estas células se mueven en forma antipersitente y por lo tanto se necesitan otros factores para la generación de una migración persistente y dirigida. Estos son factores que han de tenerse en cuenta para futuras investigaciones sobre las OECs. También hemos observado que las OECs cultivadas en substratos de PAA, con propiedades mecánicas claramente diferentes a sustratos de vidrio, aumentan su potencial migratorio. En este sentido, recientemente se ha observado un aumento de proliferación y migración de las OECs sobre varios andamios estructurados (Wang, B. et al., 2006a). Por lo tanto es posible que en el futuro se puedan utilizar estos andamios como una guía mecánica para la migración OEC ya que no sólo aumentarían la migración o proliferación de la OECs, sino que podrían ayudar a prevenir la interacción de estas células con un putativo substrado inhibidor en modelos de lesión.

Futuros experimentos con las OECs

Como se ha descrito antes sólo implantamos NgR(Ecto)-OECs en médulas no lesionadas para comprobar si las células se integrarían bien y migrarían en un sistema in

vivo. Son necesarios más estudios para observar sus comportamientos en un modelo de lesión. Podríamos combinar el uso de esta célula modificada con el uso de NPMs. Con el diseño de fuentes magnéticas específicas podremos concentrar las OECs cerca del lugar de la lesión y por lo tanto aumentar su posible interacción con los axones dañados. La secreción del NgR (Ecto) y de factores que promueven el crecimiento axonal por parte de las OECs tendría que mejorar la preservación de estos axones y enmascarar la mielina presente en el sitio para mejorar las propiedades regenerativas naturales de los axones.

Conclusiones

1. Las OECs expresan todo los elementos del complejo receptor NgR. Además, este complejo receptor es activo. De hecho, en repuesta a mielina se activa RhoA y aumenta la fosforilación ERK1-2.

2. Las OECs en presencia de mielina muestran una disminución de la capacidad migratoria. Esta capacidad está parcialmente restaurada en presencia de NEP1-40.

3. La disminución de la velocidad de las OECs paralela con una disminución en el número de FAs y de fuerza de tracción. La incubación con NEP1-40 porta a una recuperación de la organización del citoesqueleto sobre la mielina.

4. La mancada recuperación completa de la capacitad migratoria, fueza de tracion y organización del citoesqueleto sobre mielina en presencia de NEP1-40, nos sugiere que receptores diferentes del complejo NgR y/o inhibidores presentes en la mielina puedan estar ejerciendo su función sobre las OECs.

5. *In vitro*, la OECs magnetizadas pueden sobrevivir bien sin exhibir respuestas celulares asociadas al estrés. Su migración puede ser modulada por campos magnéticos, y su trasplante en cultivos organotípicos de médula espinal y los nervios periféricos muestra integración positiva en el modelo.

6. Las OECs modificadas genéticamente para expresar el ectodominio de NgR1 son capaces de expresar y secretar altos niveles ectodominio *in vitro*.

7. Las OECs modificadas migran por distancias más largas sobre un sustrato de mielina *in vitro.* Aunque la recuperación no es total.

8. Una vez implantadas en una médula espinal no lesionada estas células se integran en el tejido y migran más distancia, tanto en dirección caudal cuanto rostral desde sitio de la inyección en comparación con células normales normal.

ANEXOS

Informe del Factor de Impacto

Con la presente, hago constar el Factor de Impacto correspondiente a las revistas donde se han publicado los artículos científicos que conforman la Tesis Doctoral presentada por Sara Nocentini.

- *Cellular and Molecular Life Sciences:* **5.615**
- *International Journal of Molecular Sciences:* **2.464**

Director y Tutor de la tesis	Codirectora de la tesis
Dr. José Antonio del Río Fernández	Dra. Ana Bribian Arruego

Informe de participación

El Dr. José Antonio del Río Fernandez y la Dra. Ana Bribian Arruego, director y codirectora respectivamente de la Tesis Doctoral: "Potential of genetically modified ensheathing cells for regeneration after spinal cord injury (Potencial de la glía envolvente genéticamente modificada para la regeneración después de lesión medular)", elaborada por Sara Nocentini, informan que la participación de la doctoranda en los artículos científicos que conforman la tesis ha sido la siguiente:

En el artículo titulado "**Myelin-associated proteins block the migration of olfactory ensheathing cells: an in vitro study using single-cell tracking and traction force microscopy**", publicado en la revista *Cellular and Molecular Life Sciences,* la doctoranda ha participado en el diseño de los experimentos y es la principal responsable del desarrollo de los mismos.

Este artículo no ha sido utilizado en la elaboración de otras Tesis Doctorales.

En el artículo titulado "**Generation of magnetized olfactory ensheathing cells for regenerative studies in the central and peripheral nervous tissue**", publicado en la revista *International Journal of Molecular Sciences,* la doctoranda ha participado en el diseño de los experimentos y es la principal responsable del desarrollo de los mismos.

Este articulo ha sido utilizado en la elaboración de la Tesis Doctoral con título: "Magnetic properties of nanoparticle in nerve generation", elaborada por Cristina Riggio para optare al grado de Doctor en Bioingeniería por la Scuola Superiore Sant'Anna, Pisa, Italia.

Director y Tutor de la tesis	Codirectora de la tesis
Dr. José Antonio del Río Fernández	Dra. Ana Bribian Arruego