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Mémoire présenté en vue de l'obtention du grade de Docteur en Sciences Biomédicales et Pharmaceutiques

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Abbreviation list

Ab: Antibody
ALS: Amyotrophic Lateral Sclerosis
ATP: Adenosine Triphosphate
ATF3: Activating Transcription Factor3
BCIP: 5-Brom-4-Chlor-3-IndolylPhosphate
Bcl-2: B-Cell Lymphoma 2
Bcl-xL: B-Cell Lymphoma-Extra Large
BDNF: Brain-Derived Neurotrophic Factor
BF-2: Brain Factor-2
bp: base pair
BrdU: Bromodeoxyuridine
Brn2: Brain 2
BSA: Bovine Serum Albumin
CNS: Central Nervous System
ct: cycle threshold
DAB: 3,3'-diaminobenzidine
DAPI: 4',6'-diamidino-2-phénylindole
Dhh: Desert hedgehog
DMEM: Dulbecco's Modified Eagle Medium
DNA: Deoxyribonucleic Acid
DRG: Dorsal Root Ganglion
EC: Endothelial Cell
ECM: Extracellular Matrix
EDTA: Ethylenediaminetetraacetic Acid
Egr2: Early growth response protein 2
Epo: Erythropoietin
erbB: Erythroblastic Leukemia Viral Oncogene
FBS: Foetal Bovine Serum
FITC: Fluorescein Isothiocyanate
Flt: Fms-Like Tyrosine kinase
Flk: Fetal Liver Kinase
Fos: FBJ murine osteosarcoma viral oncogene homolog
GAP-43: Growth Associated Protein-43
GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase
GCM1: Glial Cell Missing1
GDNF: Glial Derived Neurotrophic Factor
GFAP: Glial Fibrillary Acidic Protein
GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor
gp 130: glycoprotein 130
HMG-CoA: 3-Hydroxy-3-MethylglutarylCoenzyme A
HNK-1: Human Natural Killer-1
HRG: Heregulin-β1
ICAM: Intercellular Adhesion Molecule
IF: Immunofluorescent
IFN: Interferon
IκB: Inhibitor of NF-κB
IKK: IκB Kinase
IL: Interleukin
iNOS: inducible Nitric Oxyde Synthase
i.p.: intraperitoneal
IP: immunoprecipitation
ISH: *In Situ* Hybridization
KDR: Kinase-insert Domain Receptor
Ki67: Kiel 67
KLF: Krüppel-Like Factor
Krox20: Krüppel box 20
LDL: Low-Density Lipoprotein
LFA: Lymphocyte Function-associated Antigen
LIF: Leukemia Inhibitory Factor
LRP: LDL Receptor-related Protein
MAG: Myelin-Associated Glycoprotein
MAP1B: Microtubule-Associated Protein 1B
MBP: Myelin Basic Protein
MCAO: Middle Cerebral Artery Occlusion
MCP: Monocyte Chemotactic Protein
MHC: Major Histocompatibility Complex
MIP: Macrophage Inflammatory Protein
MMP: Matrix Metalloproteinase
MTF-1: Metal regulatory Transcription Factor-1
$Nav_{1.6}$: voltage-gated sodium channels 1.6
NBT: Nitrotetrazoliumblue
N-CAM: Neural Cell Adhesion Molecule
NEMO: NF-κB Essential Modulator
NeuN: Neuronal Nuclei
NF-H: Heavy Neurofilament subunit

NF-κB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NGF: Nerve Growth Factor

Ng-CAM: Neuronglia Cell Adhesion Molecule

Nmnat: Nicotinamide mononucleotide adenylyltransferase

NO: Nitric Oxyde

NPL: Normal Print-Length

NRG: Neuregulin

NRP: Neuropilin

NS: Nervous System

NT: Neurotrophin

Oct6: Octamer binding factor 6

OMgp: Oligodendrocyte Myelin glycoprotein

OPL: Operated Print-Length

P0: Protein 0

P2R: type-2 Purinergic Receptor

p75 NGFr: low affinity nerve growth factor receptor p75

PAI: Plasminogen Activator-1 Inhibitor

PFA: Paraformaldehyde

***Pgf*$^{-/-}$:** PlGF knock-out

PLF: Print-Length Factor

PlGF: Placental Growth Factor

Pmp22: Peripheral myelin protein 22

PNS: Peripheral Nervous System

POU: Pituitary-specific 1, Octamer transcription factor proteins, neural Unc-86 transcription factor

Ptc: Patched

qRT-PCR: quantitative Real-Time Polymerase Chain Reaction

RNA: Ribonucleic acid

RT: Room Temperature

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SC: Schwann Cell

Scip: Suppressed cyclic AMP-induced POU protein

SDS: Sodium dodecylbenzenesulfonate

SE: Standard Error

SOCS: Suppressors Of Cytokine Signalling

Sox: Sex determining region Y-box

TGF: Transforming Growth Factor

TLR: Toll-Like Receptor

TNF: Tumor Necrosis Factor

Trk: Tyrosine kinase

UFD2: Ubiquitin Fusion Degradation 2

UI: Uninjured

UPS: Ubiquitin Proteasome System

UTP: Uridine-5'-triphosphate

VEGF: Vascular Endothelial Growth Factor

VLA-4: Very Late Antigen-4

vWF: von Willebrandt Factor

WD: Wallerian Degeneration

wt: wild-type

Table des matières

Section I

Introduction

The nervous system (NS) is probably the most elaborated structure of living creatures. It allows the body to receive information coming from surroundings, to analyse them and to adjust the body response to them. Since the first description of **glial cells** in the treaty of Theodor Schwann in 1839 (for review Kosinski, 2004) and **neurons** thanks to Heinrich Wilhelm Waldeyer and Santiago Ramón y Cajal at the end of the 19th /beginning of the 20th century (for review Lopez-Munoz *et al.*, 2006; Winkelmann, 2007), knowledge about the structure and functions of the NS has been constantly growing over the years, and at an exponential rate during the last decade. Nevertheless, numerous unresolved questions persist notably concerning the mechanisms of the NS's response to injury or the role of newly discovered molecules in nervous functions. Our research project was conceived with the aim to increase knowledge in these two areas by studying the role of a specific molecule in the normal and lesioned NS. This introduction is meant to set the stage and to present the state of the art in our research domain, also for readers who are not neuroscience experts.

I.1. Central and Peripheral Nervous System: anatomical and cellular differences.

The NS is made up of two parts, the central (CNS) and the peripheral (PNS) nervous system (figure I.1). The CNS includes on the one hand **the encephalon**, located in the skull and formed by the brain, the brain stem and the cerebellum, that interpret information coming from the environment and regulate complex processes such as homeostasis, perception, emotions and cognition, and on the other hand **the spinal cord,** located in the spinal canal, that conveys information coming from peripheral structures to the encephalon and transmits motor and autonomic commands coming from the encephalon to peripheral targets. The PNS allows the CNS to interact with the rest of the body and the environment. It includes cranial nerves, spinal nerves, autonomic nerves and their respective ganglia.

Two main cell types are found within the NS: neurons and glial cells. Neurons are highly specialized cells that transmit electrical signals across the NS. Glial cells are the most numerous cells in the NS, representing about 90% of the total cell population. They are vital to neurons and neuronal functions providing them support, protection, myelination and metabolic homeostasis.

Astrocytes, oligodendrocytes and microglia are the glial cells specific to the CNS, while Schwann cells (SCs) and satellite cells are specific to the PNS. This different glial cell composition has been considered responsible for one of the major functional differences

between CNS and PNS, that puzzles neuroscience researchers worldwide since many years: **axons regenerate readily in the injured PNS, whereas the regenerative capacity of injured neurons in the adult mammalian CNS is extremely limited.** The next paragraph clarifies this difference in post-injury outcome between CNS and PNS.

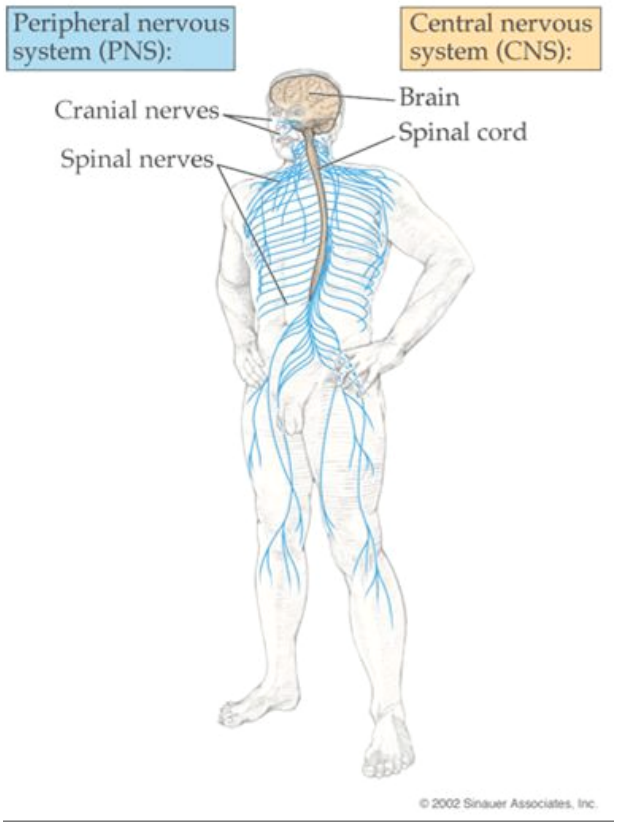


Figure I.1: *Anatomy of the nervous system (Biol 492/592 Human Neuroanatomy-Keith A.Carson)*

I.1.1. The outcome of NS injuries depends on the localization of the lesion: CNS versus PNS

Since Ramón y Cajal's famous works at the beginning of the 20th century (Ramón y Cajal, 1928), it is well known that axon regeneration in the mature mammalian CNS is extremely limited after injury, contrasting with the remarkable ability of PNS axons to regenerate and to allow for recovery of function after peripheral nerve damage. The reasons for which the outcomes of PNS and CNS injury are so strikingly different have been extensively studied. At first, the inability of CNS axons to regenerate after a lesion was attributed to **a lack of intrinsic growth capacity of mature CNS neurons**. In fact, it has been shown that CNS neurons do not up-regulate growth-associated genes such as GAP-43 to

the same extent as do PNS neurons after injury (Fernandes *et al.*, 1999). GAP-43 is a target gene of KLF-7, one of the members of the Krüppel-like factor family (Kajimura *et al.*, 2007), a group of transcription factors that regulate intrinsic axon regeneration ability. It is now established that coordinated activities of different KLFs regulate the regenerative capacity of CNS neurons (Moore *et al.*, 2009). Also, numerous experiments have shown that CNS axons are able to regenerate under certain conditions, as for example when they can grow through PNS nerve implants (Aguayo *et al.*, 1981; Lavdas *et al.*, 2008; Huebner & Strittmatter, 2009). This finding suggests that the PNS environment is permissive while the CNS environment is inhibitory for axon growth, and one of the main causes of this permissive or inhibitory environment is the difference in glial cells between PNS and CNS. Hereafter, we will discuss the essential roles of SCs in PNS regeneration and the detrimental roles of oligodendrocytes, microglia, and astrocytes on CNS regeneration. Figure I.2 summarizes the main differences between post-injury responses in PNS and CNS.

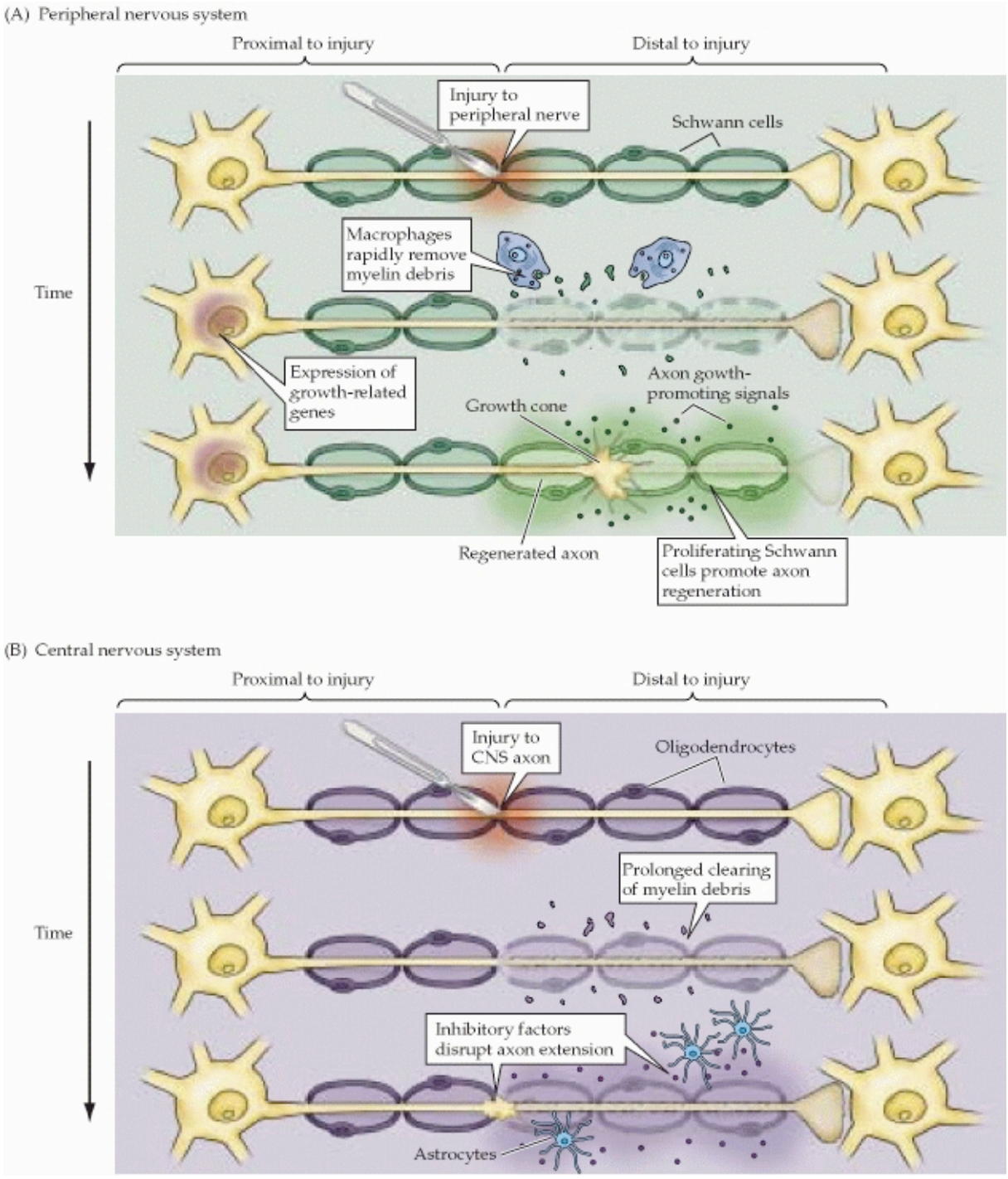


Figure I.2: *Main differences in post-injury responses between the PNS (A) and the CNS (B.) From:"Neuroscience 2nd edition" The changing brain" Chapter 25: Plasticity of Mature Synapses and Circuits" recovery from neural injury. Purves D, Augustine GJ, Fitzpatrick D, et al., editors. Sunderland (MA): Sinauer Associates; 2001.*

I.1.2. Permissive environment for axonal regeneration created by Schwann cells

After nerve injury, SCs proliferate and express a multitude of factors supporting axonal regeneration through three different mechanisms: (I) neuronal survival and neurite growth improvement, (II) axonal and myelin debris phagocytosis, and (III) axonal guidance.

To improve neuronal survival after injury, SCs secrete neurotrophic factors (see section 2.4) that are retrogradely transported towards the neuronal cell body to induce the expression of anti-apoptotic (Culmsee *et al.*, 2002; Almeida *et al.*, 2005; Pazyra-Murphy *et al.*, 2009) and regeneration-associated genes (Cafferty *et al.*, 2001; Cafferty *et al.*, 2004).

SCs play another key role after injury. They initiate the phenomenon of Wallerian degeneration (WD) (see section I.2) that leads to a rapid clearance of myelin debris, the overriding step to assure axonal regeneration. Indeed, PNS and CNS myelin sheaths contain axonal growth inhibitory proteins such as myelin-associated glycoprotein (MAG) (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994; Shen *et al.*, 1998), Nogo (Huber & Schwab, 2000) as well as oligodendrocyte myelin glycoprotein (OMgp) (Kottis *et al.*, 2002). SCs promote rapid myelin clearance via two mechanisms. First, they participate to both the breakdown of their own myelin sheath into small ovoids and the phagocytosis of the resulting debris (Stoll *et al*., 1989; Fernandez-Valle *et al*., 1995). Secondly, SCs release a wide variety of chemo- and cyto-kines (see section I.2.3) that enhance the recruitment towards the lesion site of the second cell type essential for a rapid myelin clearance, the blood-derived monocytes.

Finally, SCs also promote axonal regrowth and remyelination by providing structural guidance through their migration and alignment to form guiding tubes called “bands of Büngner” (Weinberg & Spencer, 1978) and by producing a basal lamina (Sketelj *et al.*, 1989), which contains extracellular matrix proteins that interact with integrins expressed on the surface of axons (see section I.2.4).

I.1.3. Inhibitory environment for axonal regeneration in the injured CNS: the coalition of oligodendrocytes, astrocytes and microglia.

In contrast to SCs, oligodendrocytes do not participate in the establishment of a permissive environment for axonal regrowth. Indeed, after a CNS injury, the loss of axonal contact leads to oligodendrocyte apoptosis (Shuman *et al.*, 1997; Casha *et al.*, 2001). Even if oligodendrocyte precursors are recruited at the lesion site, they possess a limited remyelination capacity (Blakemore & Keirstead, 1999). Contrary to the PNS where myelin debris are rapidly eliminated thanks to SCs and monocyte-derived macrophages, in the CNS,

oligodendrocytes do not clear myelin ovoids (Ludwin, 1990) and haematogenous macrophage recruitment is limited and occurs later than in injured PNS (Avellino *et al.*, 1995; Popovich & Hickey, 2001). This slower clearance of myelin in CNS compared to PNS results in the prolonged presence of inhibitory myelin proteins that contribute to the failure of CNS axonal regeneration (Vargas & Barres, 2007).

Resident microglia represents the main population of phagocytic cells after CNS injury (Popovich & Hickey, 2001). However, compared to macrophages, rapidly recruited and active after PNS injury, microglial cells have a weak phagocytic activity (Milligan *et al.*, 1991) and their number increases later (Lawson *et al.*, 1994) leading to an extremely slow myelin clearance. In addition, microglia is also responsible for neuronal death through the secretion of several neurotoxic agents such as reactive oxygen species, nitric oxyde (NO), and glutamate, leading to further extension of the primary lesion (Giulian *et al.*, 1993; Langmann, 2007).

After CNS injury, astrocytes proliferate and become hypertrophic. This leads to the phenomenon of astrogliosis, resulting in the formation of an astroglial scar, a physical and chemical barrier to axonal regeneration. Indeed, in addition to the physical barrier created by the cells themselves, reactive astrocytes secrete inhibitory molecules of which the main ones are chondroitin sulfate proteoglycans such as neurocan, versican, and phosphacan (Morgenstern *et al.*, 2002).

Despite this accruing knowledge on the factors facilitating PNS regeneration and those that limit CNS regeneration, many questions remain about the molecular and cellular mechanisms responsible for the success of axon regrowth in the PNS, and about possible novel therapeutic strategies to promote CNS regeneration, underlining the need for more research.

I.2. Wallerian degeneration in the PNS

Injury to the NS initiates an organized sequence of degenerative cellular and molecular events in the segment distal to the lesion site, named the Wallerian degeneration (WD), critical for successful repair (Waller, 1850). In the PNS, WD consists of axonal rupture and fragmentation, loss of contact between axon and myelin, debris removal by phagocyting SCs, which also dedifferentiate, proliferate and migrate to form the bands of Büngner along which regenerating fibres will regrow (figure I.3). Due to chemokine secretion by SCs, monocyte-derived macrophages are recruited from the circulation and participate, along with SCs, in axonal and myelin debris clearance. SCs and macrophages also secrete several inflammatory and neurotrophic factors beneficial to neuronal survival and to axonal regeneration. These main steps will be described in more details in this paragraph.

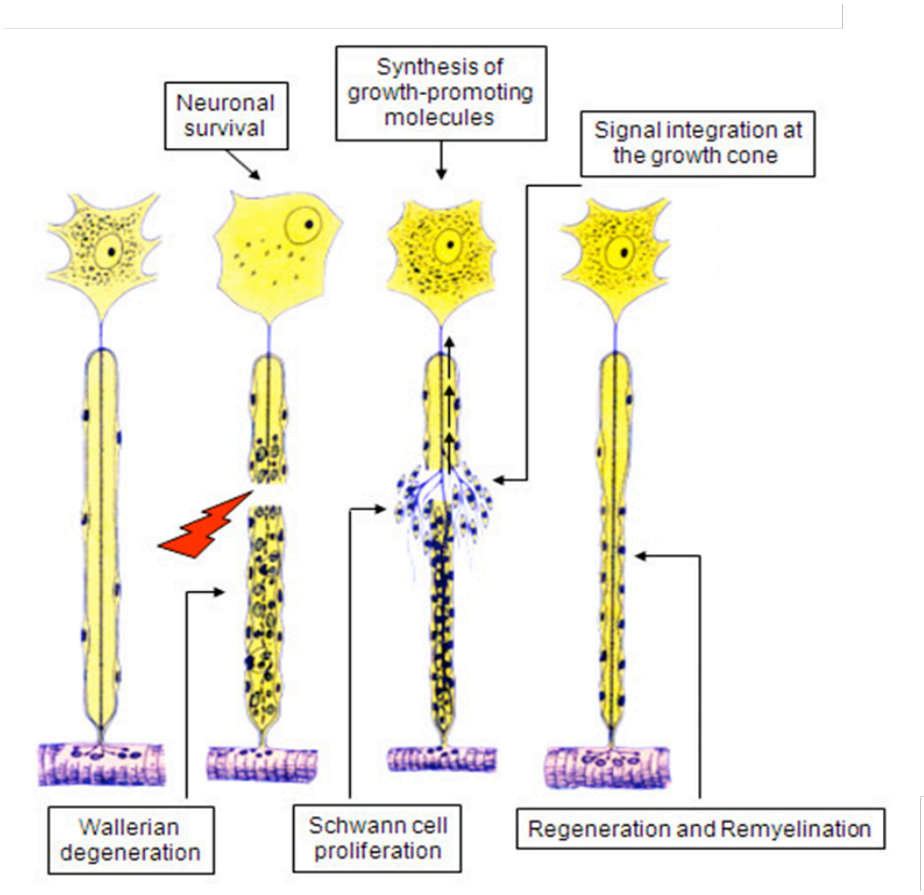


***Figure I.3**: Schematic representation of Wallerian degeneration (www.neuroanatomie.at/uploads/pics/4.jpg)*

I.2.1. Axonal breakdown

The degradation of axolemma and axoplasm is an early step of WD. Axonal breakdown is an active and rapid process that occurs as soon as 36 to 44 hours after injury, due to intrinsic properties involving homeostatic mechanisms, and is completed after 48h. WD progresses as a wave which sequentially affects adjacent regions of the axon. However its orientation depends of the lesion type; axonal degeneration is anterograde after transection, but retrograde after a crush (Beirowski *et al.*, 2005). Propagation of axon degradation is asynchronous among the axonal population of a peripheral nerve and is directly correlated with fibre diameter and internodal length (Lubinska, 1977; Veronesi & Boyes, 1988). The major early axonal changes include fragmentation of rough endoplasmic reticulum and dissolution of neurofilaments and microtubules. Nerve injury disrupts the integrity of the axoplasm and exposes it to the external ionic environment. Consequently, intra-axonal calcium increases and activates cystein proteases called calpains. These enzymes are responsible for the degradation of neurofilaments (Glass *et al.*, 2002). The ubiquitin proteasome system (UPS) is also involved in neurofilament dissolution as well as in microtubule depolymerization (Zhai *et al.*, 2003). Part of the evidence for the implication of UPS in WD comes from the discovery of the spontaneous mutant strain Wld^S in the C57Bl/6 mouse. In Wld^S mice transected axons live and thrive for weeks independently of their native cell body (Lunn *et al.*, 1989). This phenotype of slow WD is due to the expression of a novel chimeric protein consisting of the N-terminal portion of the ubiquitin regulatory enzyme UFD2 and the nicotinamide mononucleotide adenylyltransferase (Nmnat). The ability of UFD2 for multi-ubiquitination is therefore impaired, which suppresses the neurofilament and microtubule targeting by the UPS hence preventing their degradation (Mack *et al.*, 2001).

I.2.2. Glial response and myelin fragmentation

In response to this axonal degeneration, SCs undergo molecular and cellular changes called dedifferentiation. SCs downregulate myelin gene expression like P0, MBP and MAG (LeBlanc & Poduslo, 1990), fragment their own myelin sheaths into ovoids and phagocyte the resulting debris (Stoll *et al.*, 1989; Fernandez-Valle *et al.*, 1995). Concomitantly, they up-regulate several specific genes of the immature SC phenotype, such the cytoskeletal protein GFAP, the low affinity nerve growth factor receptor (p75 NGFr), as well as cell adhesion molecules like N-CAM and L1 (Taniuchi *et al.*, 1988; Thomson *et al.*, 1993; Martini, 1994). These changes of the SC phenotype require several environmental signals. The initiation of SC dedifferentiation and proliferation is consecutive to the loss of axonal contacts (Lunn *et*

al., 1989). Injured neurons also release several factors such as the type II and III isoforms of neuregulin-1 (NRG1) (Carroll *et al.*, 1997; Kwon *et al.*, 1997; Osheroff *et al.*, 1999; Guertin *et al.*, 2005), as well as TGF-β1 (Rogister *et al.*, 1993), which induce SC demyelination and proliferation. Moreover the blood-nerve barrier breakdown after the injury induces a fibrin deposit that stimulates p75 NGFr expression in SCs, maintains them in a non-myelinating state and prevents the synthesis of myelin proteins (Akassoglou *et al.*, 2002). Dedifferentiated SCs also exert an autocrine activity to maintain their unmyelinating and proliferating state by the expression of several molecules and their receptors such as Epo/Epo-R (Li *et al.*, 2005), vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (Sondell *et al.*, 1999a). In this dedifferentiated and proliferating state, SCs also induce the inflammatory response (see section I.2.3) and provide the favourable environment for axonal regeneration by organising themselves into axonal guiding tubes called bands of Büngner, and by secreting neurotrophic factors (see section I.2.4.).

I.2.3. Inflammatory reaction

In both CNS and PNS, traumatic injuries lead to a local inflammatory response regulated by numerous signalling molecules. Their production and local effects influence the outcome of trauma. In the PNS, the well-organized inflammatory process that follows axotomy plays a beneficial role for nerve repair. Indeed, it induces a rapid clearance of axonal and myelin debris by recruited macrophages and SCs, thus contributing to the establishment of a permissive environment for regrowth.

- ***Initiation of inflammatory response***

Following a nerve lesion, injured glial and neuronal cells release several molecules named “damage signals”. These endogenous molecules rapidly stimulate SCs through two types of receptors, the Toll-Like Receptors (TLRs) and type-2 Purinergic Receptors (P2Rs) (Pineau & Lacroix, 2009), leading to the activation of transcription factors NF-κB and c-Jun (figure I.4). These factors induce the synthesis and release of pro-inflammatory cyto/chemokines controlling the recruitment and the activation of both glial cells and blood-derived immune cells (Camara-Lemarroy *et al.*, 2010). The endogenous TLR ligands present within the lesion site include several heat shock proteins (Willis *et al.*, 2005; Lehnardt *et al.*, 2008), as well as self-derived RNA, DNA and associated proteins (Scaffidi *et al.*, 2002; Barrat *et al.*, 2005; Kim *et al.*, 2006; Park *et al.*, 2006). P2Rs are activated by nucleotides such as ATP and UTP, also released after injury (Inoue, 2002; Tsuda *et al.*, 2003).

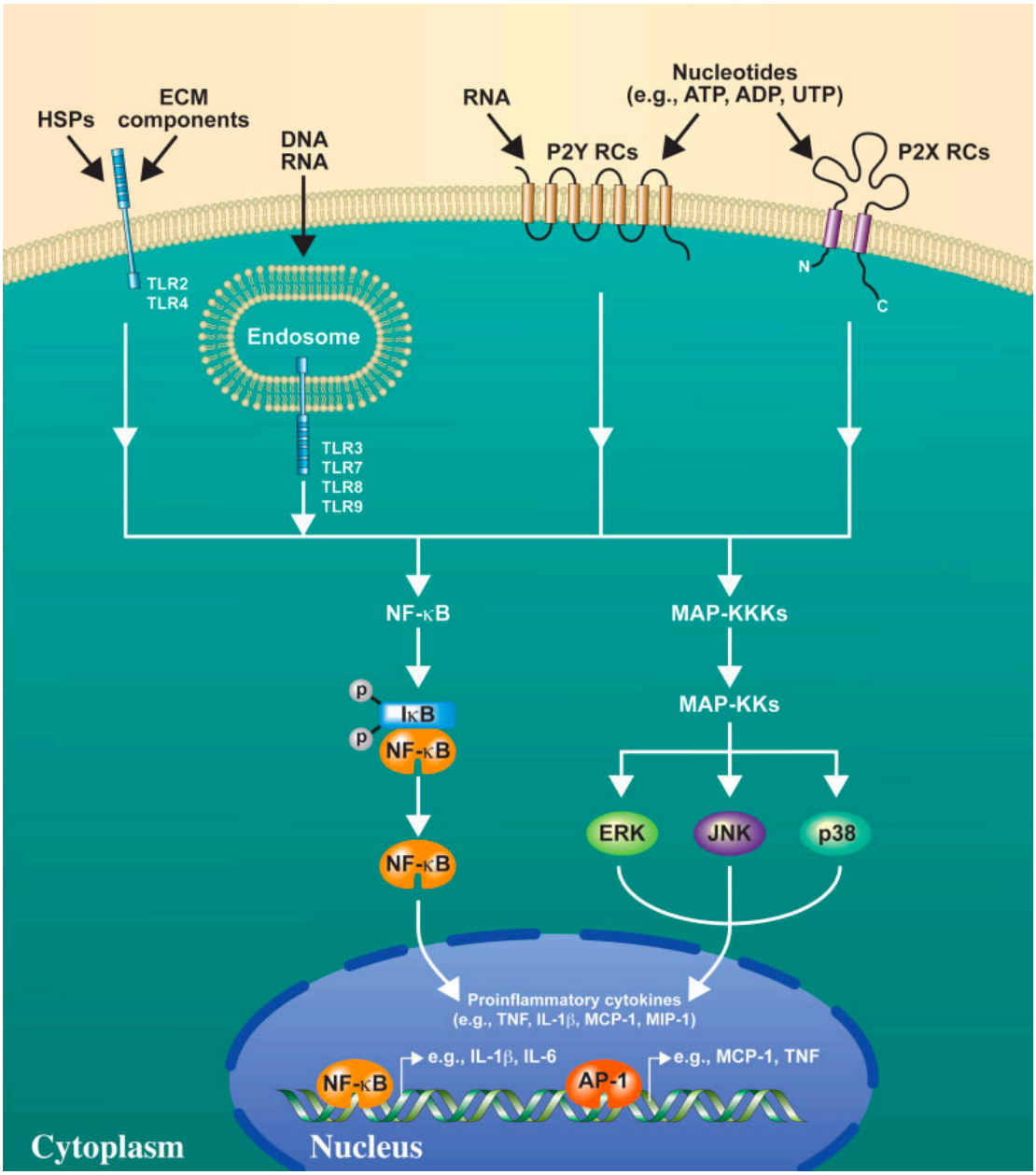


Figure I.4: *Receptors and signalling pathways initiating neuro-inflammation after injury (Pineau & Lacroix, 2009).*

❖ ***Cyto/chemokine network***

Molecules derived from damaged neurons stimulate nearby Schwann cells that initiate the process of WD by releasing pro-inflammatory cytokines and chemokines involved in leukocyte recruitment and activation. Fibroblasts and thereafter, more importantly, recruited macrophages also take part in the development of this complex cyto/chemokine network (figure I.5). The three first cytokines secreted by SCs a few hours after the nerve injury are TNF-α, IL-1α and IL-β (Taskinen *et al.*, 2000; Shamash *et al.*, 2002; Perrin *et al.*, 2005).

These cytokines are essential for WD. They initiate and control the cyto/chemokine network by the induction of several other pro-inflammatory cytokines and chemokines such as MCP-1 and MIP-1α (Taskinen & Roytta, 2000; Subang & Richardson, 2001; Tofaris *et al.*, 2002; Perrin *et al.*, 2005), but also anti-inflammatory molecules such as IL-10 (Shamash *et al.*, 2002).

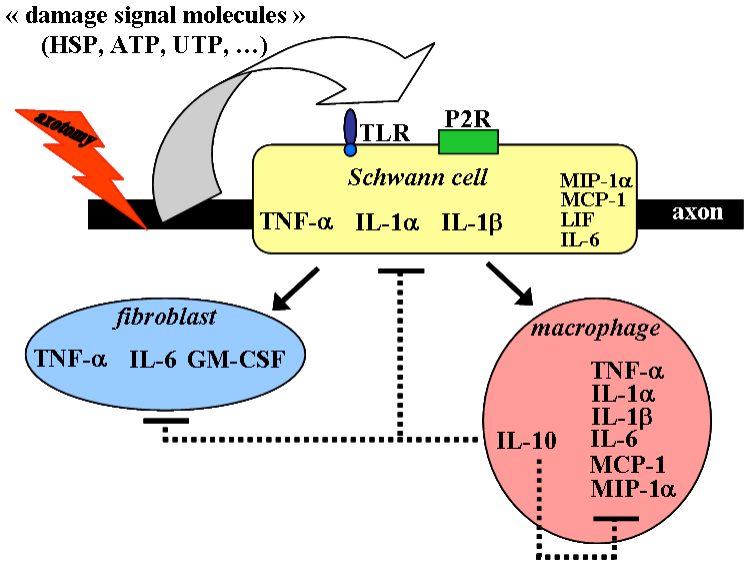


Figure I.5: *Schematic representation of the cyto/chemokine network of Wallerian degeneration (modified from Shamash et al., 2002). The early secretion of TNF-α, IL-1α and IL-β by SCs induces the expression of other pro-inflammatory cytokines and chemokines by macrophages and fibroblasts (arrows) but also the anti-inflammatory cytokine IL-10 that, in turn, downregulates the expression of pro-inflammatory molecules (dotted lines).*

Cytokines promote macrophage recruitment (see below), and, indirectly, myelin clearance; they induce the secretion of neurotrophic factors like NGF, which promote axonal regeneration (Lindholm *et al.*, 1987; Hattori *et al.*, 1994; Golz *et al.*, 2006).

The chemokines MCP-1 and MIP-1α, in addition to their chemotactic effect on macrophages (Toews *et al.*, 1998; Siebert *et al.*, 2000; Perrin *et al.*, 2005), are able to induce the expression of numerous cytokines among which IL-1β, TGF-β and IL-10 (Perrin *et al.*, 2005). GM-CSF, another cytokine induced during WD, is mainly expressed by fibroblasts in the nerve (Saada *et al.*, 1996; Be'eri *et al.*, 1998). GM-CSF induces up-regulation of galectin-3 (MAC-2) in macrophages and SCs, which enhances the phagocytic activity of these cells (Saada *et al.*, 1996). The cytokine network is also regulated by IFN-γ, that stimulates TNF-α and IL-1 production in macrophages, fibroblasts and endothelial cells (Collart *et al.*, 1986; Murwani & Armati, 1998; Ruohonen *et al.*, 2005). Finally, the upregulation of the IL-6 cytokine family members such as LIF and IL-6 contributes to neuronal survival and regenerative responses

(Curtis *et al.*, 1994; Bourde *et al.*, 1996; Kurek *et al.*, 1996; Reichert *et al.*, 1996). These neuropoietic cytokines influence the expression of neurotrophins like NGF and NT-4 (Marz *et al.*, 1999; Golz *et al.*, 2006) that are major actors in axonal outgrowth after lesioning. IL-6 and LIF also act on macrophages, by repressing the expression of TNF-α (Shamash *et al.*, 2002), itself implicated in neuropathic pain (Sommer *et al.*, 1998; George *et al.*, 1999; Leung & Cahill, 2010). Lastly, LIF is retrogradely transported towards neuronal cell bodies and improves neuronal survival by the induction of anti-apoptotic genes (Curtis *et al.*, 1994; Kurek *et al.*, 1996; Schweizer *et al.*, 2002; Moon *et al.*, 2009).

❖ ***Macrophage recruitment and myelin phagocytosis***

As mentioned above, the main functions of pro-inflammatory cytokines and chemokines are to recruit and to activate blood-derived monocytes at the lesion site, for a rapid clearance of myelin debris.

Inflammatory cytokines and chemokines, in addition to their chemotactic effect on monocytes, induce the expression of several classes of molecules involved in the recruitment of blood-derived macrophages: (I) matrix metalloproteinases such as MMP-9 by SCs that opens the blood-nerve barrier (Siebert *et al.*, 2001; Shubayev *et al.*, 2006), (II) NO via the inducible nitric oxyde synthase (iNOS) activation in both SCs and macrophages to increase permeability of the blood-nerve-barrier (Gonzalez-Hernandez & Rustioni, 1999; de la Hoz *et al.*, 2003), and (III) adhesion molecules that mediate monocyte and endothelial cell interactions to allow the trans-endothelial migration of circulating monocytes. For example, the intercellular adhesion molecule-1 (ICAM-1), expressed on endothelial cell surface, mediates migration of monocytes (Schafers *et al.*, 2002; Siebert & Bruck, 2003) through its interactions with several surface receptors like the integrin αMβ2 (= type 3 complement receptor, also called MAC-1 or CD11b), the integrin α4β1 also named VLA-4, as well as the lymphocyte function-associated antigen-1 (LFA-1) (Brown *et al.*, 1997; Avellino *et al.*, 2004).

Once arrived at the injury site, macrophages, assisted by SCs, phagocyte axonal and myelin debris. There are two main mechanisms of phagocytosis dependent or not on opsonisation. The opsonin-dependent phagocytosis is mediated by macrophages expressing the MAC-1 receptor, that recognizes particles opsonised by the complement component C3, or the Fc receptors that bind to auto-antibodies opsonising lipid-rich myelin debris. The opsonin-independent phagocytosis involves a carbohydrate receptor, expressed on

macrophages and SCs, the galactose-specific lectin called MAC-2 (Bruck & Friede, 1990; Reichert *et al.*, 1994; Hirata & Kawabuchi, 2002).

The phagocytic activity of macrophages can be enhanced by the chemokines MCP-1 and MIP-1α that induce the expression of MAC-1 (Conklyn *et al.*, 1996; Weber *et al.*, 1999; Nicholson *et al.*, 2007).

❖ ***Inflammation "turned-off"***

As described above, the inflammatory response plays an important role in Wallerian degeneration after peripheral nerve injury. For this response to be optimal, however, pro-inflammatory signals have to be "turned-off" in a timely manner in order to limit the inflammatory process and avoid adverse effects such as neuropathic pain. Inflammation is down-regulated via two mechanisms. First, macrophages are cleared from the lesioned nerve by a local apoptosis after myelin debris phagocytosis, and by their migration towards lymph nodes and spleen (Kuhlmann *et al.*, 2001). Second, pro-inflammatory cyto/chemokines are downregulated by several anti-inflammatory factors, the production of which is stimulated in two different manners. Pro-inflammatory cytokines such as TNF-α and IL-1 induce the expression of anti-inflammatory cytokines like IL-10 and TGF-β in SCs, macrophages and fibroblasts (Scherer *et al.*, 1993; Jander *et al.*, 1996; Be'eri *et al.*, 1998; Gillen *et al.*, 1998; Taskinen *et al.*, 2000; Perrin *et al.*, 2005; Sawada *et al.*, 2007), and of SOCS (suppressors of cytokine signalling) in SCs (Girolami *et al.*, 2010). In addition, myelin-laden macrophages adopt an anti-inflammatory phenotype and secrete anti-inflammatory molecules like IL-10 and TGF-β (Boven *et al.*, 2006).

I.2.4. Axonal regeneration and remyelination

Following axotomy, injured neurons try to promote their survival by the expression of neurotrophins such as BDNF and GDNF (Kobayashi *et al.*, 1996; Gordon, 2009), and their ability to regrow by the upregulation of growth-associated genes like tubulin, actin and GAP-43 (Bisby & Tetzlaff, 1992). However, the sole efforts of neurons are not sufficient to insure axonal regeneration. Denervated SCs, which dedifferentiate and secrete several molecules, are crucial to promote and guide axonal regeneration, as well as remyelination.

❖ ***Axonal regeneration***

The specific ability of SCs to provide a favourable environment for axonal regeneration is mainly due to their secretion of neurotrophic factors, their organisation in bands of Büngner, as well as their expression of cell adhesion molecules and neuronal guidance molecules.

As mentioned in section I.2.3, SCs secrete neuropoietic cytokines such as LIF and IL-6 that improve neuronal survival and axonal regeneration by binding to their gp 130 receptor. They also synthesize neurotrophins such as NGF, NT-4, BDNF, GDNF, and their low affinity receptor p75 NGFr (Heumann *et al.*, 1987; Taniuchi *et al.*, 1988; Funakoshi *et al.*, 1993; Hammarberg *et al.*, 1996). The upregulation of p75NGFr on the SC surface is accompanied by its down-regulation on axons (Zhou *et al.*, 1996) that, unlike SCs, express the neurotrophin high-affinity tyrosine kinase (Trk) receptors (Frisen *et al.*, 1993; Funakoshi *et al.*, 1993; Kobayashi *et al.*, 1996; Hammarberg *et al.*, 2000). Thus, following injury, p75NGFr expressing SCs migrate and align to form bands of Büngner. Through an autocrine mechanism, SC released-neurotrophins bind to p75NGFr on the SC surface. This creates a local concentration gradient of trophic factors along which fibres can regenerate by the displacement of neurotrophins from SC p75NGFr to axonal Trk receptors (figure I.6) (Zhou *et al.*, 1996; Ramer *et al.*, 1999).

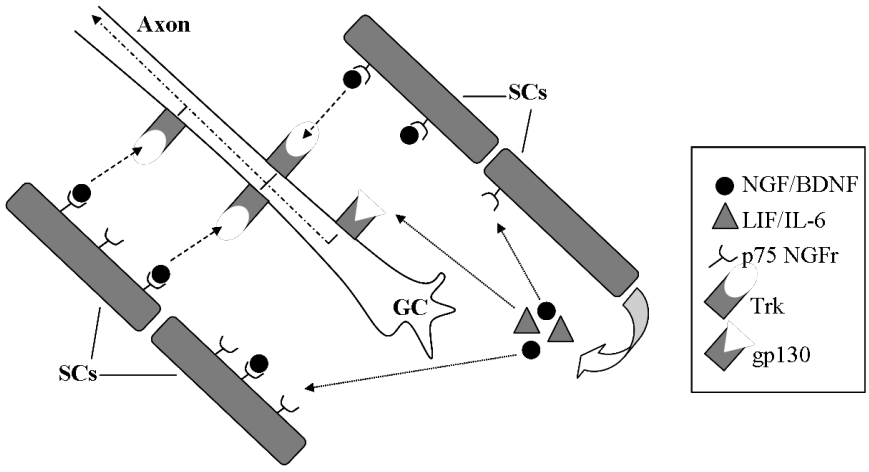


Figure I.6: *Schematic representation of axonal regeneration mechanism (simplified scheme modified from Ramer et al., 1999. Released neurotrophic factors create a local concentration gradient, allowing growth cone (GC) progression. Thereafter, they are retrogradely transported towards neuronal cell bodies to improve neuronal survival (arrow within axon).*

The formation of Büngner's bands results from a complex mechanism that involves a wide variety of ligand-receptor interactions such as NGF and its binding to p75NGFr (Anton *et al.*, 1994), MMP-9 and its LDL receptor-related protein (LRP-1) binding (Mantuano *et al.*, 2008), NRG-1 and its erbB2/3 binding (Lai, 2005; Yamauchi *et al.*, 2008), ephrin-B and its binding to EphB2 (Parrinello *et al.*, 2010) and the class 3 semaphorin receptor neuropilin-2 (NRP-2) (Ara *et al.*, 2005).

In addition, cell adhesion molecules, underlying neuron-glia interactions, are also important for axonal regeneration after nerve injury. Under regenerative conditions, SCs and neurons up-regulate adhesion molecules such as N-CAM, L1/Ng-CAM, N-cadherin and carbohydrates, like polysialic acid or L2/HNK-1, that can form "homo- or hetero-philic" interactions to promote axonal growth (Bixby *et al.*, 1988; Martini, 1994; Fu & Gordon, 1997). SCs also elaborate a basement membrane containing many ECM proteins, such as laminin, fibronectin, collagen and tenascin that can interact with integrins expressed on the surface of axons to support axonal regeneration and guidance (Sketelj *et al.*, 1989; Martini, 1994; Fu & Gordon, 1997).

Next to neurotrophic factors and cell adhesion molecules, other molecules also play a role in axonal guidance during nerve regeneration such as semaphorins and their neuropilin receptors (Scarlato *et al.*, 2003), as well as netrins that can exert both attractive and repulsive effects on regenerating axons depending on their receptors (Madison *et al.*, 2000).

- ***Remyelination***

For optimal functional recovery after peripheral nerve regeneration, axons need to reconnect with their original targets but also to be correctly remyelinated by SCs for a rapid conduction of electrical signals. The remyelination program recapitulates development in many ways and requires a large number of signals between neurons and SCs. Among the numerous axonal molecules involved in the interactions with SCs, the neurotrophin BDNF and the neuregulin NRG1 type III play important roles in axonal remyelination.

As mentioned above, injured neurons secrete the neurotrophic factor BDNF that binds to p75 NGFr on the SC surface and promotes myelin formation by inducing the expression of myelin proteins such as MAG and P0 (Cosgaya *et al.*, 2002; Tolwani *et al.*, 2004). Myelination is also regulated via the level of neuregulin NRG1 type III expressed on the axonal surface that can interact with the erbB2/B3 receptor on the SC surface. In fact, small neurons with unmyelinated axons are characterized by a very low level of NRG1 type III, whereas large neurons express high levels of NRG1 type III allowing to generate a thick

myelin sheath. NRG1 type III acts therefore as a biochemical sensor of axon size to control axonal ensheathment and myelin thickness (Michailov *et al.*, 2004; Taveggia *et al.*, 2005).

All these interaction signals between axons and SCs lead to the activation of a gene regulatory network that will drive the pro-myelinating to myelinating transition of SCs (Svaren & Meijer, 2008). The transcription factors implicated in the myelination program include the neural crest transcription factor Sox10 (Britsch *et al.*, 2001) as well as the POU domain factor Oct6/Scip and Brn2 (Jaegle *et al.*, 2003), that regulate the activation of the transcription factor Krox20/Egr2 (Svaren & Meijer, 2008). These transcription factors control the expression of genes coding for myelin proteins such as Pmp22, P0, MBP, MAG and connexin 32, and for proteins required for lipid synthesis like the3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, the rate-limiting enzyme for cholesterol biosynthesis (Pertusa *et al.*, 2007; Svaren & Meijer, 2008; Quintes *et al.*, 2010).

As mentioned in section I.2.2, SCs dedifferentiate and proliferate after nerve injury to support the growth of regenerating axons. When axons make contact with SCs, only one out of five of the latter participates in remyelination. The remaining SCs without axonal contact are eliminated by apoptosis. The induction of this apoptosis is mediated by two main mechanisms. First, SC death is promoted by the absence of the survival signal induced by the interaction between axonal NRG1 type III and SC receptor erbB2/B3 (Grinspan *et al.*, 1996; Garratt *et al.*, 2000). Moreover, an apoptotic signal can be induced in the supernumerary SCs by the binding of NGF to its p75 NGFr (Hirata *et al.*, 2001), when this neurotrophin is not displaced towards the axonal Trk receptors to promote axonal regeneration (see figure I.5).

I.3. The transcription factor "nuclear factor kappa B"

Nuclear factor kappa B (NF-κB) is an ubiquitous and inducible transcription factor. In mammals, the NF-κB/Rel family comprises five members: p50, p52, p65 (Rel-A), c-Rel and Rel-B, that share a N-terminal 300 amino acid Rel homology domain allowing DNA binding, dimerization and nuclear localization (O'Dea & Hoffmann, 2010). These proteins form homo or heterodimers that are retained inactive in the cytoplasm by interaction with inhibitory molecules, called IκBs. IκBs mask both the NF-κB nuclear localization and DNA binding domains (Zabel & Baeuerle, 1990). Upon various cellular stimulations described below in section I.3.1, NF-κB is activated once the inhibitory molecules are phosphorylated and subsequently degraded. There are two main NF-κB activation pathways: the canonical and the alternative pathways. The classical pathway, triggered by pro-inflammatory cytokines such as TNF-α, involves the recruitment and activation of the classical IκB kinase (IKK) complex, which includes the scaffold protein NF-κB essential modulator (NEMO; also named IKKγ). As for the alternative pathway, triggered by cytokines such as lymphotoxin B, B-cell activating factor or CD40 ligand, and by viruses such as human T-cell leukemia virus and the Epstein-Barr virus, it is NEMO-independent. In both pathways the IKK complex phosphorylates specific amino acids of IκBs, leading to their polyubiquitination and degradation through the proteasome pathway (figure I.7) (Viatour *et al.*, 2005).

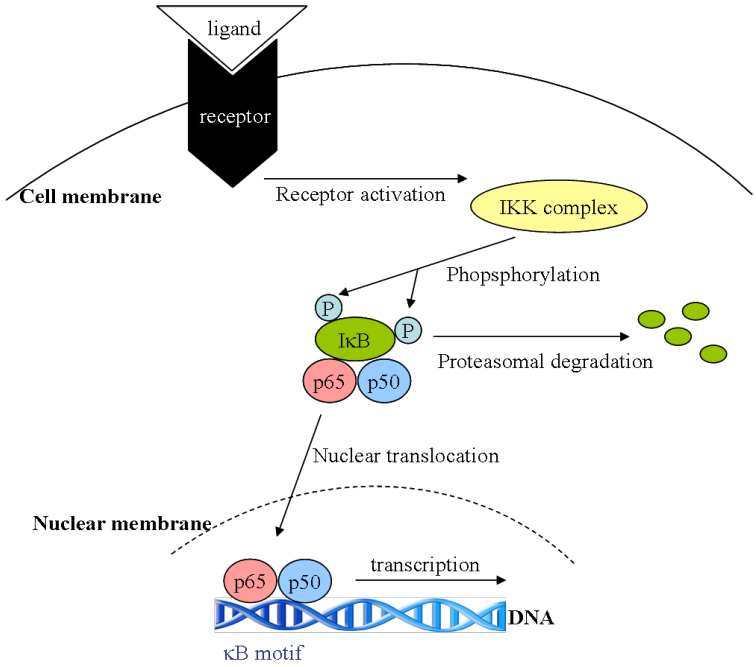


Figure I.7: *General mechanism of NF-κB activation*

The released NF-κB dimers migrate to the nucleus and bind to κB motifs whose consensus sequence is GGGRNNYYCC (C = cytosine, G = guanine, N = any base, R = purine, and Y = pyrimidine), in the promoter or enhancer regions of target genes (Ghosh *et al.*, 1998).

The NF-κB functions in the immune system and host defence mechanisms have been well characterized, contrary to its roles in the nervous system that are less well understood.

I.3.1. Activation factors and target genes of NF-κB

In 1999, Pahl compiled bravely about 150 stimuli and 150 target genes of NF-κB (Pahl, 1999). This ever growing list of activators and targets of NF-κB is updated on a website (www.nf-kb.org) created by the Dr. T.D. Gilmore who dedicated his research to the NF-κB-activating pathways.

Because its activation is induced by a wide array of stimuli including pro-inflammatory cytokines, infection, injury, ischemia, irradiations as well as oxidative stress, NF-κB represents a central regulator of stress responses (Pahl, 1999). In addition to these general NF-κB activators, some stimuli characteristic of the NS such as neurotrophins (Wood, 1995; Carter *et al.*, 1996), glutamate (Kaltschmidt *et al.*, 1995; Scholzke *et al.*, 2003), membrane depolarization (Lilienbaum & Israel, 2003), amyloid β peptide (Behl *et al.*, 1994), neural cell adhesion molecule (N-CAM) (Krushel *et al.*, 1999), as well as sleep deprivation (Brandt *et al.*, 2004) were described as NF-κB-activating molecules. Moreover, some cell type and stimulus specificities have been reported. For example, Il-1β induces NF-κB activity in astrocytes but not in neurons (Srinivasan *et al.*, 2004) while glutamate activates NF-κB only in neurons (Guerrini *et al.*, 1995). NGF activates NF-κB only in SCs (Carter *et al.*, 1996), but all neurotrophins induce its activation in microglia (Nakajima *et al.*, 1998).

In response to the stress stimulus, NF-κB activation promotes the transcription of genes whose products tend to protect against the stressor. The majority of proteins encoded by NF-κB target genes participate in the host immune response. These include, for example, 27 different cytokines and chemokines (Pahl, 1999), as well as receptors required for immune recognition, such as MHC molecules (Israel *et al.*, 1989). Other genes regulated by NF-κB, are those encoding IκBs (de Martin *et al.*, 1993; Sun *et al.*, 1993), therefore providing a negative feedback mechanism whereby resynthesized IκBs bind to DNA-bound NF-kB dimers and export them out to the cytosol.

Knowledge about the genes regulated by NF-κB in the nervous system is scarce and largely extrapolated from the immune system. However, a limited number of genes regulated

by NF-κB in the NS and with direct relevance for nervous functions were identified. These include N-CAM (Simpson & Morris, 2000), iNOS (Madrigal *et al.*, 2001), amyloid precursor protein (Grilli *et al.*, 1996), opioid receptors (Kraus *et al.*, 2003), BDNF (Lipsky *et al.*, 2001) and calcium/calmodulin-dependent protein kinase II (Kassed *et al.*, 2004).

I.3.2. NF-κB functions during Wallerian degeneration

The transcriptionally active form of NF-κB in the NS is primarily the p65/p50 heterodimer, although other dimers, containing the c-Rel subunit, have been described (Memet, 2006). After a PNS injury, there are two peaks of NF-κB activation. A first peak is observed in neurons (Fernyhough *et al.*, 2005) and SCs (Smith *et al.*, 2009). This early NF-κB activation wave is principally caused by axonal "damage signals" that activate TLRs and P2Rs (see section I.2.3.) (Ferrari *et al.*, 1997; Lee *et al.*, 2006; Boivin *et al.*, 2007), mechanical stress caused by the injury (Lan *et al.*, 1994) and SC cytokines (Moynagh *et al.*, 1993; Wood, 1995). The second peak occurs only in SCs during remyelination. This late NF-κB activation is mainly caused by the interactions of NRG1 type III with erbB2/B3 (Limpert & Carter, 2010), and of NGF with p75 NGFr (Carter *et al.*, 1996; Hirata *et al.*, 2001). The three main targets of NF-κB activation after a nerve injury are regulation of neuronal survival, inflammation and neuropathic pain, and remyelination of regenerating fibers. These three different functions of NF-κB activation will be described below.

- ***Regulation of neuronal survival***

NF-κB exhibits major and opposed functions in neurons as it can both promote and protect against cell death. These conflicting neuroprotective and neurodegenerative roles have been discussed in several articles and reviews and depend notably on the neuronal cell type and the stimulus origin and duration (Kaltschmidt *et al.*, 2002; Mattson & Meffert, 2006; Memet, 2006). Moreover, different NF-κB dimers drive opposite effects on neuronal survival. While p50/p65 acts as a transcriptional inducer of proapoptotic genes such as Bim and Noxa, c-Rel dimers promote transcription of anti-apoptotic genes like Bcl-2 and Bcl-xL (Tamatani *et al.*, 1999; Pizzi *et al.*, 2002; Inta *et al.*, 2006; Sarnico *et al.*, 2009). During WD (see section I.2.), injured neurons and SCs release several cytokines of which TGF-β1 and TNF-α. In several experimental models, these two molecules have a neuroprotective effect that requires NF-κB activation (Zhu *et al.*, 2004; Fernyhough *et al.*, 2005; Konig *et al.*, 2005). The

promotion of neuronal survival by NF-κB activation during WD improves the subsequent regeneration of injured fibers.

❖ ***Induction of cytokine expression and neuropathic pain***

PNS and CNS injuries induce the production of pro-inflammatory cyto- and chemokines, such as TNF-α, IL-1β, IL-6 and MCP-1 by glial cells. As mentioned in section I.2.3, this inflammatory response has beneficial effects on axonal regeneration. However, it has also been shown that the pro-inflammatory molecules can have negative effects like the development of neuropathic pain (Wagner & Myers, 1996; Sweitzer *et al.*, 1999; Gao & Ji, 2010). Numerous experiments have shown the role of NF-κB activation in the transcriptional induction of genes encoding inflammation- and pain-related molecules (Tegeder *et al.*, 2004; Ledeboer *et al.*, 2005; Niederberger & Geisslinger, 2008) illustrating that NF-κB, besides its beneficial effect in neuronal regeneration, is also involved in neuropathic pain. Consequently, it was suggested that selective inactivation of NF-κB in inflammatory glial cells may be a therapeutic option for neuropathic pain (Meunier *et al.*, 2007; Fu *et al.*, 2010). This novel approach for pain therapy would, however, reduce the synthesis of neuropoietic cytokines like LIF or IL-6 (Fan *et al.*, 2004) which are beneficial for axonal regeneration.

❖ **Regulation of remyelination and Schwann cell number**

As mentioned previously, during WD, SCs proliferate and migrate to form bands of Büngner that serve as guiding tubes for regenerating axons. This cellular alignment and the extra-cellular matrix deposit across the injury site allow the interaction between regenerating axons and SCs that favours axonal remyelination. Axonal signals promoting remyelination (see section I.2.4.) converge on a complex network of transcription factors, including the POU domain factors Oct6/Scip and Brn2, the high mobility group protein Sox10 and the zinc-finger protein Krox20/Egr2, that drives the transition of SCs from an unmyelinating proliferating to a myelinating state and the induction of myelin protein synthesis (see section I.2.4.). The implication of NF-κB in myelination is a more recent finding. It was shown that NF-κB is required for activation of the Oct6/Scip transcription factor in SCs (Nickols *et al.*, 2003). After remyelination, excessive SCs without axonal contact die by apoptosis that is partly mediated by the binding of NGF to its receptor p75 NGFr, which in turn activates NF-κB, leading to the induction of the apoptotic signal (Carter *et al.*, 1996; Hirata *et al.*, 2001).

I.4. The Vascular Endothelial Growth Factor Family

Recently, it was demonstrated that the nervous and the vascular systems share a common molecular basis for the control of their patterning during development but also their remodelling during pathological conditions (Carmeliet & Tessier-Lavigne, 2005). The most popular angiogenic factors belong to the VEGF family. While VEGF family proteins and their receptors are the primary mediators of the neo-vascularisation process both during development and pathological conditions, other roles, particularly in the nervous system, begin to be recognized.

The VEGF family includes seven secreted glycoproteins, designated VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF). They share a common structure of eight characteristically spaced cysteine residues in a VEGF homology domain (Muller *et al.*, 1997), but have different physical and biological properties and act through specific receptors. The receptors of the VEGF family consist of three protein–tyrosine kinases (VEGFR-1, VEGFR-2, and VEGFR-3) and two non-protein kinase co-receptors (neuropilin-1 and neuropilin-2) (figure I.8).

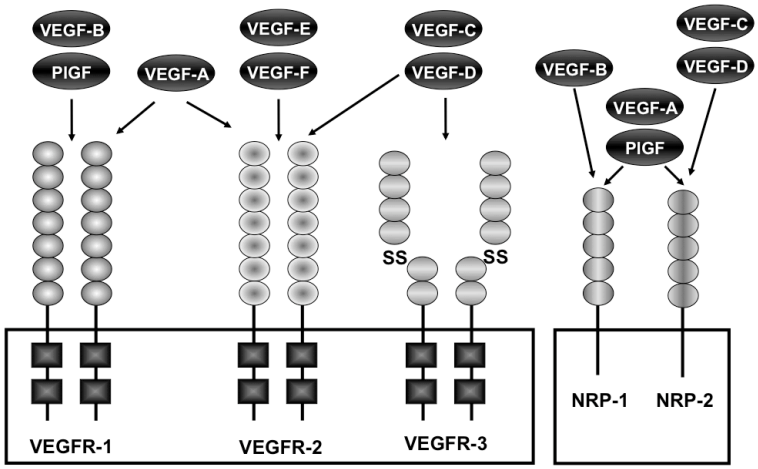


***Figure I.8**: VEGF family members and receptors. The Trk receptor VEGFR-1 binds VEGF-A, VEGF-B and PlGF with high affinity. VEGFR-2 binds VEGF-A, VEGF-C, VEGF-D, VEGF-E and VEGF-F. VEGFR-3 is a tyrosine kinase receptor with only six Ig-homology domains, which preferentially binds VEGF-C and VEGF-D. Neuropilin-1 and -2 (NRP-1 and NRP-2), the two non-tyrosine kinase-type co-receptors, respectively bind VEGF-A_{165}, VEGF-B and PlGF, and VEGF-A_{165}, VEGF-C, VEGF-D and PlGF.*

I.4.1. Members

- ***VEGF-A***

Discovered first (Senger *et al.*, 1983; Leung *et al.*, 1989), VEGF-A is one of the key regulators of angiogenesis, vasculogenesis, and developmental hematopoiesis. The deletion of the VEGF-A gene is lethal, resulting in vascular defects and cardiovascular abnormalities (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). VEGF-A exerts its effects primarily by activating VEGFR-1 (de Vries *et al.*, 1992) and VEGFR-2 (Quinn *et al.*, 1993) but its major isoform that triggers VEGF-A actions, the VEGF-A_{165}, also binds to neuropilins (Soker *et al.*, 1998; Gluzman-Poltorak *et al.*, 2000).

VEGF-A is produced in various organs such as lung, kidney, heart and liver (Berse *et al.*, 1992), and in cell types, including endothelial cells (ECs) (Ladoux & Frelin, 1993a), leucocytes (Berse *et al.*, 1992; Shen *et al.*, 1993; Freeman *et al.*, 1995), smooth muscle cells (Ferrara *et al.*, 1991; Kuzuya *et al.*, 1995), as well as neurons (Kovacs *et al.*, 1996; Sondell & Kanje, 2001), astrocytes (Stone *et al.*, 1995; Bartholdi *et al.*, 1997) and SCs (Sondell *et al.*, 1999a; Gupta *et al.*, 2005). VEGF-A expression is stimulated under pathological conditions such as hypoxia (Shweiki *et al.*, 1992; Ladoux & Frelin, 1993b), hypoglycemia (Satake *et al.*, 1998) or inflammation (Ben-Av *et al.*, 1995; Ito *et al.*, 1995).

VEGF-A has similar effects on the development of the vascular and nervous systems and on adult angiogenesis and neurogenesis. VEGF-A is a mitogen for ECs (Connolly *et al.*, 1989), vascular smooth muscle cells (Bhardwaj *et al.*, 2005), neuronal precursors (Jin *et al.*, 2002), astrocytes (Krum *et al.*, 2002) and SCs (Sondell *et al.*, 1999a). VEGF-A promotes survival of ECs (Gerber *et al.*, 1998) and neurons (Sondell *et al.*, 1999a) by inducing the expression of anti-apoptotic proteins. VEGF-A stimulates chemotaxis of ECs and vascular smooth muscle cells to induce vessel formation (Kanno *et al.*, 2000; Gerhardt *et al.*, 2003; Bhardwaj *et al.*, 2005), of monocytes during inflammatory response (Clauss *et al.*, 1990; Zhao *et al.*, 2002), of neurons during brain development (Schwarz *et al.*, 2004; Balenci *et al.*, 2007), and of SCs during WD (Sondell *et al.*, 1999b). VEGF-A also guides angiogenic (Gerhardt *et al.*, 2003) and axonal sprouting (Sondell *et al.*, 1999a; Cheng *et al.*, 2004). Finally, VEGF-A causes vasodilatation by inducing NO synthesis (Yang *et al.*, 1996) and exerts pro-coagulant activity (Clauss *et al.*, 1990).

❖ ***VEGF-B***

VEGF-B expression begins early during fetal development and is abundant particularly in heart, brain and spinal cord. During adulthood, VEGF-B is additionally expressed in kidney and testis (Lagercrantz *et al.*, 1998).

VEGF-B binds to VEGFR-1 and NRP-1 but not VEGFR-2 or VEGFR-3 (Olofsson *et al.*, 1998; Makinen *et al.*, 1999). VEGF-B modulates cell proliferation and vessel growth (Olofsson *et al.*, 1996; Silvestre *et al.*, 2003), but it has no vascular permeability activity (Abraham *et al.*, 2002; Brkovic & Sirois, 2007). Some studies indicate, however, that VEGF-B can form stable heterodimers with VEGF-A when co-expressed with the latter (Olofsson *et al.*, 1996; Tammela *et al.*, 2005; Nash *et al.*, 2006). These heterodimers might cause the angiogenic activity of VEGF-B. Studies with mice deficient in VEGF-B reported development of smaller hearts and impaired recovery after induced myocardial infarction (Bellomo *et al.*, 2000). Moreover, studies of inflammatory disease models in knock-out mice suggest a role of VEGF-B in inflammatory angiogenesis (Mould *et al.*, 2003).

In the nervous system, VEGF-B is not essential for the development or survival of neurons under normal conditions. However it exerts a neuroprotective activity in several pathological conditions (Sun *et al.*, 2004; Li *et al.*, 2009) and stimulates adult neurogenesis (Sun *et al.*, 2006).

❖ ***VEGF-C***

VEGF-C is primarily a lymphangiogenic growth factor and its lymphangiogenic effects are mediated by VEGFR-3 and NRP-2 (Oh *et al.*, 1997; Karpanen *et al.*, 2006). VEGF-C null mice show failure in lymphatic vessel development and die of oedema during the embryonic stage (Karkkainen *et al.*, 2004). During development, VEGF-C is expressed along with its receptor VEGFR-3 mainly in regions where lymphatic vessels develop. During adulthood, the expression decreases in most tissues but remains high in lymph nodes (Kukk *et al.*, 1996; Lymboussaki *et al.*, 1999).

VEGF-C has also angiogenic effects. It induces mitogenesis, migration and survival of ECs (Saharinen *et al.*, 2004) and increases vascular permeability. The latter effect is mediated by VEGFR-2 (Joukov *et al.*, 1998). VEGF-C is also chemotactic for macrophages (Skobe *et al.*, 2001).

With regard to the nervous system, VEGF-C regulates expansion of the population of oligodendrocyte precursor cells and neural progenitors in vitro and acts as a trophic factor for these cells in vivo (Le Bras *et al.*, 2006).

❖ ***VEGF-D***

VEGF-D is present in most tissues, most abundantly in the lungs and skin during embryogenesis. During adulthood, it is expressed in many tissues including the vascular endothelium, heart, skeletal muscle, lung, and bowel (Farnebo *et al.*, 1999; Roy *et al.*, 2006). Similar to VEGF-C, VEGF-D also shows lymphangiogenic and angiogenic activities through its binding to VEGFR-2 and VEGFR-3 to induce proliferation and migration of ECs (Achen *et al.*, 1998; Rissanen *et al.*, 2003).

In the nervous system, akin to VEGF-C, VEGF-D induces the proliferation of glial precursor cells and exerts a chemotactic effect on these cells during brain development (Kranich *et al.*, 2009).

❖ ***VEGF-E***

Viral VEGFs (also called VEGF-E) are found in various strains of viruses. These VEGF-E variants exhibit significant variation in their VEGFR-2 and NRP-1-binding abilities. Viral VEGFs play a critical role in viral infections because of their effects on EC proliferation and vascular permeability (Ogawa *et al.*, 1998; Wise *et al.*, 1999; Roy *et al.*, 2006; Shibuya, 2009).

❖ ***VEGF-F***

Discovered in viper venom (Komori *et al.*, 1999), VEGF-F has a unique property: it blocks specifically VEGF-A165 activity both in vitro and in vivo (Yamazaki *et al.*, 2005). However, it stimulates strongly EC proliferation and vascular permeability, and induces hypotension (Yamazaki *et al.*, 2003). VEGF-F members are classified into 3 groups: VEGF-F1 specifically binds to VEGFR-2, VEGF-F2 predominantly binds to VEGFR-1 and weakly to VEGFR-2, and VEGF-F3 binds VEGFR-1, VEGFR-2 and NRP-1 (Yamazaki & Morita, 2006).

❖ ***PlGF***

Although originally discovered in the placenta (Maglione *et al.*, 1991), PlGF is expressed in a wide variety of tissues and organs such as heart, lungs, thyroid, skeletal muscle (Maglione *et al.*, 1993), adipose tissue (Voros *et al.*, 2005), and brain (Beck *et al.*, 2002; Hayashi *et al.*, 2003), as well as in cells like activated angiogenic ECs, inflammatory cells, bone marrow cells (Autiero *et al.*, 2003a), and neurons (Beck *et al.*, 2002)**.** PlGF expression is upregulated during a number of pathological conditions including some cancers (Takahashi *et*

al., 1994; Nomura *et al.*, 1998), wound healing (Failla *et al.*, 2000), hypoxia (Simpson *et al.*, 1999; Green *et al.*, 2001), or inflammatory diseases (Perelman *et al.*, 2003; Yoo *et al.*, 2009). There are four human isoforms of PlGF (PlGF-1, -2, -3 and -4) (Maglione *et al.*, 1993; Cao *et al.*, 1997; Yang *et al.*, 2003), but only one, PlGF-2, is present in mouse (DiPalma *et al.*, 1996). The PlGF isoforms differ in size and binding affinity. They all bind VEGFR-1 (Park *et al.*, 1994) but only PlGF-2 binds NRPs and heparan sulfate proteoglycans thanks to the insertion of 21 basic amino acids at the carboxy terminal (Migdal *et al.*, 1998; Neufeld *et al.*, 2002b). Similar to VEGF-B, PlGF can form heterodimers with VEGF-A (Cao *et al.*, 1996). Loss of PlGF does not affect development, reproduction, or normal postnatal life. However, PlGF knockout mice have impaired angiogenesis and collateral vascular growth during ischemia, wound healing, cancer or inflammation (Carmeliet *et al.*, 2001).

PlGF contributes to angiogenesis via several mechanisms (figure I.9): (I) by affecting ECs directly through VEGFR-1; (II) by displacing VEGF-A from the "VEGFR-1 sink" and thus increasing the fraction of VEGF-A available to activate VEGFR-2; (III) by intermolecular transphosphorylation of VEGFR-2 via VEGFR-1 activation, increasing VEGF-A action; (IV) by PlGF/VEGF-A heterodimerization, which can act on VEGFR-1/VEGFR-2 receptors (Carmeliet *et al.*, 2001; Autiero *et al.*, 2003b; Ribatti, 2008).

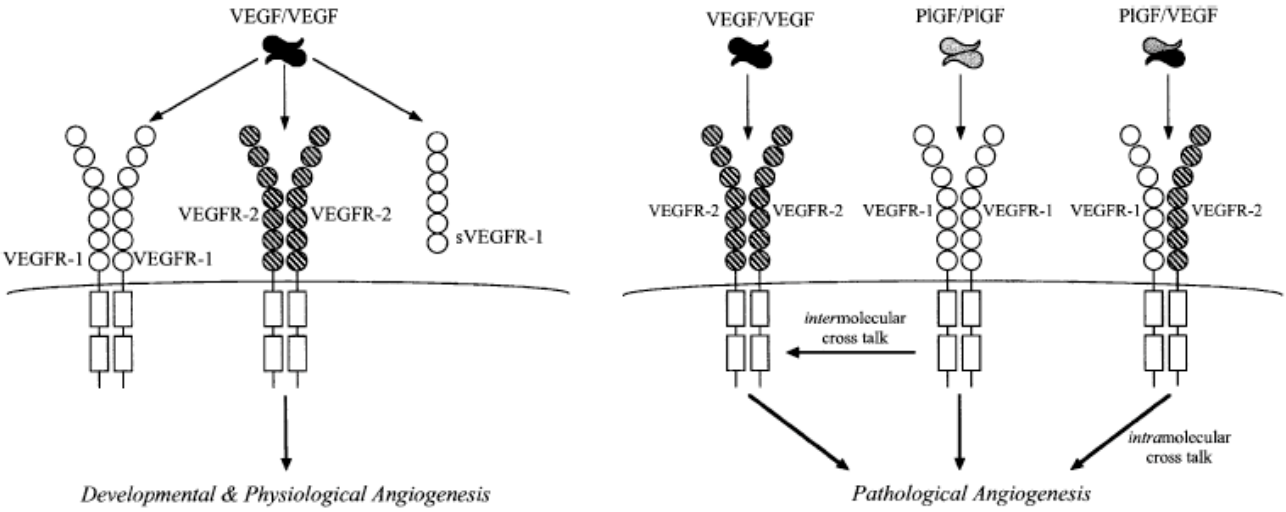


Figure I.9: *Schematic representation of the role of VEGF and PlGF in angiogenesis (from Tjwa et al., 2003).*

PlGF is also a pro-inflammatory molecule. It has a powerful chemotactic effect on monocytes (Carmeliet *et al.*, 2001; Pipp *et al.*, 2003) and induces secretion of pro-inflammatory cyto/chemokines like IL-1b, IL-8, MCP-1, and VEGF by monocytes (Bottomley *et al.*, 2000; Perelman *et al.*, 2003; Selvaraj *et al.*, 2003).

Concerning the nervous system, some studies have suggested a potential neuroprotective/neurotrophic effect of PlGF through NRP binding (Beck *et al.*, 2002; Cheng *et al.*, 2004; Liu *et al.*, 2006).

I.4.2. Receptors

- ***VEGFR-1***

VEGFR-1 (fms-like tyrosine kinase, Flt-1) is composed of seven extracellular immunoglobulin homology domains, a single transmembrane region and an intracellular tyrosine kinase domain (Shibuya *et al.*, 1990). VEGFR-1 binds VEGF-A, VEGF-B and PlGF with high affinity, however its tyrosine kinase activity is weak following stimulation by VEGF (Klagsbrun & D'Amore, 1996).

VEGFR-1 is expressed in ECs as well as in pericytes, placental trophoblasts, osteoblasts, monocytes/macrophages, renal mesangial cells, some hematopoietic stem cells (Zachary & Gliki, 2001) and SCs (Schratzberger *et al.*, 2000). Its expression is upregulated during angiogenesis (Plate *et al.*, 1993; Tsopanoglou & Maragoudakis, 1999) and by hypoxia (Gerber *et al.*, 1997). VEGFR-1 expression is also strongly upregulated in reactive astrocytes in response to CNS injury (Choi *et al.*, 2007; Krum *et al.*, 2008).

VEGFR-1 knockout mice die at early stages of embryogenesis due to disorganization of blood vessels (Fong *et al.*, 1995). Deletion of VEGFR-1 also reduces vessel proliferation and induces premature senescence of ECs (Hiratsuka *et al.*, 2005). Because of its weak tyrosine kinase activity, VEGFR-1 was believed to function as a "decoy" receptor for VEGF (Park *et al.*, 1994). However, even if VEGFR-1 transmits weak mitogenic signals to ECs, it can form heterodimers with VEGFR-2, increasing its signalling properties (Sato *et al.*, 2000).

Other studies indicated that VEGFR-1 has an active functional role in monocyte (Barleon *et al.*, 1996; Clauss *et al.*, 1996) and vascular EC migration (Kanno *et al.*, 2000).

In the nervous system, VEGFR-1 mediates astroglial and microglial proliferation (Choi *et al.*, 2007; Krum *et al.*, 2008). Recent findings reveal that VEGFR-1 exerts neuroprotective effects during pathological conditions (Poesen *et al.*, 2008).

A soluble form of VEGFR-1, which contains the six first extracellular immunoglobulin-like domains, also exists and mediates inhibition of VEGF-induced EC proliferation with high affinity (Kendall & Thomas, 1993). This soluble receptor could act as an efficient specific antagonist of VEGF-A or PlGF.

- ***VEGFR-2***

VEGFR-2 (kinase-insert domain receptor, KDR/fetal liver kinase, Flk-1) mediates the major biological activities of VEGF-A. Despite its lower affinity for VEGF than VEGFR-1, VEGFR-2 exhibits robust protein–tyrosine kinase activity when bound to its ligands (Klagsbrun & D'Amore, 1996). Like VEGFR-1, VEGFR-2 bears an extracellular region with seven immunoglobulin-like domains, a transmembrane domain and a Trk domain with a 70-amino-acid insert (Matthews *et al.*, 1991). VEGFR-2 binds VEGF-A, VEGF-C, VEGF-D and VEGF-E (Quinn *et al.*, 1993; Joukov *et al.*, 1996; Achen *et al.*, 1998; Yamazaki & Morita, 2006).

Besides by ECs (Quinn *et al.*, 1993) VEGFR-2 is also expressed by pancreatic duct cells (Oberg *et al.*, 1994), retinal progenitor cells (Yang & Cepko, 1996), hematopoietic stem cells (Matthews *et al.*, 1991), neurons (Sondell *et al.*, 2000; Ogunshola *et al.*, 2002) and some glial cells (Schratzberger *et al.*, 2000; Krady *et al.*, 2002; Lafuente *et al.*, 2006).

VEGFR-2 null mice die between embryonic days 8.5 and 9.5 as a result of defects in the development of hematopoietic and endothelial precursors (Shalaby *et al.*, 1995).

In the vascular system, the VEGFR-2 signalling pathway mediates EC migration (Gerhardt *et al.*, 2003), proliferation (Keyt *et al.*, 1996), survival (Shalaby *et al.*, 1995), and enhances vascular permeability (Prewett *et al.*, 1999) and vasodilatation (He *et al.*, 1999).

In the nervous system, VEGFR-2 also stimulates migration, proliferation, and survival of various neural cell types such as neural progenitors (Ogunshola *et al.*, 2002; Zhu *et al.*, 2003; Balenci *et al.*, 2007) or SCs (Sondell *et al.*, 1999b; a).

- ***VEGFR-3***

VEGFR-3 (fms-like tyrosine kinase 4, Flt4) is a Trk receptor with only six Ig-like domains (Pajusola *et al.*, 1993; Agnes *et al.*, 1997). VEGFR-3 preferentially binds VEGF-C and VEGF-D (Joukov *et al.*, 1996; Achen *et al.*, 1998).

VEGFR-3 is present on all endothelia during development (Kukk *et al.*, 1996) but in adulthood it becomes restricted to lymphatic ECs and certain fenestrated blood vascular ECs (Partanen *et al.*, 2000) where it is upregulated in pathological conditions such as vascular

tumors (Kubo *et al.*, 2000). During embryogenesis, VEGFR-3 plays various roles in cardiovascular development and remodelling of primary vascular networks (Dumont *et al.*, 1998). In adults, it is the primary receptor inducing lymphangiogenesis (Oh *et al.*, 1997), but it also modulates angiogenesis by transmitting sprouting signals in endothelial tip cells (Tammela *et al.*, 2008). In the nervous system, VEGFR-3 induces proliferation of oligodendrocyte precursors and certain other neural progenitors (Le Bras *et al.*, 2006).

❖ ***Neuropilins***

NRPs were identified initially as cell-surface glycoproteinic receptors for the semaphorin/collapsins (Kolodkin *et al.*, 1997), a large family of secreted and transmembrane proteins that serve as repulsive guidance signals in axonal and neuronal development (Puschel *et al.*, 1995). These non-tyrosine kinase-type receptors also bind some members of the VEGF family. NRP-1 binds VEGF-A165, VEGF-B and PlGF (Makinen *et al.*, 1999; Mamluk *et al.*, 2002) while NRP-2 binds VEGF-A165, VEGF-C, VEGF-D and PlGF (Gluzman-Poltorak *et al.*, 2000; Neufeld *et al.*, 2002b; Karpanen *et al.*, 2006).

In adults, NRPs are expressed in various organs like lung, heart, liver, kidney, pancreas, bones, skeletal muscles and brain (Otrock *et al.*, 2007). In the vascular system, NRP-1 expression is mainly restricted to arteries, whereas NRP-2 is primarily expressed in veins (Herzog *et al.*, 2001). NRP-2 is also expressed on lymphatic endothelial cells (Yuan *et al.*, 2002). In the CNS and PNS, both neuronal and glial cells express NRPs (Fujisawa *et al.*, 1997; Fujita *et al.*, 2001; Scarlato *et al.*, 2003).

NRPs have roles in immune functions (Romeo *et al.*, 2002), neuronal development and repair by the regulation of axon outgrowth, and also in angiogenesis (Neufeld *et al.*, 2002a; Bannerman *et al.*, 2008; Fantin *et al.*, 2009). NRPs enhance VEGF signalling by acting as co-receptors for VEGF receptors (Soker *et al.*, 1998; Bagnard *et al.*, 2001; Neufeld *et al.*, 2002b). However, it has recently been reported that NRP-1 mediates some types of intracellular signalling via its short C-terminal tail and its interaction with intracellular binding partners (Zachary *et al.*, 2009; Jiang *et al.*, 2010).

I.4.3. Therapeutic uses

VEGF family members have been implicated in several pathologies, in which they can trigger either negative or positive effects, depending on the context. Therefore, two types of opposite therapeutic strategies have been developed: “anti-VEGF” and “pro-VEGF” therapies.

❖ ***Anti-VEGF therapy***

Since a few years, VEGF/VEGF-receptor inhibition strategies have emerged to treat diseases associated with pathological angiogenesis like cancers (Borgstrom *et al.*, 1996; Inoue *et al.*, 2000) and age-related macular degeneration (Schmucker *et al.*, 2010), or with pathological inflammatory response like autoimmune diseases (Carvalho *et al.*, 2007), in which serum VEGF levels correlate with disease severity, e.g. rheumatoid arthritis (Harada *et al.*, 1998; Sone *et al.*, 2001) or multiple sclerosis (Su *et al.*, 2006; Zhu *et al.*, 2008). Currently there are several clinical trials testing drugs that inhibit VEGF receptors or neutralize VEGF to treat various types of cancer (Rosen, 2001; Grothey & Galanis, 2009) and rheumatoid arthritis (Khong *et al.*, 2007; Schoettler & Brahn, 2009). Some studies have also demonstrated that monoclonal antibodies directed against PlGF or its receptor VEGFR-1 can have anti-angiogenic and anti-inflammatory properties and substitute or augment the effect of anti-VEGF therapy (Luttun *et al.*, 2002c; Fischer *et al.*, 2007). VEGF/VEGF-receptor inhibition could be a useful therapy for autoimmune nervous system diseases such as multiple sclerosis by reducing the pathological angiogenic and inflammatory reaction (Kirk & Karlik, 2003; Zhu *et al.*, 2008). However, the neuroprotective and neurotrophic effects of VEGF (Sun *et al.*, 2003) will be also reduced by such treatments.

❖ ***Pro-VEGF therapy***

By contrast, the administration of VEGF might be a promising treatment for ischemic disorders like myocardial infarction (Pearlman *et al.*, 1995; Goncalves *et al.*, 2010) or brain ischemia (Zhang *et al.*, 2000; Hermann & Zechariah, 2009) by improving angiogenesis. In an animal model of brain ischemia, VEGF treatment favoured recovery via neurotrophic and neuroprotective effects, in addition to its angiogenic action (Sun *et al.*, 2003). These neurotrophic/neuroprotective effects of VEGF might also useful for the treatment of neurodegenerative disorders like amyotrophic lateral sclerosis (ALS) or Parkinson's disease. For example, in an ALS rodent model, it was shown that VEGF increases the survival of motoneurons and slows motor function decline (Lambrechts *et al.*, 2003; Azzouz *et al.*, 2004). Such a neuroprotective effect of VEGF has been also demonstrated for dopaminergic neurons in a rodent model of Parkinson's disease (Yasuhara *et al.*, 2004).

Unfortunately, because of its angiogenic properties VEGF can cause, as treatment side effects, hypotension, oedema, fibrin deposition, or formation of hemangiomas (Pettersson *et al.*, 2000; Luttun *et al.*, 2002c; Valable *et al.*, 2005). These adverse effects of VEGF may limit

its therapeutic usefulness. An alternative to VEGF-A therapy would be the use of other VEGF family members such as VEGF-B or PlGF, which have less harmful angiogenic effects than VEGF-A (Luttun *et al.*, 2002b; Poesen *et al.*, 2008; Ruiz de Almodovar *et al.*, 2009).

Section II

Objectives and Experimental approach

This research project was designed to better understand and decipher the mechanisms responsible for the successful axonal repair in the PNS, reasoning that this might also be helpful to develop therapeutic strategies to promote axonal regeneration after CNS injuries. Numerous molecules such as cytokines or neurotrophic factors are modulators of axonal regrowth. Among them, the angiogenic factors of the VEGF family play beneficial roles in nervous system trauma/disease. Most attention has been focused on the main representative member of this family, VEGF-A. As mentioned in the introduction, however, other members of this family, such as PlGF, play a role in nervous system injury and could have a more favourable side effect profile than VEGF-A in future therapeutic trials. Because of its chemoattractive effect on monocytes, and its capacity to induce cyto/chemokine secretion by monocytes, **PlGF merits indeed attention particularly as a novel actor in the complex molecular network regulating Wallerian degeneration where it has not been studied up to now**. To try to fill in this gap in scientific knowledge, the major objective of our project was to find answers to the following 3 questions:

- Is PlGF expressed in the peripheral nervous system, in physiological and pathological conditions?
- Does PlGF play a role in the cellular and molecular events of WD and its functional consequences?
- If yes, what are the underlying molecular mechanisms?

To answer these questions, we have used *in vivo* and *in vitro* experimental approaches (described in detail in section VI. “Materials and Methods”).

II.1. PlGF expression

As PlGF expression in the PNS was totally unknown when we started this project, the first part of our work has focused on the identification of the cell types and cellular structures expressing PlGF in the normal peripheral nerve and, in a second step (see below) in the injured nerve. The main method used for this study was double immunofluorescent staining, using a specific anti-PlGF antibody (Ab) and Abs for axons, myelinating and proliferating SCs, myelin sheaths, nodes of Ranvier, fibroblasts, endothelial cells and macrophages. The results on the anatomical localization of the PlGF protein were completed by the study of its mRNA expression using *in situ* hybridization (ISH) and RT-PCR.

II.2. PlGF functions

An interesting method to study the roles of a protein in the whole organism is the use of knock-out mice, wherein the gene of interest is "deleted". Thus, the function of a protein can be explored by the comparison of biological phenomena in the presence (wild-type) or absence (knock-out) of this protein. Thanks to a collaboration with the research group Professor P. Carmeliet from the Vesalius Research Centre at the Katholieke Universiteit of Leuven, we were fortunate to raise and study a colony of knock-out mice for PlGF (*Pgf*$^{-/-}$).

II.3. The model of Wallerian degeneration

The most simple and reproducible model of peripheral WD consists of the complete transection of the sciatic nerve, which is the longest and the largest sensory-motor nerve. The advantage of transection compared to compression of the nerve is the better reproducibility of the injury. Moreover, it is easier to follow the sequence of events of axonal degeneration after a transection where it is anterograde than after compression where the axonal fragmentation progresses retrogradely (Beirowski *et al.*, 2005). Finally, axotomy is a more appropriate model for the clinical situation of traumatic nerve injury in humans.

II.4. The experimental protocol

To explore the hypothesis of a potential role of PlGF in WD, we have compared the main steps of WD between wild-type (wt) and *Pgf* $^{-/-}$ mice, using the complete sciatic nerve transection model:

- SC proliferation explored *in vivo* and *in vitro* by Ki67 and bromodeoxyuridine (BrdU) immunofluorescent (IF) stainings.
- SC upregulation of p75 NGFr and c-Fos evaluated by immunostaining.
- Macrophage recruitment evaluated by CD11b immunostaining.
- Cyto/chemokine expression studied by cytokine arrays.
- Myelin sheaths degradation explored by toluidine blue staining on semi-thin sections.
- Bands of Büngner formation assessed *in vivo* by the p75NGFr expression pattern and *in vitro* by the "scratch" migration assay.
- Axonal regeneration explored by neurofilament immunostaining.
- Remyelination evaluated by toluidine blue staining and teasing.
- Functional motor recovery assessed with the behavioural footprint test.

Given our first results on PlGF expression, two additional research questions were raised and explored:

- ❖ Does PlGF play a role in the formation and/or the preservation of myelin sheaths?
- ❖ Which signalling pathway regulates PlGF expression during WD?

The potential PlGF influence on the structure of myelin sheaths was explored at the ultra-structural level by electron microscopy on cross-sections of sciatic nerves and spinal cords comparing wt and *Pgf*$^{-/-}$ mice.

Concerning the signalling pathway that regulates PlGF expression after injury, we have focused our attention on NF-κB, a transcription factor known to be involved in the regulation of cyto/chemokine expression during WD (see section I.3.2.). In particular, we have studied the NF-κB binding on *Pgf* promoter after injury by the chromatin immunoprecipitation (IP) assay.

Our objectives are summed up in the figure II.1 which represents an overview of the known implications of VEGF and NF-κB during the WD and the possible actions of PlGF that we want to study.

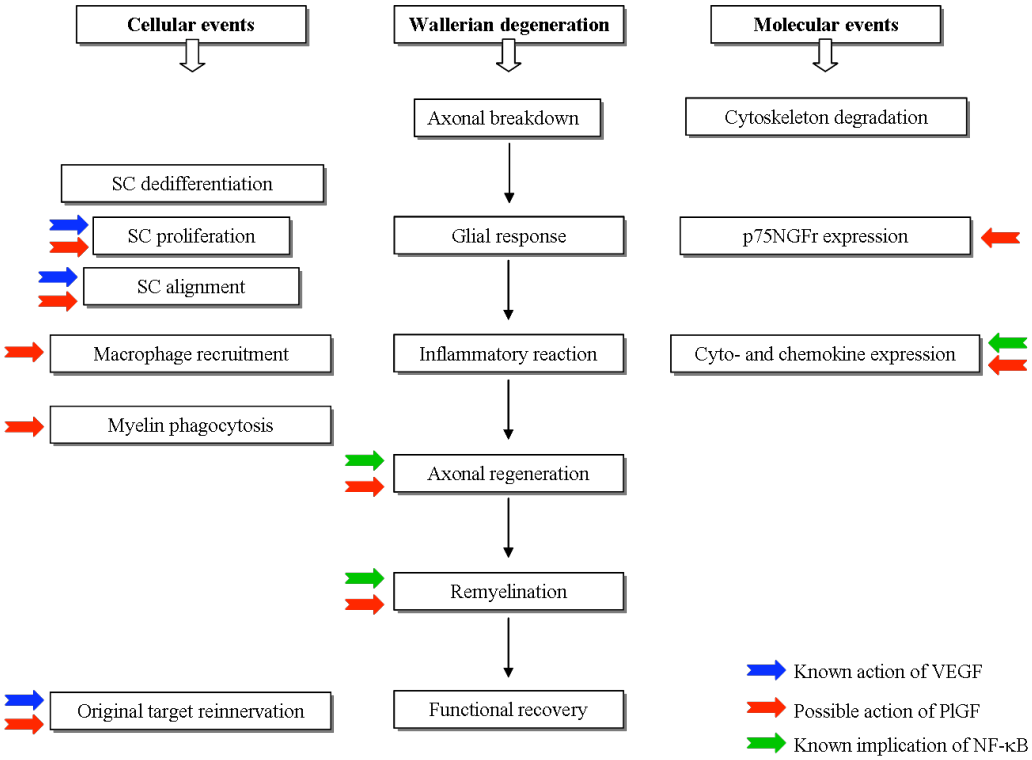


***Figure II.1**: Actions of VEGF (blue arrows) and NF-κB (green arrows) on the cellular and molecular events of the Wallerian degeneration. Hypothetic actions of PlGF (red arrows).*

Section III

Results

III.1. PlGF expression pattern in the adult murine nervous system.

The first part of our work is focused on the characterization of PlGF expression patterns in normal and injured adult mouse sciatic nerves. As the sciatic nerve contains motor fibres originating from spinal motoneurons, and sensitive fibres whose cell bodies are located in the dorsal root ganglion (DRG), we've also studied PlGF expression in those nervous structures.

This study was mainly performed by double IF stainings. To validate the specificity of the PlGF Ab, we performed various types of controls:

- Negative controls:
  - IF staining with the PlGF Ab pre-incubated or not with its specific blocking peptide prior its application on the tissue (figure III.1A and B).
  - IF staining on a tissue section negative for PlGF, the liver (Yano *et al.*, 2006) (figure III.1D).
- Positive control:
  - IF staining on tissue section positive for PlGF, the placenta (figure III.1C)

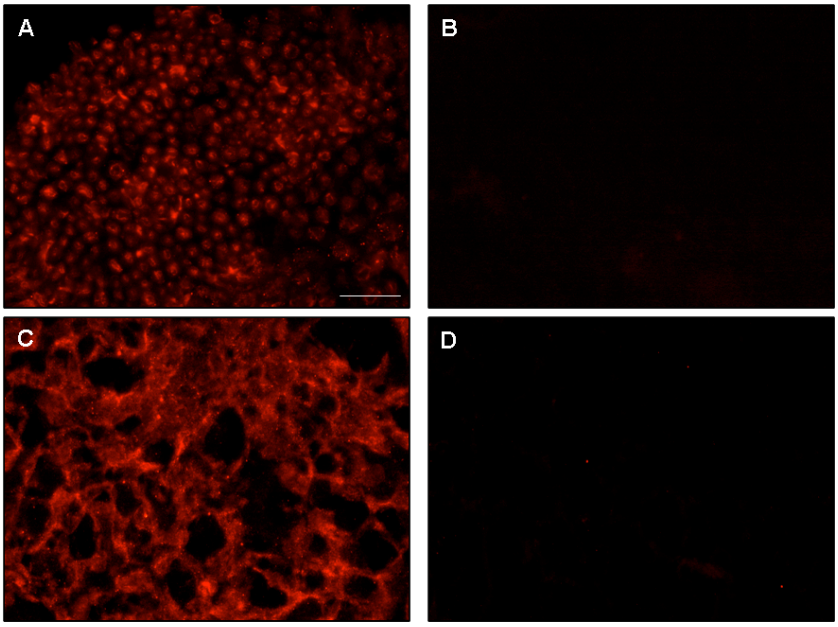


***Figure III.1*: PlGF antibody specificity.** *(A) PlGF immunostaining on transverse section of sciatic nerve. (B) Negative control: sciatic nerve section stained with PlGF Ab pre-incubated with its specific blocking peptide. (C) PlGF immunostaining on mouse placental section. (D) PlGF immunostaining on mouse liver section. Scale bar: 60 µm.*

III.1.1. PlGF expression in normal and injured adult mouse sciatic nerves

To investigate PlGF expression in the normal nerve and after axotomy, we performed various double IF stainings in order to screen all cell types and/or structures present in the nerve: **axons** (heavy neurofilament subunit (NF-H)), **myelin** (P0), **Schwann cells** (S100 or the low-affinity receptor of nerve growth factor (p75NGFr)), **nodes of Ranvier** (voltage-gated sodium channels 1.6 ($Na_{v1.6}$)), **fibroblasts** (patched-1, (Ptc-1)), **endothelial cells** (von Willebrandt factor (vWF)), and **invading macrophages** (CD11b).

Based on PlGF/NF double staining, PlGF is found on the axons of the normal sciatic nerve (figure III.2A). More precisely, it is located at the periphery of axons (figure III.2B), in close contact with, but not within the myelin sheath, as no co-localization can be seen with the P0/PlGF double staining (figure III.2C). Interestingly, PlGF/$Na_{v1.6}$ staining reveals that PlGF expression in axons is interrupted at the level of the nodes of Ranvier (figure III.2D). Myelinating SCs do not express PlGF, as shown by the PlGF/S100 staining (figure III.2E). Fibroblasts labeled with the Ptc-1 Ab (Sharghi-Namini *et al.*, 2006) express PlGF in the endoneurium, but not in epi- or perineurial areas (figure III.2G). Finally, vWF Ab, used to identify ECs, never co-localized with PlGF (figure III.2F). ISH performed on transverse sciatic nerve sections confirmed that PlGF mRNA is concentrated at the periphery of axons (figure III.2H).

After nerve transection, a progressive decrease of PlGF staining appears in the distal stump, corresponding to the degradation of axons (figure III.3C). When axonal regeneration occurs, starting as soon as 14 days post-injury, PlGF-positive fibres re-appear in the distal segment of the sciatic nerve (figure III.3D). Interestingly, the double staining with p75NGFr shows that PlGF appears in SCs as soon as 1 day post-axotomy; these cells have a dedifferentiating and proliferating phenotype. PlGF expression in SCs persists during the first 3 days after injury, and is not detectable anymore after 7 days (figures III.3E-H). PlGF in endoneurial fibroblasts, as detected with Ptc-1 immunostaining, also decreases after injury (figures III.3I-L). Finally, after injury, no PlGF was observed in invading macrophages nor in ECs (figures III.4A and B).

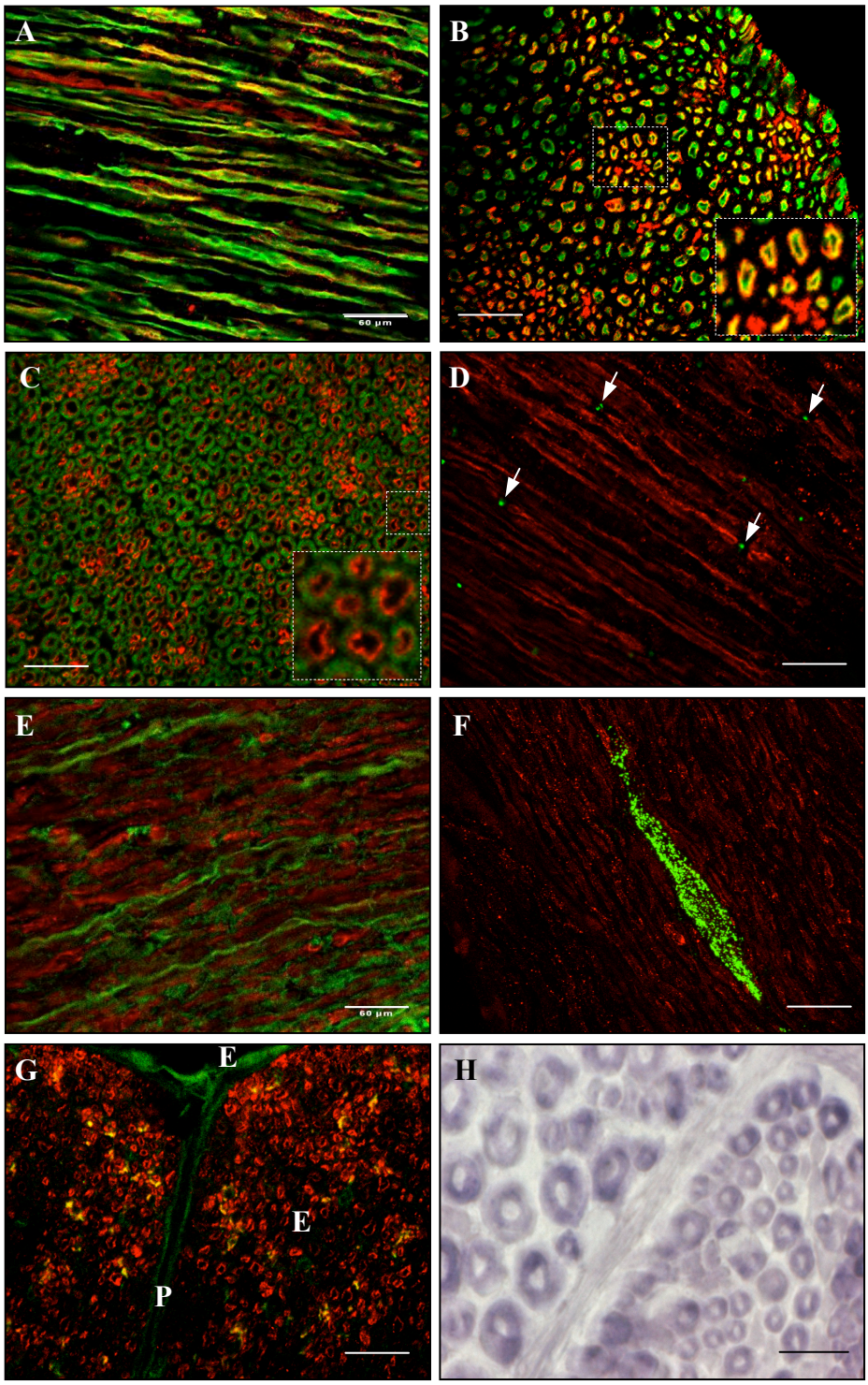


***Figure III.2*: Double IF stainings and ISH on uninjured sciatic nerve sections.** *PlGF (rhodamine) and NF (A, B), P0 (C), $Na_{v1.6}$ (D), S100 (E), vWF (F) or Ptc-1 (G) (FITC) double IF stainings, and PlGF ISH (H), on longitudinal (A, C, E and F) and transversal sections (B, D, G and H) of uninjured sciatic nerve. PlGF is expressed in axons (A) and has a periaxonal expression pattern (B and H), which is interrupted at nodes of Ranvier (C, arrows). However, PlGF is not present in myelin (D) neither in SCs (E). It is also not expressed in ECs (F). Finally, besides its axonal localization, PlGF is also expressed by fibroblasts but only in the endoneurium (G; E=endoneurium, P=perineurium, Ep=epineurium). The white dashed boxes in B and C represent enlargements of selected zones. Scale bar A-G: 60 µm; scale bar H: 10 µm.*

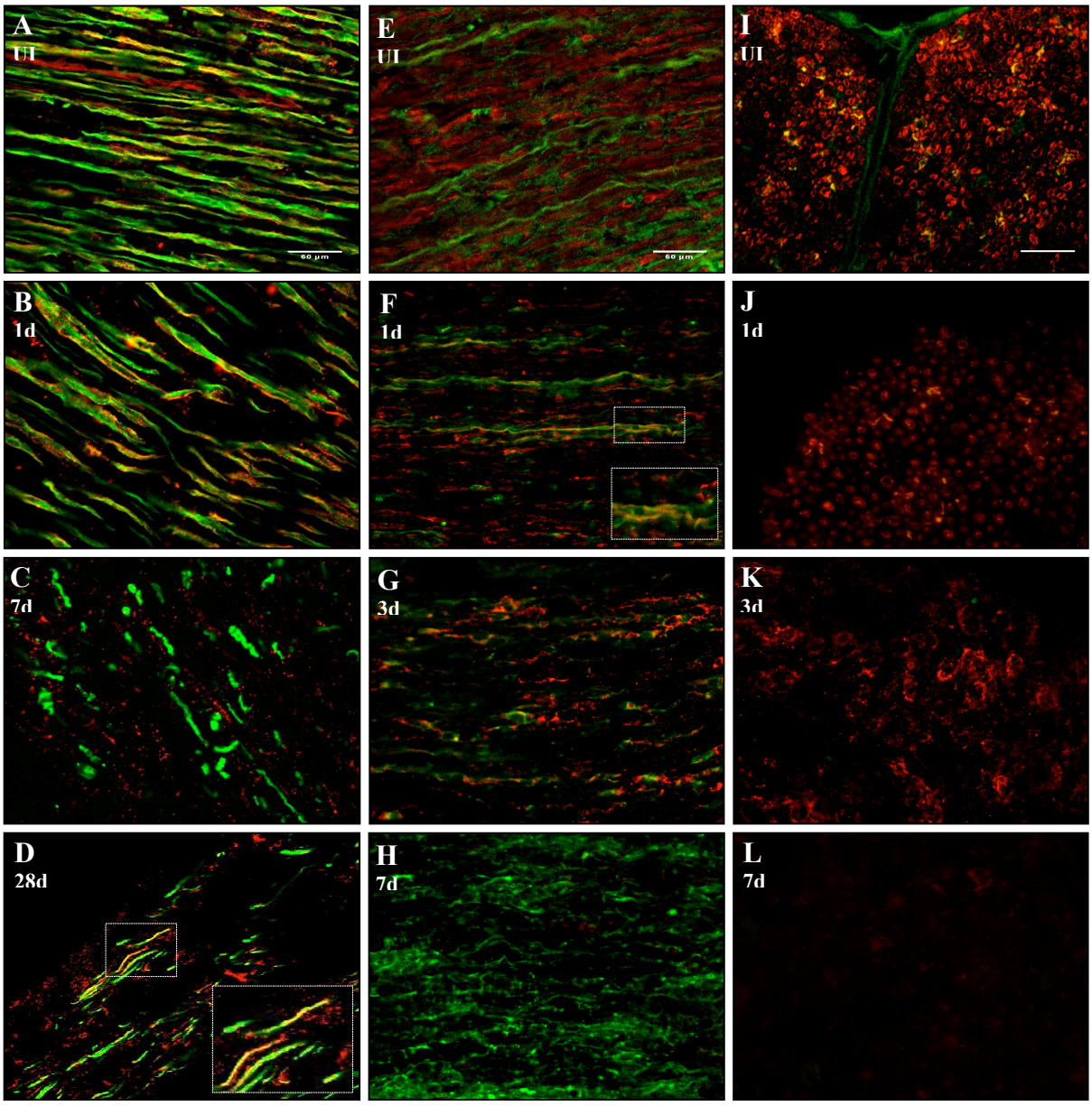


***Figure III.3*: Double IF stainings on injured sciatic nerve sections.** *PlGF (rhodamine) and NF (A-D), S100 (E), p75NGFr (F-H) or Ptc-1 (I-L) (FITC) double immunostainings, on longitudinal (A-H) and cross-sections (I-L) of uninjured (UI) (A, E, I) and injured (distal segment) sciatic nerves. Axonal PLGF expression decreases progressively after injury, following the time course of axonal degradation (B, C). 28 days post-injury, PlGF reappears in the regenerating fibres (D). SCs, which dedifferentiate and proliferate after loss of axonal contact, express PlGF from the first day after injury (F). This co-expression disappears after 7 days (H). PlGF expression in endoneurial fibroblasts also decreases with time after injury, as does Ptc-1 (J-L). The white dashed boxes in D and F represent enlargements of selected zones. Scale bar: 60µm.*

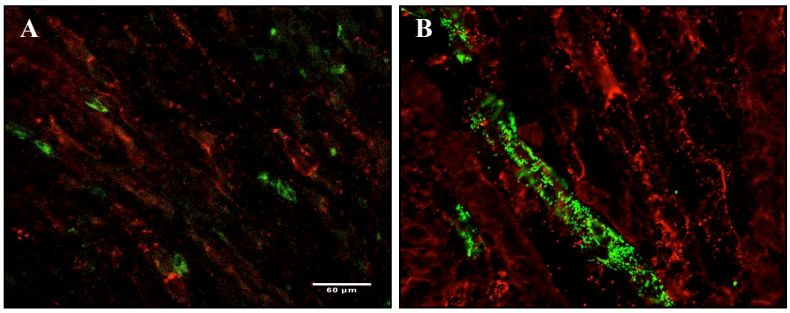


***Figure III.4*: Double IF stainings on injured sciatic nerve sections.** *PlGF (rhodamine) and CD11b (A) or vWF (B) (FITC) expression on longitudinal sections of sciatic nerves (3 days post-injury, distal segment). PlGF expression never co-localized with CD11b nor vWF during WD.*

III.1.2. PlGF expression in ventral spinal gray matter and dorsal root ganglia

Using NeuN ("neuronal nuclei") as a neuronal marker, we observed that PlGF is expressed in perikarya and neurites of several neurons in the ventral horn of the spinal cord, both in large motoneurons and small interneurons (figure III.5A). Through ISH, PlGF mRNA is detected in neurons and displays a perinuclear pattern (figure III.5B). Neuronal expression of PlGF is also seen in the DRG, in cell bodies and nerve fibres, where again its localization is peri-axonal (figures III.5C and D).

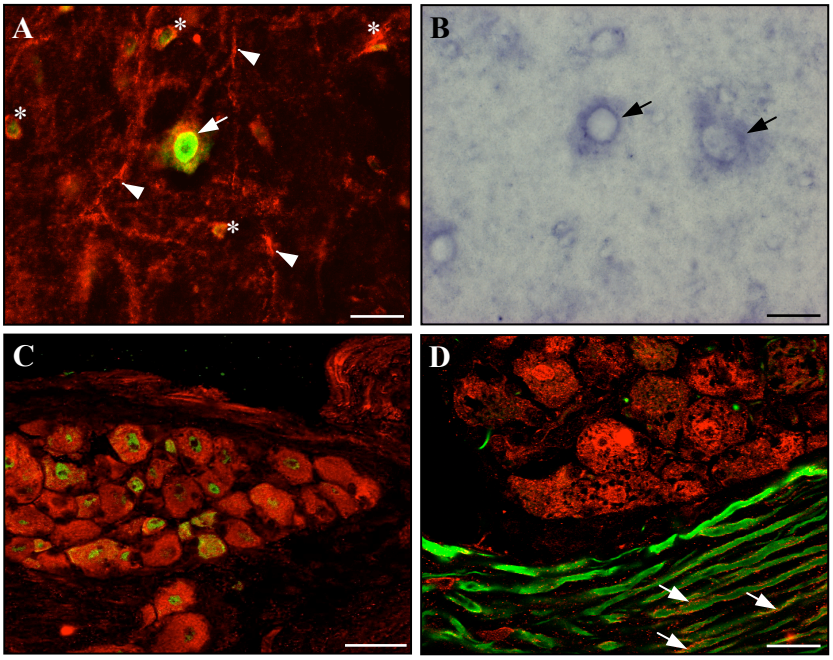


***Figure III.5*: PlGF expression in the ventral spinal gray matter and in the dorsal root ganglia.** *(A) Double IF PlGF (rhodamine)/NeuN (FITC) staining and (B) PlGF ISH of transverse spinal cord sections. PlGF is expressed in both motoneurons (arrows) and smaller interneurons (stars). Neurites also express PlGF (arrowheads). (C) PlGF (rhodamine)/NeuN (FITC) and (D) PlGF (rhodamine)/NF (FITC) in the DRG, showing its neuronal and peri-axonal localization (white arrows). Scale bars: A, B and D: 20µm; C: 50µm.*

III.2. Signalling pathways regulating PlGF expression during WD: the role of NF-κB.

During Wallerian degeneration, one of the main signalling pathways regulating the cyto- and chemokine expression implicates the transcription factor NF-κB (Subang & Richardson, 2001; Fu *et al.*, 2010). Few data are available concerning signalling pathways that regulate PlGF expression. However, it has been shown that NF-κB is involved in the induction of PlGF expression in hypoxia conditions (Cramer *et al.*, 2005). So we decided to investigate the implication of NF-κB activation in the induction of PlGF expression after nerve injury.

III.2.1. Prediction of NF-κB binding sites in mouse *Pgf* gene promoter

The κB motif localization on human *Pgf* promoter was published a few years ago (Cramer *et al.*, 2005). However, currently, nothing is known about κB motif presence on mouse *Pgf* promoter. To identify putative κB motifs on mouse *Pgf* promoter (Green *et al.*, 2001), we used four transcription factor binding site prediction programs (MatInspector, Match™, Promo and TFSEARCH). We have detected the presence of two potential NF-κB binding sites within the mouse *Pgf* promoter region (figure III.6).

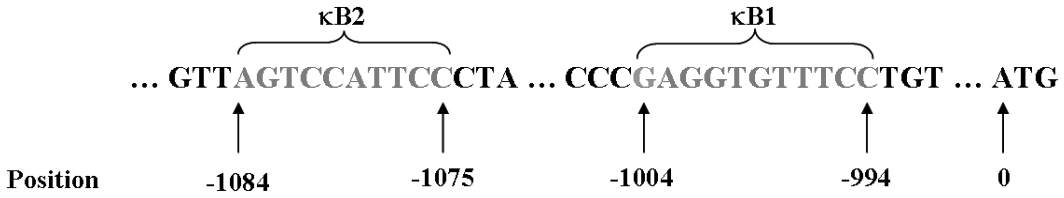


***Figure III.6*: Detection of κB motifs in the mouse Pgf gene promoter.** *The prediction of NF-κB binding sites has revealed two putative κB motifs (gray letters) located 994-1004 (κB1) and 1075-1084 (κB2) bps upstream the ATG translation initiation site of mouse Pgf gene promoter.*

III.2.2. PlGF expression regulation by NF-κB during Wallerian degeneration

In figure III.3, we have shown that SCs express PlGF as soon as 1 day after injury. Interestingly, it has been shown that NF-κB is activated in SCs within a few hours following nerve injury (Smith *et al.*, 2009). Thus, to explore the potential implication of NF-κB in the induction of PlGF expression during WD, we investigated the NF-κB binding to the two putative κB motifs (figure III.6) by IP of chromatin, from UI and one day-injured sciatic nerves, using an anti-p65 Ab. DNA fragments of immunoprecipitated chromatin were

amplified by quantitative real-time PCR (qRT-PCR), using primers specifically designed to amplify predicted κB1 or κB2 site or both (see section VI.14, table VI.4). The figure III.7 shows an about 3-fold induction of NF-κB binding for the κB2 and κB1-2 primers compared to UI nerve controls. We observed no induction for the κB1 primers.

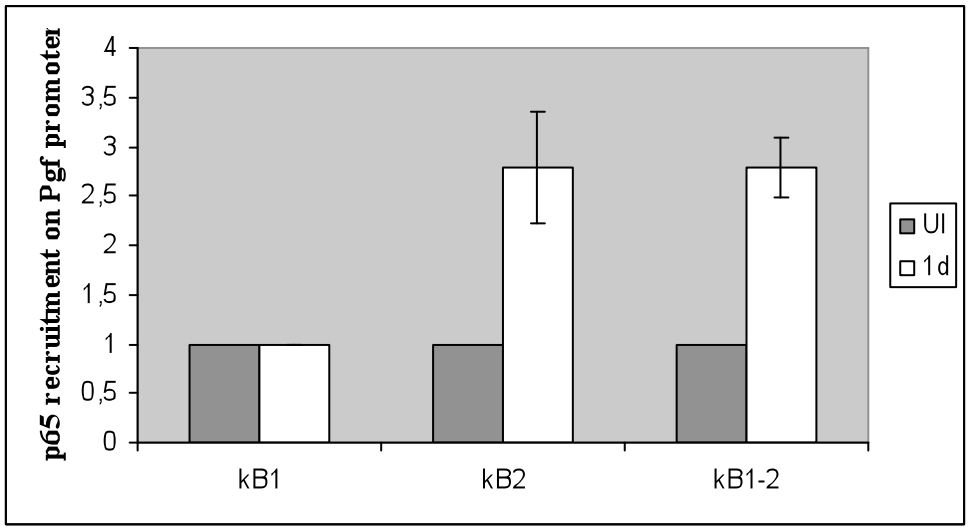


***Figure III.7*: NF-κB binding on Pgf gene promoter after sciatic nerve injury.** *p65 is specifically recruited to the Pgf gene promoter after injury, as examined by chromatin IP assays using UI sciatic nerves or 1 day-injured sciatic nerves. After normalization to inputs and to negative flag IP, signal in UI condition was set to 1 and the one obtained in injured condition was expressed relative to it. Results are from 3 independent experiments and error bars denote standard deviation. We observed no induction for the κB1 primers.*

To further prove that the sites were functional in activating gene expression, they were inserted into a luciferase reporter vector, transfected into 293 cells and NF-κB activation was assessed upon co-transfection with two NF-κB subunits, p50 and p65. As shown in the figure III.8, the κB sites found in the *Pgf* promoter (Pgf-κB) were indeed functional, as the p50/p65 heterodimer induced luciferase gene expression (about 4-5 fold).

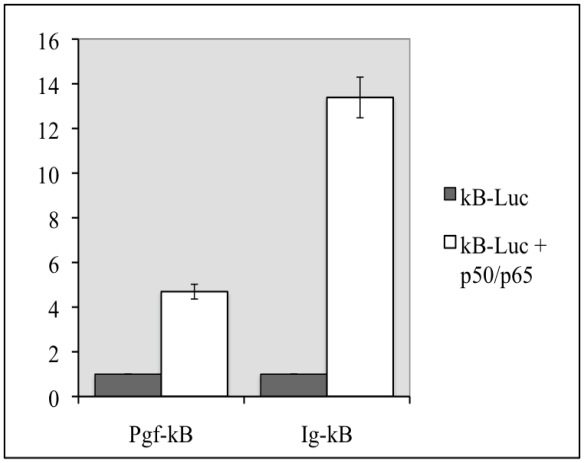


***Figure III.8:* Luciferase assay.** *The κB sites found in the Pgf promoter are functional in activating NF-κB, as examined by Luciferase assay. Pgf-kB or Ig-kB (positive control) vectors were transfected in 293 cells, with or without the p50/p65 expression vectors, as indicated. Signals were normalized by measuring Renilla activity of a co-transfected pRL vector. For each vector, the value obtained in the absence of p50/p65 was set to 1 and the other was expressed relative to it. Results are from 3 independent experiments and error bars denote standard deviation.*

III.3. PlGF and myelin sheath structure

In the section III.1.1 of this work, we have exposed our results on the expression pattern of PlGF in normal sciatic nerves. We have shown a specific PlGF localization at the periphery of axons (figure III.2B), in close contact with the myelin sheath (figure III.2C), with interruption at nodes of Ranvier (figure III.2D). This periaxonal localization could infer a function of PlGF in glia-axon interactions, as already described for other axonal molecules such as microtubule-associated protein 1B (MAP1B) (Franzen *et al.*, 2001). Thus, PlGF could play a role in the maintenance of axon/myelin structure, and a lack of PlGF could induce some modifications in myelin sheath structure. To investigate these hypotheses, we performed a morphological analysis on electronic cross-sections of wt and *Pgf* $^{-/-}$ sciatic nerves and ventral horns of spinal cords. We examined the morphology and the ultra-structure of peripheral (figures III.9A and B) and central (figures III.9C and D) myelin sheaths. This analysis reveals no obvious abnormalities of peripheral and central myelin sheath morphology in *Pgf* $^{-/-}$ mice (figures III.9B and D).

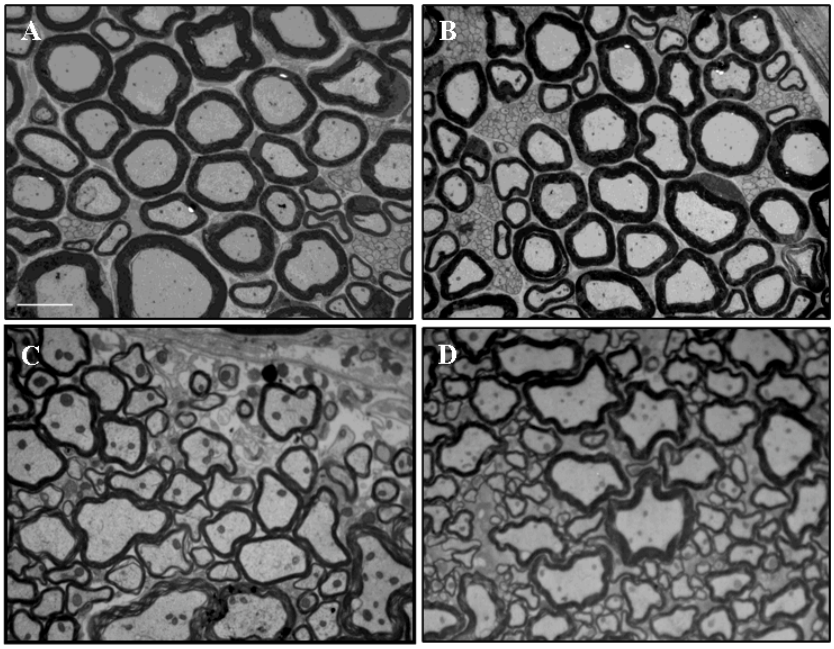


***Figure III.9*: Electronic microscopy analysis of myelin sheaths.** *Electron micrograph analysis of cross-section of wt (A, C) and Pgf$^{-/-}$ (B, D) sciatic nerves (A, B) and ventral horns of spinal cords (C, D) reveals no abnormalities of peripheral and central myelin sheath morphology in Pgf$^{-/-}$ mice (scale bar: 4µm).*

In addition to the general structure of myelin sheaths, we also examined the myelin sheath thickness that could be influenced by impairment of myelin sheath folding and/or compaction mechanisms (Martini & Schachner, 1997). Myelin sheath thickness also depends on axonal calibre (Michailov *et al.*, 2004), so we calculated the regression curves representing myelin thickness as a function of axonal diameter for wt and *Pgf*$^{-/-}$ mice (figure III.10). We observed absolutely no difference between the two groups of animals concerning the evolution of myelin thickness as a function of axonal diameter.

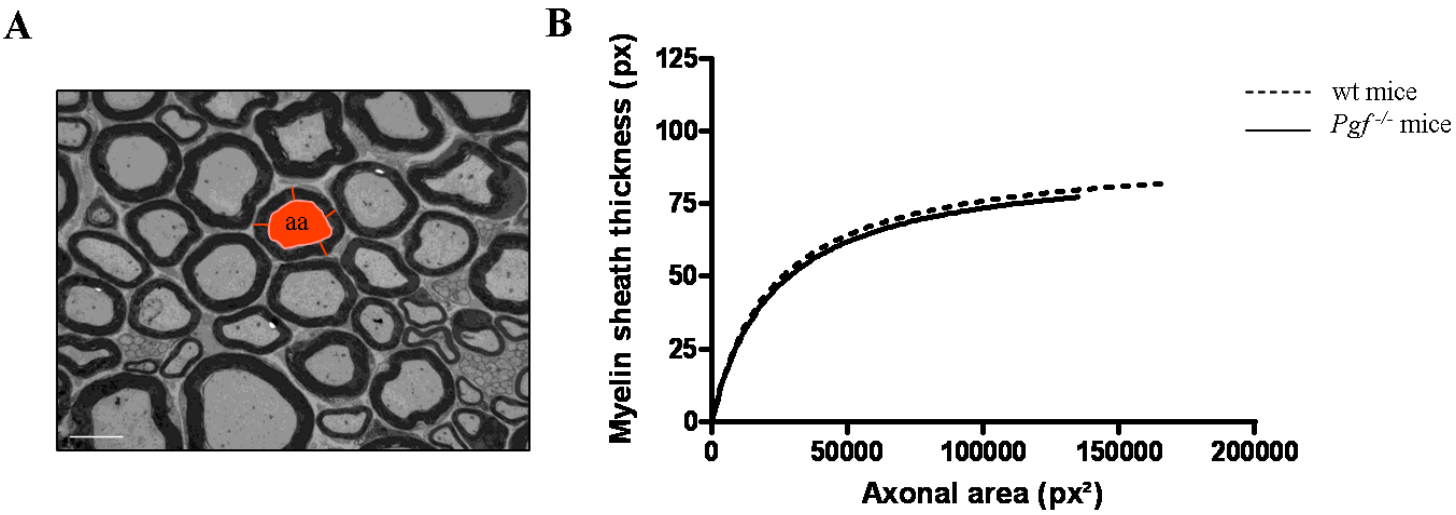


***Figure III.10*: Relationship between myelin sheath thickness and axonal area in mouse sciatic nerves.** *(A) Electronic picture of transversal section of sciatic nerve (scale bar: 4µm). The axonal area (=aa, A) and the mean myelin thickness (measurement at four random points of the myelin, red lines) were measured on wt and Pgf$^{-/-}$ ultrathin cross-sections of sciatic nerves (N=3). (B) Regression curves representing myelin thickness as a function of axonal area for wt (dotted line) and Pgf$^{-/-}$ (full line) nerves. We observed no difference between wt and Pgf$^{-/-}$ sciatic nerves.*

III.4. PlGF functional roles in Wallerian degeneration

III.4.1. PlGF role on Schwann cell proliferation and formation of bands of Büngner

❖ ***Proliferation of Schwann cells is decreased in transected Pgf$^{-/-}$ nerves***

The WD process in the distal stump of the injured nerve is associated with activation and proliferation of SCs, which form the bands of Büngner and express p75NGFr. To assess the role of PlGF in these processes, we compared the proliferation of SCs within the sciatic nerves of wt and *Pgf$^{-/-}$* mice after axotomy. Using immunofluorescent staining against Ki67, a nuclear protein expressed in cells undergoing proliferation, we quantified the number of proliferating cells at 1, 3 and 7 days after axotomy in the distal stump of nerves from wt and *Pgf$^{-/-}$* mice (figures III.11A-B). We found a significant decrease of cell proliferation in *Pgf$^{-/-}$* mice at 1 and 3 days post-injury (Fig III.11B). Because of a conflict between Abs, we were not able to perform the double immunofluorescent staining Ki67/p75NGFr to identify the proliferating cells as SCs. We can nevertheless attest that the Ki67-positive cells are not inflammatory cells, such as invading macrophages, as we have only taken into account Ki67-positive cells that do not express CD11b.

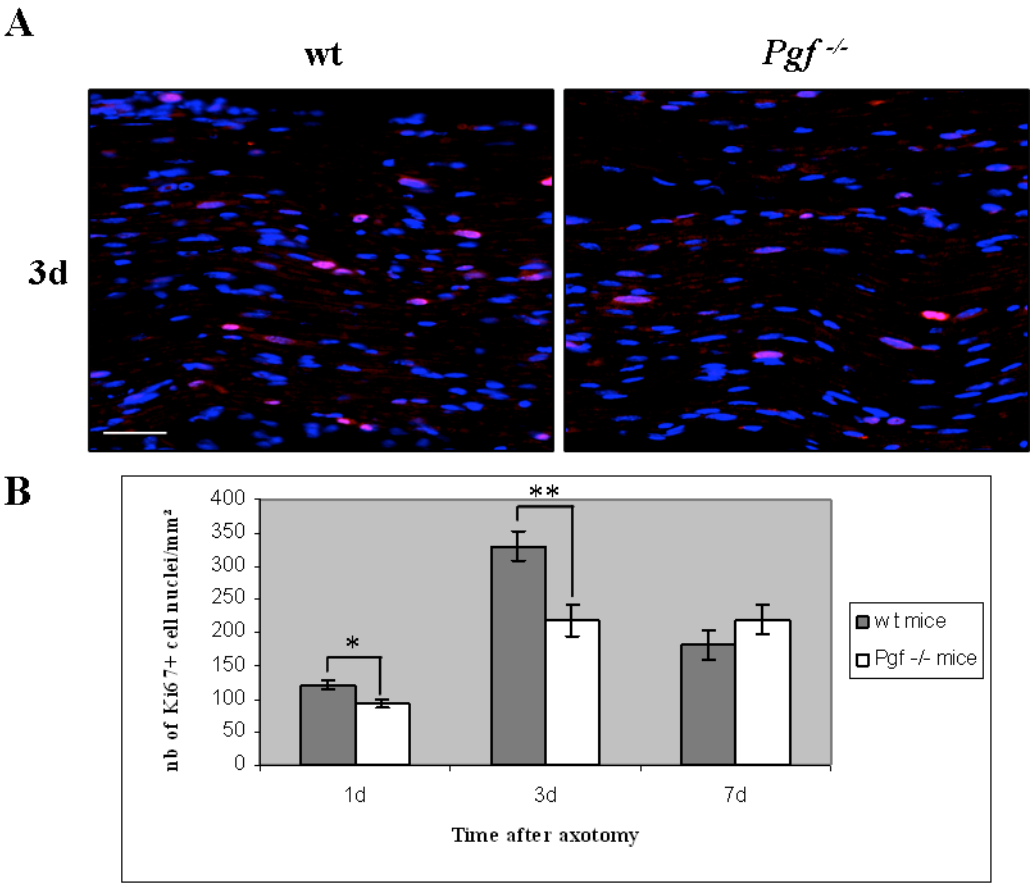


***Figure III.11*: In vivo cell proliferation.** *(A) Ki67 immunoreactivity on longitudinal sections of wt and Pgf $^{-/-}$ sciatic nerves 3 days after axotomy (scale bar: 50µm). (B) Cell quantification shows a significant difference between wt and Pgf $^{-/-}$ mice in the number of Ki67 positive cells 1 and 3 days post-injury. ** P < 0.01; * P < 0.05 (mean ± standard error (SE); N= 5 mice per group).*

- ***Proliferating Schwann cells are decreased in Pgf $^{-/-}$ SC cultures***

In order to confirm the *in vivo* finding of decreased SC proliferation in mice lacking PlGF, we compared the proliferation of primary cultures of SCs isolated from wt or *Pgf* $^{-/-}$ mice. The purity of our primary SC cultures was evaluated by a p75 NGFr immunostaining (figure III.12A) and the quantification of p75 NGFr-positive cells reveals a SC purity of about 95% (figure III.12B). Using BrdU incorporation and double immunofluorescent staining against BrdU and p75NGFr (figure III.13A), we counted the mean number of proliferating SCs. The number of BrdU positive *Pgf* $^{-/-}$ SC nuclei was decreased by about 10 % (figure III.13B).

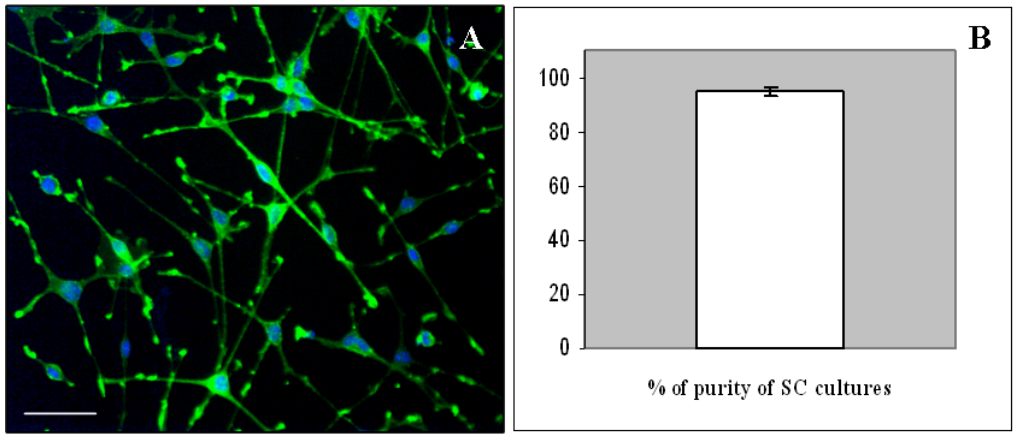


***Figure III.12*: Purity evaluation of SC cultures:** *(A) P75 NGFr (FITC)/DAPI immunostaining of SC cultures (scale bar: 50µm). (B) Quantification of the number of p75 NGFr-positive cells reveals that the purity of our SC cultures is about 95% (94.93 ± 1.41; N=2 wt cultures and 2 Pgf $^{-/-}$ cultures).*

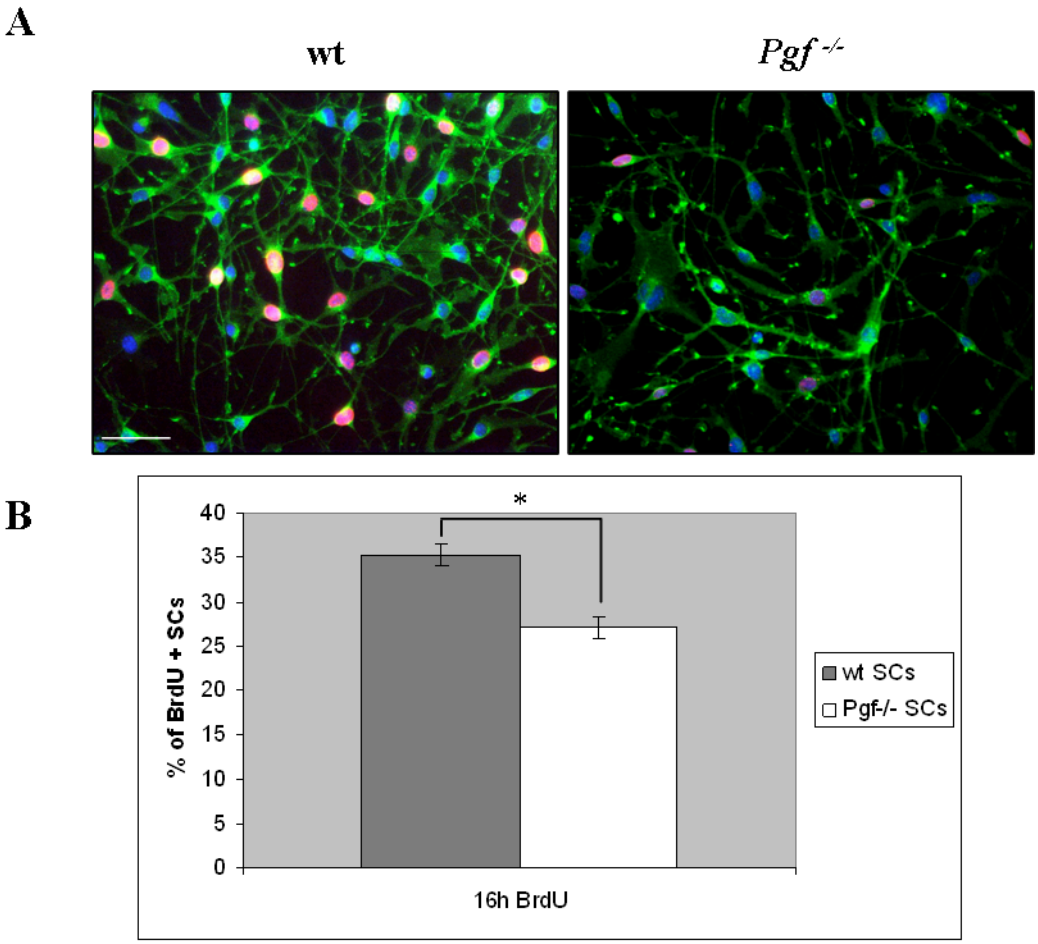


***Figure III.13*: In vitro SC proliferation.** *(A) BrdU (rhodamine)/p75NGFr (FITC) immunocytofluorescent staining of wt and Pgf $^{-/-}$ SCs after BrdU incorporation (scale bar: 50µm). (B) Quantification shows a significant decrease of Pgf $^{-/-}$ SC proliferation. * P < 0.05 (mean ±SE; N= 3 culture experiments).*

SCs in culture adopt a proliferating phenotype, as do SCs *in vivo* after injury. We thus verified that in culture conditions, SCs do also express PlGF, as observed 24 hours after sciatic nerve section. We performed a RT-PCR for PlGF (P) and GAPDH (G), as housekeeping gene, on RNA extracts from wt and *Pgf* $^{-/-}$ SC cultures, and also on DRG as positive control. The polyacrylamide gel with RT-PCR amplification products (figure III.14) shows that PlGF mRNA is expressed in DRG (as is its corresponding protein (figure III.5C-D)), and confirms a PlGF expression in wt cultured SCs and no expression in *Pgf* $^{-/-}$ SCs.

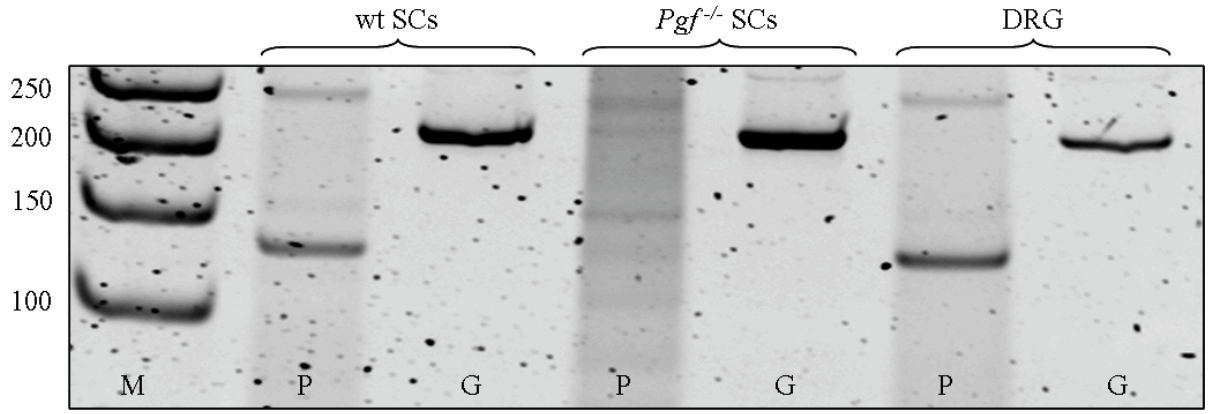


***Figure III.14*: Representative polyacrylamide gel showing PlGF (P) and GAPDH (G) RT-PCR amplification products from wt and Pgf $^{-/-}$ cultured SCs, and DRG.** *PlGF RT-PCR confirmed PlGF expression in DRG and wt SCs in culture conditions and no expression in Pgf $^{-/-}$ SCs.*

❖ ***Schwann cell alignment is delayed in transected Pgf $^{-/-}$ nerves***

We then assessed p75NGFr expression in wt and *Pgf* $^{-/-}$ mice after various post-transection survival times. As illustrated in figure III.15A, p75NGFr is weakly expressed in UI nerves. In wt animals, its expression increases 3 days after axotomy and reaches a peak at day 7 (figure III.15B). At that time, the longitudinal aspect of the p75NGFr staining is likely due to the alignment of SCs to form the bands of Büngner. In *Pgf* $^{-/-}$ mice, the peak of expression of p75NGFr is only reached 14 days after the lesion, and decreases more rapidly afterwards. Also, at day 7, the longitudinal aspect of the staining is much less pronounced than in tissue sections of wt animals.

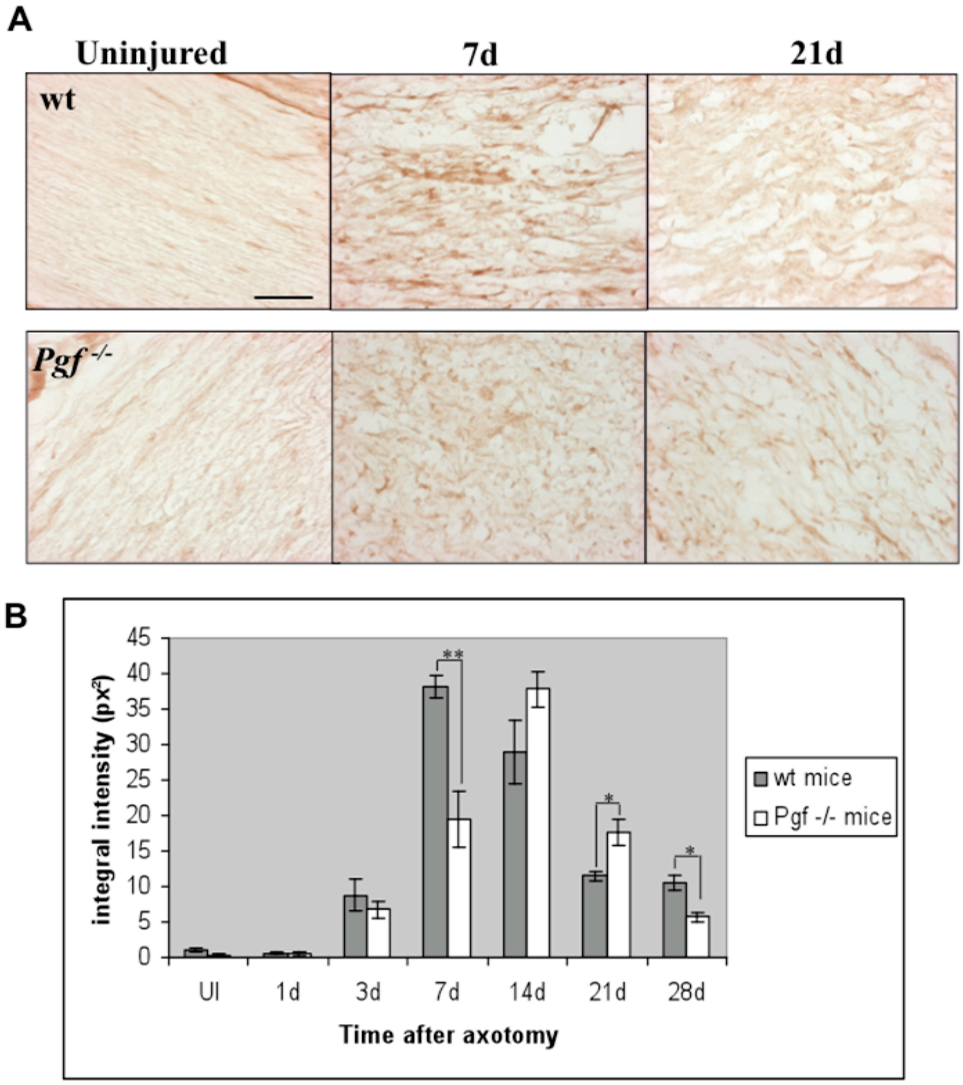


***Figure III.15*: P75NGFr immunostaining and quantification.** *(A) P75NGFr immunoreactivity on longitudinal sections of wt and Pgf$^{-/-}$ sciatic nerves before (UI) and after axotomy (scale bar: 100µm). (B) Histogram of the quantification of p75NGFr staining. A significant difference is observed between wt and Pgf$^{-/-}$ mice in the intensity of p75NGFr staining 7, 21 and 28 days post-injury. ** P < 0.01; * P < 0.05 (mean ± SE; N =5-9 mice per group).*

❖ *Migration of Pgf$^{-/-}$ Schwann cells in vitro is non significantly decreased*

As the p75NGFr staining revealed a delay in SC alignment between wt and *Pgf* $^{-/-}$ mice, we hypothesised that the migration potential of *Pgf* $^{-/-}$ SCs could be decreased. To test this, we performed a "scratch migration assay" in cultured SCs (figure III.16A). Time-lapse experimentation was performed to follow the migration of wt and *Pgf* $^{-/-}$ SCs every 2 hours during 28h. The percentage of cell-free area was quantified every 2 hours. This cell-free area was higher, at each time, in the *Pgf* $^{-/-}$ assay than in the wt one (figure III.16B), indicating a decreased migration potential for *Pgf* $^{-/-}$ SC. However, this difference was not statistically significant.

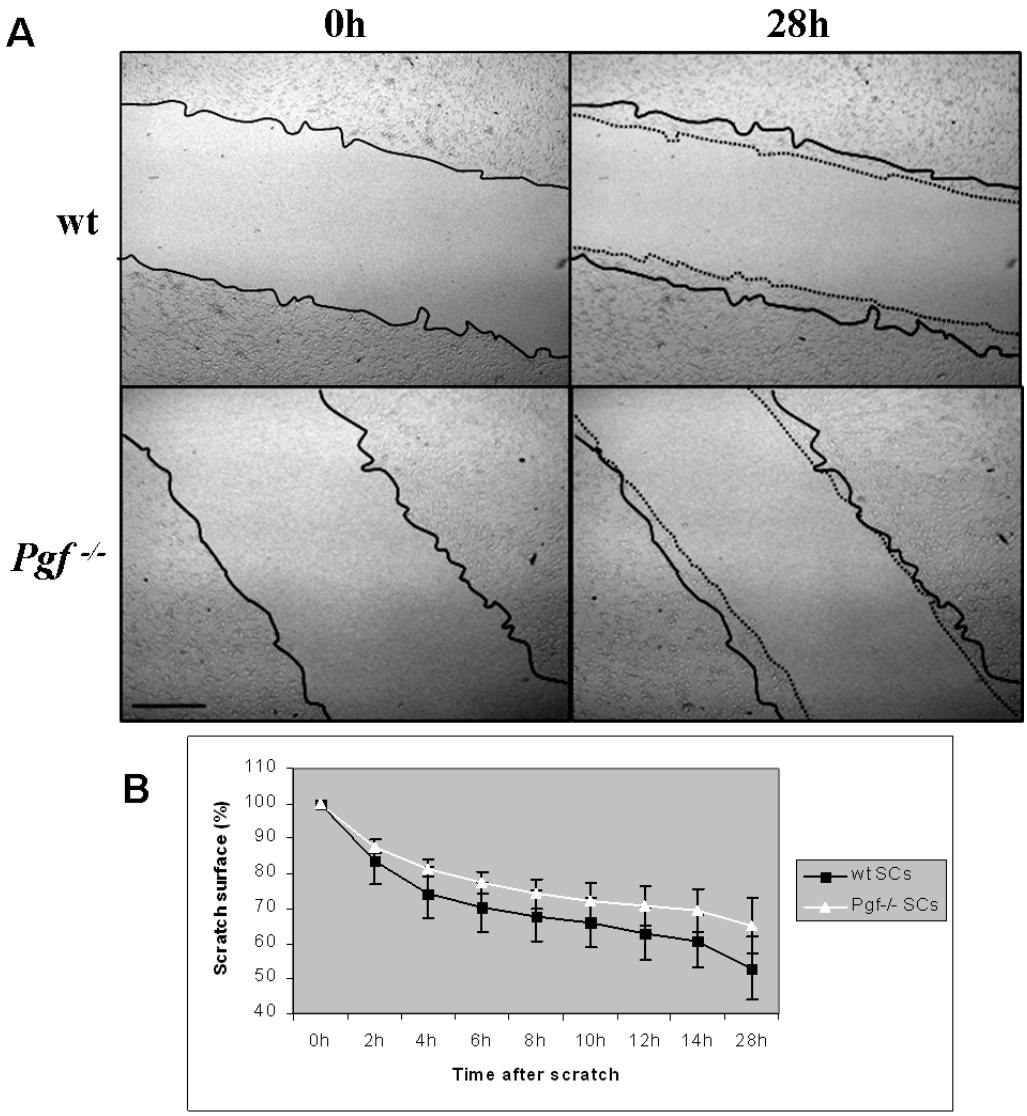


***Figure III.16*: Scratch migration assay on wt and Pgf $^{-/-}$ purified SCs.** *(A) Full black lines represent the initial edges of the scratch, while dotted lines were traced 28h later, at the front of migration of the SCs. (B) The surface of the initial scratch was measured (=100%) and compared to the cell-free surface every 2h during 28h. The percentage of cell-free area was calculated and revealed that the area from Pgf $^{-/-}$ SCs assay was always higher than the one from wt SCs, however this difference is not statistically significant (p=0.4; mean ± SE; N=4 culture experiments). Scale bar: 100µm.*

III.4.2. PlGF role on macrophage recruitment

The other main cell type involved in the WD process are macrophages, which accumulate within the degenerating nerve segment and accelerate the removal of axonal and myelin debris. As PlGF plays a role in the chemo-attraction of macrophages (Clauss *et al.*, 1996), we first quantified the number of invading macrophages within the degenerating distal segment at several post-injury delays, comparing wt and *Pgf* $^{-/-}$ mice. CD11b immunostaining of longitudinal nerve sections revealed a clear delay in the macrophage infiltration in *Pgf* $^{-/-}$

nerves respective to wt nerves (figure III.17A). While infiltration peaked between the 3rd and the 7th day post-injury in wt mice, it did so only between 14 and 21 days in *Pgf* $^{-/-}$ tissue (figure III.17B).

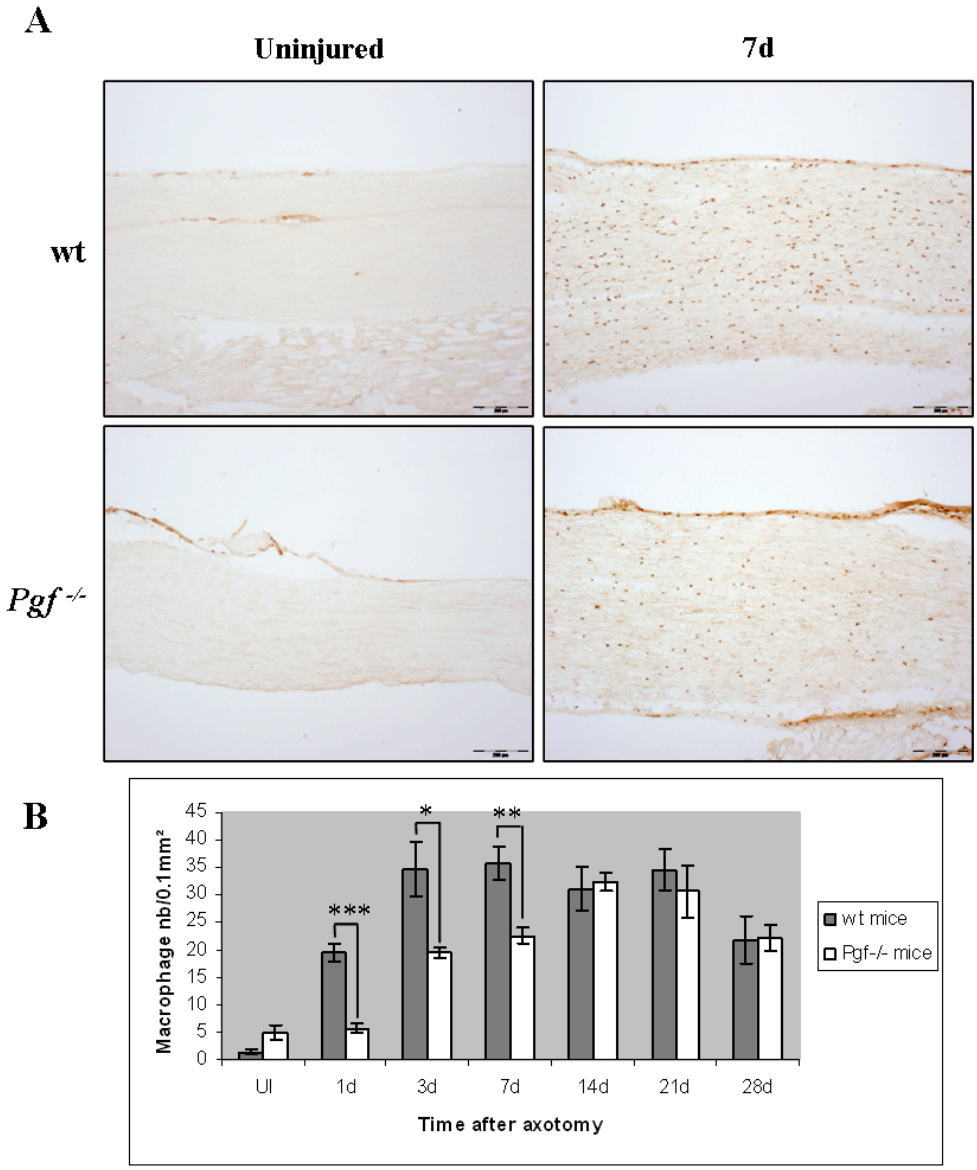
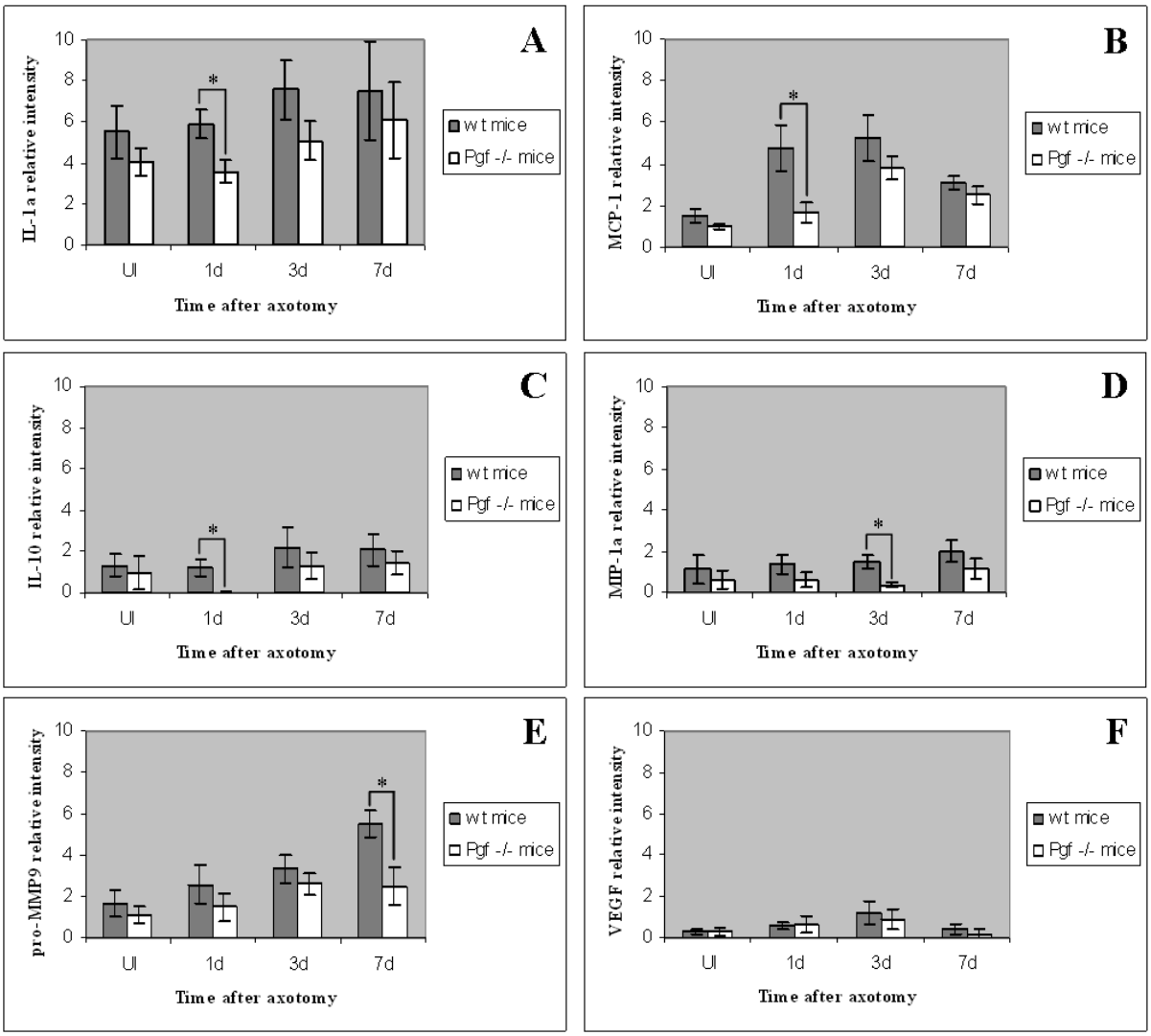


***Figure III.17*: CD11b immunostaining and quantification.** *(A) CD11b immunostaining of longitudinal sections of wt and Pgf* $^{-/-}$ *sciatic nerves before (UI) and 7 days after axotomy (scale bar: 200µm). (B) Cell quantification shows a significant difference between wt and Pgf* $^{-/-}$ *mice in the number of invading macrophages 1, 3 and 7 days post-injury. *** P < 0.001; ** P < 0.01; * P < 0.05 (mean ± SE; N =5-12 mice per group).*

III.4.3. PlGF role on cytokine and chemokine expression

WD is orchestrated by a large cyto-/chemokine network, which regulates SC and macrophage functions. As PlGF invalidation affects these two cell types, and more particularly at the early stages of WD, we compared the expression of 10 cyto/chemokines in extracts of UI, and 1, 3 and 7 days- injured sciatic nerves (distal part) from wt and *Pgf*$^{-/-}$ mice. The following molecules were studied: IL-1α, IL-1β, IL-6, IL-10, TNF-α, VEGF, VEGFR-1, MCP-1, MIP-1α and pro-MMP-9. The quantitative analysis of the signal intensity revealed significant decreases in *Pgf*$^{-/-}$ extracts of IL1-α, MCP-1 and IL-10 at 1 day (figure III.18A-C), of MIP-1α at 3 days, (figure III.18D) and pro-MMP9 at 7 days post-injury (figure III.18E).



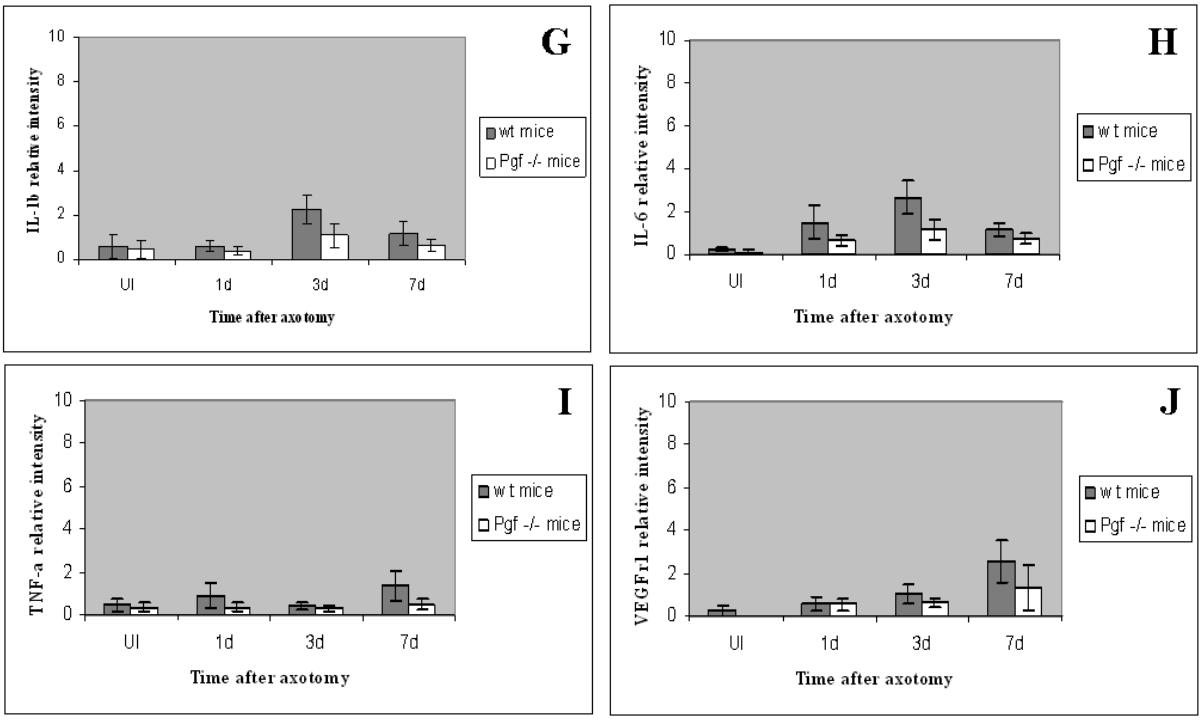


***Figure III.18*: Cytokine-array on sciatic nerve extracts from wt and Pgf -/- mice.** *(A) IL-1α,(B) MCP-1 and (C) IL-10 don't increase their expression in Pgf $^{-/-}$ nerve as much as in wt nerves 1 day after injury. (D) MIP-1α expression increases after injury in wt mice, and remains low in Pgf $^{-/-}$ mice. (E) Pro-MMP-9 peaks at 7 days post-injury only in wt nerves. (F) The VEGF expression is absolutely not affected in Pgf $^{-/-}$ during WD. Concerning (G) IL-1b, (H) IL-6, (I) TNF-a and (J) VEGFr1, their expressions after injury tend to be lesser in Pgf $^{-/-}$ mice compared to the wt one. However, these differences are not statistically significant. * P <0.05 (mean ± SE; N=6 mice per group).*

III.4.4. PlGF effect on myelin sheath degradation

To verify if the delay in SC proliferation and in macrophage recruitment affects the degradation speed of myelin sheaths, we stained these latter with toluidine blue and counted them over semi-thin transverse sections from wt and *Pgf $^{-/-}$* nerves from various post-injury delays (figure III.19A). These counts revealed that the degradation of myelin sheaths is slower in *Pgf $^{-/-}$* mice (figure III.19B).

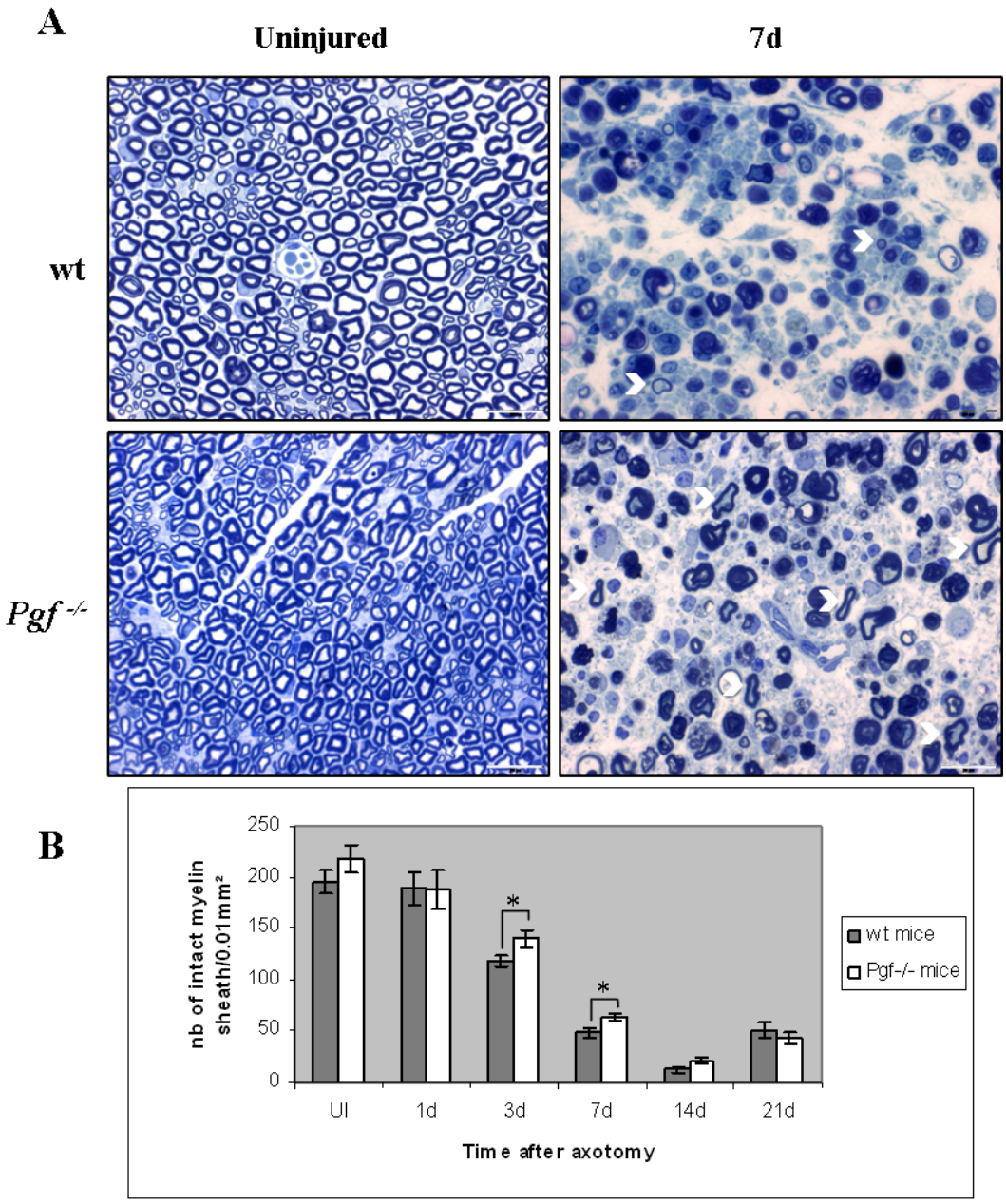


***Figure III.19*: Myelin sheath quantification.** *(A) Toluidine blue staining on semi-thin cross-sections from wt and Pgf $^{-/-}$ sciatic nerves before (UI) and 7 days after axotomy (scale bar: 20µm). (B) Quantification shows a significant difference between wt and Pgf $^{-/-}$ mice in the number of intact myelin (white arrowheads) sheaths 3 and 7 days post-injury. * $P < 0.05$ (mean ± SE; N =5-9 mice per group).*

III.4.5. PlGF effect on the phagocytic phenotype of SC as indexed by c-Fos expression

In addition to the number of phagocytic cells present in the injured site, the degradation of myelin sheaths is also influenced by the acquisition of phagocytic activity by SCs. It has been shown that this phenotype acquisition is closely associated with the expression of the cellular immediate early gene c-Fos (Liu *et al.*, 1995). Moreover, PlGF is

able to induce c-Fos expression in cells expressing its receptor Flt-1 (Holmes & Zachary, 2004). Thus, to assess the role of PlGF in SC acquisition of a phagocytic phenotype through c-Fos activation, we compared by immunofluorescent staining c-Fos expression within the sciatic nerves of wt and *Pgf*$^{-/-}$ mice one day after axotomy (figure III.20A). The quantification of the percentage of c-Fos positive cells reveals that there is no difference between wt and *Pgf*$^{-/-}$ mice concerning c-Fos expression after nerve injury (figure III.20B). Because of a conflict between Abs, we were not able to perform the double immunofluorescent staining c-Fos/p75NGFr to identify c-Fos-positive SCs. However, we can attest that the c-Fos-positive cells are neither macrophage, as they do not express CD11b, nor fibroblast, as the quantification excludes epi-perineurial c-Fos-positive cells described to be the two types of fibroblasts expressing c-Fos in the distal segment during WD (Pyykonen & Koistinaho, 1991).

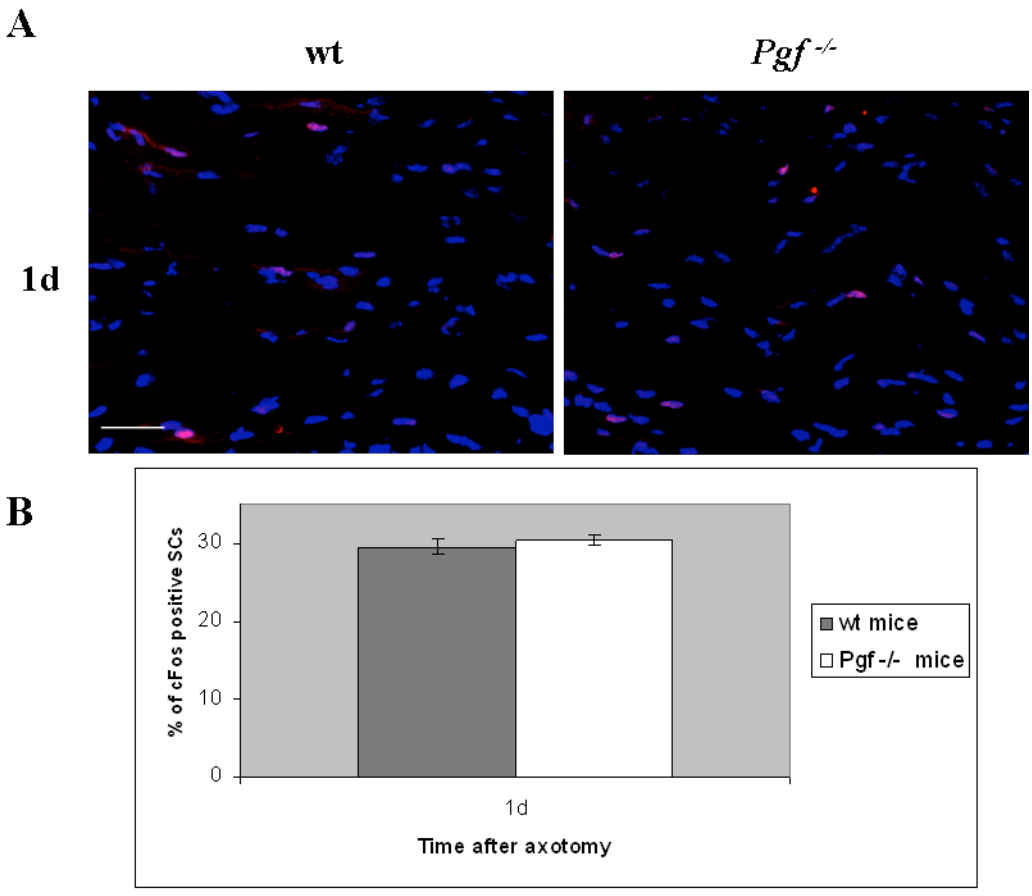


***Figure III.20*: c-Fos activation after nerve injury.** *(A) c-Fos immunoreactivity on longitudinal sections of wt and Pgf*$^{-/-}$ *sciatic nerves 1 day after axotomy (scale bar: 50µm). Cell quantification shows no difference between wt and Pgf*$^{-/-}$ *mice in the percentage of c-Fos positive SCs 1 day post-injury (mean ± SE; N= 2 mice per group).*

III.4.6. PlGF role on axonal regeneration

As SC and macrophage functions are delayed during WD in *Pgf* $^{-/-}$ animals, axonal regeneration might also be altered. To test this hypothesis, we compared the NF immunostainings of transected sciatic nerves from wt and *Pgf* $^{-/-}$ mice. At 7 days post-injury, all axons are degraded in both wt and *Pgf* $^{-/-}$ nerve distal parts, as illustrated by the high number of axonal debris (figure III.21). At 14 days, regeneration starts in wt nerves, as more longitudinal profiles can be observed in wt than in *Pgf* $^{-/-}$ nerves.

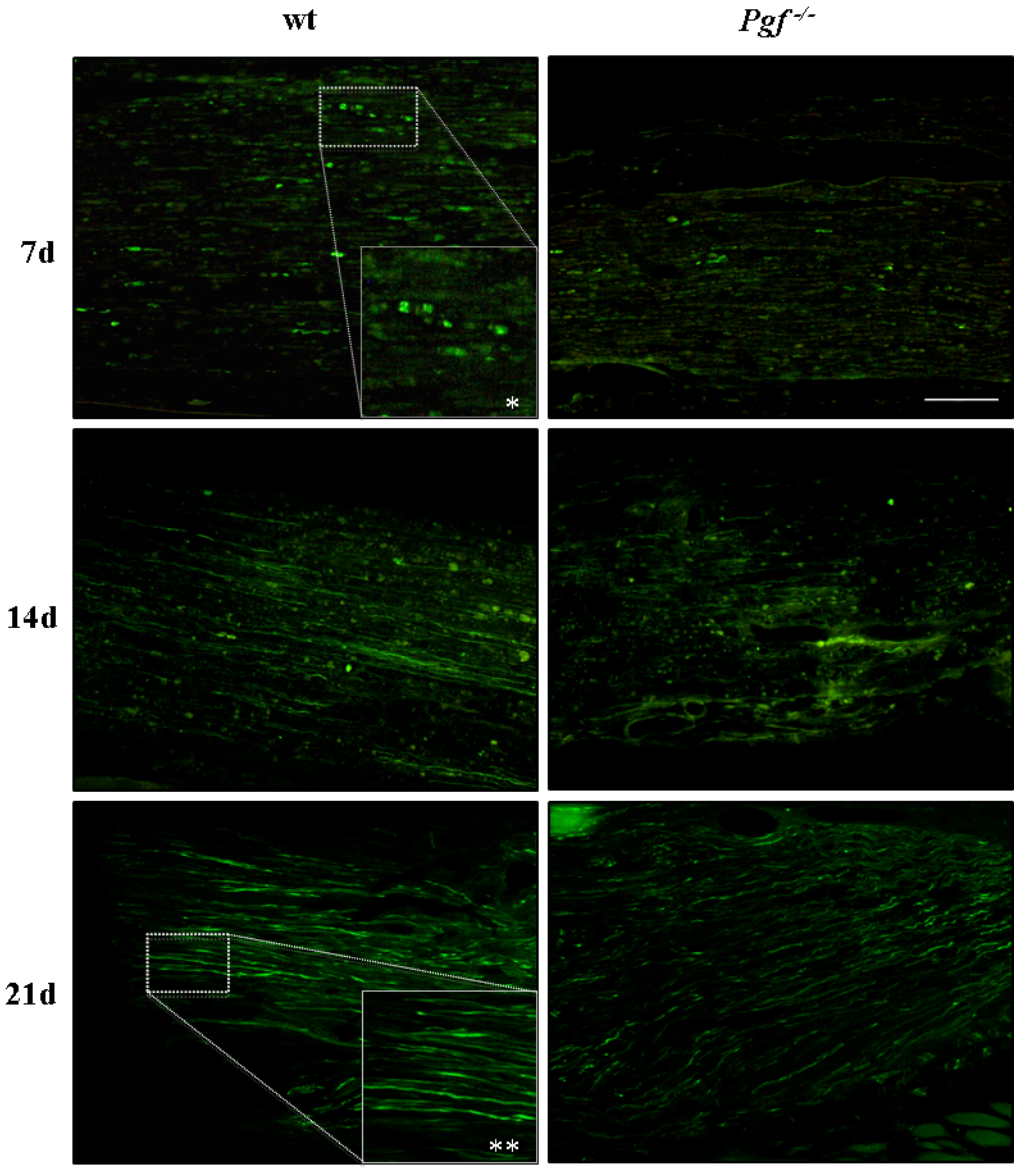


***Figure III.21*: Neurofilament immunostaining.** *NF immunostaining on longitudinal sections of wt and Pgf* $^{-/-}$ *sciatic nerves 7, 14 and 21 days after axotomy (scale bar: 100µm). Inserts illustrate representative fields considered as "debris (*)" or "fibers (**)" used for the semi-quantitative analysis illustrated in figure III.22.*

This observation was confirmed by counting the proportion of fields containing either axonal debris (dotted aspect of NF staining), axonal profiles (longitudinal profiles on NF staining), or both (figure III.22A). After 14 days post-injury, almost 60% of the total length of the nerve corresponds to fields containing only axonal debris in *Pgf* $^{-/-}$ mice, while debris-containing fields occupy less than 10% in wt mice. As a corollary, fields with higher proportions of regenerating axons occupy 50% of the nerve length in wt mice compared to less than 5% in *Pgf* $^{-/-}$ mice. No field containing only axonal profiles was observed at this time. After 21 days, regenerating profiles were more numerous, reflecting the repair progress, and there were no more difference between wt and *Pgf* $^{-/-}$ mice (figure III.22B). This finding suggests therefore that absence of PlGF leads to a transient delay of axonal regeneration.

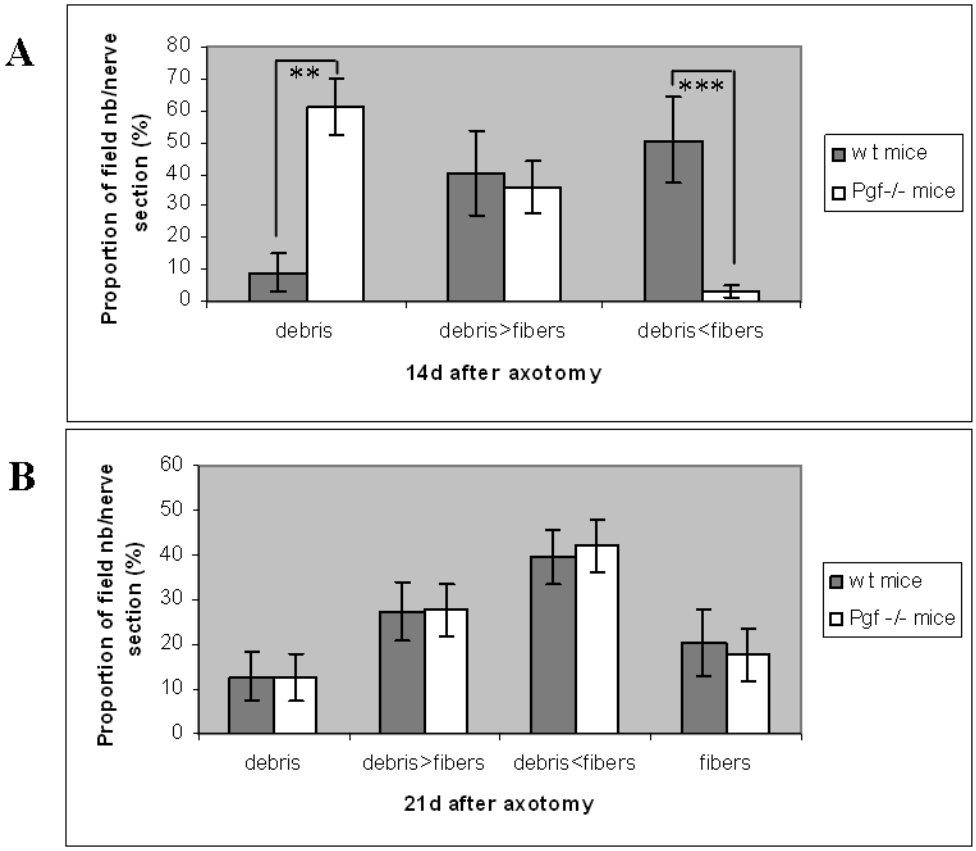


***Figure III.22*: Neurofilament semi-quantification.** *Quantification shows that after 14 days (A), Pgf* $^{-/-}$ *nerves contain a significant higher proportion of debris than wt nerves, while more regenerating profiles are observed in wt nerves than Pgf* $^{-/-}$ *ones. *** P < 0.001; ** P < 0.01 (mean ± SE; N =5-7 mice per group). (B) No more differences were found after 21 days.*

III.4.7. PlGF role on remyelination

As SC proliferation and axonal regeneration are delayed during WD in *Pgf* $^{-/-}$ animals, remyelination might also be altered. However, as observed in the figure III.19B, the number of intact myelin sheath 21 days after injury didn't differ between wt and *Pgf* $^{-/-}$ mice.

It is known that after remyelination, the distance between two adjacent nodes of Ranvier (figure III.23A) is reduced (Hildebrand *et al.*, 1985). Thus, SC proliferation could influence the internodal length during remyelination. We measured this internodal length 28 days after the nerve section: no difference was observed between wt and Pgf$^{-/-}$ nerves (figure III.23B).

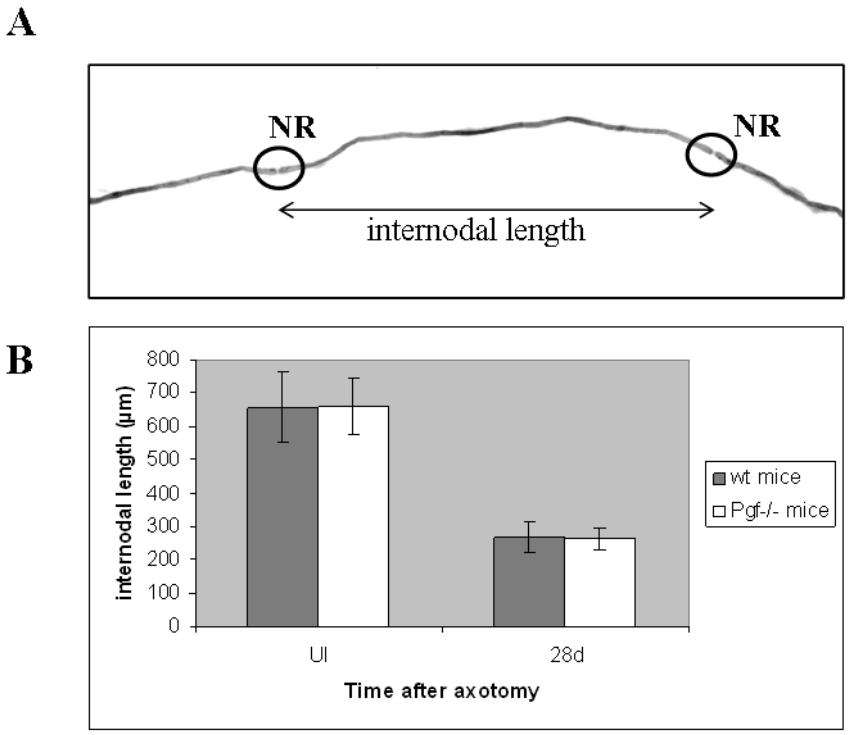


***Figure III.23*: Internodal length measure.** *(A) The distance between two nodes of Ranvier (NR) along an axon determines the internodal length. (B) Comparison of average internodal lengths from UI or regenerated axons (28 days following sciatic nerve axotomy) between wt and Pgf* $^{-/-}$ *mice shows no difference between the two groups. The means ± SE were calculated from 3-10 myelin segments (n=3 animals per group).*

III.4.8. PlGF role on motor recovery

To assess the consequence of this delayed axonal regeneration on functional recovery, we finally evaluated motor recovery after sciatic nerve axotomy in *Pgf* $^{-/-}$ compared to wt mice, using the walking track method in which foot-prints are analysed (Fig III.24A). The print-lengths of uninjured (NPL) and injured (OPL) sciatic nerves were measured preoperatively, as well as 3, 7, 10, 14, 17 and 21 days after axotomy and were used to

calculate the print-length factor (PLF, described in section VI.4). During the first week following injury, this PLF increases and reaches after 7 days the value of about 0.5 and 0.35 for wt and *Pgf*$^{-/-}$ mice respectively, indicating a marked disability in the injured paw. At day 10, the PLF of wt mice starts to decrease and reaches a value of about 0.3 at 21 days after injury, whereas the PLF of *Pgf*$^{-/-}$ mice continues to get worse and reaches a value of about 0.6 at 21 days (figure III.24B).

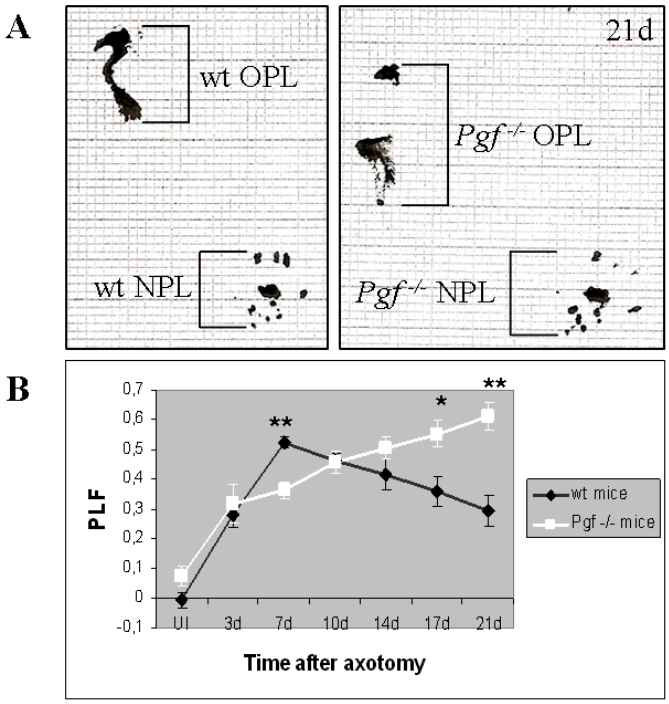


***Figure III.24*: Functional recovery.** *A) Representative footprints obtained from wt and Pgf*$^{-/-}$ *mice, 21 days after unilateral left sciatic nerve transection. Measures of OPL and the NPL were used to calculate the PLF. (B) Graph showing the recovery of motor function after sciatic nerve transection determined by the PLF. Worse recovery is evident in mice lacking PlGF compared to their wt controls. ** P < 0.01; * P < 0.05 (mean±SE; N= 5 mice per group).*

To summarize, we have shown that in *Pgf*$^{-/-}$ mice:

1. Proliferation of SCs is decreased;
2. Alignment of SCs to form Büngner's band is delayed;
3. Inflammatory response is delayed;
4. Myelin degradation is slower;
5. Axonal regeneration is delayed;
6. Motor recovery is impaired.

Section IV

Discussion

This project was designed to study the potential role of the PlGF in axonal remodeling. We hypothesized that PlGF was likely to be involved in the post-injury inflammatory process that follows an axonal lesion, i.e. the WD, because of its known effects on monocyte chemoattraction and cytokine secretion. WD in the PNS is followed by successful axon regeneration and functional recovery, while in the CNS, its slower and different progression is in part the culprit for the failure of efficient axonal regrowth.

We thus decided to study the role of PlGF in the WD process induced by injury to the PNS, and in the subsequent axonal regeneration.

As only scarce data were available on PlGF in the nervous system at the time we started this project, we have dedicated the first part of our study to expression of PlGF in the PNS under physiological and pathological conditions. We then tested the hypothesis that NF-κB might mediate PlGF transcription. The major part of our work is devoted to the study of the role of PlGF in the cellular and molecular processes involved in WD and in axonal regeneration and functional recovery, using a PlGF knock-out mouse model. We will discuss these results in sequence.

IV.1 PlGF is expressed in the normal nervous system

Since its initial discovery in the placenta in 1991 (Maglione *et al.*, 1991), PlGF has been detected in other organs, including the brain (Beck *et al.*, 2002; Hayashi *et al.*, 2003) and the spinal cord (Hedlund *et al.*, 2010).

Ten years ago, Beck et al. had shown that PlGF mRNA and protein were detectable in neurons throughout the normal mouse brain (Beck *et al.*, 2002). Using double immunofluorescent stainings, ISH and RT-PCR, we provide evidence that PlGF is also expressed in intact as well as in injured mouse sciatic nerves.

In the intact sciatic nerve, PlGF is expressed in all axons, as it is the case for the VEGF (Sondell *et al.*, 2000), and in endoneurial fibroblasts, but not in ECs, SCs or myelin sheaths. PlGF is also expressed in the perikarya of the neurons of origin of sciatic nerve axons in lumbar DRGs and spinal ventral gray matter neurons. At the subcellular level we report for the first time a peculiar periaxonal expression pattern of PlGF on sciatic nerve and DRG sections. This localization of PlGF could be due to its particular basic carboxyl terminal that allows it to bind polyanionic molecules like acidic phopholipids of the cell membranes (Persico *et al.*, 1999; De Falco *et al.*, 2002; Autiero *et al.*, 2003a). In addition to this periaxonal localization, our results show an interruption of PlGF expression at the nodes of

Ranvier. It is known that, at the nodal level, the axolemma is composed of molecules highly specialized for the electrical signal transduction. The molecular difference in membrane composition between the internodal and nodal regions, particularly in the proportion between phospholipids and ionic channels, probably explains the absence of PlGF at the nodes of Ranvier.

IV.2 PlGF expression changes during Wallerian Degeneration

Sciatic nerve transection results in axonal breakdown and disintegration, which can explain the decrease of PlGF expression observed in injured axons during the first week post-injury. Thereafter, WD in the PNS leads to successful axonal repair, explaining the re-expression of PlGF in regenerating axons. Our results also show that SCs transiently express PlGF after nerve injury.

An induction of VEGF expression in SCs was previously reported after peripheral nerve injury (Scarlato *et al.*, 2003). Axonal rupture causes a loss of contact between the axolemma and its ensheathing SCs that switch from a differentiated state to an undifferentiated, proliferating state. This SC response is, among others, due to a neuronal release of the cytokine TGF-β1, which induces SC proliferation (Rogister *et al.*, 1993). Interestingly, TGF-β1 has been shown to activate PlGF expression in cultured keratinocytes (Failla *et al.*, 2000) and in retinal pigment epithelial cells (Hollborn *et al.*, 2006). Hence, the transient expression of PlGF in SC observed after injury might be due to axonal released TGF-β1. The PlGF expression in proliferating SCs is confirmed by our RT-PCR results on primary SC cultures.

In addition to the post-injury expression of PlGF in SCs, our results reveal a decrease of PlGF expression in fibroblasts concomitantly with the down-regulation of Ptc-1, the fibroblast marker. It is known that the signalling molecule desert hedgehog (Dhh) and its receptors, Ptc-1 and 2, are down-regulated during WD (Parmantier *et al.*, 1999; Bajestan *et al.*, 2006; Sharghi-Namini *et al.*, 2006). Moreover, it has been demonstrated that the VEGF gene is a target of Ptc-1 signalling in cancers (Liao *et al.*, 2009). Hence, the reduction of Dhh signalling during WD might cause the PlGF decrease in fibroblasts.

Finally, in none of our experiments we found PlGF expression in ECs or macrophages. These results contrast with those showing that PlGF is expressed in inflammatory cells and vessels after injury (MCAO-induced stroke) to the CNS (Beck *et al.*, 2002). If these results

can be replicated, they suggest that, after injury, PlGF is expressed differently in PNS and CNS.

IV.3. PlGF is a target for NF-κB

Transcription factors, such as c-Fos, c-Jun, ATF3 and NF-κB, are upregulated during WD. They control the changes in gene expression that occur in SCs located in the distal part of the injured nerve and induce responses like pro-inflammatory molecule secretion, phagocytic phenotype acquisition and migration (Liu *et al.*, 1995; Hunt *et al.*, 2004; Camara-Lemarroy *et al.*, 2010; Fu *et al.*, 2010). It has been shown that the *Pgf* gene transcription is regulated by several transcription factors such as MTF-1, GCM1, BF-2 as well as NF-κB (Green *et al.*, 2001; Zhang *et al.*, 2003; Cramer *et al.*, 2005; Chang *et al.*, 2008). Among those factors, NF-κB is the only one involved in both WD process and regulation of PlGF expression.

It was shown previously that the human *Pgf* promotor contains some NF-κB binding sites (Cramer *et al.*, 2005). Our mouse *Pgf* promoter analysis also reveals the presence of κB motifs and our chromatin immunoprecipitation and luciferase assays confirm that PlGF transcription could be regulated by the NF-κB signalling pathway during WD. This result confirms the important role of the NF-κB signalling pathway in the induction of pro-inflammatory cyto- and chemokines by SCs during the WD (Fu *et al.*, 2010).

IV.4. Periaxonal PlGF has no role in myelin sheath formation or preservation

Because of the peculiar peripheral localisation in axons, the question arose whether PlGF plays a role in the formation and/or the preservation of myelin sheaths. In myelinated axons, PlGF is indeed in close contact with the inner lamellae of the myelin sheath and it is interrupted at the nodes of Ranvier. As mentioned above (see section IV.1.1), this PlGF expression pattern could be due to its ability to bind polyanionic molecules like acidic phopholipids of cell membranes. A similar periaxonal localization has been already shown for MAP1B, another cytoplasmic glycoprotein able to bind polyanionic molecules (Franzen *et al.*, 2001). In the latter study, Franzen et al. have shown that MAP1B is a neuronal binding partner for MAG, a myelin protein implicated in the stability of myelinated axons. Thus, as PlGF has an expression pattern similar to MAP1B, we hypothesized that PlGF might similarly play a role in the maintenance of the axon/myelin structure. This hypothesis was not

confirmed by our electron microscope analyses of myelin sheath morphology in sciatic nerves and spinal cords of *Pgf* $^{-/-}$ mice where no abnormalities could be detected. Based on these results and the fact that PlGF is also expressed in unmyelinated axons, we can conclude that PlGF is not essential for the formation and/or the preservation of myelin sheaths.

Thus, the functional implication of this peculiar periaxonal localization of PlGF remains to be determined.

IV.5. PlGF deletion delays the cellular and molecular events of Wallerian Degeneration.

The question whether PlGF has a functional role in WD was examined in *Pgf* $^{-/-}$ mice. In mice lacking PlGF we observed after nerve transection a global delay in the cellular and molecular events of WD that retarded axonal regeneration and functional motor recovery. The targets of PlGF in WD demonstrated in our work are schematized in figure IV.1

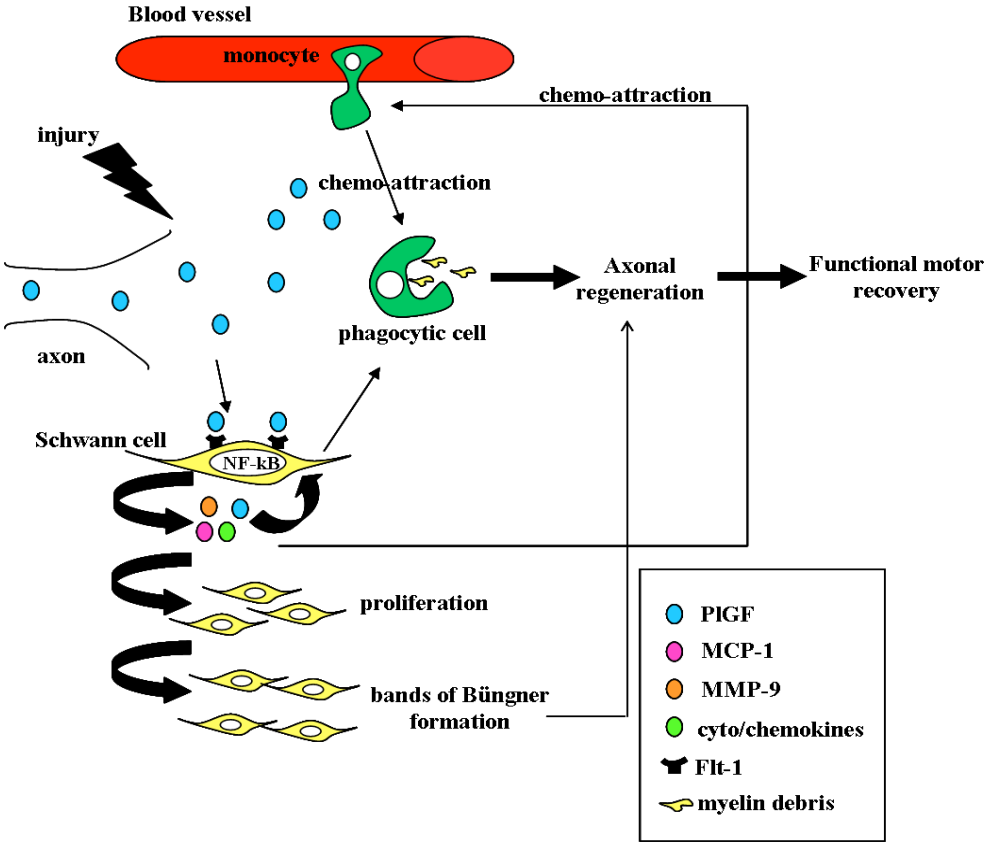


***Figure IV.1:* Model of PlGF involvement in the Wallerian degeneration.** *Following axonal breakdown, the released PlGF can bind to its flt-1 receptor on SCs, which in turn (i) produce PlGF and chemokines through the activation of the NF-κB signaling pathway, (ii) proliferate and (iii) align to form bands of Büngner, promoting axonal regeneration. PlGF also influences directly monocyte chemoattraction, thereby increasing macrophage activity of myelin debris phagocytosis, necessary to successful axonal regeneration.*

IV.5.1. Impaired Schwann cell dedifferentiation and proliferation

Nerve injury changes the phenotype of SCs that switch to a dedifferentiated, non-myelinating and proliferative state within 24h after the lesion. In *Pgf*$^{-/-}$ mice, SC proliferation rates are significantly decreased, both *in vitro* and *in vivo*. Flt-1, which binds both VEGF and PlGF, mediates VEGF-triggered proliferation of astroglial cells (Mani *et al.*, 2005; Krum *et al.*, 2008). It is thus likely that in wt animals, the PlGF released by injured axons activates Flt-1 receptors on SC membranes and induces their proliferation. This is in line with the study of Sondell et al. showing the mitogenic activity of VEGF on SC via flk-1 (Sondell *et al.*, 1999a), and confirming the biological activity of Flt-1 expressed on SCs (Schratzberger *et al.*, 2000). As other mitotic agents can act on SCs during WD (Rogister *et al.*, 1993; Kwon *et al.*, 1997; Li *et al.*, 2005; Ogata *et al.*, 2006), the sole absence of PlGF can only decrease SC proliferation, but not abolish it.

Moreover, in *Pgf* $^{-/-}$ mice, the expression of p75NGFr, a marker of SC dedifferentiation, is significantly delayed after injury. The p75NGFr expression is stimulated by the fibrin deposit occurring after the lesion (Akassoglou *et al.*, 2002). Recently, it was shown that PlGF is an inducer of the plasminogen activator-1 inhibitor (PAI) (Patel *et al.*, 2010). Thus, in *Pgf*$^{-/-}$ mice, the absence of PlGF could lead to a decrease in fibrin deposits, due to a low PAI induction, resulting in the delay of p75NGFr expression.

IV.5.2. Delayed cyto/chemokine expression

A large cyto/chemokine network, mainly produced by SCs, regulates the sequential WD cellular events. PlGF is able to induce an increase of several of these molecules like inflammatory cyto/chemokines (Bottomley *et al.*, 2000; Perelman *et al.*, 2003; Selvaraj *et al.*, 2003) or enzymes such as MMPs (Hattori *et al.*, 2002). In our cytokine arrays, PlGF has a significant effect on the expression of MCP-1 and pro-MMP-9. These molecules are essential for early WD events: MCP-1 attracts macrophages (Toews *et al.*, 1998; Siebert *et al.*, 2000; Perrin *et al.*, 2005), and MMP-9 is responsible for SC migration (Mantuano *et al.*, 2008). We also obtain statistical differences in the expression of IL-10, Il-1α and MIP-1α between wt and *Pgf*$^{-/-}$ mice. However, because of the low reproducibility of our cytokine arrays for these molecules, leading to large SE, we were not able to detect the known post-injury evolution of these cytokines (Shamash *et al.*, 2002; Perrin *et al.*, 2005) leaving open the question if they are significantly influenced by PlGF.

IV.5.3. Delayed macrophage recruitment

Macrophages play an essential role during WD. In addition to rapid clearance of axonal and myelin debris, they secrete neurotrophins (Barrette *et al.*, 2008), promoting successful axonal regeneration. PlGF is chemo-attractive for macrophages (Clauss *et al.*, 1996). It is thus not surprising to find a significant delay in macrophage invasion during the first week of WD in *Pgf*$^{-/-}$ mice. As mentioned above, our cytokine array results also showed a significant delay of MCP-1 expression in *Pgf*$^{-/-}$ mice. Post-lesional macrophage invasion can therefore be impaired directly by the lack of PlGF, or indirectly via the delay in MCP-1 expression.

IV.5.4. Delayed myelin clearance, but no effect on the phagocytic phenotype of SCs

Myelin degradation and its subsequent clearance are mediated by SCs and macrophages. The phagocytic phenotype acquired by SCs after the lesion was shown to be associated with c-Fos expression in these cells (Liu *et al.*, 1995). However, although our results show a delay in myelin clearance when PlGF is absent, they detect no difference in c-Fos expression between wt and *Pgf*$^{-/-}$ mice. Thus, the delay observed in myelin degradation and clearance is likely due to the reduced number of phagocytic cells within the lesion, consequence of the reduced SC proliferation and the delayed macrophage recruitment, rather than to a decrease in phagocytic capacity.

IV.5.5. Delayed formation of Büngner's bands

In addition to their role in the inflammatory response, SCs migrate and line up to form the bands of Büngner. SC migration involves several molecules like cell-signaling factors, integrins and proteases (Lauffenburger & Horwitz, 1996). Among them, MMP-9, which is over-expressed by SCs after nerve injury and promotes SC migration (Mantuano *et al.*, 2008), was found to be a target for PlGF (Hattori *et al.*, 2002). Concordantly, our cytokine arrays show a significant decrease of pro-MMP-9 expression 7 days after injury in *Pgf*$^{-/-}$ mice relative to wt mice.

NGF and its low affinity receptor p75NGFr are also involved in SC migration (Anton *et al.*, 1994) and in our study p75NGFr expression after injury is delayed in *Pgf*$^{-/-}$ mice. Moreover, it is known that VEGF promotes SC migration mainly through its Trk receptors flk-1 and flt-1 (Schratzberger *et al.*, 2000) and that flt-1, the PlGF receptor, mediates migration of ECs (Bae *et al.*, 2005; Li *et al.*, 2006), and retinal pigment epithelial cells (Hollborn *et al.*, 2006). The delay of SC alignment observed on p75NGFr immunostained

sections of transected *Pgf* $^{-/-}$ nerves could thus be related either to a direct effect of the PlGF deficiency, or to an indirect effect related to the delay of MMP-9 and/or p75NGFr expression or to a reduced efficiency of VEGF, of which the effects are normally amplified by PlGF via various mechanisms (Carmeliet *et al.*, 2001; Autiero *et al.*, 2003b; Ribatti, 2008).

Contrasting with the *in vivo* experiments, the *in vitro* migration assay does not reveal a significant difference between wt and *Pgf* $^{-/-}$ mice, although numerical values are systematically lower for *Pgf*$^{-/-}$ cells. This difference could be explained by the poor reproducibility of the "scratch" test, and of the size of the cell-free area at the beginning of the experiment that can influence the migration ability of cells.

IV.5.6. Delayed axonal regeneration, but normal remyelination

Since PlGF modulates the early post-axotomy SC proliferation and macrophage invasion, it came of little surprise that axonal regeneration was also delayed in *Pgf*$^{-/-}$ mice. Indeed, the delay in clearance of myelin and axonal debris by SCs and macrophages leads to an unfavorable environment for regeneration and the retarded SC p75NGFr expression and bands of Büngner formation can decrease the beneficial effect that neurotrophins exert on the regenerating axons (Taniuchi *et al.*, 1988). It must be pointed, however, that, like the other events of WD, axonal regeneration is only delayed so that it does not differ anymore between wt and *Pgf* $^{-/-}$ mice at post-injury day 21.

The remyelination of regenerating fibers, the last step of nerve repair, is not affected by the delayed early events of WD. Nevertheless, because of the decrease in SC proliferation in the absence of PlGF, we hypothesized that the internodal length could be affected in regenerated *Pgf* $^{-/-}$ nerves. It is known that, after remyelination, the distance between two adjacent nodes of Ranvier is reduced in normal nerves (Hildebrand *et al.*, 1985). In our teasing experiments we found no difference in intermodal length between regenerated wt and *Pgf*$^{-/-}$ nerve fibers. This could be due to the fact that Schwann cell proliferation may not be necessary for effective axonal regeneration and myelination after peripheral nerve injury, as shown by Yang et al. (Yang *et al.*, 2008).

IV.5.7. Lack of functional motor recovery

Although axonal regeneration and remyelination occur 21 days after injury in *Pgf* $^{-/-}$ mice, functional motor recovery is impaired compared to wt mice in our study. We have no proven explanation for this apparent contrast. A major factor that determines functional recovery, however, is the correct rewiring of regenerating axons with their peripheral target

end-organs. Regenerating motor axons are often misrouted to sensory targets and sensory axons may be misrouted to muscle, leading to abnormal sensorimotor activity (Ijkema-Paassen *et al.*, 2002; Madison *et al.*, 2007; Carlstedt, 2008).

We speculate that the favourable effect of PlGF on SC proliferation and alignment and on axonal regeneration could enhance target organ reinnervation. Such an effect has indeed been demonstrated for VEGF. In a model of axonal regeneration through an acellular conduit, it was shown that adding VEGF in the silicone chamber stimulated SC and axonal regeneration, and that this was associated with an improved target organ reinnervation (Hobson *et al.*, 2000). It remains to be demonstrated that PlGF has a similar beneficial effect on axonal rewiring.

Section V

Conclusion and Prospects

The expression and function(s) of PlGF in the intact and injured PNS were not known up to now. Our work is the first demonstration of the involvement of PlGF as a new member of the cytokine network regulating WD. We have demonstrated that in the intact mouse sciatic nerve PlGF is found in axons underneath the axolemma, while it is expressed by SCs after axotomy possibly through a NF-κB-dependent mechanism of transcription. In a second step, we have used transgenic knock-out mice for the PlGF gene to show that PlGF plays a significant role in the sequence of cellular and molecular events of WD. It stimulates proliferation and migration of SCs, as well as the inflammatory response by inducing pro-inflammatory molecule expression and macrophage recruitment. We have shown that the absence of PlGF is associated with a delay in WD events, and in axonal regeneration, as well as poor functional motor recovery.

These results may have therapeutic implications for peripheral nerve injury. PlGF is probably dispensable for effective axonal regrowth, as in our study there was only a delay in regeneration. The more important aspect in our work is the finding that, despite normal axonal regeneration at long delays in the absence of PlGF, motor recovery remains impaired compared to wt mice. This suggests that PlGF may be needed for the correct rewiring of peripheral axons and its utility for this aspect of post-injury reinnervation should be explored in future studies.

The novel observation that PlGF is a molecule required for successful and functional repair in the PNS may have important consequences and perspectives for regeneration in the CNS. As mentioned in section I.1.2, following CNS injury, axonal regeneration aborts due in part to a less vigorous macrophage invasion compared to the PNS. In the future, it seems thus of interest to explore PlGF expression after CNS injury.

Axonal regeneration within the CNS is also impaired by an astrocytic gliosis. Interestingly, PlGF is expressed by astrocytes in which its expression is increased after brain ischemia (Beck *et al.*, 2002). Astrocytes, isolated from *Pgf*$^{-/-}$ mice cortex, exhibit a higher proliferation rate under hypoxic condition than wt-isolated astrocytes (Freitas-Andrade *et al.*, 2008). These cells therefore deserve to be explored regarding their PlGF expression pattern after CNS lesion.

The question whether regeneration could be promoted by providing PlGF after a CNS lesion is of major importance. In fact, in addition to its potential beneficial effect on the post-injury inflammatory reaction, PlGF treatment could also enhance neo-angiogenesis (Takeda *et al.*, 2009), which is known to be beneficial for axonal regrowth (Hobson *et al.*, 2000; Dray *et al.*, 2009). Angiogenic effects may have drawbacks like oedema formation due to vascular

permeability increase as it was shown for the therapeutic use of VEGF in brain injury (van Bruggen *et al.*, 1999). Concerning PlGF, its effect on vascular permeability remains disputed. Some reports have shown that only VEGF analogues activating VEGFR-2 are able to increase vascular permeability (Hillman *et al.*, 2001; Brkovic & Sirois, 2007). The lack of an effect on VEGFR-2 could make PlGF a more appropriate candidate than VEGF for neurological treatment. Experiments using mutant mice in which PlGF is under- or over-expressed have not solved the question as they have revealed either a decrease (Luttun *et al.*, 2002a) or an increase (Odorisio *et al.*, 2002; Oura *et al.*, 2003) of vascular permeability under pathological conditions. One explanation for this discrepancy may be that the PlGF-induced increase in vascular permeability is an indirect effect, due to the displacement of VEGF-A from VEGFR-1 to VEGFR-2, as PlGF competes with VEGF-A for VEGFR-1 in pathological conditions (Carmeliet *et al.*, 2001).

Taken together, VEGF and PlGF, despite proven experimental advantages, could be double-sided swords as neuroprotective/neurotrophic treatments for ischemic, degenerative or traumatic neurological disorders. We can finally conclude that in future therapeutic studies, it might be worthwhile to explore the effect of PlGF combined or not with a VEGFR-2 inhibitor to better understand the mechanisms and receptors implicated in the neurotrophic and angiogenic effects mediated by VEGF and PlGF.

Section VI

Materials and Methods

VI.1. Primary antibodies

In this work, many Abs have been used. Table VI.1 summarizes information on all primary Abs: origin, dilution or quantity used, as well as their cellular or molecular specificity.

Ab Name	Reference	Company	Dilution/Quantity	Cellular/Molecular specificity
PlGF	sc-27134	Santa Cruz Biotechnology	1:50	Placental growth factor *PlGF protein*
NF-H	MAB5448	Millipore	1:500	Neurofilament heavy polypeptide *axon*
S100	ZO311	DakoCytomation	1:200	Low MW Ca^{++}- binding protein *Schwann cell*
p75 NGFr	AB1554	Millipore	1:200	Low affinity NGF receptor *proliferating Schwann cell*
vWF	AB6994	Abcam	1:2000	von Willebrand Factor *endothelial cell*
Ptc-1	sc-9016	Santa Cruz Biotechnology	1:50	Sonic Hedgehoc receptor *fibroblast*
CD11b	MCA74	Serotec	1:250	Complement receptor type 3 *macrophage*
P0	AB9352	Millipore	1:50	Glycoprotein 0 *PNS myelin*
Nav1.6	ASC-009	Alomones Labs	1:50	Voltage-gated Sodium channels *nodes of Ranvier*
NeuN	MAB377	Millipore	1:250	Neuronal nuclei *neuron*
Ki67	NCL-Ki67p	Novocastra	1:250	Nuclear antigen expressed in all proliferating cells
BrdU	OBT0030	Serotec	1:250	Bromodeoxyuridine *Cells in S phase of mitosis*
c-Fos	sc-52	Santa Cruz Biotechnology	1:100	Immediate early gene *Marker of SC activity*
p65	sc-109	Santa Cruz Biotechnology	4µg	NF-κB subunit p65
Flag M2	F3165	Sigma	4µg	synthetic peptide

Table VI.1.: *Primary antibody information.*

VI.2. Animals and tissue samples

Adult (10-12 weeks) 50%Swiss-50%129SV female wt and *Pgf* $^{-/-}$ mice were used (kind gift from Pr Peter Carmeliet, Vesalius Research Centre, VIB, KUL, Belgium). Sciatic nerves, spinal cords and DRG were harvested. The experiments were performed in accordance with the rules and regulations of the Ethical Committee for animal research of the Belgian National Fund for Scientific Research.

VI.3. Surgical procedure: sciatic nerve complete transection

Mice were anesthetized by an intraperitoneal (i.p.) injection of a mixture of ketamine (75 mg/kg; Ketalar®, Bayer HealthCare, Brussel, Belgium) and xylazine (10 mg/kg; Rompun®, Pfizer, Brussel, Belgium). Under aseptic conditions, sciatic nerves were exposed and WD induced by a complete section of the nerves at the upper thigh level (figure VI.1). Proximal and distal stumps were left in their original position to allow axonal regeneration. Muscles and skin were carefully closed in two layers.

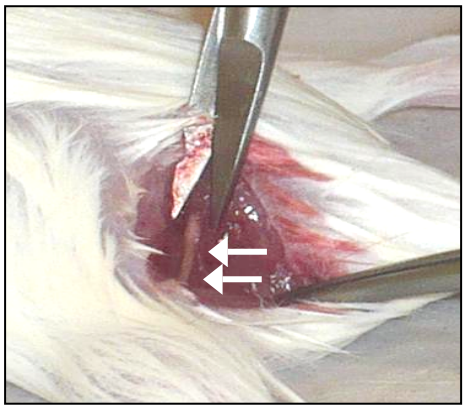


Figure VI.1.: *Complete section of mouse sciatic nerve (white arrows).*

Table VI.2 summarizes all the techniques performed in this work, the number of animals used for each one, as well as their survival times.

Experimental technique	Survival time	Animal nb
Motor recovery evaluation	21 days	5 wt and 5 *Pgf* -/- mice
Immunostainings	uninjured	6 wt and 5 *Pgf* -/- mice
	1 day	6 wt and 10 *Pgf* -/- mice
	3 days	11 wt and 7 *Pgf* -/- mice
	7 days	9 wt and 7 *Pgf* -/- mice
	14 days	10 wt and 8 *Pgf* -/- mice
	21 days	11 wt and 12 *Pgf* -/- mice
	28 days	8 wt and 6 *Pgf* -/- mice
In situ hybridization	uninjured	1 wt mouse
Toluidine blue staining	uninjured	5 wt and 5 *Pgf* -/- mice
	1 day	5 wt and 5 *Pgf* -/- mice
	3 days	8 wt and 9 *Pgf* -/- mice
	7 days	7 wt and 8 *Pgf* -/- mice
	14 days	6 wt and 7 *Pgf* -/- mice
	21 days	7 wt and 8 *Pgf* -/- mice
Cytokine arrays	uninjured	6 wt and 6 *Pgf* -/- mice
	1 day	6 wt and 6 *Pgf* -/- mice
	3 days	6 wt and 6 *Pgf* -/- mice
	7 days	6 wt and 6 *Pgf* -/- mice
Teasing	uninjured	3 wt and 3 *Pgf* -/- mice
	28 days	3 wt and 3 *Pgf* -/- mice
Electronic microscopy	uninjured	3 wt and 3 *Pgf* -/- mice
Schwann cell culture	uninjured	16 wt and 16 *Pgf* -/- mice
RT-PCR	uninjured	1 wt mouse
Chromatin Immunoprecipitation	uninjured	12 wt mice
	1 day	12 wt mice

Table VI.2.: *Techniques, number of animals and survival times used for this work.*

VI.4. Motor recovery evaluation

Prior to the lesion and at 3, 7, 10, 14, 17 and 21 days following unilateral left sciatic nerve section, mice had their hind paws inked and were then allowed to walk down a 60cm long corridor lined with graph paper. For each animal (5 wt and 5 *Pgf* $^{-/-}$ mice), at every time point, at least four clear footprints were obtained for each foot. Measurements of the printed

foot length were then made on the operated side (OPL) and the normal side (NPL). A mean of four values was then calculated for the OPL (xOPL) and NPL (xNPL), and a print-length factor (PLF) was calculated as follows: PLF = (xOPL – xNPL)/xNPL (George *et al.*, 2003).

VI.5. Immunostainings

VI.5.1. Tissue processing

Mice were sacrificed after different survival times (1, 3, 7, 14, 21 and 28 days post-injury) by an overdose of Nembutal (150 mg/kg, i.p, CEVA Santé Animale, Brussel, Belgium). The distal parts of the transected sciatic nerves (a 12 to 15 mm long portion) were freshly harvested, embedded in Tissue-Tek® O.C.T.™ Compound (Labonord SAS, Templemars, France) and directly frozen. Tissues were cut on a cryostat at 10µm thickness. Longitudinal or cross-sections were collected onto gelatin-coated slides and stored at -20°C until used.

UI sciatic nerves, DRGs and lumbar spinal cords were also freshly dissected out, embedded in Tissue-Tek® and cut at 10 µm (sciatic nerves and DRGs) or 20 µm thickness (spinal cords). Sections were collected onto gelatin-coated slides and stored at -20°C until used.

VI.5.2. Immunofluorescent staining protocol

To characterize PlGF expression, we used double immunofluorescence stainings for PlGF and several specific cellular markers such as NF-H, S100, p75 NGFr, vWF, Ptc-1, P0, Nav1.6, CD11b and NeuN (see table VI.1). After drying, tissue sections were fixed in cold acetone for 10 min at 4°C. Nonspecific binding was prevented by 1h incubation in a 10% normal serum solution in 0.1% triton-PBS (0.1M, pH 7.4). After overnight incubation at room temperature (RT) with the specific primary Abs, sections were rinsed 3 times with PBS and incubated for 1h at RT with their respective secondary Abs coupled to rhodamine or FITC (1:500, Jackson ImmunoResearch). They were then rinsed twice in PBS, twice in distilled water and mounted under coverslips using the “vectashield” (Vector Laboratories) solution. Negative controls were obtained by incubation of the primary Ab with its specific blocking peptide (sc-27134 P; Santa Cruz Biotechnology) before applying it on the tissue sections.

VI.5.3. DAB immunostaining protocol

Tissue sections, after drying, were fixed with 4% paraformaldehyde (PFA) for 5 min, then incubated in a 0.3% H_2O_2, 0.1% Na azide solution in PBS for 20 min at RT to reduce endogenous peroxydase activity. Non-specific binding was prevented by 1h incubation in 3% normal serum and 1% bovine serum albumin solutions in 0.1% triton-PBS. Sections were then incubated overnight at RT with the specific primary Abs: anti-p75NGFr to study SC dedifferentiation or anti-CD11b to study macrophage recruitment (see table VI.1). After 3 PBS rinses, they were incubated for 1h at RT with their respective secondary biotinylated Abs (Vector Laboratories) diluted and centrifuged in a 3% normal mouse serum and 1% bovine serum albumin (BSA) in 0.1% triton-PBS solution. After 1h incubation with the avidin-biotin-peroxydase complex (Vector Laboratories) diluted 1:1000 in PBS, the immunostaining was revealed with 3,3'-diaminobenzidine (DAB).

VI.6. Morphometric analyses

VI.6.1. Quantification of *in vivo* Schwann cell proliferation

To study SC proliferation, longitudinal sections of sciatic nerves were stained with anti-Ki67 Ab (see table VI.1). Because of a conflict between Abs, we were not able to perform the double Ki67/p75NGFr immunofluorescent staining. Therefore, longitudinal sections of distal parts of sciatic nerves were double-stained with anti-Ki67 and anti-CD11b Abs (see table VI.1) and counter-stained with DAPI to detect all cell nuclei. The total number of Ki67-positive/CD11b-positive cells (proliferating macrophages) or Ki67-positive/CD11b-negative cells (proliferating SCs) were manually counted within non-overlapping successive fields covering the entire nerve section captured with an Olympus DP50 digital camera connected to an Olympus AX-70 microscope (magnification 20 x). The results were expressed as a mean number (± SE) of Ki67 positive SC nuclei per mm² of nerve tissue.

VI.6.2. Quantification of Schwann cell c-Fos expression

As for Ki67 Ab, because of a conflict between Abs the expression of the early-expressed protein c-Fos in SCs could not be studied with double c-Fos/p75NGFr immunofluorescent staining. Instead, longitudinal sections of distal parts of sciatic nerves were double-stained with anti-c-Fos and anti-CD11b Abs (see table VI.1) and counter-stained with DAPI to detect all cell nuclei. The total number of nuclei and c-Fos-positive/CD11b-

positive cells (macrophages expressing c-Fos) or c-Fos-positive/CD11b-negative cells (SCs expressing c-Fos) (magnification 20 x) were manually counted within non-overlapping successive fields covering the entire nerve section, however we didn't count c-Fos-positive nuclei located in the epi-/perineurium to avoid bias due to fibroblasts (Pyykonen & Koistinaho, 1991). The results were expressed as the mean percentage (± SE) of c-Fos-positive SCs amongst the total number of cells.

VI.6.3. Quantification of axonal regeneration

To evaluate axonal regeneration, longitudinal sections from the distal part of injured sciatic nerves were fluorescently stained with anti-NF-H Ab as described above. For the quantification, non-overlapping successive fields covering the entire tissue sections (which means the whole length of the distal part) were captured at magnification 10x. The mean number of fields/section was similar between wt and *Pgf* $^{-/-}$ mice (7.47±0.76 and 7.54±0.64 respectively), resulting in comparable total tissue length. Each field was then allocated to one of the following group: (i) field containing only debris of degenerating axons; (ii) field containing a majority of debris and few regenerating profiles; (iii) field containing scarce debris and a majority of regenerating fibres; and (iv) field containing only regenerating fibres. Data were expressed as a mean proportion of field numbers in each of those 4 groups per total nerve section (%) ± SE.

VI.6.4. Quantification of Schwann cell dedifferentiation

To quantify SC dedifferentiation, digitalized images obtained with a 20 x microscopic magnification were converted to gray scale. A gray colour threshold intensity was set for each section. The intensity of immunostaining was then quantified within non-overlapping successive fields covering the total nerve tissue area using the Olympus AnalySIS computer program. Data were expressed as the mean integral intensity (± SE) of total stained area (pixel²) per total area of the nerve section (pixel²).

VI.6.5. Quantification of recruited macrophages

In order to quantify the recruitment of macrophages, CD11b-positive cells were manually counted at 10x magnification within non-overlapping successive fields covering the entire nerve section. Results were expressed as the mean number (± SE) of CD11b-positive cells per 0.1 mm² of nerve tissue.

VI.7. Primary Schwann cell culture

Adult SCs were isolated from wt and *Pgf* $^{-/-}$ sciatic and trigeminal nerves, which are both an important source of SCs easily accessible, as described previously by others (Bouquet *et al.*, 2007). After dissection, the nerves were left for 2 weeks in a degeneration medium: DMEM, 10% foetal bovine serum (FBS), Fungizone (2.5µg/ml; GIBCO®, Invitrogen™), Forskolin (2µM Calbiochem®), Gentamycine (50µg/ml; GIBCO®) and Heregulin-β1 (10 ng/ml, HRG; R&D Systems GmbH) at 37°C, 5% CO_2. After enzymatic and mechanical dissociation, cells were plated on T25 culture dishes pre-coated with poly-$_L$-lysine (50 µg/ml, Sigma-Aldrich), laminin (10µg/ml, Sigma-Aldrich), and DMEM supplemented with 10% FBS. They were then incubated at 37°C, 5% CO_2 in $N2^{HRG}$ medium: 50% DMEM 50% F12 supplemented with N2 (dilution 100 x; GIBCO®), gentamycin (50µg/ml), fungizone (2.5 µg/ml), forskolin (2µM), and HRG (10ng/ml). At confluence, SCs were purified by magnetic cell-sorting using the anti-p75 NGFr Ab (see table VI.1), according to the manufacturer's instructions (Miltenyi Biotech GmbH, Germany). To assess the degree of purity of our SC cultures, we performed p75 NGFr/DAPI immunofluorescent staining and we evaluated the percentage of p75 NGFr-positive cells.

VI.8. In vitro BrdU assay

SCs were plated on coated glass coverslips at a concentration of $25x10^3$cells/300µl. 20 µM BrdU (Sigma-Aldrich) were directly added to the cultures for 16h before fixation in 4% PFA for 10min. Cells were treated in HCl 2N for 10min at 37°C and then washed in borate buffer (0.1M; pH 8.5). Cells were permeabilised and nonspecific binding was prevented by 1h incubation in a 10% normal serum - 0.1% triton-PBS solution. They were incubated overnight with anti-BrdU and anti-p75NGFr Abs (see table VI.1). After 3 PBS washes, cells were incubated for 1h at RT with the respective secondary Abs coupled to rhodamine or FITC (1:500, Jackson ImmunoResearch). The coverslips were then rinsed twice in PBS, twice in distilled water, and mounted on slides using the vectashield solution containing DAPI in order to visualise all nuclei of the total cell population. Double-stained p75NGFr/BrdU cells and total number of p75NGFr positive cells were manually counted at the 20x magnification within non-overlapping successive fields and results were expressed as mean % (± SE) of proliferating SCs.

VI.9. Schwann cell migration assay

Purified wt or *Pgf* $^{-/-}$ SCs were plated on coverslips coated with poly-$_L$-lysine and laminin at a density of 25x10³ cells in a 50µl drop of $N2^{HRG}$ medium. After cell attachment, culture medium was added and cells were left for 24h without further treatment. A cell-free area was generated by a scratch with the tip of a plastic pipette. Migration of SCs into the cell-free area was monitored every 2h for 28h under a phase-contrast inverted microscope (magnification 10 x) equipped with a digital camera. As described by Sakurai and Osumi, (Sakurai & Osumi, 2008), the migration ability of SCs was estimated by the size reduction of the cell-free area every 2 hours after the scratch.

VI.10. In situ hybridization

Mice were deeply anesthetized by an i.p. injection of Nembutal (60 mg/kg) and perfused with 4% PFA in 0.1 M phosphate buffer, pH 7.4. Sciatic nerves and spinal cord were harvested, post-fixed with 4% PFA for 24 h at 4°C and kept for 48 h in 30% sucrose at 4°C for cryoprotection. They were then cut transversally at 10 µm on a cryostat and mounted onto SuperFrost®Plus slides (Prosan, Merelbeek, Belgium). Sections were post-fixed with 4% PFA for 10 min and treated with acetylated triethanolamine 100 mM, PH 8, for 15 min. After 3 washes with TBS-Tween 0.1%, sections were pre-hybridized with hybridization buffer (Amresco, Ohio, U.S.A.) for 1h at 70°C and then hybridized with a digoxygenin-labeled antisense PlGF RNA probe overnight at 70°C (the plasmid containing the mouse PlGF-2 cDNA was provided by P. Carmeliet). Two negative controls were used: sense RNA probes and nerve sections from *Pgf* $^{-/-}$ mice. The next day, sections were washed twice with pre-warmed washing buffer (formamide 50%, 0.1% Tween, SSC 20× in H_20) for 1h at 70°C, twice with Tris-Saline buffer (100mM Tris-HCl, 150M NaCl, 0.1% Tween) for 15 min at RT and then blocked with 10% goat serum in Tris-Saline buffer for 30min at RT. Sections were then incubated overnight at 4°C with an anti-digoxigenin Ab coupled to alkaline phosphatase (1:2000, Roche Applied Science) in blocking buffer. After 3 Tris-Saline buffer washes, sections were incubated twice with a 100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$ and 0.1% Tween 20 buffer for 30min each at RT. Then sections were overlaid at RT in the dark with a 200 µl filtered NBT/BCIP/Tween-20 0.1% solution (Sigma-Aldrich) between coverslips for 4 to 6 hours. The reaction was blocked by washes with PBS followed by post-fixation with 4%PFA for 15 minutes.

VI.11. Semi-quantitative reverse transcription PCR

RNA from DRGs or cultured SCs was extracted with TRIzol® (Invitrogen) and isolated according to the manufacturer's protocol. mRNAs for PlGF and GAPDH, taken as internal control, were amplified with aliquots of 20 ng of total RNA using a RT-PCR kit (GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit; Applied Biosystems, Foster City, CA) and pairs of primers (oligonucleotide sequences shown in table VI.3; Eurogentec, Seraing, Belgium). Reverse transcription was performed at 70°C for 15 minutes followed by 2 minutes of incubation at 95°C for denaturation of RNA-DNA heteroduplexes. Amplification consisted in cycles of 15 seconds at 94°C, 20 seconds at 58°C, and 20 seconds at 72°C. RT-PCR products were resolved on 10% polyacrylamide gel, stained with GelStar (Lonza, Belgium).

Gene	Primer type	Oligonucl. sequence (5'- 3')	PCR product size (bp)	Cycle nb
mPlGF	reverse	TTCCCCTTGGTTTTCCTCCTT	130	45
	forward	AGATCTTGAAGATTCCCCCCA		
GAPDH	reverse	GCCTTCTCCATGGTGGTGAAGAC	210	25
	forward	GACCCCTTCATTGACCTCAACTACATG		

Table VI.3.: *RT-PCR probe information.*

VI.12. Total protein extraction from sciatic nerves

Fresh sciatic nerves were homogenized in a lysis buffer containing 80% PBS, 10% 500mM NaF, 0.1% 100mM Na_3VO_4, 1% triton X-100 and protease inhibitors (Complete™; Roche) on ice. The tissue weight/buffer volume ratio was 1/10. After homogenization, samples were centrifuged at 13000 rpm for 15 min at 4°C. Clarified cell lysates were collected and total protein concentrations were quantified with the BCA protein assay kit (ThermoScientific).

VI.13. Cytokine array

Expressions of IL-1α, IL-1β, IL-6, TNF-α, IL-10, VEGF, VEGFR-1, MCP-1, MIP-1α and pro-MMP-9 were measured in tissue extracts of normal and injured sciatic nerves from wt and *Pgf*$^{-/-}$ mice using RayBio® Cytokine Antibody Arrays (RayBiotech, Inc.). 200µg of total protein were used per sample. The cytokine array membranes were scanned with a Las-4000 luminescent image analyser (Fujifilm). The intensity of the signal was measured

and compared to that of a positive control with the Quantity One 1-D Analysis software (Bio-RAD Laboratories, Inc.). The signal intensities of positive controls and samples were normalized using background substraction. The results were expressed as the mean relative signal intensity, i.e. the ratio of sample intensity/positive control intensity.

VI.14. Prediction of NF-κB binding sites

The mouse *Pgf* gene sequence was obtained from the www.ensembl.org web site. To identify NF-κB binding sites (referred to as "κB motifs"), computational analysis of the promoter sequence of mouse *Pgf* (Green *et al.*, 2001) gene was performed using four different programs designed to look for transcription factor binding sites:

- MatInspector program (www.genomatix.de/matinspector.html)
- Match™program (www.generegulation.com/cgi-bin/pub/programs/match/bin/match.cgi)
- Promo (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)
- TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html)

VI.15. Chromatin immunoprecipitation assay

VI.15.1. Immunoprecipitation and DNA purification

Sciatic nerves (from intact and 24h post-injured adult wt mice) were surgically removed from four adult mice per condition, minced and immediately fixed in PBS containing 1% PFA for 25 min at RT to promote the protein-DNA cross-linking. To stop the formaldehyde action, 1.25 M glycine was diluted 10 times in the cross-linking solution, and then nerves were washed twice with cold PBS containing protease inhibitors (Roche). Nerves were homogenized into 1.5 ml of lysis buffer (1% SDS, 10 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, protease inhibitors). The lysate was sonicated for 15 min with alternating 30-s pulses at high power with a Bioruptor sonicator (Diagenode), and then centrifuged at 13000 rpm for 5 min at 4°C. To check the fragment size of sheared chromatin (optimal size between 250-500 bp), 10µl of sheared chromatin was boiled for 5 min to denature proteins, and DNA fragments were separated on a 1.5% agarose gel stained with GelRed™ (Biotium). The protein concentration of lysates was measured using the BCA protein assay kit (Pierce). 30 µg of chromatin was set aside as a tenth of total chromatin input used for IP. Lysates were diluted 10 times into dilution buffer (1% Triton X-100, 150mM NaCl, 2mM EDTA, 20mM Tris-HCl pH 8.0, protease inhibitors). The sheared chromatin (300 µg per IP) was pre-cleared with 100

µl of Protein A-agarose (Santa Cruz Biotechnology), diluted in a solution of dilution buffer (1% triton X-100, 150 mM NaCl, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH8.0, protease inhibitors) and lysis buffer in 9/1 proportion and subsequently blocked with BSA (100 µg/ml) and sheared salmon sperm DNA (500 µg/ml; Invitrogen), for 2h at 4°C under agitation. After the Protein A-agarose removal, "cleared" lysates were incubated with 4µg of anti-p65 or anti-Flag (used as negative control Ab, see table VI.1) at 4°C on a rotating platform overnight. The immune complexes were incubated with 100 µl of Protein A-agarose solution for 2h at 4°C. The immune complexes were then washed three times with 1 ml low salt washing buffer (1% triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, protease inhibitors), once with 1 ml high salt washing buffer (same buffer but containing 500 mM NaCl), once with 1 ml LiCl buffer (0.25 M LiCl, 1% IGEPAL CA630 (Sigma), 1 mM EDTA, 1% deoxycholic acid, 10 mM Tris-HCl pH 8.0), and finally once 1 ml with TE buffer (10 mM Tris-HCl, 1 mM EDTA, final pH 8.0). Immunoprecipitated chromatin was eluted in 250 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at RT, twice. Chromatin inputs were also diluted in elution buffer. Protein-DNA cross-links were reversed by incubation with 20 µl of 5 M NaCl at 65°C for 4h and then we added 10µl of 0.5 M EDTA, 20µl of 1M Tris-HCl pH 6.5 and 20µg of proteinase K (Promega), for 1h at 45°C to digest proteins. DNA was purified with phenol/chloroform (Sigma) on Phase Lock Gel™ tube (Eppendorf). After a 5 min-centrifugation at 13000 rpm, the aqueous phase was incubated 1h at -20°C in a solution containing 45 µl of 3 M NaAC pH 5.5, 30 µg of glycogen (Roche) and 1 ml of ethanol, and then samples were centrifuged 5 min at 13000 rpm. The DNA pellet was washed with 70% ethanol, then resuspended in 100 µl of DNase-free distilled water and stored at -80°C until use for PCR.

VI.15.2. Quantitative real-time PCR

Quantitative RT-PCR amplifications were performed in a 96-well plate in the ABI Prism® 7000 sequence detector (Applied Biosystems) in a total volume of 20 µl, which included 1 µl of DNA sample coming from the Chromatin IP assay and 19 µl of a reaction mixture (10 µl of Power SYBR® Green PCR Master Mix [Applied Biosystems], 8.2 µl of DNase-free distilled water, and 0.4 µl of each primers [designed to amplify detected κB motifs, table VI.4; Eurogentec, Belgium]). Each qRT-PCR amplification was performed in triplicate. The cycle conditions were 2 min at 50°C, 10 min at 95°C, and then 45 temperature cycles (15 sec at 95°C and 1 min at 60°C).

Amplicon name	Gene localization	Primer type	Oligonucl. Sequence (5'-3')
PlGF κB1 site	-908 to -1015	reverse	ACGTGCCTCCAGAACCGTCC
		forward	CCAGGTGCCCCGAGGTGTTT
PlGF κB2 site	-1041 to -1120	reverse	CTCCTGTCAGGTCAGGCCAGC
		forward	CCTCTTCTGCTTGGGCTCGGG
PlGF κB1-2 sites	-996 to -1104	reverse	AAACACCTCGGGGCACCTGG
		forward	TCGGGAGGTGATGCCAGGTTAGT
PlGF gene	624 to 753	reverse	TTCCCCTTGGTTTTCCTCCTT
		forward	AGATCTTGAAGATTCCCCCCA

Table VI.4.: *PCR probe information.*

VI.15.3. Relative quantification of NF-κB binding

SYBR Green is a fluorescent molecule that binds double-stranded DNA. During the PCR reaction, fluorescence increases proportionally to the amount of amplified DNA product. For each qRT-PCR amplification, we obtained a sigmoid curve (figure VI.2) and the cycle threshold (ct) is determined. The ct corresponds to the cycle number from which the curve of amplified product apparition becomes exponential.

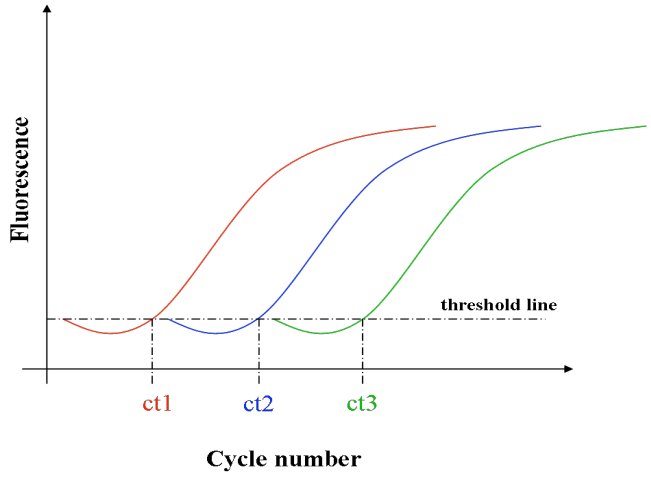


Figure VI.2.: Graph representing the qRT-PCR reaction.

The relative quantification of NF-κB binding on κB sites is based on the difference between the ct of DNA amplification coming from one day-injured sciatic nerves immunoprecipitated with p65 Ab and the DNA amplification from uninjured sciatic nerves.

The complete analysis to quantify NF-κB binding brings into play several normalization steps described below.

For each pairs of primers used (see table VI.4), we amplify immunoprecipitated DNA, from UI or injured (I) sciatic nerves, with p65 or Flag Ab, and the two DNA inputs coming from also uninjured and injured nerves. After the qRT-PCR, we obtain the ct in triplicate and

calculate the mean ct for each condition: ct1 p65 UI, ct1 p65 I, ct1 Flag UI, ct1 Flag I, ct1 input UI and ct1 input I. Then, the first normalization to apply concerns the amount of chromatin used for the IP. So, to remove the possible bias due to protein concentration measurement, we substract the ct of inputs of the others ct:

- Ct1 p65 or Flag UI – ct1 input UI = ct2 p65 or Flag UI
- Ct1 p65 or Flag I – ct1 input I = ct2 p65 or Flag I

The second normalization concerns the specificity of the IP. As negative control of IP, we used anti-Flag Ab that recognizes a synthetic peptide naturally absent in cells. To obtain the specific IP with p65 Ab, we calculate the difference between ct2 Flag and ct2 p65:

- Ct2 Flag UI – ct2 p65 UI = ct3 UI
- Ct2 Flag I – ct2 p65 I = ct3 I

Then, we can calculate the δ ct = ct3 I – ct3 UI. The fold induction value of NF-κB binding after nerve injury compared to the uninjured condition is then calculated with the following formula:

- Fold induction = $2^{\delta ct}$

Finally, the last normalization concerns the specific NF-κB binding to a κB site located in the gene promoter. We have used a pair of primers to amplify a DNA region comprised in the gene and not in the promoter of PlGF (see table VI.3, amplicon name: PlGF). The fold induction for the RT-PCR using these primers should be close to 1, corresponding to the control condition where there is no NF-κB binding to a promoter. The ratio (R) 1: fold induction value obtained with PlGF primers is used to normalize the real fold induction and the formula is the following:

- Real fold induction = $2^{\delta ct}$ x R

VI.16. Luciferase assay

The *Pgf* promoter region including the two κB sites was inserted into the pGL3-Promoter Vector (Promega). 293 cells (4×10^5 cells per well) were seeded in 6-well (35 mm) plates. After 12 h, cells were transfected as described previously (Leonardi *et al.*, 2000) with 0.5 μg of either the Pgf-κB-luciferase or the Ig-κB-luciferase and with expression plasmids as indicated. The total amount of transfected DNA was kept constant by adding empty

expression vector DNA as needed. Cell extracts were prepared 24 h after transfection, and reporter gene activity was determined by using Dual-Luciferase Reporter Assay System (Promega). The pRL Vector (40ng), which provides constitutive expression of Renilla luciferase, was used to normalize for transfection efficiencies.

VI.17. Myelin sheath morphology

VI.17.1. Tissue processing

Mice were perfused with Karnovski buffer containing 1% PFA and 1.25% glutaraldehyde in 0.1M Sörensen buffer (solution of Na_2HPO_4 and NaH_2PO_4 in proportion 4/1 diluted in distilled water, pH 7.4). Sciatic nerves were carefully dissected out and post-fixed with Karnovski buffer overnight at 4°C. After two washes in 0.1M Sörensen buffer, nerves were post-fixed with 1% osmium in 0.1M Sörensen buffer for 1h at 4°C.

- ***Toluidine blue staining and electronic microscopy***

Sciatic nerves were progressively dehydrated in successive ethanol baths and then soaked in epoxy-propane twice during 10 min. Tissues were embedded in epon resin by soaking them in a epoxypropane/epon mixture of successive 2/1, 1/1 and ½, proportions, 1h each. The resin was harden at 265°C for 2 days. The blocks were trimmed, and semi-thin (1 µm) and ultra-thin (75 nm) cross-sections were cut with a Leica Ultracut UCT microtome. Semi-thin sections were collected onto glass slides, and then dried on a hot plate at 60°C for 1h. To optimise dye impregnation, sections were treated with 1% potassium tetraborate before staining with 0.5% toluidine blue. Ultra-thin sections were collected on copper grids and contrasted with 5% uranyle-acetate and lead citrate.

- ***Teasing***

Karnovski-fixed sciatic nerves were treated with 30%, 60%, 90% and 100% glycerine, each for 24h at 37°C. The nerves were placed on a slide with a few drops of 100% glycerine under a dissecting microscope. Using ultra-fine forceps, the nerves were then separated from proximal to distal into smaller bundles of fibers until individual axons could be identified. Slides were then prepared for light microscopy.

VI.17.2. Morphometric analysis of myelin sheaths

❖ ***Number of intact myelin sheaths***

On semi-thin toluidine blue stained 1 µm sections, intact myelin sheaths (magnification 40 x) were manually counted within non-overlapping successive fields covering the entire nerve section, and results were expressed as the mean number (± SE) of intact myelin sheaths per 0.01 mm² of nerve tissue.

❖ ***Myelin sheath thickness***

The myelin sheath thickness in individual axons was determined on ultra-thin cross-sections at the electron microscopic level. Two sections were selected for each animal (N=3 per group) and three images per section were taken at a 1000x magnification using a Zeiss LEO 906 transmission electronic microscope equipped with a ProgRes MF camera (JENOPTIK, Germany) and the ProgRes CapturePro 2.6. software. The axonal area and the mean thickness of myelin sheath, measured at four random points of its circumference, were determined on five myelinated axons randomly selected per field using the Olympus AnalySIS computer program. Results were represented in graphs showing the myelin sheath thickness as a function of axonal area.

❖ ***Internodal lengths***

Images of each teased axon were taken at the 10x or 20x magnifications. The Olympus AnalySIS computer program was used to measure internodal length and results were expressed as mean length (± SE) of internodes.

VI.18. Statistical analyses

Means and SEs for each experimental time point were calculated**.** Statistical significance was set at $P < 0.05$.). To evaluate the group effect in motor recovery (section VI.4) and in the migration assay (section VI.9) during time, we used Zerbe's non-parametric model, which compares both the global evolution of two groups and the values at the different time point separately. For the other experiments, the statistical significance of differences between wt and *Pgf* $^{-/-}$ mice was tested separately at each time points using a Student t-test. All quantifications were performed blind to the genotype. Statistical analyses were supervised by the Department of Biomedical Statistics of the University of Liege (Pr. A Albert).

Section VII

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Section VIII

Appendices

GLIA

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Involvement of Placental Growth Factor in Wallerian Degeneration

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KEY WORDS
PNS injury; inflammation; Schwann cell; cytokine; NF-κB

ABSTRACT
Wallerian degeneration (WD) is an inflammatory process of nerve degeneration, which occurs more rapidly in the peripheral nervous system compared with the central nervous system, resulting, respectively in successful and aborted axon regeneration. In the peripheral nervous system, Schwann cells (SCs) and macrophages, under the control of a network of cytokines and chemokines, represent the main cell types involved in this process. Within this network, the role of placental growth factor (PlGF) remains totally unknown. However, properties like monocyte activation/attraction, ability to increase expression of pro-inflammatory molecules, as well as neuroprotective effects, make it a candidate likely implicated in this process. Also, nothing is described about the expression and localization of this molecule in the peripheral nervous system. To address these original questions, we decided to study PlGF expression under physiological and degenerative conditions and to explore its role in WD, using a model of sciatic nerve transection in wild-type and *Pgf*$^{-/-}$ mice. Our data show dynamic changes of PlGF expression, from periaxonal in normal nerve to SCs 24h postinjury, in parallel with a p65/NF-κB recruitment on *Pgf* promoter. After injury, SC proliferation is reduced by 30% in absence of PlGF. Macrophage invasion is significantly delayed in *Pgf*$^{-/-}$ mice compared with wild-type mice, which results in worse functional recovery. MCP-1 and proMMP-9 exhibit a 3-fold reduction of their relative expressions in *Pgf*$^{-/-}$ injured nerves, as demonstrated by cytokine array. In conclusion, this work originally describes PlGF as a novel member of the cytokine network of WD.

INTRODUCTION

Wallerian degeneration (WD) is the active process of degradation of the nerve segment distal to the lesion site. First described in 1850 (Waller, 1850), WD is critical for successful axonal regeneration and occurs more rapidly in the peripheral than in the central nervous system, where axonal regeneration fails (Lawson et al., 1994; Perry et al., 1987). The sequence of degenerative events comprises cellular and molecular changes, and requires effective Schwann cell (SC) and macrophage responses (Stoll et al., 1989). Indeed, following injury, SCs dedifferentiate, proliferate, and align in "bands of Büngner" providing structural guidance and growth-promoting substrates to regenerating axons (Vargas and Barres, 2007). SCs also participate in the clearance of myelin debris, which occurs during the first few days postinjury (Stoll et al., 1989) and release a wide variety of chemokines and cytokines, which in turn recruit circulating macrophages to the degenerating nerve (Shamash et al., 2002; Siebert et al., 2000; Tofaris et al., 2002). Macrophages mediate the second phase of myelin debris removal, a type-3 complement receptor-dependent mechanism (CR-3/Mac-1/CD11b) (Slobodov et al., 2001). All these events are orchestrated by a network of cytokines and chemokines, of which the induction is partly regulated by the activation of the transcription nuclear factor kappa B (NF-κB) signaling pathway (Fu et al., 2010; Subang and Richardson, 2001), as well as by neurotrophic factors, secreted both by SCs and macrophages: interleukin-1beta (IL1-β) (Perrin et al., 2005; Shamash et al., 2002), IL-6 (Bolin et al., 1995), tumor-necrosis factor alpha (TNF-α) (Shamash et al., 2002), monocyte chemotactic protein-1 (MCP-1) (Perrin et al., 2005), macrophage inflammatory protein-1 alpha (MIP-1α) (Perrin et al., 2005), nerve growth factor (NGF) (Funakoshi et al., 1993) and vascular endothelial growth factor (VEGF) (Scarlato et al., 2003). Placental growth factor (PlGF), belonging to the VEGF family, has not been implicated in WD up to now. Identified in 1991 in the placenta (Maglione et al., 1991), PlGF has also been detected in heart, lung, thyroid, skeletal muscle (Persico et al., 1999) and brain (Beck et al., 2002). There are 3 isoforms of PlGF in humans, but only one, PlGF-2, is present in mice (DiPalma et al., 1996). Mice

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in which the PlGF gene has been deleted (*Pgf* $^{-/-}$ mice) do not exhibit any defects in development or reproduction, but show impaired angiogenesis in pathological conditions (Autiero et al., 2003a,b; Carmeliet et al.,2001; Ribatti, 2008). PlGF is produced by activated endothelial cells (ECs), inflammatory cells, bone marrow cells, as well as neurons (Beck et al., 2002; Luttun et al., 2002). Interestingly, PlGF displays pro-inflammatory properties: it mobilizes macrophages, has a chemotactic activity on blood monocytes (Clauss et al., 1996) and increases mRNA expression of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-8, MCP-1, MIP-1α, and VEGF (Bottomley et al., 2000; Perelman et al., 2003; Selvaraj et al., 2003). Recently, PlGF was found to have neuroprotective and angiogenic effects in cerebral ischemia (Liu et al., 2006), but its precise role in the nervous system remains elusive.

Considering the above-described properties of PlGF, we decided to study its expression in peripheral nerves under physiological and degenerative conditions, to investigate the signaling pathway that regulates its expression and to explore its role in the inflammatory context of WD, using a model of sciatic nerve transection in adult wild-type (wt) and *Pgf* $^{-/-}$ mice.

MATERIALS AND METHODS

Animals

Adult (10–12 weeks), 50%Swiss-50%129SV female wt and *Pgf* $^{-/-}$ mice were used (Vesalius Research Center, Pr Carmeliet, KUL, Belgium). The experiments were performed in accordance with the rules and regulations of the Ethical Committee for animal research of the Belgian National Fund for Scientific Research.

Surgical Procedure: Sciatic Nerve Complete Transection

Mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine (75 mg/kg; Ketalar®, Bayer HealthCare, Brussel, Belgium) and xylazine (10 mg/kg; Rompun®, Pfizer, Brussel, Belgium). Under aseptic conditions, sciatic nerves were exposed and WD induced by a complete axotomy of the nerves at the upthigh level. Proximal and distal stumps were left in their original position to allow axonal regeneration. Muscles and skin were carefully closed in two layers.

Tissue Processing

Mice were killed after different survival times (1, 3, 7, 14, 21, and 28 days postinjury) by an overdose of Nembutal (150 mg/kg, i.p., CEVA Santé Animale, Brussel, Belgium) and the distal parts of the transected sciatic nerves were freshly harvested, embedded in Tissue-Tek® O.C.T.™ Compound (Labonord SAS, Templemars, France) and directly frozen. Ten micrometer-thick longitudinal or transverse sections were collected onto gelatin-coated slides, and stored at −20°C until used.

Dorsal root ganglions (DRG) from the lumbar segment were harvested from uninjured mice, directly frozen, before being cryosectioned for further immunofluorescent staining.

Primary Schwann Cell Culture

Adult SCs were isolated from sciatic and trigeminal nerves of wt and *Pgf* $^{-/-}$ mice as described (Bouquet et al., 2007). After careful dissection under sterile conditions, nerves were left for 2 weeks in degeneration medium: DMEM, 10% foetal bovine serum, Fungizone (2.5 mg/mL, GIBCO®, Invitrogen™, Carlsbad, CA), Forskolin (2 mM, Calbiochem®, Darmstadt, Germany), Gentamycine (50 mg/mL, GIBCO) and Heregulin-β1 (10 ng/mL, HRG; R&D Systems GmbH, Wiesbaden, Germany) at 37°C, 5% CO_2. After enzymatic and mechanical dissociation, cells were plated on precoated poly-L-lysine (50 μg/mL, Sigma-Aldrich, Saint Louis, MO) and laminin (10μg/mL, Sigma-Aldrich) T25 culture dishes and incubated at 37°C, 5% CO_2 in $N2^{HRG}$ medium: 50% DMEM 50% F12 supplemented with N2 (GIBCO®), gentamycin (50μg/mL), fungizone (2.5 μg/mL), forskolin (2μM), and HRG (10 ng/mL). At confluence, SCs were purified by magnetic cell-sorting using the anti-low affinity NGF receptor (p75NGFr; AB1554; 1/200; Millipore, Temecula, CA) antibody (Ab), according to the manufacturer's instructions (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany).

Immunofluorescent Stainings

To characterize PlGF expression, double immunofluorescenct stainings for **PlGF** (sc-27134; 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, Taylor and Goldenberg, 2007) and **NF-H** (MAB5448/clone TA51; 1:500; Millipore, De Girolamo et al., 2000) for axons, **Neu-N** (MAB377/clone A60; 1:250; Millipore, Borsani et al., 2010) for neuronal cell nuclei, **S100** (ZO311; 1/200; DakoCytomation, Gould et al., 1986) for quiescent SCs, **p75NGFr** (AB1554; 1/200; Millipore, Runyan and Phelps, 2009) for proliferating SCs, von Willebrand factor (**vWF**; AB6994; 1/2000; Abcam, Yin et al., 2010) for endothelial cells, patched-1 (**Ptc-1**; sc-9016/H-267; 1/50; Santa Cruz Biotechnology, Chen et al., 2007) for fibroblasts, **CD11b** (MCA74; 1/250; Serotec, Springer et al., 1979) for macrophages, **P0** (AB9352; 1/50; Millipore) for peripheral myelin, were performed. After drying, tissue sections were fixed in cold acetone for 10 min at 4°C. Nonspecific binding was prevented by 1h incubation in 10% normal serum solution in 0.1% triton-PBS (0.1 M, pH 7.4). Sections were then incubated overnight at room temperature (RT) with the specific primary Abs, rinsed 3 times with PBS and incubated with their respective secondary Abs coupled with rhodamine or FITC (1/500, Jackson ImmunoResearch Laboratories, West Grove, Pennsylva-

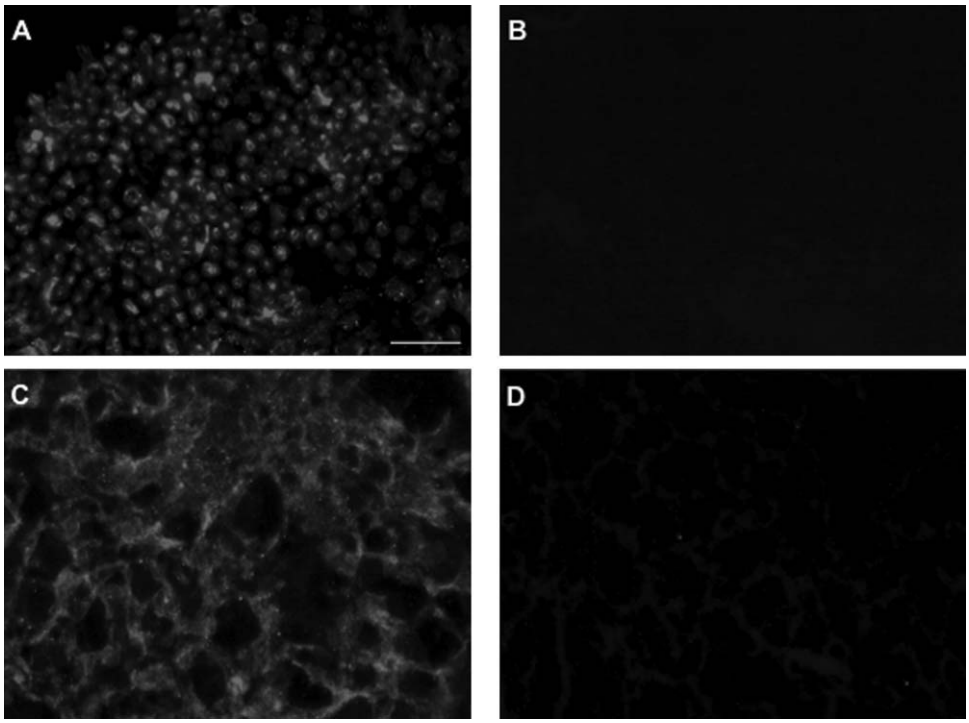

Fig. 1. Confirmation of PlGF Ab specificity. (**A**) PlGF immunostaining on transverse section of sciatic nerve. (**B**) Negative control: nerve section stained with anti-PlGF Ab preincubated with its specific blocking peptide. (**C**) PlGF immunostaining on placenta used as positive control tissue and (**D**) on liver used as negative control tissue. Scale bar: 60μm.

nia), for 1h at RT. Sections were then rinsed twice in PBS, twice in distilled water, and mounted under coverslips using vectashield solution (Vector Laboratories, Burlingame, CA). To assess the specificity of the anti-PlGF Ab, negative controls were obtained by incubation of the primary Ab with its specific blocking peptide (sc-27134 P; Santa Cruz Biotechnology) before applying it on the tissue sections. Also, specific positive (placenta) and negative (liver) tissues for PlGF were used to confirm this specificity (Fig. 1).

Prediction of NF-κB Binding Sites and Luc Assay

To identify NF-κB binding sites, computational analysis of the promoter sequence of mouse *Pgf* gene (Green et al., 2001) was performed using four different programs designed to look for transcription factor binding sites (MatInspector program, Match™ program, Promo, TFSEARCH).

For luciferase assays, the region including the two κB sites were inserted into the pGL3-Promoter Vector (Promega). 293 cells (4×10^5 cells per well) were seeded in 6-well (35 mm) plates. After 12 h, cells were transfected as described above with 0.5 μg of either the Pgf-κB-luciferase or the Ig-κB-luciferase (Leonardi et al., 2000) and with expression plasmids as indicated. The total amount of transfected DNA was kept constant by adding empty expression vector DNA as needed. Cell extracts were prepared 24 h after transfection, and reporter gene activity was determined by using Dual-Luciferase Reporter Assay System (Promega). The pRL Vector (40 ng), which provides constitutive expression of *Renilla* luciferase, was used to normalize for transfection efficiencies.

Chromatin Immunoprecipitation (ChIP) Assay

Sciatic nerves (from uninjured [UI] and 24h postinjured wt mice) were surgically removed from four adult mice per condition, minced and immediately fixed in PBS containing 1% paraformaldehyde (PFA) for 25 min at RT. The nerves were washed twice with cold PBS containing protease inhibitors (Complete™, Roche Applied Science, Mannheim, Germany) and then homogenized into 1.5 mL of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, protease inhibitors). The lysates were sonicated for 15 min with alternating 30-s pulses at high power with a Bioruptor sonicator (Diagenode, Liege, Belgium), and then centrifuged at 13,000 rpm for 5 min. The protein concentration of lysates was measured using the BCA protein assay kit (ThermoScientific, Rockford, Illinois). Inputs were kept for later analysis. Lysates were diluted 10 times into dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-

TABLE 1. Quantitative PCR probe informations

Amplicon name	Gene localization	Primer type	Oligonucl. Sequence (5′-3′)
PlGF kB1 site	−908 to −1015	Reverse	ACGTGCCTCCAGAACCGTCC
		Forward	CCAGGTGCCCCGAGGTGTTT
PlGF kB2 site	−1041 to −1120	Reverse	CTCCTGTCAGGTCAGGCCAGC
		Forward	CCTCTTCTGCTTGGGCTCGGG
PlGF gene	624 to 753	Reverse	TTCCCCTTGGTTTTCCTCCTT
		Forward	AGATCTTGAAGATTCCCCCCA

HCl pH 8.0, protease inhibitors). The sheared chromatin was incubated with 4μg of anti-NF-κB subunit p65 (p65; sc-109, Santa Cruz Biotechnology) or anti-Flag (used as negative control; F3165, Sigma-Aldrich) at 4°C on a rotating platform overnight. The immune complexes were incubated for 2 hours at 4°C with Protein A-agarose beads (Santa Cruz Biotechnology) blocked with BSA (100 μg/mL) and shared salmon sperm DNA (500 μg/mL; Invitrogen™). The immunoprecipitates were then washed three times with 1 mL low salt washing buffer (1% triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, protease inhibitors), once with 1 mL high salt washing buffer (same buffer but containing 500 mM NaCl), once with 1 mL LiCl buffer (0.25 M LiCl, 1% IGEPAL CA630 (Sigma-Aldrich), 1 mM EDTA, 1% deoxycholic acid, 10 mM Tris-HCl pH 8.0), and finally once 1 mL with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Immunoprecipitated (IP) chromatin was eluted in 250 μL elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at RT, twice. Chromatin inputs were also diluted in elution buffer. Protein-DNA cross-links were reversed by incubation with 20 μL of 5 M NaCl at 65°C for 4h and proteins were digested by addition of 10 μL of 0.5 M EDTA, 20 μL of 1M Tris-HCl pH 6.5 and 20μg of proteinase K (Promega, Madison, Wisconsin) and incubation 1h at 45°C. DNA was purified with phenol/chloroform (Sigma-Aldrich) using Phase Lock Gel™ tube (Eppendorf, Hamburg, Germany). The aqueous phase was incubated 1h at -20°C after the addition of 45 μL of 3 M NaAC pH 5.5, 30 μg of glycogen (Roche Applied Science) and 1 mL of absolute ethanol and then centrifuged 5 min at 13,000 rpm. The DNA pellets were washed with 70% ethanol, resuspended in 100 μL of DNase-free distilled water. Quantitative PCR (using SYBR green PCR master mix; Applied Biosystems) was performed to analyse the IP DNA. Signals were normalized to respective inputs and compared with negative control (anti-Flag IP). The primers (Eurogentec, Seraing, Belgium) used to amplify specific κB sites of *Pgf* gene are resumed in Table 1.

Axonal Regeneration Study

To evaluate axonal regeneration, longitudinal sections from the distal part of injured sciatic nerves were fluorescently stained with anti-NF-H Ab as described above. For the quantification, nonoverlapping successive fields covering the entire tissue sections (which means the whole length of the distal part) were taken using Olympus DP50 digital camera connected to an Olympus AX-70 microscope (magnification 10x). The mean number of fields/section was similar between wt and *Pgf* $^{-/-}$ mice (7.47 ± 0.76 and 7.54 ± 0.64, respectively), resulting in comparable total tissue length. Each field was then allocated to one of the following group: (i) field containing only debris of degenerating axons; (ii) field containing a majority of debris and few regenerating profiles; (iii) field containing scarce debris and a majority of regenerating fibers; and (iv) field containing only regenerating fibers. Data were expressed as a mean proportion of field numbers in each of those 4 groups per total nerve section (%)± standard error.

Motor Recovery Evaluation

Before lesion and at 3, 7, 10, 14, 17 and 21 days following unilateral left sciatic nerve axotomy, mice were allowed to walk down a 60cm long corridor lined with graph paper after inking their hind paws. For each animal (5 wt and 5 *Pgf* $^{-/-}$ mice), at every time point, at least four clear footprints were obtained for each foot. Measurements of print length were then made on the operated side (OPL) and the normal side (NPL). A mean of four values was then calculated for the OPL (xOPL) and NPL (xNPL), and a print-length factor (PLF) was calculated as follows: PLF = (xOPL – xNPL)/xNPL (George et al., 2003).

Quantification of Schwann Cell Proliferation

In vivo

Longitudinal sections of sciatic nerves were stained with anti-Ki67 Ab according to the above-described protocol (NCL-Ki67p; 1/250; Novocastra™, Leica Microsystems GmbH). Ki67 positive SC nuclei (magnification 20x) were manually counted within nonoverlapping successive fields covering the entire nerve section, and results were expressed as a mean number ± standard error of Ki67 positive SC nuclei per mm^2 of nerve tissue.

In vitro

Cultured SCs were plated on coated glass coverslips at a concentration of 25,000 cells/300 μL. 20μM bromodeoxyuridine (BrdU; Sigma-Aldrich) was directly added to the cultures for 16h before fixation in 4% PFA for 10min. Cells were treated in HCl 2N for 10min at 37°C and then washed in borate buffer (0.1M; pH 8.5). Cells were permeabilised and nonspecific binding was prevented by 1h incubation in 10% normal serum 0.1% triton-PBS solution. Then cells were incubated overnight with anti-BrdU Ab (OBT0030; 1/250; Serotec) and anti-p75NGFr Ab to assess the purity of SC culture. After 3 PBS washes, cells were incubated with their respective

TABLE 2. RT-PCR probe informations

Gene	Primer type	Oligonucleotide sequence (5′-3′)	PCR product size (bp)	Cycle nb
mPlGF	Reverse	TTCCCCTTGGTTTTCCTCCTT	130	45
	Forward	AGATCTTGAAGATTCCCCCCA		
GAPDH	Reverse	GCCTTCTCCATGGTGGTGAAGAC	210	25
	Forward	GACCCCTTCATTGACCTCAACTACATG		

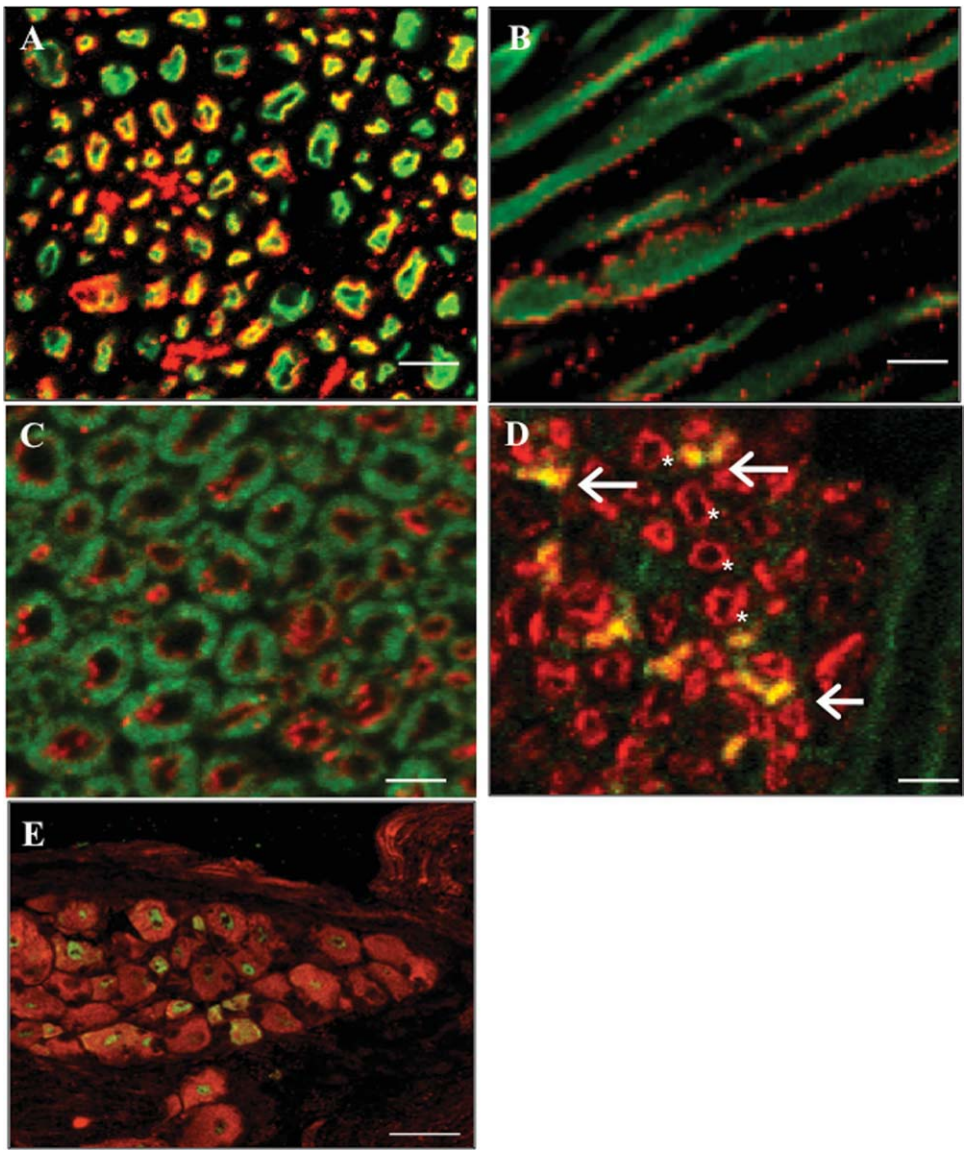

Fig. 2. Double immunofluorescent stainings on uninjured sciatic nerve (**A–D**) and dorsal root ganglion (**E**) sections. PlGF (Rhodamine) and NF (A, B), P0 (C), Ptc-1 (D), NeuN (E) (FITC) double immunofluorescent stainings, on transverse (A, C, D) and longitudinal (B) sections of uninjured sciatic nerve. PlGF is expressed in axons and has a peri-axonal expression pattern (A, B). However, PlGF is not present in the myelin sheath, as no co-localization could be seen with P0 (C). Finally, besides its periaxonal localization, PlGF is also expressed by fibroblasts in the endoneurium (D, arrows show PlGF/Ptc-1 labelled fibroblasts, and * design axons). Scale bar: A, C, D: 20μm; B: 10μm, E: 50 μm.

secondary Abs coupled with rhodamine or FITC, for 1h at RT. Coverslips were then rinsed in PBS and distilled water, and mounted on slides using vectashield solution containing DAPI to visualize all nuclei for the determination of the total cell number. Double-stained p75NGFr/BrdU cells and total number of p75NGFr positive cells (magnification 20x) were manually counted within nonoverlapping successive fields, and results were expressed as a mean % ± standard error of proliferating SCs.

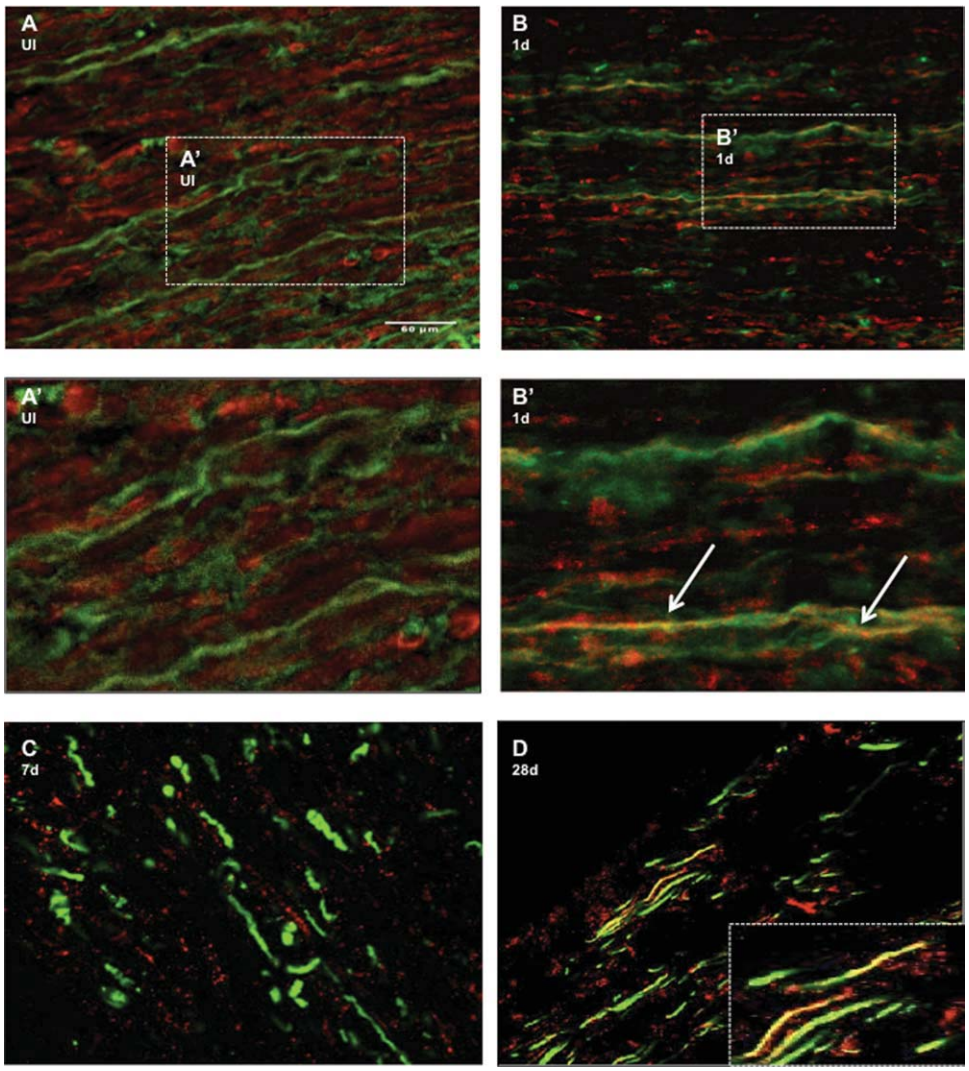

Fig. 3. Double immunofluorescent stainings on normal and injured sciatic nerve sections. PlGF (Rhodamine) and S100 (**A, A**′), p75NGFr (**B, B**′) or NF (**C, D**) (FITC) double immunostainings, on longitudinal sections of normal (A, A′) and injured (distal segment, B, B′, C, and D) sciatic nerves. PlGF is not expressed by myelinating SCs, as no co-localization is observed with S100 (A, A′). SCs, which dedifferentiate and proliferate after loss of axonal contact, express PlGF from the first day after injury (B, B′). Axonal PlGF expression decreases after injury, following the time course of axonal degradation (C). 28 days postinjury, PlGF reappears in the regenerating fibres (D). Scale bar: 60μm.

RT-PCR

RNA from cultured SCs was extracted with TRIzol® (Invitrogen™) and isolated according to the manufacturer's protocol. PlGF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, as internal control), mRNAs were amplified with aliquot of 20 ng of total RNA using a RT-PCR kit (GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit; Applied Biosystems, Foster City, CA) and appropriate "forward" and "reverse" primers (oligonucleotide sequences shown in Table 2; Eurogentec, Seraing, Belgium). Reverse transcription was performed at 70°C for 15 minutes followed by 2 minutes of incubation at 95°C for denaturation of RNA-DNA heteroduplexes. Amplification consisted in cycles of 15 seconds at 94°C, 20 seconds at 58°C, and 20 seconds at 72°C. RT-PCR products were resolved on 10% polyacrylamide gel, stained with GelStar (Lonza, Rockland, Maine).

DAB Immunostainings

Tissues were fixed with 4% PFA for 5 min, then incubated in a 0.3% H_2O_2, 0.1% Na azide solution in PBS for 20 min at RT to reduce endogenous peroxydase activity. Nonspecific binding was prevented by 1h incubation in 3% normal serum and 1% bovine serum albumin solutions in 0.1% triton-PBS. Sections were then incubated overnight at RT with the specific Abs: anti-p75NGFr (AB1554; 1/200; Millipore) to study SC dedifferentiation or anti-CD11b (MCA74; 1/250; Serotec) to study macrophage recruitment. After 3 PBS rinses, they were incu-

bated with their respective secondary biotinylated antibodies (Vector Laboratories) diluted and centrifuged in a 3% mouse normal serum and 1% BSA in 0.1% triton-PBS solution, for 1h at RT. Then sections were incubated 1h with the avidin-biotin-peroxydase complex (Vector Laboratories), diluted 1/1000 in PBS and the immunostaining revealed with 3,3'-diaminobenzidine.

Image Analysis

To quantify SC dedifferentiation, pictures (magnification 20x) were converted to gray scale. A threshold intensity of gray-colored staining was set and applied to each slice. Then, immunostaining was quantified within nonoverlapping successive fields covering the total nerve tissue area using the Olympus AnalySIS computer program. Data were expressed as the mean integral intensity ± standard error of total stained area ($pixel^2$) per total nerve tissue area ($pixel^2$). For the macrophage recruitment quantification, CD11b-positive macrophages (magnification 10x) were manually counted within nonoverlapping successive fields covering the entire nerve section, and results were expressed as a mean number ± standard error of CD11b-positive macrophages per 0.1 mm^2 of nerve tissue.

Toluidine Blue Staining

Mice were perfused with Karnovski solution containing 1% PFA and 1.25% glutaraldehyde in 0.2M Sorensen buffer (~ 750 mOsm). Sciatic nerves were carefully harvested and postfixed with Karnovski overnight at 4°C. After two washes in 0.1M Sorensen buffer, nerves were postfixed with 1% osmium in Sorensen buffer for 1h at 4°C. Sciatic nerves were progressively dehydrated in successive ethanol baths and then soaked in epoxypropane twice during 10 min each. Tissues were embedded in epon resin by soaking in a mixture of epoxypropane/epon in proportions 2/1, then 1/1 and finally 1/2, 1h each. The resin was harded at 265°C for 2 days. The blocks were trimmed and semi-thin transverse sections (1 μm) were cut with a Leica Ultracut UCT microtome, picked up onto glass slides, and then dried on a hot plate at 60°C for 1h. To have a good dye impregnation in the tissue, sections were treated with 1% potassium tetraborate before staining with 0.5% toluidine blue. Intact myelin sheaths (magnification 40x) were manually counted within nonoverlapping successive fields covering the entire nerve section, and results were expressed as a mean number ± standard error of intact myelin sheaths per 0.01 mm^2 of nerve tissue.

Cytokine Array

Expressions of IL-1α, IL-1β, IL-6, TNF-α, IL-10, VEGF, VEGF receptor-1 (VEGFr1), MCP-1, MIP-1α and the pro-matrix metalloprotease-9 (proMMP-9) were measured in tissue extracts of UI and injured sciatic nerves from wt and *Pgf* $^{-/-}$ mice using RayBio® Cytokine Antibody Arrays (RayBiotech, Norcross, Georgia). 200μg of total protein were used per sample. The cytokine array membranes were scanned with a Las-4000 luminescent image analyser (Fujifilm, Tokyo, Japan). The intensity of the signal was measured with the Quantity One 1-D Analysis software (Bio-RAD Laboratories, Hercules, CA). The results were expressed as the mean relative signal intensity, i.e. the ratio of sample signal intensity/positive control signal intensity. Each signal intensity of positive controls and samples was normalized by the background substraction.

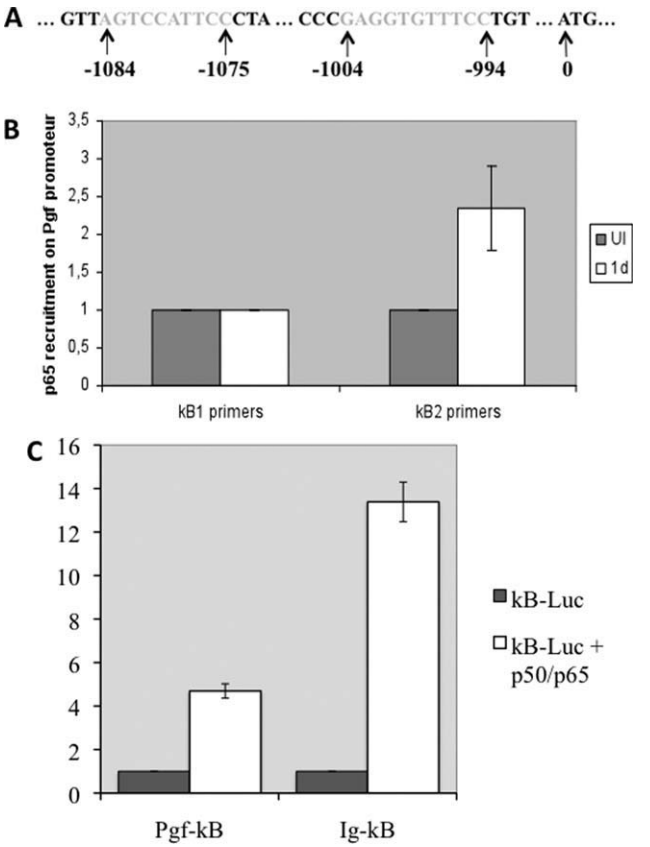

Fig. 4. NF-kB binding on Pgf gene promoter after sciatic nerve injury. (**A**) The prediction of NF-κB binding sites has revealed two putative κB motifs (grey letters) located at positions 994-1004 (κB1) and 1075-1084 (κB2) bps upstream the ATG translation initiation site of the mouse *Pgf* gene. (**B**) p65 is specifically recruited to the *Pgf* gene promoter after injury, as examined by chromatin immunoprecipitation assays using normal uninjured sciatic nerves (UI) or 1 day-injured sciatic nerves (1d). After normalization to inputs and to negative flag IP, signal in UI condition was set to 1 and the one obtained in injured condition was expressed relative to it. Results are from three independent experiments and error bars denote standard deviation. We observed no fold induction for the kB1 primers. (**C**) The κB sites found in the *Pgf* promoter are functional in activating NF-κB, as examined by Luciferase assay. Pgf-kB or Ig-kB (positive control) vectors were transfected in 293 cells, with or without the p50/p65 expression vectors, as indicated. Signals were normalized by measuring *Renilla* activity of a co-transfected pRL vector. For each vector, the value obtained in the absence of p50/p65 was set to 1 and the other was expressed relative to it. Results are from three independent experiments and error bars denote standard deviation.

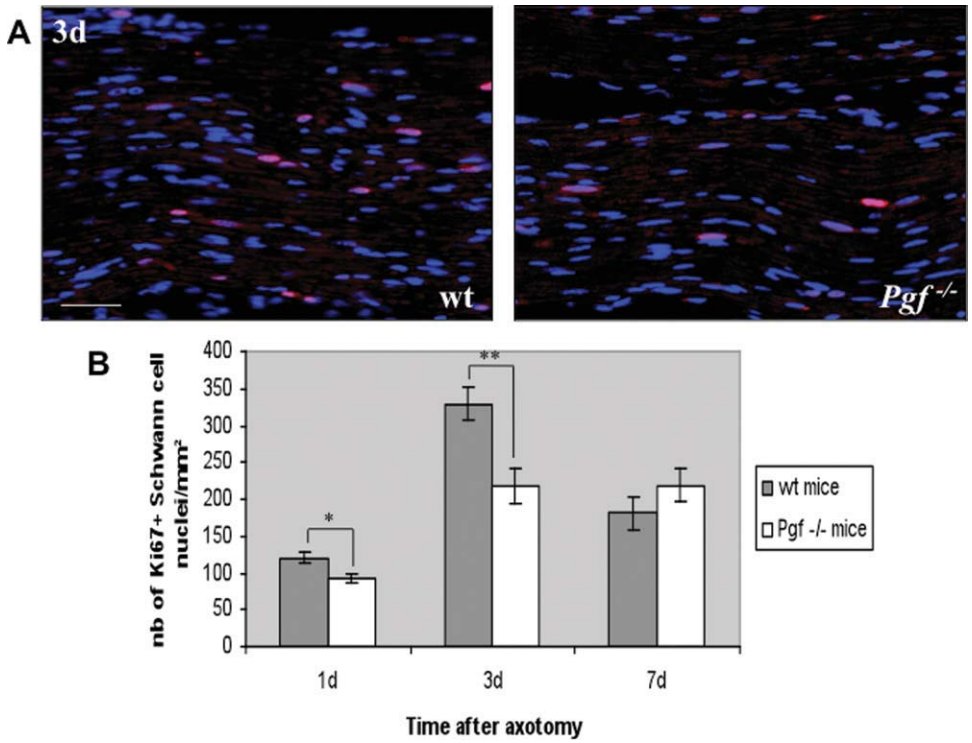

Fig. 5. *In vivo* cell proliferation. (**A**) Ki67 immunoreactivity in longitudinal sections of wt and $Pgf^{-/-}$ sciatic nerves 3 days after axotomy (scale bar: 50 μm). (**B**) Cell quantification shows a significant difference between wt and $Pgf^{-/-}$ mice in the number of Ki67 positive cells 1 and 3 days postinjury. $^{**}P < 0.01$; $^{*}P < 0.05$ (mean ± SE; $N = 5$ mice per group).

Statistical Analysis

Means and standard errors for each experimental time point were calculated. Statistical significance was set at $P < 0.05$. The statistical significances between wt and $Pgf^{-/-}$ mice were tested separately at each time points using a Student t-test (for CD11b, NGFr immunostaining and Toluidine blue quantifications).

To evaluate the group effect in motor recovery during time (foot-print data), we used Zerbe's nonparametric model, which compares both the global evolution of two groups and the values at the different time point separately. All quantifications were performed blind to the genotype. Statistical analyses were supervised by the Department of Biomedical Statistics of the University of Liege.

RESULTS

PlGF Expression in Normal and Injured Adult Mouse Sciatic Nerve

To investigate PlGF expression in the normal nerve and after axotomy, we performed various double immunofluorescent stainings to screen all cell types and/or structures present in the nerve: axons, myelin, myelinating and proliferating SCs, fibroblasts, ECs, and invading macrophages.

Based on PlGF/NF double staining, PlGF is found within the axons of the normal sciatic nerve and more precisely at the periphery of axons (Fig. 2A,B). This expression is found in close contact with, but not within the myelin sheath, as no co-localization can be seen with the P0/PlGF double staining (Fig. 2C). Fibroblasts within the endoneurium, labeled with the Ptc-1 Ab, express PlGF (Fig. 2D). to further assess neuronal expression of PlGF, we also performed PlGF/NeuN double immunofluorescent staining on lumbar dorsal root ganglion sections, where neuronal cell bodies are located. This staining clearly shows that PlGF is expressed within the neuronal cell body (Fig. 2E). Finally, vWF Ab, used to identify endothelial cells, never co-localized with PlGF (data not shown). PlGF/S100 staining confirms that myelinating Schwann cells (SCs) do not express PlGF (Fig. 3A,A′). After nerve transection, the detection of proliferating SCs by immunostaining with p75NGFr interestingly shows that PlGF is seen in these cells as soon as 1 day postaxotomy (Fig. 3B,B′) and persists during the first week after injury (data not shown). Concomitantly, PlGF is no more detectable in the degenerating fibres (Fig. 3C). When axonal regeneration occurs, starting as soon as 14 days postinjury, PlGF-positive fibres reappear in the distal segment of the sciatic nerve (Fig. 3D). Finally, after injury, no PlGF was observed in invading macrophages nor in ECs (data not shown).

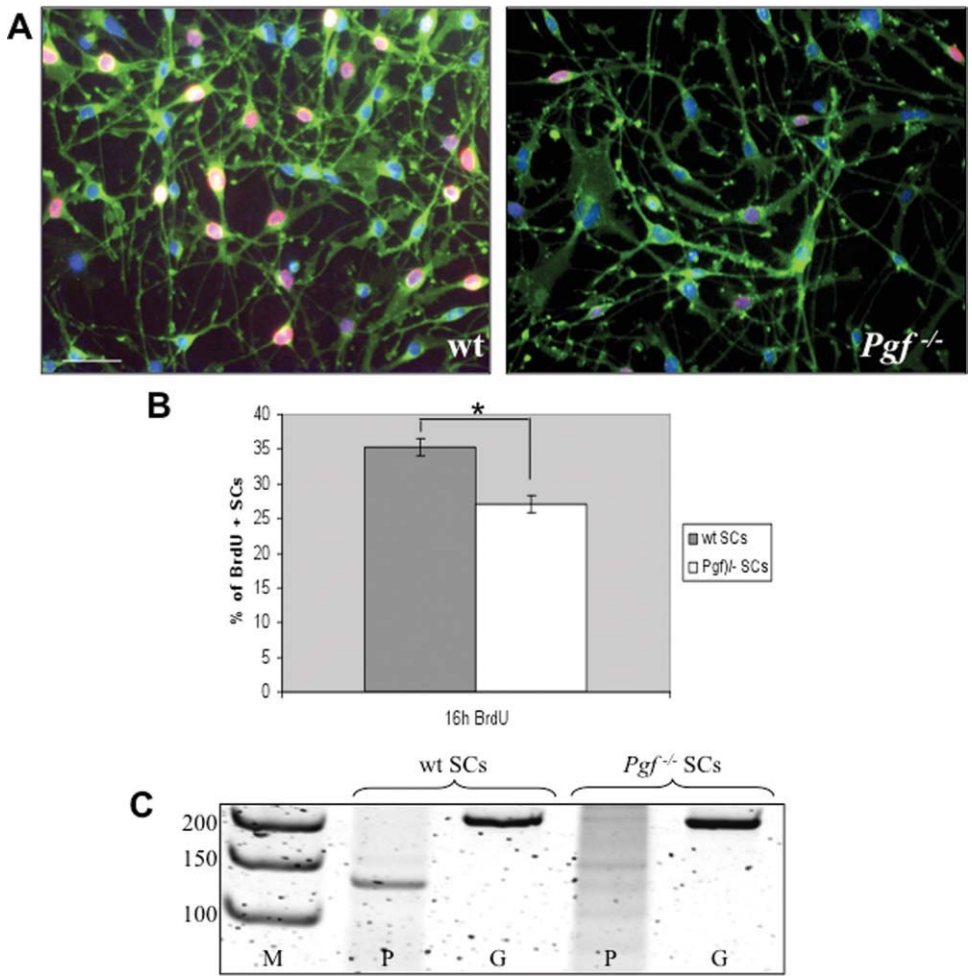

Fig. 6. *In vitro* Schwann cell proliferation. (**A**) BrdU (rhodamine)/p75NGFr (FITC) immunostaining of wt and $Pgf^{-/-}$ SCs after BrdU incorporation (scale bar: 50μm). (**B**) Quantification shows a significant decrease of $Pgf^{-/-}$ SC proliferation. $^{*}P < 0.05$ (mean ± SE; $N = 3$ culture experiments). (**C**) Polyacrylamide gel showing PlGF (P) and GAPDH (G) RT-PCR amplification products from wt and $Pgf^{-/-}$ cultured SCs. RT-PCR confirmed PlGF expression in wt SCs in culture conditions and, as expected, no expression in $Pgf^{-/-}$ SCs.

PlGF Expression Induction: Role of NF-κB

During WD, one of the main transcription factor that regulates the cyto- and chemo-kine expression by SCs is NF-κB (Fu et al., 2010; Subang and Richardson, 2001). As we demonstrated that PlGF is expressed by SCs in a postinjury inflammatory context, we investigated the potential implication of NF-κB in the induction of PlGF expression during WD by ChIP assay. Firstly, we analysed the *Pgf* promoter sequence, using transcription factor binding site prediction programs, to identify putative κB motifs on the mouse *Pgf* promoter. We detected the presence of two putative NF-κB binding sites within the mouse *Pgf* promoter region (Fig. 4A). To investigate the NF-κB binding to these two κB motifs, we immunoprecipitated the chromatin, from UI and one day-injured sciatic nerves, using an anti-p65 Ab. DNA fragments of IP chromatin were amplified by quantitative PCR, using primers specifically designed to amplify predicted κB1 or κB2 site (Table 1). The Fig. 4B shows a threefold increase in NF-κB binding to the κB2 site after injury compared with UI nerve controls, whereas no significant recruitment of NF-κB was observed to the κB1 site. To further prove that the sites were functional in activating gene expression, they were inserted into a luciferase reporter vector, transfected into 293 cells and NF-κB activation was assessed upon co-transfection with two NF-κB subunits, p50 and p65. As shown in the Fig. 4C, the κB sites found in the *Pgf* promoter (Pgf-κB) were indeed functional as the p50/p65 heterodimer induced luciferase gene expression (about 4-5 fold).

PlGF in WD

Role of PlGF on Schwann cell proliferation and formation of Büngners' bands

The WD process in the distal stump of the injured nerve is associated with activation and proliferation of SCs, which form the bands of Büngner and express

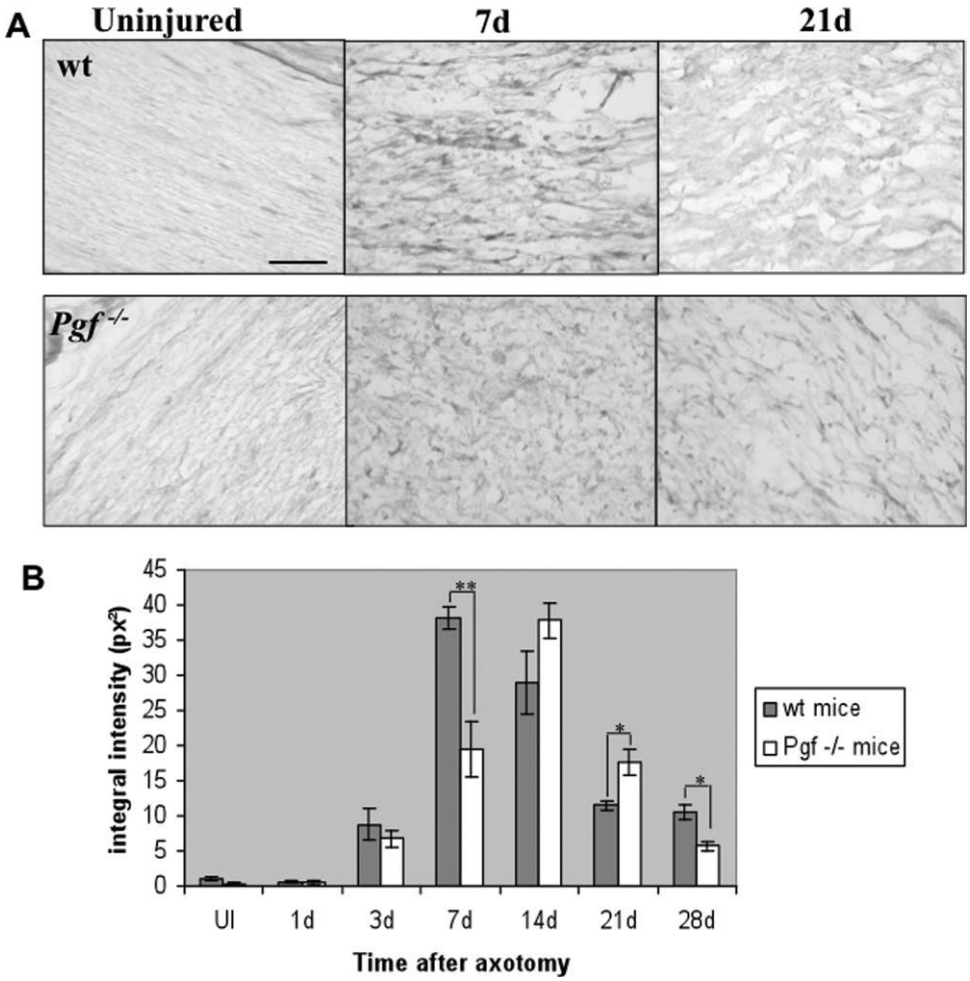

Fig. 7. P75NGFr immunostaining and quantification. (**A**) P75NGFr immunoreactivity in longitudinal sections of wt and *Pgf* $^{-/-}$ sciatic nerves before (UI) and after axotomy (scale bar: 100μm). (**B**) Image analysis and staining quantification show a significant difference between wt and *Pgf* $^{-/-}$ mice in the intensity of P75NGFr staining 7, 21 and 28 days postinjury. $^{**}P < 0.01$; $^{*}P < 0.05$ (mean ± SE; $N = 5$–9 mice per group).

p75NGFr. To assess the role of PlGF in these processes, we compared the proliferation of SCs within the sciatic nerves of wt and *Pgf* $^{-/-}$ mice after axotomy. Using immunofluorescent staining against Ki67, a nuclear protein expressed in cells undergoing proliferation, we quantified the number of proliferating cells at 1, 3 and 7 days after axotomy in the distal stump of nerves from wt and *Pgf* $^{-/-}$ mice (Fig. 5A,B). We found a significant decrease of cell proliferation in *Pgf* $^{-/-}$ mice at 1 and 3 days postinjury (Fig. 5B). Because of a conflict between Abs, we were not able to perform the double immunofluorescent staining Ki67/p75NGFr to identify the proliferating cells as SCs. We can nevertheless attest that the Ki67-positive cells are not inflammatory cells, such as invading macrophages, as they do not express CD11b (data not shown).

To confirm the *in vivo* finding of decreased SC proliferation in mice lacking PlGF, we compared the proliferation of primary cultures of SCs isolated from wt or *Pgf* $^{-/-}$ mice. Using BrdU incorporation and double immunofluorescent staining against BrdU and p75NGFr (Fig. 6A), we counted the mean number of proliferating SCs. The number of BrdU positive *Pgf* $^{-/-}$ SC nuclei was significantly decreased (Fig. 6B). To attest that SCs express PlGF in culture condition, which corresponds to the proliferating state they adopt after a nerve injury, we performed a RT-PCR for PlGF on RNA extracts from wt and *Pgf* $^{-/-}$ SC cultures, using *GAPDH* as housekeeping gene. The polyacrylamide gel showing RT-PCR amplification products (Fig. 6C) confirmed a PlGF expression in proliferating wt SCs. We then assessed p75NGFr expression in wt and *Pgf* $^{-/-}$ mice after several post-transection survival times. As illustrated in Fig. 7, p75NGFr is weakly expressed in UI nerves (Fig. 7A). In wt animals, its expression increases 3 days after axotomy and reaches a peak at day 7 (Fig. 7B). At that time, the longitudinal aspect of the p75NGFr staining is likely due to the alignment of SCs to form the bands of Büngner, still observed 14 days after the lesion. In *Pgf* $^{-/-}$ mice, the peak of expression of p75NGFr is only

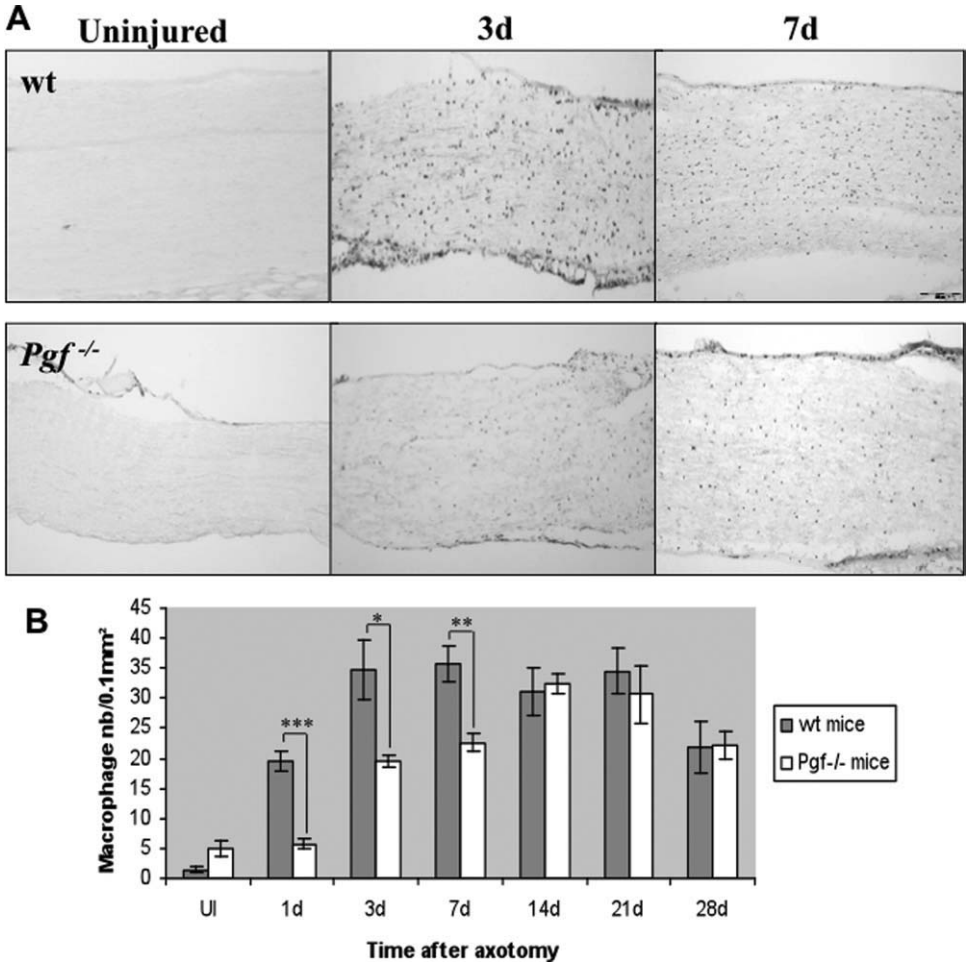

Fig. 8. CD11b immunostaining and quantification. (**A**) CD11b immunostaining of longitudinal sections of wt and $Pgf^{-/-}$ sciatic nerves before (UI) and after axotomy (scale bar: 200μm). (**B**) Cell quantification shows a significant difference between wt and $Pgf^{-/-}$ mice in the number of invading macrophages 1, 3, and 7 days postinjury. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ (mean ± SE; N = 5–12 mice per group).

reached 14 days after the lesion, and decreases more rapidly afterwards. Also, at day 7, the longitudinal aspect of the staining is much less pronounced than in tissue sections of wt animals (Fig. 7A).

Role of PlGF on macrophage recruitment and myelin sheath degradation

The other main cell type involved in the WD process are macrophages, which accumulate within the degenerating nerve segment and facilitate the removal of axonal and myelin debris. As PlGF plays a role in the chemo-attraction of macrophages (Clauss et al., 1996), we first quantified the number of invading macrophages within the degenerating segment at several postinjury times, comparing wt and $Pgf^{-/-}$ mice. CD11b immunostaining of longitudinal nerve sections revealed a clear delay in the macrophage infiltration in $Pgf^{-/-}$ nerves respective to wt nerves (Fig. 8A). While infiltration peaked between the 3rd and the 7th day postinjury in wt mice, it did so only between 14 and 21 days in $Pgf^{-/-}$ tissue (Fig. 8B). To verify if this delay affects the degradation of myelin sheaths, we stained the latter with toluidine blue and counted them on semi-thin transverse wt and $Pgf^{-/-}$ nerve sections at various survival times (Fig. 9A). These counts clearly confirmed that the clearance of myelin sheaths is slower in $Pgf^{-/-}$ mice (Fig. 9B).

Role of PlGF on cytokine and chemokine expression

WD is orchestrated by a large cyto-/chemo- kine network, which regulates SC and macrophage functions. As PlGF absence affects more particularly early events of WD, we compared the expression of 10 cyto/chemokines in extracts of UI, and 1, 3 and 7 days- injured sciatic nerves from wt and $Pgf^{-/-}$ mice. The following molecules were studied: IL-1α, IL-1β, IL-6, IL-10, TNF-α, VEGF, VEGFr1, MCP-1, MIP-1α and proMMP-9. The

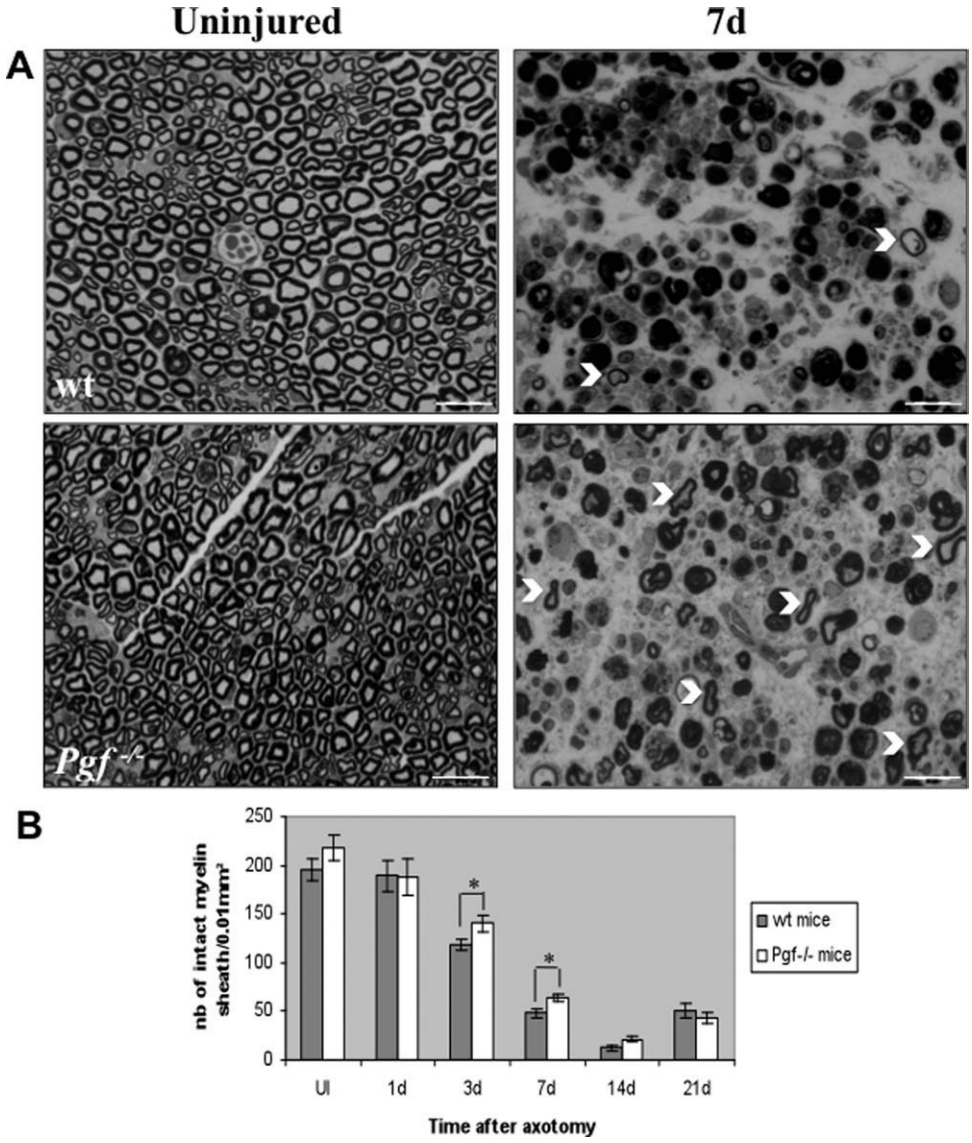

Fig. 9. Myelin sheath quantification. (**A**) Toluidine blue staining on semithin transverse sections from wt and *Pgf* $^{-/-}$ sciatic nerves before (UI) and 7 days after axotomy (scale bar: 20 μm). Quantification shows a significant difference between wt and *Pgf* $^{-/-}$ mice in the number of intact myelin sheaths (white arrowheads) 3 and 7 days postinjury. *$P < 0.05$ (mean ± SE; $N = 5$–9 mice per group).

quantitative analysis of the signal intensity revealed significant decreases in *Pgf* $^{-/-}$ extracts of MCP-1 at 1 day, and proMMP-9 at 7 days postinjury ($P < 0{,}05$) (Fig. 10).

Role of PlGF in axonal regeneration and functional recovery

As SC and macrophage functions appear to be delayed during WD in *Pgf* $^{-/-}$ animals, axonal regeneration might also be altered. To test this hypothesis, we compared the NF-H immunostainings of transected sciatic nerves in wt and *Pgf* $^{-/-}$ mice. At 7 days postinjury, all axons are degraded in both wt and *Pgf* $^{-/-}$ nerve distal parts, as illustrated by the high number of axonal debris (Fig. 11A). At 14 days, regeneration starts in wt nerves, as more longitudinal profiles can be observed in wt than in *Pgf* $^{-/-}$ nerves. This observation was confirmed by counting the proportion of fields containing either axonal debris (dotted aspect of NF staining), axonal profiles (longitudinal profiles on NF staining), or both (Fig. 11B). Almost 60% of the total length of the nerve corresponds to fields containing only axonal debris in *Pgf* $^{-/-}$ mice, while debris-containing fields occupy less than 10% in wt mice. As a corollary, fields with higher proportions of regenerating axons occupy 50% of the nerve length in wt mice compared with less than 5% in *Pgf* $^{-/-}$ mice. No field containing only axonal profiles was observed 14

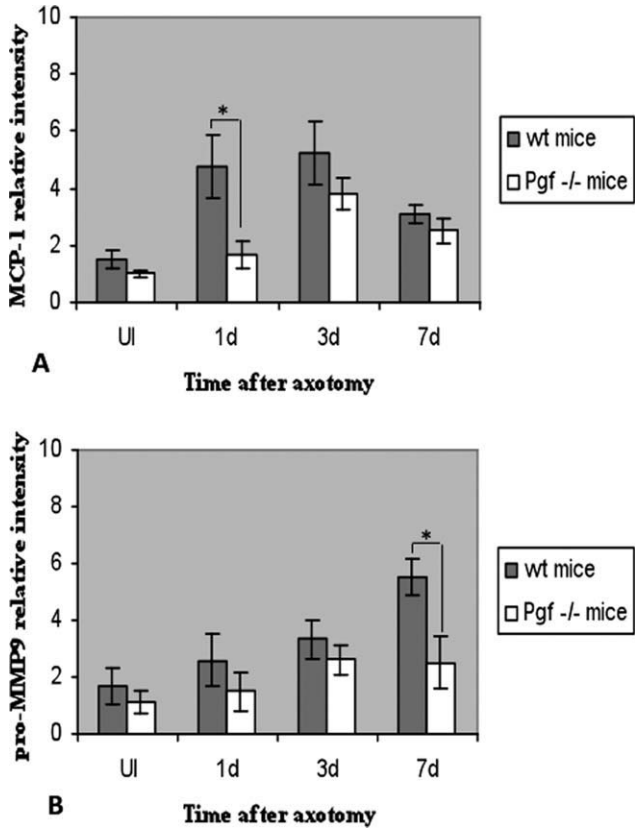

Fig. 10. Cytokine arrays on sciatic nerve extracts from wt and Pgf $^{-/-}$ mice. (**A**) MCP-1 doesn't increase its expression in *Pgf* $^{-/-}$ nerve as much as in wt nerves 1 day after injury. (**B**) Pro-MMP-9 peaks at 7 days postinjury only in wt nerves. $^{*}P < 0.05$ (mean ± SE; $N = 6$ mice per group).

days postinjury. After 21 days, regenerating profiles were more numerous, reflecting the repair progress, and there were no more difference between wt and *Pgf* $^{-/-}$ mice. This finding suggests therefore that absence of PlGF leads to a transient delay of axonal regeneration. To assess the consequence of this delayed axonal regeneration on functional recovery, we further evaluated motor recovery after sciatic nerve axotomy in *Pgf* $^{-/-}$ compared with wt mice, using the walking track method in which footprints are analysed (Fig. 12A). The print-length of uninjured (NPL) and injured (OPL) sciatic nerves were measured preoperatively, as well as 3, 7, 10, 14, 17 and 21 days after axotomy and were used to calculate the print-length factor (PLF, described in Methods section). During the first week following injury, this PLF increases and reaches after 7 days the value of about 0.5 and 0.35 for wt and *Pgf* $^{-/-}$ mice, respectively, indicating a marked disability in the injured paw. At day 10, the PLF of wt mice starts to decrease and reaches a value of about 0.3 at 21 days after injury, whereas the PLF of *Pgf* $^{-/-}$ mice continues to get worse and reaches a value of about 0.6 at 21 days. Figure 12B illustrates the recovery of motor function determined by the PLF. We observe no functional recovery for the *Pgf* $^{-/-}$ mice. $^{**}P < 0.01$; $^{*}P < 0.05$ (mean ± SE; $N = 5$ mice per group).

DISCUSSION

This study provides evidence of a new function for PlGF within the PNS, where its expression had never been described. Using immunohistochemistry, double immunofluorescent stainings, *in vivo* and *in vitro* cell proliferation assays, cytokine arrays, RT-PCR, foot-prints, Luc assay and ChIP assay, we propose an original model for PlGF role in the inflammatory context of WD (Fig. 13).

PlGF is Expressed in the Peripheral Nervous System

Since its initial discovery in the placenta in 1991, PlGF has been found in other organs, including the brain (Beck et al., 2002). Until now, nothing was known about its distribution in the PNS. As the goal of our study was to investigate its potential role in WD in a model of sciatic nerve injury, the first part of our work focused on the expression of PlGF in the sciatic nerve. Using immunostaining on intact nerve and DRG, we are providing evidence that PlGF is expressed in neuronal cell bodies and axons, but not in SCs, nor in myelin sheaths. The peculiar peri-axonal localization of PlGF could be due to its particular basic carboxyl end that confers him the ability to bind polyanionic molecules like acidic phopholipids of cell membranes (Autiero et al., 2003a; De Falco et al., 2002; Persico et al., 1999).

PlGF Expression During WD

Axotomy results in axonal breakdown and disintegration, which can explain the decrease of PlGF expression observed in injured axons during the first week postinjury. Thereafter, WD in the PNS leads to successful axonal regeneration, explaining the re-expression of PlGF in axons. Our results also show that SCs transiently express PlGF after nerve injury. An induction of VEGF expression in injured peripheral nerves has been previously reported (Höke et al., 2001; Scarlato et al., 2003). Axonal rupture causes a loss of contact between the axolemma and its corresponding SC that switches from a differentiated state to an undifferentiated, proliferating state. This SC response is, among others, due to a neuronal release of the cytokine TGF-β1, which induces SC proliferation (Rogister et al., 1993). Interestingly, TGF-β1 has been shown to activate PlGF expression in cultured keratinocytes (Failla et al., 2000) and in retinal pigment epithelial cells (Hollborn et al., 2006). Hence, the transient expression of PlGF in SC observed after injury might be due to axonal released TGF-β1.

PlGF: A Target of NF-κB

The NF-κB factor plays important roles in immune, inflammatory and apoptotic responses through the induction of the transcription of numerous genes coding

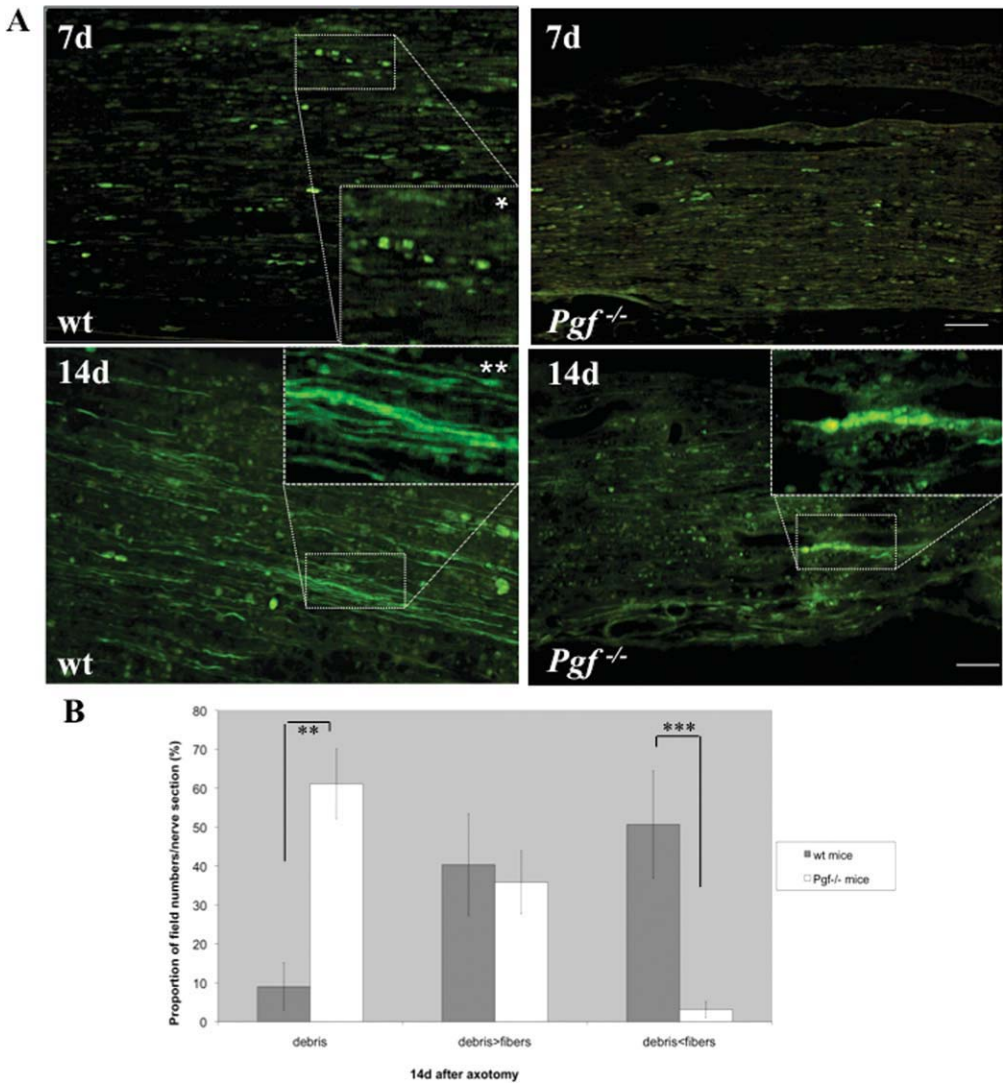

Fig. 11. Neurofilament immunostaining and semiquantification. (**A**) NF immunostaining on longitudinal sections of wt and *Pgf* $^{-/-}$ sciatic nerves 7 and 14 days after axotomy (scale bar: 100 μm). Inserts illustrate representative fields considered as "debris (*)" or "fibres (**)." (**B**) Quantification shows that after 14 days, *Pgf* $^{-/-}$ nerves contain a significant higher proportion of debris than wt nerves, while more regenerating profiles are observed in wt nerves than *Pgf* $^{-/-}$ ones. ***$P <$ 0.001; **$P < 0.01$ (mean ± SE; $N = 5$–7 mice per group).

for pro-inflammatory cytokines (Ledeboer et al., 2005; Vallabhapurapu and Karin, 2009), chemokines (Giraud et al., 2010; Grove and Plumb, 1993), matrix metalloproteases (Hnia et al., 2008; Rhee et al., 2007), and adhesion molecules (Haddad et al., 2010; Moynagh et al., 1994). After axotomy, activation of NF-κB in SCs is also implicated in the regulation of the inflammatory response occurring during WD (Fu et al., 2010; Subang and Richardson, 2001). Previous study has revealed that human PlGF expression during hypoxia was regulated by NF-κB (Cramer et al, 2005). Our results of chromatin immunoprecipitation assay agree with the fact that PlGF transcription is regulated by the NF-κB signalling pathway. By Luciferase assay, we further confirmed that the κB sites found in the *Pgf* promoter are functional in activating NF-κB.

PlGF Deletion Causes a Delay in Cellular and Molecular Events of WD

Lack of PlGF delays Schwann cell dedifferentiation and decreases their proliferation

Nerve injury stimulates the generation of proliferative nonmyelinating SCs within 24h after the lesion. In *Pgf* $^{-/-}$ mice, SC proliferation rates were shown, both *in vitro* and *in vivo*, significantly delayed. Flt-1, which binds both VEGF and PlGF-2, mediates VEGF-triggered proliferation of astroglial cells (Krum et al., 2008; Mani et al., 2005). It is thus possible that in wt animals the PlGF released by injured axons activates Flt-1 receptors on SC membranes hence inducing their proliferation. As

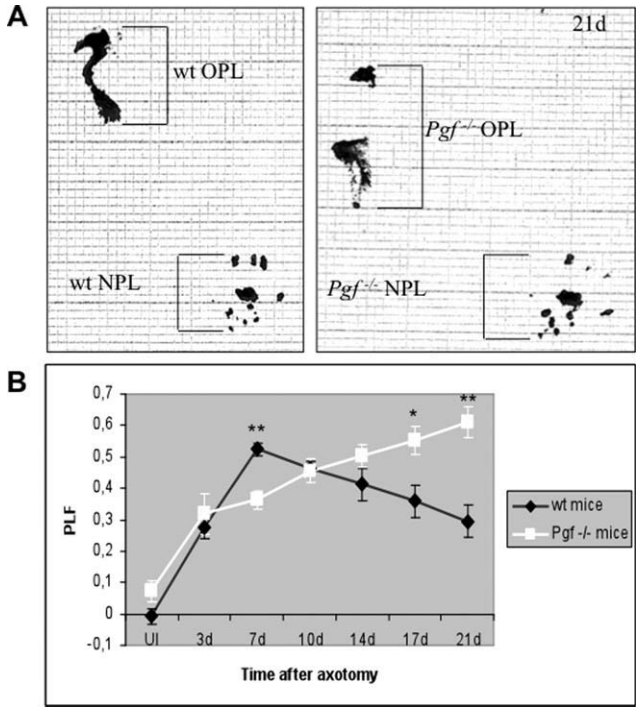

Fig. 12. Functional recovery. (**A**) Representative footprints obtained from wt and $Pgf^{-/-}$ mice, 21 days after unilateral left sciatic nerve transection. Measures of the length of the operated footprints (OPL) and the normal footprints (NPL) were used to calculate the print-length factor (PLF). (**B**) Graph showing the recovery of motor function after sciatic nerve transection determined by the PLF (described in the Methods section). Worse recovery is evident in mice lacking PlGF compared with their wt controls.

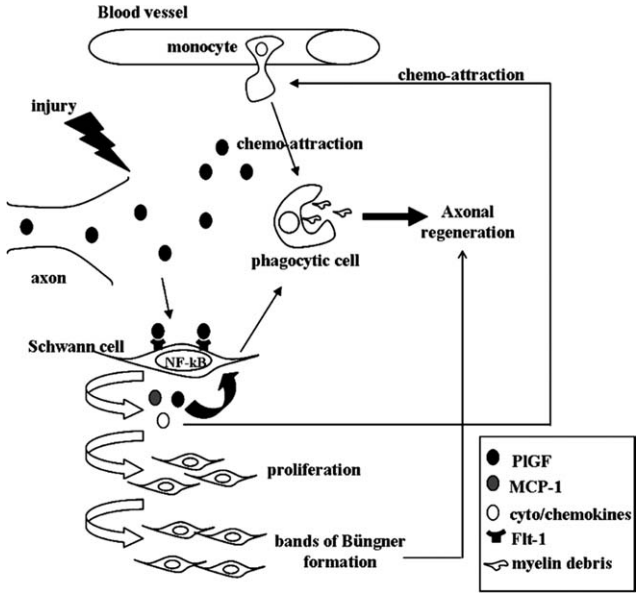

Fig. 13. Model of PlGF function in the injured peripheral nerve. Following axonal breakdown, the released PlGF can bind to its flt-1 receptor on SC, which in turn (*i*) produces PlGF and chemokines through the activation of the NF-κB signaling pathway, (*ii*) proliferate, and (*iii*) align to form bands of Büngner, promoting axonal regeneration. PlGF also influences directly monocyte chemoattraction, thereby increasing macrophage activity of myelin debris phagocytosis, necessary to successful axonal regeneration.

other mitotic agents act on SCs during WD (D'Antonio et al., 2006; Kwon et al., 1997; Li et al., 2005; Ogata et al., 2006), the sole absence of PlGF can only decrease SC proliferation, but not abolish it.

Lack of PlGF delays macrophage recruitment and myelin clearance

Macrophages play an essential role during WD. In addition to rapid clearance of axonal and myelin debris, they secrete neurotrophins (Barrette et al., 2008), promoting successful axonal regeneration. PlGF is chemoattractive for macrophages (Clauss et al., 1996), and stimulates chemokine secretion (Selvaraj et al., 2003). It is therefore not surprising to observe a significant delay in macrophage invasion during the first week of WD in $Pgf^{-/-}$ mice. This observation is corroborated by our cytokine array results, where significant difference of MCP-1 expression was observed between wt and $Pgf^{-/-}$ mice. Indeed, while MCP-1 rapidly increases after injury in wt nerves, it takes 2 more days before an increase occurs in $Pgf^{-/-}$ nerves. MCP-1 mRNA is known to be induced very early after transection in the distal portion of the sciatic nerve (Perrin et al., 2005; Toews et al., 1998). MCP-1 is necessary for specific and full recruitment of monocytes during WD (Perrin et al., 2005; Siebert et al., 2000).

The delay observed in myelin degradation and clearance, which is dependent on SCs and macrophages, is therefore the consequence of the delayed recruitment and reduced activity of these two cell types.

Lack of PlGF delays the formation of Büngner's bands

In addition to their role in the inflammatory response, SCs migrate and line up to form the bands of Büngner. SC migration involves several molecules like cell-signaling factors, integrins, as well as proteases (Lauffenburger and Horwitz, 1996). Among them, MMP-9, which is over-expressed by SCs after nerve injury and promotes SC migration (Mantuano et al., 2008), was found to be a target for PlGF (Hattori et al., 2002). Indeed, our cytokine arrays show a significant decrease of proMMP-9 expression 7 days after injury in $Pgf^{-/-}$ mice relative to wt. NGF and its low affinity receptor p75NGFr are also involved in SC migration (Anton et al., 1994) and in our study p75NGFr expression is delayed in $Pgf^{-/-}$ mice after injury. Interestingly, mice lacking the neuropilin-2 receptor for PlGF-2 do also show a delayed regeneration after sciatic crush injury, due to a delayed reestablishment of contact between axons and SCs (Bannerman et al., 2008). Moreover, it is known that VEGF has migratory effects on SCs mainly through its tyrosine kinase receptors flk-1 and flt-1 (Mani et al., 2010;

Schratzberger et al., 2000) and that flt-1, the PlGF receptor, mediates migration of endothelial cells (Bae et al., 2005; Li et al., 2006), and retinal pigment epithelial cells (Hollborn et al., 2006). The delay of SC alignment could thus also be related to a dampened action of VEGF, of which the effects are normally amplified by PlGF via various mechanisms (Autiero et al., 2003b; Carmeliet et al., 2001; Ribatti, 2008).

Lack of PlGF delays cyto/chemokine expression

A large cyto/chemokine network regulates the sequential WD cellular events described above. PlGF is able to induce an increase of several of these molecules like inflammatory cyto/chemokines (Bottomley et al., 2000; Selvaraj et al., 2003) or enzymes such as metalloproteases (Hattori et al., 2002). In our cytokine arrays PlGF has a significant effect on the expression of MCP-1 and proMMP-9. These molecules are essential for early WD events: MCP-1 attracts macrophages (Perrin et al., 2005), and MMP-9 is responsible for SC migration (Mantuano et al., 2008). Thus, PlGF lack of expression can directly affect macrophage invasion as well as MCP-1 and MMP-9 levels of expression. Additionally, as SCs increase their expression of both MCP-1 (Taskinen and Röyttä, 2000) and MMP-9 (Chattopadhyay and Shubayev, 2009) after injury, their delayed proliferation in the absence of PlGF can also trigger a decrease in MCP-1 and MMP-9 expression, leading indirectly to a decrease of macrophage activation and a delayed axonal regrowth.

In conclusion, we show that PlGF is involved in the early postaxotomy SC proliferation and macrophage chemoattraction, which can explain that in *Pgf*$^{-/-}$ mice, axonal regeneration and functional recovery are delayed. Indeed, the delay in myelin and axonal debris clearance by SCs and macrophages leads to an unfavorable environment for regeneration. Also, the delay of SC p75NGFr expression and bands of Büngner formation could decrease the beneficial effect that neurotrophins have on the regenerating axons (Taniuchi et al., 1988). This work is the first demonstration of the implication of a new member in the cytokine network regulating WD, the cytokine PlGF, whose function(s) in the intact and injured nervous system were not known up to now. The original observation that PlGF is in particular instrumental in the early events of WD like Schwann cell proliferation and macrophage invasion raises important questions and perspectives for regeneration in the CNS. It is well known indeed that following injury in the CNS, axonal regeneration partly fails because of a less pronounced macrophage invasion compared with PNS. What about PlGF expression after a CNS injury? Could regeneration be promoted by providing PlGF after a CNS lesion? In addition to its potential beneficial effect on the postinjury inflammatory reaction, PlGF treatment could also enhances neo-angiogenesis (Takeda et al., 2009), which is also known to be beneficial to axonal regrowth (Dray et al., 2009; Hobson et al., 2000).

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Placental growth factor: a tissue modelling factor with therapeutic potentials in neurology?

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Abstract

Placental growth factor (PlGF) is an angiogenic factor that belongs to the vascular endothelial growth factor (VEGF) family. Besides its well known capacity to potentiate the angiogenic action of VEGF, PlGF also participates in inflammatory processes by attracting and activating monocytes; it plays therefore more specifically a role in pathological conditions. PlGF and its two receptors, VEGFR-1 and neuropilins (NRPs), are expressed in the brain and increase after experimental stroke, but their precise functions in the nervous system remain underexplored. In this review article, we summarize present knowledge on the role of PlGF in various nervous system disease processes. Given the available data, PlGF has neuroprotective and neurotrophic properties that make it an actor of considerable interest in the pathophysiology and potentially in the therapy of degenerative and traumatic brain or spinal cord diseases.

Key words: PlGF; neuroprotection; trauma; neurodegeneration; neuropilins; VEGFR-1.

Introduction

From Andreas Vesalius' anatomical drawings to current research, the number of studies highlighting the parallelism between vessel and nerve patterning is constantly increasing (1-8). The best known angiogenic factors belong to the vascular endothelial growth factor (VEGF) family. The VEGF family includes seven secreted glycoproteins, designated VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF). They possess different physical and biological properties and act, as dimeric glycosylated proteins, through specific receptors: three protein–tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3), present on most vascular endothelial cells and two non-protein kinase co-receptors, neuropilin-1 and neuropilin-2 (Fig. 1). Discovered first (9, 10), VEGF-A is the best characterized VEGF family member. VEGF-A mediates its effects by interacting with VEGFR-1 (also referred to as fms-like tyrosine kinase, Flt-1), and VEGFR-2 (also referred to as fetal liver kinase, Flk-1/KDR). In addition, $VEGF_{145}$ and $VEGF_{165,}$ two isoforms of VEGF-A, can also bind to neuropilins (NRPs) (11, 12). Numerous reports have described the role of VEGF in the nervous system (NS), both during development (3, 6, 8, 13-17) and in neurogical disorders such as amyotrophic lateral sclerosis (18-28), Alzheimer's (29-31) or Parkinson's diseases (32, 33), brain ischemia (29, 34-43) or spinal cord injury (44, 46).

While VEGF is important under physiological and pathological conditions, PlGF (47), much less studied than VEGF-A, appears to be more specifically involved in pathological states (15). Although scientific data are scarce, we thought it timely to summarize our present knowledge on PlGF and the nervous system, as some of the pathological processes that are influenced by PlGF are relevant for the pathophysiology of various neurological disorders, and, possibly in the future, for their therapy. We will review the available information coming from direct studies of PlGF's functions in the nervous system from effects observed after its binding to its two receptors, VEGFR-1 and NRPs. The major findings on the known respective roles of VEGF and PlGF in experimental models of nervous system disorders are shown in table 1.

PlGF: biochemistry and functions

PlGF is a ~46 KDa dimeric glycoprotein that occurs in 3 isoforms in humans (131, 152 and 203 amino acids). Only one isoform, PlGF-2, is expressed in mice due to alternative mRNA splicing from a single gene (48). While PlGF was originally identified in the placenta (47), it has been detected

Table 1

VEGF and PlGF neurobiological effects in experimental models of neurological disorders.
Normal: beneficial effect; *Italic*: deleterious effect

Growth factor	Experimental models of neurological disorders	Neurobiological effect	References
VEGF	ALS (Mouse, SOD-1)	neuroprotection and neurotrophic action	19; 20; 22; 24; 27; 28
		reduction of astrogliosis and increased formation of neuromuscular junctions	26
	Cerebral ischemia (Rat, MCAO)	neuroprotection	37; 38; 42; 43
	Spinal cord injury (rat, clip injury)	increased angiogenesis, proliferation of glial progenitors, neuroprotection, tissue sparing, improvement of locomotor recovery	44; 45; 46
		exacerbation of lesion volume	86
	Parkinson (Rat, 6-OHDA)	neuroprotection, neovascularization and astroglial proliferation (anti-oxidant, GDNF source)	32; 33
	Alzheimer (Mouse, APP23Tg)	*maintenance of the chronic inflammatory response*	31
	Peripheral nerve injury	increased nerve regeneration, neurotrophic action, mitogenic activity on Schwann cells	82-84
PlGF	Cerebral ischemia	neuroprotection	50; 72
	Peripheral nerve injury	pro-inflammatory effects, mitogenic activity on Schwann cells	85

in several other organs including heart, lung, thyroid gland, skeletal muscles and more recently in brain. Numerous cell types, like endothelial cells, pericytes, macrophages, bone marrow cells, tumour cells, astrocytes and neurons, produce PlGF especially when they are activated or stressed like in hypoxia, inflammation or trauma (49-51).

PlGF potentiates the angiogenic effects of VEGF such as stimulation of endothelial cell proliferation and migration, increase of endothelial cell survival, induction of neoangiogenesis and vessel maturation (51). PlGF is also involved in inflammatory processes where it stimulates chemotaxis and activation of monocytes, and increases their secretion of pro-inflammatory cyto- and chemokines such as TNF-α, IL-1β and MCP-1 (52-54).

PlGF: a neuroprotective and neurotrophic factor

Contrasting with VEGF (55-59), there are only few reports on the potential neurotrophic/neuroprotective role of PlGF. The first study showing that PlGF might play an important role in the nervous system was performed by Beck *et al.* (50), who explored the expression pattern of NRPs, known to regulate both angiogenesis and neuronal axon guidance (3, 5, 8), in an experimental model of cerebral ischemia. Because NRPs have short intracellular segments without cytoplasmic signal transduction domains, they require an association with other receptors to be functional. These putative co-receptors include plexin A (52) as well as VEGFR-1 and VEGFR-2 (11, 13). Thus, in addition to NRPs, Beck *et al.* (50) also studied the expression of their co-receptors VEGFR-1 and 2, and their ligands VEGF-A and PlGF (61, 62). They demonstrated for the first time that both PlGF mRNA and its protein are expressed in neurons in the normal brain, but not in astrocytes, nor in vessels. They further showed that 3 days following a middle cerebral artery occlusion (MCAO), the PlGF transcript and protein are upregulated in vessels inside and around the infarcted area, as well as in neurons and astrocytes located at the ischemic border zone. These results were confirmed one year later in another report that assessed the expression of angiogenic genes after experimental brain ischemia (63). Thus, in addition to providing further support for the potential neurotrophic and neuroprotective roles of VEGF, these data suggested for the first time that PlGF may be implicated in pathological processes of the nervous system. This hypothesis was established later, notably thanks to Cheng *et al.* (64), who demonstrated that PlGF is able to exert an anti-chemorepulsive effect on axons through its NRP binding. NRPs are known to be

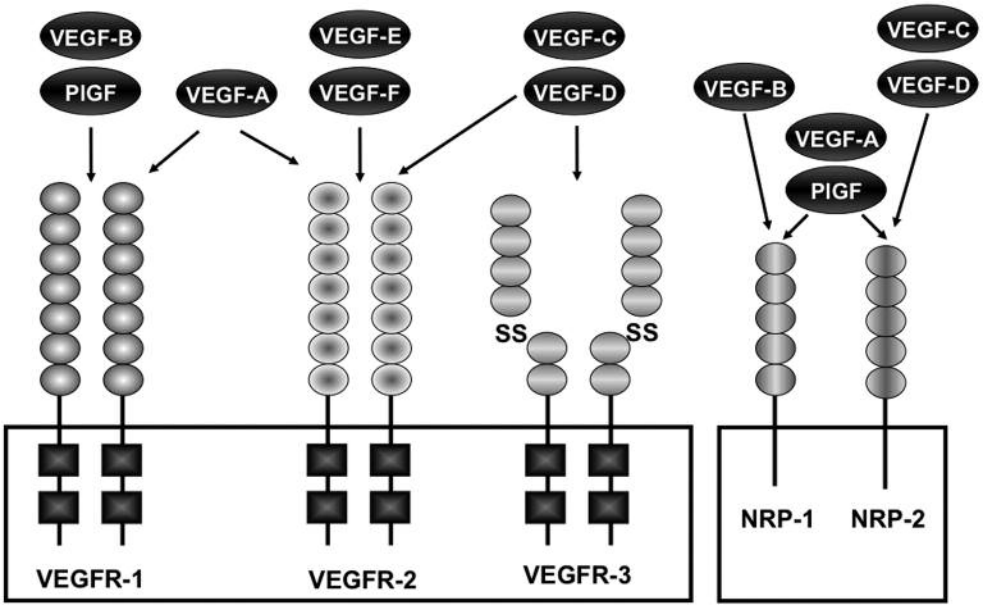

FIG. 1. — VEGF family members and receptors. The tyrosine kinase receptor VEGFR-1 binds VEGF-A, VEGF-B and PlGF with high affinity. VEGFR-2 binds VEGF-A, VEGF-C, VEGF-D, VEGF-E and VEGF-F. VEGFR-3 is a tyrosine kinase receptor with only six Ig-homology domains, which preferentially binds VEGF-C and VEGF-D. Neuropilin-1 and -2 (NRP-1 and NRP-2), the two non-tyrosine kinase-type co-receptors, respectively bind VEGF-A_{165}, VEGF-B and PLGF and VEGF-A_{165}, VEGF-C, VEGF-D and PLGF.

involved in axonal guidance during development, and the balance between their ligands, semaphorins and VEGF, plays a crucial role. On the one hand, NRPs, in conjunction with their co-receptors plexins, bind Sema3A and transduce repulsive signals to axons (60). On the other hand, when associated with VEGFR, NRPs bind VEGF and mediate attractive signal (13). Cheng *et al.* (64) analyzed neurite outgrowth in DRG explants and the effect of the NRP ligands, semaphorins, VEGF and PlGF, on the collapse of growth cones. Only $VEGF_{165}$ and PlGF were able to counteract the chemorepulsive effect of sema3A which supports a specific role for NRPs in mediating the neurotrophic actions of $VEGF_{165}$ and PlGF. More recently, a neuroprotective effect of PlGF was confirmed *in vitro* by a study showing that it promotes the survival of cultured primary cortical neurons under conditions of oxygen and glucose deprivation (65).

The neurotrophic and neuroprotective effects of PlGF, in addition to its already known angiogenic properties, are of great interest for the treatment of cerebral ischemia. The beneficial effects of cell therapy in experimental models of cerebral ischemia were shown in several studies (66-69). In particular, bone marrow derived mesenchymal stem cell (MSC) transplantations have beneficial neuroprotective and angiogenic effects, which are notably mediated by paracrine mechanisms (70). Among the various factors released by MSCs, VEGF and PlGF are indeed good candidates to explain these effects (71). This hypothesis is supported by a study (72) comparing the efficacy of systemically delivered human MSCs transfected or not with an adenoviral vector coding for PlGF in a rat MCAO model of cerebral ischemia. Transplantation of both cell types 3 hours after MCAO reduced the lesion size, induced angiogenesis and improved motor function, but greater effects were observed when rats were treated with the PlGF-transformed MSCs. These results suggest that PlGF might be an interesting candidate for neuroprotective treatment in ischemic stroke. Moreover, PlGF, probably via its binding to NRPs, could also be of therapeutic interest in neurodegenerative disorders where decreased vascularisation was described, or in spinal cord trauma where angiogenesis and neuroprotection are necessary to support and enhance axonal regeneration.

PlGF: a glial activator?

The glial response plays an important role after nervous injury. In the injured central nervous system, the proliferation and migration of astrocytes result in a gliotic scar that can exert both beneficial and detrimental effects. As a matter of fact, this glial scar isolates the injury site by re-establishing the glia limitans, thus restricting the propagation of secondary

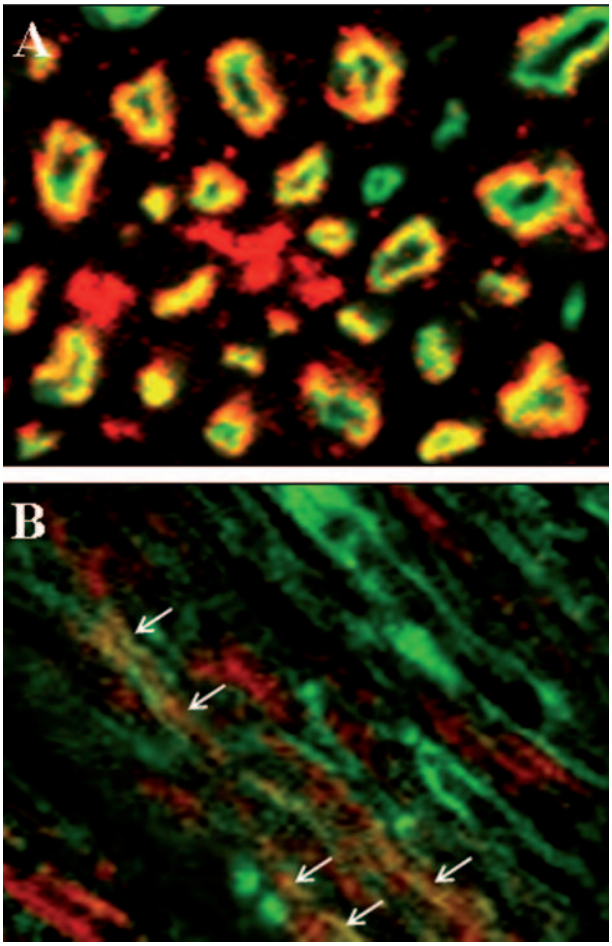

FIG. 2. — Double immunofluorescent stainings on normal and injured mouse sciatic nerve sections. PlGF (Rhodamine) and NF (A) or S100 (B) (FITC) double immunostainings, on cross-section of normal (A) and longitudinal section of injured (distal segment, B) sciatic nerves. In normal sciatic nerve, PlGF is found in axons underneath the axolemma (A). Schwann cells, which dedifferentiate and proliferate after loss of axonal contact, express PlGF from the first day after injury (B). Arrows point to Schwann cells expressing PlGF 24h after injury.

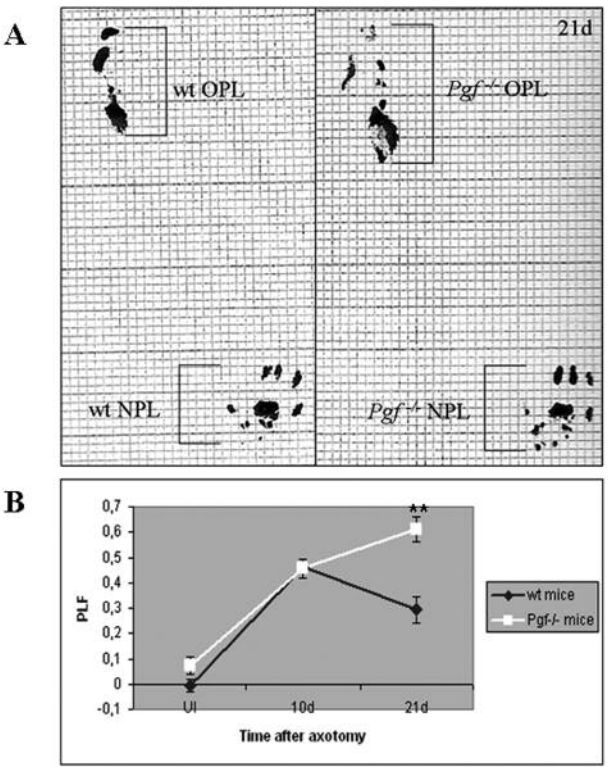

FIG. 3. — Functional recovery. (A) Representative footprints obtained from wt and *Pgf*$^{-/-}$ mice, 21 days after unilateral left sciatic nerve transection. Measurements of print length were made on the operated side (OPL) and the normal side (NPL). A mean of four values was then calculated for the OPL (xOPL) and NPL (xNPL), and a print-length factor (PLF) was calculated as follows: PLF = (xOPL – xNPL)/xNPL. (B) Graph showing the recovery of motor function after sciatic nerve transection determined by the PLF. During the first week following injury, PLF increases, indicating a marked disability in the injured paw. Later on, the PLF in wt mice progressively decreases, indicative of functional recovery. Worse recovery is evident in mice lacking PlGF compared to their wt controls.

lesions, but it also creates a physical and chemical barrier to axonal regeneration (73, 74). In central nervous system injury, the role of VEGF in the glial response has already been assessed. Several studies show that VEGF stimulates astroglial proliferation and migration via its flt-1 receptor, but also that it facilitates the expression of several growth factors, such as ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF) (75-77). By contrast, the influence of PlGF in the formation of the astroglial scar remains unknown. However, highlights have been recently brought by recent studies showing that the early growth response (Egr-1) factor, which regulates the astrocytic expression of phosphacan, a glial scar component, after experimental stroke (78), is a target gene for PlGF (79).

After peripheral nervous system injury, VEGF is expressed by Schwann cells (80, 81), stimulating their proliferation and migration (82-84) which is a crucial step for efficient axonal regeneration. Recently, we have published the first description of the expression and function of PlGF in the peripheral nervous system under normal physiological situation and after nerve injury (85). In the intact mouse sciatic nerve, PlGF is found in axons underneath the axolemma, while it is expressed by Schwann cells after axotomy (Fig. 2). Using transgenic knock-out mice for the PlGF gene (*Pgf*$^{-/-}$) and a multidisciplinary approach, we were able to demonstrate that PlGF plays a role in Wallerian degeneration: it stimulates proliferation and migration of Schwann

cells, as well as the inflammatory response by inducing MCP-1 expression and macrophage recruitment, essential for successful repair. Thus, PlGF depletion in *Pgf* $^{-/-}$ results in delayed axonal regeneration and impaired functional recovery (Fig. 3).

Conclusion

Currently, PlGF has received much less attention than VEGF-A as a potential treatment for neurological disorders. However, this careful analysis of the literature highlights the fact that the neuroprotective and neurotrophic effects of VEGF-A are mainly due to its binding to neuropilins and to a lesser extent to VEGFR-1, both of which being also PlGF receptors. It is only recently that specific studies have been devoted to PlGF demonstrating that it has its proper neuroprotective/neurotrophic properties.

A major drawback of the therapeutic use of VEGF-A in brain or spinal cord injury is increase in vascular permeability and oedema formation (87) because of its pro-angiogenic properties (9, 86). The effect of PlGF on vascular permeability remains disputed. Some reports have shown that only VEGF-A analogues activating VEGFR-2 are able to increase vascular permeability (88, 89). The lack of such an effect for PlGF could thus make it a more appropriate candidate than VEGF-A for neurological treatment. However, experiments using mutant mice in which PlGF is under- or over-expressed have respectively revealed a decrease (90) or an increase (91, 92) of the vascular permeability in pathological conditions. One explanation, according to Carmeliet *et al.* (93) is that the PlGF-induced increase in vascular permeability is an indirect effect, consequence of the displacement of VEGF-A from VEGFR-1 to VEGFR-2, as PlGF competes with VEGF-A for VEGFR-1 in pathological conditions.

Taken together, VEGF and PlGF, despite proven experimental advantages, could be double-sided swords as neuroprotective/neurotrophic treatments for ischemic, degenerative or traumatic neurological disorders. In future therapeutic studies, it might be worthwhile to explore the effect of a combination of PlGF to induce beneficial tissue modelling effects and a VEGFR-2 inhibitor to block the VEGF-dependent angiogenic effect.

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