THE MYELINATION PUZZLE: DO FIBROBLAST GROWTH FACTORS AND THEIR RECEPTORS HAVE REGULATORY ROLES IN PERIPHERAL NERVE MYELINATION?

by

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To my three lovely families…

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ABSTRACT

THE MYELINATION PUZZLE: DO FIBROBLAST GROWTH FACTORS AND THEIR RECEPTORS HAVE REGULATORY ROLES IN PERIPHERAL NERVE MYELINATION?

Axon-Schwann cell interaction is the key step in myelination and critical in the pathogenesis of peripheral neuropathies. Molecules mediating this interaction to promote the complex mechanism of myelination have not been elucidated properly yet. However, recent studies emphasized the importance of tyrosine kinase receptor signaling in the process. Although expression of FGFs and their tyrosine kinase receptors FGFRs have been shown in PNS, little information is available about their regulation during the myelination process. In this study, we aimed to investigate whether FGF1, FGF2, FGF9 that were previously implicated in CNS development, involved in peripheral nerve myelination and regulate axon-Schwann cell interactions, through their high affinity receptors FGFR1-3. For this purpose, we used dorsal root ganglia (DRG) and the sciatic nerve from mice as *in vivo* models and Schwann cell-neuron co-culture developed from fetal mouse DRG tissue as an *in vitro* model. The expression patterns and localization of the molecules were investigated both *in vivo* and *in vitro*. Among the three FGFs analysed, FGF1 was chosen as a candidate for further investigation because of its high level of expression in all tested tissue types with an axonal localization. In contrast, FGFR1-3 were found to be expressed by Schwann cells. Protein expression levels of FGF1 and FGFR1-3 were examined through the developmental stage to adulthood from sciatic nerves by Western blotting. Both FGF1 and its receptors were found to be modulated at key time points of the myelination route. Immunolabeling studies showed that FGF1 expression in neurons and FGFR1-3 expression in Schwann cells continued throughout the process. When FGF1 was blocked in DRG culture, a reduction in the levels of myelin proteins and in the number of myelinated axonal segments was observed. Our findings provide evidence for the first time for the involvement of FGF1 in peripheral nerve myelination and suggest that FGF1 signaling through FGFR1-3 have regulatory roles at the onset of myelination, in myelin compaction and protection of the stability of mature structure.

ÖZET

MİYELİNİZASYON BULMACASI: FİBROBLAST BÜYÜME FAKTÖRLERİ VE RESEPTÖRLERİNİN PERİFERAL SİNİR MİYELİNİZASYONDA DÜZENLEYİCİ GÖREVLERİ VAR MIDIR?

Akson ile Schwann hücresinin etkileşimi miyelinizasyon için gerekli anahtar adımdır ve periferal nöropatilerin patogenezinde kritik öneme sahiptir. Bu etkileşimde rol alan ve miyelinizasyonun karmaşık mekanizmasını kontrol eden moleküller hala tam olarak aydınlatılamamıştır. Yeni çalışmalar tirozin kinaz reseptör sinyallerinin bu süreçte önemli olduğunu göstermiştir. Periferal sinir sisteminde anlatıldığı bilinen FGF'lerin ve tirozin kinaz reseptörü olan FGFR'lerin, miyelinizasyon sürecinde modülasyonları hakkında çok az bilgi mevcuttur. Bu çalışmada, daha önce merkezi sinir sistemi gelişiminde rol aldığı gösterilen FGF1, FGF2 ve FGF9 ve yüksek affinite reseptörleri FGFR1-3'ün periferal sinir miyelinizasyonu sürecinde akson-Schwann hücresi etkileşimini düzenleyici olası görevlerinin araştırılması hedeflenmiştir. Bu amaçla, fare dorsal kök ganglionu (DRG) ve siyatik siniri *in vivo*, fetal fare DRG dokusu kullanılarak oluşturulan Schwann hücresi-nöron ko-kültürü ise *in vitro* model olarak kullanılmıştır. İlgili moleküllerin lokalizasyonu ve anlatım profilleri araştırılmıştır. İncelenen üç FGF arasından FGF1, test edilen bütün dokulardaki yüksek anlatımı ile birlikte, aksonal lokalizasyona sahip olmasından dolayı sonraki çalışmalar için aday olarak seçilmiştir. Diğer yandan, FGFR'lerinin Schwann hücrelerinde anlatıldığı gösterilmiştir. Farklı gelişim aşamalarındaki farelerden ve yetişkin fareden alınan siyatik sinir örneklerinde, FGF1 ve FGFR1-3'ün protein seviyeleri Western blot tekniği ile incelenmiş ve miyelinizasyon sürecindeki kritik noktalarda bu moleküllerin anlatımlarında bir düzenlenme olduğu belirlenmiştir. FGF1'in nöronlardaki, reseptörlerinin FGF1-3'ün ise Schwann hücrelerindeki anlatımının bu süreç boyunca devamlılığını koruduğu immün boyamalarla belirlenmiştir. DRG ko-kültüründe FGF1 bloke edildiğinde, miyelin proteinlerinin anlatımları ve miyelin kılıf sayısı azalmıştır. Bu çalışma, FGF1'in periferal sinir miyelinizasyonunda rolü olduğunu ilk defa ortaya koymakla birlikte, FGF1'in FGFR1-3 aracılığıyla miyelinizasyonun başlamasında, miyelin kompaktlaşmasında ve miyelin yapısının korunmasında düzenleyici işlevleri olabileceğini önermektedir.

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LIST OF SYMBOLS

ml	Mililiter
mM	Milimolar
ng	Nanogram
v	Volume
w	Weight
µg	Microgram
µm	Micrometer
µl	Microliter

LIST OF ACRONYMS/ABBREVIATIONS

A	Acidic box
AKT	V-akt murine thymoma viralonco gene
APS	Ammonium persulfate
BC	Boundary cap
BDNF	Brain-derived neurotropic factor
BF	Bright field
Brn-2	POU class 3 homeobox 2
BSA	Bovine serum albumin
CASPR	Contactin-associated protein
CDC42	Cell division control protein 42 homolog
CHD	Cell adhesion molecule homology domain
CMT1A	Charcot-Marie-Tooth type 1A
CNP	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	Central nervous system
DABCO	1,4-diazabicyclo[2.2.2]octane
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DCX	Double-cortin
div	Day/s *in vitro*
dpi	Day/s post induction
DR	Dorsal root
DREZ	Dorsal root entry zone
DRG	Dorsal root ganglion
E10	Embryonic day 10
E13	Embryonic day 13
E15	Embryonic day 15
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

ErbB2/3	v-erb-b2 a*via*n erythroblastic leukemia viral oncogene homolog 2/3
ERK	Extracellular signal-regulated kinases
ERK1/2	Extracellular signal-regulated kinases 1/2
ERM	Ezrin, radixin and moesin
FABP	Fatty acid binding protein
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGF-1	Fibroblast growth factor 1
FGF-2	Fibroblast growth factor 2
FGF7	Fibroblast growth factor 7
FGF-9	Fibroblast growth factor 9
FGF-23	Fibroblast growth factor 23
FGFR	Fibroblast growth factor receptor
FGFR1	Fibroblast growth factor receptor 1
FGFR2	Fibroblast growth factor receptor 2
FGFR3	Fibroblast growth factor receptor 3
FGFR4	Fibroblast growth factor receptor 4
FRS2	FGF receptor substrate 2
Gab1	Grb2 associated binding protein 1
GAF	Glia-activating factor
GFAP	Glial fibrillary acidic protein
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HNPP	Hereditary neuropathy with liability to pressure palsies
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycans
Ig	Immunoglobulin
IGF2	Insulin-like growth factor 2
JNK	c-Jun NH2-terminal kinase
KCh	Potassium channel
Krox-20	Egr2 early growth response 2
LIF	Leukaemia inhibitory factor

LPA	Lysophosphatidic acid
MAG	Myelin associated glycoprotein
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
min	Minute
MPZ	Myelin protein zero
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
NaCh	Sodium channel
NB	Neurobasal
NF-100	Neurofilament-100
NF-H	Neurofilament H
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
Nrg1	Neuregulin-1
NT3	Neurotrophin 3
OCT	Optimum cutting temperature
Oct-6	Octamer transcription factor 6
OL	Oligodendrocyte
P1	Postnatal day 1
P4	Postnatal day 4
P7	Postnatal day 7
P10	Postnatal day 10
P15	Postnatal day 15
P20	Postnatal day 20
$p75^{NTR}$	p75 neurotrophin receptor
pAKT	Phospho-AKT
PBS	Phosphate buffered saline
PC12	Pheochromocytoma 12
PDGF-β	Platelet-derived growth factor-β
PDL	Poly-D-lysine
pERK	Phospho-extracellular signal-regulated kinases

pERK1/2	Phospho-extracellular signal-regulated kinases 1/2
PFA	Paraformaldehyde
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLCγ	Phospholipase Cγ
P2	Myelin protein 2
PMP22	Peripheral myelin protein 22
PNS	Peripheral nervous system
PTB	Phospho-tyrosine binding domains
PVDF	Polyvinyl difluoride
Rac1	RAS-related C3 botulinum substrate 1
RIPA	Radio-immuno-precipitation Assay
RNA	Ribonucleic acid
RPM	Revolutions per minute
RTK	Receptor tyrosine kinase
SC	Schwann cell
SDS	Sodium dodecyl sulfate
SH2-3	Src homology domain 2-3
Shp2	Protein tyrosine phosphatase, non-receptor type 11
SOS	Son of sevenless
SP	Signal peptide
TBS	Tris-Buffered Saline
TBST	TBS with Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	Type II transforming growth factor-β
TK	Intracellular domain with tyrosine kinase domain
VR	Ventral root

1. INTRODUCTION

Myelination is provided by Schwann cells in peripheral nervous system (PNS) and by oligodendryocytes in the central nervous system (CNS). Myelination insulates the axons and provides rapid saltatory conduction through axons. Continuous axon-Schwann cell interaction is the key event in peripheral nerve myelination and critical in the pathogenesis of peripheral neuropathies [1, 2]. Neurotrophins and growth factors have been proposed to act in these interactions [3]. Recent studies demonstrated that Neuregulin-1 (Nrg1) is an important molecule regulating Schwann cell proliferation, migration, myelination and also remyelination through ErbB2/3 receptors [4-9]. However, molecular mechanisms of the complex myelination process and all the signaling molecules that regulate axon-Schwann cell interactions are still not well understood. Revealing the molecules acting in the myelination process is an urgent need to understand the pathology of neuropathies and develop efficient therapeutic tools. Fibroblast growth factors (FGFs) are also candidates to act in peripheral nerve myelination. So far, they have been mostly studied in the CNS but to a lesser extent in the PNS. Although FGF and FGF receptor (FGFR) expression have been shown in murine dorsal root ganglion (DRG) previously, little information is available about their regulation during the myelination process [10, 11]. Out of 22 FGFs, only FGF2 has been studied elaborately and shown to be involved in sciatic nerve regeneration and remyelination following nerve damage [11]. Thus, in this study, FGF1, FGF2, FGF9 and FGFR1-3 were investigated in order to explore their possible involvement in peripheral nerve myelination.

1.1. Myelin Structure and Function

Myelin is formed by tight wrapping of a thin sheet of highly specialized Schwann cell membrane around an axon. If the two μm thick myelin sheath around a large axon with a diameter of seven μm or more is unwrapped, its length reaches to about 4000 μm in an open configuration. The axonal length, that one Schwann cell can cover, in other words the

length of one internode can be one mm, and even longer and the space between two internodes is about one μm at the end of this process [12].

Nearly all of the myelin sheath throughout the internode is compact myelin whereas the noncompact myelin is found in paranodes and Schmidt–Lanterman incisures, which provide transport between Schwann cell cytoplasm and wrapped membranes. Myelin is composed predominantly of water, lipids, where lipids comprise up to 70-80% of the myelin dry weight. Lipids, including cholesterol and glycolipids, galactocerebroside and sulfatide are concentrated in the compact myelin, while noncompact myelin is composed of the proteins that form adherens, tight, and gap junctions to join the layers of the myelin sheath [13].

1.1.1. Myelin Proteins

Most of the myelin proteins including myelin protein zero (MPZ), peripheral myelin protein 22 (PMP22), and myelin associated glycoprotein (MAG) are glycosylated. Other myelin proteins including myelin basic protein (MBP) and protein P2 are basic proteins.

MPZ is a 30-kDa transmembrane glycoprotein that belongs to the immunoglobulin (Ig) superfamily with a single extracellular immunoglobin -like domain. MPZ represents over half of the total protein in compact myelin and acts as a cell adhesion molecule [13]. The Ig-like domains of MPZ protein form tetramers and establish homophilic adhesive interactions between Schwann cell membrane layers [14]. In rodents, MPZ expression is at a maximum during active myelination occurring at the first three postnatal weeks. Thereafter, it is preserved at lower steady state levels until adulthood. MPZ expression decreases upon Wallerian degeneration or demyelination, but it increases again upon initiation of remyelination [15].

PMP22 is a 22 kDa integral membrane glycoprotein consisting of four hydrophobic and two extracellular domains. PMP22 comprises less than five percent of PNS myelin protein and is found in the compact region [13]. PMP22 is mainly expressed by

myelinating Schwann cells but is also found in many neuronal and non-neuronal tissues. PMP22 is one of the molecules regulating neuron-glia interactions at the initial phases of myelination and later in the maintenance of the structure. In addition to various point mutations, abnormal expression levels of PMP22 cause the most common hereditary demyelinating peripheral neuropathies including Charcot-Marie-Tooth-1A (CMT1A) or hereditary neuropathy with liability to pressure palsies (HNPP) and Dejerine-Sottas Syndrome [16-18].

MAG is a 100 kDa glycoprotein and belongs to the sialic acid-binding subgroup of immunoglobulin superfamily. MAG is composed of five extracellular Ig-like domains, a single transmembrane domain, and a cytoplasmic domain where alternative mRNA splicing occurs that generates two developmentally regulated isoforms (L- and S-MAG). In the PNS, S-MAG is the predominant form both at developmental stages and in the adult [19, 20]. In PNS, MAG is localized to noncompact myelin including the periaxonal membrane, paranodal loops, Schmidt-Lanterman incisures, and the inner/outer mesaxons. MAG represents 0.1% of the total myelin proteins. The protein is thought to regulate Schwann cell-axon adhesion and promote axon-glia communication. MAG expression is specific to myelin-forming cells and starts very early in the myelination process and continues at a relatively high level in mature animals. Thus, MAG has roles both in the initial Schwann cell-axon interactions and in the maintenance of myelin [21-23].

P2, belonging to the fatty acid binding protein (FABP) family, is found on the cytoplasmic face of compact myelin. P2 is proposed to function in forming and maintaining the lipid composition of the myelin membrane by its lipid binding capability [24].

MBP contains a very high percentage (25%) of basic residues. Four isoforms of MBP with molecular weights of 14, 17, 18.5, 21.5 kDa are expressed in the PNS. MBP is thought to be involved in the maintenance of the major dense line and the compaction of the PNS myelin sheath [19, 24].

The 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) is a membrane associated enzyme that belongs to 2H phosphoesterase superfamily. The reaction CNP catalyzes is the

production of 2′-nucleotide from 2′,3′-cyclic substrates. It is abundant in the myelin sheath and is present in the noncompact myelin, namely in the periaxonal region, the outer mesaxon, Schmidt-Lanterman incisures, and Schwann cell cytoplasm. CNP is thought to be involved in RNA trafficking, splicing, or metabolism in the myelinating glial cell [25]. The physiological function or the physiological substrate of CNP has not been determined yet. In tissues positive for CNP, 2′- or 2′,3′-cyclic nucleotides could not be found [19, 24, 25].

1.1.2. Function of the Myelin Sheath

The myelin sheath insulates the axon and provides faster transmission of the action potentials along the axons that it ensheaths. Unmyelinated axons do not have this insulation and propagate nerve impulses at much slower rates [26, 27]. The transmission mechanism in myelinated axons is called saltatory conduction [28]. The cytoplasm of the axon is electrically conductive, and the nonconductive material of myelin prevents charge leakage through the membrane. Sodium (Na^+) channels are clustered at the nodes of Ranvier and this clustering is provided by Schwann cell derived signals. Thus, depolarization at one node with high concentration of Na^+ channels is enough to elevate the voltage at the next node to the threshold for firing action potential.

The charge generated at the first node passively diffuses and depolarizes the next node to generate another Na^+ wave, this cycle is repeated until the last node. As action potentials do not propagate as waves, but are conducted directly to the next node likely by jumping, they travel faster than they would otherwise (Figure 1.1) [26, 29]. In addition, it is proposed that small diameter axons do not require such myelin sheaths as the speed of impulse transmission in those axons would not be improved [3].

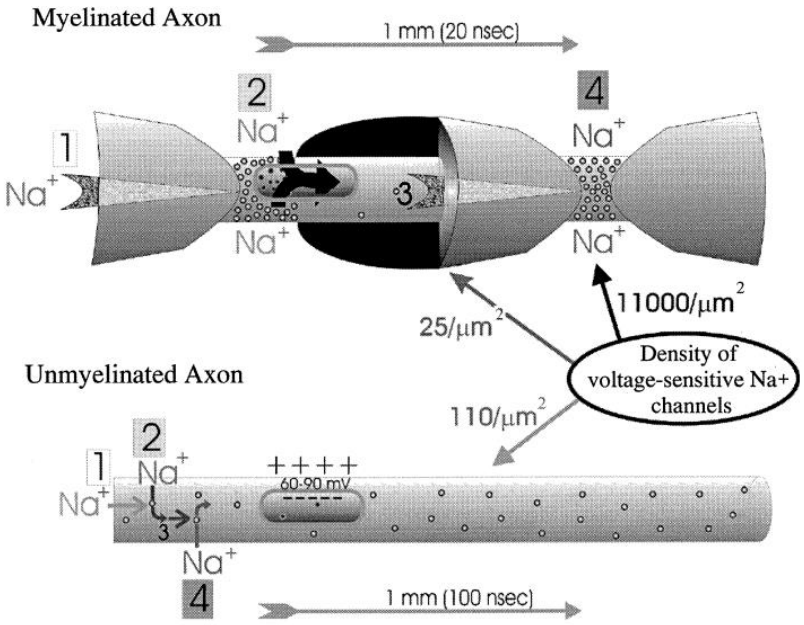

Figure 1.1. Nerve conduction in myelinated and unmyelinated axons. 1. Arrival of action potential in a form of a wave of Na^+ ions. 2 and 4. Entry of Na^+ ions by the activation of Na^+ channels. 3. Passive diffusion of the wave of Na^+ ions along the axon following the concentration gradient [26].

1.2. Dorsal Root Ganglion

The peripheral nervous system is composed of sensory, motor, sympathetic and parasympathetic neurons. The cell bodies of the sensory neurons are located in the dorsal root ganglia (DRG) outside the spinal cord (Figure 1.2). Sensory neurons are pseudo-unipolar neurons, with only one process extending from the cell body. After a short distance the process bifurcates into two, where the peripheral branch (dendrites) goes to the periphery to innervate a target tissue like skin and muscle, whereas the central branch (axons) enters the spinal cord (Figure 1.3).

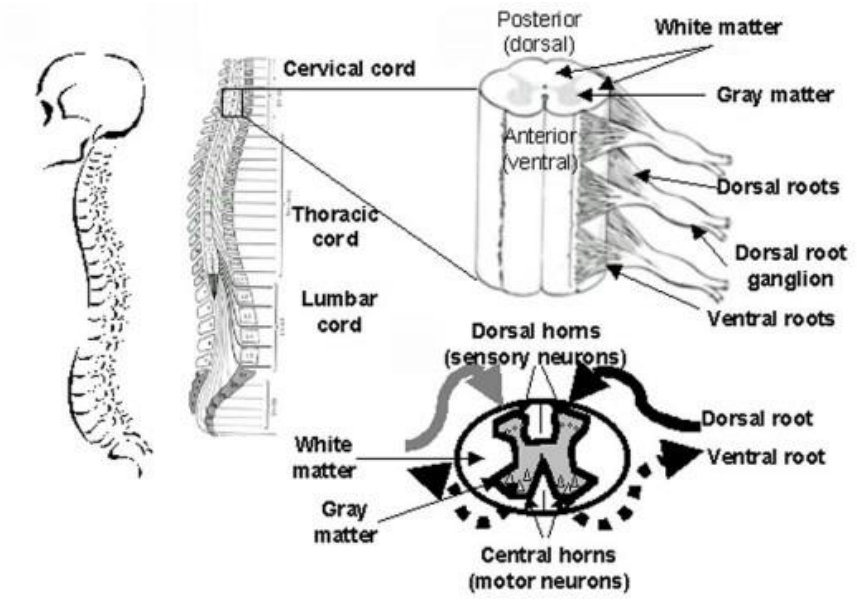

Figure 1.2**.** The spinal cord and localization of dorsal root ganglion [30].

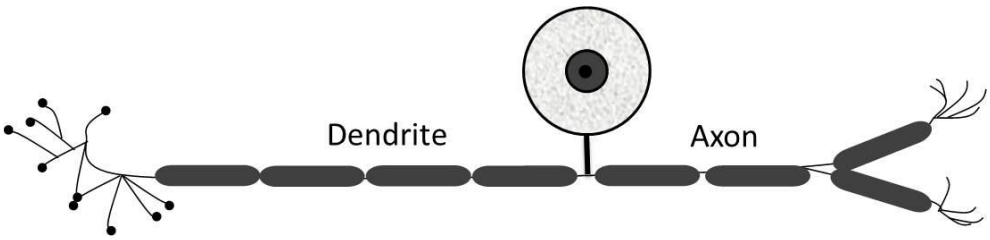

Figure 1.3**.** Pseudo-unipolar neuron.

The peripheral branch conveys sensory information from the body to the soma of the neuron and the central branch from the soma to the nerve junction in the dorsal horn of the spinal cord (Figure 1.4) [31]. The sciatic nerve, used as the *in vivo* peripheral nerve model in experiments is the longest peripheral branch of DRG neurons. DRG tissue is frequently used to establish DRG cultures as the *in vitro* myelinated peripheral nerve model. Because DRG tissue is readily accessible and serves as a source of both neurons and glia, thus is itself enough to generate a myelinating neuron-Schwann cell co-culture.

Moreover, the tissue provides satellite and other kinds of cells found *in vivo,* that makes the culture conditions closer to *in vivo* conditions.

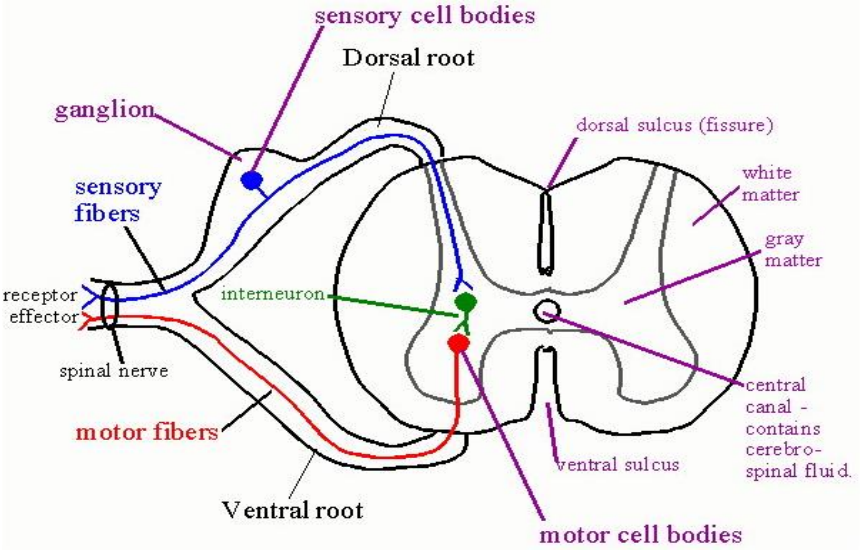

Figure 1.4**.** Sensory neurons. Localization of sensory neurons and connections in the gray matter of the spinal cord [31].

1.3. Schwann Cells and Myelination

Glial cells are the other constituents of the nervous system. Glial cells are indispensable for the survival and function of neurons as they surround, insulate and support neurons, supply nutrients and oxygen to neurons, destroy pathogens and remove neuronal and other debris. In the PNS, Schwann cells are the main glia myelinating the axons of PNS neurons. Furthermore, they are the responding elements to axonal injury as they quickly enter mitosis upon damage. Schwann cells are associated with various demyelinating disorders, such as Charcot–Marie-Tooth, Guillain–Barré syndrome and also leprosy and are responsible for the tumours seen in patients with neurofibromatosis type 1 and 2 [26, 27].

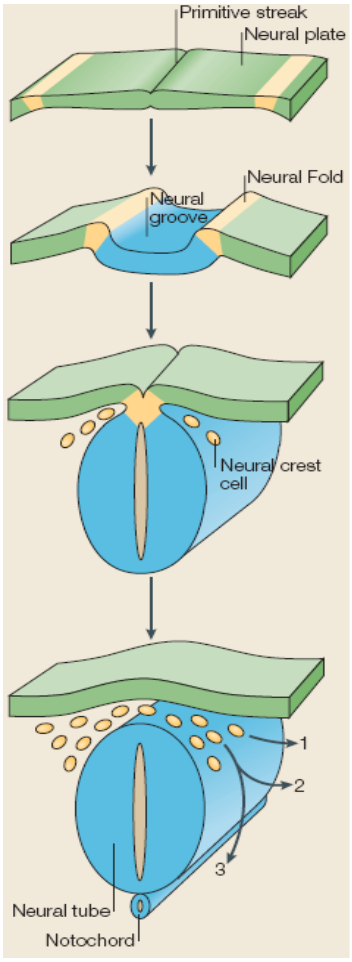

Figure 1.5. Neurulation and neural crest cells. After the formation of neural tube, neural crest cells migrate along one of the two major streams: in lateral direction (1) to give rise to melanocytes in the skin and in ventral direction (2,3) to give rise to neurons in dorsal root sensory ganglia and glia (2), or glia and autonomic neurons (3) [29].

Both neurons and Schwann cells in DRG originate from the neural crest cells, which migrate away from the dorsal side of the closing neural tube during the neurulation process (Figure 1.5) [32]. Neural crest cells give rise to Schwann cell precursor cells (around embryonic day 12-E12- in mice), which in turn give rise to immature Schwann cells (E15 in mice) [33]. Finally after birth, immature Schwann cells differentiate into one of the two types of mature Schwann cells; myelinating Schwann cell on larger diameter axons that are thicker than one micrometer (μm) or non-myelinating Schwann cells that

associate with smaller diameter axons and ensheath them to form C-fibres in Remak bundles (Figure 1.6) [34, 35].

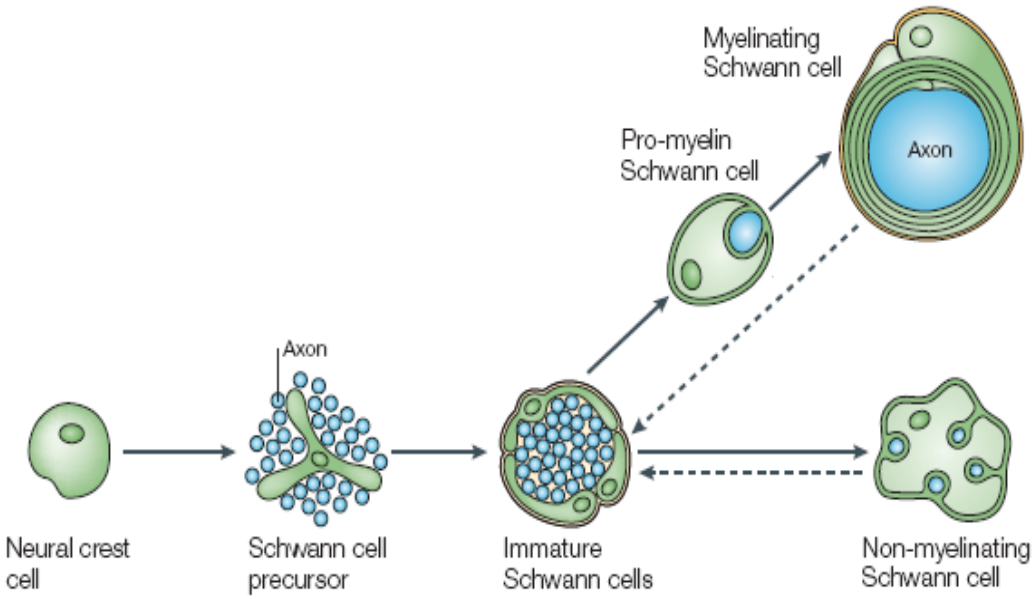

Figure 1.6. Schwann cell lineage [29].

The myelination process of PNS axons by Schwann cells can be categorized into three stages due to presence of different types of nerve fibres. In the immature, developing nerve, a large bundle of naked axons is surrounded by a single thin layer of Schwann cell cytoplasm, forming fetal nerve fibres (Figure 1.7). The establishment of direct axonal contact induces Schwann cell proliferation. As Schwann cells proliferate and send their processes deeper into the bundle to surround smaller bundles of axons, axons are gradually segregated. Subsequently, radial sorting is achieved and a one-to-one axon-Schwann cell relationship is formed. At the same time, the Schwann cell elongates laterally along the axon (Figure 1.8). By this way, promyelin nerve fibres are formed, where each Schwann cell-axon unit is surrounded by a basal lamina (Figure 1.9). Thereafter, the Schwann cell plasmalemma spirally wraps the axon, establishing the periaxonal space and the mesaxon. Subsequently, myelinated fibres occur: compact myelin lamellae can be distinguished from the non-compacted ones where internal (adaxonal) and external (abaxonal) mesaxons are composed of 2-3 membrane spirals [29, 36].

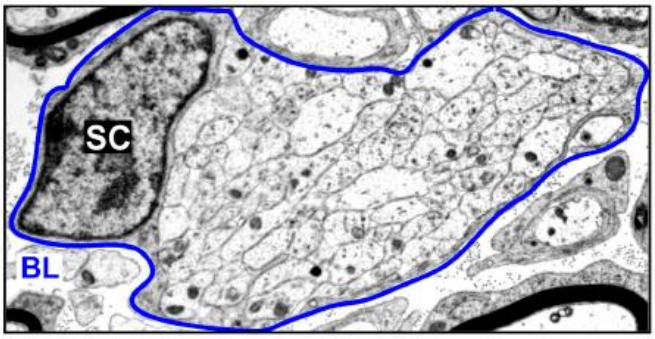

Figure 1.7. Schwann cell ensheathment of axon bundles to form a fetal nerve fibre. SC: Schwann cell nucleus, BL: Basal lamina [37].

Figure 1.8. Promyelin structure. This longitudinal view shows the one-to-one associated axon and Schwann cell. Schwan cell is aligned with the axon.

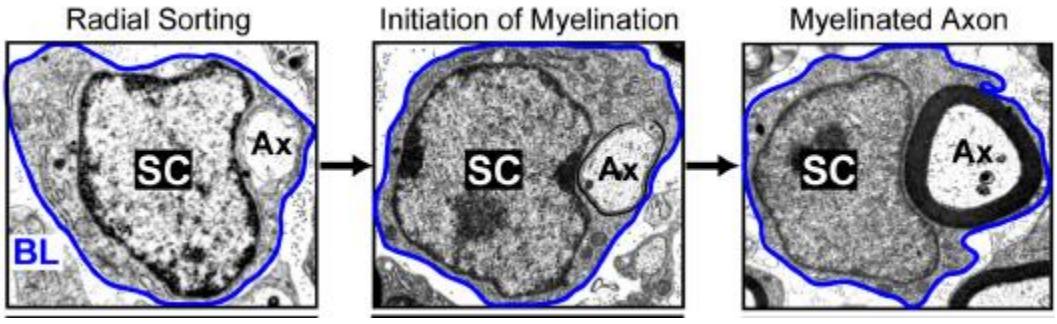

Figure 1.9. Radial sorting of an individual axon and myelination. The image on the left shows the promyelin (transverse section) formed during the radial sorting process. After then, the myelination is initiated (middle image) and this Schwann cell wraps the axons to form compact myelin (image on the right). SC: Schwann cell nucleus, Ax: Axon, BL: Basal lamina [37].

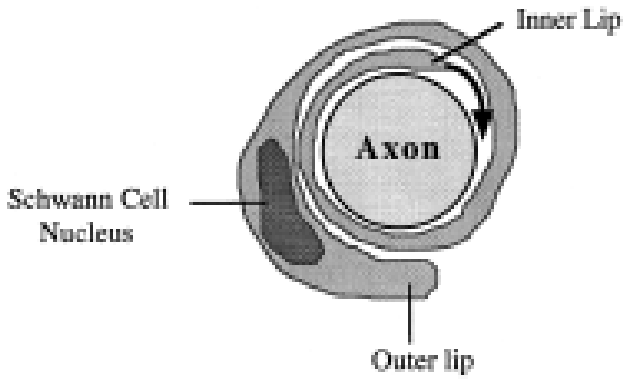

Figure 1.10. Myelin formation by progression of the inner lip [26].

Without ignoring the fact that the progress through these three steps does not occur synchronously, for mice it can be said that fetal nerve fibres start to be established at around E15. Before this event, during early development, axonal contact induces Schwann cell proliferation and Schwann cell precursors migrate and proliferate along the developing nerves. At E15, mostly Schwann cell precursors differentiate into immature Schwann cells and in contrast to precursors, immature cells have a basal lamina and their processes interact with axons, separating them into large bundles to form fetal nerve fibres. After birth, one-day-old mouse Schwann cells are proliferating at a high rate, segregating the axons into groups and are establishing one-to-one Schwann cell-axon association (radial sorting). Peripheral nerves at P1 have mostly fetal nerve fibres, but a few promyelin fibres and some rare myelinated fibres with a few lamellae of compact myelin. The Schwann cell in the fetal nerve fibre may commit itself to myelinate one of the axons in condition that one-to-one association is achieved and myelination signals are received or may ensheath a smaller bundle of axons as a nonmyelinating Schwann cell. During the following 4-5 days, due to the radial sorting event, the number of promyelin and myelinated fibres increases progressively and the number of fetal fibres decreases. Around the end of the first postnatal week active myelination period starts and Schwann cells start rapid synthesis of myelin proteins to form mature myelin sheath. Active myelination period covers approximately the first two postnatal weeks. At the age of one week mice have approximately 25 compact lamellae around the myelinated fibres. As the inner lip of Schwann cell membrane form new layers around the axon, the outer layers get compacted, which spares the membrane at

the periphery of each segment, forming the nodes of Ranvier, and the narrow channels that form the Schmidt-Lanterman incisures (Figure 1.10, Figure 1.11).

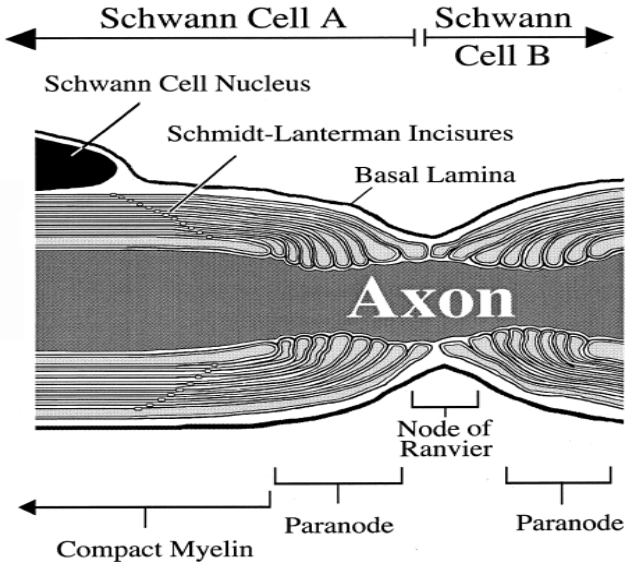

Figure 1.11**.** Schematic representation of a myelinated axon (longitudinal section).

Until P15, lamallae formation and lipid incorporation is very fast. After 14 days, there are only a few promyelin fibres left, and the number and size of the fetal fibres decrease. A large majority of the axons are thus ensheathed by their own Schwann cells and the myelinated fibres have an average of 36 lamellae of compact myelin [26, 38]. After this period, due to maturation of the animal, the myelination rate decreases. Axons get thicker slowing down the myelination radially, while the animal size and axon length increases slowing down myelination longitudinally. Thus, a slower myelin compaction period follows, after nearly two weeks of fast myelination process [39].

1.4. Molecular Control of Schwann Cells

During development, differentiation and myelination, Schwann cells proliferate, migrate, sort and ensheath axons. The whole process is controlled by specific transcription factors expressed at different times. Precursor Schwann cell survival depends on axonal

signals, but control of immature Schwann cell survival depends on autocrine signals. Immature Schwann cells can support their own survival by secreting some factors, such as insulin-like growth factor 2 (IGF2), neurotrophin 3 (NT3), platelet-derived growth factor-β (PDGF-β), leukaemia inhibitory factor (LIF) and lysophosphatidic acid (LPA) [29, 40]. This autocrine fashion is most probably required in the survival of Schwann cells upon nerve injury and degeneration. When postnatal nerves are injured, the switch from paracrine dependence on axonal signals to autocrine fashion guarantees the survival of Schwann cells for supporting regeneration [29].

Recent studies have shown some of the mechanisms involved in the complex myelination process, mostly regulated by by tyrosine kinase receptors, integrins, and G-protein-coupled receptors [8, 41, 42]. Neuregulin (Nrg1) acting *via* ErbB2/3 tyrosine kinase receptors is one of the axonal signals controlling the transcriptional events at the onset of myelination, during the myelination process, and in remyelination after nerve injury. Also at earlier stages, Nrg1 accelerates the transition of Schwann cell precursors to Schwann cells, as well as promoting precursor and immature Schwann cell survival and proliferation. The Nrg1 levels secreted from axons is known to control the decision of the axon to be myelinated or not [3, 9, 43, 44]. Nrg1 also up-regulates expression of transcription factors such as NFκB, Oct-6, and Brn2, that promote the promyelinating stage. At this stage, radial sorting takes place and early myelin markers are expressed due to upregulation of Krox-20, thus Schwann cells differentiate into myelinating phenotype. This differentiation is reversible as myelinating Schwann cells remain plastic. Upon axon transection, c-Jun is up regulated to provide Krox-20 down regulation so that rapid Schwann cell dedifferentiation (i.e., down regulation of myelin protein expression and up regulation of non-myelin markers) and proliferation occur, which in turn provide nerve regeneration and remyelination (Figure 1.12) [29, 45]. Nrg1/ErbB system is also involved in this remyelination process after injury [8].

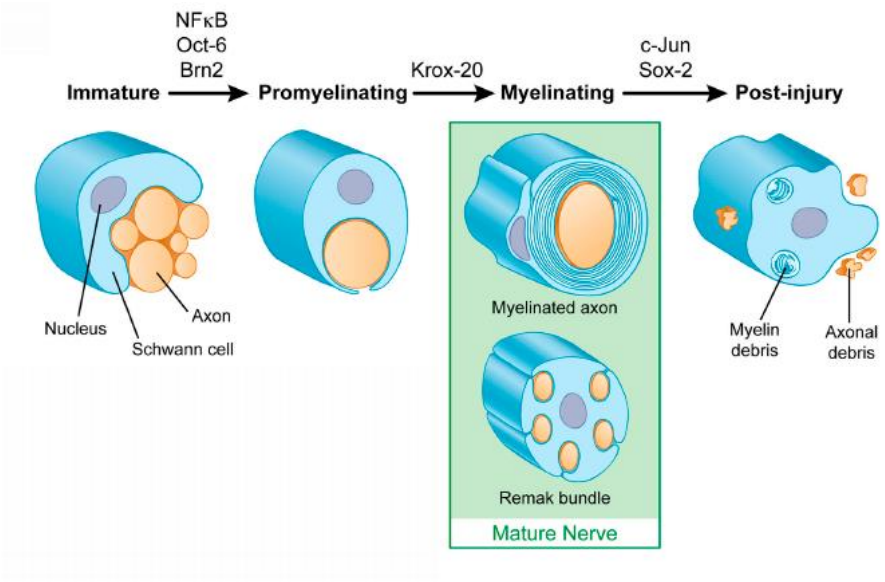

Figure 1.12. Regulation of Schwann cell myelination and dedifferentiation [12].

Downstream molecules activated *via* Nrg1 signaling include mitogen-activated protein kinase (MAPK-MAP kinase), phosphatidylinositol-3-kinase/v-Akt murine thymoma viral oncogene (PI3K/Akt), phospholipase Cγ (PLC), and Rac1 and all of which have been implicated in myelination [8]. Specifically, mutation of the MAP kinases extracellular regulates kinases1/2 (Erk1/2) or disruption of MAPK signaling due to lack of the cytoplasmic tyrosine phosphatase Shp2, negatively effects Schwann cell development and myelination [46, 47]. Mammalian target of rapamycin (mTOR) is also one of the other molecules recently implicated in the myelination process [48].

Type II transforming growth factor-β (TGF-β) is another mitogen acting on Schwann cells. In contrast, nerve growth factor (NGF) signaling through p75 neurotrophin receptor ($p75^{NTR}$) acts on Schwann cell death in newborns following injury [3, 49].

Schwann cell migration is also affected by many factors in cell culture and probably *in vivo* these signals regulate Schwann cell movements during radial sorting. These molecules include Nrg1, insulin-like growth factor (IGF), Neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF). P38-MAPK pathway is also implicated in the early myelination events regarding Schwann cell motility such as alignment of Schwann

cells with axons [29]. Lastly, the basal lamina extracellular matrix component, laminin, is implicated in Schwann cell migration. Laminin signaling is proposed to activate the Rho family GTPase Rac1 in Schwann cells *via* the laminin receptors β1-integrins present on Schwann cells. This process is thought to promote radial sorting and subsequent myelination of axons [41, 50]. The basal lamina also forms cellular asymmetry and a polarity axis, that determines the orientation of the Schwann cell to the extracellular environment and to the axonal membrane to be myelinated [51].

1.5. Fibroblast Growth Factors

Fibroblast growth factors (FGFs) comprise 17-34 kDa proteins involved in angiogenesis, wound healing, and embryonic development [52]. Currently 22 different types of mammalian FGFs (FGF-1 through FGF-23) are known. Mammalian FGFs are grouped into seven subfamilies according to their sequence similarities and functional properties (Figure 1.13) [53].

FGFs exert their effects through fibroblast growth factor receptors (FGFRs) and are known to bind heparan sulfate proteoglycans (HSPGs) in order to provide receptor dimerization. Most FGFs (FGFs 3-8, 10, 17-19, 21 and 23) are secreted *via* their amino-terminal signal peptides. On the other hand, FGF- 9, 16, and 20 lack an amino-terminal signal peptide but are still secreted. FGF1 and FGF2 also lack signal peptides and are secreted by non-canonical pathways [53]. FGFs 11–14 are not secreted and do not activate FGF receptors. However, they localize to the cell nucleus. Moreover, FGF1, FGF2, and FGF3 can be translocated to the nucleus *via* their nuclear localization signals [52, 54].

Fibroblast growth factors are expressed both in embryonic and adult tissues. FGF-3, 4, 8, 15, 17 and 19 are expressed during embryonic development, while others are expressed both in the embryo and the adult. During mammalian embryonic development, FGFs regulate cell proliferation, migration, and differentiation as well as nervous system development [55].

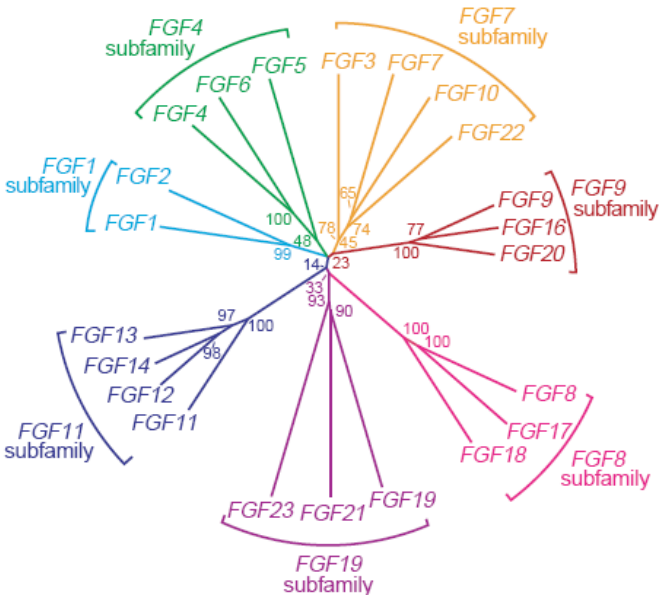

Figure 1.13**.** The evolutionary relation of the human FGF members. Evolutionary distances between each gene are symbolized by branch lengths. The percentage of times that a node was supported in 1000 bootstrap pseudo-replications is given at the branch-points [53].

1.6. Fibroblast Growth Factor Receptors

FGF receptors are members of the receptor tyrosine kinase (RTK) superfamily. There are four types of FGFRs encoded by different genes: FGFR1, FGFR2, FGFR3, FGFR4. FGF receptors consist of a single transmembrane domain, an extracellular domain containing three Ig-like domains (D1-3), a signal peptide (SP), an acidic box (A), a heparin binding region, a cell adhesion molecule homology domain (CHD) and an intracellular domain with tyrosine kinase domain (TK), which is subdivided into TK1 and TK2 (Figure 1.14).

Alternative splicing generates FGFR isoforms, some of which are secreted due to loss of TM domains. Some isoforms lack one of the Ig-like domains. More importantly, alternative splicing within the third extracellular immunoglobulin loop in FGFR1–3 generates IIIb or IIIc isoforms (Figure 1.15) [52].

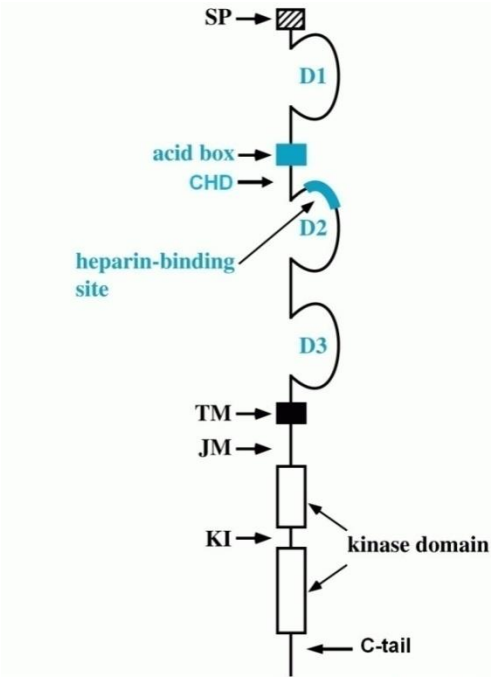

Figure 1.14**.** FGFR structure (modified from Powers *et al.*, 2000) [54].

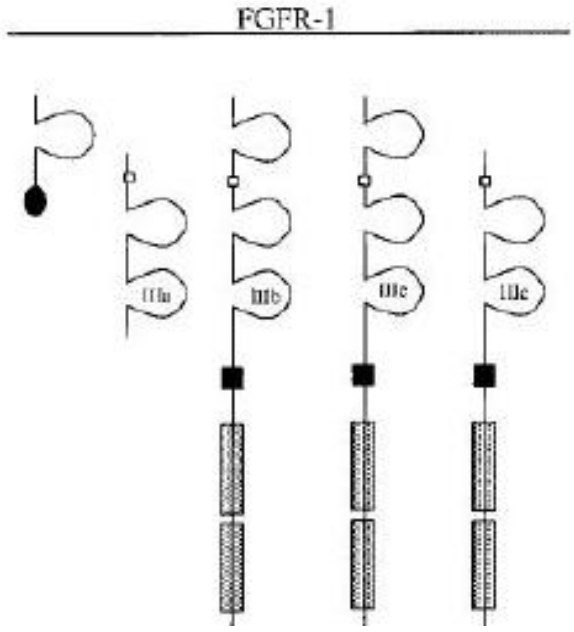

Figure 1.15**.** FGFR1 isoforms. Isoforms are generated by alternative splicing of Ig-III domain [54].

Table 1.1. Ligand specificity of fibroblast growth factor receptors.

FGF	Preferred FGFR
FGF1	All
FGF2	R1, R3 > R1-IIIb, R2 >> R4
FGF9	R3 > R2>R3-IIIb >> R1-IIIc

FGFs interact with the Ig-like domains II and III. DIII structure determines the ligand-binding specificity of the receptor. Therefore, isoforms of FGFRs spliced within the Ig-III domain, differ in their specifities to bind different ligands [54]. Ligand binding preferences of FGFR isoforms are shown in Table 1.1 [52, 56-59]. It should be noted that FGF1 can bind to all isoforms of all FGFRs with high affinity. Binding of heparan sulfate that is the low affinity receptor of FGFs, is mediated through the DII domain. The acid-box, a short string of 7-8 acidic residues interacts with the heparan sulfate binding site and interferes with receptor activation in the absence of FGFs.

1.7. FGF/FGFR Signaling

All FGFs require to bind heparan sulfate proteoglycans located on the cell surface, to provide receptor dimerization and form stabilized FGF-FGFR complex (Figure 1.16) [60, 61]. Heparan sulfate binding was suggested to stabilize and prevent degradation of FGFs, serve as storage reservoirs where FGF can be freed to bind FGFRs, and to facilitate FGF-FGFR interaction by causing conformational change of FGF [54, 62, 63].

FGF/FGFR signal transduction is initiated upon binding of FGF ligand to FGFR in conjunction with heparan sulfate to form a ternary complex (FGF:FGFR:HPSG - 2:2:1) (Figure 1.16) [52, 62, 64]. Upon binding of the ligand, FGFR monomers dimerize and tyrosine kinase domains autophosphorylate each other. Phosphorylation of different tyrosine residues on the cytoplasmic site creates different docking sites for downstream proteins. Proteins with Src homology domain 2-3 (SH2-3) and phospho-tyrosine binding domains (PTB) can bind to this cytoplasmic site for further propagation of the signal [65].

A

B

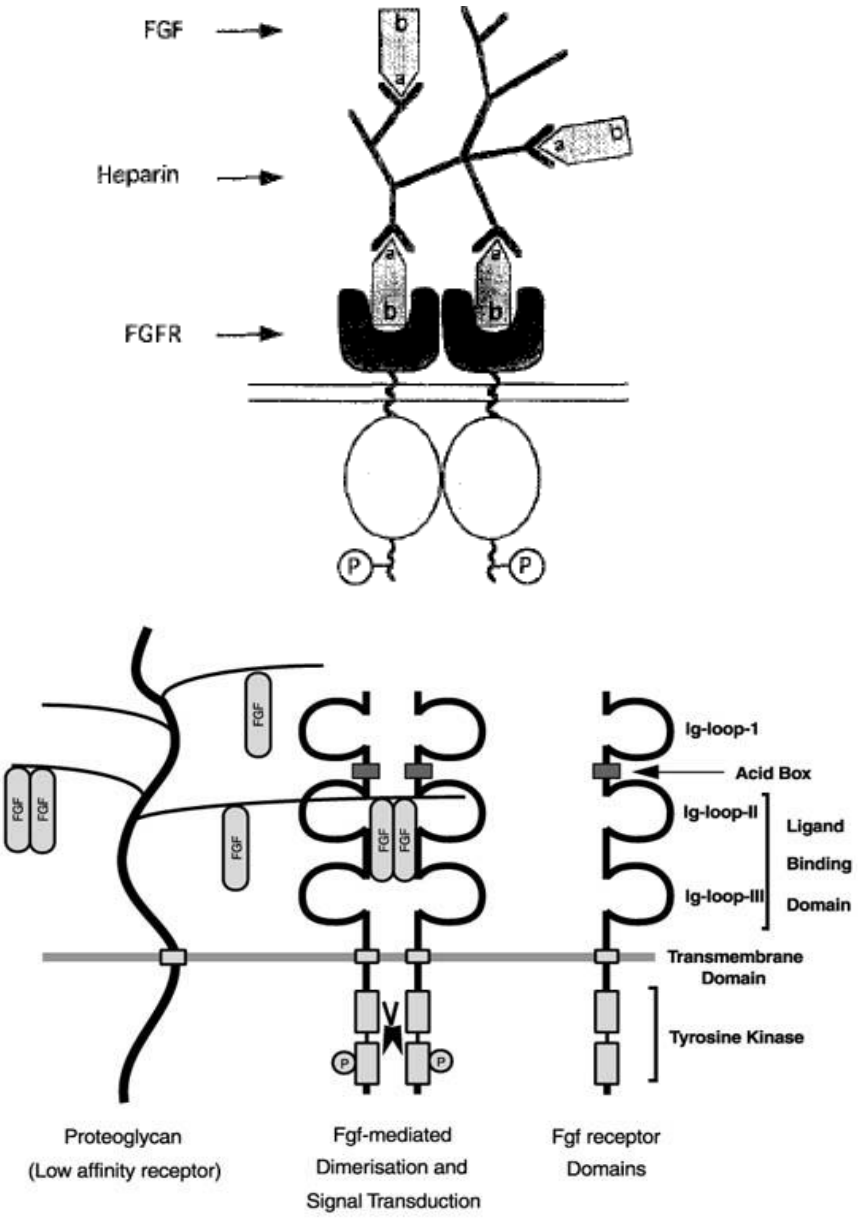

Figure 1.16. FGF-heparin induced activation of FGFRs. A) Heparin induced dimerization of FGFRs. B) Activation of FGFRs (Modified from Spivak-Kroizman *et al.*, 1994) [64].

The three major signal transduction pathways activated by FGFRs include RAS/MAPK, phospholipase Cγ/protein kinase C (PLCγ/PKC) and PI3 kinase pathways (Figure 1.17). Upon FGFR activation, the docking protein FGF receptor substrate 2 (FRS2), that contains a PTB domain, binds FGFR and recruits growth factor receptor-bound protein 2 (Grb2) and son of sevenless (SOS) complex, which in turn activates the

MAP kinases Erk1/2. Erk1/2 translocate to nucleus and activate transcription factors [52]. Transient activation of ERK *via* FGF signaling is known to promote proliferation and migration of PC12 cells, while its sustained activation causes their differentiation [66].

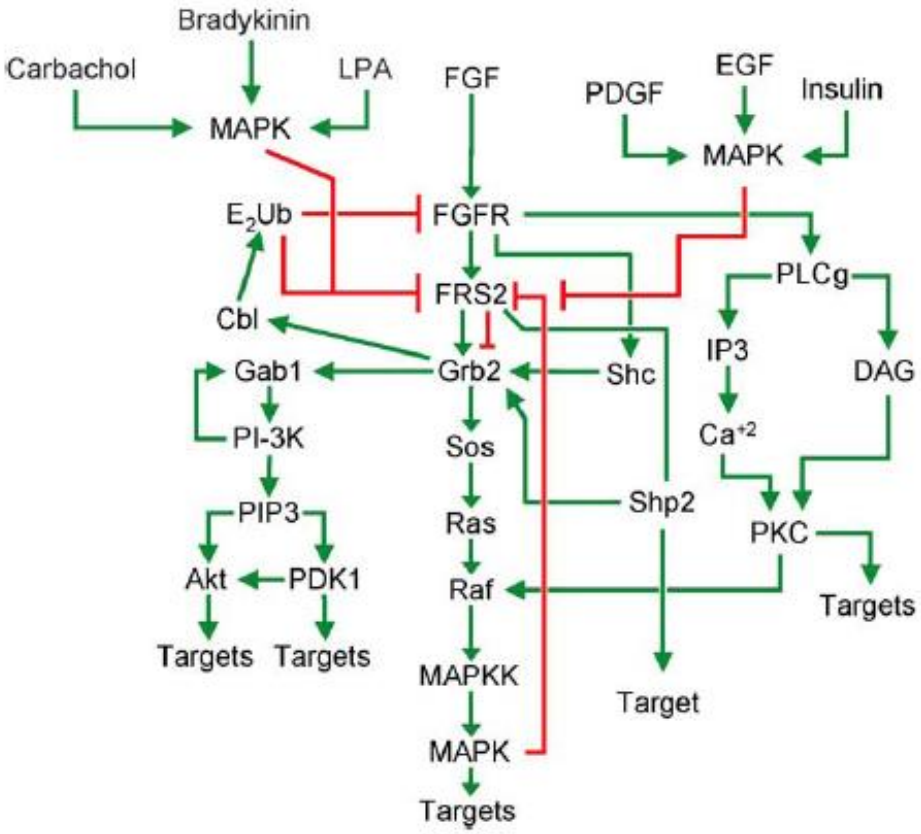

Figure 1.17**.** FGF signal transduction pathways. The negative signals are shown by red lines [57].

FGF signaling can also activate p38 and c-Jun N-terminal kinase (JNK) cascades in a cell type specific manner. Erk1/2 pathway is involved in cell proliferation and migration but p38 and JNK MAPKs inducing apoptosis, are generally associated with inflammatory or stress responses [67]. Grb2 can also associate with Grb2 associated binding protein 1 (Gab1) and recruit PI3K, which in turn activates the AKT/PKB pathway. AKT/PKB is the main pathway for preventing apoptosis and providing growth factor dependent cell survival [52]. PLC/PKC pathway is activated by direct interaction of PLC that has Src

Homology (SH) 2 domain and FGFRs. This results in phosphatidylinositol (PI) hydrolysis and the generation of the two secondary messengers, diacylglycerol (DAG) and Ins(1,4,5)P3 (IP3). IP3 provides mobilization of Ca^{2+} from the intracellular stores that influence calcium/calmodulin dependent protein kinases and DAG activates PKC which in turn promotes MAPK activation [57].

Inhibition of signaling *via* FGFR is achieved by the negative regulators of growth factor-mediated MAPK pathway: FRS2, Sprouty and Sef proteins and Cbl recruited by Grb2 [52, 68].

1.8. Fibroblast Growth Factors and Receptors in the Nervous System

Although FGFs have been studied extensively in the CNS, there is limited information about their expression and modulation in the PNS. In the CNS, FGFs have been shown to be involved in neurogenesis, differentiation, axonal branching, and neuron survival. FGFs also act in repair processes upon different brain and peripheral nerve lesions and degenerations. In addition, in the adult brain FGFs function in cognitive processes such as learning and memory [69].

1.8.1. Expression of Fibroblast Growth Factors and Receptors in the Nervous System

In the CNS, FGF2 was shown to be expressed by both neuronal and glial cells, while FGF1 and FGF9 were predominantly localized to neurons. FGFR1 is expressed in both neurons and glia, whereas FGFR2 and FGFR3 is expressed primarily in glial cells [10, 58, 69-74]. FGFR4 was shown to be strongly expressed only during early stages of development, and was not detectable other than a small region of the lateral habenular nucleus in the adult CNS [69].

Fibroblast growth factors have been studied less extensively in the PNS, except FGF2 that was linked to peripheral nerve regeneration upon injury. FGF1, FGF2, FGF7 and FGFR3 were shown to be up regulated in the neuron bodies after peripheral nerve

lesion, while the levels of FGFR1 and FGFR2, that were always expressed at high levels, did not change [11, 75]. FGF2 is proposed to trigger neuronal cell death, after nerve lesion *via* FGFR3 and to promote neuronal survival and Schwann cell proliferation and inhibition of myelination *via* FGFR1/2 [11]. Exogenous application of FGF2 was also shown to promote neurite outgrowth through FGFR3 [75].

In the developing nervous system, FGF2 was shown induce proliferation of the neuroepithelial cells that give rise to mature neurons and glia. It was also shown that FGF1 and FGF2 effect proliferation of neural crest cells. FGF2 mRNA was detected in the neural tube and in newly formed sensory ganglia of E10 mouse, where neural crest cell precursors are proliferating [10].

FGF1 expression was examined in rat DRG, where FGF1 and FGFR1-2 transcripts were detected in DRG neurons but not in Schwann cells [76]. FGF1 mRNA and protein were detectable starting from the embryonic day 18 (E18), and thereafter their levels increased progressively up to postnatal levels. In DRG neuron culture (from E15 mouse), FGF1 was detectable after 3 days *in vitro* (div).

Although FGF9 was originally isolated from the human glioma cell culture, numerous studies showed that FGF9 is predominantly expressed in neurons in the CNS, and that, FGF9 functions as a glia-activating factor (GAF) to promote the differentiation and survival of glia [58]. FGF9 was also shown to act in maintenance of the CNS [77, 78]. FGF9 transcript was shown to be present in ventral horn motor neurons of adult rat as well as E14 and new born mice [79, 80] and motor neuron derived FGF9 was proposed to promote survival of motor neurons in an autocrine and/or paracrine mechanism [80-82]. Moreover, neuronally secreted FGF9 was proposed to be mutagenic for retinal glia [83].

In DRG, presence of FGF9 transcript and protein were demonstrated by cDNA array and immunohistochemistry, respectively. By *in situ* hybridization, FGF9 transcript was localized to some large DRG neurons but not to glial cells [79, 84]. However, glial expression of FGF9 was reported in the CNS by an *in vivo* study [85].

FGF9 dependent up regulation of FGFR1 and FGFR3 and down regulation of FGFR2 and myelin proteins in oligodendrocyte cultures were also reported in a study [86]. Developmentally expressed FGFR1 and FGFR3 were found to be absent in myelin, while FGFR2 was shown to be present in myelin compartments. Thus, it was proposed that FGF9 blocks myelination and promotes oligodendrocyte development *via* FGFR1 and FGFR3. A newer study in the cerebellum localized FGF9 transcripts to neurons and FGF9 protein to both neurons and glia [87]. This study proposed that neuronal FGF9 acts on the development of Bergmann glia *via* FGFR1 and FGFR2. Moreover, FGF9 was shown to have no effect on DRG neuron survival *in vitro* [80].

1.8.2. Implications of Fibroblast Growth Factors and Receptors in Myelination

Three recent studies published focused on FGFR1 and FGFR2 and implicated FGF signaling in myelination. Conditionally double mutant mouse lacking FGFR1/2 in nonmyelinating Schwann cells was found to have axonal degeneration in C-fibres [88]. In another study, FGF receptor signaling was implicated in neurodegenerative hearing loss *via* loss of myelinated spiral ganglion [89]. A newer study reported that active myelination taking place at the first two weeks was inhibited in double mutant mice that lack FGFR1/2 expression in oligodendrocytes [90]. Consequently, in adulthood, the myelin sheath was thinner and although the number of oligodendrocytes was normal, the production of major myelin genes was reduced. This hypomyelination was linked to reduced Erk1/2-MAPK activity by further analysis.

1.9. Previous Studies Performed in Our Laboratory

FGF1, FGF2, FGF4, FGF9 and FGFR1-3 are known to be expressed in the nervous system and implicated in CNS development. Dr. Erkut from our laboratory, investigated transcript levels of these molecules in mouse DRG tissue through the postnatal developmental period to adulthood [91]. He showed that FGF1, 2, 9 and FGFR1-3 are produced in the DRG tissue. FGF1 transcript level was the highest among the three, followed by FGF9 and FGF2 levels. FGF1 and FGF2 transcripts increased during early myelination and then remained stable. FGF9 increased in early myelination and continued

in a lower steady state levels in adulthood. He also performed Western blotting for FGF1 from DRG tissue and showed that FGF1 protein expression started at the onset of active myelination reaching to a maximum at the point where myelination rate was the highest.

FGFR1 transcript levels were comparably higher than other FGFRs. Its level significantly increased at the time of fast myelination and was stable at the same level thereafter. FGFR2 expression was unchanged during development and adulthood. FGFR3 transcript level was also stable during the developmental period and continued at a lower level in adulthood.

These findings suggested that expression of these molecules were modulated in parallel with the progression of myelination and changes in their levels occurred at key time points of this process. Thus, in our study, we investigated the expression and possible functions of FGF1, FGF2, FGF9, and FGFR1-3 during peripheral nerve myelination.

2. AIM OF THE STUDY

Our aim in this study was to investigate:

(i) Whether FGF1, FGF2, FGF9 and their high affinity receptors FGFR1-3 are expressed in the peripheral nervous system;

(ii) Localizations of FGF1 and FGFR1-3 in the time course of peripheral nerve myelination and in the myelinated nerve;

(iii) Possible roles of FGF1 in the myelination process, using DRG neuron-Schwann cell cultures.

To accomplish the aims stated, we asked the following questions and used experimental approaches described briefly to find their answers:

(i) Which PNS model can be used to investigate their expression? We used DRG and the sciatic nerve from mice as *in vivo* models and Schwann cell-neuron cultures developed from fetal mouse DRG tissue as an *in vitro* model.

(ii) We knew that these molecules and the three receptors are expressed at the RNA level in DRG from our initial studies. Are they expressed at the protein level in DRG and sciatic nerve tissues and in DRG cultures? If they are expressed, where are they localized? Do their expression profiles fit with the proposed expectation in which an effector molecule is expected to be expressed by the neuron while its receptor by the Schwann cell? These questions were answered *via* immunolabeling analyses.

(iii) FGF1 was chosen as a candidate for the myelination process and /or maintenance of the myelin sheath because of its high level of expression in all tested tissue types with an axonal localization and investigated for its possible function in myelination.

(iv) Are FGF1 and its high affinity receptors modulated in parallel with the progress of myelination? We examined protein expression levels through the development to adulthood from sciatic nerves by Western blotting.

(v) What is the function of FGF1 in the myelination process? We investigated the effect of FGF1 blockage on the expression levels of the myelin proteins and different downstream molecules of FGF/FGFR pathway in DRG cultures.

3. MATERIALS

3.1. Mice

Mice were provided by the animal facility in Bogazici University Center for Life Sciences and Technologies. C57BL/6J mice were maintained on a 12 hour light / 12 hour dark cycle at 21 - 25 °C. Food and water were provided *ad lib*. The study was approved by Bogazici University Ethics Committee an all use and handling of animals were in accordance with the committee guidelines.

C57BL/6-Tg (ACTbEGFP)1Osb/J mouse strain that produces beta-actin-EGFP was used to prepare DRG cultures in the short term study performed in the frame of this Ph.D thesis at Åsa Fex Svenningsen's laboratory, at Southern Denmark University, Odense, Denmark.

3.2. Chemicals, Buffers and Solutions

Chemicals used in this study were obtained from Sigma Aldrich (USA), Riedel de-Häen (Germany) or Merck (Germany), solutions and mediums used in DRG culture experiments were obtained from Invitrogen (USA), unless stated otherwise. Glassware and plastic consumables were sterilized by autoclaving at 121 °C for 15 minutes unless they were provided as sterile.

Compositions of buffers and solutions are explained in Table 3.1-3.2.

Table 3.1. Buffers and solutions for Western blot analysis.

10% Sodium dodecyl sulfate (SDS) polyacrylamide gel (running gel)	375 mM Tris(hydroxymethyl)aminomethane (Tris) Cl (pH 8.8) 10% Acrylamide:Bisacrylamide (37.5:1) 0.1% SDS 0.1% Ammonium persulfate (APS) 0.1% N,N,N',N'-tetramethylethylenediamine (TEMED)
4.5% SDS-polyacrylamide gel (stacking gel)	125 mM Tris-Cl (pH 6.8) 4.5% Acrylamide:Bisacrylamide (37.5:1) 0.1% SDS 0.1% Ammonium per sulfate (APS) 0.1% TEMED
6X Sample Buffer	300 mM Tris-Cl (pH 6.8) 12 mM Ethylenediaminetetraacetic acid (EDTA) 60% Glycerol 12% SDS 6% 2-mercaptoethanol 0.04% Bromophenol blue
Running Buffer	25 mM Tris-Cl 250 mM Glycine 0.2% SDS
Transfer Buffer	25 mM Tris-Cl 200 mM Glycine 15% Methanol
Coomassie Blue Solution	50% Methanol 10% Acetic acid 0.05% Coomassie R250
Destaining Solution	7% Acetic acid 5% Methanol
Fixing Solution	10% Acetic Acid 20% Methanol

Table 3.1. Buffers and solutions for Western blot analysis (cont.).

Stripping Solution	62,5 mM Tris-Cl (pH 6.8) 2% SDS 0.7% beta-mercaptoethanol
Radio-immuno-precipitation Assay (RIPA) Buffer	1 M NaCl 1% Triton X-100 0,5% Deoxycholate 0,1% SDS 50 mM Tris 2 mM EDTA Complete mini protease inhibitor cocktail (Roche, Germany)
Blocking Solution	1% skimmed milk powder or 1% bovine serum albumin (BSA) in Tris-buffered saline-Tween-20 (TBST)
TBST	0.1% Tween-20 in TBS
Tris-Buffered Saline (TBS)	20 mM Tris-Cl (pH 8.0) 150 mM sodium chloride (NaCl)

Table 3.2. Buffers and solutions used for tissue processing and immunolabelings.

Blocking Solution	0.25% Triton X-100 0,25 % BSA in PBS
Dabco mounting medium	2.5% DABCO 50 mM Tris pH8 90% Glycerol
DAPI (Roche, Germany)	0,02 µg/ml in TBS
Paraformaldehyde Solution (PFA)	4% PFA in PBS
Sucrose Solution	20% Sucrose in PBS
Washing Solution	0.25% Triton X-100 in PBS

3.3. Antibodies

Primary antibodies used against mouse proteins and secondary antibodies used in this study are given through Table 3.3-3.6.

Table 3.3. Primary antibodies used for Western analysis.
* shows the antibodies that are gift from Åsa Fex Svenningsen's laboratory, from Denmark and ** shows the antibodies that are gift from Ibrahim Yaman laboratory from Bogazici University, Turkey

Antigen	Host	Dilution	Brand
Actin	Goat	1000	Santa Cruz Biotechnology
AKT**	Rabbit	1000	Cell Signaling Technology
CNP	Mouse	10000	Abcam
ERK**	Rabbit	1000	Cell Signaling Technology
FGF1	Rabbit	1000	Abcam
FGFR1	Rabbit	1000	Santa Cruz Biotechnology
FGFR2	Rabbit	1000	Santa Cruz Biotechnology
FGFR3	Rabbit	1000	Santa Cruz Biotechnology
MAG	Rabbit	1000	Santa Cruz Biotechnology
MBP	Mouse	1000	Stern-Berger
MBP*	Rabbit	2000	Abcam
MBP	Chicken	1000	Millipore
MBP *	Rabbit	2000	Åsa Fex Svenningsen's laboratory, Denmark
MPZ	Rabbit	1000	Santa Cruz Biotechnology
pAKT**	Rabbit	1000	Cell Signaling Technology
pERK**	Rabbit	1000	Cell Signaling Technology

Table 3.4. Primary antibodies used for immunolabelings.

* shows the antibodies that are gift from Åsa Fex Svenningsen's laboratory, from Denmark

Antigen	Host	Dilution	Brand
CNP	Mouse	4000	Abcam
DCX	Guinea pig	1000	Abcam
FGF1	Rabbit	400	Abcam
FGF2	Rabbit	400	Abcam
FGF9	Goat	200	Santa Cruz Biotechnology
FGF9	Rabbit	100	Abcam
FGF9	Goat	200	Santa Cruz Biotechnology
FGFR1	Rabbit	200	Santa Cruz Biotechnology
FGFR2	Rabbit	200	Santa Cruz Biotechnology
FGFR3	Rabbit	200	Santa Cruz Biotechnology
GFAP*	Rabbit	4000	Dako
GFAP*	Chicken	1000	Abcam
MAG	Mouse	2000	Chemicon
MBP	Mouse	1000	Stern-Berger Monoclonals
MBP*	Rabbit	1000	Abcam
MBP	Chicken	300	Millipore
MBP *	Rabbit	1000	Åsa Fex Svenningsen's laboratory, Denmark
Nestin*	Mouse	400	Biotrunsduction Labs
NF-200	Mouse (IgM)	250	Santa Cruz Biotechnology
NF-200*	Rabbit	500	Sigma Aldrich
S100-ß*	Mouse	200	InRo BioMedTek

Table 3.5. Secondary antibodies used for Western analysis.

Target	Host	Tag	Dilution	Brand
Goat	Donkey	Horse radish peroxidase	5000	Santa Cruz Biotechnology
Rabbit	Goat	Horse radish peroxidase	5000	Santa Cruz Biotechnology
Mouse	Donkey	Horse radish peroxidase	5000	Santa Cruz Biotechnology

Table 3.6. Secondary antibodies used for immunolabelings.

* shows the antibodies that are gift from Åsa Fex Svenningsen's laboratory, from Denmark

Target	Host	Fluorescent Tag	Dilution	Brand
Chicken*	Goat	Alexa 488	500	Dako
Chicken*	Donkey	Rhodamine Red-X	400	Jackson Laboratories
Chicken	Donkey	FITC	200	Jackson Laboratories
Chicken	Goat	Dye 488	500	Abcam
Goat	Donkey	Alexa 555	500	Molecular Probes
Goat	Bovine	Rhodamine Red-X	200	Santa Cruz Biotechnology
Guinea pig*	Donkey	Rhodamine Red-X	400	Jackson Laboratories
Mouse	Donkey	Alexa 488	500	Molecular Probes
Mouse	Donkey	Alexa 555	500	Molecular Probes
Mouse*	Donkey	FITC	400	Jackson Laboratories
Mouse-IgM	Donkey	FITC	400	Jackson Laboratories
Mouse-IgM	Goat	FITC	400	Santa Cruz Biotechnology
Rabbit	Donkey	Alexa 555	500	Molecular Probes
Rabbit	Donkey	Alexa 488	500	Molecular Probes
Rabbit*	Donkey	Rhodamine Red-X	400	Jackson Laboratories

3.4. Equipment

Equipment used in this study are given in Table 3.7.

Table 3.7. Equipment used in this study.

Autoclave	Model MAC-601, Eyela, Japan
Blotting apparatus	Mini Trans-Blot Cell, Bio- Rad, Italy
Balance	DTBH 210, Sartorius, Germany

Table 3.7. Equipment used in this study (cont.).

Centrifuge	Centrifuge 5415 R, Eppendorf, USA Spectrafuge 16M, Labnet, USA
Cryostat	Leica CM3050 S, USA
Deep Freezers	-20 ^{0}C Arçelik, Turkey
Documentation System	Raytest Stella, Bio-Rad, USA
Electrophoresis	Mini-Protean III Cell, Bio-Rad, Italy
Heat blocks	DRI-Block BD-2A, Techne, UK
Illuminator	41723-Series High Intensity Illuminator, Cole Parmer, USA
Homogenizor	MagNa Lyser, Roche, Germany
Magnetic Stirrer	Speed Safe Hanna Instruments, USA MK 418, Nüve, Turkey
Magnetic stirrers	KMO 2 Basic, IKA, Germany RCT Basic, IKA, Germany
Microscopes	S2026, Prior, UK Leica TCS SP5 Confocal Microscopy System, USA
Microwave oven	M1733N, Samsung, Malaysia
Power Supplies	PowerPac Basic, Bio-Rad, Italy
Refrigerator	+4 ^{0}C Arçelik, Turkey
Shaker	SL 350 Nüve, Turkey
Spectrophotometer	NanoDrop ND-1000 NanoDrop, USA
Vortex	Nuvemix Nuve, Turkey

4. METHODS

4.1. Poly-D-Lysine and Laminin Coating

Positive charge coating of 6-well plates (JetBiofil, China) was achieved by adding 1mg/ml Poly-D-lysine (PDL) (Sigma Aldrich, USA, P6407) solution into the wells and incubating the plates at 37 ^{0}C for one hour. Next, the plates were washed twice with dH_2O, and laminin solution (0.75 μg/cm^2) (Invitrogen, USA, 23017-015) was added to the wells. Plates were incubated at 37 ^{0}C for one hour and then washed twice with L15 medium (Gibco, USA, 11415-049). Plates were not let dry and kept at room temperature, with L15 medium inside until cells are seeded or for longer storage up to three weeks they were kept at 4 ^{o}C.

Round, glass coverslips (13 mm diameter) (Sarsted, Germany, 83.1840.002) were placed in a 10 cm Petri dish (JetBiofil, China) and coated with PDL as explained above. Then, coverslips were placed into 4 well plates (Nunc, USA, 179820) and coated with laminin following the same procedure explained previously.

4.2. Primary DRG Cultures

DRG cultures were prepared as described previously by Svenningsen *et al.* (2003) [92]. Pregnant mice were sacrificed by carbon dioxide inhalation. After the belly was rinsed with 70% ethanol, it was cut open and the whole uterus carrying the embryos was taken out into a sterile 10 cm Petri dish on ice. All procedures following this step were performed in cold L15 medium. Embryos were taken out of the uterus and transferred into a new Petri dish. The spinal cord, with the DRGs attached was dissected from each embryo and collected in another petri dish. DRGs were cut one by one using fine scissors and collected in the same petri dish. In order to dissociate the tissue, 0.25% trypsin (Invitrogen, 25200-072, USA) was added and the DRGs were incubated at 37 °C for 5 minutes. Tissue clumps were dissociated and placed back for an additional incubation of 10 minutes at 37 °C. Trypsin was inactivated by 100% FBS (Gibco, 10500-064, USA), cells were triturated completely and collected by 5 minute centrifugation at 800 rpm with a benchtop

ultracentrifuge. The pellet was resuspended in 1 ml of L15 medium. A sample was taken in and the cell density (cells/ml) was determined by the aid of a heamocytometer. The volume was completed to 10 ml with L15 medium and cells were recollected by centrifugation at 800 rpm for 5 minutes. The pellet was resuspended in the culture medium (NB, 2% B27, 1 mM L-glutamin, 0,1 µg/ml NGF 2,5S) (NB: Gibco, 21103-049, USA; B27: Gibco, 17504-044, USA; L-glutamin: Gibco, 25030032, USA; NGF 2.5S: Chemicon, NC011, USA) and seeded at a density of 100.000 cells/ml and incubated at 37 °C in the presence of 5% CO2. After allowing Schwann cell proliferation for 4-5 days, 50 µg/ml ascorbic acid (Sigma Aldrich, A4544, USA) was added to the culture medium to induce myelination. The culture medium including ascorbic acid was replenished every four days for six weeks, to obtain myelinating cultures.

4.3. Antibody Mediated FGF1 Blocking

For functional analysis, DRG cultures were prepared in 6-well plates. FGF1 activity was blocked in the cultures by addition of 0.5 µg/ml rabbit-anti-FGF1 neutralizing antibody (Abcam, ab9588) to the culture medium, together with ascorbic acid induction of myelination. Control cultures were treated with 0.5 µg/ml rabbit-IgG (Abcam, ab37415) targeting no specific epitope. Both experiment groups were maintained for six weeks and the culture medium containing ascorbic acid and FGF1 antibody or ascorbic acid and control antibody was replenished every four days during the experiment period. At the end of the experiment, both groups of cells were fixed and immunolabelled, where myelin sheaths were stained by anti-CNP antibody and axons were stained with anti-NF-H antibody. The complete area of each plate from each group was monitored and myelinating cells and axons in the whole plates were counted. The ratio of myelinating cell per total axon number (myelination index) of the experiment group was compared to that of the control grup. Nine plates of cultures were prepared for each experimental group.

A third group of cells was prepared for Western blot analysis. This group served as a control, representing the basal levels of the investigated proteins, on the start day of induction/treatment. At the time of the treatment of the first two groups explained above, this third group did not get any ascorbic acid or neutralizing/control antibody but instead

were collected directly for Western blot analysis. By Western blot analysis, the levels of myelin proteins, namely MAG, CNP, MBP, MPZ, PMP22 and also pAKT, pERK levels of the experiment group was compared to that of the two control groups. Nine plates of cultures were prepared for each experimental group. The experiment was performed in triplicates.

4.4. Preparation of Cell Lysates

Culture medium was aspirated and cells were washed with PBS containing protease inhibitor cocktail (Roche, 11873580001, Germany) and phosphatase inhibitors (Roche, 04906837001, Germany). Cells were scraped from the surface by a sterile cell scraper. The cell suspension was collected in 1.5 ml eppendorf and pelleted by centrifugation at 13000 rpm for 15 min at 4 ^{0}C in a bench-top microcentrifuge. Supernatant was discarded and cells were resuspended in RIPA buffer. Cells were lysed using ceramic beads (Roche, 03358941001, Germany) in MagneLyser homogenizor at 6500 rpm for 30 seconds. Cell debris was spinned down at 13,000 rpm for 15 min at 4 ^{0}C and the supernatant containing the total protein extract was transferred to a new tube. Total protein concentration was determined by bicinchoninic acid protein assay kit (Pierce, USA).

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4.5. Preparation of Sciatic Nerve Lysates

Sciatic nerves from P1, P7, P10, P15, P20 and adult mice were dissected. The tissue was homogenized in RIPA buffer (100 µl/100 mg tissue) by the aid of ceramic beads using MagneLyser homogenizor with the setting of 6500 rpm - 60 seconds. Cell debris was removed by centrifugation at 13,000 rpm for 20 min at 4 ^{0}C. Supernatant containing the total protein extract was collected in a new tube. Total protein concentration was determined using bicinchoninic acid protein assay kit (Pierce, USA).

4.6. Western Blot Analysis

Fifty µg of sciatic nerve tissue lysate or DRG culture lysate was mixed with 6X protein sample buffer. Protein denaturation was achieved by 5 min incubation at 70 °C. Samples were loaded in 10% SDS-polyacrylamide gels. Gels were run at constant current

(20 mA/gel) for 2 hours. Proteins were electro-transferred to polyvinyl difluoride (PVDF) membranes (Roche, Germany) in transfer buffer at 100 V for 15-120 minutes depending on the size of the protein of interest (1 min per 1 kDa). The membrane was washed briefly in TBS and blocked at RT for 1 hour. Primary antibody incubation was performed overnight at 4 °C. Excess antibody was washed away three times with TBST for 10 minutes each. Horseradish peroxidase (HRP)-conjugated secondary antibody incubation was performed at room temperature for 1 hour. After washing 10 min with TBST for three times, blots were incubated in Lumi-Light Western blotting substrate (Roche, Germany) for 5 minutes and were visualized by chemiluminescence detection system (Stella). For analysis, protein levels were normalized to corresponding actin levels.

4.7. Preparation of Silanized Slides

The glass microscope slides were positively charged using 3-aminopropyltriethoxysilane (Sigma Aldrich, USA). For this purpose, slides were washed in detergent for 30 minutes. They were rinsed under running tap water for 30 minutes and washed twice with distilled water for 5 minutes. They were incubated in 95% alcohol twice for 5 minutes each and air dried for 10 minutes. Slides were immersed in a freshly prepared 2% solution of 3-aminopropyltriethoxysilane in acetone for 5 seconds. The excess liquid was discarded and slides were briefly washed twice in distilled water. Slides were dried overnight at 42 ^{o}C and stored at room temperature.

4.8. Cell, Tissue Processing and Immunolabeling

DRG cultures were fixed with 4% PFA and washed three times for 10 minutes with PBS and immunolabeled.

DRG and sciatic nerve tissues from P1, P10, P20 and three months old adult mice were dissected, fixed with 4% PFA for one hour and washed three times for 10 minutes with PBS. Tissues were cryoprotected by overnight incubation in 20% sucrose and frozen. Sections of 12 μm thickness were taken onto silanized or commercially available positively charged slides, air dried and frozen until immunolabeling.

For experiments to analyze FGF9, 15 days old mouse embryo was fixed for three hours in 4% PFA, washed in PBS overnight, cryopreserved in 20% sucrose for two days, embedded in the OCT medium, frozen and sectioned at a thickness of 20 μm.

Sciatic nerves were dissected from adult mice and transferred to cold PBS solution. Short pieces were cut and individual nerves in the bundles were separated on positively charged slides. Tissues were air-dried, fixed with 4% PFA and washed three times for 10 minutes with PBS. Prepared samples were either cryoprotected in 20% sucrose and frozen or directly immunolabeled.

For immunolabeling experiments, frozen tissues or cells were rehydrated by washing three times with PBS for 10 minutes each. Afterwards, samples were made permeable and blocked at the same time in blocking solution for one hour at room temperature. Samples were incubated overnight in primary antibody diluted in blocking solution, at 4 °C. Excess antibody was washed away three times with washing solution for ten minutes each. Samples were incubated with the fluorescent dye conjugated secondary antibody diluted in blocking solution for two hours at RT, at dark. DAPI labeling was performed using 0.01 μg/ml DAPI in TBS for 5 min. Samples were washed three times with washing solution for 10 minutes each. Samples were mounted using DABCO mounting medium.

The antibodies against marker proteins used in immunolabeling experiments and their target structures are given in Table 4.1.

Table 4.1. Marker proteins used in this study.

Marker protein:	To visualize:
Neurofilament-H (NF-H)	Axons and mature neurons
GFAP	Nonmyelinated Schwann cells
S100	Glial cells
Double-cortin (DCX)	Young neurons
Nestin	Stem cells in CNS
CNP	Mature myelin sheath

Table 4.1. Marker proteins used in this study (cntd.).

Marker protein:	To visualize:
MBP	Mature myelin sheath
MAG	Myelinating Schwann cell

4.9. Statistical Analysis

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software version 16. The distribution of the data was analyzed for normality and equality of variances between groups was tested. Differences between experiment groups were analyzed using Student's T-test or one-way Anova. Confidence interval of 95% was used as the criterion of statistical significance ($p<0.05$).

5. RESULTS

A previous study performed by Dr. Cihan Erkut indicated that FGF1, FGF2, FGF9 and FGFR1-3 mRNA were expressed in mouse dorsal root ganglion tissue [91]. In this thesis study, to confirm these findings at the protein level, DRG cultures from E13 mice were prepared. Young, premyelinating cultures were analyzed by immunocytochemistry. FGF1 was selected for further investigation since it was, localized to axons, whereas its receptors, FGFR1, -2 and -3 were found on Schwann cells. Because, this would mean that FGF1, produced in the neuron could potentially be released and act on Schwann cells.

5.1. FGF2 Expression and Localization in DRG Culture

To investigate the *in vitro* localization of FGF2, DRG cultures from fetal mice were prepared and immunolabeled. DRGs harbor both neurons and a certain amount of Schwann cells, thus this tissue itself enables to obtain a neuro-glia co-culture and eliminates additional separate purification steps of neurons and Schwann cells.. In DRG culture, FGF2 protein was detectable in both neurons and Schwann cells (Figure 5.1A). Its expression continued on Schwann cells that aligned with axons (Figure 5.1B). Moreover, FGF2 expression increased on both these Schwann cells and axons upon induction of myelination (Figure 5.1B). Initiation of myelination requires the contact of Schwann cells and axons and the alignment of Schwann cells with axons. However, since FGF2 was expressed by both neurons and glia, and as it has previously been implicated in nerve regeneration and remyelination after injury [75], it was not included in further investigations.

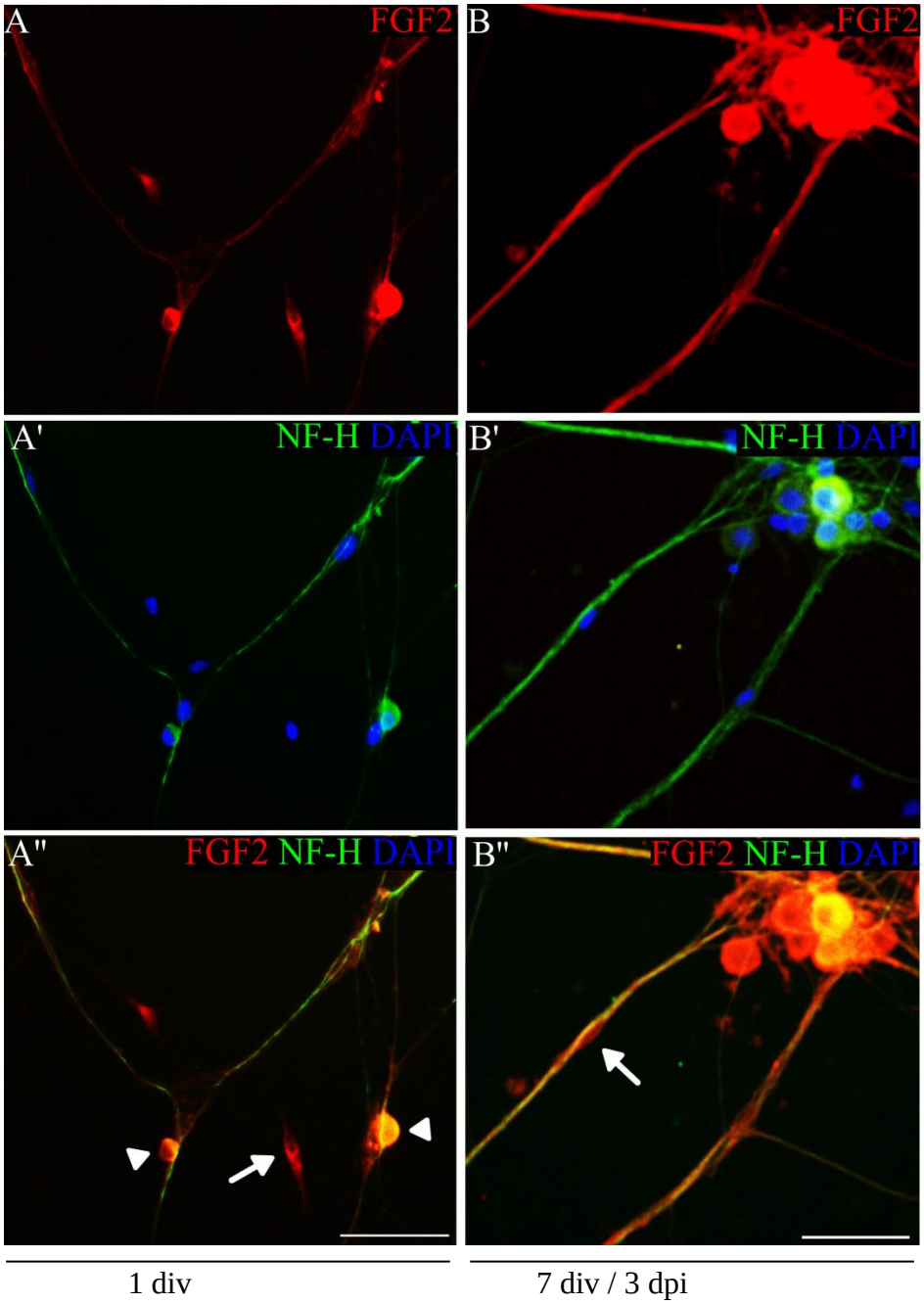

Figure 5.1. FGF2 immunolabeling in DRG culture. A-A'') 1 div (1 day *in vitro*) DRG culture. Arrow, a migrating Schwann cell; arrowheads, neuron bodies; B-B'') 7 div culture, (3 dpi; 3 days post induction). Arrow, a Schwann cell aligned with an axon. NF-H labels neuron bodies and axons; DAPI labels nuclei. A, B) FGF2 (red); A', B') Merge of NF-H (green) and DAPI (blue); A'', B'') Merge of FGF2, NF-H and DAPI. Scale bars: 50 µm.

5.2. Developmental FGF9 Expression and Localization

To investigate the localization and expression pattern of FGF9, DRG cultures, mouse embryonic (E15) frozen sections, and adult mouse sciatic nerve sections were immunolabeled.

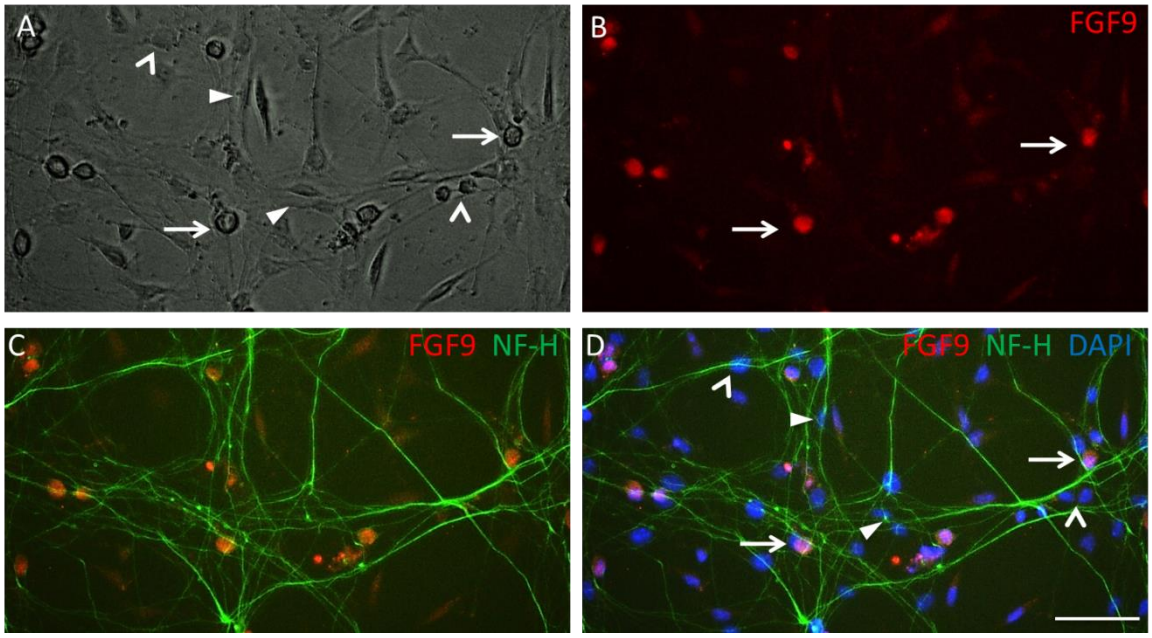

Figure 5.2. Localization of FGF9 in 1-div DRG-Schwann cell culture. A) Brightfield image; B) FGF9 labeling (red); C) Merge of FGF9 and NF-H (green) labeling; D) Merge of FGF9, NF-H and DAPI (blue) labeling. (→), Neuron bodies positive for FGF9; (>), Neuron bodies negative for FGF9; (►),Schwann cells that ceased FGF9 expression upon aligning with axons. Scale bar corresponds to 50 µm.

In 1-div DRG cultures, FGF9 was present in a population of neurons (predominantly in the neural cell bodies, Figure 5.2, arrows), as well in some Schwann cells (Figure 5.2, arrowheads). Interestingly, Schwann cells aligned with axons had lower FGF9 signal (Figure 5.2, filled arrowhead). The alignment of Schwann cells with axons is an initial step that prepares Schwann cells to differentiate and start their myelination program. Our finding suggest that FGF9 may be expressed by undifferentiated/precursor or nonmyelinating Schwann cells and its expression may be down regulated when a closer contact of axon and Schwann cell is established. Thus, it may be absent in myelinating or

premyelinating cells. This suggestion may explain lower FGF9 signal in Schwann cells aligned with axons in our culture. Secondly, the finding that FGF9 was present in a population of some neuron bodies in 1 div old culture could mean that FGF9 expression may depend on the maturity of the neurons. To test these suggestions neuronal (Figure 5.3), and glial expression of FGF9 was analyzed in more details (Figure 5.4).

To explore the neuronal FGF9 expression, E15 mouse cryo-sections were prepared and immunolabeled with antibodies against Double cortin (DCX-marker of immature neurons) and NF-H (marker for mature neurons). FGF9 expression in DRG was investigated. Interestingly, both mature (NF-H positive, Figure 5.3A) and immature neurons (DCX positive, Figure 5.3B) expressed FGF9 (Figure 5.3). Thus, FGF9 was concluded to be produced by all sensory neurons in early development but this expression is probably regulated at a later time in development.

To investigate the glial expression of FGF9, Nestin (CNS stem cell marker) and S100 (glial marker) immunolabelings were performed on E15 mouse cross sections. On the ventral end of the spinal cord, FGF9 was found to partly colocalize with Nestin (Figure 5.4A). Thus, FGF9 is expressed by a population of stem cells located in this region. FGF9 expression was also observed on the walls of guts, where enteric stem cells are located (Figure 5.4E).

In the same region (ventral part of the spinal cord), FGF9 was observed to colocalize with S100 protein, indicating glial expression of FGF9 (Figure 5.4B). FGF9 expression was also found in a region between the DRG and the spinal cord, namely dorsal root entry zone (DREZ), where boundary cap (BC) cells are located at this embryonic stage (Figure 5.3B). BC cells are a transient group of cells originating from neural crest. These cells include multipotent stem cells and generate functional sensory neurons of different subclasses as well as peripheral glia including nonmyelinating and myelinating Schwann cells [93-95]. Thus, our results from cell culture and embryonic mouse cryosection stainings imply that FGF9 may be an important factor for cells that have stem cell property and more specifically for glial precursors including undifferentiated Schwann cells.

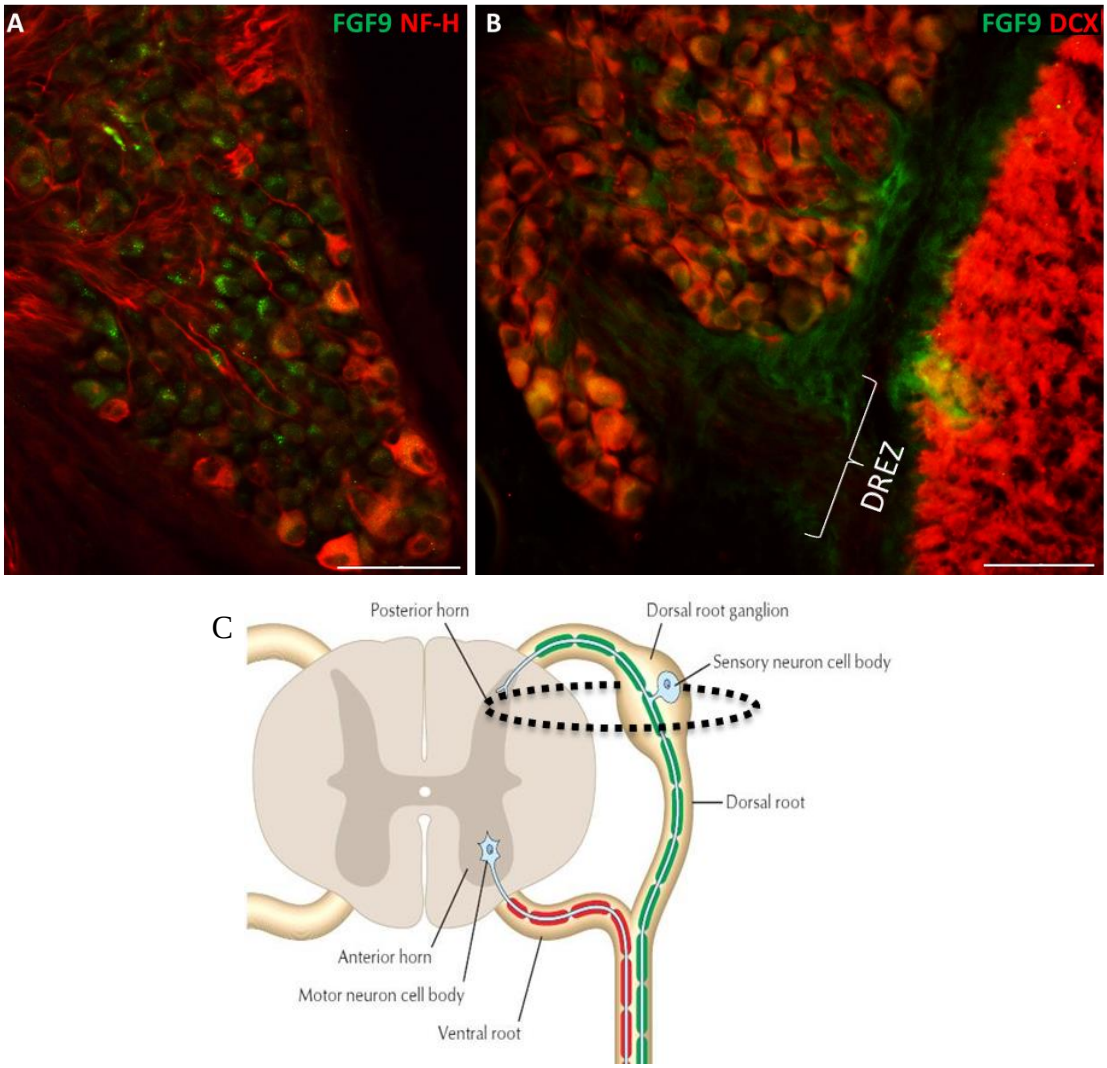

Figure 5.3. Localization of FGF9 in cross sections of E15 mouse DRG. FGF9 (green) expressing cells in DRG are NF-H (red) labeled mature neurons and DCX (red) labeled immature neurons). FGF9-NF-H (A) and FGF9-DCX (B) co-immunolabelings. C) The scheme showing the region visualized in this experiment. DREZ: Dorsal root entry zone. Scale bars correspond to 50 μm.

To further investigate this hypothesis, longitudinal and cross sections of the adult (three months old) mouse sciatic nerve, were labeled with antibodies to FGF9 and nonmyelinating Schwann cell marker glial fibrillary acidic protein (GFAP) (Figure 5.4C, D).

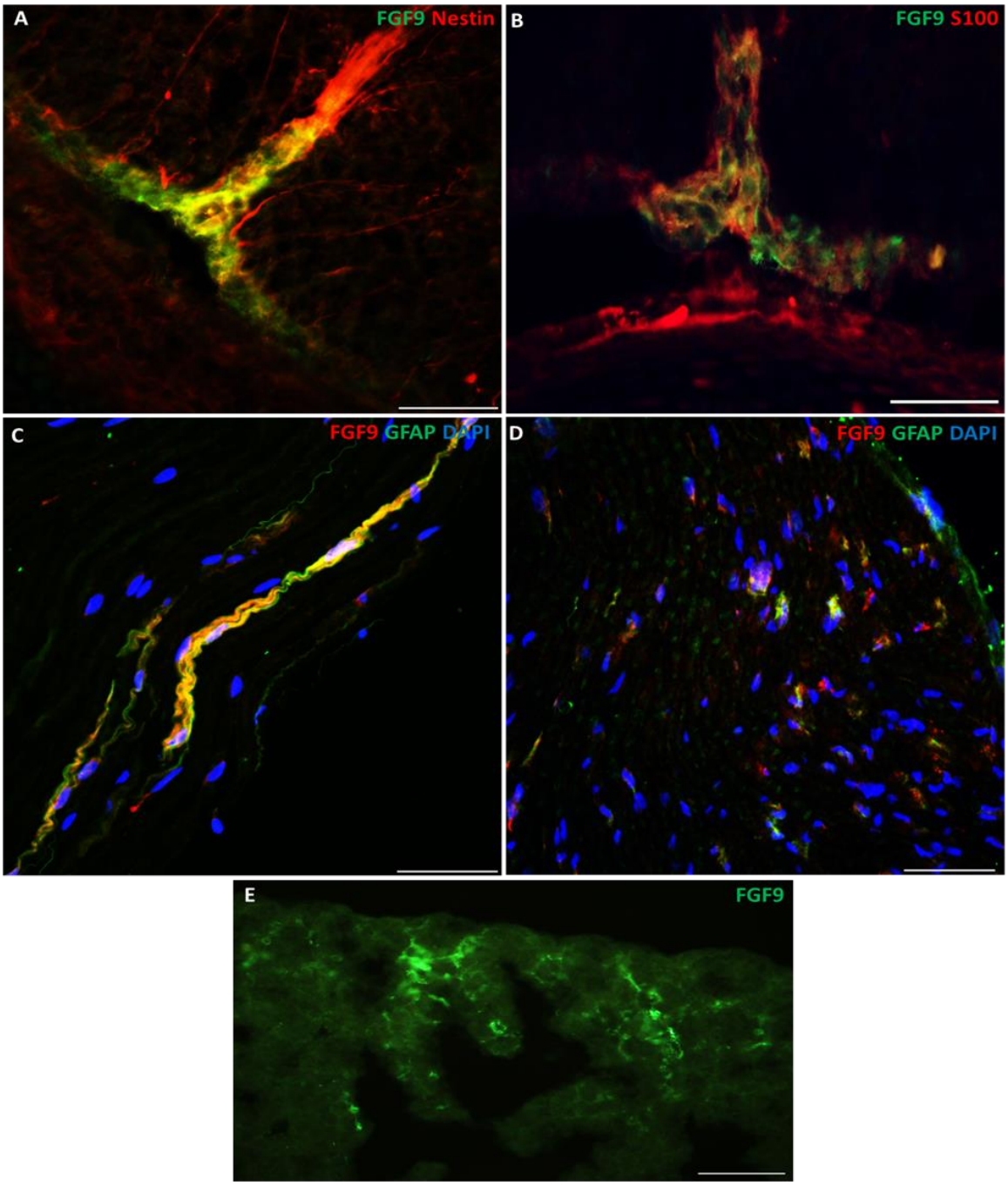

Figure 5.4. Localization of FGF9 in cross sections of E15 mouse nervous system and adult mouse sciatic nerve. Mouse E15 embryonic cross section (A, FGF9 (green) and Nestin labeling; B, FGF9 (green) and S100 labeling; E, FGF9 (green)); adult mouse sciatic nerve longitudinal and cross sections (C and D, FGF9 (red), GFAP (green) and DAPI (blue) labeling). Nestin antibody labels CNS stem cells; S100, glial cells and GFAP nonmyelinating Schwann cells. Scale bars correspond to 50 μm.

Sciatic nerve is formed by axonal extensions of DRG neurons, where thicker axons are myelinated while the rest is gathered in Remak bundles by nonmyelinating Schwann cells. These nonmyelinating Schwann cells express GFAP and some populations of them still possess precursor cell characteristics. FGF9 staining in adult sciatic nerve showed that GFAP expressing Schwann cells also expressed FGF9, supporting previous findings (Figure 5.4C-D).

In conclusion, FGF9 could have importance for stem cells or precursors during early development and later in particular for Schwann cell precursors. When this suggestion is considered together with FGF9 down regulation in premyelinating Schwann cells, it can be proposed that FGF9 may have an inhibitory effect in the myelination process. Further analysis is required to investigate this suggestion. Regarding these findings and suggestions, FGF9 was not involved in our further study.

5.3. FGF1 and FGFR1-3 are Expressed in DRG Tissue

Depending on the results of FGF2 and FGF9, we focused on FGF1 in this thesis. To investigate the presence and distribution of FGF1 and FGFR1-3, adult mouse DRG sections were immunolabeled using antibodies specific to these proteins. Both neuronal bodies (arrows) and satellite glial cells (arrowheads) that surround the neuronal bodies were FGF1 and FGFR1-3 positive, indicating that both DRG neurons and satellite cells produce FGF1 and its receptors (Figure 5.5).

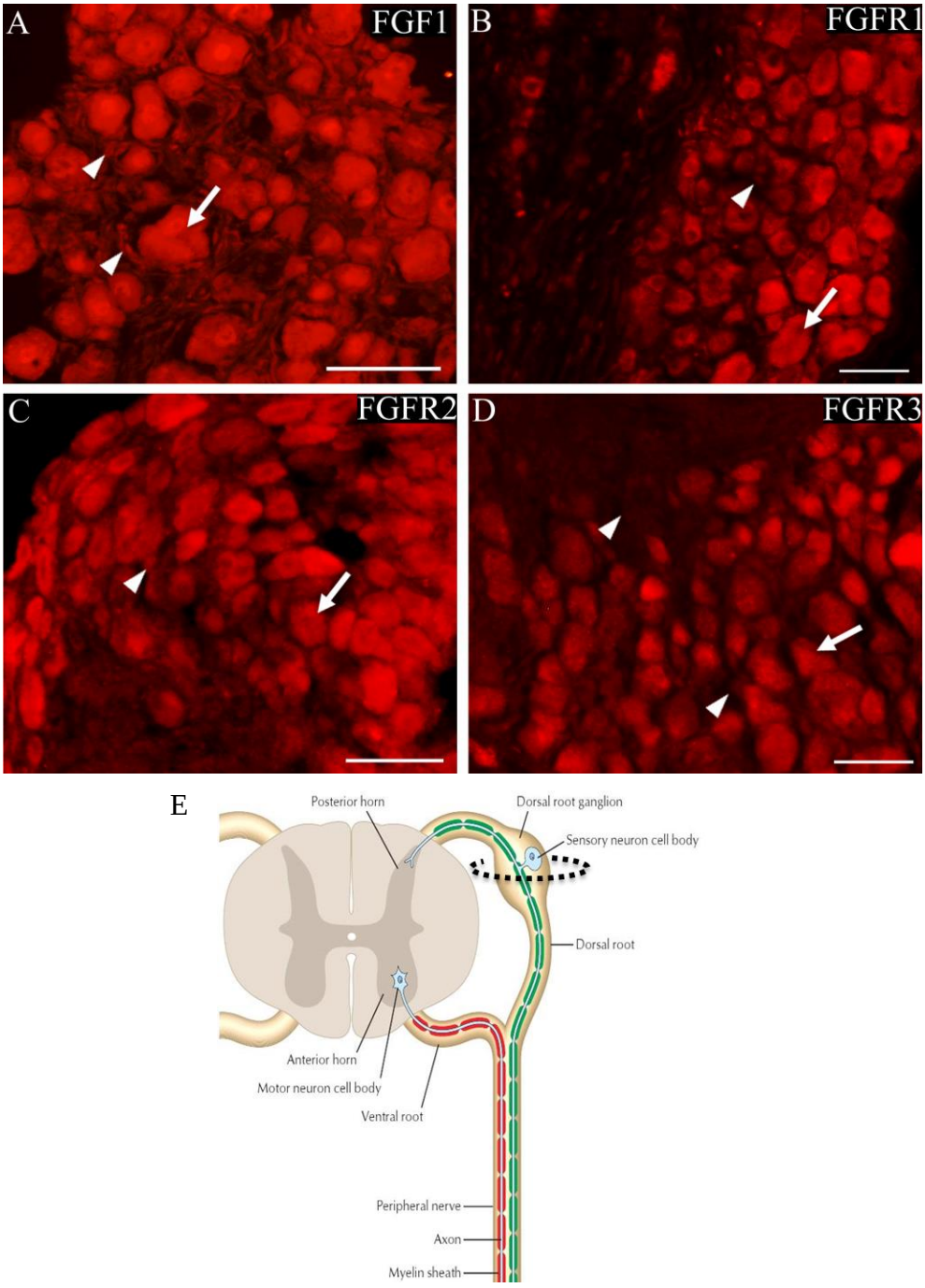

Figure 5.5. Localization of FGF1 and FGFRs in adult mouse DRG tissue. Red shows in A, FGF1; B, FGFR1; C, FGFR2; D, FGFR3 labeling. Arrows show neuron bodies, arrowheads show glial cells surrounding neuron bodies. E) The scheme showing the region visualized in this experiment. Scale bars correspond to 50 μm.

5.4. FGF1 and FGFR1-3 are Expressed in the Sciatic Nerve

As explained earlier, DRG contains sensory neuron bodies and satellite cells supporting these neurons. Even though some myelination can be seen in the DRG, the majority of the myelin is seen distal to the ganglia, in the sciatic nerve. For this reason, the sciatic nerve was used as the *in vivo* model of myelinated peripheral nerve. In order to investigate the presence and quantity of FGF1 and FGFR1-3 in the sciatic nerve, total tissue lysates of the sciatic nerve from mice in different postnatal developmental stages were prepared and analyzed by Western blotting.

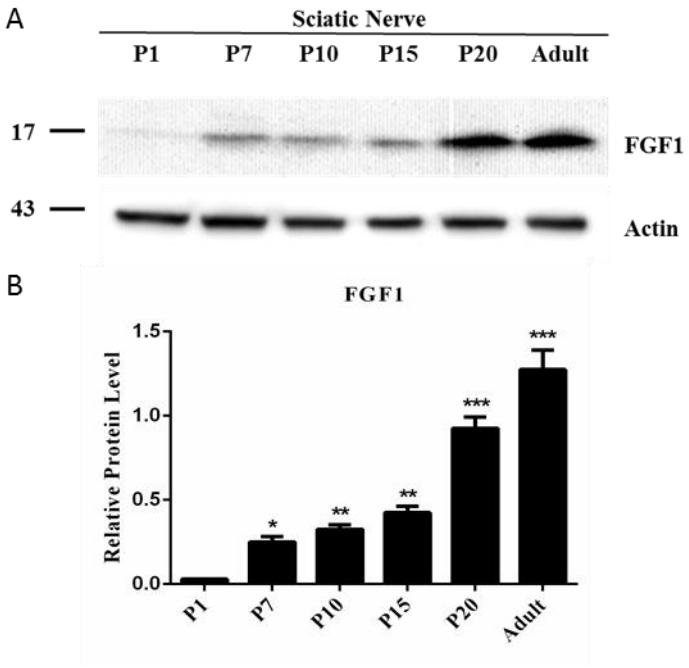

Figure 5.6. Western blot analysis of FGF1 in the sciatic nerve. A) Immunoblotting of FGF1 and actin from P1, P7, P10, P15, P20 and adult mouse sciatic nerve tissue. B) FGF1 protein level relative to corresponding actin levels. * p<0,05 compared to P1; ** p<0,01 compared to P1; *** p<0,01 compared to P1-P15; (One-way Anova - Post-hoc, Dunnett's T3 method was used to analyze the data). Y-error bars represent SEM.

5.4.1. FGF1 Expression Increases During Myelination in the Sciatic Nerve

In sciatic nerve, FGF1 protein level gradually increased with age, clearly during the time of active myelination (P1-P20). The first increase was around postnatal day-seven

(P7), around when active myelination starts. Its expression was up regulated for a second time at P20, when myelin compaction starts. FGF1 expression was also high in the adult mouse (Figure 5.6).

5.4.2. FGFR1-3 Expression is Modulated During Myelination

Western blot analysis from sciatic nerve showed that similar to FGF1, FGFR1 protein level increased at P7. After a decrease at P10, it was again up regulated around P15-P20 when the rate of myelination is at a maximum. Then, the level of FGFR1 decreased to a lower level in adult animals (Figure 5.7A).

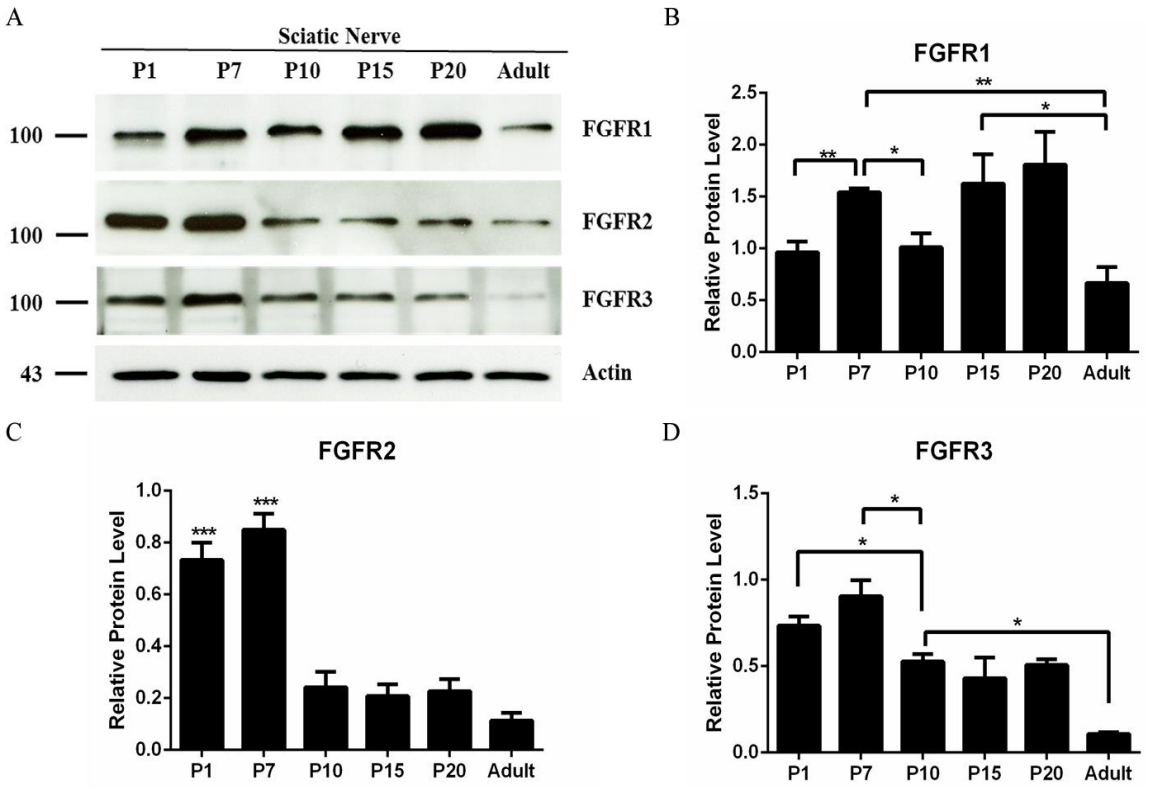

Figure 5.7. Western blot analysis of FGFR1-3 in the sciatic nerve. A) Immunoblotting of FGFR1-3 from P1, P7, P10, P15, P20 and adult mouse sciatic nerve tissue. B-D) FGFR1, FGFR2 and FGFR3 protein levels relative to corresponding actin levels. * p<0,05 ; ** p<0,01; *** p<0,001 (Student T-test method was used to analyze the data). Y-error bars represent SEM.

Both FGFR2 and FGFR3 were highly expressed just after birth (P1). Unlike FGFR1, there was no change in their levels at the beginning of myelination (P7). However, similar to FGFR1, they were both down regulated starting by P10, but FGFR3 protein was still much higher than FGFR2 between P10 and P20. After the active myelination period, both FGFR2 and FGFR3 were stabilized at a lower expression level in older animals (Figure 5.7B, C).

5.5. FGF1 is Localized to Myelinated Axons and Nonmyelinating Schwann Cells in the Sciatic Nerve

After FGF1 and the three FGFRs were confirmed to be expressed in DRG and the sciatic nerve, their localization in the nerve was investigated using immunochemistry. For this purpose, transverse sections of P1, P20 and adult mouse sciatic nerve and teased sciatic nerves from adult mice were prepared and labeled for FGF1 and FGFR1-3.

5.5.1. Transverse Sciatic Nerve Sections

5.5.1.1. Transverse Sections of P1 Mouse Sciatic Nerve. Immunolabelling of transverse P1 sciatic nerve sections showed that neurons were the main source of FGF1 at this time point. Interestingly, high level of FGF1 was observed on axons that were segregated by one Schwann cell cytoplasm to form a fetal nerve fiber. Such a fiber can be observed in Figure 5.8, where arrow shows a MAG positive Schwann cell cytoplasm that had clearly penetrated within FGF1 positive axons. Unlike the axons, this Schwann cell in the fibre expressed less or no FGF1. On the other hand, Schwann cells that were in an earlier developmental stage in this process and were likely penetrating inside axonal groups expressed more FGF1 (Figure 5.8, arrowhead). Axonal immunolabeling confirmed these findings, where FGF1 positive axons in fetal nerve fibres are shown by NF-H staining (Figure 5.9, arrowhead).

It should be noted that, thicker axons had higher FGF1 expression, implicating a direct link to myelination, since axon diameter is critical in the decision of myelination.

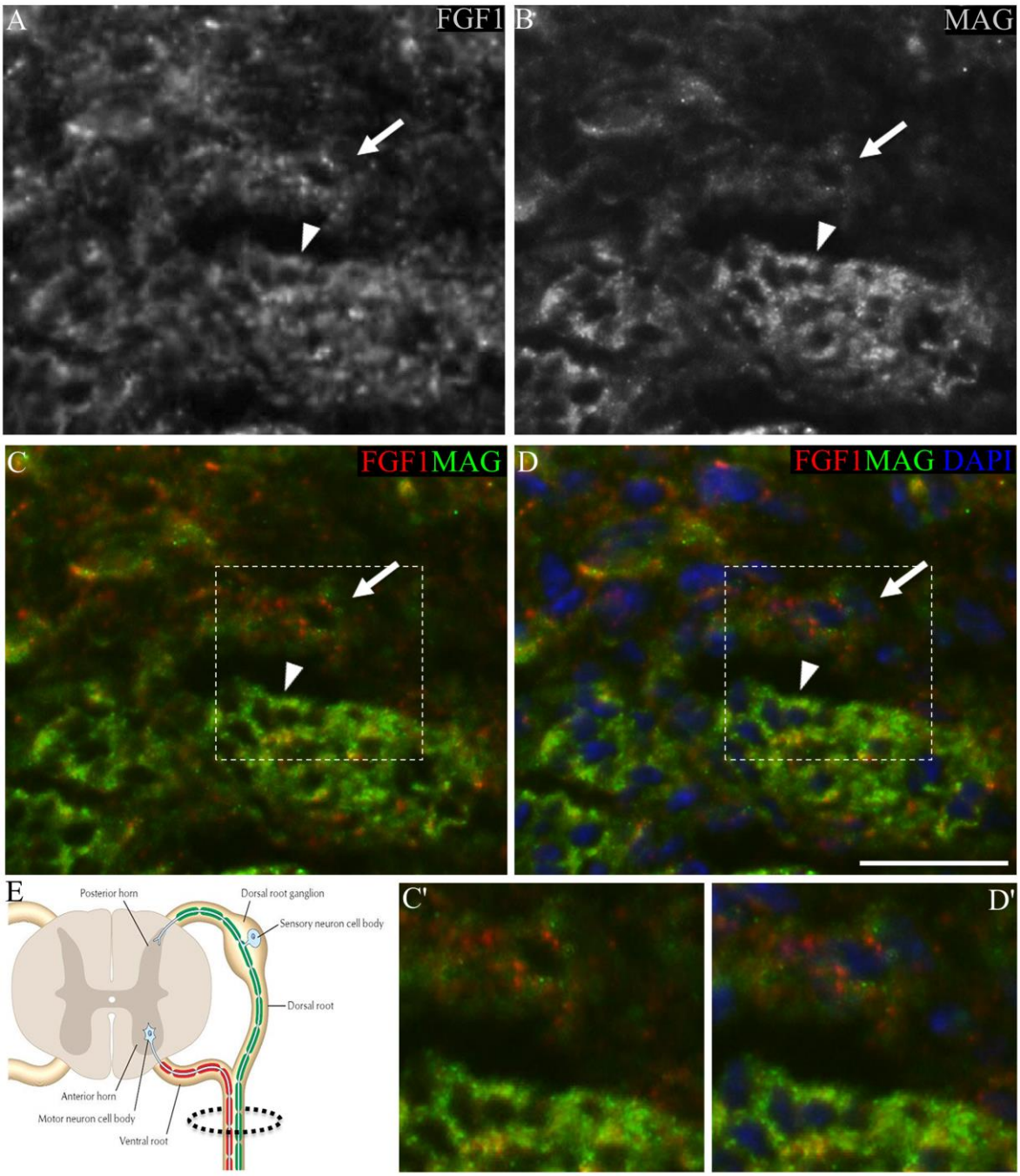

Figure 5.8. FGF1 expression and localization in P1 mouse sciatic nerve cross section. A, FGF1; B, MAG; C, merge of FGF1 (red) and MAG (green); D, Merge of FGF1, MAG and DAPI (blue). C', D') Closer views of the marked areas in C and D. E) The scheme showing the location the cross section labeled in this experiment. MAG antibody labels Schwann cells. Scale bar corresponds to 20 μm.

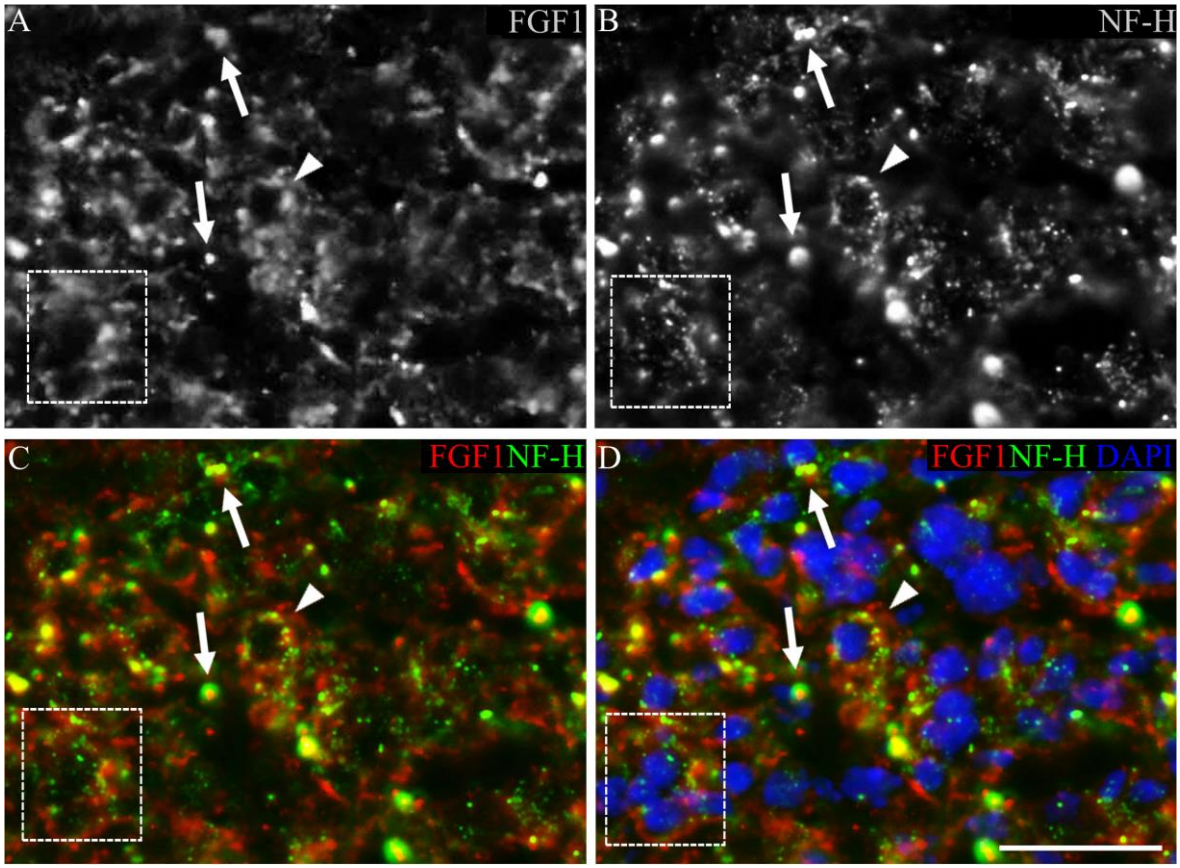

Figure 5.9. FGF1 expression and localization in P1 mouse sciatic nerve cross section. A, FGF1; B, NF-H; C, merge of FGF1 (red) and NF-H (green); D, merge of FGF1, NF-H and DAPI (blue) labeling. FGF1 is localized to thick caliber axons (arrows) and axons in fetal nerve fibres (arrowhead). Thinner axons are negative for FGF1 (square). Schwann cells associated with thick axons do not have FGF1 (arrows). Scale bar: 20 μm.

These axons were likely to have been isolated by Schwann cells that are in contact with them (Figure 5.9, arrows).These Schwann cells can be observed by their nuclei; interestingly in such Schwann cells FGF1 expression was absent, like the ones in fetal nerve fibers. At this time point, there were also some populations of thinner axons that would probably not myelinated and these axons were FGF1 negative (Figure 5.9, area marked by the square). FGF1 was also observed in the extracellular region in a diffused pattern that may be due its secretion to the extracellular space.

5.5.1.2. Transverse Sections of P20 Mouse Sciatic Nerve. By the postnatal day 20, Schwann cells have already differentiated to a myelinating or nonmyelinating phenotype.

Concerning myelination route, the event signifying this time point is the compaction of the myelin sheaths. As seen on Figure 5.10, in cross sections of the P20 mouse sciatic nerve, Schwann cells that produced MBP positive myelin sheaths did not express FGF1 (arrows), whereas the wrapped axons expressed FGF1. In contrast, MBP negative nonmyelinating Schwann cells can also be clearly observed to express FGF1 (arrowheads).

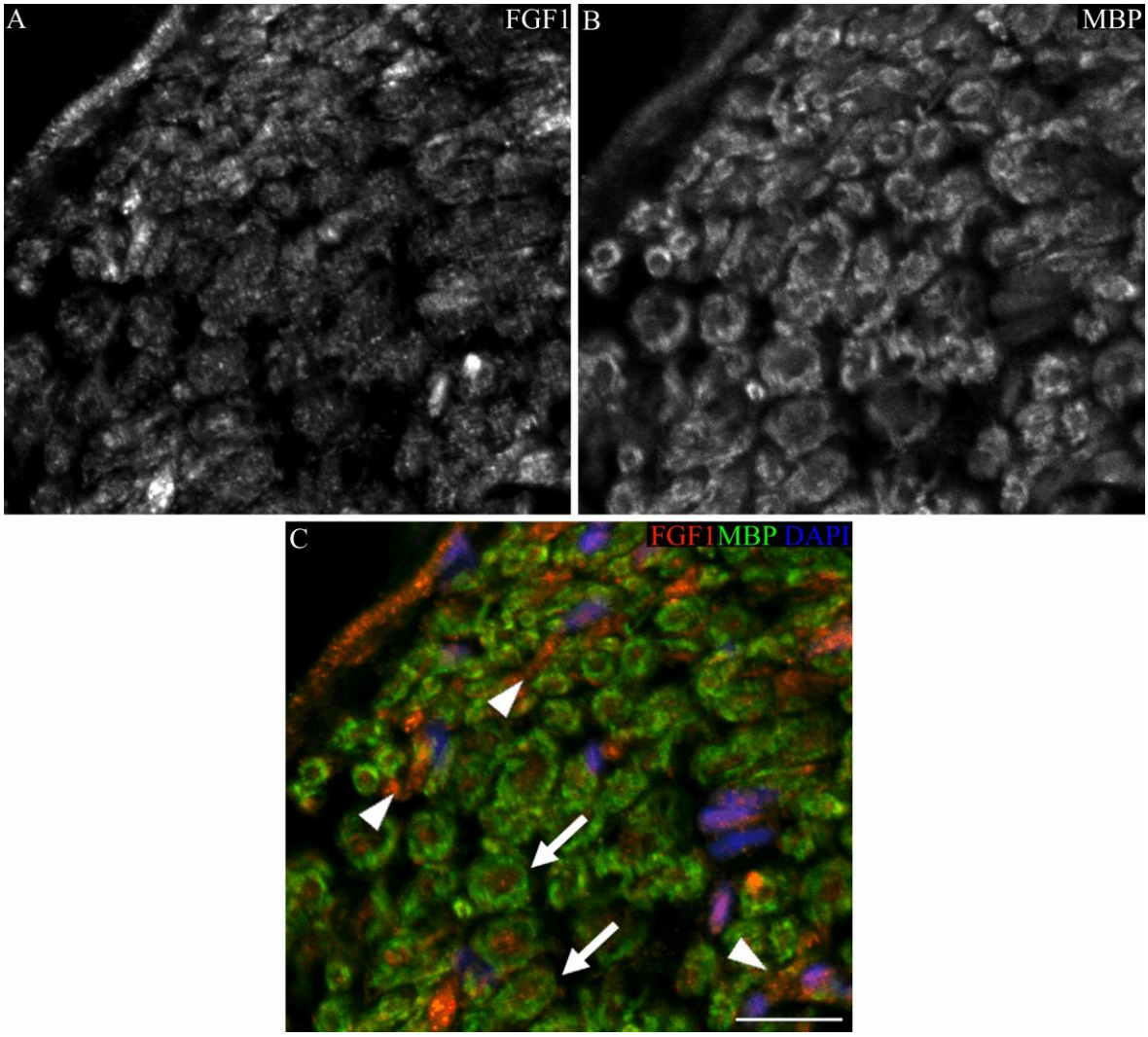

Figure 5.10. FGF1 expression and localization in P20 mouse sciatic nerve cross section. A, FGF1; B, MBP; C, Merge of FGF1 (red), MBP (green) and DAPI (blue) labeling. FGF1 was expressed by myelinated axons, where the myelin sheaths were visualized by MBP staining (arrows). These myelinating Schwann cells did not express FGF1, whereas nonmyelinating Schwann cells did (arrowheads). Scale bar corresponds to 20 μm.

5.5.1.3. Transverse Sections of Adult Mouse Sciatic Nerve. In agreement with the data acquired from P1 and P20 sections, FGF1 expression in the axons persisted in myelinated adult axons (Figure 5.11A). The same result was produced where FGF1 colocalized with NF-H in the myelinated axons (Figure 5.11B). Interestingly, unlike P20 sciatic nerve, not all myelinated axons expressed FGF1 in the adult mouse. The arrow shows a thick axon that is possibly myelinated but not expressing FGF1 in Figure 5.11B-B''. Nonmyelinating Schwann cells also continued to express FGF1 as previously shown in earlier time points examined (Figure 5.11A'', B'', arrowheads). These FGF1-positive nonmyelinating Schwann cells can be seen ensheathing bundles of thin caliber NF-H positive axons (Figure 5.11B''). It should be noted that these thin caliber axons do not express FGF1, in agreement with the findings from earlier time points.

5.5.1.4. Does FGF1 Expression Differ Between Motor and Sensory Neurons? In adulthood, some of the thick caliber axons did not express FGF1 (Figure 5.11B, arrow). The sciatic nerve is a mixed neuronal population of both sensory and motor neurons. To investigate if FGF1 expression was restricted to one of these two populations, DRGs were dissected with the dorsal and ventral roots attached. The dorsal roots correspond to the branches of sensory neurons that extend to the spinal cord and the ventral roots correspond to axons of motor neurons emerging from the spinal cord. The tissue was sectioned and labeled for FGF1.

FGF1 was found to be expressed in both dorsal and ventral roots (Figure 5.12). Thus, the expression difference among the population of myelinated axons does not depend on the neuron subtype. Further investigation is required to understand the absence of FGF1 from some of the myelinated axons.

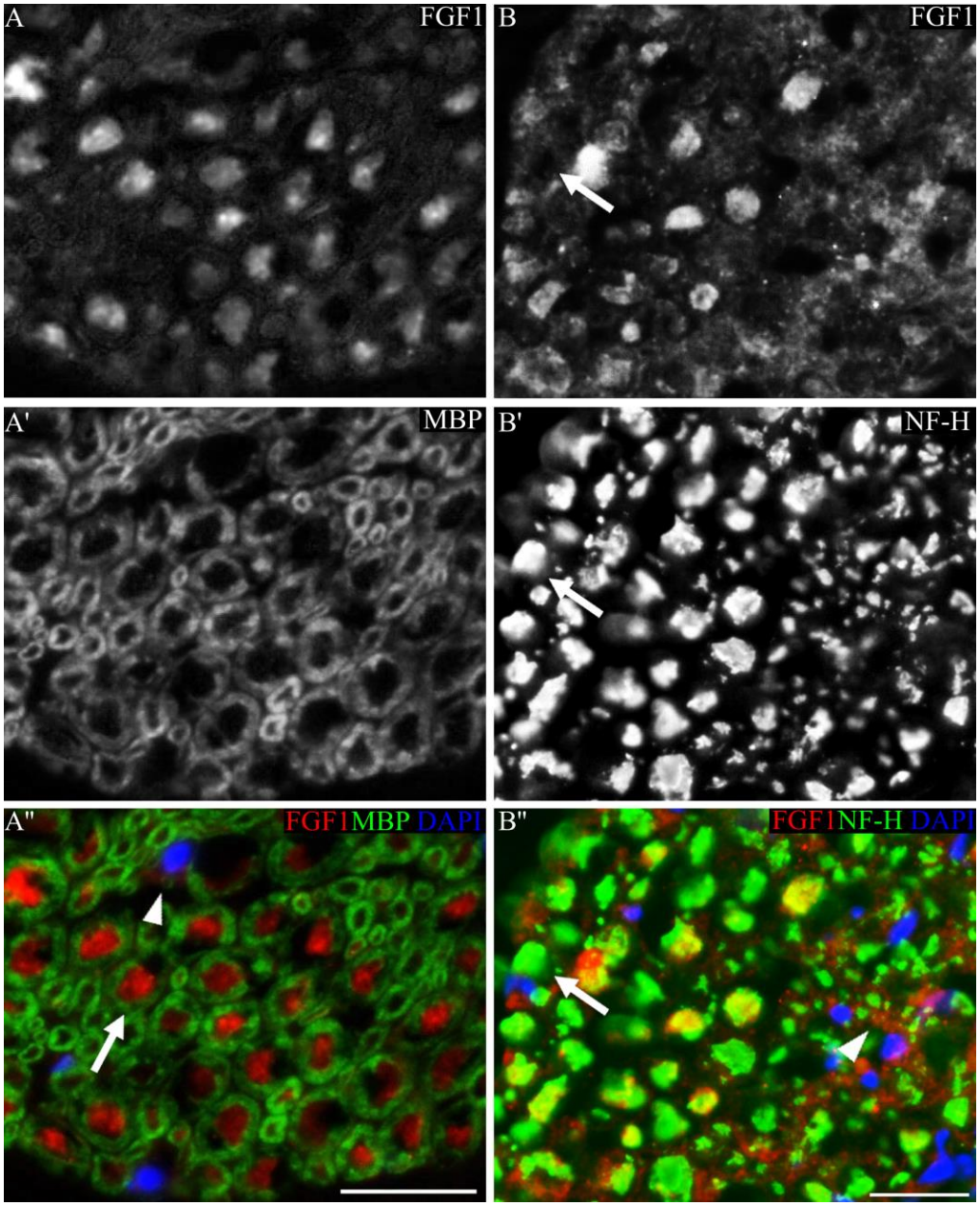

Figure 5.11. FGF1 expression and localization in the adult mouse sciatic nerve cross section. Co-immunolabelings of FGF1 (red) with MBP (green) (A-A'') and NF-H (green) (B-B''). FGF1 is localized to myelinated axons and abundant in thicker axons (B'', arrow). Some thick axons that are possibly myelinated do not express FGF1 (arrow, B-B''). Nonmyelinating SCs continue to express FGF1 (A'', B'', arrowheads). Scale bars: 20 µm.

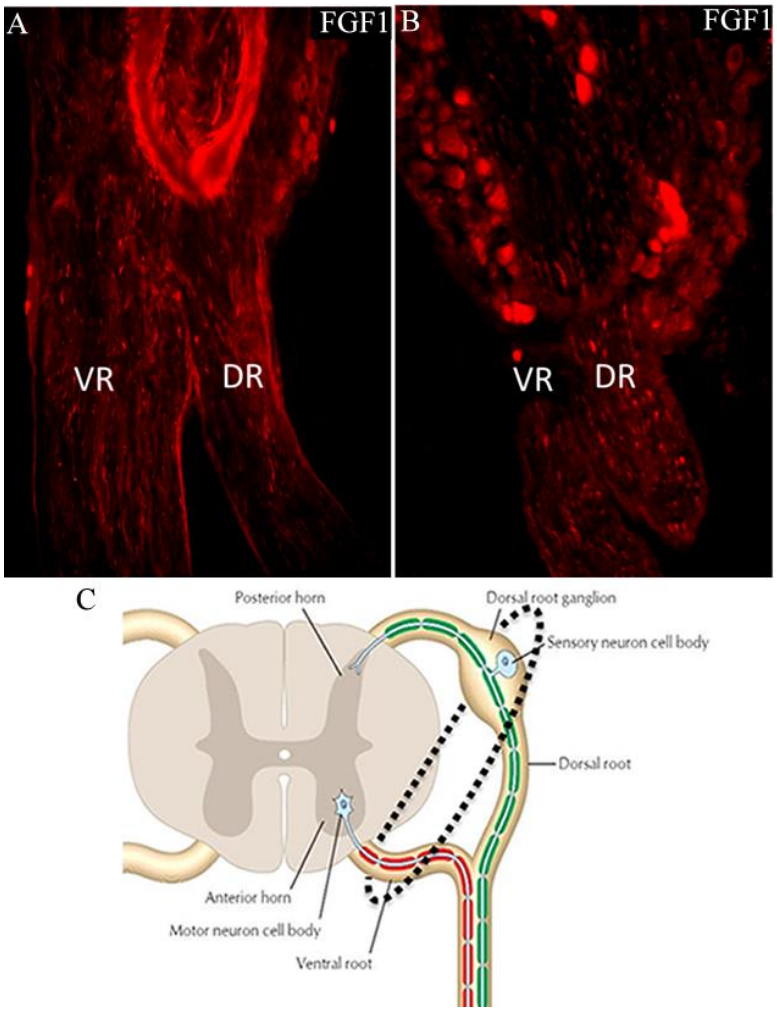

Figure 5.12. Expression and localization of FGF1 in DRG dorso-ventral roots. A, B) FGF1 expression does not differ between the ventral and dorsal roots, indicated by VR and DR, respectively. C) The scheme showing the location the cross section labeled in this experiment.

5.5.2. Teased Sciatic Nerve Preparations

To further examine the distribution of FGF1 throughout a whole nerve, teased sciatic nerve samples were prepared and labeled. Working with teased sciatic nerve provides investigation of the whole structure of a myelinated fibre together with its domains and also creates the opportunity to compare myelinating and nonmyelinating Schwann cells on the same sample. On these samples, FGF1 was found on the myelinated

axons and a higher expression level of FGF1 was observed on large caliber axons supporting the previous findings (Figure 5.13A, C).

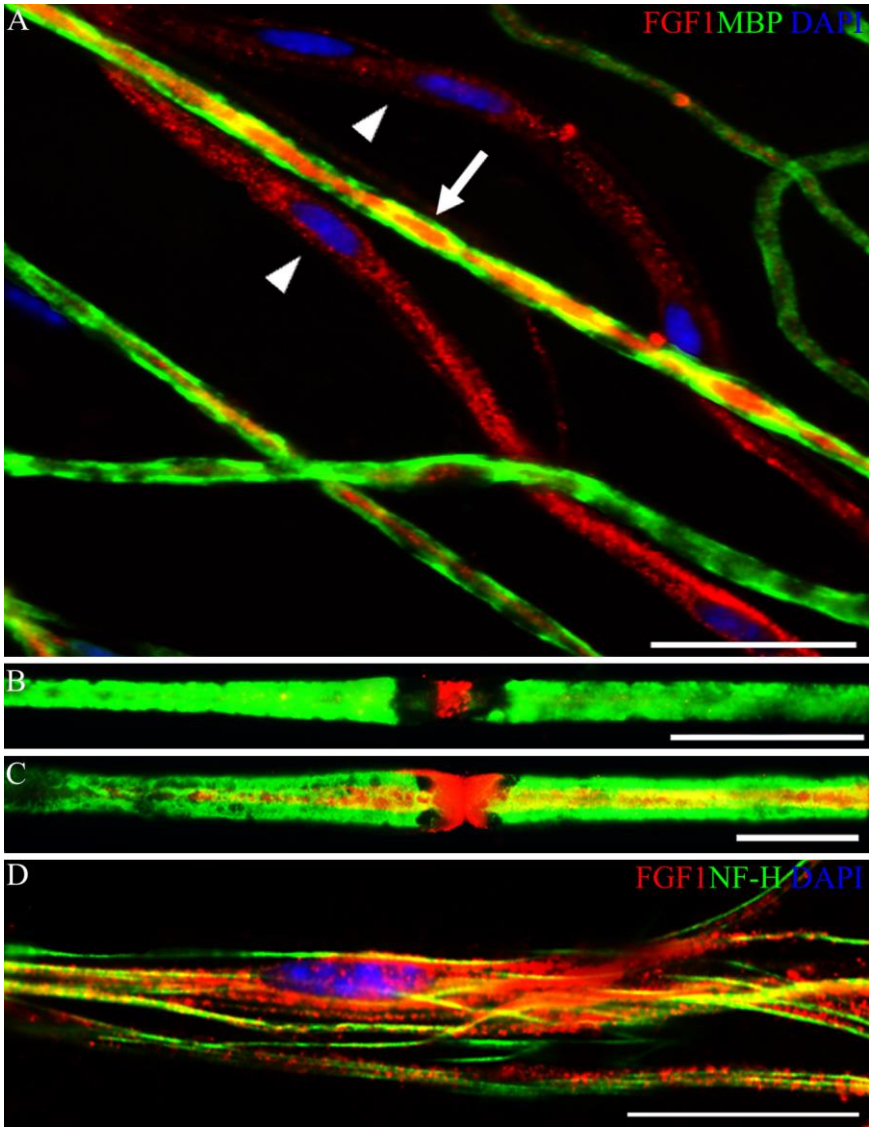

Figure 5.13. Expression and localization of FGF1 in teased adult mouse sciatic nerve. A) Merge of FGF1 (red), MBP (green) and DAPI (blue) labeling; B, C) Merge of FGF1 and MBP labeling; D) Merge of FGF1, NF-H (green) and DAPI (blue) labeling. FGF1 was localized to myelinated axons (arrow), nonmyelinating Schwann cells (arrowheads) (A, D), nodes of Ranvier (B) and microvilli (C). Scale bars correspond to 20 μm.

Interestingly, FGF1 protein was also detected at the nodes of Ranvier and on the microvilli providing attachment of the lamellae to the nodes (Figure 5.13B, C). Nonmyelinating

Schwann cells were also verified to express FGF1 (Figure 5.13A, arrowheads). In Figure 5.13D, a FGF1 positive nonmyelinating Schwann cell can be seen enveloping NF-H positive thin caliber axons. These thin axons should be noted to be negative for FGF1 expression, as shown earlier.

5.6. FGF1 is Expressed by Neurons and Migrating Schwann cells in DRG Culture

DRG neuron-Schwann cell culture system was used to establish an *in vitro* model system of myelinating peripheral nerve for investigation of the possible functions of FGF1 molecule and its receptors during development and myelination.

Before studying function, it was important to evaluate the *in vitro* system to understand if it was a good model for functional analysis and this was done by confirming that the *in vivo* findings were also valid for this culture system. For this purpose, DRG cultures were established, induced by ascorbic acid and fixed at different time points in development. The cultures were then stained to investigate the expression and distribution of FGF1 and FGFR1-3. These experiments verified *in vivo* results, reproducing the results showing FGF1 expression in large caliber neurons, and axonal FGF1 localization (Figure 5.14, arrows). In addition, the level of FGF1 increased in the axons after induction of myelination (Figure 5.14C).

Migrating Schwann cells, that could be thought of as immature Schwann cells (present *in vivo)*, had a low expression of FGF1 (Figure 5.14A, arrowheads). Just as *in vivo*, the FGF1 expression was down regulated in these Schwann cells, when they established axonal contact (Figure 5.14C, arrowheads).

To further support these results, DRG cultures from beta actin-EGFP mice were prepared and investigated. In Figure 5.15A, neuronal bodies and axons can be clearly seen to express FGF1 protein. On the other hand, Schwann cells that did not have axonal contact expressed FGF1 (Figure 5.15B), while Schwann cells with clear neuronal contact had no FGF1 expression (Figure 5.15A, arrows).

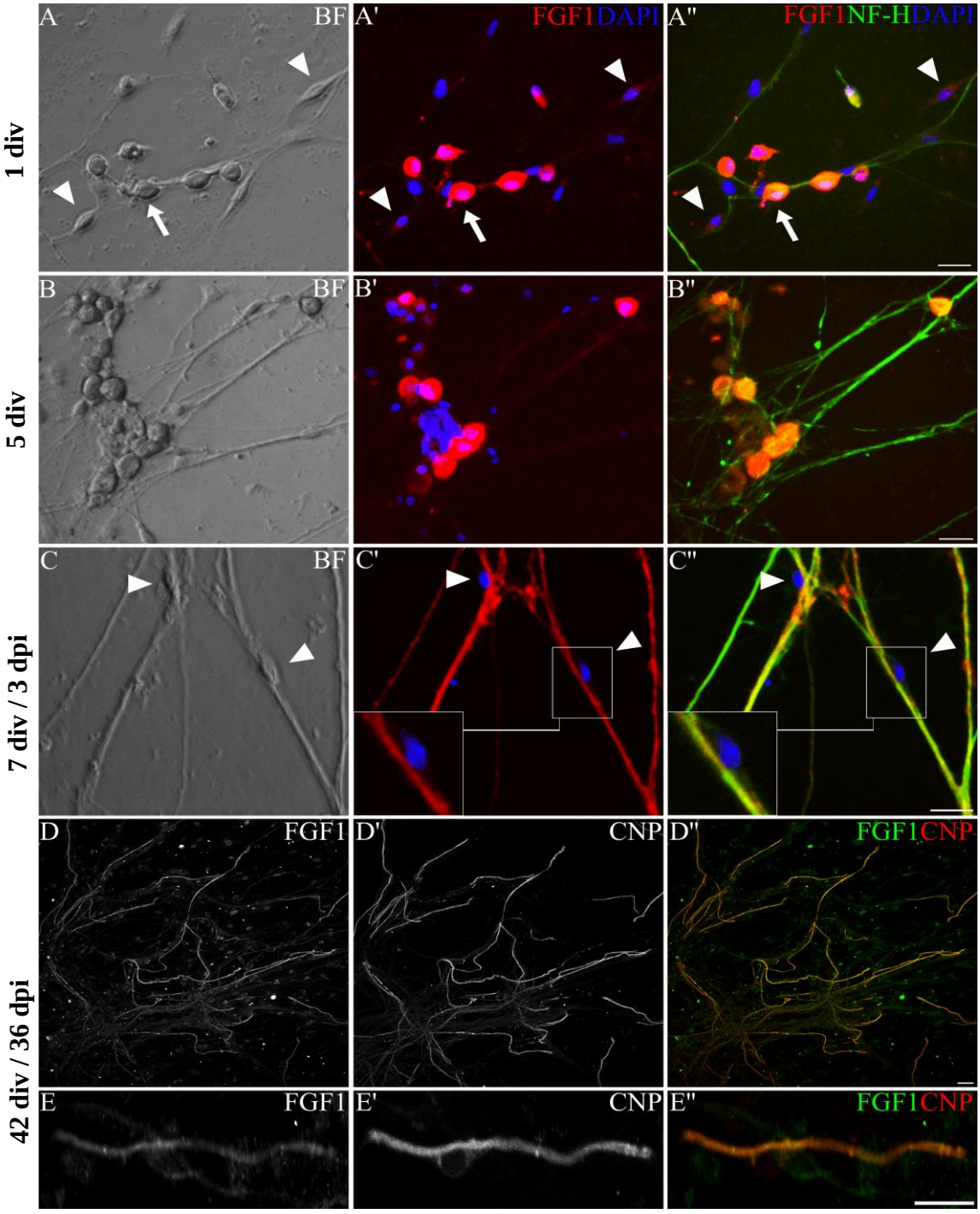

Figure 5.14. Localization and expression of FGF1 in DRG culture. FGF1 immunolabeling of 1 div (A), 5 div (B), induced 7 div (3 dpi) (C) and 42 div (36 dpi) (D, E) DRG cultures. FGF1 is depicted by red, green shows NF-H labeling and blue shows DAPI labeling (A-C); FGF1 is labeled green and red shows CNP that labels myelin sheath (D, E). Scale bars correspond to 20 μm.

As previously shown *in vivo*, in cultures with thick myelinated segments, FGF1 was found in the axons that were myelinated (Figure 5.14D, E). Compact myelin sheaths were visualized by using an antibody against the myelin marker protein CNP.

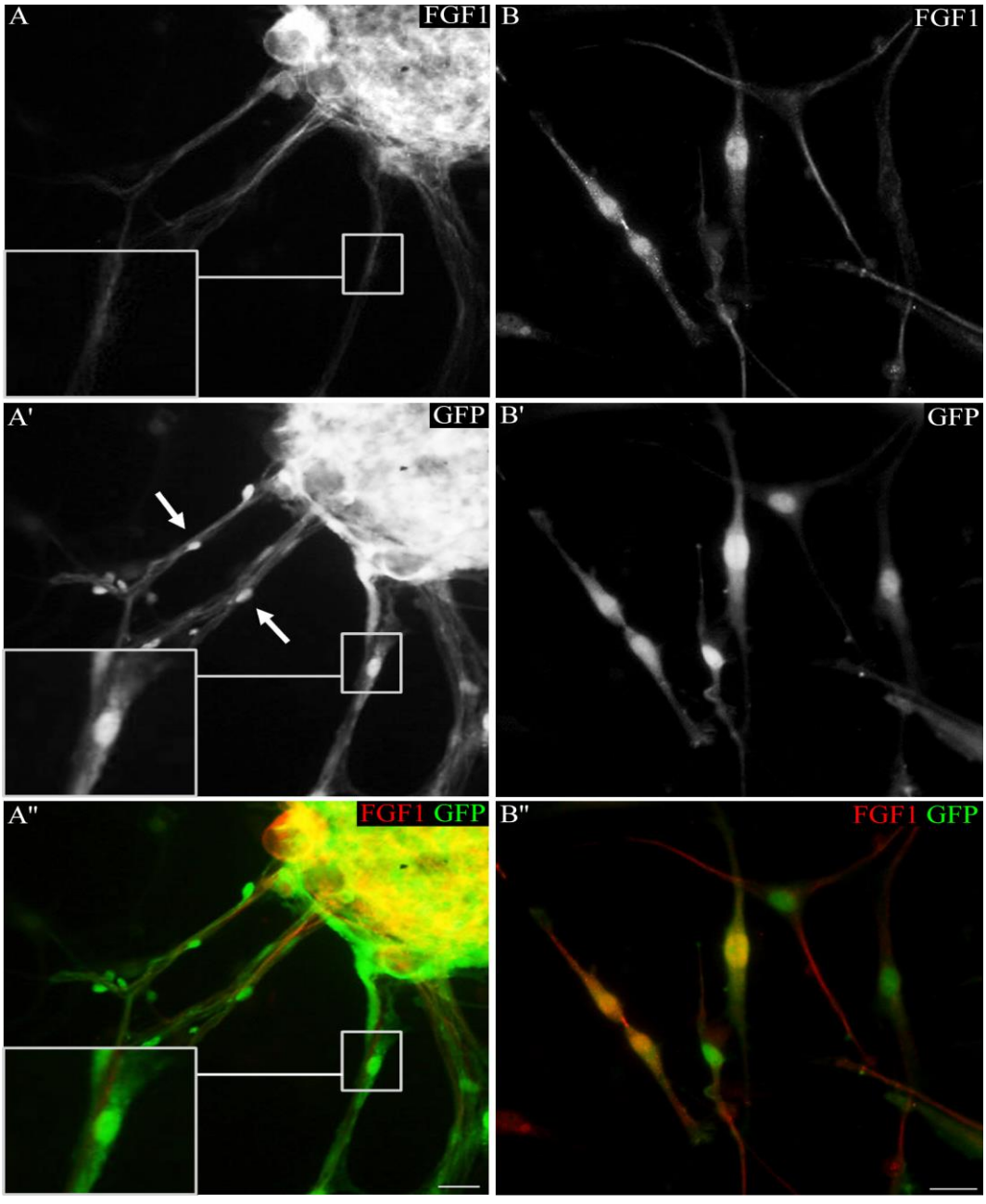

Figure 5.15. Localization of FGF1 in 18-div DRG culture from EGFP mice. A, B) FGF1; A', B') GFP; A', B'') merge of FGF1 (red) and GFP (green). The insets in A-A'' show closer views of the marked areas. Scale bars correspond to 20 µm.

FGF1 protein was also localized to the filopodia of migrating Schwann cells (Figure 5.16). This finding suggests a possible role of FGF1 in Schwann cell motility. This is also consistent with the previous finding localizing FGF1 to Schwann cell microvilli (Figure 5.13C). The microvilli enable the motility necessary when Schwann cells wrap the axons during the myelination process [96, 97].

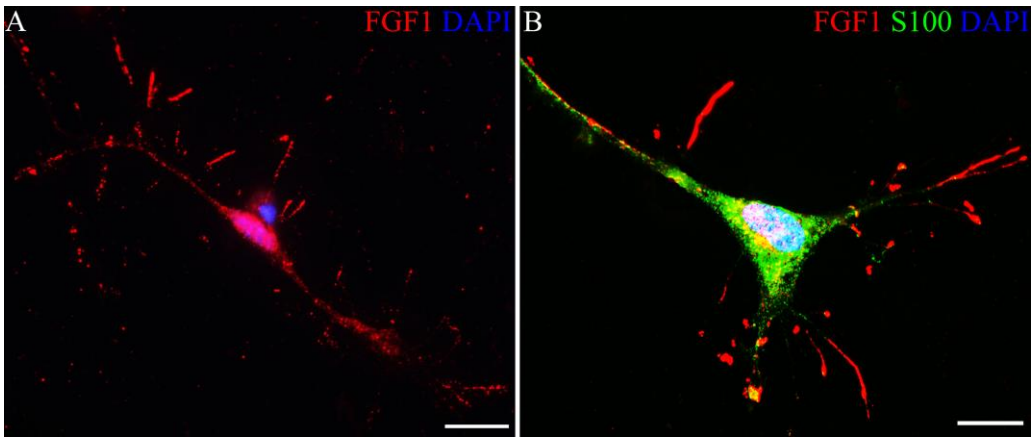

Figure 5.16. FGF1 localization in 4 div DRG culture. A) Merge of FGF1 (red) and DAPI (blue); B) Merge of FGF1 (red), S100 (green) and DAPI (blue) labeling. FGF1 is expressed in filopodia of migrating Schwann cells. S100, marker of glial cells. Scale bars correspond to 20 µm.

In conclusion, FGF1 was expressed by neurons and by Schwann cells that were not in contact with axons. The FGF1 expression in axons was up regulated when myelination was induced, while Schwann cells that aligned with these axons down regulated their intrinsic FGF1 expression. These results were in agreement with *in vivo* findings. In the early development of the sciatic nerve, FGF1 was similarly found in axons. Higher FGF1 expression was present in thicker axons that were in one-to-one association with Schwann cells. As seen *in vitro*, Schwann cells in earlier phases of development in the sciatic nerve expressed FGF1, while Schwann cells in fetal nerve fibres and myelinating Schwann cells down regulated this expression. The expression of FGF1 continued in the myelinated axons throughout the development. In addition, the FGF1 localization in the microvilli *in vivo* and in the filopodia of the Schwann cells *in vitro* may be parallel findings. Both results point to motility of Schwann cells required for migration *in vitro* and enwrapment of axons *in vivo.*

5.7. FGFR1-3 are Localized to the Myelin Sheath and Nonmyelinating Schwann Cells in the Sciatic Nerve

To further understand the role of FGF1 in myelination, it is also necessary to investigate the expression and localization of its receptors. Thus, the same tissues previously used for FGF1 were also immunolabeled for FGF receptors 1, 2 and 3.

5.7.1. Transverse Sciatic Nerve Sections

To investigate the localization of FGF receptors in a myelinated nerve, sciatic nerve cross sections from adult mouse were labeled with antibodies to the receptors. These experiments showed that all three receptors were localized to the myelin sheaths as seen in Figure 5.17 - 5.19.

5.7.2. Teased Sciatic Nerve Preparations

Cross sections of the sciatic nerve indicated that all three FGF receptors were present in the myelin sheath. To better understand their distribution throughout the nerve, we investigated teased preparations of the adult mouse sciatic nerve. The finding from these preparations, verified the data that we obtained from the cross sections. The FGFR1-3 were present in the myelin sheath. However, we have also observed that FGFR1 was present in the perinuclear region of the myelinating cells (Figure 5.20, white arrowheads) and at the nodes of Ranvier very likely on the glial side (Figure 5.20, yellow arrowheads). Nonmyelinating Schwann cells also expressed FGFR1 (Figure 5.20, yellow arrowheads).

FGFR2 was intensely labeled in the nuclei of myelinating Schwann cells. FGFR2 was also localized to some of the nodes (Figure 5.21A, arrowhead) and microvilli, but not commonly (Figure 5.21A, B, arrows). Nonmyelinating Schwann cells also expressed FGFR2 (Figure 5.21C, arrow). The distribution of FGFR3 was similar to that of FGFR2. FGFR3 was also localized to the nucleus of myelinating (Figure 5.21D) and nonmyelinating Schwann cells (Figure 5.21E, arrow).

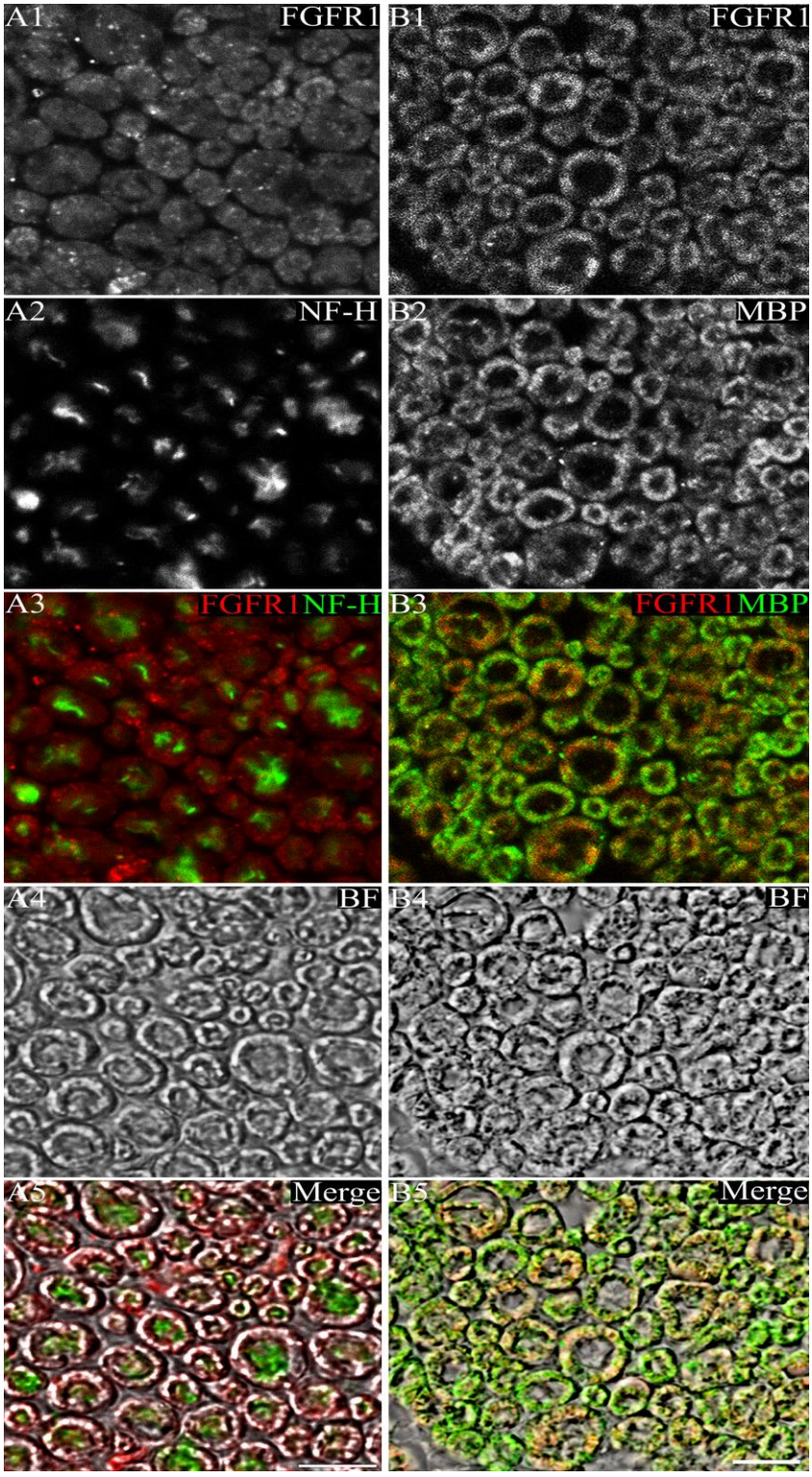

Figure 5.17. Localization of FGFR1 in cross sections of adult mouse sciatic nerve. A1, B1: FGFR1; A2: NF-H; A3: Merge of FGFR1 (red)-NF-H (green); A4: Brightfield; A5: Merge of FGFR1-NF-H -brightfield. B2: MBP; B3: Merge of FGFR1-MBP (green); A4: Brightfield; A5: Merge of FGFR1-NF-H-brightfield. FGFR1 is expressed in myelin sheath (A). FGFR1 colocalizes with MBP that labels the myelin sheath (B). Scale bars: 20 μm.

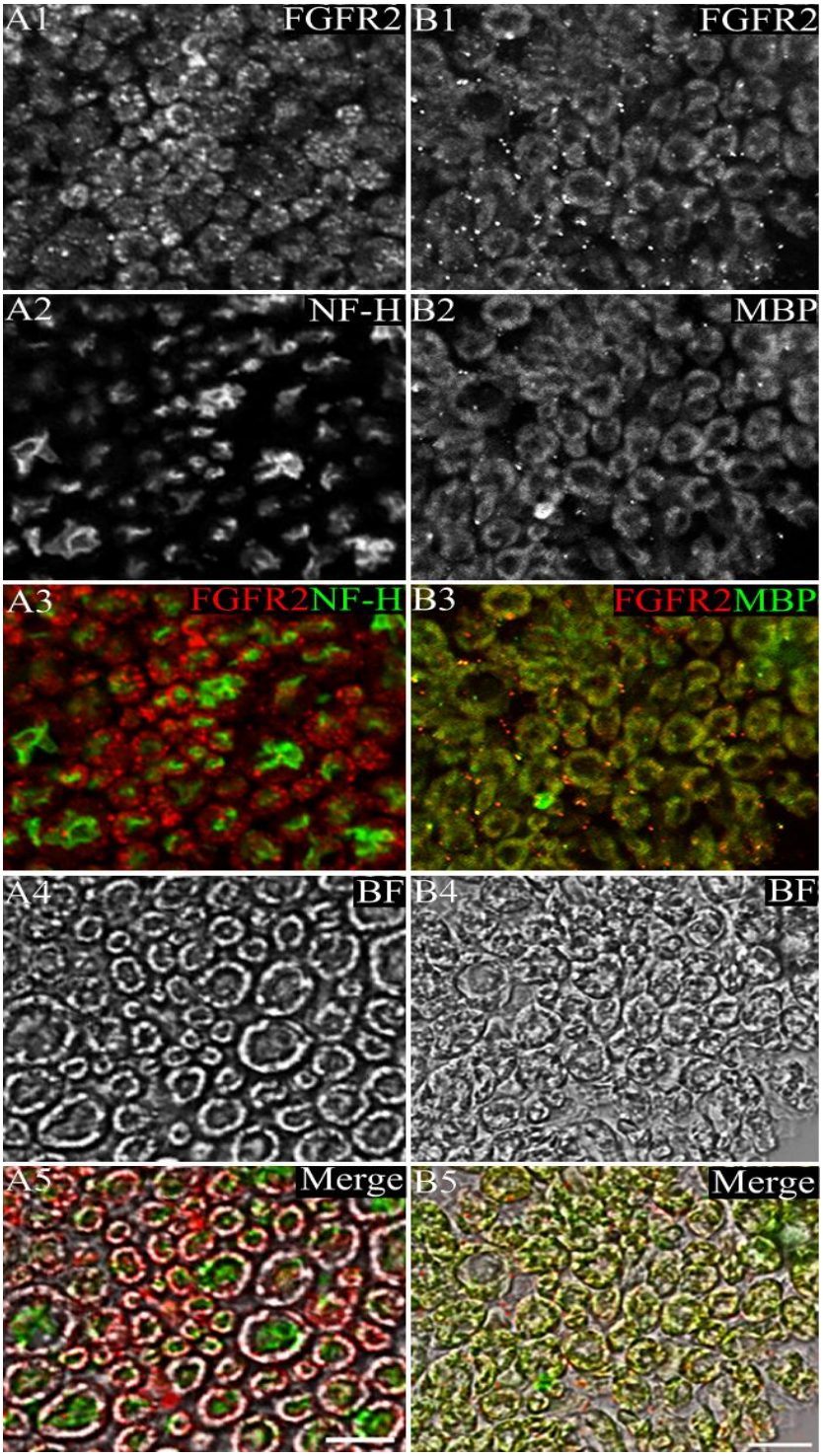

Figure 5.18. Localization of FGFR2 in cross sections of adult mouse sciatic nerve. A1, B1: FGFR2; A2: NF-H; A3: Merge of FGFR2 (red)-NF-H (green); A4: Brightfield; A5: Merge of FGFR2-NF-H -brightfield. B2: MBP; B3: Merge of FGFR2-MBP (green); A4: Brightfield; A5: Merge of FGFR2-NF-H-brightfield. FGFR2 is expressed on the myelin sheath (A). FGFR2 colocalizes with MBP in the myelin sheath (B). Scale bars: 20 μm.

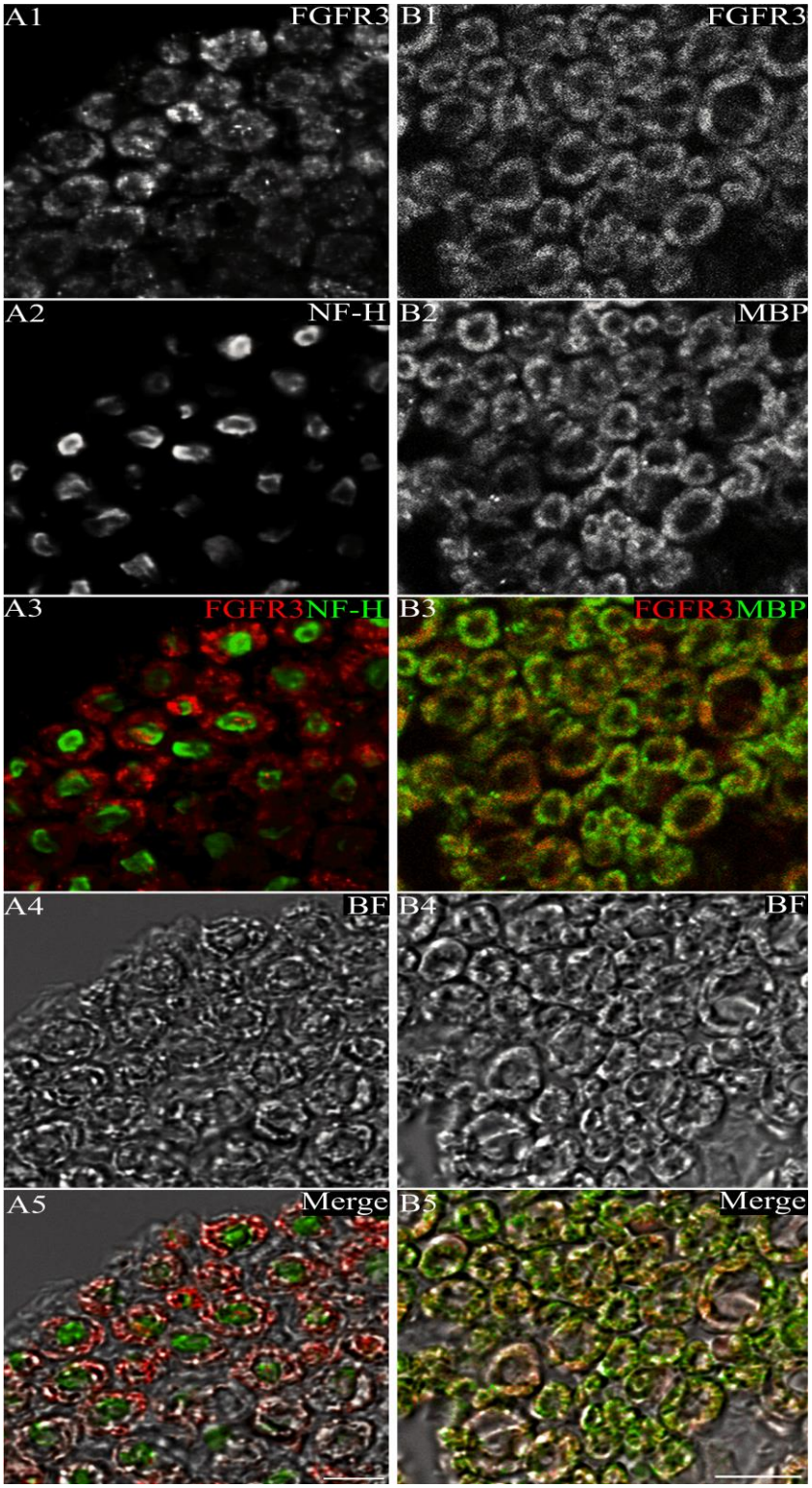

Figure 5.19. Localization of FGFR3 in cross sections of adult mouse sciatic nerve. A1, B1: FGFR3; A2: NF-H; A3: Merge of FGFR3 (red)-NF-H (green); A4: Brightfield; A5: Merge of FGFR3-NF-H -brightfield. B2: MBP; B3: Merge of FGFR3-MBP (green); A4: Brightfield; A5: Merge of FGFR3-NF-H-brightfield. FGFR3 is expressed on the myelin sheath (A). FGFR3 colocalizes with MBP in the myelin sheath (B). Scale bars: 20 μm.

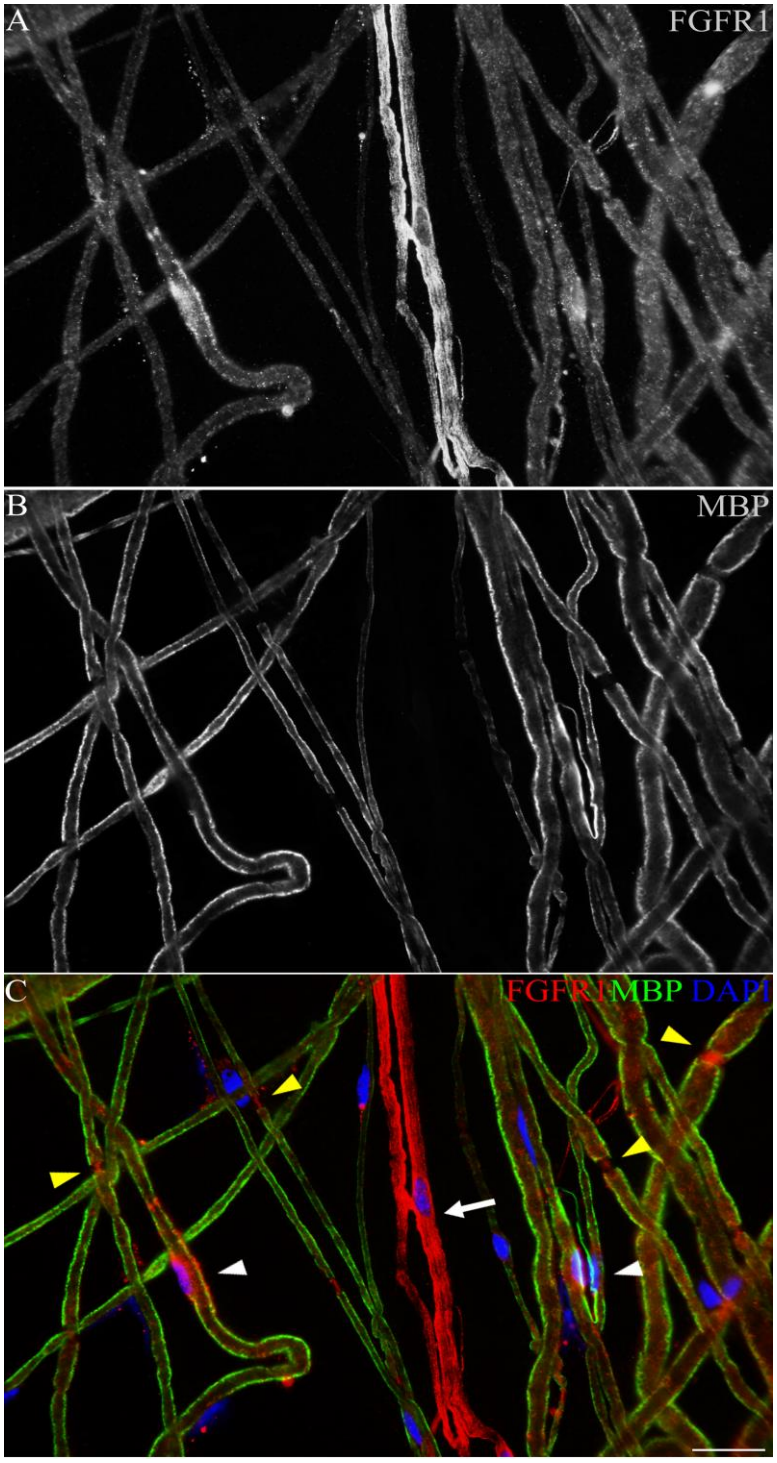

Figure 5.20**.** Localization of FGFR1 in teased adult sciatic nerve. A) FGFR1, B) MBP and C) merge of FGFR1 (red), MBP (green) and DAPI (blue) labeling. FGFR1 is present throughout the myelin sheath, at perinuclear region (white arrowheads) and nodes (yellow arrowheads). Nonmyelinating Schwann cells also express FGFR1 (arrow). Scale bar corresponds to 50 µm.

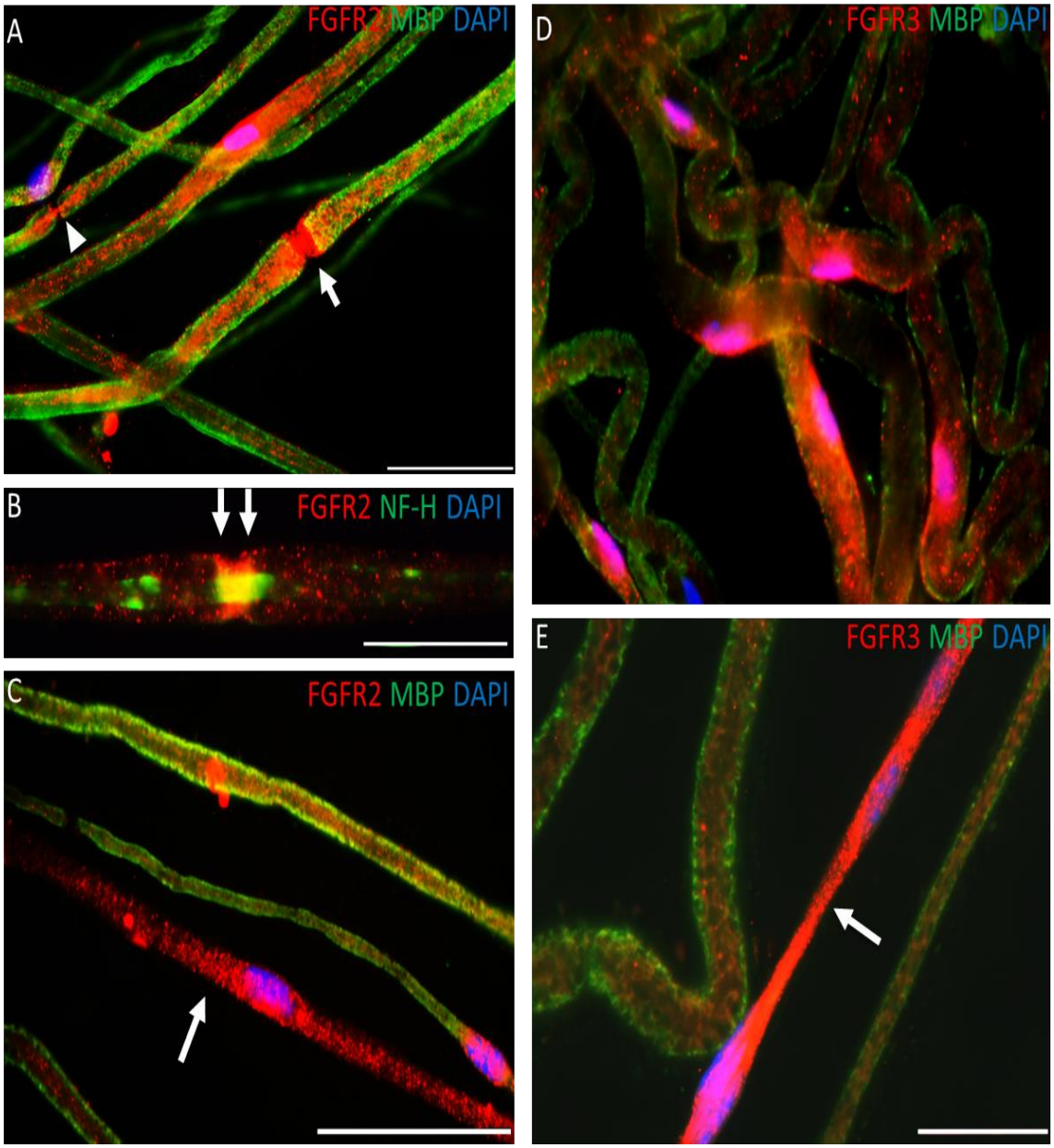

Figure 5.21. Localization of FGFR2 and FGFR3 in teased adult sciatic nerve. FGFR2 (red) and FGFR3 (red) are expressed throughout the myelin sheath. FGFR2 is also localized to the microvilli (A, B; arrows) and to the node (A, arrowhead). Both FGFR2 and -3 are also expressed by nonmyelinating Schwann cells, investigated by absence of MBP (green) immunoreactivity (C, E; arrows). Scale bars correspond to 50 µm.

5.8. Expression of FGFRs is Restricted to Schwann Cells in DRG Culture

To investigate the distribution of the FGFRs during development and the myelination process, fetal mouse dissociated primary cultures were fixed at different stages of development and myelination. In these cultures, FGFR1 was present in the Schwann cells at an early stage and continued to be expressed during the alignment with axons (Figure 5.22A). In older cultures with more advanced myelination, FGFR1 was clearly localized to the myelin sheaths (Figure 5.22B), as previously shown *in vivo*.

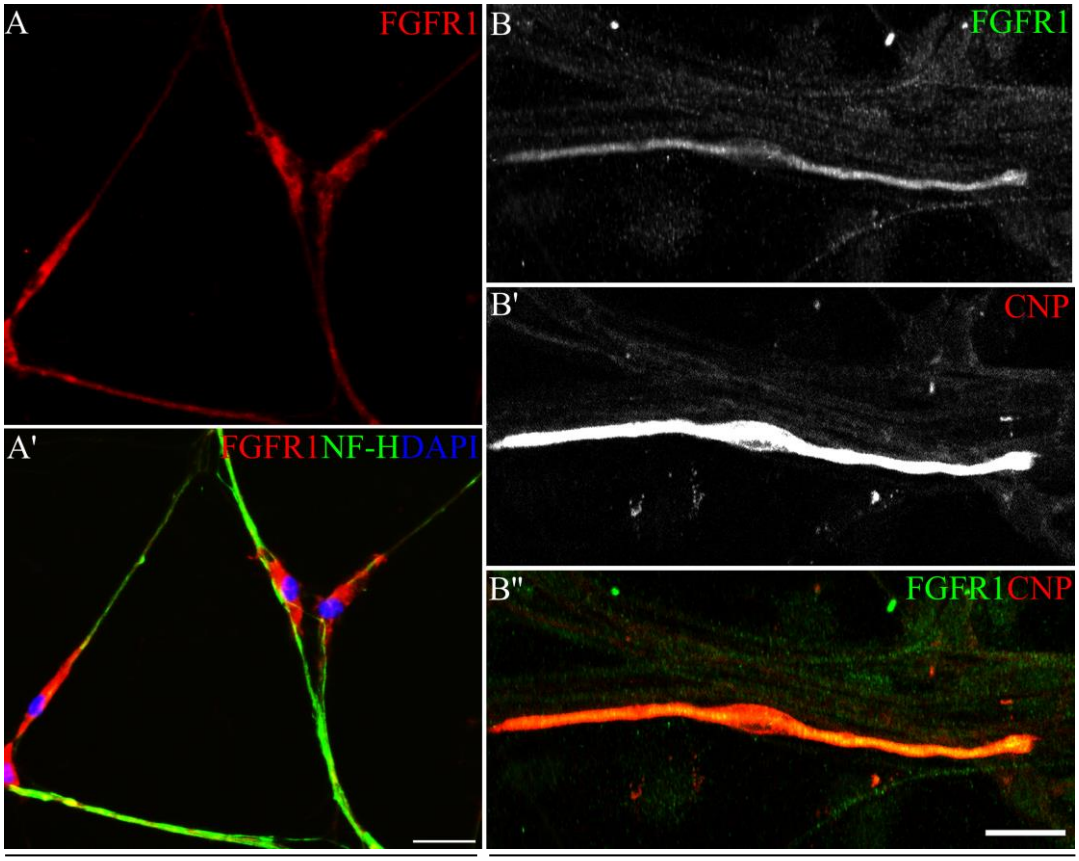

Figure 5.22. Localization of FGFR1 in DRG culture. FGFR1 immunolabeling on induced 7 div (3 dpi) (A) and 42 div (36 dpi) (B) DRG cultures. A) FGFR1 (red), A') merge of FGFR1 (red), NF-H (green) and DAPI (blue) labeling; B) FGFR1, B') CNP and B'') merge of FGFR1 (green) and CNP (red) labeling. FGFR1 is expressed by Schwann cells (A-A') and in the myelin sheath (B-B''). Scale bars correspond to 20 µm.

FGFR2 was expressed in the cell processes and nuclear region of Schwann cells at the earlier developmental stage (Figure 5.23A). FGFR2 then continued to be expressed on the Schwann cells throughout the myelination process and then primarily in the myelin sheath itself (Figure 5.23B).

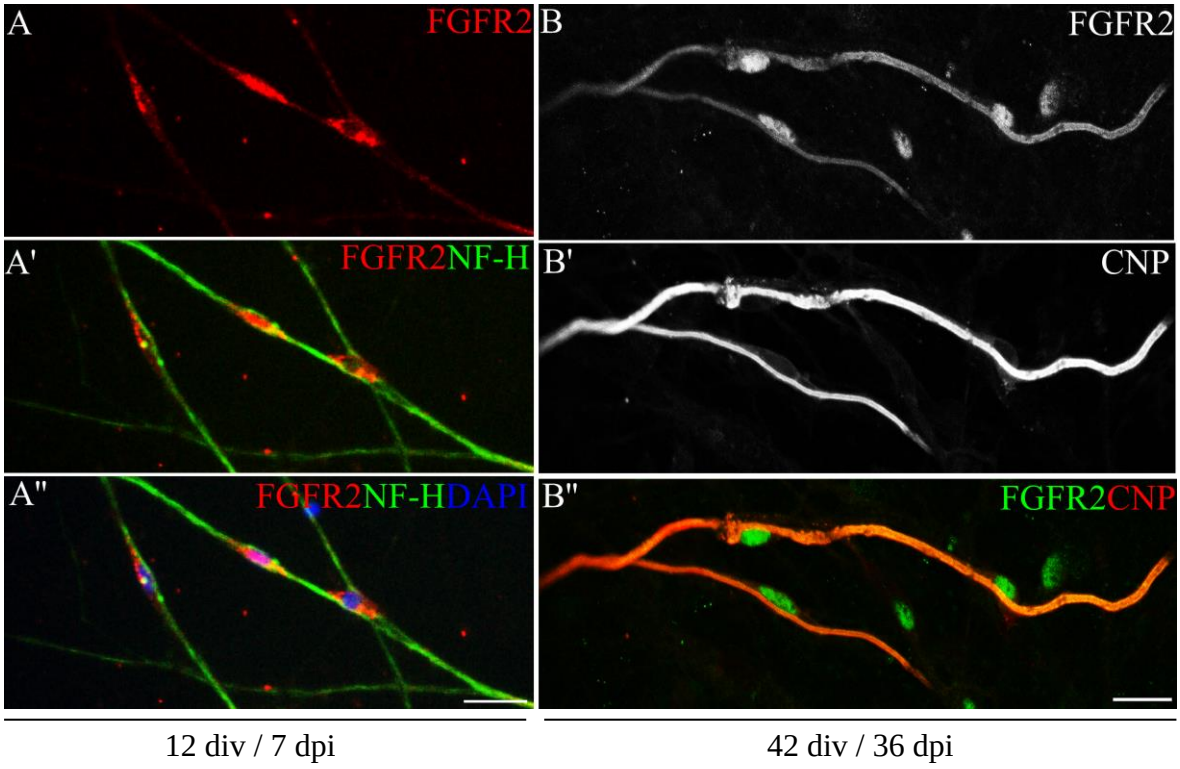

Figure 5.23. Localization of FGFR2 in DRG culture. FGFR2 immunolabeling on induced 7 div (3 dpi) (A) and 42 div (36 dpi) (B) DRG cultures. A) FGFR2 (red), A') merge of FGFR2 (red), NF-H (green), A'') merge of FGFR2 (red), NF-H (green) and DAPI (blue) labeling; B) FGFR2, B') CNP and B'') merge of FGFR2 (green) and CNP (red) labeling. Scale bars correspond to 20 µm.

The Schwann cells in culture also expressed FGFR3, primarily in nuclear regions (Figure 5.24A). The intensity of the FGFR3 labeling decreased somewhat on the Schwann cells after they had aligned with axons (Figure 5.24A, arrow). Similar to FGFR1 and FGFR2, FGFR3 was expressed in the myelin sheath in myelinating cultures (Figure 5.24B).

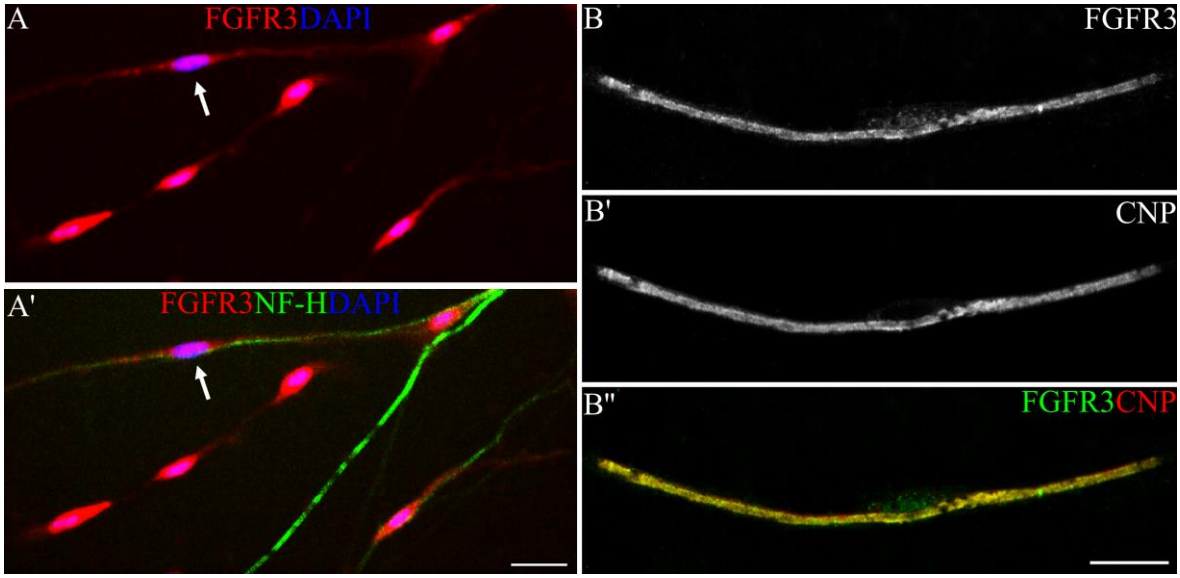

Figure 5.24. Localization of FGFR3 in DRG culture. FGFR3 immunolabeling on induced 7 div (3 dpi) (A) and 42 div (36 dpi) (B) DRG cultures. A) Merge of FGFR3 (red) and DAPI (blue), A') merge of FGFR3 (red), NF-H (green) and DAPI (blue) labeling; B) FGFR3, B') CNP and B'') merge of FGFR3 (green) and CNP (red) labeling. FGFR3 is expressed by Schwann cells (A-A') and in the myelin sheath (B-B''). Scale bars correspond to 20 µm.

In conclusion, versus the axonal FGF1, all three FGF receptors were expressed by Schwann cells during both the early and late stages of the myelination process (*in vitro* and *in vivo*). However, their expression was differentially modulated during development. During the development and in adulthood, FGFR1 expression was always higher compared to FGFR2 and FGFR3. FGFR2 and FGFR3 were expressed at birth and at the start of myelination at high and constant levels, whereas FGFR1 expression was gradually increasing starting at birth with a peak at the onset of myelination. All three receptors were down regulated at P10, but FGFR1 was up regulated again at P15 that coincides with the start of myelin compaction period. Additionally, FGFR2 and FGFR3 expression levels were preserved during this compaction period, but with a higher FGFR3 expression compared to FGFR2.

5.9. Blocking of FGF1 Causes Decrease in Myelination

To better understand the function of FGF1 in peripheral nerve myelination, FGF1 was blocked in the DRG culture *via* neutralizing antibody treatment for six weeks (42 days). Total protein from these cultures and controls were used for Western blot analysis. The results from this experiment are shown in Figure 5.25 and Figure 5.26. The first panel shows immunoblotting from one week old DRG culture. The protein levels shown on this panel are basal levels before any myelination event takes place. The second and third panels show six weeks old myelinating cultures. The myelinating cultures seen in panel two were control cultures, thus treated with rabbit-IgG targeting no specific epitope, while cultures in panel three were treated with FGF1 neutralizing antibody produced in rabbit. MAG, CNP, pERK1/2 and pAKT protein levels were normalized to actin levels and the difference between three cell culture groups was statistically analyzed.

The relative protein levels of MAG, CNP, pERK and pAKT normalized to actin levels are shown in the graph Figure 5.27. Cell cultures treated with FGF1 neutralizing antibody (panel three) had a significant decrease in MAG and CNP expression compared to control cultures (panel two) (Figure 5.25, Figure 5.27). Surprisingly, ERK1/2 and AKT phosphorylation levels increased in cultures where FGF1 was blocked (Figure 5.25, Figure 5.27). When FGF1 was blocked, a clear decrease was also seen in MPZ levels (Figure 5.26). Due to the presence of various isoforms of MBP and MPZ, the analyses of these two myelin proteins could not be statistically evaluated.

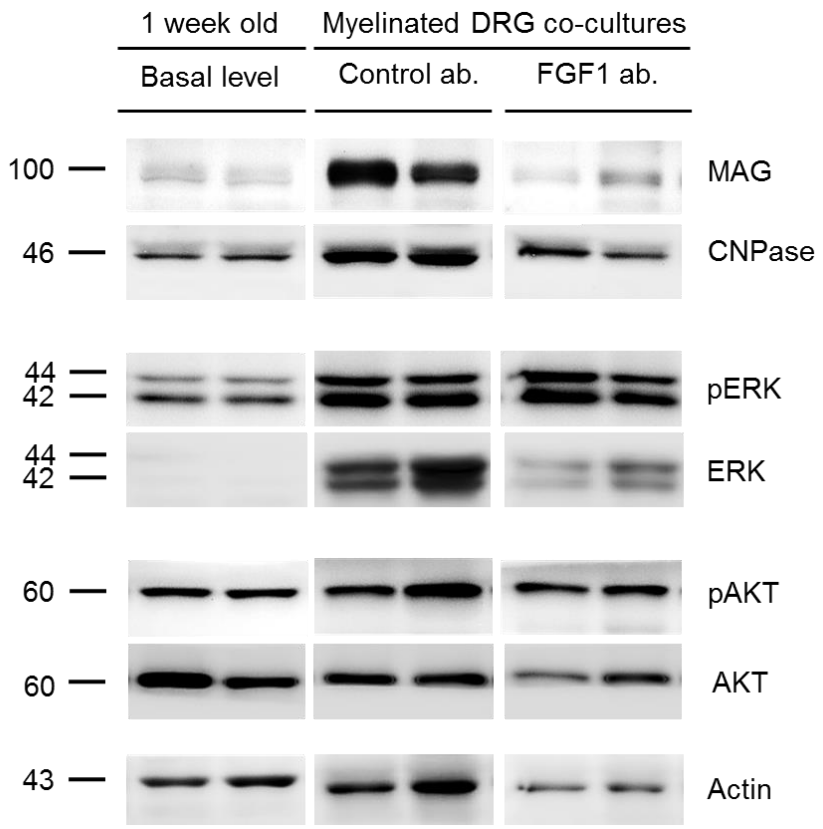

Figure 5.25. Western blot analysis of DRG cultures with FGF1 blocking. Blotting of myelin proteins (MAG, CNP), pERK/ERK, and pAKT/AKT of one week old DRG cultures (first panel), and myelinating DRG cultures treated with rabbit-IgG (control antibody - second panel) and FGF1 neutralizing antibody produced in rabbit (third panel). Two representative images of the three experimental sets are given in each panel.

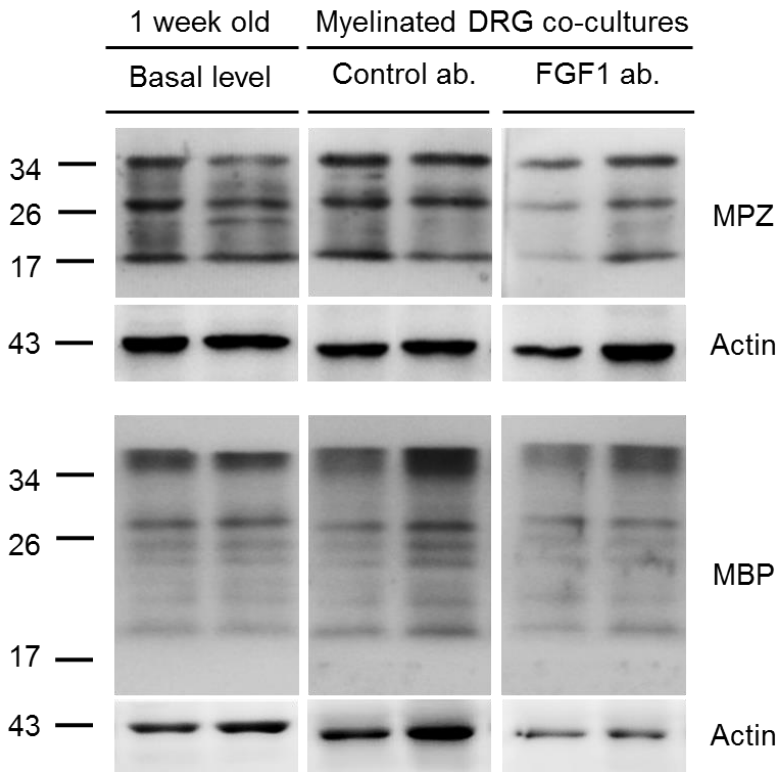

Figure 5.26. Western blots of DRG cultures with FGF1 blocking. Western blotting of myelin proteins MPZ and MBP from DRG cultures of one week old DRG cultures (first panel), and myelinating DRG cultures treated with rabbit-IgG (control antibody - second panel) and FGF1 neutralizing antibody (third panel). Two representative images of the three experimental sets are given in each panel.

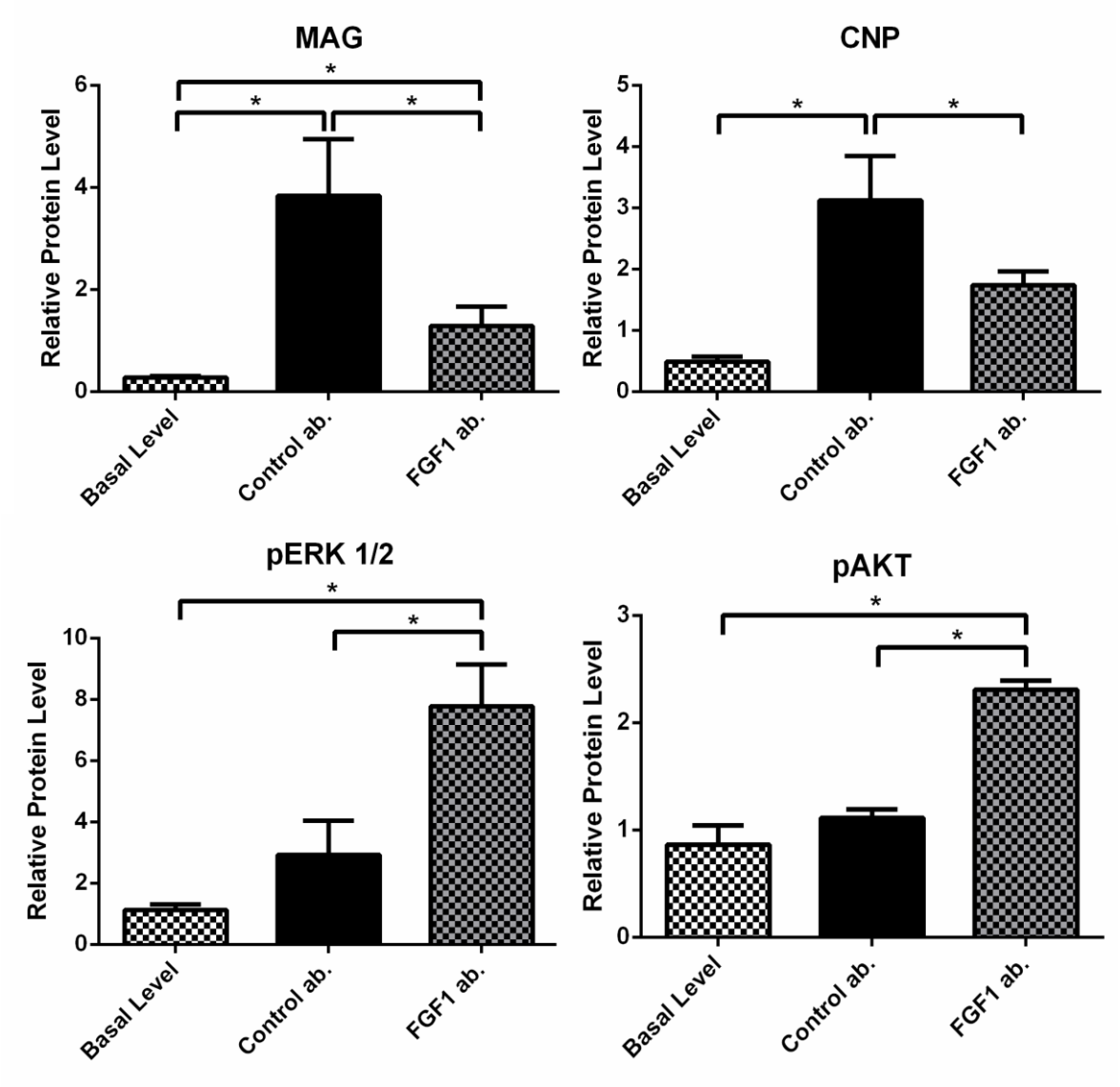

Figure 5.27. Analysis of Western blotting from FGF1 blocked DRG cultures. Average protein levels of MAG, CNP, pAKT and pERK1/2 relative to corresponding actin levels in one week old DRG cultures (basal level) and myelinating cultures treated with rabbit-IgG (control ab.) or treated with FGF1 neutralizing antibody (FGF1 ab.) are shown in the graphs. * $p<0,05$ (Student T-test). Y-error bars represent SEM.

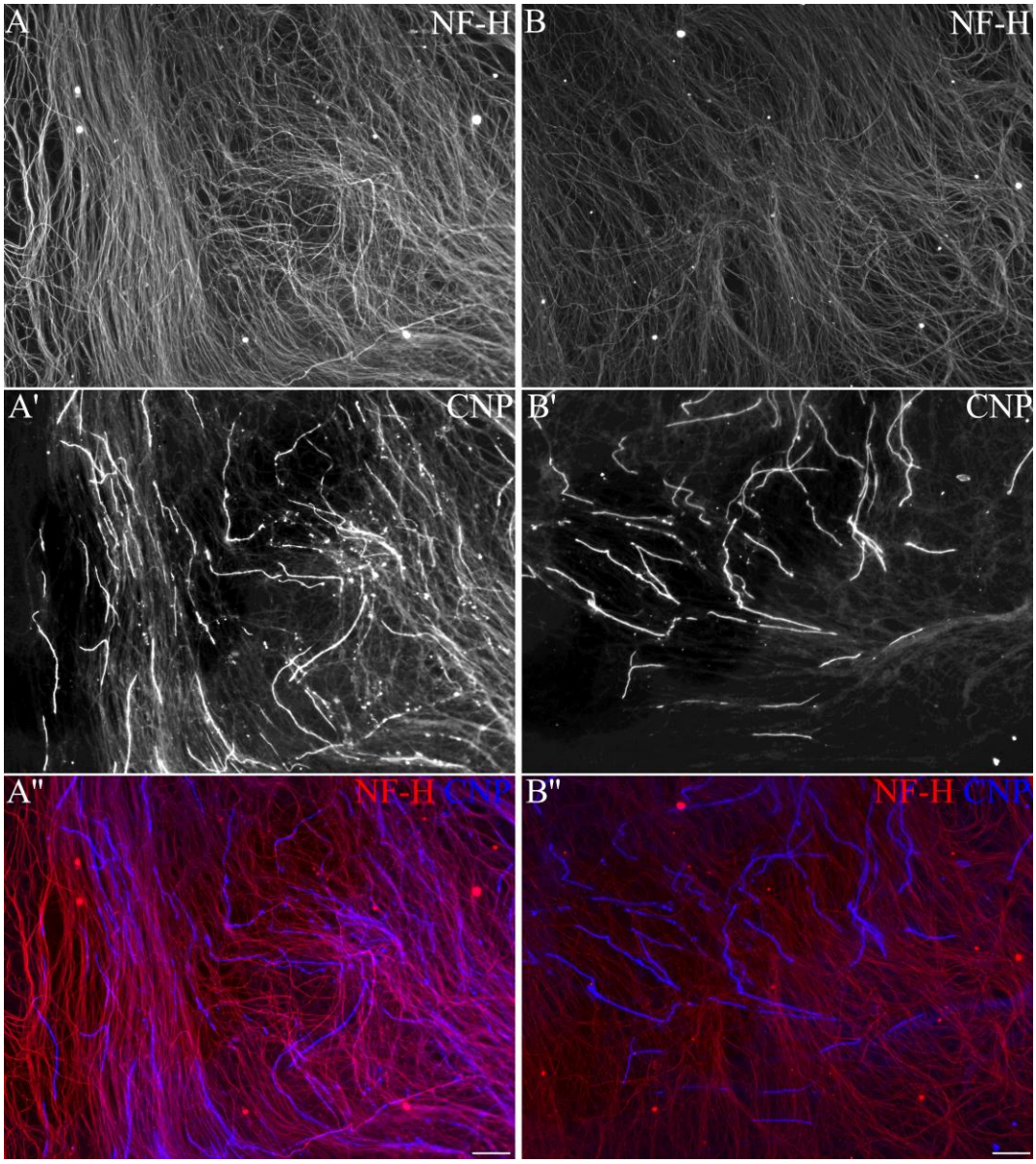

Figure 5.28. Effect of FGF1 blockage in the phenotype. Immunolabeling of myelinating DRG cultures treated with rabbit-IgG (control antibody) (A) and treated with FGF1 neutralizing antibody (B) for NF-H and CNP. A, B) NF-H, A', B') CNP, A'', B'') merge of NF-H (red) and CNP (blue) labeling. Scale bars correspond to 100 μm.

To further investigate the effect of the down regulation of myelin proteins on Schwann cell phenotype, six weeks old myelinating DRG cultures treated with control antibody or FGF1 blocking antibody were immunostained for NF-H and CNP (Figure 5.28). Myelination indexes of these cultures were calculated as the ratio of the number of

CNP positive myelin sheaths between the number of NF-H immunoreactive axons. When these ratios obtained from cultures with FGF1 blockage and control cultures were compared, myelination was found to decrease 15% in the presence of FGF1 blockage (Figure 5.29). However, this reduction in myelination was not statistically significant.

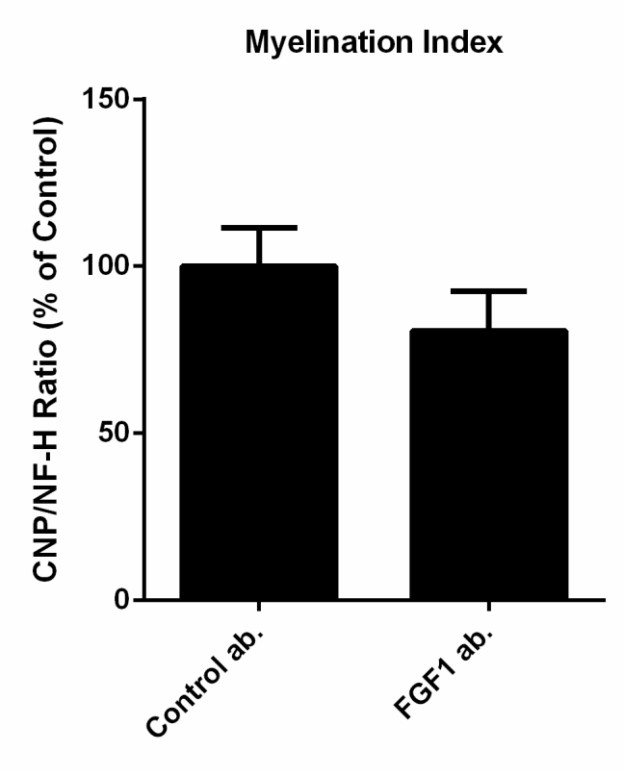

Figure 5.29. Evaluation of the effect of FGF1 blockage in the phenotype. Myelination index of myelinating DRG cultures treated with control antibody or FGF1 neutralizing antibody is shown in the graph. Myelination index is given as the ratio of number of CNP immunoreactive myelin sheaths and number of NF-H immunoreactive axons. In FGF1 blocked cultures, a 15% decrease in the number of myelinated segments was found.

6. DISCUSSION

Patients affected with peripheral neuropathies have defects in the maintenance of the axon-Schwann cell interaction [98]. It is conceivable that the signaling molecules involved in this interaction are expressed by the neurons and evoke the response in the Schwann cells. The aim of this thesis study was to test whether FGF1, FGF2, FGF9 and their high affinity receptors FGFR1-3 were among these signaling molecules and were involved in the myelination process and/or maintenance of the axon-Schwann cell interaction. To delineate this, firstly the expression and localization of FGF and FGFR molecules were investigated in DRG tissue as well as the sciatic nerve during postnatal development and *in vitro* in DRG neuron-Schwann cell culture. Secondly, to better understand their possible relevance to the myelination process, correlation of their expression with the main events taking place in the time course of peripheral nerve myelination was investigated. At the next step, possible roles of the final candidate molecule FGF1 in the peripheral nerve myelination process were analyzed in myelinating DRG cultures. Regarding the results of the first molecule investigated, this study supported previous studies implicating a continuous FGF2 expression that may increase following nerve injury. Secondly, FGF9 was localized to undifferentiated cell types and it was found that FGF9 was probably not involved in the myelination process. Depending on these results, and its axonal localization, FGF1 was selected as the final candidate molecule and further investigated. The findings suggested that FGF1 derived from neurons, may be involved in initiation of myelination as a contributory but not essential factor. It was also shown that FGF1 may be involved in myelin compaction and preservation, as well. In addition, it was demonstrated that FGFR1-3 were differentially expressed by the Schwann cells during development and FGF1 signaling through these receptors may lead to different cellular responses at different time points that are proposed in this study.

6.1. Expression of FGF2 and FGF9

FGF2 expression was investigated using DRG cultures and it was shown that both axons and Schwann cells expressed FGF2. Schwann cells continued to express FGF2 after

aligning with axons and FGF2 signal increased on both Schwann cells and axons upon this contact (Figure 5.1). These results suggest that, FGF2 could modulate the axon-Schwann cell interaction at the onset of myelination, *via* bidirectional signaling. Further analysis related to FGF2 was not performed, since both axons and Schwann cells were expressing this molecule and it was already known to have a role in axon regeneration after nerve injury [11].

FGF9 was investigated in the same culture system and found to be expressed mainly during early development and later in adulthood in nonmyelinating Schwann cells (Figure 5.2). Since it was in glial precursor cells and absent in premyelinating Schwann cells, we suggest that FGF9 may have a blocking effect in the initiation of myelination process. Further analysis is required to analyze this proposed role. After this initial study, the focus was set on FGF1, and its expression and possible roles during development and myelination were examined.

6.2. Blocking FGF1 Results in Delayed Myelination

The results obtained in this project, suggest direct involvement of FGF1 signaling in peripheral nerve myelination. When FGF1 was blocked in DRG cultures, the level of myelin proteins, namely MAG and CNP were decreased (Figure 5.25). In addition, FGF1 blockage lead to a 15% reduction in the number of myelinated segments positive for CNP that was used as a marker of myelin compaction period in this study (Figure 5.28, Figure 5.29). Interestingly, despite FGF1 blockage, phosphorylation of ERK1/2 and AKT increased compared to untreated controls (Figure 5.27). It was unexpected to find an increase in their phosphorylation levels, as these molecules are known to act downstream of the FGF pathway. The active myelination period, taking place at the first two postnatal weeks in mice is characterized with fast membrane synthesis. It is followed by a myelin compaction period coincident with the maturation of the organism. During myelin compaction, myelination slows down radially as axons get thicker and longitudinally as the animal size and axon length increase. It has been shown that murine pERK1/2 and pAKT levels gradually decrease starting from P15, at this maturation stage [39]. The lower pERK1/2 and pAKT levels in our myelinating control cells in culture are thus in parallel

with this information, suggesting that compaction of myelin is proceeding in these cells. In the cultures where FGF1 was blocked, the change in pAKT and pERK levels could be considered as an increase. However, high levels of these molecules are probably due to the presence of the cells that are still at an earlier stage of myelination, where compaction has not been initiated or progressed, yet. Thus, it is conceivable that instead of an increase in phosphorylation levels, it is more probable that the phosphorylation levels have not decreased yet, when FGF1 is blocked. In conclusion, these results suggest that FGF1 blockage cause a delay in the myelination program.

In the expression analysis, FGF1 protein level in the sciatic nerve increased at the start of this compaction period (Figure 5.6), supporting the requirement of FGF1 in compaction. Another observation was the reduced number of the myelinated fibers with CNP labeling, which is the marker for myelin compaction, due to FGF1 blockage. In the light of these findings, it is thus plausible that, blocking of FGF1, did not hinder the initiation of the myelination, but prevented the compaction and probably lead to its retardation.

It has been previously shown that a conditional ERK1/2 mutation in mouse oligodendrocytes and oligondendrocyte precursors resulted in a decrease in the myelin sheath thickness [99]. A similar decrease in the myelin sheath thickness was also observed in mice with FGFR1/2 double mutant oligondendrocytes. This was suggested to be associated with the reduction in ERK1/2 phosphorylation [90]. A reduced peripheral myelin thickness was also reported in Schwann cells mutant for ERK1/2 [47]. In our experiments, neither ERK phosphorylation nor FGFR activation were completely blocked. In fact, only FGF1, one of the ligands of the FGFR pathway was blocked. Therefore, other FGFs activating the FGFR pathway and/or molecules other than FGF/FGFRs upstream of ERK could have contributed to ERK phosphorylation. The same assumption can be made for the higher AKT phosphorylation upon FGF1 blockage. However, the levels of myelin proteins and the number of CNP positive myelinated segments were indeed lower. These findings may suggest that FGF1 could exert its effect on peripheral myelination *via* other downstream molecules, controlling the transcription of myelin proteins. When FGF1 is not available, other myelination signaling molecules cannot efficiently provide the right scheduling for this biological process.

6.3. Axonal FGF1 vs. Glial FGFR1-3

It is widely accepted that signaling molecules promoting axon-Schwann cell interactions are expressed by neurons and activate downstream pathways through their signaling partners localized in Schwann cells. Nrg1 is such a molecule, expressed in neurons, with its ErbB2/3 receptors that are localized in Schwann cells. Nrg1-ErbB2/3 system has been widely investigated in recent studies and shown to have critical functions in Schwann cell development and myelination. Similar to Nrg1-ErbB2/3 system, this study localized FGF1 to axons whereas FGF receptors were localized to Schwann cells in both developing and adult nerves. Supporting their proposed functions, it was found that, the FGFR1-3 distribution was kept stable during the maturation of the PNS, where they were all first expressed in premyelinating Schwann cells during development and later in the myelin sheath itself. Both FGF1 and FGFR1-3 were produced by DRG neurons. Our results suggest that FGF1 is produced in the DRG neurons and is transported out into the axons. Here, the FGF1 can be released, bound to heparin to be kept in a reservoir and signal through FGFR1-3 that are present on the Schwann cells. Interestingly, although FGFR1-3 were also present in DRG neuronal bodies, they were not presented in the axons. Thus, neuronally produced FGFR1-3 are possibly not transported out into axons but must be important in signaling in the cell bodies.

6.4. FGF1 and FGFR1-3 in Early Phases of Myelination

Schwann cell-axon interactions start at an early stage in development and continue throughout the whole myelination process. Stability and maintenance of these interactions are at high importance for myelin formation, maturation and preservation of the entire structure. Our study suggests that neuronal FGF1 is involved in axon-Schwann cell interactions at the onset of myelination. It should be considered that, since Schwann cells in our DRG cultures could start the myelination process without FGF1 signaling, it may not be the only factor required at this stage or its role may be taken over by other FGFs in its absence.

FGF1 and its three receptors FGFR1-3 were expressed in the DRG tissue and in the sciatic nerve during development. The FGFR1 expression pattern follows that of FGF1. FGFR1 protein was low at birth but was clearly up regulated at the start of myelination, similar to FGF1 suggesting that FGFR1 is more likely to contribute to the FGF1 dependent processes occurring at the onset of myelination. FGFR2 and FGFR3 were already expressed at high levels at birth and initiation of myelination (Figure 5.7) and thus, these two receptors may act at the earlier stages of Schwann cell development, for example in the radial sorting and establishment of one-to-one Schwann cell-axon association. In this case, it is possible that other FGFs are part of this process, since FGF1 expression increases later, around P7 (Figure 5.6).

In cross sections of the P1 mouse sciatic nerve, FGF1 was primarily expressed on the axons of the fetal nerve fibre. Thicker axons that were in a more advanced developmental stage, in one-to-one association with a Schwann cell, expressed higher levels of FGF1. Thinner caliber axons were FGF1 negative (Figure 5.8, Figure 5.9). Axon thickness is one of the factors influencing the commitment of a Schwann cell to a myelinating phenotype. It is thought that the amount of axonal signals secreted from the thicker axons increases, as the diameter and surface of the axon increases. It is already known that the level of Nrg1 secreted from the axons has a direct effect on the myelination decision of the Schwann cell [9]. These findings strongly support that FGF1 could also be a mediatory molecule in the decision of being myelinated or not, taken by the Schwann cell.

In DRG culture from fetal mouse, FGF1 had the same distribution as *in vivo*. Both axonal FGF1 and FGFR1-3 located in Schwann cells were up regulated upon the alignment of Schwann cells with axons and induction of myelination. Thus, it is conceivable that this up regulation could be due to reciprocal signaling where axonally secreted FGF1 serves as a signal for the Schwann cell to initiate myelination (Figure 5.14, Figure 5.15). Overall, the data presented so far support the involvement of FGF1 and FGFR1-3 at the onset of myelination and Schwann cells' differentiation to a myelinating phenotype.

6.5. FGF1 and FGFR1-3 in the Compaction and Late Stages of Myelination

To support the proposed functions of FGF1, it is important to combine the results with expression patterns of its receptors. Our findings showed that all three FGF receptors are all expressed at high levels at the onset of myelination as explained before. But later, at P10 all FGF receptors analyzed are down regulated. Out of the three receptors, FGFR2 is down regulated the most and is then kept at a stable low level throughout the myelination process. The FGFR3 expression is kept rather high through P10-20 when, myelin compaction occurs. Importantly, following the decrease at P10, FGFR1 is up regulated again at P15-20. This expressional modulation suggests that FGF1 signaling is likely to be mediated primarily *via* FGFR1 and FGFR3 in the axon-Schwann cell interactions during myelin compaction. In a conditionally mutant mice where Schwann cells lack FGFR1/2 expression, C-fibres were found to be affected, but the myelin was observed to be intact [88]. This could mean that the myelin was thinner or less compacted, which can not be observed by immunolabeling. When our observations are considered together with these previous findings, it can be hypothesized that FGFR3 is likely to be the major FGF receptor responsible for compaction and loss of FGFR3 rather than FGFR1 or FGFR2 could lead more severe effects on myelination.

In the adult mouse, all three receptors are expressed throughout the myelin sheath even though their expression levels are lower. Interestingly both FGFR1 and FGFR2 are also expressed at the node of Ranvier. Nodes of Ranvier are important for the stability and function of the myelin sheath [100, 101]. Thus, it is suggested that the continued expression of FGF1 and FGFR1-3 in the adult nerve could regulate the preservation of the whole nerve structure including the nodal regions, where the signaling in the nodes of Ranvier is achieved through FGFR1 and FGFR2.

6.6. Immature and Nonmyelinating Schwann Cells Expressing FGF1

FGF1 is expressed by glial cells both *in vivo* in the sciatic nerve and *in vitro* in DRG culture. In cross sections of the P1 mouse sciatic nerve, only Schwann cells that were

in early stages of development expressed FGF1. This is the time period in development when Schwann cells migrate along axons and segregate them. Schwann cells in more advanced developmental stages, like the ones in fetal nerve fibres and the ones in one-to-one association with thicker axons were not expressing FGF1 at P1. In later phases of myelination, at P20, myelinating Schwann cells were FGF1 negative (Figure 5.8, Figure 5.9, Figure 5.10). The data obtained from cultures were also in agreement with the *in vivo* results. In cell culture at early stages, Schwann cells that were not in contact with axons expressed FGF1. This FGF1 expression was down regulated, when these cells aligned with axons (Figure 5.14, Figure 5.15).

It is known that survival of Schwann cells depend on autocrine signals at developmental stages [29, 40]. After association with axons, they start to depend on axonal signals. Thus, one hypothesis may be that immature Schwann cells in earlier developmental time points express FGF1 as a proliferative/survival autocrine factor. However, FGF1 expression is likely to be down regulated, when Schwann cells get the signals from the large caliber axons, to start their myelination program. A continuance of FGF1 expression in these Schwann cells may have a preventive effect on Schwann cell differentiation to a myelinating phenotype.

About half of the Schwann cells in the sciatic nerve do never myelinate. Instead, they become nonmyelinating Schwann cells and ensheath one or several thin caliber axons to form Remak bundles. These cells in our experiments continued to express FGF1 during their differentiation and maturation (Figure 5.11, Figure 5.13). This glial derived FGF1 expression may be mediating interactions between Schwann cells and the axons that they ensheathe. In conditionally mutant mouse where Schwann cells lack FGFR1/2 expression, axonal degeneration occurred primarily in C-fibres [88]. Similarly, ErbB signaling was shown to mediate Schwann cell-axon interactions in the Remak bundle [36, 41]. Taken together, these data suggest that nonmyelinating Schwann cells may sustain themselves and thin caliber axons using their intrinsic FGF1. As a second hypothesis, it may be suggested that other FGFs derived axonally could signal through FGF receptors in these nonmyelinating Schwann cells and act in Remak bundle stability.

6.7. FGF1 Expression Depends on Axon Caliber

Supporting the role of FGF1 in myelination, FGF1 expression in axons was found to depend on their diameter. While thick caliber axons express high concentrations of FGF1, thin caliber axons express very little or seemingly no FGF1 at all. At P1, the expression of FGF1 was not detectable in most of the axons. However, as Schwann cells aligned, the FGF1 expression increased in the thicker axons while the Schwann cells that were associated with those axons stopped their FGF1 expression. The thin caliber axons that never started to express FGF1 were instead ensheathed by nonmyelinating Schwann cells. There was also a population of axons, that were FGF1 negative or expressed less FGF1 even though they were myelinated (Figure 5.11). This difference did not depend on the subtype of the neuron (Figure 5.12). Further investigations are required to understand the absence of FGF1 in this neuronal population.

6.8. FGF1 in Schwann Cell Migration and Motility

Interestingly, FGF1 was found in the filopodia of Schwann cells that were not in contact with axons and that were likely migrating in the DRG cultures (Figure 5.16). This finding suggests that glial FGF1 expressed during development may be involved in Schwann cell motility.

FGF1 was present in the microvilli at the node of Ranvier in the adult sciatic nerve (Figure 5.11). FGFR2 had a similar distribution localized to the node of myelinating Schwann cells (Figure 5.18). This type of microvilli is required for wrapping the axons [96, 97]. These findings therefore implicate FGF1 in the axonal wrapping that is generated by myelinating Schwann cells. The signal is likely mediated through the FGFR2 that is specifically localized to the glial microvilli.

6.9. Suggestions for Molecular Mechanisms of FGF1 in Schwann Cell Development and Myelination

Several possible functions for FGF1 have been proposed in this thesis. Both Schwann cells and neurons express FGF1 and Schwann cells show different cellular responses to signaling of this molecule. Different outcomes observed during differentiation using the same molecule and receptors is not unique to our hypothesis since it is a common theme in many tissues as well as in the nervous system [5, 6, 43, 44, 65, 98, 102-110, 113]. Firstly, FGF1 signaling through different receptor subtypes (FGFR1-3) may lead to different outcomes. Secondly, the expression of FGF1 and its receptors at different developmental time points may generate completely different signaling responses. Thirdly, the activation of the signaling pathways through different isoforms of the receptors, as well as different durations of activation of the receptor may also result in different outcomes. The latter situation, was previously shown for rat pheochromocytoma cell line, PC12 cells where transient ERK activation by epidermal growth factor (EGF) provides proliferation of the cells, whereas sustained ERK activation provided by FGF and nerve growth factor (NGF) signaling results in differentiation of the PC12 cells [66, 102-105]. These different responses was shown to depend on the FGF signaling recruiting more Grb2–SOS complex to the plasma membrane through the phosphorylation of FRS2 [105].

The mechanism of action for FGF1 may be similar to that of Nrg1 that also signals through tyrosine kinase receptors and can generate several different responses in Schwann cells. Nrg1 mediates activities of both myelinating and nonmyelinating Schwann cells and regulates axon-Schwann cell interactions at every stage of the development and myelination process. Nrg1 is expressed in DRG sensory, motor and autonomic neurons, while its receptor ERB2/3 is expressed by Schwann cells as for FGF1 and its receptors. Nrg1 is known to be involved in the differentiation of neural crest cells into Schwann cells, maturation of Schwann cell precursors, survival, proliferation, motility and migration of Schwann cells, *in vitro*. Nrg1 also controls Schwann cell proliferation and myelination, *in vivo*. In adult nerve, axonal Nrg1 represses Schwann cell proliferation and acts as a survival factor for Schwann cells [5, 6, 43, 106-109]. Nrg1 also regulates myelin sheath thickness [44]. Thus, the suggested functions for FGF1 in this thesis show similarity to those of Nrg1 during Schwann cell development, myelination and its maintenance. These

two factors may act together providing evidence for the widely accepted view that coordinated action of several factors is required in this complex process.

Recent studies linked a number of downstream pathways of Nrg1 to myelination like Shp2/MAPK, PI3K/Akt, PLC, and Rac1 [8]. Interestingly, mTOR (mammalian target of rapamycin), the serine threonine kinase activated by insulin and growth factors, has recently been implicated in the myelination process. A lack of mTOR signaling resulted in reduced axonal growth and myelination [48]. FGF signaling through FGFRs also activates the same downstream molecular pathways, thus axonally secreted FGF1 may activate the very same signaling molecules in the Schwann cells. It is actually unclear how FGF1 may act as it is a secreted factor, in comparison to axon resident Nrg1. In fact, in this study, in addition to FGF1 present in the axons, a lower level of FGF1 outside the axons was also observed (Figure 5.10, Figure 5.11). As a possible explanation to this question, these findings suggest that this secreted FGF1 could possibly bind to the heparan sulfate proteoglycans on the cell surface to form a reservoir and efficiently provide the signaling [63].

In early development, FGF1 expressed by Schwann cells could act in an autocrine or paracrine manner for their own survival/proliferation through similar pathways explained above. In addition, this thesis study showed FGF1 expression in the filopodia of Schwann cells and microvilli in the myelin sheath and suggested that FGF1 could also control motility of these Schwann cells. There are two Rho family GTPases, Cdc42 and Rac1 known to be involved in Schwann cell migration and motility. Nrg1 was shown to control the activity of these molecules in Schwann cells. These molecules are upstream of c-Jun N-terminal kinase (JNK) and the p38 mitogen-activated protein kinase pathways (MAPK). Moreover, disruption of these pathways found to interfere with myelination [8]. JNK and p38 pathways are also downstream of FGF signaling [110]. These findings suggest that FGF1 may be acting through JNK and p38 pathways and provide Schwann cell motility for their migration during development and/or for wrapping axons in the myelination process that should be further tested in our system.

6.10. Suggested Model for the Roles of FGF1 and FGFR1-3 in Peripheral Nerve Myelination

The suggested model comprising the possible outcomes of FGF1-FGFR1-3 signaling during development and myelination is schematized in Figure 6.1.

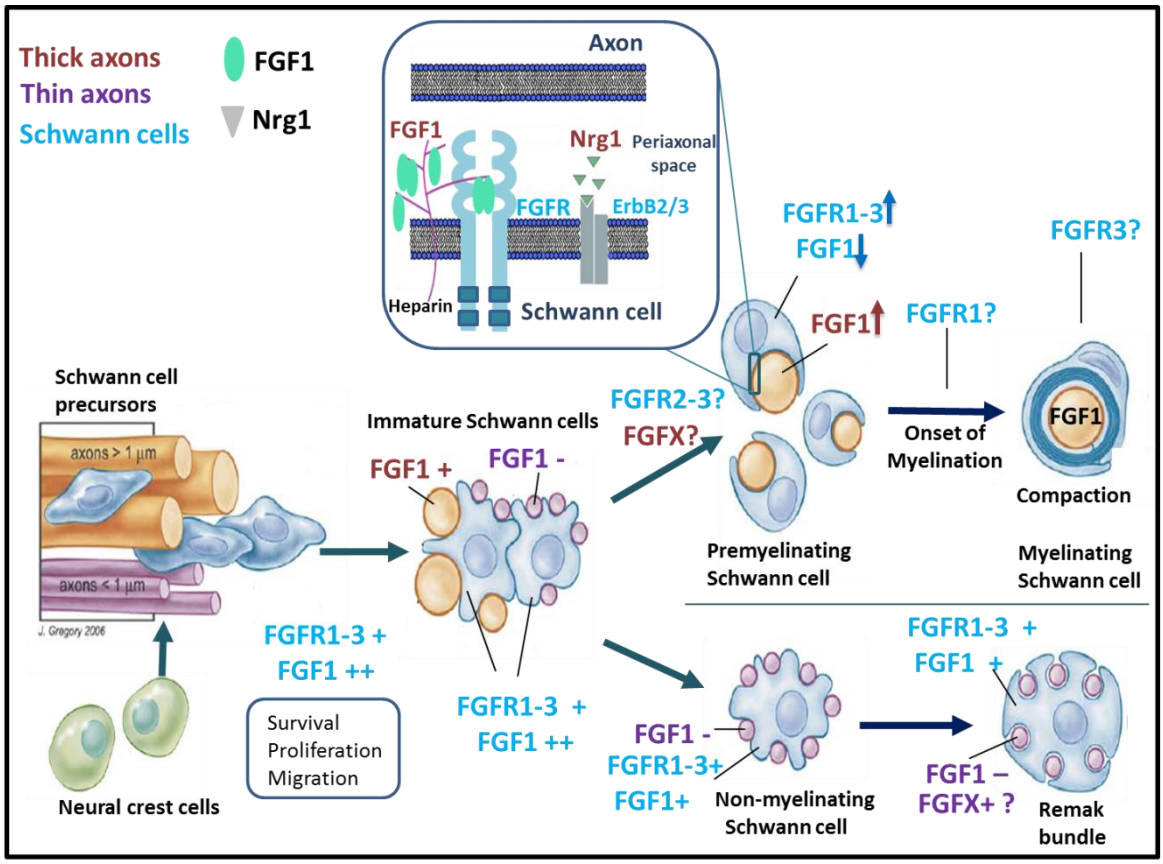

Figure 6.1. The model for proposed functions of FGF1. Factors produced by thick axons are colored red, factors related with thin axons are colored purple and factors related with Schwann cells are colored blue. Modifed from Nave and Salzer, 2006 [111].

In early phases of development, immature Schwann cells express FGF1 and its three receptors, acting mainly as an autocrine and paracrine survival factor as well as regulating proliferation and motility of Schwann cells. When these Schwann cells and axons get into closer contact, neurons with larger caliber axons (with a diameter thicker

than one micrometer) up regulate FGF1 expression and start to secrete FGF1 as well as other myelination signals to the Schwann cells in the vicinity. These Schwann cells contact the axons, establish one-to-one association and down regulate their intrinsic FGF1 expression. In this transition stage, before FGF1 is up regulated in axons, other FGFs may possibly act through FGFRs on Schwann cells. The Schwann cells then start myelination. At this stage, it is suggested that FGF1, together with other myelination signals like Nrg1, is secreted from axons, bound by heparin and signal through FGFRs on Schwann cells to initiate myelination.

After myelination is initiated, axonal FGF1 regulates the compaction of the myelin sheath most probably *via* controlling the expression of myelin proteins and lipids that are deposited to the Schwann cell membrane. FGFR1 is likely to be the major FGF receptor acting at the onset of myelination, whereas FGFR3 is most likely to be involved in the compaction process. Later, when all myelination is completed, axonal FGF1 serves as a factor in the maintenance of the myelinated structure

Thinner axons never express FGF1 or other factors that serve as myelination signals. Because they lack myelination signal production and/or because they excrete other signals, the immature Schwann cells that are in contact with these axons differentiate into nonmyelinating Schwann cells and form Remak bundles. These Schwann cells do not stop expressing FGF1 during their differentiation and continually express this protein throughout development and maturation. In adulthood, FGF1 produced in nonmyelinating Schwann cells functions for their own stability, thus the stability of the Remak bundle. As a second hypothesis, since FGFRs are expressed on Schwann cells, most probably other FGFs derived from axons exert their effects through these receptors and act in Remak bundle maintenance.

7. CONCLUSION

In this study, we provided evidence for our hypothesis stating that neuronal FGF1 is one of the axonal signals regulating peripheral nerve myelination through its high affinity receptors FGFR1-3. Our results suggest that axonally derived FGF1 has important roles at the onset of myelination, during myelin compaction and also in the maintenance of the myelin sheath. In addition, glial FGF1 is suggested to act through FGFR1-3 as an autocrine factor for proliferation/survival/motility for immature Schwann cells and as a regulatory molecule in nonmyelinating Schwann cell-axon interactions in Remak bundles.

As future prospects, co-immunolabeling of FGF1/ FGFR1-3 and marker proteins on the sciatic nerve could be performed to support their localizations in the domains of the myelinated fibre, namely at the nodes and microvilli. Further functional analyses are already being performed in our laboratory to support the suggested roles for FGF1 and to identify through which receptor it exerts its effect in different developmental points.

To our knowledge, for the first time in literature this study implicates the involvement of FGF1 in peripheral nerve myelination. Understanding the molecules and the pathways controlling Schwann cell development and myelination serves as an important tool to unravel the pathology of peripheral neuropathies and develop novel and efficient therapeutic agents.

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