EXPRESSION OF FIBROBLAST GROWTH FACTORS AND THEIR RECEPTORS IN DEVELOPING MOUSE DORSAL ROOT GANGLIA

by

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To my dear mother and lovely sister...

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ABSTRACT

EXPRESSION OF FIBROBLAST GROWTH FACTORS AND THEIR RECEPTORS IN DEVELOPING MOUSE DORSAL ROOT GANGLIA

Myelin is a key component of the nervous system where it discontinuously insulates axons and increases the speed of action potential propagation. The absence or insufficiency of myelin sheathing causes hereditary neuropathies. For the initiation and maintenance of myelin sheathing, axon – Schwann cell contact seems to be essential and growth factor-mediated signaling has been proposed to mediate intercellular communication. Nerve regeneration paradigms and in vitro models point to the importance of FGFs and neurotrophins in myelination. However, their roles in the initiation and progression of myelination in vivo are not yet clear.

Therefore, we studied expression of FGF1, FGF2, FGF9, FGFR1-4, and BDNF in adult and postnatal DRG during the period of active myelination. Our results show a gradual increase in FGF1 and FGF2 transcripts during early myelination, there after no modulations were evident. In contrast, FGF9 transcript levels increased until P10, then gradually decreased to basal levels in adulthood. FGF1 protein was first observed on P4, its levels increased for 10 days, then gradually decreased to basal levels in adults. Transcript levels of FGFR1 increased until P10 and then stabilized while FGFR2 transcript was present at all time points with no significant change. FGFR3 and BDNF transcript levels were unchanged during active myelination period, followed by a significant decrease. FGFR4 transcript levels were barely detectable. The expression of myelin proteins PMP22 and MPZ at the transcript level, and MAG both at transcript and protein levels were followed in the same samples. The results agree with the literature.

The observed profiles suggest, if these factors have any roles at all in myelination, FGF1 and FGF2 might be important for both the formation and maintenance, while BDNF and FGF9 might be implicated primarily in the formation of the myelin sheath.

ÖZET

GELİŞEN FARE ARKA KÖK GANGLİYONLARINDA FİBROBLAST BÜYÜME FAKTÖRLERİ VE RESEPTÖRLERİNİN ANLATIMI

Miyelinasyon, çevresel sinir sistemi işlevinin önemli bir parçasıdır ve oluşumundaki bozukluklar periferal nöropatilerle sonuçlanmaktadır. Miyelin kılıfın oluşumu ve korunmasında Schwann hücrelerinin aksonlarla etkileşiminin elzem olduğu ve büyüme faktörlerinin hücreler arası iletişimde kritik rol oynadıkları düşünülmektedir. PNS rejenerasyon ve in vitro miyelinasyon modelleri, FGF ve nörotrofin aile üyelerinin myelin kılıf oluşumuna katkılarının önemine işaret etmektedir. Ancak bu faktörlerin miyelinasyon sürecindeki ve miyelin kılıfın korunmasındaki in vivo rolleri ile ilgili bilgi bulunmamaktadır.

Bu nedenle çalışmamızda, erişkin ve postnatal arka kök gangliyonlarında FGF1, FGF2, FGF9, FGFR1-4 ve BDNF anlatımları incelenmiştir. Verilerimiz FGF1 ve FGF2 transkript düzeylerinin aktif miyelinlenme döneminde arttığını, sonrasında bu düzeyin sabit kaldığını göstermektedir. Buna karşın P10'a kadar artan FGF9 transkript düzeyleri, daha sonra düşmektedir. FGF1 proteini ilk olarak P4 – P10'da artma göstermiş, sonrasında azalarak erişkinde bazal seviyeye inmiştir. FGFR1 transkript düzeyleri P10'a kadar artmış, sonrasında sabit kalmıştır. FGFR2 transkripti tüm örneklerde düşük düzeydedir ve değişim göstermemektedir. FGFR3 ve BDNF transkript düzeyleri ise aktif miyelinlenme döneminde sabit kalıp, erginlerde anlamlı düşüş göstermiştir. FGFR4 düzeylerinin çok düşük olduğu görülmüştür. Miyelin proteinlerinin anlatımı PMP22 ve MPZ için transkript, MAG içinse hem protein hem transkript düzeyinde irdelenmiştir. Verilerimiz literatürle uyumludur.

Tanımlanan anlatım profilleri, FGF1 ve FGF2'nin myelin kılıfın oluşumu ve sonrasında korunması aşamalarında önemli olabileceklerini düşündürmektedir. Öte yandan, aktif miyelinasyon sonrasında FGF9 ve BDNF düzeylerinde benzer bir azalma görülmesi, bu faktörlerin daha çok, sürecin oluşum aşamasında yer alabileceklerine işaret etmektedir.

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BDNF	Brain-derived neurotrophic factor
bp	Base pairs
cAMP	Cyclic AMP
cDNA	Complementary DNA
CMT	Charcot-Marie-Tooth
CNS	Central nervous system
CO_2	Carbondioxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRG	Dorsal root ganglion
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FGFRL1	Fibroblast growth factor receptor-like 1
FRS2	Fibroblast growth factor receptor substrate 2
HNPP	Hereditary neuropathy with liability to pressure palsies
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycan
Ig	Immunoglobulin
kDa	Kilo Dalton
L-MAG	Large myelin-associated glycoprotein
MAG	Myelin-associated glycoprotein
$MgCl_2$	Magnesium chloride
M-MLV	Moloney murine leukemia virus
MPZ	Myelin protein zero
mRNA	Messenger RNA
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride

NGF	Nerve growth factor
NP-40	Nonidet P-40
NRG1	Neuregulin-1
NT3	Neurotrophin-3
OD	Optical density
OL	Oligodendrocyte
P	Postnatal day
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PMP22	Peripheral myelin protein 22
PNS	Peripheral nervous system
PVDF	Polyvinyl difluoride
qRT-PCR	Reverse transcription-coupled quantitative real-time PCR
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RIPA	Radio-immuno-precipitation assay
RPII	RNA polymerase II
S-MAG	Small myelin-associated glycoprotein
SC	Schwann cell
SDS	Sodium dodeacyl sulfate
SEM	Standard error of the mean
shRNA	Small hairpin RNA
SOS	Son of sevenless
SPSS	Statistical package for social sciences
TAE	Tris acetate ethylenediaminetetraacetic acid
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine

1. INTRODUCTION

Myelin is a key component of the nervous system where it discontinuously insulates axons from the outer environment and increases the speed of action potential propagation. The absence or insufficiency of myelin sheathing causes hereditary neuropathies such as Charcot-Marie-Tooth disease (CMT). CMT patients suffer from a wide range of symptoms such as muscle weakness and atrophy, partial or complete loss of deep tendon reflexes, sensory loss and skeletal deformation. More than 20 genes are held responsible for different subtypes of this disease (Parman, 2007).

Myelin is formed by oligodendrocytes (OLs) in the central nervous system (CNS) and Schwann cells (SCs) in the peripheral nervous system (PNS). The major triggering signal for myelination is the contact between the axon and the glial cell (Wood and Bunge, 1975). It has been suggested that this event not only involves direct interaction between cell surface proteins but also utilizes growth factor-mediated signal transduction through neurotrophins and fibroblast growth factors (FGFs). Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) are proposed to be active in the initiation of myelination. FGF2 is known to be upregulated upon axonal injury, and FGF2-overexpressing mice regenerate faster after sciatic nerve crush (Chan *et al*., 2001; Grothe *et al*., 2001; Tolwani *et al*., 2004; Jungnickel *et al*., 2006).

In this project, we aimed to investigate the possibility of FGFs taking part in PNS myelination. Instead of degeneration / regeneration models, we focused on the *in vivo* myelination process. For this purpose, we followed the modulations in the steady state levels of different FGF and FGFR transcripts in dorsal root ganglia (DRG) on the day of birth, before the initiation of myelination, until 20 days when the myelination impetus is decreasing, as well as the adult stage when myelin formation is finished.

1.1. Dorsal Root Ganglia

Various receptors on distal parts of the body transmit sensory information to the spinal cord. Sensory neurons, which are involved in this transmission, enter the spinal cord through dorsal roots. Distal to dorsal roots, cell bodies of sensory neurons accumulate inside intervertebral foramina and form DRGs (Figure 1.1).

Like all nervous system tissues, DRG is composed of neurons and glia. While majority of the glia are SCs, there are also satellite glial cells unique to peripheral sensory neurons which wrap them and bind them together into clusters (Hanani, 2005).

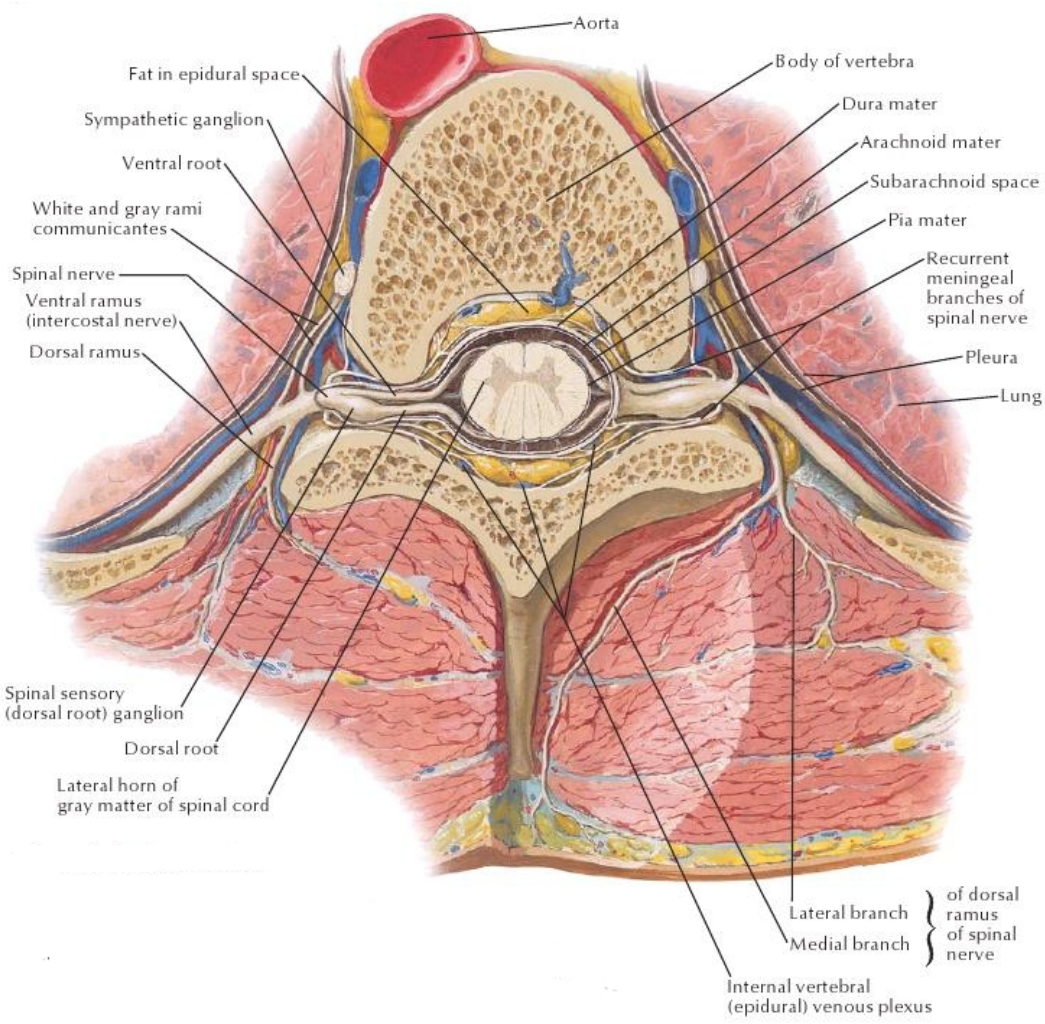

Figure 1.1. A cross section through thoracic vertebra. DRGs are shown lateral to dorsal roots, as extensions of the ventral rami (Netter *et al*., 2002).

SCs play essential roles in PNS development and regeneration. Myelination is one of their most important functions and it is carried out only by myelinating SCs. There are also non-myelinating SCs in the DRG, which provide physical and metabolic support to neurons (Jessen, 2004).

Both neurons and SCs in the DRG originate from migrating neural crest cells during embryonic development. Some of these cells differentiate into SC precursors, which further differentiate into immature SCs. All immature SCs have equal developmental potential but the diameter of the axon they contact with determines their fate. Those which associate with larger diameter axons (> 1 μm) display myelinating phenotype while the others develop into non-myelinating SCs (Figure 1.2) (Windebank *et al*., 1985; Jessen and Mirsky, 2005).

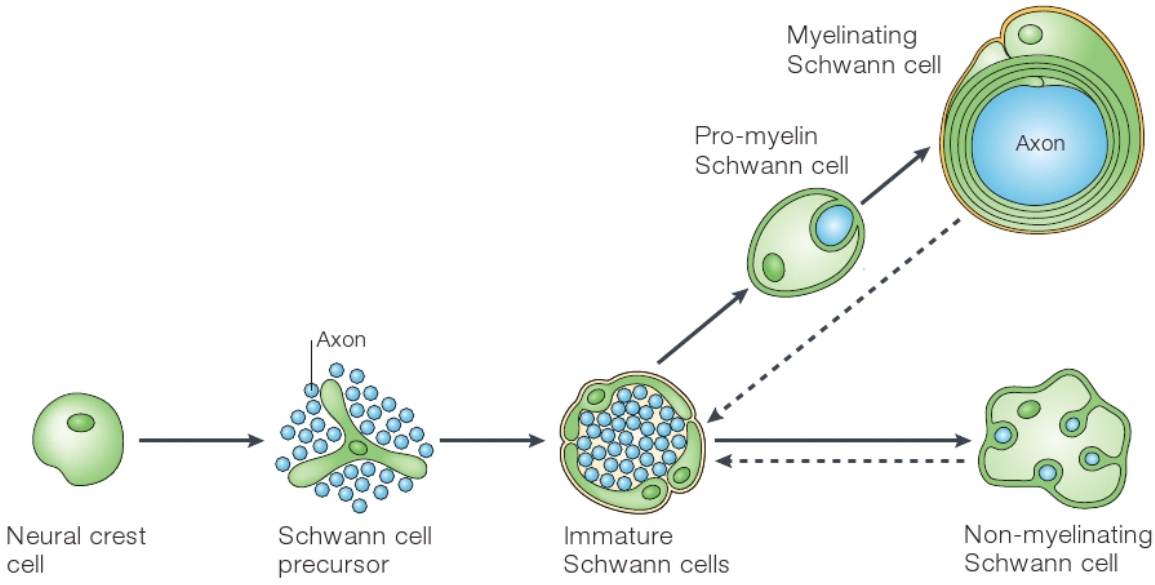

Figure 1.2. Fate of a Schwann cell. As neural crest cells differentiate into SC precursors, they start associating with axons and develop into immature SCs. Eventually, those which are in contact with larger-diameter axons mature into myelinating SCs, while the others become non-myelinating (Jessen and Mirsky, 2005).

1.2. Structure and Function of Myelin

Myelin is a complex structure insulating axons from the outer environment and thereby increasing the velocity of action potential conduction by 15 to 300-fold (Figure 1.3) (Augustine, 2004).

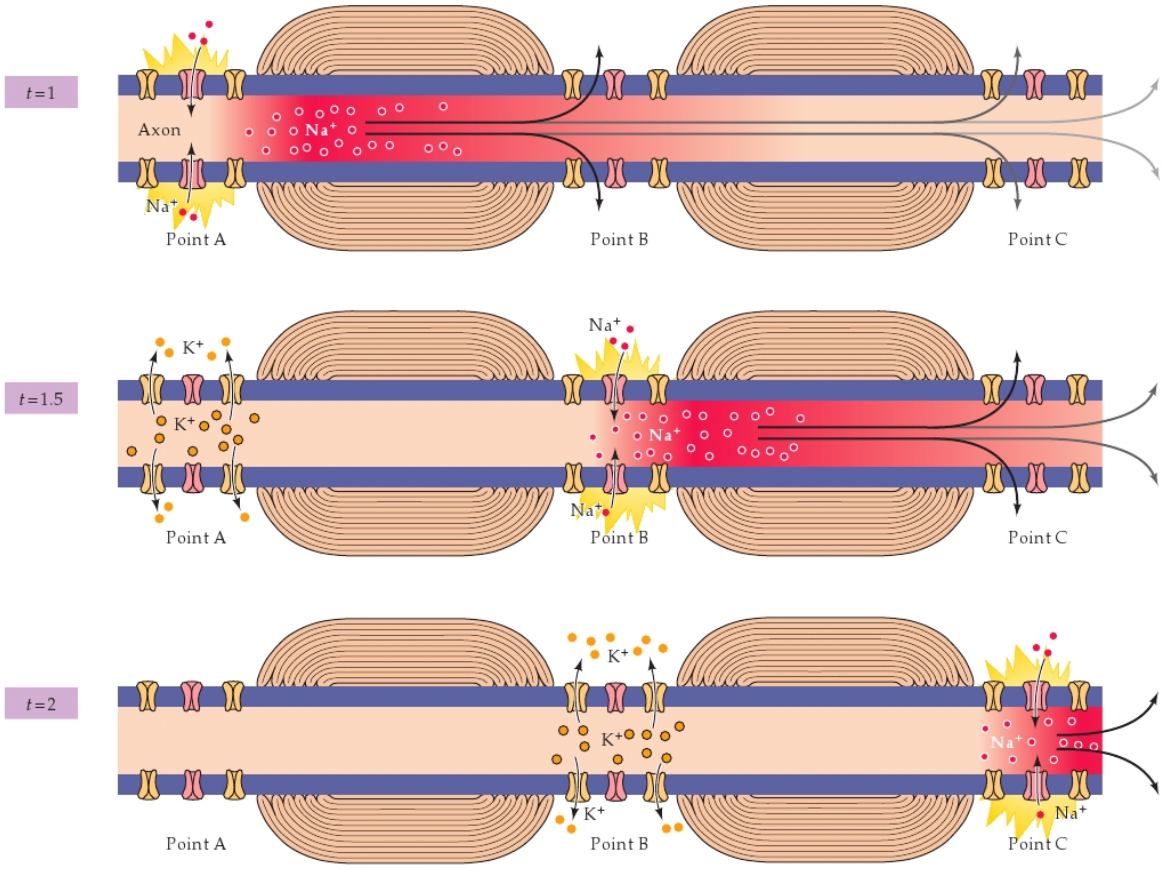

Figure 1.3. Propagation of an action potential through a myelinated axon. At $t = 1$, the first Na^+ wave is generated at the first node and conducted to the second one. Upon arrival of this wave at $t = 1.5$, axonal membrane at the node undergoes a local depolarization which triggers sodium channels. Then these channels are activated and another Na^+ wave is generated. At $t = 2$, the latter wave is conducted to the third node. Since action potential seems to be jumping from node to node, this process is called saltatory conduction (Augustine, 2004).

The composition of myelin is different in both nervous systems. Isolated PNS myelin consists of 70 – 80 per cent lipid and 20 – 30 per cent protein. Lipid composition of myelin includes various galactolipids and phospholipids as well as cholesterol (Garbay *et al*., 2000).

More than 60 per cent of myelin proteins are glycosylated. The major ones in this group are myelin protein zero (MPZ), peripheral myelin protein 22 (PMP22), myelin-associated glycoprotein (MAG), periaxin and epithelial cadherin. The second group of myelin proteins are basic proteins including myelin basic protein and protein P2. Finally, there are others such as proteolipid and gap junction proteins which constitute less than 1 per cent of total myelin protein (Garbay *et al*., 2000). In this study, only MPZ, PMP22 and MAG were used as myelin markers.

1.2.1. Myelin Protein Zero

MPZ represents more than half of the total myelin protein in the PNS (Everly *et al*., 1973) and it is specific to myelinating SCs (Garbay *et al*., 2000). It is a 28 kilo Dalton (kDa) single-pass transmembrane glycoprotein with an extracellular immunoglobulin (Ig) and an intracellular domain (Eichberg, 2002).

Evidence suggests that extracellular domains of MPZ interact with each other (Filbin *et al*., 1990) and the proteins exist as stable dimers or tetramers on the cell membrane (Thompson *et al*., 2002). Therefore, it is widely accepted that MPZ is involved in myelin compaction in the PNS (Kursula, 2008).

Point mutations in MPZ cause different subtypes of CMT, such as CMT1B (dominant demyelinating form), CMT2I (dominant axonal form) or CMTDI3 (dominant intermediate form) (Berger *et al*., 2006).

1.2.2. Peripheral Myelin Protein 22

PMP22 is a 22 kDa tetraspan glycoprotein. It accounts for 5 per cent of the total myelin protein in the PNS. Like MPZ, it is found in the compact myelin but its expression is not restricted to the PNS (Quarles, 2002). Although expression levels are much higher in SCs, PMP22 was also observed in other tissues like lung, brain and colon (Spreyer *et al*., 1991).

D'urso *et al*. showed that PMP22 colocalizes with MPZ on the myelin membrane (D'urso *et al*., 1999). Since PMP22 protein level is much lower as compared to MPZ, it was proposed that PMP22 assists MPZ in myelin formation and maintenance.

An imbalance of PMP22 expression results in CMT1A or hereditary neuropathy with liability to pressure palsies (HNPP) depending on gene dosage. A heterozygous intrachromosomal duplication of the genomic region spanning PMP22 gene results in CMT1A whereas its heterozygous deletion results in HNPP (Gabriel *et al*., 1997). Furthermore, many point mutations were identified in this gene. Most of them are dominantly inherited and cause CMT1A or Dejerine-Sottas Syndrome, which is a more severe subtype of CMT1 with earlier onset (Parman *et al*., 2004). These mutations usually impair intracellular trafficking of PMP22 (Naef and Suter, 1999).

1.2.3. Myelin-Associated Glycoprotein

MAG is a single-pass transmembrane protein with an apparent mass of 100 kDa, one third of which is carbohydrate (Quarles *et al*., 1983). It has five extracellular Ig-like domains and two alternatively spliced cytoplasmic domains (Kursula, 2008). These isoform are known as large and small MAG (L-MAG and S-MAG, respectively). Although temporal expression of these isoforms show a complex pattern in the CNS, S-MAG is the predominan isoform found in the PNS (Butt *et al*., 1998; Quarles, 2007). The intracellular domain of L-MAG can bind to S100β and various kinases while S-MAG can bind to the cytoskeleton via tubulin (Kursula, 2008). Therefore, it was suggested that L-MAG may act in glial signal transduction whereas S-MAG functions in preserving myelin integrity (Garbay *et al*., 2000).

1.2.4. Formation of Myelin in the Peripheral Nervous System

Myelination requires a coordinated interplay between neurons and SCs. Before birth, fetal nerve fibers are surrounded by a thin layer of SCs. Subsequently, SCs associate with individual axons in one-to-one stoichiometry through a process called radial sorting, and form promyelin fibers (Figure 1.4) (Chan, 2007; Garbay *et al*., 2000). At birth (postnatal day 0, P0), mouse PNS has mostly fetal nerve fibers. During the following week, promyelin and myelinated fibers increase while fetal nerve fibers decrease. In mice, myelination is initiated at P4. Until P15, lamellae formation and lipid incorporation progress very fast. After two weeks (P14), most of the fibers are myelinated, there are very few fetal nerve fibers left. Subsequently, this impetus is lost and myelin formation is much slower. Although myelination is very rapid during the first two weeks, the process may continue until the end of the first month after birth (Garbay *et al*., 2000).

Expression of myelin-related genes also correlate with this timing (Garbay *et al*., 2000). After the decision on myelination is made, SCs sorted on axons start synthesizing myelin by incorporating myelin-associated lipids and proteins like PMP22 and MPZ. Meanwhile, they wrap the axon by rotating around it and form myelin lamellae, which subsequently exclude their cytoplasms to compact the myelin sheath (Garbay *et al*, 2000). Mutant PMP22 and MPZ proteins or abnormal levels of PMP22 protein are thought to interfere with this process and eventually cause different subtypes of CMT disease (Berger *et al*., 2006).

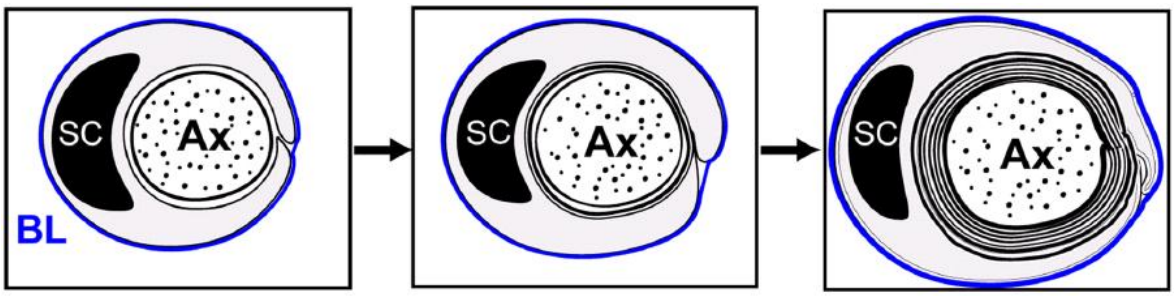

Figure 1.4. Radial sorting of SCs. After a SC ensheaths an individual axon, it starts wrapping around it and initiate the formation of myelin. BL: Basal lamina of the SC, depicted in blue; SC: Schwann cell; Ax: Axon (Chan, 2007)

The major signal triggering the initiation of myelination is thought to be the axon – SC contact (Wood and Bunge, 1975). However, very little is known about the mechanism of this interaction. On the molecular level, recent evidence shows that neuregulin-1 (NRG1) type III expressed on the axon is critical in whether the axon will be myelinated or not (Taveggia *et al*., 2005). Basal lamina, or at least laminin, is also required by SCs to start myelination. This is thought to be achieved through integrin receptors and focal adhesive kinase (Feltri *et al*., 2002; Grove *et al*., 2007). Some transcription factors such as Oct-6, Krox-20 and Sox-10 have also been implicated in SCs during myelination (Monuki *et al*., 1990; Topilko *et al*., 1994; Kuhlbrodt *et al*., 1998).

It has been postulated that signals other than axon - SC contact are necessary for the initiation of this process. Neurotrophins, especially neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF), have been identified in this context. Chan *et al*. (2001) proposed a model in which decrease in NT3 levels allowed the initiation of myelination whereas a high level of BDNF was required during the early stages of myelin formation (Chan *et al*., 2001). Subsequently, BDNF levels were reduced, probably by the upregulation of TrkB-T1, which is a truncated TrkB receptor acting as a BDNF scavenger in this case (Cosgaya *et al*., 2002). Furthermore, BDNF overexpressing mice showed accelerated myelin formation and increased axon diameter (Tolwani *et al*., 2004) whereas NT3 mutant mice underwent normal radial sorting but myelination was not initiated, myelin proteins were downregulated and apoptosis was induced in SCs (Woolley *et al*., 2008).

In addition to these two, nerve growth factor (NGF) was proposed as a regulator of myelination *in vitro*, for a class of DRG neurons that express TrkA receptors. Interestingly in neuron / SC cocultures, NGF induces myelination in contrast to its inhibitory effects on OLs (Chan *et al*., 2004).

The FGF family has also been proposed to play important roles in the development and functioning of mammalian nervous system (Slack, 1994; Powers *et al*., 2000). Though their *in vivo* roles are not clear at this time, they appear to be strong candidates to take part in myelination.

1.3. Fibroblast Growth Factors

FGFs constitute a large family of growth factors with at least 22 members in vertebrates. They are arranged in seven subfamilies by phylogenetic similarity (Figure 1.5) (Mason, 2007).

Molecular weights of FGFs range between 17 to 34 kDa. They share a conserved core region which is important for receptor recognition (Ornitz and Itoh, 2001; Reuss, 2003). FGFs also have a high affinity for heparan sulfate proteoglycans (HSPGs), which are proposed to stabilize the receptor – ligand complex (Ornitz, 2000).

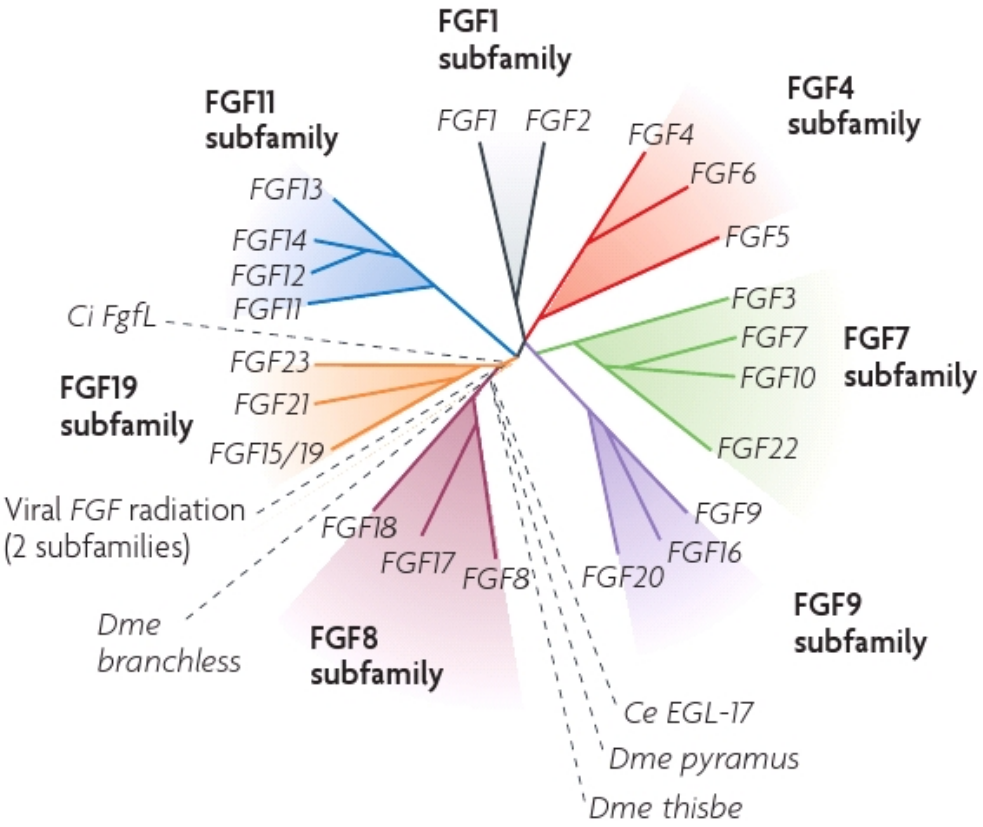

Figure 1.5. Phylogenetic relation of FGF family members. Each subfamily is depicted in a different color. Orthologues in different organisms are also shown. Ci: *Ciona intestinalis*, Ce: *Caenorhabditis elegans*, Dme: *Drosophila melanogaster* (Mason, 2007).

FGFs play significant roles in embryonic development, tumorigenesis, angiogenesis as well as cellular processes such as proliferation, survival, differentiation and motility (Mason, 1994; Powers *et al*., 2000; Presta *et al*., 2005). Our lab has recently shown that FGF2 and FGF9 can induce proliferation of cultured Müller glia of retina (Cinaroglu *et al*., 2005; Candaş, 2007). Earlier reports suggest that FGF1 and FGF2 may have neurotrophic effects on neurons of both CNS and PNS (Unsicker *et al*., 1987). On the tissue level, FGF2, 3, 4, 8, 17 and 18 have been implicated in the patterning of CNS structures (Ford-Perriss *et al*., 2001; Dono, 2003). In this study, only FGF1, FGF2 and FGF9 were investigated.

1.3.1. Fibroblast Growth Factor 1

FGF1 is a 17.5 kDa protein composed of 155 amino acids in mice. The mature protein has a nuclear localization motif but no signal peptide and appears to be secreted through non-canonical pathways (Powers *et al*., 2000). Expression of FGF1 has been demonstrated in different rat CNS and PNS regions, such as DRG, spinal cord, mesencephalon and cauda equina (Oellig *et al*., 1995).

1.3.2. Fibroblast Growth Factor 2

FGF2 has four isoforms with apparent weights of 18, 22.5, 23.1 and 24.2 kDa. The 18 kDa isoform has a high homology with FGF1. Similarly, it has a nuclear localization signal but no secretory signal sequence (Powers *et al*., 2000). It can be phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) (Feige and Baird, 1989). Expression of FGF2 was investigated in rat PNS where it was shown in intact sciatic nerve and DRG (Grothe *et al*., 2001).

1.3.3. Fibroblast Growth Factor 9

Since it was originally purified from glial cells, FGF9 is also known as glia-activating factor (Miyamoto *et al*., 1993). It is a 29/30 kDa protein expressed in several nervous system tissues such as brain, retina, motor neurons and DRG (Tagashira *et al*., 1995; Nakamura *et al*., 1997; Li *et al*., 2002; Cinaroglu *et al*., 2005). Knock-out studies

also reveal that it is important in lung and testes development (Colvin *et al*., 2001a, 2001b).

1.4. Fibroblast Growth Factor Receptors

1.4.1. Structural Properties

Secreted FGFs are ligands for receptor tyrosine kinases known as FGF receptors (FGFRs). These are single-pass transmembrane proteins with three Ig-like domains and an acidic domain on the extracellular side as well as a split tyrosine kinase domain and several regulatory sequences on the intracellular side. The Ig-like domains are designated as IgI, IgII and IgIII. Acidic domain, also known as the acid box, resides between IgI and IgII (Figure 1.6) (Powers *et al*., 2000; Eswarakumar *et al*., 2005). IgII and IgIII domains, together with the linker region in between, constitute the ligand binding site for FGFs (Plotnikov *et al*., 1999).

There are four FGFRs (FGFR1-4) as well as a recently characterized FGFR-like membrane protein (FGFRL1) which has a similar extracellular structure but no intracellular kinase domain (Trueb *et al*., 2003; Eswarakumar *et al*., 2005).

There are also several isoforms of FGFRs except for FGFR4, all generated by alternative splicing. Some of them lack one of the extracellular Ig-like domains and some are not localized to the membrane but instead secreted (Figure 1.7). Differential exon usage on the third Ig-like domain generates FGFR-IIIb and -IIIc isoforms which seems to be important in defining ligand specificity. Preferred ligands of these FGFR isoforms are presented in Table 1.1.

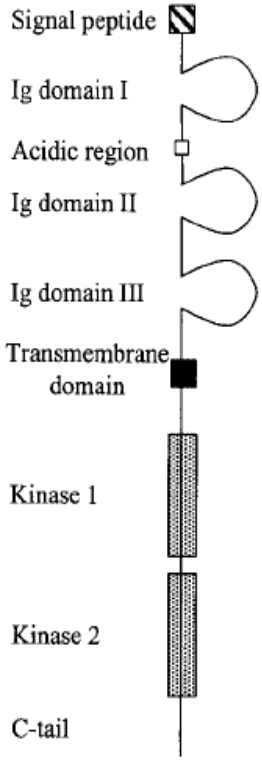

Figure 1.6. Schematic representation of a FGFR (Powers *et al*., 2000)

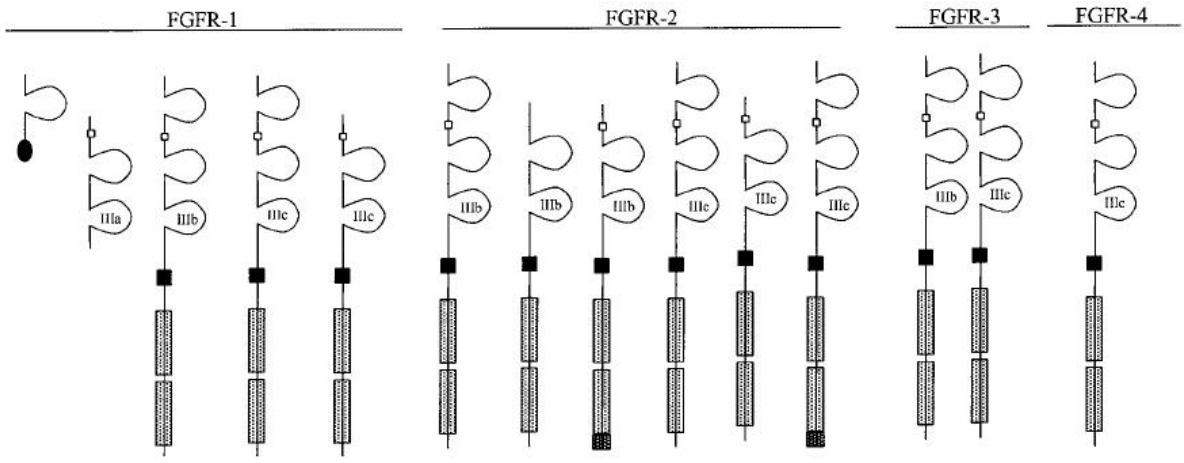

Figure 1.7. Schematic representations of various FGFR isoforms. Black oval represents early truncation and a dashed box below the kinase domain represents alternative carboxy terminus (Powers *et al*., 2000).

Table 1.1. FGFR isoforms and their preferred ligands (modified from Mason, 2007).

Receptor	Preferred ligands
FGFR1-IIIb	FGF1, 2, 3, 10, 21 and 22
FGFR1-IIIc	FGF1, 2, 3, 4, 5, 6, 8, 15/19, 17, 20, 21 and 23
FGFR2-IIIb	FGF1, 3, 7, 10, 15/19, 21, 22 and 23
FGFR2-IIIc	FGF1, 2, 4, 5, 6, 8, 9, 15/19, 16, 17, 18, 20, 21 and 23
FGFR3-IIIb	FGF1, 8, 9, 15/19, 20, 21 and 23
FGFR3-IIIc	FGF1, 2, 4, 8, 9, 15/19, 16, 17, 18, 20 21 and 23
FGFR4	FGF1, 2, 4, 6, 7, 8, 15/19, 17, 18, 20, 21 and 23

1.4.2. Signaling Through Fibroblast Growth Factor Receptors

Upon ligand binding, FGFRs dimerize and their kinase domains are activated by transphosphorylating each other. Moreover, binding of HSPG to this complex is essential for the stabilization of FGFs in the extracellular matrix and complete activation of FGFRs. (Harmer, 2006). Generally, the functional complex consists of FGF:FGFR:HSPG in a 2:2:1 stoichiometry (Stauber *et al*., 2000).

Several pathways are activated by FGFRs (Figure 1.8), which include Grb2 as a common component. Upon ligand binding, FGF receptor substrate 2 (FRS2) as a docking protein binds to FGFR and recruits Grb2 as well as son of *sevenless* (SOS). Downstream to this complex, Erk1/2 is activated, which in turn activates transcription factors, thus regulates gene expression. Grb2 can also interact with FGFR directly and activate phosphatidylinositol-3-kinase (PI3K) pathway upstream of Akt / protein kinase B (PKB). In addition, Grb2 can promote FGFR turnover by recruiting the ubiquitin ligase Cbl (Mason, 2007).

Another important cascade is Ca^{2+} / PKC pathway which is activated upon phospholipase Cγ (PLCγ) docking directly to activated FGFR. Furthermore, p38, Jun and Rho also reside downstream of FGFR in some cells (Mason, 2007).

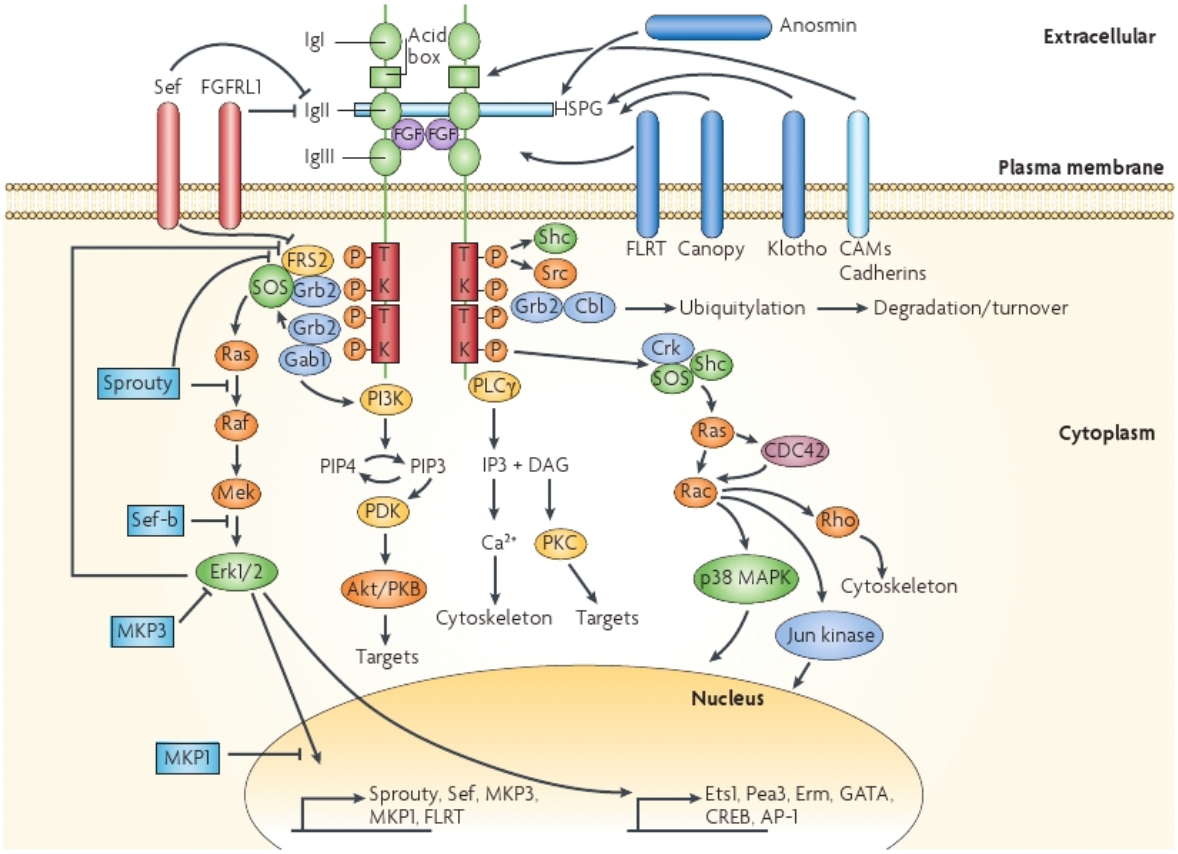

Figure 1.8. A summary of main pathways activated by FGFRs (Mason, 2007).

The FGF signaling pathways are associated with diverse cellular responses such as proliferation, differentiation, survival, apoptosis, cytokine production and migration (Powers *et al*., 2000). It is also known that there are cross-talks between different pathways like TrkA and WNT (Ong *et al*., 2000; Katoh and Katoh, 2006), and complexities are introduced by the cell-type dependent expression of many of the signaling protein elements and transcription factors as well as activation kinetics.

1.5. Roles of Fibroblast Growth Factors in the Nervous System

In the CNS, FGF1 is expressed mostly in neurons whereas FGF2 and FGF9 can be found in glia as well. They are distributed widely in the cerebrum, medulla oblangata and retina (Bugra and Hicks, 1997; Nakamura *et al*., 1999; Reuss and von Bohlen und Halbach, 2003).

FGFR1-3 are also expressed in CNS tissues. While FGFR1 is usually neuronal, FGFR2 and FGFR3 are predominantly glial (Yazaki *et al*., 1994). FGFR4 expression is strong in embryos but diminished significantly in adults (Fuhrmann *et al*., 1999).

FGFs are implicated in various processes in the CNS. FGF2 can induce neurogenesis and act as a neuroprotective agent for neurons as well as a differentiation factor for both neurons and glia (Reuss and von Bohlen und Halbach, 2003). It also enhances axonal growth and branching of cortical neurons, survival and neurite extension of hippocampal neurons (Walicke *et al*., 1986; Kalil *et al*., 2000). On the other hand, FGF2 is postulated to induce apoptosis in chick retina (Yokoyama *et al*., 1997). FGF8 is another neuroprotective factor in oxidative stress conditions (Mark *et al*., 1999). FGF9 was shown to modulate the expression of myelin-related proteins in oligodendrocyte cultures, thus it is proposed to be important for glial cell differentiation (Cohen and Chandross, 2000).

Although FGFs in the CNS were investigated in detail, little is known about their functions in the PNS. Expression of FGF1, 2, 5, 7, 9, 10, 13 and 14 mRNAs were demonstrated in rat DRG by Northern blotting and microarray studies (Oellig *et al*., 1995; Li *et al*., 2002). The sciatic nerve was shown to express only FGFR1-3 whereas in DRG all FGFRs have been detected (Grothe et al, 2001).

Upon sciatic nerve lesion, FGF2 and FGFR expressions were up-regulated in the corresponding DRG, as well as at the site of damage. While FGF2 and FGFR3 were upregulated in both tissues, FGFR1 and FGFR2 levels increased only in the crush site (Grothe *et al*., 2001). The regeneration process in FGF2-overexpressing mice was found to be accelerated after similar treatments (Jungnickel *et al*., 2006). Additionally, FGF2 and FGFR3 were suggested to be involved in lesion-induced but not developmental apoptosis (Jungnickel *et al*., 2005). Therefore, a bimodal mechanism was proposed for the effects of FGF2 during nerve regeneration, such that it may promote neuronal survival via FGFR1/2 while mediating apoptosis through FGFR3 (Grothe *et al*., 2006).

Though not as elaborately as FGF2, other FGFs were also studied in the PNS. A microarray screening by Giambonini-Brugnoli *et al*. (2005) indicates a strong increase in FGF1 and FGF4 levels from P4 to P60 in wild-type animals while they remained

unchanged in PMP22 mutants, which may suggest a potential role for these FGFs in myelination (Giambonini-Brugnoli *et al*., 2005). Furthermore, FGF9 promotes the survival of motoneurons but not DRG neurons in cultured cells. This survival effect is thought to be via an autocrine or paracrine mechanism of action (Kanda *et al*., 1999; Garcès *et al*., 2000). Although this evidence is not strong enough to assign a role on FGF9 in myelination, it can be considered as a promising candidate to study.

2. PURPOSE

Myelination is one of the key events in the development and functioning of the nervous system. Defects in this process lead to peripheral neuropathies. In mice PNS, myelination starts after birth (P0), proceeds rapidly between P4 and P20, then gradually slows down until it is completed in adulthood. Some neurotrophins such as BDNF have been implicated in the initiation and formation of myelin *in vitro* but they were not investigated during *in vivo* myelination. Besides, FGFs were suggested to play roles in PNS remyelination after nerve damage, yet their roles in developmental myelination is unclear at this time.

In order to gain a further insight into their functions in myelination, we aimed to determine the temporal expression patterns of FGF1, 2 and 9, FGFR1-4 as well as BDNF in mouse DRG during the active myelination period and in adulthood.

3. MATERIALS AND METHODS

3.1. Chemicals, Buffers, Primers and Antibodies

All chemicals used in this study were obtained from Sigma Aldrich (USA) or Merck (Germany) unless stated otherwise. All antibodies were purchased from Santa Cruz Biotechnology (USA). All solutions, glassware and plastic consumables were sterilized by autoclaving at 121 °C for 15 minutes unless they were provided as sterile. Solutions used in RNA-related protocols were treated with diethylpyrocarbonate for 1 hour and autoclaved twice when applicable.

Compositions of all buffers and solutions as well as information about gene-specific primers and antibodies are presented in Table 3.1 through Table 3.4.

Table 3.1. Buffers and solutions for RT-PCR

Moloney Murine Leukemia Virus (M-MLV) Reverse Transcription Buffer (Promega, USA)	50 mM Tris·Cl (pH 8.3) 75 mM KCl 3 mM $MgCl_2$ 10 mM Dithiothreitol
Taq DNA Polymerase Buffer (Fermentas, Lithuania)	10 mM Tris·Cl (pH 8.8) 50 mM KCl 0.08 % NP-40
Tris Acetate EDTA (TAE) Buffer	40 mM Tris·Cl, 1 mM Ethylenediaminetetraacetic acid (EDTA) 0.1 % (v/v) Acetic acid
6X Loading Buffer	10 mM Tris·Cl (pH 7.6) 60 mM EDTA 60 % Glycerol 0.03 % Bromophenol blue 0.03 % Xylene cyanol FF

Table 3.2. Buffers and solutions for Western analysis

Radio-immuno-precipitation Assay (RIPA) Buffer	150 mM NaCl 50 mM Tris·Cl (pH 7.5) 1 mM Na_3VO_4 1 mM EDTA 1 % Nonidet P-40 (NP-40) 0.1 % Sodium dodeacyl sulfate (SDS) Cømplete mini protease coctail (Roche, Germany)
10 % SDS-polyacrylamide gel (running gel)	375 mM Tris·Cl (pH 8.8) 10 % Acrylamide:Bisacrylamide (29:1) 0.1 % SDS 0.1 % Ammonium persulfate (APS) 0.1 % N,N,N',N'-tetramethylethylenediamine (TEMED)
12.5 % SDS-polyacrylamide gel (running gel)	375 mM Tris·Cl (pH 8.8) 12.5 % Acrylamide:Bisacrylamide (29:1) 0.1 % SDS 0.1 % APS 0.1 % TEMED
5 % SDS-polyacrylamide gel (stacking gel)	125 mM Tris·Cl (pH 6.8) 5 % Acrylamide:Bisacrylamide (29:1) 0.1 % SDS 0.1 % APS 0.1 % TEMED
6X Protein Sample Buffer	300 mM Tris·Cl (pH 6.8) 12 mM EDTA 60 % Glycerol 12 % SDS 6 % 2-mercaptoethanol 0.04 % Bromophenol blue
Running Buffer	25 mM Tris·Cl 250 mM Glycine 0.2 % SDS

Table 3.2. Buffers and solutions for Western analysis (continued)

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
Tris-Buffered Saline (TBS)	20 mM Tris·Cl (pH 8.0) 150 mM NaCl
TBS with Tween-20 (TBST)	0.1 % Tween-20 in TBS
Blocking Solution	1 % skimmed milk powder in TBST

Table 3.3. Antibodies used for Western analysis

Epitope	Origin - Modification	Dilution
FGF1	Goat	1:500
FGF2	Goat	1:500
FGF9	Goat	1:500
MAG	Rabbit	1:1000
Actin	Goat	1:1000
Secondary	Donkey anti-goat IgG-HRP	1:5000
	Goat anti-rabbit IgG-HRP	1:5000

Table 3.4. Primer sequences, product sizes and PrimerBank ID numbers.

Gene	F / R*	Sequence (5' – 3')	Product Size	PrimerBank ID
FGF1	F	CAGCTCAGTGCGGAAAGTG	102 bp	6753850a1
	R	TGTCTGCGAGCCGTATAAAAG		
FGF2	F	GCGACCCACACGTCAAACTA	101 bp	7106315a3
	R	TCCATCTTCCTTCATAGCAAGGT		
FGF9	F	TCTTCCCCAACGGTACTATCC	124 bp	7305057a3
	R	CCGAGGTAGAGTCCACTGTC		
FGFR1	F	GCAGAGCATCAACTGGCTG	163 bp	6753856a2
	R	GGAGAAGTAGGTGGTATCGCTG		
FGFR2	F	AATCTCCCAACCAGAAGCGTA	142 bp	6753858a1
	R	CTCCCCAATAAGCACTGTCCT		
FGFR3	F	GGACGTGGCTGAAGACACAG	287 bp	6679787a2
	R	GGATGCTGCCAAACTTGTTCT		
FGFR4	F	CGTGGTCGTCACTGGTACAAA	68 bp	6679789a3
	R	CCTCTCCAACCCCGTACTC		
PMP22	F	CCGTCCAACACTGCTACTCC	192 bp	6679395a2
	R	CACTCATCACGCACAGACCA		
MAG	F	CAGCCGCCTATTGGGAGAC	159 bp	6754614a3
	R	GGGGTGTTGACGATGTCCA		
MPZ	F	CCGAGATGCCATTTCGATTTTC	219 bp	6678928a2
	R	ACATAGAGCGTGACCTGAGAG		
BDNF	F	TCATACTTCGGTTGCATGAAGG	137 bp	34328442a1
	R	AGACCTCTCGAACCTGCCC		
RPII	F	CAAGAGAGTGCAGTTCGGAGT	154 bp	6677795a2
	R	GCCAGTCCGCTCAATCACC		

* F: Forward primer, R: Reverse primer

3.2. Animals

Mice (*Mus Musculus*, C57BL/67 strain), originally purchased from TÜBİTAK MAM, were bred in our animal room on a 12 hour light / 12 hour dark cycle at 21 – 25 °C. Six to nine adults of the same gender were kept in each cage. Food and water were provided *ad libitum*.

Pregnant mice were isolated into single cages a week prior to the anticipated day of delivery. When pups on different postnatal days were collected, special care was taken to leave the last pup with the mother until the end of the lactation period. Pups or adults with visible developmental defects were not included in any of the experimental groups.

Euthanasia was performed with diethyl ether inhalation. All use and handling of animals were performed in accordance with Boğaziçi University Ethics Committee guidelines.

3.3. Dorsal Root Ganglion Dissection

Four independent groups of postnatal animals were used in the experiments. Each group consisted of a set of animals at P0, P4, P6, P10, P15, P20 and adult (older than two months).

Following decapitation, peripheral blood was drained from the body to prevent the loosening of the spinal cord due to contact with blood. Dissection was performed under a stereomicroscope at 10X – 20X final magnification. The dorsal skin and dorsal muscles were opened starting from the posterior end until the vertebral column was visible along the anteroposterior axis. Subsequently, using curved eye scissors, each vertebra was cut from the dorsal side. This was achieved by inserting a scissor into the space between the dura mater of the medulla spinalis and the lamina of the spine, while leaving the other scissor outside the vertebra, as lateral to the vertebral column as possible. Cutting out the lamina and the corresponding spinous process was required to be done at both sides. The laminae and the spinous processes were removed sequentially. Once the spinal cord was exposed, DRGs were snipped one by one and collected in a 2 ml tube on ice.

Tissues damaged during any of the dissection steps were discarded to minimize RNA or protein degradation. Approximately 30 – 40 intact ganglia were obtained from each animal.

3.4. Reverse Transcription-Coupled Quantitative Real-Time PCR

3.4.1. RNA Isolation

Total RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). In order to extract sufficient amount of RNA, tissues from three animals at P0 stage or two animals at P4, P6, P10, P15 and P20 stages were pooled.

Tissues were homogenized in QIAzol Lysis Reagent and chloroform was added. After mixing thoroughly, samples were centrifuged at 12000 g for 15 minutes at 4 °C and the aqueous layers were transferred into separate tubes. Following addition of equal volume of 70 per cent ethanol, samples were applied to columns and spinned shortly at 8000 g. Finally, after extensive wash with provided buffers, RNA was eluted in 60 µl RNase-free water.

RNA concentration and purity was determined by spectrophotometry. RNA integrity was checked by agarose gel electrophoresis.

3.4.2. cDNA Synthesis

cDNA was synthesized by using M-MLV reverse transcriptase. Each reaction mixture was composed of 2 µg RNA, 30 nmol deoxyribonucleotide triphosphate (dNTP), 0.5 µg random hexamer primers, 32 U RNasin Plus RNase Inhibitor and 200 U M-MLV reverse transcriptase in reverse transcriptase buffer. The final volume of a reaction mixture was 30 µl. Before adding the enzyme, samples were denatured at 70 °C for 5 minutes followed by immediate cooling on ice. A negative control without RNA was also prepared similarly. All reagents were purchased from Promega (USA).

Synthesis was carried out in three steps. First, mixtures were kept at 20 °C for 10 minutes to allow the annealing of primers. Then they were incubated at 37 °C for 2 hours for the reaction to take place. Finally, the enzyme was inactivated by a 95 °C incubation for 10 minutes. After the reaction, each product was diluted by 1:12 with PCR-grade water.

3.4.3. Primers

Gene-specific primer pairs for fibroblast growth factors (FGF1, FGF2 and FGF9), fibroblast growth factor receptors (FGFR1-4), myelin-related proteins (PMP22, MAG and MPZ), BDNF and RNA polymerase II (RPII) were purchased from Integrated DNA Technologies (Belgium). Primer sequences were obtained from Primer Bank (Wang and Seed, 2003).

Since all primers target common regions among alternate transcripts, they do not distinguish between isoforms. Primer sequences, length of the amplified regions and unique Primer Bank ID numbers are presented in Table 3.4.

3.4.4. Polymerase Chain Reaction

Polymerase chain reaction (PCR) mixture was composed of 6 µl of the cDNA dilution, 3 µM $MgCl_2$ (Fermentas, Lithuania), 0.2 mM dNTP (Promega, USA), 0.2 µM primers and 0.6 U Taq DNA polymerase (Fermentas, Lithuania) in Taq DNA polymerase buffer. The final volume of a reaction mixture was 25 µl.

PCR reaction was performed as follows: Initial denaturation at 95 °C for 2 minutes; 30 cycles of 95 °C, 60 °C and 72 °C, each held for 30 seconds; and final extension at 72 °C for 5 minutes.

Products were run on 2 per cent agarose gel at 130 V and visualized by ethidium bromide staining.

3.4.5. Real-Time Polymerase Chain Reaction

Real-time PCR was performed on LightCycler 1.5 instrument (Roche, Germany). Reactions were run in triplicates. Each reaction mixture was composed of SYBR Premix Ex Taq (TaKaRa, Japan), 250 nM primers and 6 µl cDNA in a final volume of 20 µl.

PCR reaction program was composed of four stages: Initiation (50 °C for 2 minutes), initial denaturation (95 °C for 10 seconds), amplification (35 cycles of 95 °C for 5 seconds, 60 °C for 10 seconds and 72 °C for 10 seconds, single acquisition after each cycle) and melting curve construction (heating to 95 °C and immediate cooling to 55 °C followed by slowly heating to 95 °C with a ramping rate of 0.2 °C/s, continuous acquisition).

Raw real-time PCR data was exported by LightCycler Software 4, and analyzed computationally according to the method proposed by Ramakers *et al*. (2003) as an alternative to conventional analysis methods (Ramakers *et al*., 2003). In order to correct for deviations in cDNA amounts, RPII was used as an internal control. Expression of this gene was shown to be extremely stable in different tissues (Radonić *et al*., 2004).

3.4.6. Agarose Gel Electrophoresis

One µg of RNA or whole volume of PCR product were mixed with 6X loading buffer. RNA samples were loaded onto 1 per cent agarose gel containing 0.5 µg/ml ethidium bromide and run at 80 V. PCR products were loaded onto 2 per cent agarose gel and run at 130 V. In all cases, 1X TAE was used as a running buffer.

3.4.7. Statistical Analysis

All statistical analyses were performed on Statistical Package for Social Sciences (SPSS) software version 16. Distribution of data was determined by Shapiro – Wilk's test of normality. Student's t-test or Mann – Whitney U test were applied for normally and non-normally distributed data, respectively. Level of statistical significance was set as $\alpha = 0.05$ for all tests and error bars represent 1 standard error of the mean (SEM) in all charts.

3.5. Western Blot Analysis

Tissues were homogenized in RIPA buffer and centrifuged at 800 g for 35 minutes. The supernatant was used as the tissue lysate. In order to have enough protein, tissues from 2 animals at P0, P4 and P6 stages were pooled. Protein concentrations were determined by bicinchoninic acid protein assay kit (Pierce, USA).

Twenty-five µg of DRG tissue lysate was mixed with 6X protein sample buffer and heated at 70 °C for 5 minutes before loading onto 10 or 12.5 per cent SDS-polyacrylamide gels. Gels were run in running buffer at 130 V until marker bands were properly resolved. Fractionated proteins were electroblotted to polyvinyl difluoride (PVDF) membranes (Roche, Germany) in transfer buffer at 100 V for 30 – 90 minutes depending on gel concentration and the size of the protein of interest. Membranes were washed briefly in TBS and incubated in blocking solution at room temperature for 1 hour. Subsequently, they were incubated in primary antibody solution overnight at 4 °C. On the next day, blots were washed thrice with TBST for 30 minutes and incubated in horseradish peroxidase (HRP)-conjugated secondary antibody solution at room temperature for 1 hour. Following this incubation, blots were again washed thrice with TBST for 30 minutes and once with TBS for 5 minutes. Finally, they were incubated in Lumi-Light Western blotting substrate (Roche, Germany) for 5 minutes before exposure to chemiluminescence detection system (Stella, Germany).

3.6. List of Equipments

Autoclave	ASB270NT, Astell, UK
Balance	DTBH 210, Sartorius, GERMANY
Blotting apparatus	Mini Trans-Blot Cell, Bio-Rad, ITALY
Centrifuges	Spectrafuge 16M, Labnet, USA
	Genofuge 16M, Techne, UK
	Centrifuge 5415 R, Eppendorf, USA
Deep freezers	-20 °C, Arçelik, TURKEY
	-86 °C ULT Freezer, ThermoForma, USA
Dissection tools	Harvard Apparatus, USA

Documentation systems	Gel Doc XR System, Bio-Rad, ITALY
	Raytest, Stella, GERMANY
Electrophoresis systems	Easy-Cast System, Hybaid, UK
	Mini-Protean III Cell, Bio-Rad, ITALY
Heat blocks	DRI-Block BD-2A, Techne, UK
	StableTemp Dry Bath Incubator, Cole Parmer, USA
Homogenizer	Pellet Pestles Tissue Grinder, Kimble Kontes, USA
Illuminator	41723-Series High Intensity Illuminator, Cole Parmer, USA
Magnetic stirrers	KMO 2 Basic, IKA, GERMANY
	RCT Basic, IKA, GERMANY
Micropipettes	Gilson, FRANCE
Microscope	S2026, Prior, UK
Microwave oven	M1733N, Samsung, MALAYSIA
pH meter	SympHony, VWR, GERMANY
Power supplies	PowerPac Basic, Bio-Rad, ITALY
	PS250, Hybaid, UK
Real-time PCR	LightCycler 1.5, Roche, GERMANY
Refrigerator	2082C, Arçelik, TURKEY
Shaker	SL350, Nüve, TURKEY
Softwares	LightCycler 4.0 Analysis Software, Roche, GERMANY
	ImageJ, Image Analysis Software, NIH, USA
	XStella 1.0, Stella, GERMANY
	Aida 4.19, Stella, GERMANY
	SPSS 16, SPSS Inc, USA
Spectrophotometer	NanoDrop ND-1000, Thermo, USA
Thermocyclers	MyCycler, Bio-Rad, ITALY
	TC-312, Techne, UK
Vortex	Vortexmixer VM20, Chiltern Scientific, UK
Water bath	TE-10A, Techne, UK
Water purification	WA-TECH Ultra Pure Water Purification System, GERMANY

4. RESULTS

4.1. RNA Quality, Primer Specificity and Reverse Transcription

Relative levels of the transcripts were measured by qRT-PCR during the active myelination period and adulthood. Before cDNA synthesis, RNA quality was determined by agarose gel electrophoresis. No RNA degradation (Figure 4.1) and no indication of genomic DNA contamination (data not shown) were evident on the gels. Spectrophotometric readings revealed an OD_{260} / OD_{280} ratio of 2.07 ± 0.03 for all samples.

Primer specificity was tested by running PCR reactions using adult cDNA samples. As shown in Figure 4.2, we obtained products with the expected sizes in all cases.

cDNAs were checked by standard PCR using RPII primers. No bands were observed in negative controls. In all other samples, 154 bp single amplicons were obtained (Figure 4.3).

Although primer dimers were observed in conventional PCR, melting curves of real-time PCR reaction products showed single peaks without shoulders (data not shown). To confirm the presence of single amplicons, these products were also run on 2 per cent agarose gel and no primer dimers were observed (data not shown). This clearly implies that very specific amplifications were obtained in real-time PCR reactions and no primer dimers interfered with the dsDNA measurement via SYBR Green I.

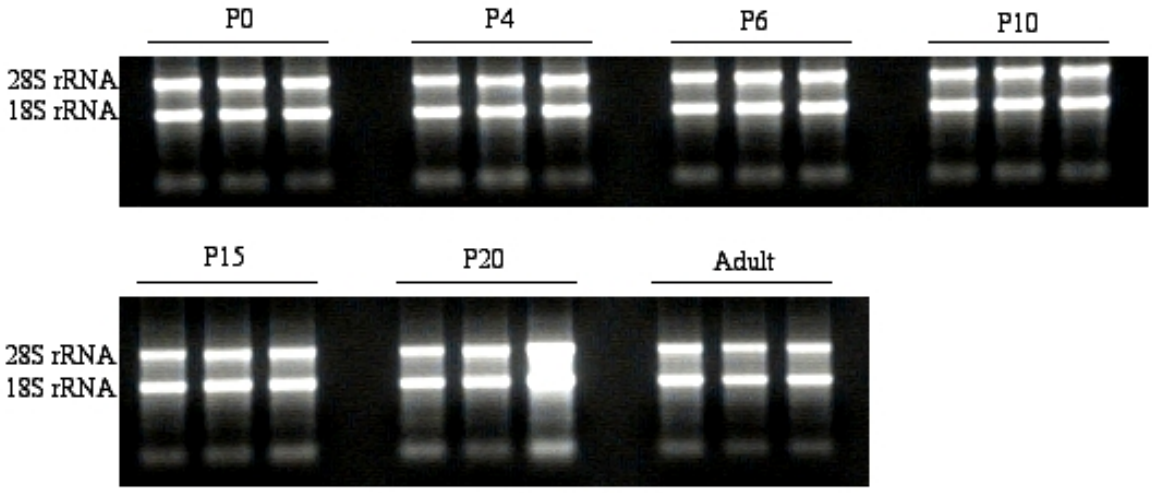

Figure 4.1. Quality of RNA extracted from different samples. 18S and 28S rRNA bands were sharp and no small molecular weight smear was evident.

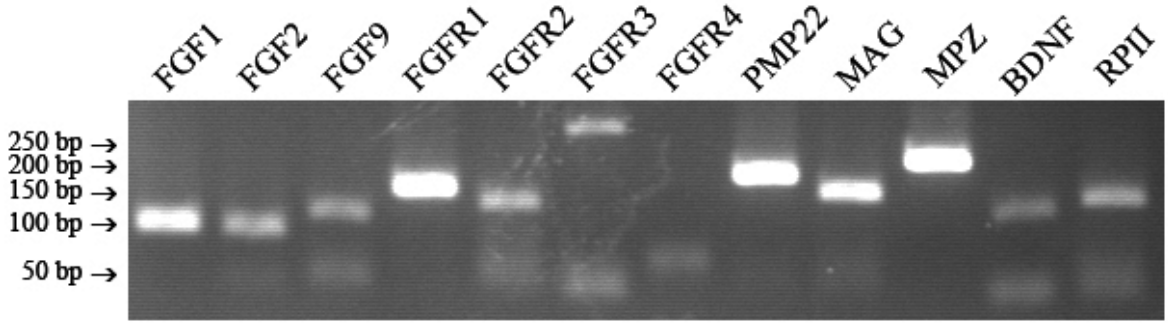

Figure 4.2. Amplification products of different primer pairs. In each reaction, adult DRG cDNA was used as a template and 35 cycles of amplification was performed with specific primers. All product sizes were in agreement with the predicted ones.

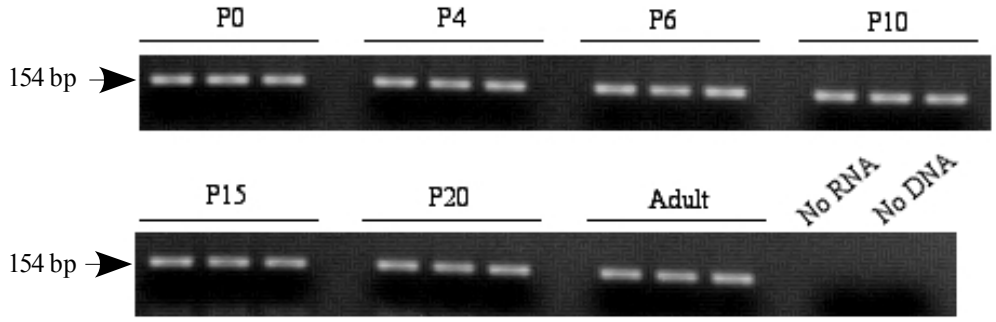

Figure 4.3. PCR products of cDNAs. All products were amplified with RPII primers. Single products of 154 bp were observed for all samples except for negative controls.

4.2. Expression of Myelin-Related Proteins

In order to confirm that the DRG tissue was in the myelination process between postnatal days 0 and 20, relative transcript levels of myelin-related proteins PMP22, MAG and MPZ were measured by qRT-PCR (Figure 4.4 through Figure 4.6). Additionally, protein levels of MAG were determined by Western blot analysis (Figure 4.7).

As a general observation, P4 transcript levels of all myelin proteins showed an increase of more than 2-fold as compared to P0. For PMP22, steady state transcript levels continued to increase until P15, reaching 2.9-fold of P0 (Figure 4.4). Similarly, MPZ levels increased until P20, reaching a peak level of 5.1-fold of P0 (Figure 4.5). Transcript levels of these genes were then stabilized until adulthood. MAG transcript levels increased until P10 and did not change until P20. At adulthood, the levels decreased to near-P0 levels (Figure 4.6). In contrast, protein levels of MAG increased until P20 and stabilized thereafter (Figure 4.7).

For all 3 genes, mean relative transcript levels normalized to P0 are presented in Figure 4.8. Among all the proteins investigated in this study, PMP22 and MPZ were those with the highest transcript levels. Average MAG levels were several fold lower than the others. This finding is consistent with the reported data, since PMP22 and MPZ proteins are more abundant than MAG in the PNS myelin (Garbay *et al*., 2000).

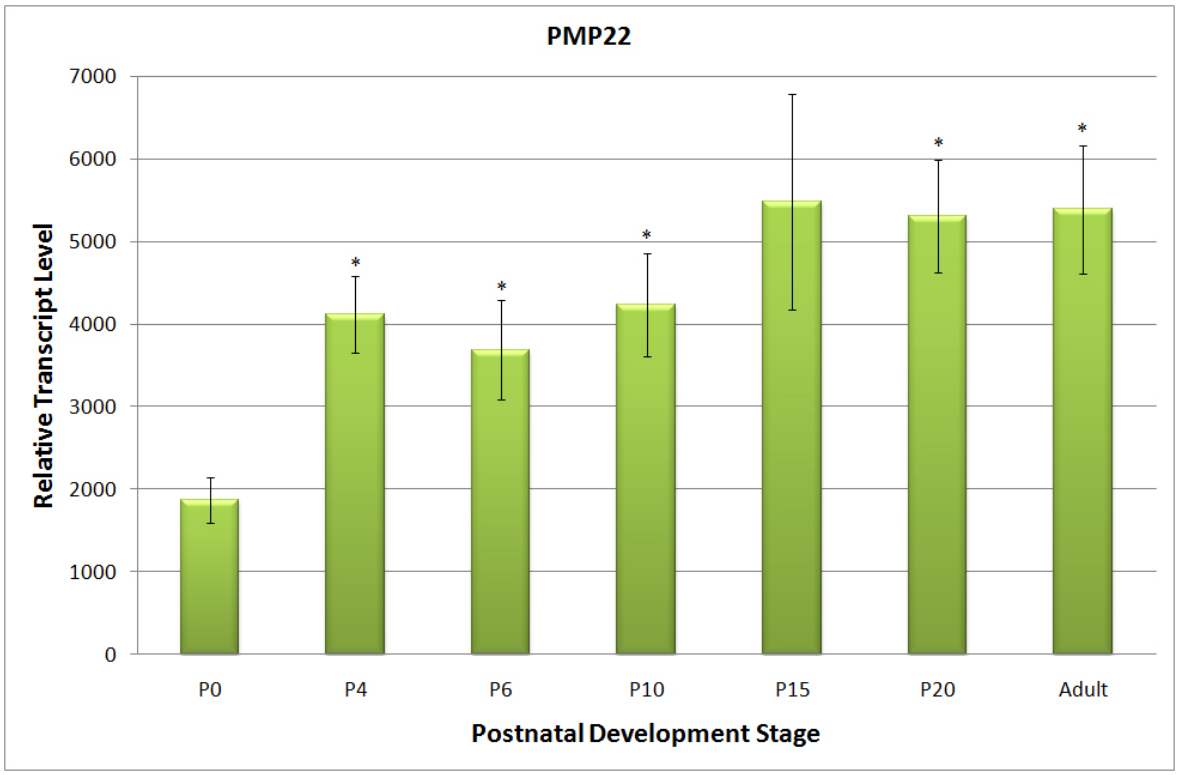

Figure 4.4. Relative transcript levels of PMP22 during the active myelination period and adulthood. Transcript levels of PMP22 were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0

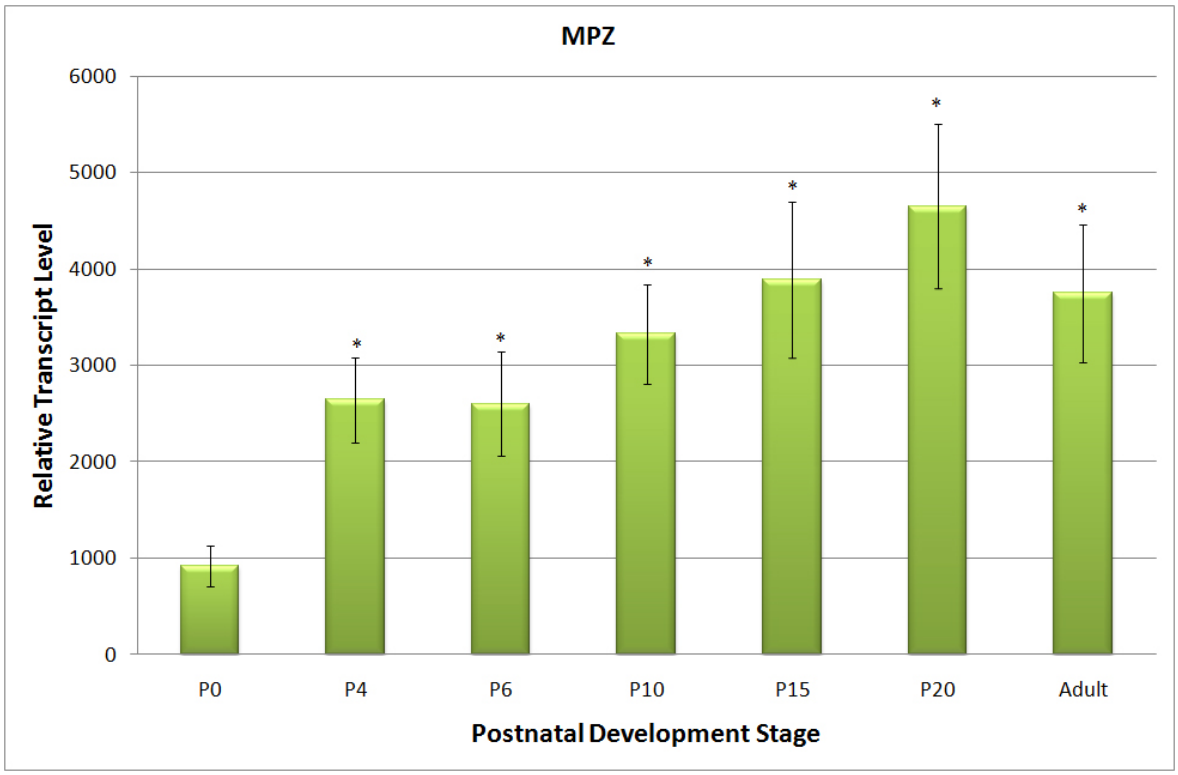

Figure 4.5. Relative transcript levels of MPZ during the active myelination period and adulthood. Transcript levels of MPZ were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0

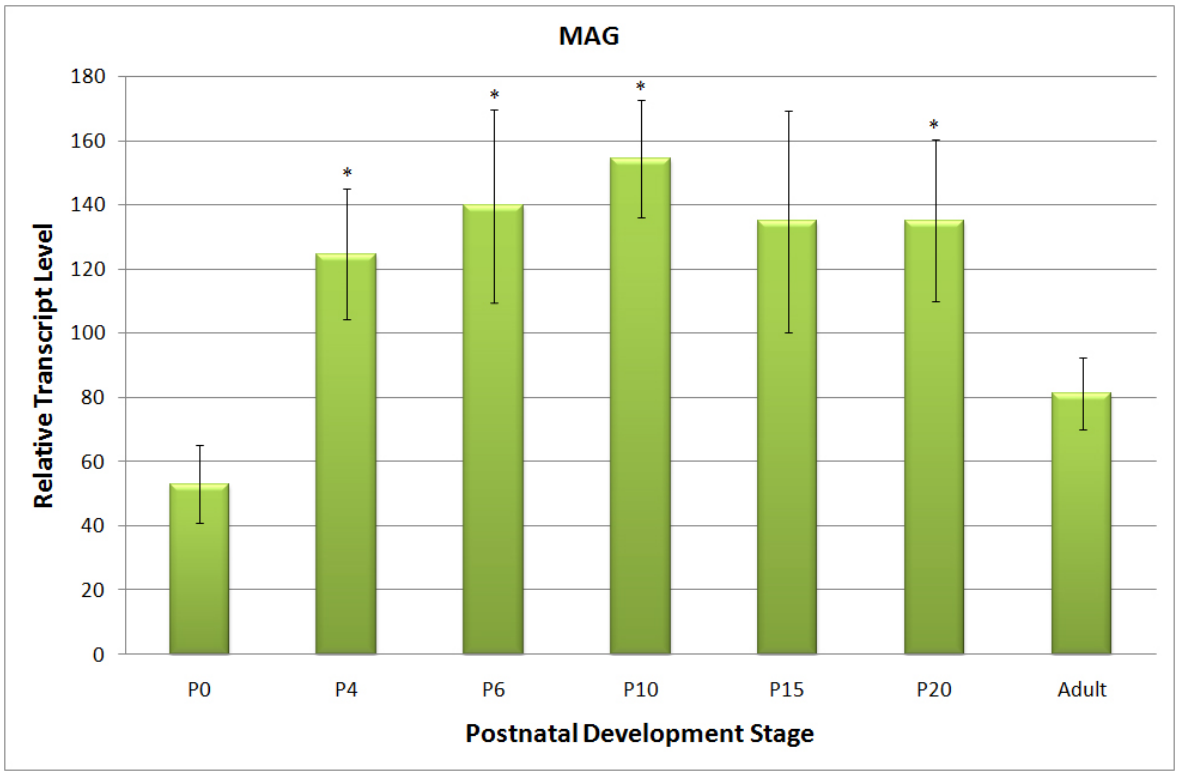

Figure 4.6. Relative transcript levels of MAG during the active myelination period and adulthood. Transcript levels of MAG were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0

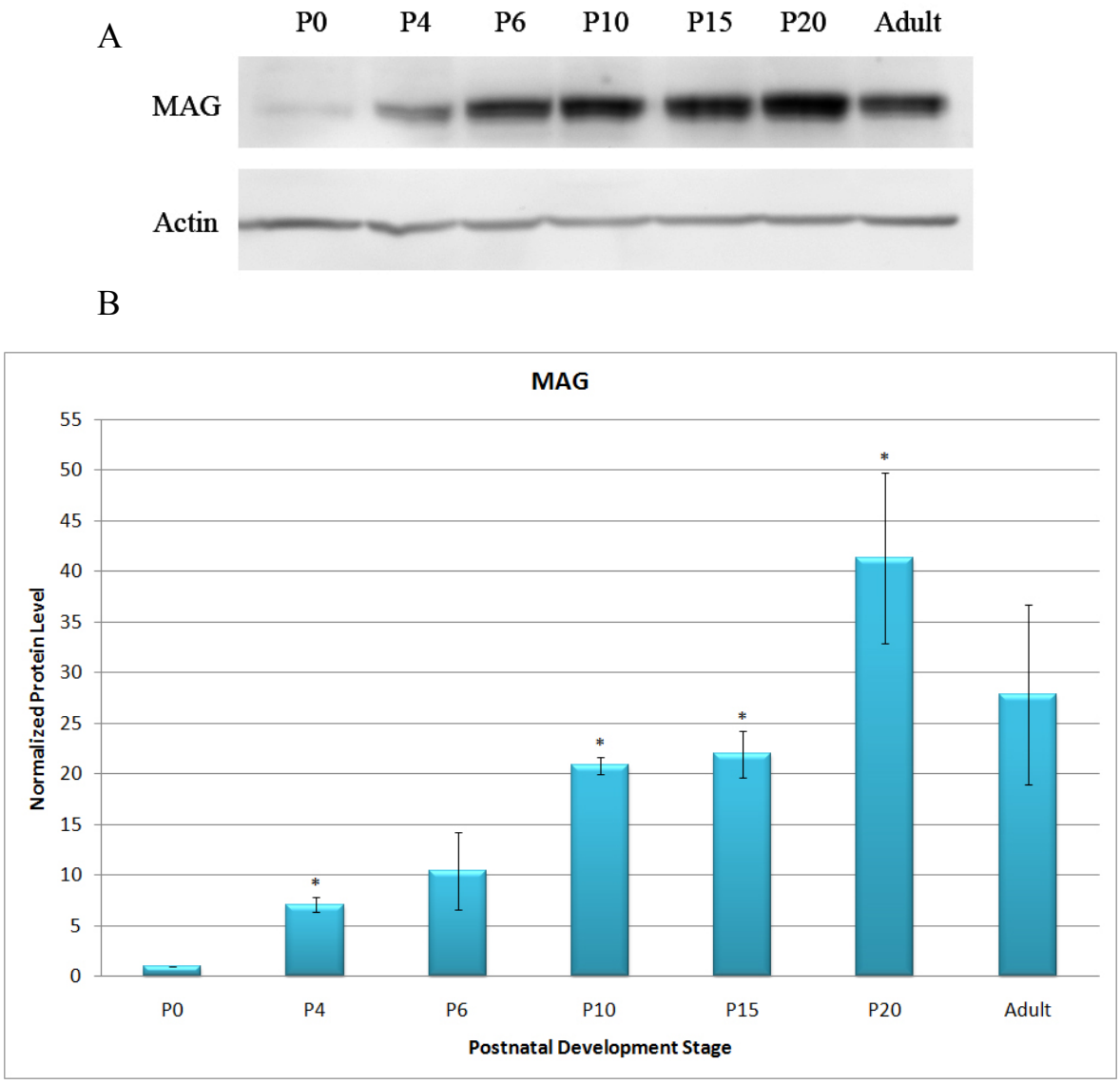

Figure 4.7. Expression of MAG in developing DRG. (A) Western blot analysis of MAG protein during the active myelination period and adulthood. Twenty-five µg protein, extracted from DRG samples at different time points, was resolved on 10 per cent polyacrylamide gel, electroblotted on PVDF membrane and incubated with relevant antibodies. Actin was used as a control. (B) Relative protein levels of MAG during the active myelination period and adulthood. Protein levels of MAG were determined at different time points by Western blot analysis and normalized to corresponding actin levels and P0 values. Three independent samples were included for each time point. Means of normalized values were calculated with their standard errors. All groups were compared to 1 by one-sample t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0.

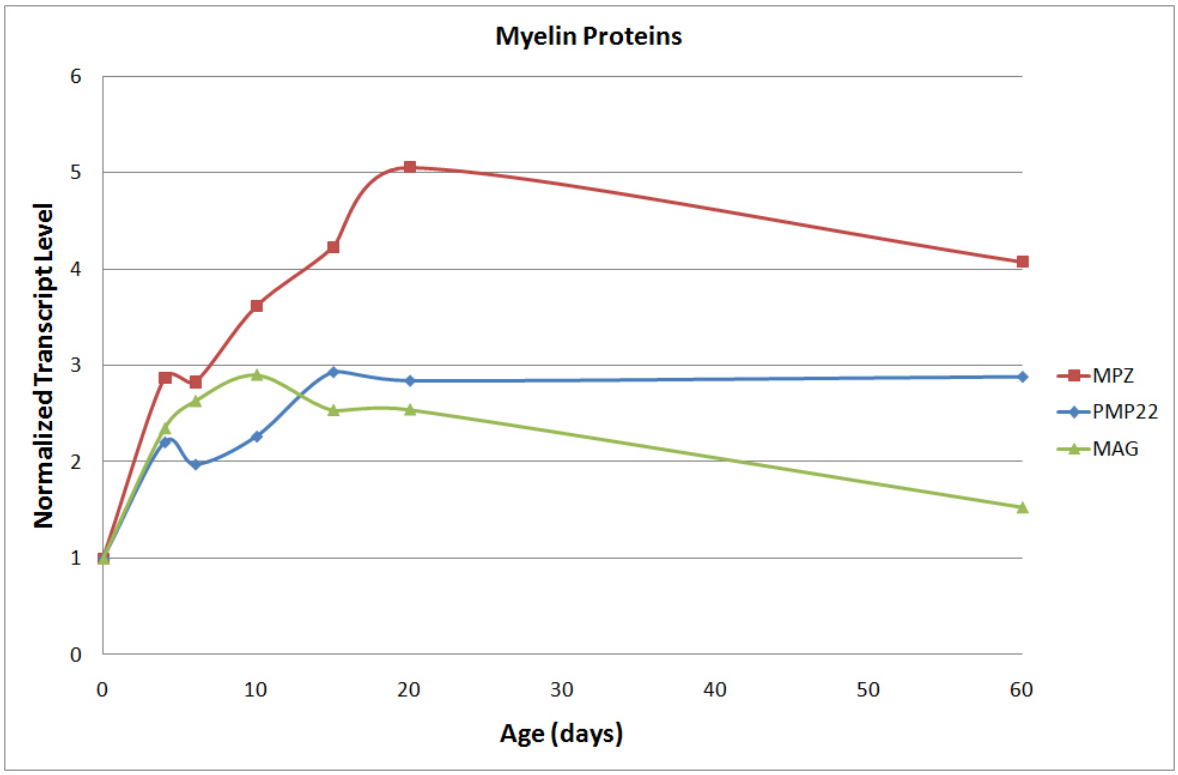

Figure 4.8. Normalized transcript levels of MPZ, PMP22 and MAG. Mean relative transcript levels were normalized to the P0 value. MPZ, PMP22 and MAG levels are indicated in red, blue and green, respectively.

4.3. Expression of Fibroblast Growth Factors

qRT-PCR analysis indicated a two stepwise increase in FGF1 transcript levels until P15. The first significant increase was observed between P0 and P4, and the second one was between P10 and P15. As compared to each other, P4, P6 and P10 levels were not statistically different. Similarly, P15, P20 and adult levels did not show a significant difference (Figure 4.9).

FGF1 protein could also be detected in developing DRG tissues starting from the postnatal 4^{th} day. It seems to be increasing until P10, reaching a peak level, then gradually decreasing to a very low level at adulthood (Figure 4.10). Since this result has not been reproduced yet, the significance of these differences is not clear at this time.

Unlike FGF1, FGF2 transcript levels showed a gradual increase until P10, after when they were stabilized until adulthood (Figure 4.11). The transcript level on P15 is not significantly different as compared to P10 or P20.

Transcript levels of FGF9 were modulated in a different pattern than those of FGF1 and FGF2. After a gradual increase until P10, FGF9 levels reached a peak of 3.1-fold as compared to P0. Then they gradually decreased until P20. Adulthood levels were similar to P0 levels, where the difference was not statistically significant (Figure 4.12).

FGF2 and FGF9 proteins could not be detected in 50 µg total protein extracted from P15 DRG (Figure 4.13). In the same blots, we were able to detect corresponding recombinant proteins.

Mean relative transcript levels normalized to P0 for all 3 FGF family members are presented in Figure 4.14. Among the FGFs investigated, overall levels of FGF1 transcripts were higher than those of FGF2 and FGF9.

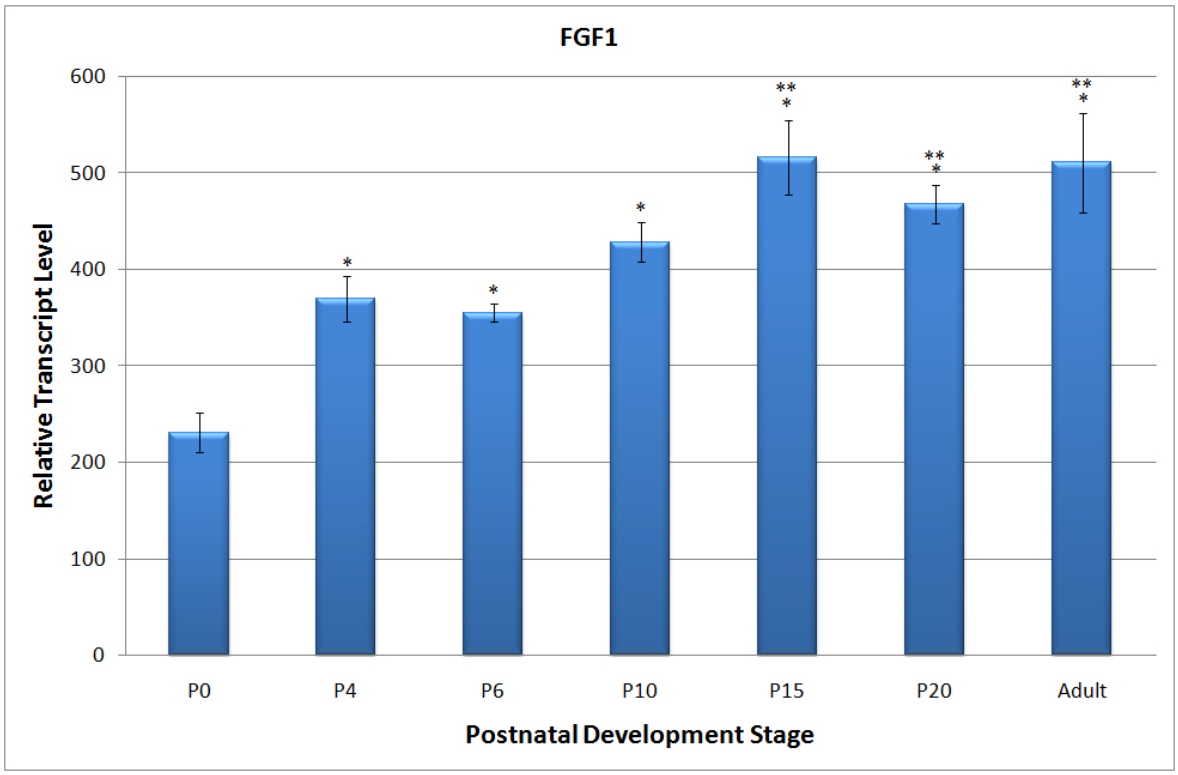

Figure 4.9. Relative transcript levels of FGF1 during the active myelination period and adulthood. Transcript levels of FGF1 were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 and P4 by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0; ** $p < 0.05$ compared to P4

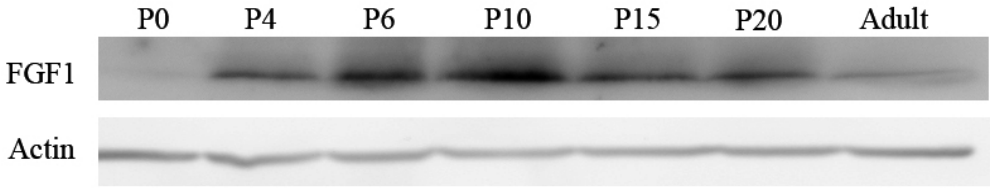

Figure 4.10. Western blot analysis of FGF1 protein during the active myelination period and adulthood. Twenty-five µg protein, extracted from DRG samples at different time points, was resolved on 12.5 per cent polyacrylamide gel, electroblotted onto PVDF membrane and incubated with relevant antibodies. Actin was used as a control.

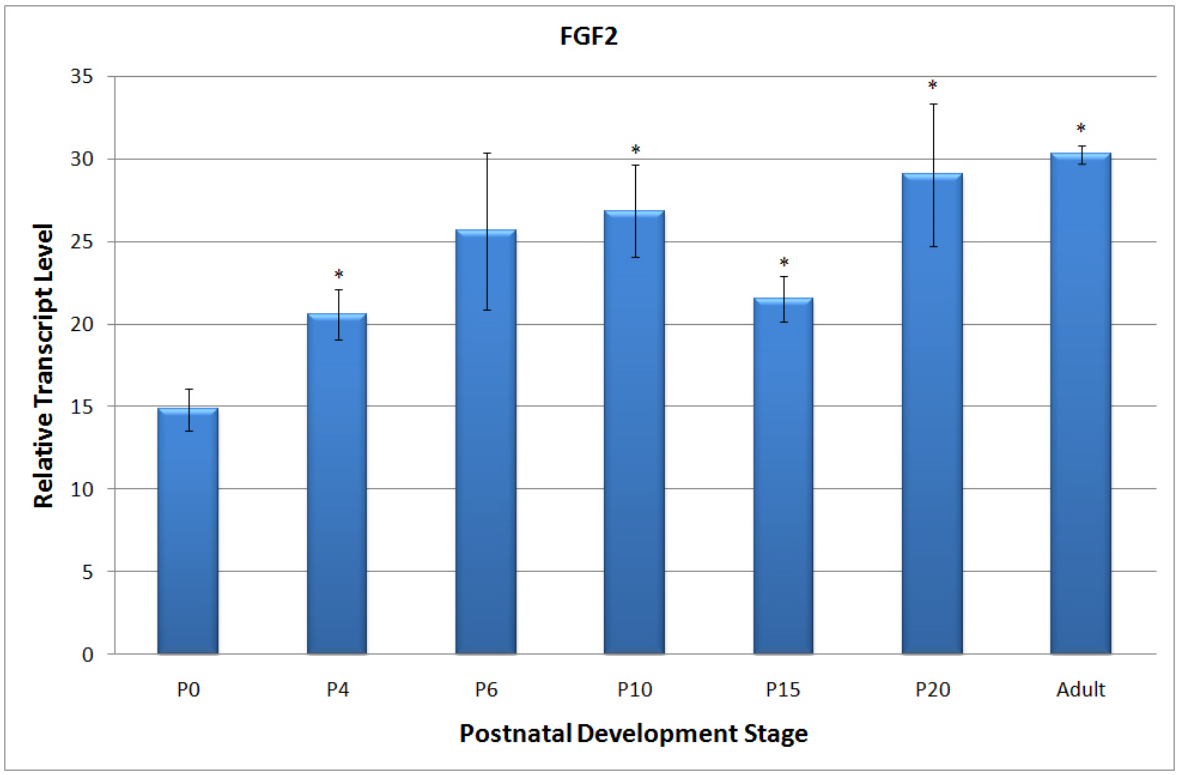

Figure 4.11. Relative transcript levels of FGF2 during the active myelination period and adulthood. Transcript levels of FGF2 were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0

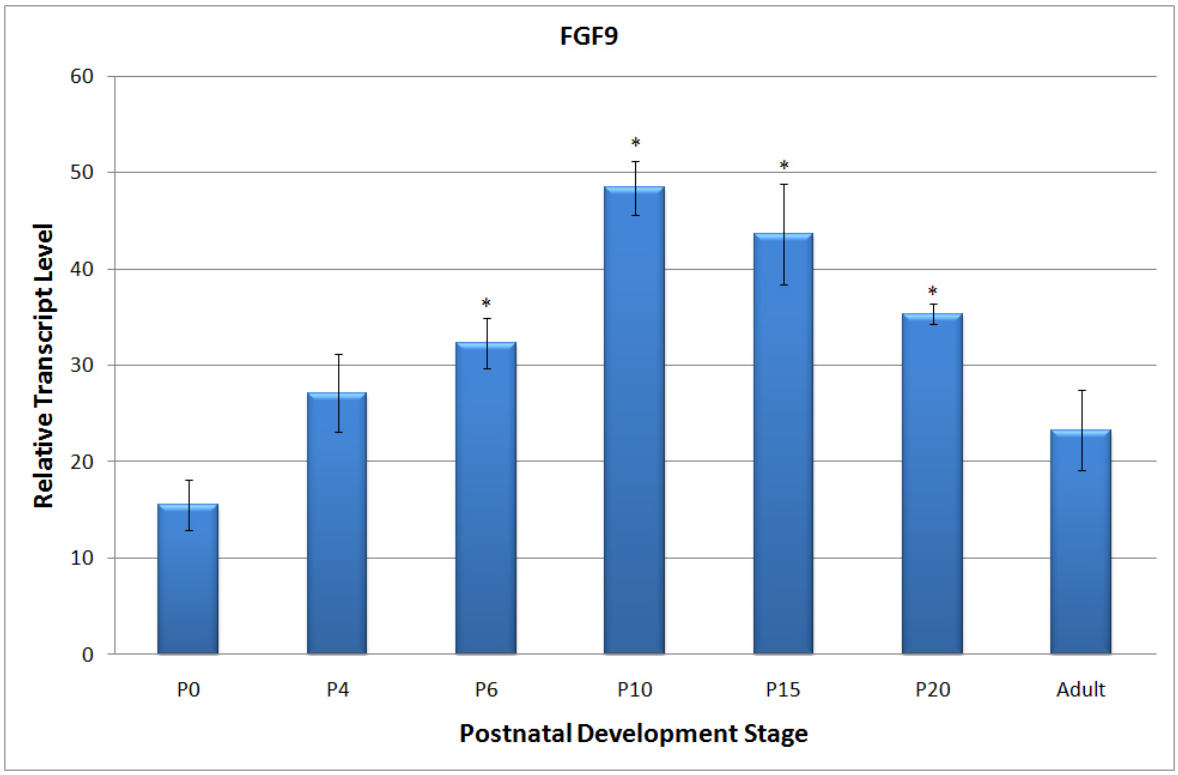

Figure 4.12. Relative transcript levels of FGF9 during the active myelination period and adulthood. Transcript levels of FGF9 were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0

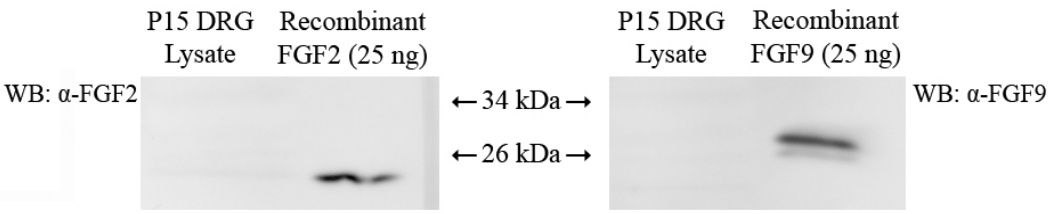

Figure 4.13. Expression of FGF2 and FGF9 in P15 DRG. Fifty µg P15 DRG total protein was resolved on 12.5 per cent polyacrylamide gel, electroblotted onto PVDF membrane and incubated with relevant antibodies. Twenty-five ng recombinant FGF2 or FGF9 as positive controls.

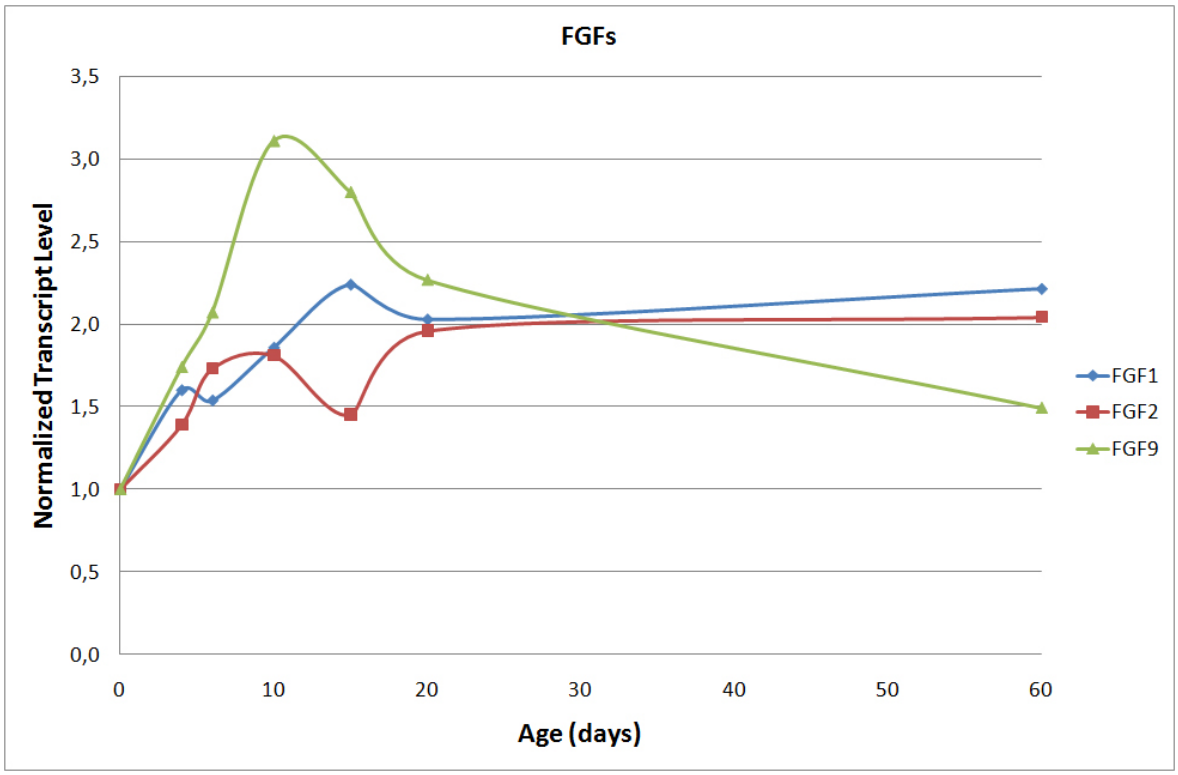

Figure 4.14. Normalized transcript levels of FGF1, FGF2 and FGF9. Mean relative transcript levels were normalized to the P0 value. FGF1, FGF2 and FGF9 levels are indicated in red, blue and green, respectively.

4.4. Transcript Levels of Fibroblast Growth Factor Receptors

Among all FGF receptors, average levels of FGFR1 were the highest. Its levels did not show a significant increase during the first week after birth. At P10 we observed a slight increase which was stabilized until adulthood (Figure 4.15).

On the other hand, FGFR2 levels remained steady and we observed no statistically significant changes at any age as compared to P0 levels (Figure 4.16).

FGFR3 levels did not show a significant change between P0 and P20. Interestingly, the only difference was a 2-fold decrease in adulthood as compared to P0 (Figure 4.17).

FGFR4 levels were lower than detectable limits in general, and were not included in further analyses.

Since FGFR primers targeted common regions, these data show the cumulative levels of all isoforms. No information on the levels of different isoforms is available at this time.

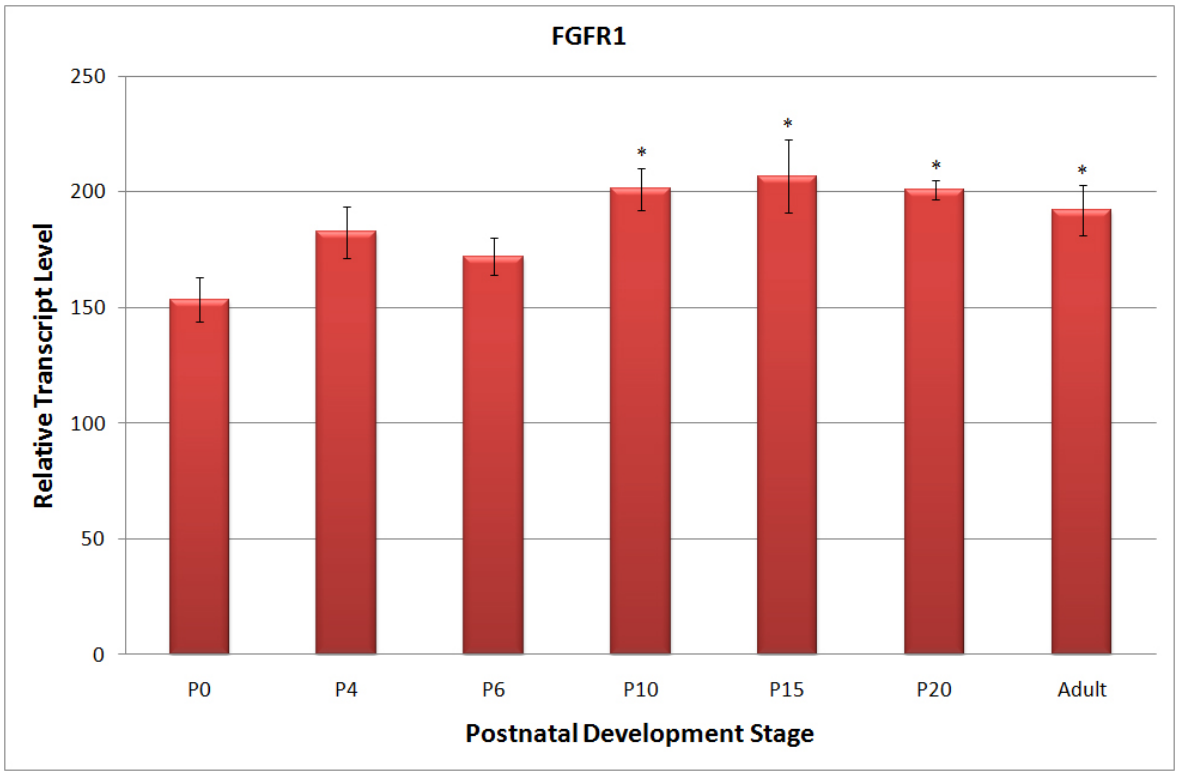

Figure 4.15. Relative transcript levels of FGFR1 during the active myelination period and adulthood. Transcript levels of FGFR1 were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0

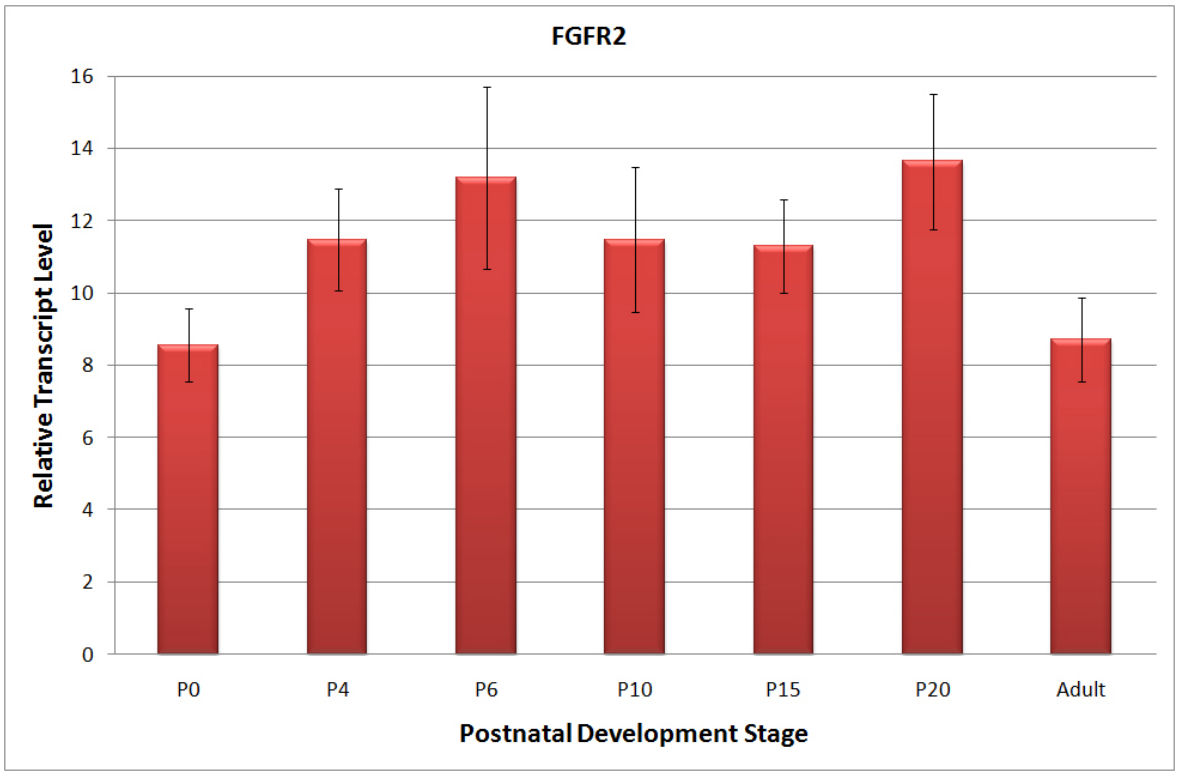

Figure 4.16. Relative transcript levels of FGFR2 during the active myelination period and adulthood. Transcript levels of FGFR2 were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 by independent samples t-test. Error bars indicate 1 standard error of the mean.

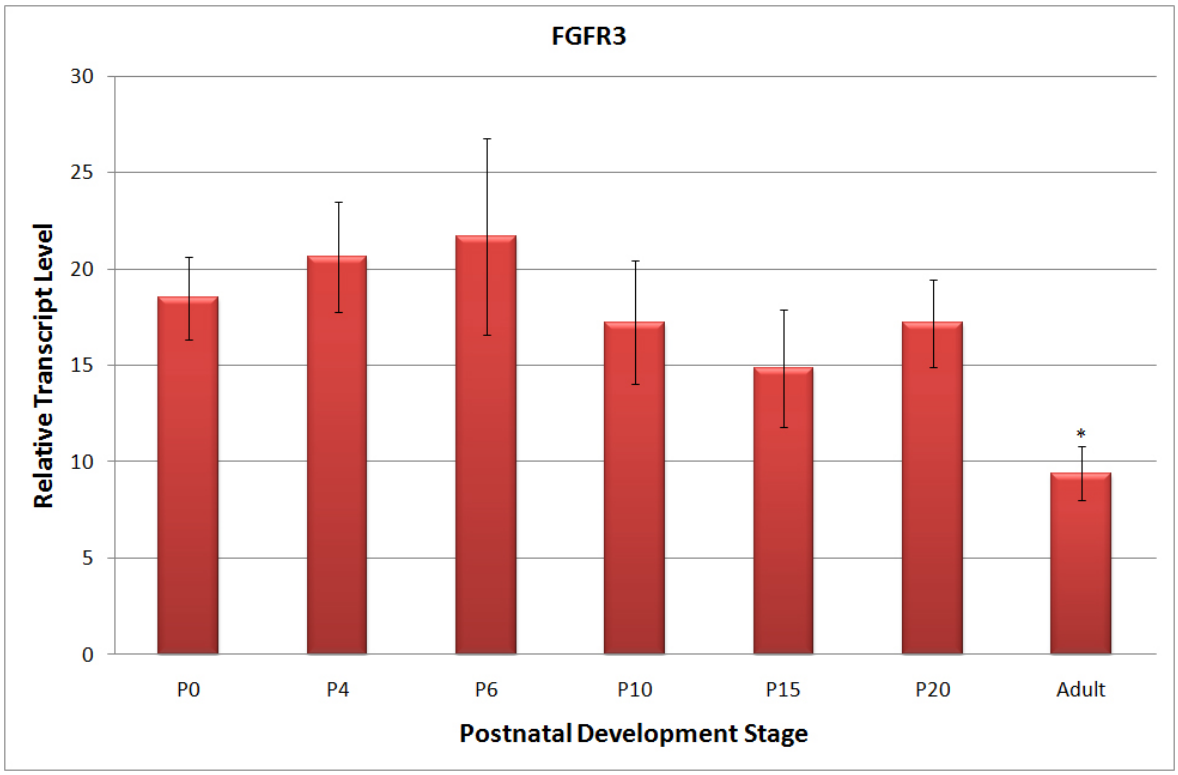

Figure 4.17. Relative transcript levels of FGFR3 during the active myelination period and adulthood. Transcript levels of FGFR3 were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to P0

4.5. Transcript Levels of Brain Derived Neurotrophic Factor

BDNF expression in myelinating DRG neuron – SC co-culture has already been demonstrated (Ng *et al*., 2007). However, its temporal expression pattern in the *in vivo* process has not been reported. For this reason, we measured the relative transcript levels of BDNF during the active myelination period and in adulthood.

Although average BDNF levels were modulated during the time course examined, none of these changes were significantly different as compared to P0. However, P6, P10 and P20 levels were significantly higher than adulthood levels (Figure 4.18)

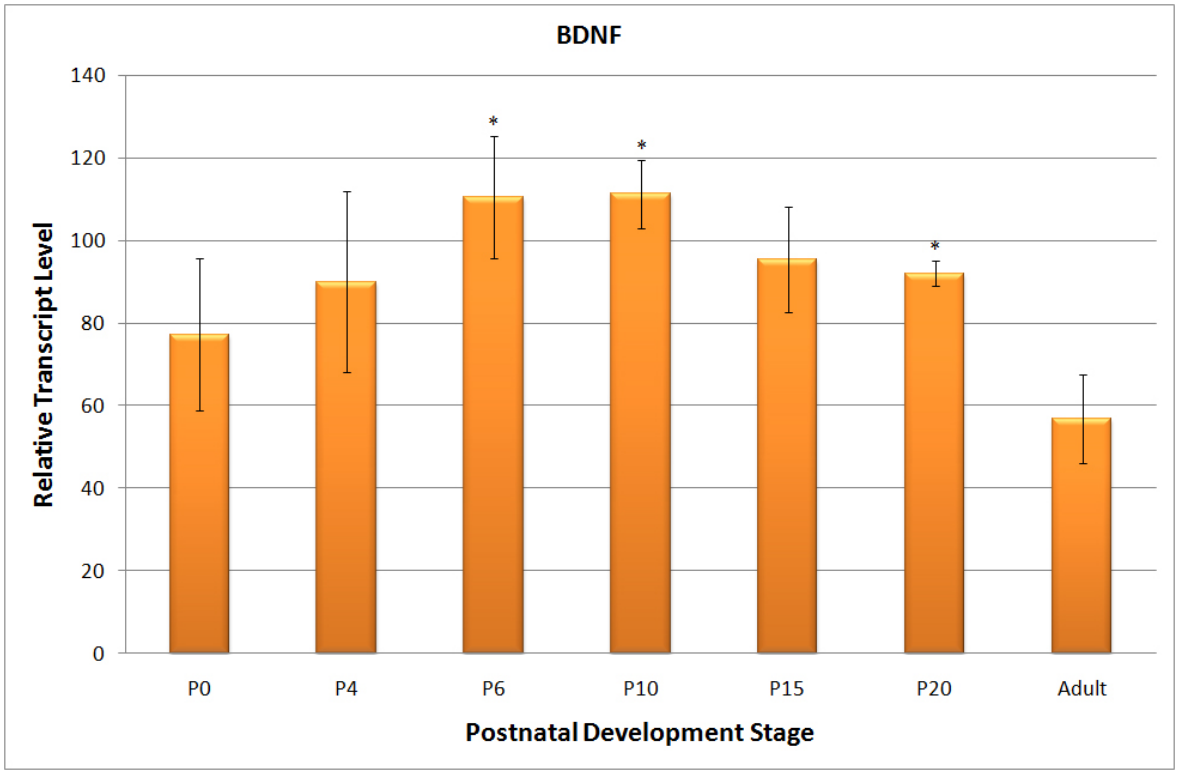

Figure 4.18. Relative transcript levels of BDNF during the active myelination period and adulthood. Transcript levels of BDNF were measured at different time points by qRT-PCR and normalized to corresponding RPII levels. Four independent samples were included for each time point. Mean values were calculated with their standard errors. All groups were compared to P0 and adults by independent samples t-test. Error bars indicate 1 standard error of the mean. * $p < 0.05$ compared to Adult

5. DISCUSSION

Myelination is one of the key events in the development and functioning of the nervous system. Myelin sheath, formed by Schwann cells in the PNS, greatly enhances the velocity of action potential conduction. The failure of proper myelination results in debilitating neuromuscular disorders.

The process is postnatal, and requires neuron – SC contact for initiation. The information on the elements of the communication between these cells is scarce. NRG1 / Erb2 and laminin / integrin interactions, as well as signaling by neurotrophins NT3 and BDNF have been proposed to be involved in the initiation of myelination (Chan *et al*., 2001; Feltri *et al*., 2002; Tolwani *et al*., 2004; Taveggia *et al*., 2005). Another neurotrophic family implicated in the development and physiology of the nervous system is the FGF family. They have been proposed to act as mitogens, survival and differentiation factors for neurons and glia (Powers *et al*., 2000). A number of studies emphasize the role of FGF2, the prototypic member of the family, in remyelination following nerve damage (Walicke *et al.*, 1986; Unsicker *et al.*, 1987; Grothe *et al*., 2001; Jungnickel *et al*., 2006). Thus, it is conceivable that FGFs might contribute to the orchestration of myelin formation and / or the maintenance of the mature myelin sheath.

As a first step towards the elucidation of potential roles of FGFs in the PNS myelination program, we studied the temporal expression profiles of the selected members of the family as well as their receptors, and compared the patterns with the known timing of myelination. In this study, we used DRG as an *in vivo* model for PNS myelination since the tissue is readily accessible. It is also very suitable for setting up a co-culture system for future studies. We focused on FGF1, FGF2 and FGF9 which show postnatal expression in the PNS (Powers *et al.*, 2000).

The expression of myelin proteins PMP22 and MPZ at the transcript level, and MAG both at transcript and protein levels were followed in P0 – P20 and adult mice to indicate that myelination was in progress. MAG is thought to be required for the initiation of myelination and its predominant isoform in PNS, S-MAG, connects myelin to cytoskeleton

via tubulin (Owens and Bunge, 1991). PMP22 and MPZ are associated with myelin formation and maintenance (Martini and Schachner, 1997). The first significant increase in the steady state transcript levels of all three genes was detected with the accelerated myelination at P4. PMP22 and MPZ transcript levels reached maximum levels at P15 and P20, respectively, and did not change significantly in adults. MAG transcript levels peaked at P10, remained at that level until P20 and decreased to that of P0 by adulthood. The observed profiles are in agreement with the published data (Owens and Bunge, 1991; Martini and Schachner, 1997). Expression at the protein level was studied only in the case of MAG. The results indicate that the highest protein level was attained by P20. In contrast to the transcript profile, MAG protein levels remained high into adulthood, which might indicate that the stability of the deposited protein is high. It is also conceivable that the rate of translation of MAG is increased after myelination is completed.

FGF1 transcript levels increased steadily until P15, and no modulation was evident in later time points. In our Western blots, FGF1 was not detectable at P0. Between P4 and P10 we see a gradual increase. At P15 the protein level started to decrease and in adulthood it was low but still detectable. In an earlier study, FGF1 transcript was detected in DRG at P5 and P120 by Northern blot analysis and RNase protection assay (Oellig *et al*., 1995). In contrast to our finding, the protein levels were found to continuously increase in postnatal mouse DRG up to P120, the latest time point examined. The underlying reasons for this discrepancy is not clear at this time. At any rate, we report a comparative study with a higher resolution of temporal profile of FGF1 expression at the transcript level in DRG. The observed protein profile of FGF1 follows general progress of myelination. The findings lend support to the proposed potential roles of FGF1 in both the formation and maintenance of myelin.

FGF2 has long been known to promote neurite outgrowth and regeneration of motor neurons (Walicke *et al*., 1986; Unsicker *et al*., 1987). Recent data demonstrate upregulation of FGF2 mRNA in axotomized sciatic nerve and the corresponding DRG (Grothe *et al*., 2001). In the same vein, overexpression of FGF2 leads to accelerated regeneration (Jungnickel *et al*., 2005). Clearly, these data point to the importance of FGF2 in remyelination. Hereby, we postulated that the factor might be implicated in the developmental myelin formation as well. FGF2 transcript levels peak at P10 and remain

stable thereafter. This observation is in line with our hypothesis and may point to different functions at different stages of the myelination process.

FGF9 is known to be expressed in motor neurons and DRG of adult mice (Nakamura *et al*., 1999; Li *et al*., 2002). Here we show that FGF9 expression is detectable in newborn mouse DRG, and within 10 days transcript levels triple. In contrast to the other members of the family studied, FGF9 transcript levels gradually decrease back to that of P0 in adulthood. These data suggest that, if FGF9 has any role, it might be primarily in the formation of myelin sheath but not in its maintenance.

We detect FGFR1-3 expression in postnatal DRG. FGFR4 transcript, however, was barely detectable in all stages examined, reminiscent of illegitimate transcription. Grothe *et al*. (2001) reported the presence of the receptor transcripts in adult rat DRG (Grothe *et al*., 2001). Similar to our findings, FGFR4 levels were very low. Our results show that transcript levels of FGFR1 did not display modulation during the first week after birth. The subsequent 30 per cent increase on P10 was conserved at later time points. FGFR2 transcript levels did not show any significant change in the study and were always lower than that of FGFR1 and FGFR3. A similar pattern was observed for FGFR3 during the period of active myelination, but in adulthood the levels decreased by 2-fold.

Thus, at all stages examined in DRG, FGFR1 and FGFR2 are present as high affinity receptors for FGF1 and FGF2 signaling. FGF9 interacts with higher affinity to FGFR3, and with lower affinity to FGFR2-IIIc. It is striking that both FGF9 and FGFR3 decrease after active myelination period. Taken together with the observed increase in FGFR1-3 levels in peripheral nerve lesion paradigms (Grothe *et al*., 2001), our results suggest that FGF signaling is important in myelination.

In an *in vitro* induced myelination study, it was proposed by Chan *et al*. (2001) that the interplay between BDNF and NT3 is important in the process. Before induction, BDNF and NT3 coexist, where NT3 suppresses myelin initiation. After induction by ascorbic acid, NT3 is downregulated rapidly and myelination starts. Once the process is on its way, BDNF levels also decrease (Chan *et al*., 2001). However, no *in vivo* expression data was available. Our results indicated a stable expression of BDNF during active myelination

period and after P20 the transcript levels decreased significantly. This agrees with the model explained above in that BDNF expression is necessary for myelin formation but not in later stages.

Our results also indicate parallel modulation patterns of BDNF, FGF9 and FGFR3 transcripts, supporting our hypothesis that FGF9 and FGFR3 may play a role during myelination. Since myelination is a complex process, it is not expected to be orchestrated by a single mechanism. The interplay between BDNF and NT3 mentioned above can well be expanded with the contribution of FGFs *in vivo*. Cross-talk between FGF and neurotrophin families through FRS2 is known in different processes (Ong *et al*., 2000).

Future experiments are required to further test and develop our hypothesis. Localizations of FGFs and FGFRs by immunostaining and *in situ* hybridization studies will identify the sources and targets of the signals. DRG neuron / SC co-culture system where myelination can be induced will be valuable in initial functional studies. In this system, cell-specific shRNA-mediated silencing of FGFs and FGFRs will be instrumental in dissecting the role of these factors in myelination.

6. REFERENCES

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