Mechanisms promoting myelin formation and maintenance under normal and pathological conditions

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ABSTRACT

Oligodendrocytes (OLs) are the myelinating cells of the central nervous system (CNS). They develop from oligodendrocyte precursor cells and differentiate into mature myelin forming OLs. Once myelin is formed, myelinated axons are segregated into different domains around the myelin-devoid nodes of Ranvier. Maintenance of these domains is essential for efficient saltatory conduction, and several myelin proteins contribute to the maintenance of myelin structure and function. Netrin-1 and its receptor DCC were shown to be involved in the maintenance of paranodal axoglial junctions *in vitro*, but their role *in vivo* is not established. Myelin is also a major source of inhibition in the injured CNS, and several myelin proteins have been shown to inhibit axonal regeneration. In this thesis, different aspects of OL biology will be considered.

First, signaling pathways involved in OL differentiation will be studied. I demonstrate a synergistic combination of growth factors that promotes late stages of OL differentiation via the PI3K/Akt/mTor pathway *in vitro*. In the second part, I present *in vivo* evidence of the involvement of DCC in paranodal and myelin maintenance, and in myelin protein composition. Furthermore, absence of DCC expression by OLs leads to the development of a balance and coordination deficit in mice. These results show that expression of DCC by OLs is required for proper maintenance and stability of myelin *in vivo*. Finally, I investigate the source of netrin-1 expression in the injured CNS. Our findings demonstrate that netrin-1, in addition to being a potential myelin-associated inhibitor, is also expressed by fibroblasts and some reactive astrocytes in the injured CNS. Netrin-1 expression might contribute to the inhibition of regeneration and failure of remyelination in the injured CNS.

Understanding the mechanisms of myelin formation and maintenance will help to develop therapeutic strategies for the treatment of demyelinating diseases like multiple sclerosis, and to promote functional recovery following CNS injury.

RÉSUMÉ

Les oligodendrocytes (OLs) sont les cellules myélinisantes du système nerveux central (SNC). Les OLs matures formant la myéline sont dérivés des précurseurs d'OLs, qui se différencient pendant le développement. La myélinisation engendre la formation de différents domaines axonaux autour des nœuds de Ranvier, zones non-myélinisées de l'axone. Le maintien de ces domaines est essentiel pour la conduction axonale saltatoire, et plusieurs protéines de la myéline contribuent au maintien de la structure et de la fonction de la myéline. La nétrine-1 et son récepteur DCC sont impliqués dans le maintien des jonctions paranodales *in vitro*, mais leur rôle *in vivo* n'est pas encore établi. La myéline est également une source majeure d'inhibition après une lésion du SNC. Dans cette thèse, différents aspects de la biologie des OLs seront couverts.

Premièrement, les voies de signalisation impliquées dans la differentiation des OLs seront étudiées. Dans la première partie, je démontre une synergie de facteurs de croissance sur la voie de signalisation PI3K/Akt/mTor, agissant sur la différentiation morphologique des OLs *in vitro*. Dans la seconde partie, en utilisant des souris knockout conditionnelles, je présente des preuves de l'implication de DCC dans le maintien des paranodes, de la myéline, et de la composition protéique de la myéline. De plus, l'absence d'expression de DCC par les OLs mène au développement d'un déficit moteur de coordination et d'équilibre. Ces résultats montrent que l'expression de DCC par les OLs est requise pour le bon maintien et la stabilité de la myéline *in vivo*. Finalement, nous avons investigué la source de l'expression de la nétrine-1 dans les lésions du SNC. Nos résultats démontrent que la nétrine-1, en plus d'être potentiellement un inhibiteur associé à la myéline, est aussi exprimée par les fibroblastes et certains astrocytes réactifs dans les lésions de la moelle épinière. L'expression de nétrine-1 pourrait donc contribuer à l'inhibition de la regénération et l'échec de la remyélinisation après une lésion du SNC.

Une meilleure compréhension des mécanismes de formation et de maintien de la myéline peut contribuer à développer des stratégies thérapeutiques pour le traitement de maladies démyélinisantes comme la sclérose en plaques, ou à promouvoir le rétablissement des fonctions après les lésions du SNC.

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LIST OF FIGURES

LIST OF ABBREVIATIONS

A2B receptor	Adenosine 2B receptor
AD	Alzheimer's disease
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
C. elegans	*Caenorhabditis elegans*
CGT	ceramyl galactosyl transferase
CNP	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	Central nervous system
CREB	cAMP responsive element
CSPG	chondroitin sulphate proteoglycan
CST	ceramyl sulfatyl transferase
DCC	Deleted in Colorectal Cancer
DAPK	Death associated protein kinase
DIV	days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DSCAM	Down's syndrome cell adhesion molecule
DWM	Dorsal white matter
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	extracellular signal regulated kinase
FAK	Focal Adhesion Kinase
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FNIII	Fibronectin type III
GalC	Galactocerebroside

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GEF	guanine exchange factor
GFAP	glial fibrillary acidic protein
GPI	gylcophosphatidyl inositol
HSPG	Heparin sulphate proteoglycan
Ig	immunoglobulin
IGF-1	Insulin-like growth factor
IGF1R	Insulin-like growth factor receptor
MAG	Myelin associated protein
MAL	Myelin and lymphocyte protein
MAPK	mitogen-activated protein kinase
MBP	Myelin basic protein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
mTor	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
NCAM	Neural cell adhesion molecule
NF155	Neurofascin 155
NF186	Neurofascin 186
NFH	high molecular weight neurofilament
NFM	medium molecular weight neurofilament
NgR	Nogo Receptor
NGS	Normal goat serum
NrCAM	neural-glial-related cell adhesion molecule
NRG	Neuregulin
NT-3	Neurotrophin-3
OPC	Oligodendrocyte precursor cell

OL	Oligodendrocyte
OMgp	Oligodendrocyte-myelin glycoprotein
PAK	p21 activated kinase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PDK1	Phosphinoside-dependent kinase 1
PDL	poly-D-lysine
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PKA	Protein kinase A
PLP	Proteolipid protein
pMN	Motor neuron progenitor domain
PP2A	Protein phosphatase A2
PNS	Peripheral nervous system
PSA-NCAM	Polysialylated neural cell adhesion molecule
PTEN	Phosphatase and Tensin homolog
RGC	Retinal ganglion cell
RGM	Repulsive Guidance Molecule
ROCK	Rho kinase
RT	room temperature
Shh	Sonic hedgehog
TBs	Transverse bands

CONTRIBUTION OF AUTHORS

Chapter I to V
All chapters of this thesis were edited by Jenea Bin and Katherine Horn.

Chapter I:
Sarah-Jane Bull: wrote the manuscript and drew all figures

Chapter II:

Sarah-Jane Bull: developed rationale, performed all the experiments except the culture and treatment of oli-neu cells used for the luminex assay (Figure 2-2A and 2-4A) and wrote the manuscript.

Ajit Singh Dhaunchak: developed rationale and edited the manuscript

Sathyanath Rajasekharan: developed rationale, provided guidance with the luminex technology.

Justin Im: performed oli-neu cell culture and treatments (Figure 2-2A and 2-4A)

Timothy E. Kennedy: developed rationale and edited the manuscript.

Chapter III:

Part I :

Sarah-Jane Bull: isolated OPCs from newborn pups, genotyped the pups, collected and processed the retina, analyzed the paranodes, wrote the manuscript

Mohammadali Almasieh : performed the transplantation surgeries

Timothy E. Kennedy: developed the rationale and edited the manuscript

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Jenea M. Bin: assisted in performing the grip test (Figure 3-8B), quantified blindly the domain organisation of paranodes (Figure 3-6), and edited the manuscript.

Jeannie Mui : prepared ultra-thin sections for electron microscopy

Alexandre Boutet: performed the rotarod test (Figure 3-8E).

Timothy E. Kennedy : developed rationale and edited the manuscript.

Chapter IV:

Kenneth Adam Baker: developed rationale, performed immunohistochemistry on spinal cord and acquire all the images, quantified β-gal intensity staining, co-wrote the manuscript.

Sarah-Jane Bull: isolated glial cells from netrin-$1^{-/+}$ mice, performed immunocytochemistry, quantified β-gal intensity staining, performed statistical analysis, and co-wrote the manuscript.

Hiba Kazak: performed the surgeries.

Timothy E. Kennedy: developed rationale, co-wrote manuscript.

Chapter V:
Sarah-Jane Bull: wrote the manuscript

GENERAL PREFACE AND RATIONALE

Myelin, the insulating sheath surrounding axons, was for a long time a forgotten and dismissed structure of the nervous system. However, with the identification of the cells responsible for its production both in the central and peripheral nervous system, the oligodendrocytes and the Schwann cells respectively, came the realization of the importance of myelin for CNS function. This importance is highlighted in diseases that affect myelin such as multiple sclerosis in the central nervous system, or peripheral demyelinating neuropathies such as Guillain-Barré syndrome or chronic inflammatory demyelinating neuropathy.

In the last twenty years, tremendous advances have been made in the understanding of oligodendrocyte development and myelin formation. Our laboratory pioneered studies revealing the role of the secreted guidance cue netrin-1 and its receptor DCC in oligodendrocyte precursor cells migration, mature oligodendrocyte maturation, and myelin maintenance. Work carried out during my PhD aimed at building on that knowledge and I investigated different aspect of myelin biology, focusing on the mechanisms promoting myelin formation and maintenance, as well as myelin-derived inhibition following CNS injury.

CHAPTER I
LITERATURE REVIEW

This thesis investigates oligodendrocyte and myelin biology, building on previous studies conducted by our laboratory and others. The introduction presents a detailed review of the literature, and is separated into three sections. The first section details the state of knowledge of netrin-1 and DCC function in the central nervous system. The second section describes the formation of myelin by oligodendrocytes during development. The last section presents some examples of myelin-associated pathologies.

I.1 NETRIN-1 AND ITS RECEPTORS

The nervous system undergoes complex wiring during development, and intricate networks of axons form as a result of guided axonal growth. The question of how these axons navigate and find their targets fascinated scientists for decades. More than an century ago, Ramón y Cajal proposed that growth cones, sensitive structures at the tip of growing axons, could be either attracted or repulsed by secreted molecules along their developmental journey (Ramón y Cajal and Azoulay, 1909). Since then, scientists have been searching for such guidance factors. Commissural neurons, which extend their growing axons from their cell body in the dorsal spinal cord toward the ventral floor plate, constituted a system that played a pivotal role in proving that axon guidance could be achieved by secreted molecules in the central nervous system (CNS). In fact, it was demonstrated that the floor plate cells were a source of secreted factor(s) that were specifically attracting the commissural axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990).

1.1.1 The netrin family

Netrin was named after the Sanskrit word Netr, which means "the one who guides", and it was first identified in mammals during a search for a secreted molecule emanating from the floor plate that could attract commissural axons in the developing spinal cord (Kennedy et al., 1994; Serafini et al., 1994). Netrin was later found to be a vertebrate homologue of a previously identified guidance molecule in nematode worm *Caenorhabditis (C.) elegans*, UNC-6. The UNC genes were identified in a screening of molecules regulating neural development (Hedgecock et al., 1990; Ishii et al., 1992). UNC-6 mutations in the nematodes resulted in uncoordinated behaviour, which was also seen following mutations of two other genes: UNC-40 and UNC-5 (Hedgecock et al., 1990), later identified as UNC-6/netrin-1 receptors (Keino-Masu et al., 1996; Leonardo et al., 1997). Six members of the netrin family have been identified in vertebrates: four secreted netrins (1 to 4) and two GPI-linked netrins (G1 and G2). Of these, only netrin-2 is absent in mammals, but has been identified in chicken and zebrafish. Netrin-Gs

are evolutionarily distinct from the other netrins, and bind to the netrin-G ligands NGL-1 and NGL-2 (Moore et al., 2007; Rajasekharan and Kennedy, 2009).

Netrins have a molecular mass of ~70-75 kDa and possess an amino-terminal signal peptide and three domains: domain V, VI and C (Figure 1-1A). Domains V and VI are homologous to laminins, with netrin 1-3 similar to the γ1 chain of laminin, and netrin-4, -G1 and –G2 similar to the β1 chain of laminin. Domain C, present in all secreted netrins, is rich in basic amino acids and can bind to cell surface molecules like heparan sulphate proteoglycans (Barallobre et al., 2005). Studies in *C. elegans* demonstrated that mutation of domain VI disrupted all UNC-6/netrin functions, while mutation of domain V-3 selectively disrupted UNC40/DCC mediated functions, and mutation of domains V-2 and V-3 disrupted UNC-5 mediated functions (Lim and Wadsworth, 2002). Other evidence demonstrates that netrin-1 can bind to both UNC5 and DCC in the absence of domain V or domain VI, suggesting that netrin-1 binds to its receptors via multiple domains (Kruger et al., 2004). In mammals, netrin-1 is widely expressed throughout the developing CNS, and is required for normal migration of commissural axons in the brain and spinal cord. Mice lacking functional netrin-1 die within hours after birth and exhibit multiple commissural guidance defects (Serafini et al., 1996). However, even if it was first identified as a guidance cue, the role of netrin-1 goes well beyond guidance in the CNS, and it is now appreciated that netrin-1 is expressed widely outside of the nervous system and serves several different functions.

Netrin-1 expression

In the developing nervous system, netrin-1 is expressed at the midline in the ventral neuroepithelium, but also in other structures of the developing forebrain, such as the retina, the striatum and the cerebellum (Livesey and Hunt, 1997). Netrin-1 is also expressed outside the nervous system in the developing embryo, and netrin-1 transcripts are detected in mammary glands, cardiac muscle, lungs, intestine and pancreas (Sun et al., 2011a). Netrin-1 levels decrease after birth, but netrin-1 is still expressed in the adult mature nervous system. Striatal cholinergic

and projection neurons express netrin-1 in the adult brain (Shatzmiller et al., 2008). Netrin-1 mRNA expression is also high in the cerebellar granular layer, in the hippocampus and in the cortex (Allen Mouse Brain Atlas). In the adult rat spinal cord, *in situ* hybridization and immunohistochemistry revealed expression of netrin-1 by both neurons and oligodendrocytes, but not astrocytes. More specifically, netrin-1 was found to be membrane-associated in the adult spinal cord, and in white matter, concentrated in non-compact myelin fractions (Manitt et al., 2001).

1.1.2 The netrin-1 receptors

As established previously with its *C. elegans* homologue UNC-6, netrin-1 can act as a bifunctional cue, attracting some axons and repelling others (Colamarino and Tessier-Lavigne, 1995). The nature of the netrin-1 response is dependent on the type of receptors a cell expresses. Multiple proteins have been identified as putative netrin-1 receptors, but the most characterized ones are the UNC-40 homologue Deleted in Colorectal Cancer (DCC), the DCC paralogue neogenin, and the UNC-5 homologues: UNC5A-D (Figure 1-1B).

The DCC family

In vertebrates, the DCC family of proteins consists of Deleted in Colorectal Cancer (DCC) and neogenin, two proteins from the immunoglobulin (Ig) superfamily, whose orthologs are UNC-40 in *C. elegans* and Frazzled in *Drosophila*. They both share homology to Neural Cell Adhesion Molecule (NCAM) (Cho and Fearon, 1995; Keino-Masu et al., 1996).

Deleted in Colorectal Cancer (DCC)

DCC is a single pass transmembrane protein with an extracellular domain composed of four Ig domains, six fibronectin type III (FNIII) repeats, and an intracellular C-terminus characterized by conserved P1, P2 and P3 regions (reviewed in Moore et al., 2007). Netrin-1 was shown to bind to DCC via its fourth and fifth FNIII repeats (Geisbrecht et al., 2003; Kruger et al., 2004). The *dcc* gene is located on chromosome 18q, spanning 1.4Mb and containing 29 exons (Cho et al., 1994). It was first identified as a candidate tumor suppressor gene

inactivated in colorectal cancer (Fearon et al., 1990). DCC absence from tumors was identified as a poor prognostic marker in colorectal cancer patients (Shibata et al., 1996). However, follow up studies shed doubt on the functional relevance of this deletion in the development of colorectal cancer (Fazeli et al., 1997), and exactly how DCC functions in tumourigenicity is not known (Sun et al., 2011a). The role of DCC in netrin-1 function was established very early using blocking antibodies that were seen to inhibit the netrin-1 induced axon outgrowth of commissural axons *in vitro* (Keino-Masu et al., 1996). The phenotype of mice lacking functional DCC is reminiscent of that of netrin-1 mutants, and they too die shortly after birth (Fazeli et al., 1997). *In vitro* studies have shown that DCC is required not only for netrin-1 mediated attraction (Keino-Masu et al., 1996; Stein et al., 2001), but also for netrin-1 induced repulsion (Hong et al., 1999; Keleman and Dickson, 2001). The P3 domain is required for DCC multimerization and attraction to netrin-1 (Stein et al., 2001), while repulsion depends on association of the P1 domain of DCC and the intracellular domain of UNC5 (Hong et al., 1999). Work with chimeric proteins showed that the intracellular domains of DCC and UNC5 are central to the response to bound netrin (Hong et al., 1999; Keleman and Dickson, 2001). Interestingly, a spontaneous *dcc* mutant, the *kanga* mouse, is missing exon 29 coding for domain P3. This DCC mutant lacks CNS commissures, but survives into adulthood (Finger et al., 2002). DCC expression is very high in the developing CNS, but its levels decrease after birth. Expression is low in the adult rat spinal cord, but DCC was shown to be expressed by both neurons and oligodendroglial cells (Manitt et al., 2004).

Neogenin

Neogenin and DCC emerged from a common ancestral gene (Cole et al., 2007), and they share around 50% homology (Vielmetter et al., 1994). In embryogenesis, neogenin expression is high in the developing mesoderm, but contrary to DCC, its expression in the developing nervous system is limited. While DCC expression tends to decrease during development, neogenin levels increase in an opposite manner, suggesting complementary functions of these two receptors (Gad et al., 1997). Netrin-3 was shown to bind to neogenin with higher affinity than netrin-1;

however, the Repulsive Guidance Molecule (RGM) proteins were shown to have even higher affinity to neogenin than netrins (Wilson and Key, 2007). RGMs are GPI-linked membrane proteins that by binding to the neogenin receptor, mediate axon guidance events and neuronal survival (Severyn et al., 2009). More recently, several members of the Bone Morphogenetic Proteins (BMPs) were also shown to bind neogenin receptors and activate RhoA (Hagihara et al., 2011). Although multiple roles of neogenin have been described in different developmental processes, its precise role in guidance and migration in response to netrin signals is still not well understood (Wilson and Key, 2007). However, adhesive functions of netrin-1 and neogenin have been described in the developing mammary gland (Srinivasan et al., 2003).

The UNC5 family

The UNC5 homologues are all single pass transmembrane proteins, expressed in different cell types and tissues. There are four UNC5 homologues in vertebrates: UNC5A, B, C, and D. UNC5s are composed of two Ig domains and two thrombospondin-like domains in their extracellular region. Their intracellular region contains a zona occludens 5 (ZO5) domain, a DCC binding domain, and a death domain (Moore et al., 2007). Netrin-1 interacts with UNC5 proteins through their Ig domains (Leonardo et al., 1997; Geisbrecht et al., 2003).

In *C. elegans*, UNC-5 mutants exhibit defective ventro-dorsal axon guidance (Hedgecock et al., 1990). In vertebrates, UNC5 protein was shown to mediate the chemorepellent action of netrin-1 on trochlear axon guidance (Colamarino and Tessier-Lavigne, 1995). The expression of an UNC5 homologue is required for the repulsive response to netrin-1 to occur, both in cell migration and axon guidance (Keleman and Dickson, 2001; Jarjour et al., 2003). While association with DCC seems required for netrin-1 mediated long range repulsion, DCC-independent UNC5 responses can mediate short-range repulsion (Hong et al., 1999; Keleman and Dickson, 2001).

During development, UNC5A expression is restricted to the CNS. UNC5B expression is the most widespread, with expression in developing blood vessels, eyes, limb buds, and lungs (Engelkamp, 2002; Liu et al., 2004). UNC5C is expressed on developing limb buds, in hindbrain and in cerebellar neurons (Kim and Ackerman, 2011). UNC5D is the last homologue to turn on its expression during development, and is found on developing epithelial buds (Engelkamp, 2002). In the adult mouse brain, all UNC5 homologues are expressed, albeit at different levels in different regions (Allen Mouse Brain Atlas). In the adult rat spinal cord, expression of UNC5 homologues is increased relative to developmental levels, with UNC5B being the homologue with the highest expression (Manitt et al., 2004). Concomitant with a decrease in DCC expression, these enhanced levels of UNC5 expression might contribute to the inhibitory environment of the mature adult nervous system (see section 1.3.3).

The wide range of phenotypes seen in UNC5 mutants highlights the diversity of functions of each homologue. UNC5A knockout mice show decreased developmental apoptosis of neurons, and altered embryonic spinal cord morphology, but nevertheless survive after birth (Williams et al., 2006). UNC5B knockout mice do not survive after birth and exhibit a severe vascular phenotype (Lu et al., 2004). UNC5C knockout mice survive through adulthood, but exhibit a severe cerebellar phenotype and ataxia (Ackerman et al., 1997). UNC5C was shown to regulate dorsal guidance of cerebellar axons (Kim and Ackerman, 2011). No known reports have described UNC5D knockout mice.

Other netrin receptors

Adenosine 2B (A2B) receptor, a G-protein coupled receptor, was described first as the netrin-1 receptor which, by complexing with DCC, mediates netrin-1 induced axon outgrowth (Corset et al., 2000). However, subsequent reports questioned the specificity of the methods used and argued against a role of A2B receptors in netrin function (Stein et al., 2001). More recently, Down's syndrome Cell Adhesion Molecule (DSCAM), was shown to act as a netrin receptor (Andrews et al., 2008; Ly et al., 2008; Liu et al., 2009). Evidence shows that

DSCAM can act together with, and in parallel to, DCC to promote netrin-1 induced axon turning (Ly et al., 2008; Liu et al., 2009) and midline crossing in the developing spinal cord (Andrews et al., 2008). More detailed examination is needed to determine the exact contribution of DSCAM to netrin-1 function.

Similarities between netrin-1 and laminin structures suggest that netrin-1 could bind integrins, the laminin receptors. Indeed, binding of netrin-1 to α6β4 and α3β1 integrins was demonstrated in pancreatic epithelial cells, although surprisingly through its non-laminin homologous C domain (Yebra et al., 2003). Netrin-1 interaction with α3β1 integrins was also shown to regulate the migration of interneurons in the developing cortex (Stanco et al., 2009).

In addition to conventional transmembrane receptors, netrin-1 can be sequestered by heparin sulphate proteoglycans (HSPGs) on the cell surface. It has long been known that netrin-1 binds to heparin, as heparin affinity chromatography was used in its initial purification (Serafini et al., 1994). Binding studies demonstrated that netrin-1 could bind heparin and HSPGs (Shipp and Hsieh-Wilson, 2007) and that this binding occurs through netrin's C domain (Kappler et al., 2000). HSPG expression was in fact shown to be required in a cell autonomous fashion for netrin-1 induced commissural axons pathfinding (Matsumoto et al., 2007). Syndecan, a HSPG expressed in the CNS, was shown to regulate UNC5 function in *C. elegans* by modulating the distribution of extracellular cues (Schwabiuk et al., 2009). Together, these findings suggest a role for HSPGs in sequestering netrin protein and presenting it to its receptors present on the cell surface.

Signaling downstream of DCC

Migration of cells or axons is dependent on the reorganization of the actin cytoskeleton. In the recent years, much has been learned about the molecular mechanisms linking DCC to the actin cytoskeleton. Netrin-1 binding to DCC triggers the formation of a multimeric intracellular complex composed of the adaptor protein nck1 (Li et al., 2002b), which recruits N-WASP and pak1 (Shekarabi et al., 2005). Members of the Rho family of small GTPases cdc42 and

Rac1, whose activation is essential for the remodelling of the actin cytoskeleton and netrin-1 mediated attraction (Li et al., 2002a), are in turn recruited to the complex via association with pak1 and N-WASP (Shekarabi et al., 2005). The Rho guanine nucleotide exchange factor (GEF) responsible for the activation of these Rho GTPases is currently being investigated, but substantial evidence supports a major role for β-Pix upstream of Cdc42 and Rac1 activation (Rodrigues and Kennedy, unpublished). Evidence supporting roles of two different GEFs for Rac acting downstream of DCC, Trio (Briancon-Marjollet et al., 2008) and Dock180 (Li et al., 2008), has recently been described. Netrin-1 also induces DCC phosphorylation by the Src family kinase Fyn, and causes the recruitment of Focal Adhesion Kinase (FAK), both necessary for the attractive response to netrin-1 (Li et al., 2004; Meriane et al., 2004).

Other signalling pathways have been implicated downstream of DCC in response to netrin-1 signals. Phospholipase Cγ and phosphoinositide 3-kinase (PI3K) were shown to mediate axon outgrowth in response to netrin (Ming et al., 1999; Xie et al., 2006). Protein kinase A (PKA) activity is also a key modulator of the response to netrin-1. In *Xenopus* spinal neurons, decreasing cAMP levels and PKA activity causes the response to netrin-1 to switch from attraction to repulsion (Ming et al., 1997). However, that switch was not observed in rodent commissural axons, but a decreased sensitivity was observed when PKA activity was inhibited (Moore and Kennedy, 2006), which could be explained by a PKA-dependent increase of DCC insertion in plasma membrane following netrin-1 treatment (Bouchard et al., 2004).

DCC cleavage events can also regulate DCC signalling and modulate responses to netrin-1. The DCC extracellular domain can be cleaved by metalloproteases, and inhibiting this DCC shedding potentiates the response to netrin-1 (Galko and Tessier-Lavigne, 2000). Ubiquitination and proteolysis of DCC was also shown to occur following netrin-1 treatment of embryonic cortical neurons, inducing a down-regulation of DCC expression on the cell surface (Kim et al., 2005).

Localization of DCC to membrane microdomains is also critical for netrin-1 responses. DCC palmitoylation induces its translocation into lipid rafts, whose integrity is essential for netrin-1 induced axon outgrowth (Guirland et al., 2004; Herincs et al., 2005). Interestingly, while evidence shows that netrin-1 binding does not affect DCC localization to rafts, DCC signalling complexes located in raft and non-raft regions were shown to differ, suggesting a localization-dependant activation of signalling pathways downstream of DCC (Petrie et al., 2008).

Recent findings have linked the intracellular domain of DCC to the regulation of local translation. DCC associates physically with translation initiation factors and ribosomal subunits through its P1 domain. Following netrin-1 binding to DCC, the translation machinery is released and translation can proceed (Tcherkezian et al., 2010). These findings are consistent with observations that local translation at the growth cone is required for attraction to netrin-1 (Campbell and Holt, 2001).

1.1.3 Functions of DCC/netrin-1

Both long range and short range functions of netrin-1 have been described. In general, evidence of long-range functions of netrin-1 is found in the developing nervous system, where netrin-1 acts as a repellent or an attractant far from its expression site. Netrin-1, produced by floor plate cells, was shown to diffuse and form a gradient in the embryonic spinal cord (Kennedy et al., 2006). In the adult nervous system, netrin-1 most likely serves more short range functions, as it is found associated with cell membranes and extracellular matrix (Manitt et al., 2001; Baker et al., 2006).

Guidance functions

Netrin-1 expression at the ventral midline and by floor plate cells in the developing spinal cord mediates a number of long-range guidance functions (Figure 1-2). While netrin-1 acts as a long-range attractant for commissural axons (Serafini et al., 1996), it repels growing trochlear motorneuron axons (Colamarino and Tessier-Lavigne, 1995). In addition to directing axons to their targets, netrin-1

can also mediate cell migration during development, both by attracting and repelling precursor cells in the CNS. Netrin-1 regulates chemoattraction of migrating cerebellar neural precursor cells through DCC (Alcantara et al., 2000). Netrin-1 also acts as a repellent for migrating oligodendrocyte precursor cells, (OPCs) which express both UNC5A and DCC receptors (Jarjour et al., 2003). These effects on axon guidance and cell migration observed during development can also be replicated in the adult CNS, as it was demonstrated that netrin-1 can act as a repellent for adult neural progenitor cells in the injured spinal cord (Petit et al., 2007).

Netrin-1 also functions as a guidance and branching factor during developmental angiogenesis. Formation of blood vessels involves similar regulated pathfinding events to what regulates developmental axon guidance, and many guidance cues have been implicated in both systems. Netrin-1 is such a factor; however, its precise function during angiogenesis is still unclear (Adams and Eichmann, 2010). Netrin-1 was associated with both chemorepulsion of nascent vessel branches and chemoattraction of migrating endothelial cells. A key modulator of netrin-1 response in angiogenesis may be the presence of UNC5B on endothelial cells (Castets and Mehlen, 2010).

Survival functions

Multiple lines of evidence point to the possibility that netrin-1 acts as a survival factor (Mehlen and Furne, 2005). The mechanism by which netrin-1 promotes survival is thought to follow the dependence receptor hypothesis. According to this view, cells expressing DCC and/or UNC5 undergo apoptosis in the absence of netrin-1. The dependence receptor hypothesis has revived the possibility that DCC inactivation may be implicated in colorectal cancer progression, as absence of DCC would release the cells from the apoptotic pathway (Mehlen and Fearon, 2004). However, evidence from knockout mice argues against that hypothesis, since netrin-1 knockout mice do not exhibit increased cell death (Williams et al., 2006). Furthermore, DCC knockout mice display increased cell death in the

retina, which argues against its function as a dependence receptor (Shi et al., 2010).

UNC5 homologues have also been categorized as dependence receptors. However, even if UNC5A mediates neuronal apoptosis *in vivo*, this effect seems to be independent of netrin-1 expression (Williams et al., 2006). Nonetheless, it is possible that netrin-1 modulates UNC5 mediated apoptosis under certain conditions. Interaction of UNC5B with death associated protein kinase (DAPK) and its subsequent activation through the protein phosphatase A2 (PP2A) were shown to be increased in the absence of netrin-1, and to induce apoptosis (Llambi et al., 2005). Netrin-1 binding to UNC5B inhibits DAPK activation by recruiting CIP2A (cancerous inhibitor of PP2A) to the complex and neutralizing apoptotic signals (Guenebeaud et al., 2010).

Cell adhesion functions

DCC's structural homology to NCAM suggests a role for the netrin receptor in adhesion. Recruitment of adhesive complex components to DCC and UNC5 also suggests that netrin-1 binding to its receptors is involved in the formation of adhesive complexes (Li et al., 2004; Meriane et al., 2004; Li et al., 2006). Importantly, such short-range actions of netrin-1 have been shown to regulate morphogenesis of several organs including mammary glands (Srinivasan et al., 2003) and developing lungs (Liu et al., 2004; Strickland et al., 2006). DCC was shown to increase cell-cell adhesion, an effect that could be mediated through ezrin association with the intracellular portion of DCC (Martin et al., 2006). Independent of DCC, netrin-1 mediates adhesion of epithelial cells in the developing pancreas through interaction with integrins (Yebra et al., 2003). Netrin-1 and DCC were also shown to regulate the adhesion between the noncompact glial loops of myelin and the axonal membrane at paranodal junctions (Jarjour et al., 2008).

Functions in oligodendrocytes

In the developing spinal cord, OPCs originate in the ventral ventricular zone, and

migrate dorsally (Noll and Miller, 1993). Netrin-1, expressed by the floor plate, acts as a repellent for OPCs that express both Unc5A and DCC (Jarjour et al., 2003; Tsai et al., 2003). This repellent response is dependent on the activation of RhoA by netrin-1 (Rajasekharan et al., 2010). Netrin-1 starts to be expressed by oligodendrocytes at the initiation of myelination in the CNS (Rajasekharan et al., 2009), and adult oligodendrocytes still express netrin-1 at high levels (Manitt et al., 2001). Subcellular fractionation of adult rat spinal cord white matter revealed the presence of netrin-1 in the non-compact myelin fraction (Manitt et al., 2001). More specifically, netrin-1 and DCC were later shown to be enriched at the paranode, a non-compact myelin compartment, in adult rat spinal cord (Jarjour et al., 2008; Low et al., 2008). *In vitro*, DCC and netrin-1 do not seem to be required for formation of myelin and paranodes per se, but rather play an important role in the maintenance of adhesive contacts between the paranodal loops and the axolemma. More detailed description of oligodendrocyte and myelin biology follows in section I.2.

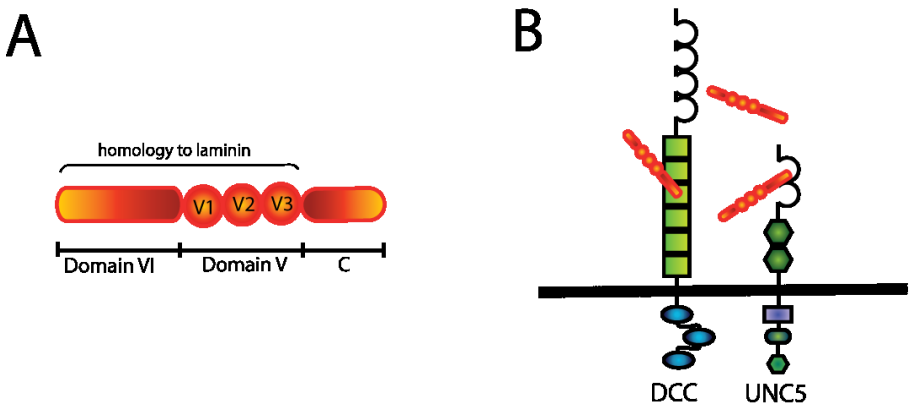


FIGURE 1-1: **Netrin-1 and its receptors**. A) Netrins are composed of three domains: domain VI, V, and C. Domain VI and V are homologous to domains in laminin. B) DCC and UNC5 homologues are the most well characterized netrin-1 receptors. Netrin-1 binds to DCC through its fibronectin domains and to UNC5 through an Ig domain.

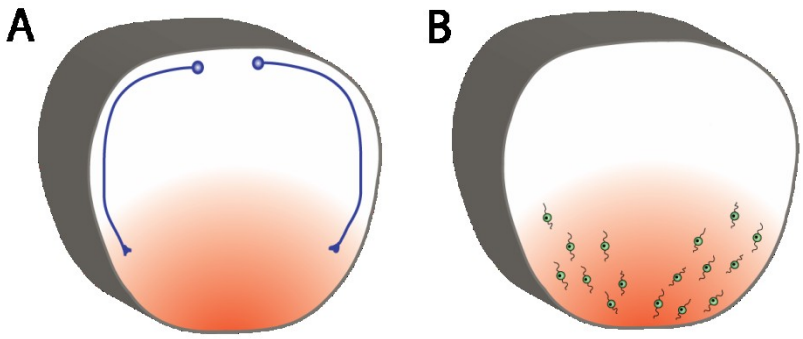


FIGURE 1-2: **Netrin-1 mediated guidance in the developing spinal cord**. Netrin-1 secreted by floor plate cells establishes a ventro-dorsal gradient in the developing spinal cord. A) Growth cones of commissural axons are attracted by netrin-1 and will reach and cross the ventral midline. B) OPCs, born in the ventral part of the spinal cord, migrate dorsally in response to netrin-1.

I.2 OLIGODENDROCYTE DEVELOPMENT AND MYELIN FORMATION

Unlike his descriptions of neuronal anatomy and structure, Cajal's reports of myelin were highly imprecise. He described myelin as a fatty substance surrounding the axon, most likely secreted by the axon itself (Ramón y Cajal and Azoulay, 1909). Notably, Wilder Penfield, following the initial description of oligodendrocytes by Rio Hortega, characterized these cells as the myelin producing cells of the CNS, and proved to Cajal their existence (Gill and Binder, 2007). Further studies highlighted the tremendous advantage that myelination procures. Throughout evolution, the nervous system has adapted to achieve more rapid and efficient conduction of action potentials. Myelination of axons allows for increased speed of nerve transmission at a lower energy expense for neurons. Myelin is an insulating membrane sheath produced by oligodendrocytes (OLs) in the CNS, and by Schwann cells in the peripheral nervous system (PNS). While some commonalities exist between the two types of myelinating cells, some features differ dramatically. For example, one OL can myelinate several axon segments, whereas Schwann cells myelinate axons following a 1:1 ratio (Poliak and Peles, 2003). This section will focus on OLs, their biology, and the anatomy of CNS myelin. When relevant, differences between the CNS and PNS myelin will be highlighted.

I.2.1 Oligodendrocyte development and differentiation

Oligodendrocyte specification during development

Early studies of cell specification in the CNS suggested that OLs are the last cell type to be specified, after neurons and astrocytes (Altman and Bayer, 1984). OPCs arise from the subventricular zone neuroepithelium in the ventral side of the developing neural tube, although there is evidence for a dorsal source of OPCs (Cai et al., 2005; Vallstedt et al., 2005). As with other cells generated at the floor plate, the specification of OPCs is dependent on signals from the notochord that will induce the expression of transcription factors in a gradient-like fashion (Trousse et al., 1995; Orentas and Miller, 1996; Pringle et al., 1996).

Transcriptional control of specification

Sonic hedgehog (Shh), a signalling molecule secreted by the notochord and the floor plate (Echelard et al., 1993), was the first induction signal described for OPC specification (Pringle et al., 1996; Orentas et al., 1999; Davies and Miller, 2001). Shh signaling was shown to be necessary and sufficient for the expression of the Olig class of transcription factors in the ventral spinal cord (Lu et al., 2000). However, Shh-independent Olig expression in the dorsal spinal cord was described, revealing a different class of OLs originating from the dorsal part of the CNS (Nery et al., 2001; Cai et al., 2005). Olig 1 and 2 are expressed in the motor neuron progenitor domain (pMN) of the ventral spinal cord (Sun et al., 1998), that gives rise to both OPCs and motor neurons. Loss of Olig2 expression results in a complete loss of all OPCs and spinal motor neurons. Recent findings demonstrate that the phosphorylation state of Olig2 is responsible for the switch from a motor neuron fate to an OPC fate (Li et al., 2011; Sun et al., 2011b). Changes in the phosphorylation state of serine 147 appears to regulate the preferred dimerization partners of Olig2 (Li et al., 2011) that will ultimately control the transcriptional activation. Olig2 is activated not only during OPC specification, but is continuously expressed in differentiated and myelinating OLs (Lu et al., 2002). There have been conflicting findings on the role of Olig1 in OPC specification, with some reporting no effect on developmental myelination (Lu et al., 2002), and others describing myelination defects (Xin et al., 2005) in Olig1 deficient mice. Nevertheless, the same group that described no role of Olig1 in developmental myelination (Lu et al., 2002) reported that Olig1 deficient mice failed to remyelinate following cuprizone-induced demyelination (Arnett, 2004), arguing for a role of Olig1 in OL maturation under certain circumstances.

Coexpression of the transcription factors Nkx2.2 and Olig2 in cells of the pMN domain is necessary for OPC differentiation (Zhou et al., 2001) and gives rise to Sox10 expression and commitment to the OL lineage (Sun et al., 2001). Sox10 was identified as a transcription factor specifically expressed in myelinating glia (Kuhlbrodt et al., 1998), and was shown to be essential for terminal differentiation

of OLs (Stolt et al., 2002). Together with Sox9, Sox10 regulates the expression of Platelet-derived growth factor receptor alpha (PDGFR-α) in OPCs (Finzsch et al., 2008) and is able to induce expression of several myelin genes essential in the terminal differentiation of OLs (Wegner, 2008).

Many transcription factors active during developmental OPC specification continue to be expressed in mature OLs (Wegner, 2008). Some of them were shown to regulate specific aspects of OL and myelin structure, like Nkx6.2, which controls axo-glial interactions at the paranode in fully formed myelin (Southwood, 2004). Other transcription factors, like Olig2, Nkx2.2 and Sox10, control broader activation of myelin genes throughout life (Wegner, 2008).

Dorsal origins of OLs

Whether dorsally generated OLs exist has been a matter of debate for many years (Cameron-Curry and Le Douarin, 1995; Richardson et al., 2006). However, recent knockout studies have provided evidence of a dorsal source of OLs in the spinal cord. In the absence of Nkx6 transcription factors, whose expression are induced by Shh signalling, no OLs or motor neurons develop in the ventral ventricular zone. This complete loss of ventral pMN domain permits the observation of Olig2 and PDGFRα positive cells in the dorsal spinal cord (Cai et al., 2005; Vallstedt et al., 2005). A dorsal origin of OLs in the forebrain has also been shown (Kessaris et al., 2006). A commonality between dorsally derived OLs in spinal cord and forebrain is their late appearance in development, as they are specified after ventral OLs have started to migrate (Richardson et al., 2006).

Proliferation and migration

Proliferation and migration are intertwined, as OPCs will start to differentiate when they reach their destination. Thus, signals affecting migration often have roles in proliferation and vice versa. For example, PDGFRα activation was identified as a proliferative signal for OPCs (Collarini et al., 1991; Pringle et al., 1992), while PDGF was also shown to increase general motility and migration of OPCs (Armstrong et al., 1990). However, since PDGF is present uniformly in the

developing CNS (Yeh et al., 1991), it is unlikely to guide OPCs toward a precise target. Other factors contribute to more than one aspect of OL development. Shh, in addition to controlling OPC specification, was shown to promote migration and proliferation of OPCs (Merchan et al., 2007). Netrin-1, in addition to its role in OPC migration (Tsai et al., 2003), was also shown to regulate branching in later stages of OL development (Rajasekharan et al., 2009).

Paths of OPC migration in the cortex are not well defined, but mechanisms of OPC dispersal are better understood in spinal cord and optic nerve, where the paths of migration are easier to follow (Miller, 2002). OPCs are thought to migrate along pre-existing tracts, most likely through interactions with adhesion molecules and extracellular matrix components. A distinction is made between short range cues, which are substrate bound, and long range cues, which can diffuse and function far from the cells that secrete them. Short range cues can either promote OPC migration, like fibronectin and laminin, or inhibit migration, like tenascin-C. Long range cues include growth factors like PDGF and Fibroblast growth factor (FGF), and guidance cues like netrins and semaphorins. Most evidence indicates that netrin-1 and class 3 semaphorins act as repellent cues for migrating OPCs (Jarjour and Kennedy, 2004). Migration arrest of OPCs at the correct destination is also important to achieve a uniform concentration of OPCs throughout the CNS. The chemokine CXCL1, whose expression is temporally regulated during development, acts on OPCs through CXCR2 receptor to stop their migration (Tsai et al., 2002).

Survival

Once OPCs have stopped their migration, survival mechanisms come into play and it is estimated that around 50% of OPCs and pre-myelinating OLs undergo programmed cell death (Richardson et al., 2006). Evidence tends to show that this cell death is caused by competition for a limited amount of trophic factors provided by axons (Barres et al., 1992; Raff et al., 1993). Survival of OLs and differentiation seem to be connected events as several factors promoting survival were also shown to favour differentiation. Many factors were shown to promote

survival of OPCs, such as PDGF, NT-3 and IGF-1 (Barres et al., 1992). Several factors were shown to act through the Akt pathway to increase survival *in vitro* (see section below on signalling pathways). The final result of the massive OL death happening prior to myelination is that the number of differentiating OLs matches the number of axons that must be myelinated.

Oligodendrocyte differentiation

The different steps described above, from OPCs specification to the initiation of myelination, are characterized by differential expression of protein and surface markers, as well as morphological changes (Figure 1-3). These discrete phases of differentiation can be observed both *in vivo* and *in vitro*. At the progenitor phase, highly motile bipolar OPCs express PDGFRα and are A2B5-positive *in vitro*. As differentiation proceeds, pre-OLs/immature OLs start to express 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP or RIP) (Bradl and Lassmann, 2010) and are characterized by a dense branching network; however, they do not yet exhibit myelin sheets. Maturation of these immature cells is characterized by appearance of myelin membrane domains. Mature myelinating OLs start to express myelin specific proteins such as myelin binding protein (MBP), proteolipid protein (PLP) and myelin-associated glycoprotein (MAG) and extend myelin sheaths around axons *in vivo*, and myelin-like sheets *in vitro* (Baumann and Pham-Dinh, 2001).

Myelin formation and compaction

Myelination occurs mostly postnatally in rodents and humans, and follows a spatiotemporal sequence that is highly reproducible within a species (Baumann and Pham-Dinh, 2001). Axons with a caliber exceeding a certain limit (~1 μm) will be myelinated, whereas smaller caliber axons will remain unmyelinated. Likewise, myelin thickness is also dependent on axon caliber, such that larger axons are surrounded by a thicker layer of myelin. Axonal signals most likely regulate myelin thickness, as a single OL can myelinate several axons with distinct diameters (Waxman and Sims, 1984). In the PNS, the amount of neuregulin1 (NRG1) type III on the axon is a critical signal triggering myelination by Schwann cells, as it determines not only if the axon will be myelinated

(Taveggia et al., 2005), but also the number of wraps of myelin produced (Michailov et al., 2004). However, NRG1 signals do not seem to be required in the CNS (Brinkmann et al., 2008), and the mechanisms underlying the recognition of axon caliber by OLs are still not well defined (Sherman and Brophy, 2005).

Initiation of myelination relies on the establishment of an adhesive contact between the extending myelin-producing OL process and the axon to be myelinated. It is thus not a surprise that several proteins that mediate the formation of adhesive complexes were found to be implicated in the process of myelination. For example, FAK regulates the timing of myelin initiation through OL morphological remodelling (Forrest et al., 2009). Of all the proteins implicated in the formation of adhesion complexes, integrins received a lot of attention in studies looking for membrane-associated initiators of myelin formation. Laminin-2, expressed on axons, was shown to promote myelin formation through OL integrin α6β1 (Colognato et al., 2002). The laminin-2 mutant exhibited hypomyelination (Chun et al., 2003), suggesting a role for integrin signalling in myelin formation. However, subsequent studies on the role of β1 integrins yielded puzzling and apparently contradicting results. β1 integrin knockout mice showed no defect in CNS myelination (Benninger et al., 2006). Taking a dominant negative strategy by overexpressing the extracellular domain of β1 integrin, Lee et al, (2006), described hypomyelination of several CNS axon tracts. In contrast, overexpression of the intracellular domain of β1 demonstrated a myelination delay of small caliber axon (Camara et al., 2009). Thus, while integrins might have a role in the initiation of myelination, myelin formation per se is not an integrin-dependent mechanism. Interestingly, recent findings provide a link between α6β1 integrin and localized translation of myelin proteins such as MBP through the interaction of the mRNA binding protein hnRNP-K (Laursen et al., 2011), providing additional evidence for a role of integrins in the initiation of myelination and myelin gene expression.

Negative regulation of myelination could also have a role in the timing and choice of axons to myelinate. Polysialylated neural cell adhesion molecule (PSA-NCAM) was shown to inhibit myelination when present on the axonal surface (Charles et al., 2000; Jakovcevski et al., 2007). Importantly, demyelinated axons start to re-express PSA-NCAM, which could contribute to remyelination failure in several pathological conditions (Charles et al., 2002a). Another negative regulator of myelination is LINGO-1, which is expressed on OLs and inhibits differentiation and myelination through RhoA activation (Mi et al., 2005).

The exclusion of cytoplasm from the wrapped layers of OL membrane is termed "compaction". Localized translation of MBP protein, which mediates the adhesion of the intracellular membrane leaflet, is central to the compaction of CNS myelin. The role of cytoskeletal remodelling in myelin compaction is highlighted in the RhoGTPase cdc42 and Rac1 conditional mutants, which exhibit aberrant myelin compaction (Thurnherr et al., 2006). Other signalling pathways have been implicated in different stages of OL development, and this aspect is further discussed in the next section.

Signaling pathways involved in OL development and differentiation

Several signalling pathways are implicated in OL maturation, differentiation and myelination. While some pathways act primarily on cell remodelling through their action on the cytoskeleton, some signalling pathways exert their action through gene activation and protein synthesis. Even though significant progress has been made in recent years in the identification of signalling molecules involved in OL differentiation, the exact function of each pathway is still unclear, especially since *in vitro* and *in vivo* studies have not always agreed. Collaboration and synergy of multiple signalling pathways is probably regulating the different stages of OL development.

PI3K/Akt pathway

Akt (also known as protein kinase B or PKB) is a serine/threonine kinase that can mediate numerous cellular functions. Phosphoinositide 3-kinase (PI3K) is a lipid

kinase that can be activated downstream of the majority of receptor tyrosine kinases and other plasma membrane receptors. Upon activation by receptors, PI3K phosphorylates the specialized lipid PtdIns-4,5-P_2 generating PtdIns-3,4,5-P_3 which recruits Akt and phosphinoside-dependent kinase 1 (PDK1) to the plasma membrane. Akt is in turn activated by PDK1 and can phosphorylate a variety of targets implicated in different cell functions including survival, proliferation, glucose metabolism and protein synthesis. To turn off the signal, PTEN (Phosphatase and Tensin homolog) antagonizes the action of PI3K and dephosphorylates PtdIns-3,4,5-P_3 (reviewed in Katso et al., 2001).

Growth factor dependent Akt signalling has been implicated in survival of cells of the OL lineage after growth factor deprivation (Flores et al., 2000; Cui et al., 2005), TNFα exposure (Takano et al., 2000; Pang et al., 2007) and glutamate toxicity (Ness and Wood, 2002). Akt signalling in cells of the OL lineage has been mostly investigated downstream of IGF-1 receptor (insulin-like growth factor-1 receptor). IGF-1 induces a sustained Akt activation and provides long term protection against cell death (Ness and Wood, 2002; Zaka et al., 2005). Transgenic mice overexpressing IGF-1 display increased brain weight, OL number and myelin sheet thickness, as well as an upregulation of myelin protein gene expression (Carson et al., 1993; Ye et al., 1995). However, the specificity of the IGF-1 response is unclear. Early reports describe a hypomyelination phenotype in IGF-1 knockout mice (Beck et al., 1995). However, follow-up studies suggested an indirect effect on OLs secondary to loss of projection neurons, as IGF-1 knockout mice display normal myelination in areas where the axon tracts are normal (Cheng et al., 1998). These results are in concordance with a case report of a boy bearing a homozygous IGF-1 deletion that displayed normal myelination (Woods et al., 1996). Signaling through the IGF1R receptor has been shown to play a role in remyelination following a cuprizone demyelinating challenge (Mason et al., 2003). Thus, the physiological role of IGF-1 in OLs development and myelination is unclear, and it may be involved more in remyelination events than in initial myelination.

Whereas the Akt pathway seems to be central to OPC and OL survival in culture systems (Ebner et al., 2000; Baron et al., 2003; Jaillard et al., 2005), the expression of constitutively active Akt in OLs using a PLP promoter did not increase the number of OLs *in vivo* (Flores et al., 2008). Rather, overexpression of constitutively active Akt caused hypermyelination in the CNS that persisted through adulthood. This hypermyelination was found to be mediated by mTor activation (mammalian target of rapamycin), as it was prevented by administration of rapamycin (Narayanan et al., 2009). mTor is a serine/threonine kinase that can be activated downstream of Akt and that mediates protein synthesis. mTor activation of its downstream targets S6K1 and 4E-BP seems to be central to the OL differentiation process, mediating differentiation from the precursor stage (A2B5+) to immature OL stage (GalC+) (Tyler et al., 2009). The mTor pathway was also shown to mediate IGF-1 induced protein synthesis and OL development *in vitro* (Bibollet-Bahena and Almazan, 2009). Thus, Akt activation in OLs might have a survival effect *in vitro* or in the context of survival following injury, but its primary action *in vivo* seems to be on differentiation and myelin formation.

Consistent with a predominant role of the PI3K/Akt pathway in morphological differentiation of OLs and myelin wrapping, PTEN inactivation in OLs caused a hypermyelination phenotype similar to what was seen in the Akt overexpression model (Flores et al., 2008; Goebbels et al., 2010). Thus, a sensitive balance between PI3K and PTEN, the brake turning off PI3K signalling, seems to be necessary for normal myelination and myelin thickness. Knocking-out PTEN in the nervous system or specifically in OLs produced hypermyelination and myelin compaction abnormalities (Fraser et al., 2008; Goebbels et al., 2010). Interestingly, transgenically removing PTEN expression in OLs later in development using an inducible conditional knockout also caused hypermyelination and increased myelin thickness (Goebbels et al., 2010), which indicates that the signalling pathways governing myelination during development

are still active later in life. However, following a demyelination insult to the CNS, PTEN inactivation did not increase remyelination efficiency (Harrington et al., 2010).

MAPK/ERK pathway

The mitogen-activated protein kinases (MAPK) are serine/threonine kinases that are activated downstream of multiple types of receptors. The kinase cascade comprised of Ras, Raf, and MEK, in turn phosphorylates and activates MAPKs. Conditional ablation of B-Raf, a Raf kinase family member, in neuronal precursors highlighted the importance of the MAPK pathway in OL development, as these mice had impaired OL differentiation and substantial hypomyelination. In addition, in cells of the OL lineage, B-Raf is the critical upstream activator of ERK1/2 (Galabova-Kovacs et al., 2008).

Multiple components of the MAPK pathway have been implicated in OL differentiation. p38 MAPK is essential for morphological maturation of OLs, expression of stage specific myelin markers (Bhat et al., 2007) and myelination in a co-culture system (Fragoso et al., 2007). cAMP responsive element (CREB) was shown to be activated by p38 in the regulation of differentiation (Bhat et al., 2007). More recently, the MAPK-activated protein kinase 2 (MK2) was shown to be a critical effector downstream of p38 for the regulation of OL differentiation (Haines et al., 2010).

ERK1 and ERK2 (p42 and p44 MAPK respectively) are solely activated by MEK MAPK. They are thought to compensate for one another; however, while ERK1 knockout mice have no overt phenotype (Adams and Sweatt, 2002), ERK2 knockout mice develop numerous deformities and are embryonic lethal (Satoh et al., 2007). ERK1/2 activation was shown to mediate differentiation of OLs from neural stem cells in response to NT-3 (Hu et al., 2004) and PDGF-AA (Hu et al., 2008). MAPK activation is also critical for differentiation in response to brain-derived neurotrophic factor (BNDF)(Du et al., 2006; Van't Veer et al., 2009). ERK1/2 was shown to be activated downstream of IGF-1 and to promote

proliferation of OPCs. Interestingly, this activation is dependent on PI3K and Src-like tyrosine kinase activation, in addition to MEK1 activation, raising the possibility of the convergence of multiple signalling pathway onto a common effector (Cui and Almazan, 2007). MAPKs thus seem to play a major role in proliferation, survival, and differentiation of OLs. In adult myelin, the MAPK pathaway is also linked to myelin maintenance and stability. For example, MAPK phosphorylation confers stability to MBP and this phosphorylation was altered in cases of the demyelinating disease multiple sclerosis (Yon et al., 1996).

Small Rho GTPases

The Rho family of small GTPases consists of 3 subfamilies: Rho, Rac and Cdc42. They have emerged in recent years as key regulatory molecules linking membrane receptors to the actin cytoskeleton. Current models propose antagonistic action between Rho and Cdc42/Rac1 in the regulation of process extension in several different cell types, with Rho inhibiting and Cdc42/Rac1 promoting process extension (reviewed in Ridley, 2006). Their central role in the regulation of cell morphology makes them appealing candidates for the regulation of differentiation and myelination in OLs.

As one might predict, expression of dominant negative Rho increased process extension in OLs (Wolf, 2001), whereas expression of dominant negative Rac1 or Cdc42 inhibited differentiation and process extension *in vitro* (Liang, 2004). However, conditional deletion of Rac1 or Cdc42 from OLs *in vivo* appears to impair myelin compaction only, with no effect on differentiation or initiation of myelination (Thurnherr et al., 2006). Actin remodelling molecules downstream of Rac1 and Cdc42 have also been linked to OL morphological maturation. Neural Wiskott-Aldrich syndrome protein (N-WASP) activation leads to process extension in OLs (Bacon et al., 2007), while knockout mice for WASP family Verprolin-homologous protein 1 (WAVE1) are hypomyelinated (Kim et al., 2006).

Membrane specialization in OLs is crucial for differentiation into myelinating OLs. Neuronal signals were shown to trigger redistribution of PLP-containing membranes from late endosomes and lysosomes to the cell membrane (Trajkovic et al., 2006). This process was shown to be regulated by Rho activity, and the authors suggest that development of membrane specialization and myelin in OLs is dependent on Rho inactivation (Kippert et al., 2007). Furthermore, RhoA activity was shown to decrease as differentiation was triggered in an oligodendrocyte cell line, which is consistent with previous results suggesting the requirement for inactivation of Rho for differentiation to proceed (Liang et al., 2004). This is particularly relevant in a disease context, where inhibition of the differentiation of OPCs is thought to contribute to the failure of remyelination in demyelinated lesions (section I.3.2). Downstream of Rho, Rho kinase (ROCK) and its target myosin II have also been implicated in myelination. The activation levels of both ROCK and myosin II are reduced during myelination, and inhibition of myosin II leads to increased branching and myelin formation (Wang et al., 2008). Several factors were shown to act through Rho GTPases in OLs. Laminin-2, a component of the extracellular matrix that promotes OL process outgrowth and myelin formation, exerts its effect through the activation of Cdc42/Rac1 and deactivation of RhoA downstream of its receptor integrin α6β1 (Liang et al., 2004). In this case, signalling to the GTPases is dependent on the kinase activity of fyn, a member of the Src family kinases.

Another cue that acts on RhoGTPases in OL is netrin-1. As mentioned in section I.1, netrin-1 acts as a chemorepellent for OPCs and thus triggers process retraction (Jarjour et al., 2003; Tsai et al., 2003). This process retraction was shown to be dependent on RhoA and ROCK activation (Rajasekharan et al., 2010). In differentiated OLs grown *in vitro*, netrin-1 induced morphological differentiation and process elaboration (Rajasekharan et al., 2009). This effect was mediated by the netrin-1 mediated recruitment and activation of fyn and concomitant reduction of RhoA activity (Rajasekharan et al., 2010). Thus, distinct effects of netrin-1 along OLs differentiation path were shown to rely on a molecular switch resulting

in differential regulation of RhoA activity. This switch could be caused by different receptor expression on OLs, as UNC5 homologs expression changes throughout development (Manitt et al., 2004). Alternatively, the switch could result from increased association of fyn with DCC as the OL mature, which could decrease RhoA activation through its action on p190RhoGAP (Wolf et al., 2001).

Other signalling partners and pathways

Fyn kinase activity is upregulated early during OL differentiation and morphological maturation is impared when fyn function is inhibited (Osterhout et al., 1999). *In vitro*, IGF-1 (Sperber and McMorris, 2001) and netrin-1 (Rajasekharan et al., 2009) failed to stimulate morphological maturation in fyn knock out OLs. Fyn is thus required for the activation of multiple signalling pathways in OLs. Another signalling pathway, the Notch pathway, was shown to inhibit OL differentiation during development (Wang et al., 1998; Genoud et al., 2002), but more recent studies disagree on the importance of Notch in OPC differentiation in development and following demyelination insult (Fancy et al., 2010). More recently, the Wnt/β –catenin pathway was shown to contribute to the timing of myelination (Fancy et al., 2009; Feigenson et al., 2009) and inhibiting this pathway blocked expression of several myelin genes, such as PLP, in OLs (Tawk et al., 2011).

I.2.2 Myelin architecture

Nerve impulses in myelinated fibres jump between each myelin segment from one node of Ranvier to another, allowing for rapid saltatory conduction. This is based on the complex domain architecture along the myelinated fiber. The internode is the area of the axon surrounded by compact myelin, and its length depends on axon caliber. Compact myelin is formed of multiple wraps of compacted membrane bilayers. When observed through an electron microscope, one can distinguish myelin as alternating dark and light lines. The major dense line, or dark line, corresponds to the intracellular membrane portion that is brought in close apposition through proteins like MBP and the exclusion of cytoplasm. The

intraperiodic line corresponds to the extracellular portion of myelin. When an OL wraps its membrane around an axon, and cytoplasm is excluded from the myelin, cytoplasm-filled channels still remain on each side of the compacted membrane. These channels – or loops – contact the axons at the end of each myelin section. The loops are called paranodes, because they are located each side of the unmyelinated portion of the axons, the nodes of Ranvier (Baumann and Pham-Dinh, 2001). As a consequence of this complex architecture, myelin structure is highly organized around different domains: the node, the paranode and the juxtaparanode (Figure 1-4).

Nodes of Ranvier

Interaction complexes at the node are different between the CNS and the PNS. In the PNS, nodes are contacted by the Schwann cells' microvilli, whereas CNS nodes are surrounded by perinodal astrocytes, an NG2 positive astrocytes subtype (Poliak and Peles, 2003). Nodes contain high densities of voltage gated sodium (Na^+) channels, essential for the rapid depolarization required for saltatory conduction. These channels form a complex with the neuronal 186 kDa isoform of neurofascin (NF186) through a direct interaction and with the neural-glial-related cell adhesion molecule (NrCAM) indirectly (Davis et al., 1996; McEwen and Isom, 2004). NF186 is essential for the formation of Na^+ channel complexes, but NrCAM is dispensable (Sherman et al., 2005). AnkyrinG, an adaptor protein linking transmembrane proteins to spectrins and the actin cytoskeleton, is the first protein to appear at the node. It is thought to recruit the Na^+ channel complex components and to provide a link to the cytoskeleton (Kordeli et al., 1995; Jenkins and Bennett, 2002; Dzhashiashvili et al., 2007), along with βIV spectrin (Berghs et al., 2000).

In the PNS, microvilli of Schwann cells express gliomedin, a transmembrane protein that associates with NrCAM and NF186 and mediates the assembly of the nodal complex (Eshed et al., 2005). In contrast, work on retinal ganglion cells *in vitro* showed that a diffusible factor secreted by OLs is responsible for Na^+ channel clustering (Kaplan et al., 1997) and formation of paranodal junctions

(Susuki and Rasband, 2008) in the CNS. It thus appears that the mechanisms of node assembly differ between the PNS and CNS.

Paranode

The paranode is located on each side of the node of Ranvier, where the compact myelin membranes terminate in cytoplasm-filled loops that contact the axolemma (Figure 1-4). The paranode is believed to act as a barrier against the diffusion of protein between the node and the juxtaparanode, and it electrically isolates the myelinated from the unmyelinated portions of the axon (Pedraza et al., 2001). The paranode is also considered to act as a path for the trafficking of protein and for communication between the axon and the glial cell (Trapp and Kidd, 2000). The points of contact between the oligodendroglial loops and the axolemma are characterized ultrastructurally by the presence of transverse bands (TBs) (Schnapp et al., 1976). In the last decade, multiple proteins located at the paranode were characterized, but whether or not these proteins form the core of the TBs or are only required for the assembly of TBs is still under debate.

The glial loops of the paranode contain the glial 155 kDa isoform of neurofascin (NF155), which is necessary for the recruitment of the axonal complex partner, formed by Caspr and contactin, to the paranodal domain (Tait et al., 2000; Sherman et al., 2005). NF155 can be differently glycosylated, and the most glycosylated form was shown to be required for paranodal integrity (Pomicter et al., 2010). On the axonal side of the paranode, contactin, a GPI-linked protein, forms a complex in cis with Caspr (Peles et al., 1997), and its presence is necessary for the expression of the complex at the paranode (Faivre-Sarrailh et al., 2000). NF155 was shown to bind to Caspr and contactin on the axonal membrane, and this complex is thought to be the molecular basis of the formation of axoglial junction (Charles et al., 2002b). However, some evidence contradicts this tripartite complex model. Gollan et al. (2003) demonstrated through binding studies that Caspr inhibits NF155 binding to contactin. Nonetheless, these three proteins have been shown to play a pivotal role in paranode organization. NF155 function is dependent on its immunoglobulin domains 5 and 6 (Thaxton et al.,

2010), and NF155 mutants show motor defects, paranodal disorganization and early lethality (Sherman et al., 2005; Pillai et al., 2009). Contactin mutants exhibit neurological defects, absence of TBs and die by P18. Caspr is absent from paranodes in this mutant, demonstrating the need for contactin for proper transport of the Caspr/contactin complex to the paranode (Boyle et al., 2001). Caspr mutants exhibit paranodal abnormalities, irregular TBs and have a relatively longer life span than the contactin mutant (Bhat et al., 2001). Notably, severity of the phenotype of paranodal mutants is related to the integrity of TBs (Mierzwa et al., 2010b) suggesting a crucial role for these adhesive complexes in the function of myelinated fibers. Interaction with the axonal cytoskeleton is essential to the domain organization of paranodes, and the intracellular portion of Caspr acts as a scaffold that mediates this interaction (Gollan et al., 2002). Protein 4.1B acts as a linker between Caspr and the actin cytoskeleton (Ohara et al., 2000; Denisenko-Nehrbass et al., 2003). Other cytoskeletal components were identified at the paranode, like ankyrinB, αII spectrin and βII spectrin (Garcia-Fresco, 2006; Ogawa et al., 2006), but their exact contributions to the stabilization of paranodal junctions are still unclear.

Lipid composition and organization of OL membranes were also shown to be critical for the maintenance of paranodal organization. Myelin galactolipids, galactocerebroside (GalC) and sulfatide, are essential for the establishment and maintenance of functional axoglial adhesive complexes (Dupree et al., 1998; Marcus and Popko, 2002). Mutating the enzyme responsible for the formation of both GalC and sulfatide (CGT mutant) led to myelin abnormalities and the absence of TBs (Dupree et al., 1998). In contrast, mice lacking only sulfatide (CST mutant) develop normally, but exhibit myelin and paranode maintenance defects as they age (Ishibashi et al., 2002; Marcus et al., 2006). Abnormal galactolipid composition has been shown to inhibit NF155 partitioning into lipid rafts (Schafer et al., 2004) and to alter expression of cytoskeletal proteins in OLs (Fewou et al., 2010). This highlights the highly complex interplay between transmembrane proteins, molecular bridges to the cytoskeleton and membrane

microdomains in the establishment of functional axoglial junctions and myelin domains.

A common interpretation of paranodes' function is that it serves as a tight seal preventing short circuiting and loss of power of travelling impulses. However, a recent study demonstrated that molecules can reach the space between the axon and the myelin sheath at the juxtaparanodes and the internodes by travelling through the paranodes. This transport channel is believed to lie between the glial loops (Mierzwa et al., 2010a). Thus, paranodes are believed to serve different functions. First, they provide electrical isolation while leaving a small communication channel to transport small molecules and metabolites to the internode. Second, it acts as a barrier against the diffusion of proteins and ions channels along the different domains of the axon. Third, it maintains the nodal domain to ensure proper conduction from node to node (Rosenbluth, 2009).

Juxtaparanode

The juxtaparanodes are located next to the paranodes, under the compact myelin sheet. They are characterized by clusters of voltage-gated potassium (K^+) channels (Wang et al., 1993), but also contain adhesive molecules such as Tag1 (Traka et al., 2002) and Caspr2 (Poliak et al., 1999) that are required for the clustering of channels at the juxtaparanode (Traka et al., 2003). Tag1 is expressed on both the OL and axonal plasma membrane; however, only glial Tag-1 is required for the assembly of the K^+ channel complex at the juxtaparanode (Savvaki et al., 2010). Caspr2 is a scaffold protein that mediates the K^+ channel complex assembly through its intracellular interaction with protein 4.1B (Horresh et al., 2010). The role of K^+ channels under the myelin sheath at the juxtaparanode is not fully understood, but some propose that they serve to maintain the internodal resting potential and act as a mediator of axoglial communication (Poliak and Peles, 2003).

Compact myelin composition

Myelin is composed primarily of lipids, in particular the galactolipids GalC and sulfatide, and cholesterol. Compact myelin also contains several proteins, of which PLP/DM20 and MBP are the most abundant in the CNS.

Proteolipid Protein (PLP)/DM20

PLP is the most abundant protein in CNS myelin, while its expression in PNS myelin is minimal. The PLP gene is highly conserved and is alternatively spliced. It encodes both PLP and its shorter isoform DM20, which is expressed early in development (Wight and Dobretsova, 2004). Both proteins consist of four transmembrane domains. PLP transport to the cell surface is controlled by a secreted neuronal signal, which activates a cAMP- and RhoGTPase-dependent signalling pathway that leads to the redistribution of PLP from late endosomes/lysosomes to the cell surface (Trajkovic et al., 2006; Kippert et al., 2007). Because of its molecular structure, its location in compact myelin and its abundance, it was previously believed that PLP mediated adhesion and compaction of extracellular membranes. However, mutant mice that lack the expression of PLP/DM20 do not exhibit any myelin formation defects, and mice appear phenotypically normal until one year of age. A destabilization of myelin in older mutant mice was observed (Klugmann et al., 1997), followed by axon degeneration (Griffiths et al., 1998). This suggests a role of PLP in the maintenance of myelin integrity rather than myelin formation and membrane compaction.

Myelin Basic Protein (MBP)

MBP is the second most abundant myelin protein, but it is considered the most important one. Moreover, it is the only protein essential for formation of myelin in the CNS. The shiverer mutant mice carry a naturally-occurring deletion of part of the MBP gene. Although OLs wrap their membrane around axons in this mutant, there is no compact myelin, leading to severe neurological deficits and death by 3 months of age (Readhead et al., 1987; Readhead and Hood, 1990). MBP is an intracellular protein bound to the OL membrane through electrostatic interactions,

and it mediates intracellular membrane reorganization and adhesion, and myelin compaction (Fitzner et al., 2006). Several isoforms are formed by alternative splicing, and their expression is developmentally regulated (Boggs, 2006). Interestingly, MBP is not required for myelin formation in the PNS, and it is thought that other proteins expressed in PNS myelin, such as PMP22 and P0, compensate for the absence of MBP in these mutants (Martini et al., 1995; Boggs, 2006). Local translation of MBP occurs via assembly of MBP mRNA in RNA granules, which include proteins of the translation machinery and transport of these granules via the vesicular transport pathways (Colman et al., 1982; Barbarese et al., 1995; Baron and Hoekstra, 2010). In addition to ensuring that membrane compaction does not occur in domains other than myelin, this allows for spatiotemporal control of MBP translation through the activation of signals such as integrin or fyn signalling (White et al., 2008; Laursen et al., 2011).

Non-compact myelin composition

Non-compact myelin includes the paranodal loops, the innermost layer of OL membrane contacting the axon (also named adaxonal membrane), and the outermost layer of OL membrane. Pockets of uncompacted myelin along the internodes, named the Schmidt-Lanterman incisures, are common in the PNS, but are rarely observed in the CNS (Baumann and Pham-Dinh, 2001). In addition to the paranodal and juxtaparanodal proteins expressed by OLs (described above), several other proteins are specially localized to those non-compact myelin domains.

Myelin associated glycoprotein (MAG)

MAG is a transmembrane protein of the Ig superfamily, and has an apparent molecular weight of 100 kDa, of which 30% is due to its heavy glycosylation (Baumann and Pham-Dinh, 2001). MAG is expressed predominantly on the adaxonal OL membrane, facing the axonal membrane, but is also present in other non-compact compartments. MAG axonal receptors include gangliosides GD1a and GT1b, and the Nogo receptor NgR1 (Schnaar and Lopez, 2009). Mice deficient for MAG exhibit normal myelin formation, but develop axon and myelin

degeneration in late adulthood, suggesting a role of MAG in the maintenance of myelinated fibers (Li et al., 1994; Fruttiger et al., 1995). Interestingly, MAG was shown to be critical for axonal neurofilament phosphorylation, central to the phenomenon of myelination-induced axonal expansion (Yin et al., 1998). Thus, MAG could exert its functions through the maintenance of the axonal cytoskeleton and the preservation of efficient axonal transport in myelinated fibers (Sousa and Bhat, 2007).

2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP)

CNP is an abundant myelin protein and is localized specifically in the cytoplasm of non-compact regions such as inner mesaxon and paranodes (Braun et al., 1988; Trapp et al., 1988). There are two isoforms of CNP resulting from distinct translation start sites (Monoh et al., 1989). The precise function of CNP is still elusive, as *in vitro* and *in vivo* results are hard to reconcile. *In vitro*, CNP was shown to mediate process outgrowth in OLs (Lee et al., 2005) and was able to induce microtubule polymerization (Bifulco et al., 2002). Despite its abundance and function *in vitro*, CNP knockout mice show no myelination defects (Lappe-Siefke et al., 2003). However, paranodes start to become disorganized at 3-4 months of age (Rasband et al., 2005), followed by axonal swelling, degeneration, and premature death (Lappe-Siefke et al., 2003), implying a role for CNP in glial support of axonal integrity. Interestingly, a protein microarray identified CNP as a binding partner of the intracellular domain of Nogo-A in OLs, suggesting a role for CNP in stabilizing myelin transmembrane receptors (Sumiyoshi, 2010).

Oligodendrocyte myelin glycoprotein (OMgp)

Oligodendrocyte myelin glycoprotein (OMgp) is a glycosylphosphotidylinositol (GPI)-anchored protein expressed both in neurons and OLs in the mature CNS. Its precise localization and role in myelin are still under debate. Early reports of OMgp mutants described a role for OMgp in nodal integrity, either by restricting axonal sprouting at the node (Huang et al., 2005) or by controlling nodal length and structure (Nie et al., 2006). However, more recent studies disagree with these results. Chang et al. (2010) demonstrated the absence of OMgp immunostaining at

nodes as previously reported (Huang et al., 2005; Nie et al., 2006), and found no effect of OMgp absence on nodes, paranodes or myelin thickness in optic nerve. Another group recently showed decreased myelin thickness in spinal cord of OMgp mutants (Lee et al., 2011). Clearly, more evidence is needed to draw a firm conclusion on the role of OMgp in OL biology and myelin formation.

I.3 MYELIN-ASSOCIATED CNS PATHOLOGIES

I.3.1 Normal and pathological aging

Aging is associated with a decline in cognitive performance, which can be exacerbated by pathological conditions such as Alzheimer's disease (AD) or other types of dementias. Previous models have focused on neuronal loss with aging and its effect on cognitive performance. However, a magnetic resonance imaging (MRI) study described a decrease in white matter volume with age, while the grey matter areas were unchanged (Guttmann et al., 1998). In fact, imaging studies and histopathological observations have led several groups to link the integrity of white matter tracts to age-related cognitive decline (Hinman and Abraham, 2007). With aging, myelin integrity is compromised, and loss of myelin correlates with age in humans (Svennerholm et al., 1994), and cognitive decline in aged monkeys (Luebke et al., 2010). Ultrastructural defects of myelin, such as decompaction or redundant myelination, have been described in myelinated fibers of aged monkeys (Sloane et al., 2003; Peters, 2009). Paranodal junctions undergo substantial reorganization with aging. Paranodal loops detach from the axon, and Caspr and K^+ channels expression profiles are abnormal in paranodes of aged monkeys and rats (Sugiyama et al., 2002; Hinman et al., 2006). More specifically, this reorganization of paranodes leads to the disruption of TBs (Shepherd et al., 2010), whose integrity is related to the severity of the phenotypes observed in transgenic mice models (Mierzwa et al., 2010b).

Pathological conditions developed in aged individuals such as AD have also been linked to myelin function. The myelin model of AD states that AD results from the failure of the brain to maintain and repair its myelin with age (Bartzokis, 2009). Evidence in favour of this model is at the most circumstantial. However, several reports have described white matter abnormalities in patients with AD, contributing to cognitive impairment (Roth et al., 2005; Heo et al., 2009; Agosta et al., 2011). AD is characterized by accumulation of β-amyloid, forming

extracellular plaques toxic both for neurons and OLs (Roth et al., 2005). Interestingly, MBP binds to β-amyloid and promotes its degradation (Liao et al., 2009), which raises the possibility that myelin breakdown could potentiate the assembly of toxic β-amyloid plaques and accelerate the AD course.

Altogether, these recent findings shake preconceptions about cognitive impairment, which was thought to be the result of neuronal loss, and bring to light the possibility that a primary defect in myelin maintenance and integrity can affect cognitive performance. Future research in this area will possibly identify new targets for future studies on age related and pathological cognitive decline.

I.3.2 Multiple Sclerosis

The most prevalent demyelinating disease, multiple sclerosis (MS), is characterized by inflammation and degeneration of myelin in the CNS, and ultimately a failure of remyelination (Prat and Antel, 2005). The most common type of MS is the relapsing-remitting form, characterized by the appearance of symptoms, followed by total remission. In the majority of cases, a secondary progressive form of MS develops, where symptoms worsen in between attacks, and this form of MS is sometimes referred to as chronic MS (Reynolds et al., 2011). The cause leading to the development of MS remains unknown. While several studies suggest that the primary event is a dysregulation of the immune system (McFarland and Martin, 2007), others argue that a primary defect in myelin stability causes an exposure of antigenic material that subsequently triggers an immune response (Mastronardi and Moscarello, 2005).

Remyelination failure

The fact that MS patients can recover from their relapse symptoms, along with the presence of shadow plaques, was an early indication that remyelination could take place in MS (Prineas et al., 1984; Prineas et al., 1993). Remyelination can lead to functional recovery, and protection of axons from transection and subsequent

neurodegeneration (Trapp et al., 1988). However, most studies indicate that remyelination does occur at early stages of the disease, but after a certain point in the course of the disease, remyelination fails, which can ultimately cause neuronal death (Lassmann et al., 1997). As immunomodulatory therapeutic agents are becoming more effective at preventing relapses in MS patients, the secondary progressive course caused by the death of chronically demyelinated neurons is still hardly treatable. Finding ways to promote endogenous myelination is thus a focus of MS research.

To achieve successful remyelination, OPCs need to migrate to the lesion, differentiate, and remyelinate the axons. Several factors expressed in myelin could be present in MS plaques and could act as repellent for migratory OPCs, such as netrin-1 or semaphorins (Manitt et al., 2001; Williams et al., 2007). Nevertheless, absence of remyelination in chronic MS is not caused by a lack of precursors, since OPCs are found within chronic lesions (Chang et al., 2002; Wilson et al., 2006), but rather lack of remyelination seems to be due to blocked differentiation of OPCs (Kuhlmann et al., 2008). MS plaques thus seem to constitute an inhibitory environment for OL differentiation. Inhibition of differentiation can occur through exposure of OPCs to myelin debris (Baer et al., 2009), LINGO-1 (Mi et al., 2005) or PSA-NCAM re-expression on demyelinated axons (Charles et al., 2002a). Evidence points to the convergence of inhibition signals through Rho activation in OPCs. *In vitro*, pharmacological inhibition of RhoA, ROCK or PKC signalling was able to overcome the halt in differentiation provoked by myelin debris (Baer et al., 2009). In further agreement of Rho inactivation in OLs differentiation, inhibition of myelination by LINGO-1 has been linked to its ability to activate RhoA (Mi et al., 2005). Other pathways, such as the Wnt/β-catenin pathway, were also shown to regulate OPCs differentiation following demyelination (Fancy et al., 2009). The causes and mechanisms of OL differentiation block in MS are still being investigated, and therapeutic interventions that aim to improve endogenous remyelination are key strategies both to alleviate the symptoms and to promote the recovery of MS patients.

Paranodal dysfunction in MS

Some models of MS implicate an initial disruption of myelin stability as the event that triggers the disease process. This instability, which can be caused by an imbalance of myelin protein expression, could lead to decompaction of myelin and exposure of antigenic material (Mastronardi and Moscarello, 2005). In addition to changes in compact myelin, paranodal abnormalities have also been observed in MS patients. Expression and localization of Caspr is altered both in chronic lesions and in normal appearing white matter, raising the possibility that paranodal dysfunction could precede the appearance of a demyelinating plaque (Wolswijk and Balesar, 2003). NF155 expression has also been found to be altered in and near demyelinated MS lesions, causing juxtaparanodal K^+ channels to migrate into the paranodal regions (Howell et al., 2006). More specifically, the glycosylated form of NF155, termed NF155 high, is decreased in MS (Pomicter et al., 2010). These findings suggest that paranodal disorganization may precede demyelination in MS, and investigating the consequences of this alteration in paranodal integrity is an important step in identifying the mechanisms that lead to demyelination events in MS.

I.3.3 Spinal cord injury

The inherent inhibitory nature of the adult CNS is apparent after spinal cord injury, where severed axons fail to regenerate. In comparison, severed PNS axons are capable of regrowth and reinnervation of peripheral targets after peripheral nerve transection. This distinction between the PNS and CNS injury environment was highlighted in the early 1980s, in studies showing that spinal cord axons can regenerate into peripheral nerve grafts after injury (David and Aguayo, 1981). The major source of inhibition in the CNS results from the breakdown of myelin, and proteins expressed by OLs were shown to mediate this inhibition (Caroni and Schwab, 1988).

Three main myelin-derived inhibitors were identified as having an effect on the failure of neurite regeneration: Nogo, MAG, and OMgp (Xie and Zheng, 2008). These three inhibitors exert their effect by binding to a common receptor, the Nogo Receptor 1 (NgR1), which forms a complex with co-receptors p75NTR, TROY, and LINGO-1 (Yiu and He, 2006). Evidence suggests that activation of RhoA and its effector ROCK, downstream of this receptor complex, mediates the inhibitory effect on axonal growth (Niederost et al., 2002; McGee and Strittmatter, 2003). This signaling pathway has been a target for the development of therapeutic agents to enhance regeneration (Dergham et al., 2002; Walmsley and Mir, 2007). However, the absence of these three inhibitors does not produce strong regeneration following injury, as there is little to no effect on regeneration in single Nogo, MAG, or OMgp knockout mice, or triple knockout mice (Bartsch et al., 1995; Lee et al., 2010). Moreover, NgR1 knockout mice also exhibit a limited effect on neurite outgrowth and do not show long distance regeneration after injury (Kim et al., 2004; Zheng et al., 2005). This suggests that more inhibitors are present within the lesion, and that they act on other receptors on regenerating axons. Of note, OLs also express several repulsive guidance molecules, which could repel growing axons and prevent outgrowth. Such factors include ephrin-B3, sema4D, sema5A, RGM, and netrin-1 (Xie and Zheng, 2008). In the injured nervous system, netrin-1 expression increases in mouse spinal cord and cerebellum (Wehrle et al., 2005), whereas expression in the injured rat spinal cord was shown to decrease (Manitt et al., 2006). When present at lesion sites, netrin-1 acts as a myelin-associated growth inhibitor and can impede axonal regeneration (Low et al., 2008). Moreover, this inhibitory effect of netrin-1 on regenerating axons is specific for UNC5-expressing axons (Low et al., 2008).

Some myelin proteins acting as inhibitors are specific to OLs, and are not expressed by Schwann cells, like Nogo, which provide a partial explanation as to why the CNS environment may be functionally different (Xie and Zheng, 2008). Another difference is the immune system response and the clearing of myelin

debris after injury, which is substantially more efficient in the PNS than in the CNS (Hirata and Kawabuchi, 2002).

In addition to myelin-derived inhibitors, glial scar formation at the site of injury can also be a source of inhibitory molecules and is believed to be another barrier to regeneration in the CNS. The scar is composed mostly of reactive astrocytes, which secrete chondroitin sulphate proteoglycans (CSPGs), an extracellular matrix component that inhibits neurite outgrowth (McKeon et al., 1999). Treatment of the injury with chondroitinase ABC, which degrades CSPGs, leads to improvement of axonal regeneration and functional recovery (Moon et al., 2001; Bradbury et al., 2002). Fibroblasts within the glial scar also express semaphorin class 3, which repels regenerating growth cones (De Winter et al., 2002). However, a growing body of evidence tends to demonstrate that glial scar formation is beneficial in mediating different aspects of CNS repair after injury, such as toxicity buffering, immune modulation and promotion of angiogenesis (Rolls et al., 2009). Deciphering both the myelin-associated proteins and the glial scar-derived molecules involved in promoting and inhibiting regeneration could thus help in identifying specific targets for therapeutic intervention after spinal cord injury.

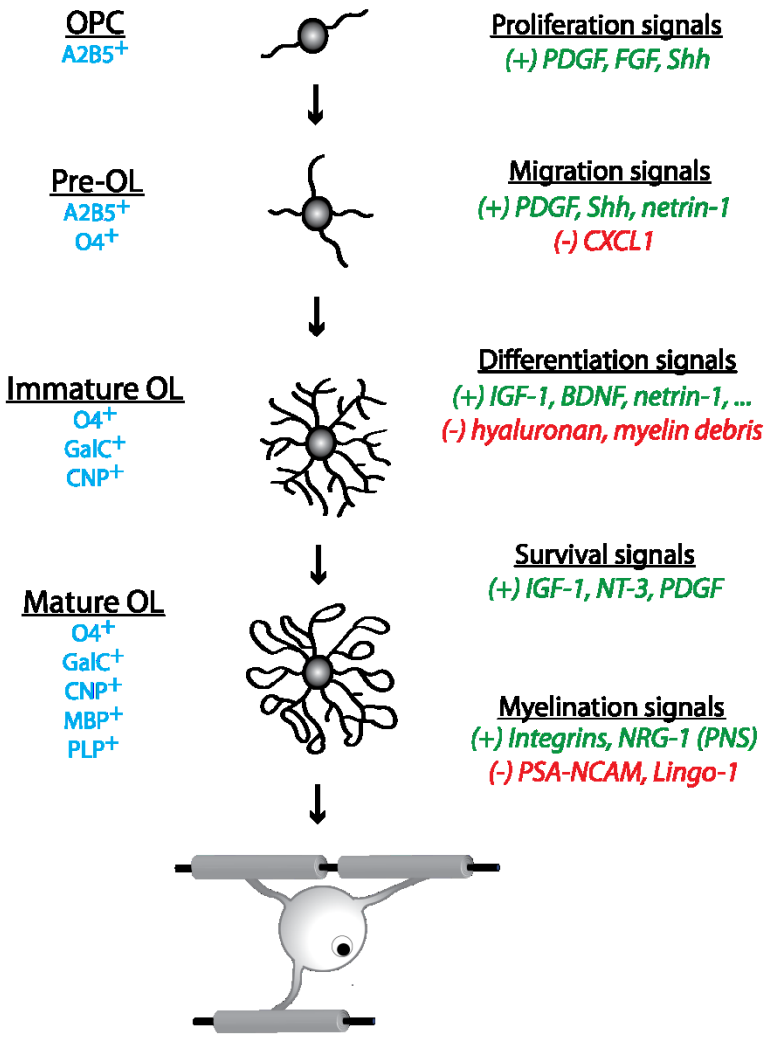


FIGURE 1-3: The life of an oligodendrocyte. OLs develop from bipolar and highly motile OPCs, which follow an ordered path of differentiation characterized by the expression of different markers (in blue). Cells are influenced by different signals, both positive (green) and negative (red).

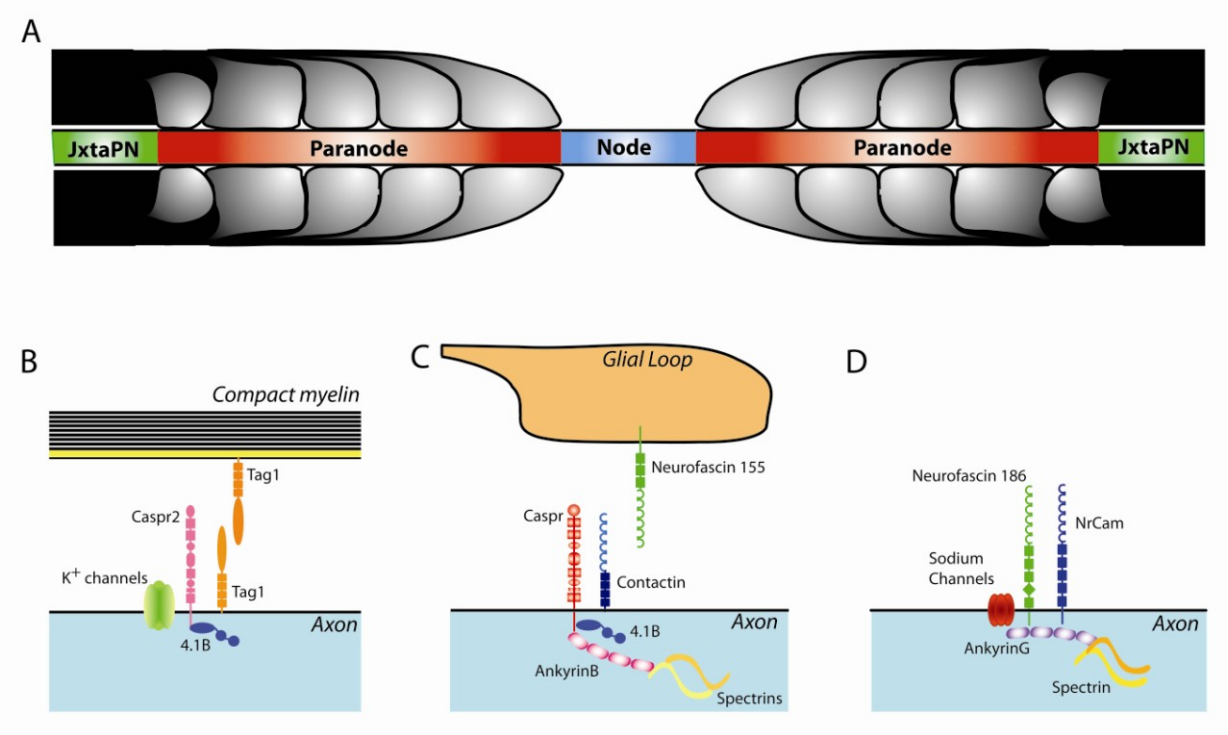


FIGURE 1-4: Domain segregation and organization around the Node of Ranvier. A) The compact myelin sheath terminates in cytoplasm-filled loops around the node of Ranvier. This leads to the formation of the paranode domain. The juxtaparanodes are located under the compact myelin sheaths adjacent to the paranodes. B) Juxtaparanodes contain K^+ channel complexes formed by Caspr2 and Tag1 proteins. C) A glial form of neurofascin NF155 on the paranodal loop membrane interacts with the the Caspr and Contactin complex on the axonal membrane. The Caspr intracellular domain interacts with the actin cytoskeleton through protein 4.1B, AnkyrinB and spectrins. D) Sodium channels at the nodes complex with the neuronal form of neurofascin NF186 and NrCam, which links the sodium channel clusters with the cytoskeleton through AnkyrinG and spectrins.

RESEARCH RATIONALE AND OBJECTIVES

As described in chapter I, myelin formation and maintenance result from the complex interplay of extracellular signals and the signalling pathways activated. During the course of my PhD, a body of literature emerged describing a preponderant role of Akt and mTor in OL development and myelin formation (Narayanan et al., 2009; Tyler et al., 2009). We hypothesized that screening for molecules that could activate Akt in OLs could provide insight into the role of this signalling pathway in OL maturation. This is addressed in chapter II.

Previous work carried on in the lab described a role for DCC and netrin-1 in paranodal maintenance in organotypic cerebellar slice cultures *in vitro* (Jarjour et al., 2008). However, the cellular location where DCC is required to function at paranodes still had to be determined. In addition, we did not know whether the abnormal phenotype observed *in vitro* also occurs *in vivo*. These questions are addressed in chapter III, using intraretinal OPCs injection and a conditional knock out strategy.

Finally, our lab and others provided evidence that netrin-1 is a myelin-associated inhibitor of axon growth after injury (Wehrle et al., 2005; Manitt et al., 2006; Low et al., 2008). However, conflicting results were reported, depending on the study. To resolve this issue, and to test whether oligodendroglial netrin-1 was the only source of netrin-1 at sites of injury in the CNS, we examined netrin-1 gene activation in a model of spinal cord injury. This study is presented in chapter IV.

CHAPTER II
NT-3, BNDF, NGF AND NRG1 SYNERGIZE VIA AKT/MTOR PATHWAY TO REGULATE OLIGODENDROCYTE MORPHOLOGICAL DIFFERENTIATION

The results presented in this chapter are part of a manuscript prepared in collaboration with members of Dr David Colman's laboratory and members of the Experimental Therapeutic Program (ETP)

PREFACE AND RATIONALE

Recent findings have identified Akt/mTor signaling as a key pathway in OL differentiation and myelin formation (Flores et al., 2008; Narayanan et al., 2009; Tyler et al., 2009). Activation of the Akt pathway, either by ablation of PTEN (Goebbels et al., 2010) or overexpression of constitutively active Akt (Flores et al., 2008) in OLs induces hypermyelination via the key downstream effector of Akt mTor, and subsequent activation of protein synthesis (Narayanan et al., 2009; Tyler et al., 2009).

The upstream activators of Akt and mTor that are responsible for promoting myelination *in vivo* are not fully characterized. Akt signalling in OLs has been mostly investigated downstream of the IGF-1 receptor (insulin-like growth factor-1 receptor). However, the physiological role of IGF-1 in OL development and myelination is unclear (see section I.2.1), and it may be involved more in remyelination events than in initial myelination. Signaling downstream of multiple factors converges on Akt, and loss of IGF-1 could thus be compensated for by other factors present in the developing CNS, such as growth factors. Nerve growth factor (NGF), brain–derived neurotrophic factor (BDNF) and neurotrophins-3 (NT-3) are neurotrophins highly expressed in the developing CNS and they bind preferentially to TrkA, TrkB and TrkC, respectively. In addition to their preferred Trk receptors, they all bind to p75 (Huang and Reichardt, 2001). Most studies agree on the expression of p75 by OLs, but the described pattern of expression of different Trks varies. Overall, expression of the three Trks has been reported in OLs (Althaus et al., 2008). Neuregulin 1 (NRG1) is also a growth factor highly enriched in the nervous system. NRG1 binds to the ErbB class of receptors that includes members ErbB2, ErbB3 and ErbB4, and can activate several signalling pathways, including the Akt pathway (Falls, 2003).

All these growth factors were examined for their capacity to promote myelination or remyelination *in vitro* and *in vivo*. Neuregulin 1 (NRG1) regulates myelination

in the PNS (Taveggia et al., 2005), but its role in OL differentiation and myelination in the CNS is unclear (Brinkmann et al., 2008). Neurotrophins have also received considerable attention as they are potent regulators of many different aspects of nervous system development (Huang and Reichardt, 2001). BDNF and NT-3 promote differentiation of OLs (Lachyankar et al., 1997; McTigue et al., 1998; Heinrich et al., 1999; Du et al., 2006; Xiao et al., 2011). NGF inhibits differentiation and myelination through axonal signals (Chan et al., 2004; Lee et al., 2007), but was also identified as a remyelination promoting factor following lysolecithin demyelination of the corpus callosum (Althaus, 2004). Extracellular factors can elicit distinct effects depending on the downstream signalling pathways they activate. In addition, convergence of signalling pathways can contribute to the different effects described in some studies. Here we aimed to identify factors that would stimulate Akt activation and increase OL differentiation.

MATERIALS AND METHODS

Animals

Sprague Dawley newborn pups were obtained from Charles River Canada (Montreal, Québec, Canada). All procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

Oli-neu cell culture

Oli-neu cells, originally generated by Dr Jacqueline Trotter (Johannes Gutenberg University, Mainz, Germany) (Jung et al., 1995), were provided by Dr. Ajit-Singh Dhaunchak (McGill University) and cultured in Sato medium containing 1% heat-inactivated horse serum. Cells were plated onto cell culture substrates coated with poly-D-lysine (PDL). Prior to treatment of oli-neu cells with different factors (see below), media was changed to DMEM alone.

Rat OPC isolation and treatments

Primary OLs were cultured as previously described (Armstrong, 1998; Jarjour et al., 2003). In brief, P0 rat pups were decapitated, neocortices removed, chopped with a razor blade, and treated with trypsin-EDTA (Invitrogen) for 20 minutes at 37°C. The tissue was then triturated with 18G and 22G syringes, and plated in T75 flasks containing 10% heat-inactivated foetal bovine serum, 1% penicillin/streptomycin in DMEM. Cultures were allowed to proliferate for 10-12 days, with the media being changed every 2 days. Microglia were removed by a 1hr shake-off at 150 rpm. OPCs were then obtained by an overnight shake off at 180 rpm, and purified by differential adhesion. Cells were plated in OL defined media (DMEM, 100ug/ml transferrin, 30nM sodium selenite, 30nM T3, 100 µg/ml penicillin/streptomycin, 2mM glutamax) onto PDL coated chamber slides for immunocytochemistry analysis and onto PDL coated 6 well dishes for biochemical analysis. When present in the media, insulin was at a concentration of 5 µg/ml.

Cells were treated with either NRG1 ectodomain (Sigma), NT-3 (Invitrogen), BDNF (Invitrogen), NGF (Invitrogen), or N1 supplement (Sigma). For all experiments with primary OLs, when used alone, NRG1, NT-3, BDNF and NGF were used at 10 ng/ml. When combined, these factors were used at a concentration of 5 ng/ml. The following inhibitors were used: Rapamycin (15 nM; Calbiochem), Wortmannin (0,5 μM; Sigma), and Pi-103 (0,5 μM; Biovision).

Luminex assay

Treated oli-neu cells were lysed using the Bio-Plex Cell lysis kit (Bio-Rad). Levels of Akt phosphorylation (pAkt) were assessed using bead based Luminex xMAP technology (Bio-Rad) described in Figure 2-1. A similar assay was run in parallel to assess total levels of Akt protein in each sample. Levels of pAkt were compared to total Akt levels in the lysate.

Western blot

Cells were rinsed with cell washing buffer (Bio-Rad) and lysed with Tris-HCl RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors. Proteins were separated by SDS-PAGE and submitted to standard western blotting using BSA as a blocking agent. Phospho-Akt was detected using a rabbit monoclonal p-Akt directed against phosphor-Ser473 (Cell Signaling). Total protein levels were assessed using a rabbit polyclonal anti-Calnexin (Biovision).

Immunocytochemistry and morphological analysis

Cells were fixed with 4% paraformaldehyde (PFA) and stained either with chicken anti-MBP (AvesLabs) or mouse anti-PLP (provided by Dr. David Colman, McGill University), and with phalloidin-488 (Invitrogen) to visualize actin. Images were captured with a Magnafire CCD camera (Optronics, Goleta, CA) and an Axiovert 100 microscope (Carl Zeiss Canada, Toronto, ON).

Morphological maturation was assessed using the Sholl analysis plugin in ImageJ (National Institutes of Health (NIH) Bethesda, MD, USA), using concentric circles 12 μm apart around the cell body of mature PLP-positive oligodendrocytes. The total number of intersects was used as a branching index.

Cell Survival Assay

Purified OLs were plated in media with or without insulin for 5 days in 48 well plates. After fixation and staining with MBP and phalloidin, 9 pictures from each well were taken and MBP positive cells counted using ImageJ cell counter.

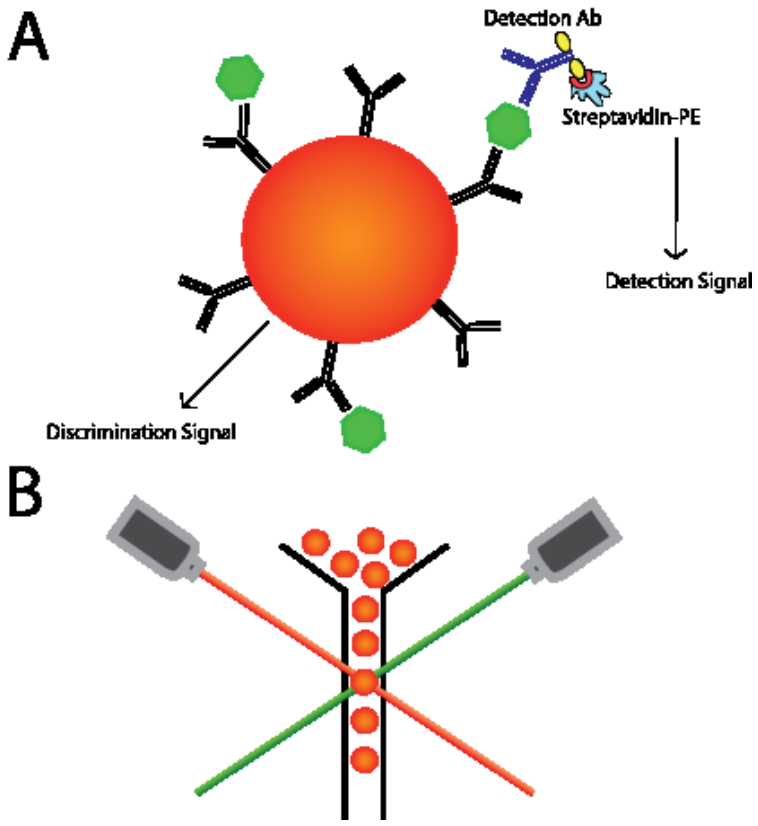


FIGURE 2-1: Overview of the Luminex Assay. A) Cell lysates are incubated with fluorescent beads coated with antibodies against a specific target. A second antibody to the specific target linked to a fluorescent protein is then added. **B)** The beads flowing through the luminex are excited by two different lasers to detect the bead signal and the detection signal. A value proportional to the amount of protein bound to each bead is then generated.

RESULTS

Insulin-independent OL survival and differentiation

Cell culture protocols typically use insulin to promote OL proliferation and differentiation *in vitro*. Insulin binds to IGF1R and insulin receptors expressed by OLs, and activation of these receptors produces a strong and sustained activation of Akt in OLs (Baron-Van Evercooren et al., 1991; Ness and Wood, 2002; Cui and Almazan, 2007), promoting OL survival (Barres et al., 1993; Ness and Wood, 2002; Cui and Almazan, 2007 and Figure 2-2). This strong Akt activation could easily mask the effect of neurotrophins on Akt activation. This was confirmed by examining Akt activation in cells grown in insulin-free media, after treatment with insulin. Not surprisingly, treatment of oli-neu cells with N1 supplement, which contains insulin (Figure 2-2A) or treatment of differentiated OLs with insulin (0,5 μg/ml; Figure 2-2B) produced a strong and sustained activation of Akt. Furthermore, OLs cultured in insulin-containing media exhibit high baseline Akt phosphorylation levels (Figure 2-2C), which could mask the effect of adding insulin or other factors that could activate Akt.

We, therefore, analyzed OL viability when grown in absence of insulin. For this, OLs were isolated and allowed to differentiate in OL-defined medium in the presence or absence of insulin (5 μg/ml). After 4 days *in vitro* (DIV), MBP-positive cells were observed in both conditions (Figure 2-2D), demonstrating that the presence of insulin in the media is not essential for differentiation *in vitro*. However, approximately 50% fewer MBP-positive cells were present in the insulin-free condition (Figure 2-2E). Apart from minimal contamination by astrocytes and fibroblasts that did not differ between the two conditions, no other phalloidin+/MBP- or CNP+/MBP- cells were detected in the cultures. This indicates that while insulin promotes OL survival, differentiation to a mature myelinating MBP-expressing state is insulin-independent. We subsequently performed all of the following experiments using insulin free media.

Akt activation during OL differentiation *in vitro*

To investigate Akt activation during OL differentiation, we examined Akt phosphorylation levels at different time points during OL differentiation *in vitro*. After isolation, plated OPCs rapidly began to differentiate and after 4 DIV, they displayed MBP and PLP positive membrane sheets. Akt activation during differentiation *in vitro* was assessed by lysing the cells at different time points after plating. As a screening method, we used the Luminex Multiplex Technology (Luminex Corporation, Austin, TX), a highly sensitive antibody based detection method adapted to phosphoprotein detection.

Mature OLs, at 4 DIV, exhibited higher Akt activation levels than did OPCs and immature OLs after 12 hours and 24 hours in culture respectively (Figure 2-3). This is consistent with a key role for Akt activation in the differentiation process, in agreement with previous studies (Flores et al., 2008; Tyler et al., 2009). The implication of Akt in myelin membrane formation highlighted by activated Akt overexpressing mice (Flores et al., 2008) prompted us to screen for factors that could activate Akt and promote morphological differentiation at a late stage of differentiation. Thus, we examined the effect of different treatments on the level of Akt phosphorylation and on the cellular morphology of 4 DIV OLs.

Only collectively applied factors induce prolonged activation of Akt

To identify putative factors that would trigger Akt activation in OLs, and thus contribute to the differentiation process, we first analyzed Akt activation in serum and growth factor starved oli-neu cells with the luminex assay. We assayed Akt activation 10 min and 60 min after treatment with factors that are highly expressed in the developing nervous system, including NT-3, BDNF, NGF, and NRG1, either applied individually or collectively. When applied individually, NT-3, BDNF, NGF and NRG1 produced a weak and transient Akt activation. Interestingly, when applied collectively, these four factors induce a prolonged and robust activation of Akt (Figure 2-4A).

Next, we examined whether these factors exert similar Akt activation profiles in differentiated primary rodent OLs. We treated OLs grown 4 DIV in the absence of insulin with either individual neurotrophins or NRG1 at a concentration of 10 ng/ml, or with a combination of NRG1/NT-3/BDNF/NGF, each added at a concentration of 5 ng/ml. Whole cell lysates obtained from OLs treated with the growth factors individually or collectively were then analyzed using immunoblotting against pAkt. We found that neurotrophins and NRG1 only induced a strong and sustained Akt activation when applied collectively (Figure 2-4B), in agreement with our findings with oli-neu cells (Figure 2-4A). These results demonstrate that the combination of growth factors mediates a similar Akt activation pattern in both serum and growth factor starved oli-neu cells and primary OLs.

Prolonged Akt activation induces OL differentiation via mTor that can be inhibited by pharmacological inhibitors

Akt/mTor activation in OLs promotes their differentiation from the precursor stage (A2B5+ cells) to the immature OL stage (GalC+) *in vitro* (Tyler et al., 2009). After 4 DIV, our cultures consisted only of PLP-positive mature OL cells. We thus examined the level of OL morphological differentiation induced by treatment with NRG1/NT-3/NGF/BDNF. Overexpression of Akt in OLs by two independent groups using two independent approaches was shown to induce hyper-myelination *in vivo*. Therefore, we determined if prolonged Akt activation mediated by neurotrophins and NRG1 also results in elevated morphological differentiation and branching, *in vitro*. Since OLs differentiate into MBP and PLP positive sheet forming cells *in vitro*, we examined the branching index of OLs 24 hrs after treatment with neurotrophins and NRG1, either applied individually or collectively. Cells were stained with phalloidin to visualize all OL branches, since the actin filament network extends from the cell body to peripheral branches and small villi in OLs (Song et al., 2001). Branching index was obtained by summing the intersect values of each level of a Sholl analysis (see methods; Rajasekharan et

al., 2009; Figure 2-5C). In agreement with luminex and immunoblotting data, we found that during prolonged Akt activation, upon collective application of NRG1/NT-3/NGF/BDNF, a significantly higher degree of morphological differentiation in OLs was observed. Treatment with NT-3, BDNF, NGF, or NRG1 individually had no effect on OL morphological differentiation (Figure 2-5D).

PI3K activation by plasma membrane receptors induces the recruitment and activation of Akt. Akt can phosphorylate a variety of targets implicated in different cell functions including survival, proliferation, glucose metabolism and protein synthesis (reviewed in Katso et al., 2001). Amongst the known targets of Akt in OLs is mTor (Narayanan et al., 2009; Tyler et al., 2009). We thus wanted to determine if the effect on differentiation of collectively applied factors was dependent on PI3K/Akt/mTor activation by application of various pharmalogical inhibitors. The morphological differentiation induced by collectively applied neurotrophins and NRG1 is dependent on the activation of PI3K, as it was not observed when cells were treated in conjunction with PI3K inhibitors Wortmannin and Pi-103 (Figure 2-6). Furthermore, this differentiation is dependent on mTor activation, since addition of the mTor inhibitor Rapamycin inhibited the increased branching induced by the combination of neurotrophins. The inhibitors alone did not have any significant effect on morphological differentiation. This might suggest that baseline morphological differentiation and branching *in vitro* at that stage of OL maturation is not regulated by the PI3K/Akt/mTor axis. Alternatively, baseline branching triggering events in the absence of any external cues could happen prior to 4 DIV, time when the inhibitors were added along with the factors. Our findings support the conclusion that the increased maturation caused by NRG1/NT-3/NGF/BDNF is dependent on PI3K/Akt/mTor activity.

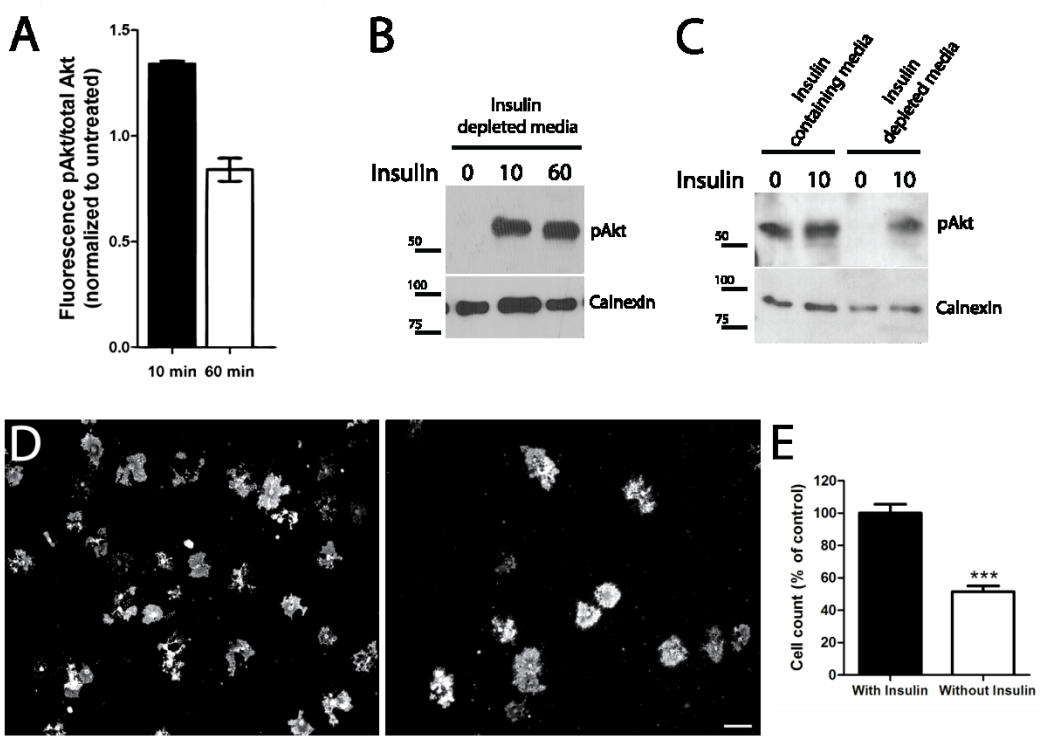


FIGURE 2-2: Using insulin-free media unmasks Akt signalling activation in OLs A) Using the luminex assay, Akt phoshorylation was assessed following application of N1 supplement, which contains insulin, on oli-neu cells. pAkt/total Akt values of untreated controls were set to 0. Insulin was shown to induce a strong and sustained increase in Akt phosphorylation, as it remained elevated 60 mins after treatment. **B)** A similar effect was detected using immunoblotting of whole cell lysates of primary OLs treated with insulin (0,5 µg/mL) for either 10 or 60 min. **C)** Activation of Akt following insulin treatment was masked by high baseline Akt phosphorylation levels produced by the presence of insulin in the media. **D)** OLs survive and differentiate in insulin-free media. MBP-positive cells were visible after 4 DIV in the presence (left panel) or absence (right panel) of insulin in the media. **E)** In the absence of insulin, the number of OLs was reduced by 50%. ***: $p < 0.001$. Scale bar = 200 µm.

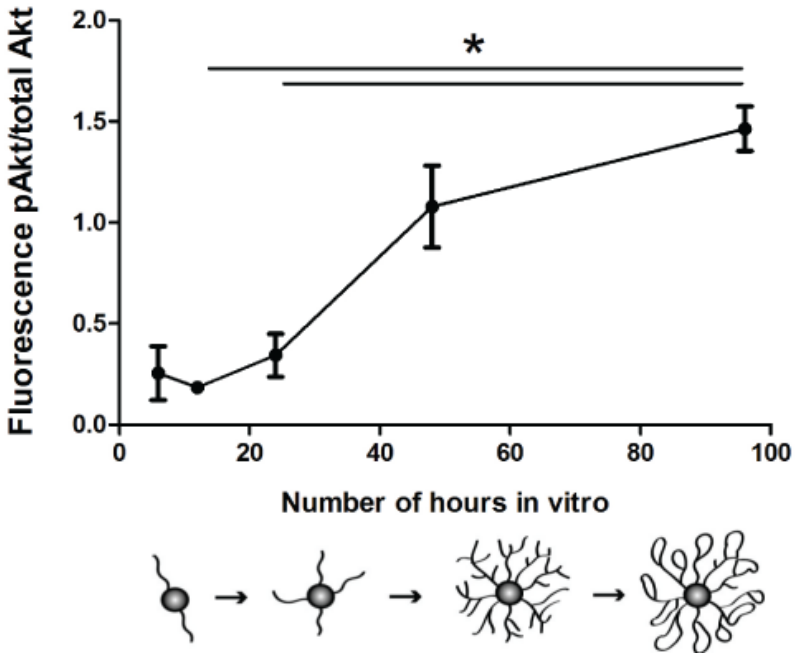


FIGURE 2-3: Akt activation during OL differentiation *in vitro.* Akt activation during OL differentiation *in vitro* was assessed with the luminex assay. Mature OLs at 4 days *in vitro* exhibited higher Akt activation levels than OPCs and immature OLs at 12 hours and 24 hours *in vitro* respectively. Each time point represents 3 separate replicates. *:$p<0,05$

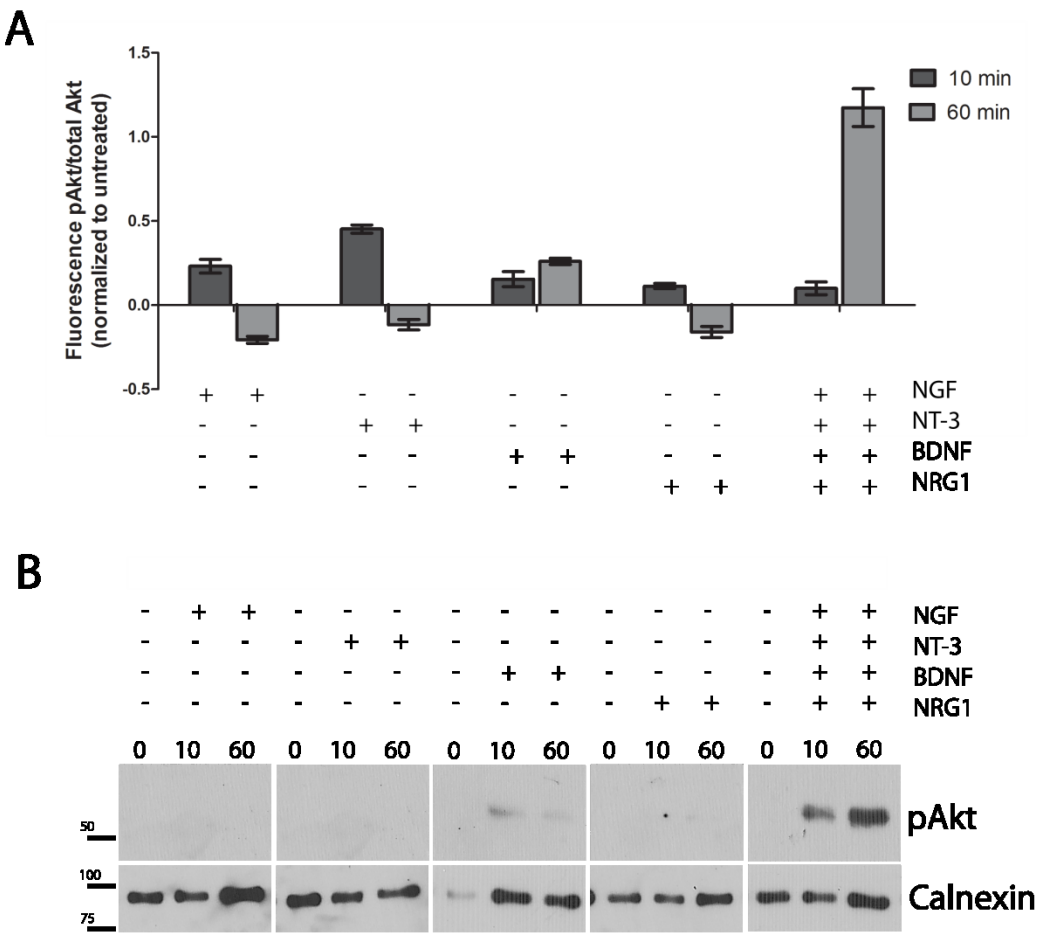


FIGURE 2-4: Synergistic effect of NRG1/NT-3/NGF/BDNF on Akt activation. **A)** Differentiated oli-neu cells were assessed for pAkt levels after different treatments using the luminex assay. PhosphoAkt/totalAkt values were normalized to untreated values, which were set to 0. Treatment with NRG1, NT-3, NGF or BDNF induced moderate Akt activation. However, the same factors used collectively induced a strong and sustained Akt activation, and phosphoAkt/totalAkt levels were still elevated 60 mins after treatment **B)** This effect was confirmed in rat primary differentiated OLs *in vitro* by immunoblotting. The same amount of protein was loaded for each condition (5µg).

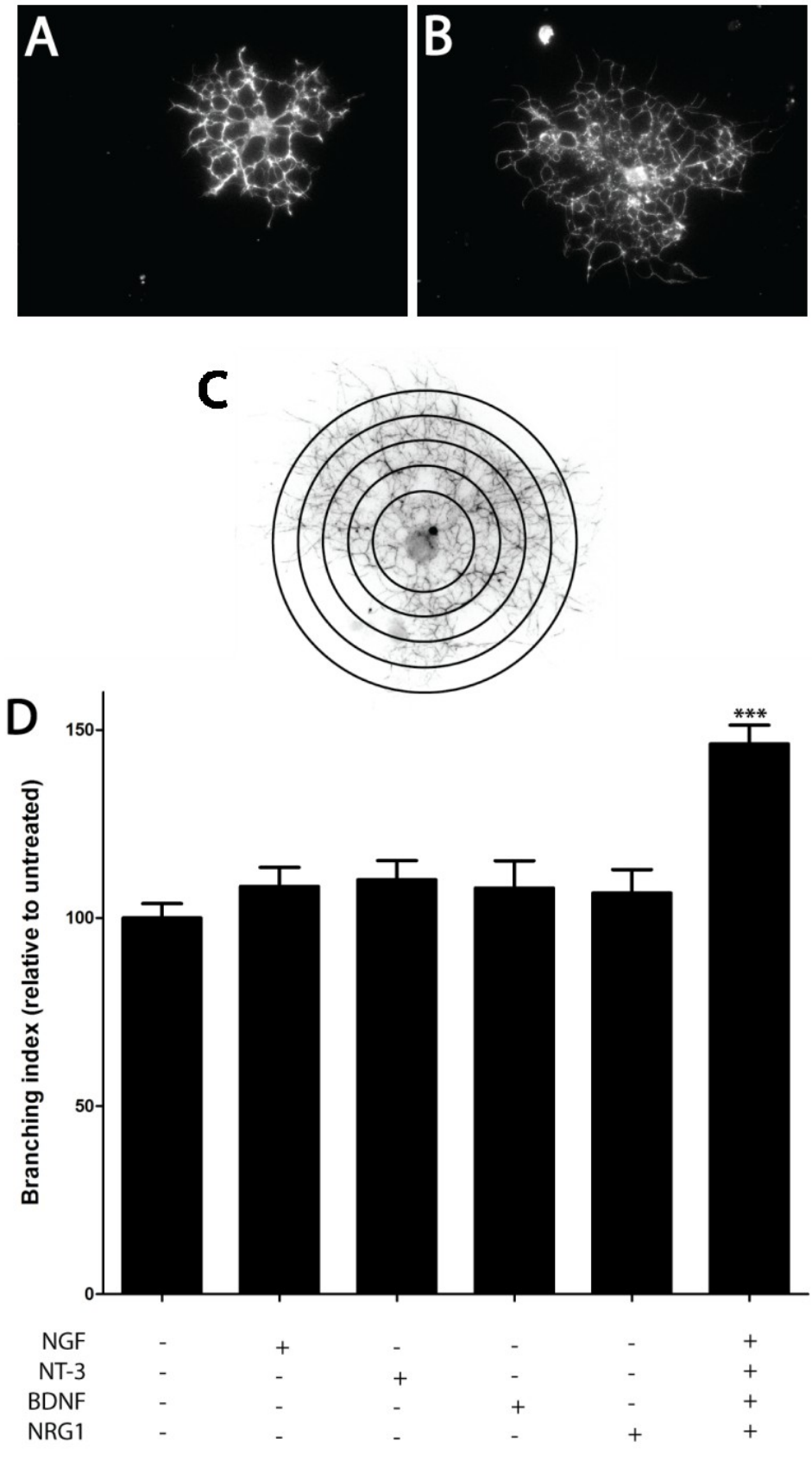

A
B
C
D
Branching index (relative to untreated)
150
100
50
0
***
NGF - + - - - +
NT-3 - - + - - +
BDNF - - - + - +
NRG1 - - - - + +

FIGURE 2-5: Synergistic effect of NRG1/NT-3/NGF/BDNF on morphological maturation of OLs *in vitro*. Different treatments were applied to rat primary OLs at 4 DIV and morphology was assessed 24 hrs later by staining with phalloidin and PLP. **A)** Example of phalloidin stained untreated OLs. **B)** Example of a phalloidin stained OL treated with NRG1/NT-3/NGF/BDNF. **C)** Morphological analysis was performed using Sholl analysis and the total number of intersects made by OL branches was used as a branching index. **D)** Treatment with either factor alone (10 ng/mL) did not induce any morphological differentiation. However, treatment with NRG1/NT-3/NGF/BDNF (5 ng/mL) in combination induced a strong morphological differentiation ***: $p< 0.001$ except **: $p< 0.01$ when compared to BDNF.

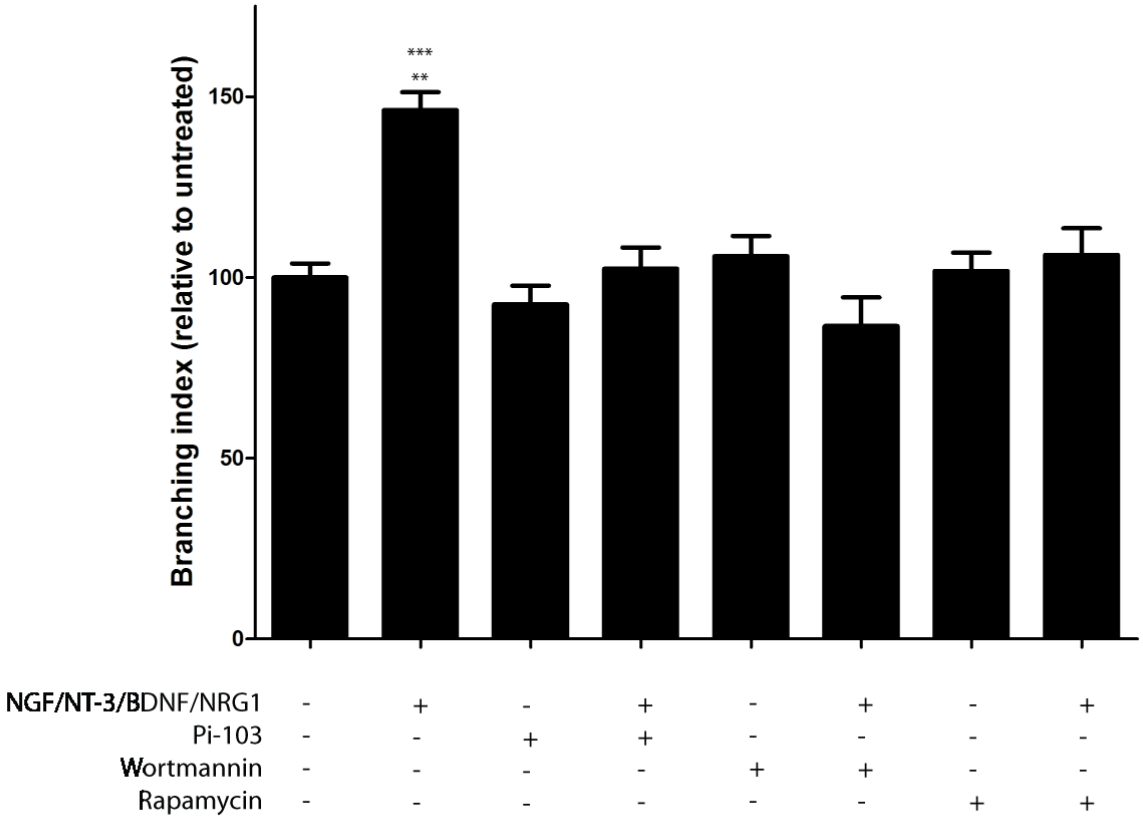


FIGURE 2-6: Synergistic effect of NRG1/NT-3/NGF/BDNF on morphological maturation of OLs *in vitro* is mediated by PI3K/Akt/mTor axis activation. The morphological differentiation induced by NRG1/NT-3/NGF/BDNF is abolished when cells are treated with PI3K inhibitors (Wortmannin and Pi-103) or an mTor inhibitor (Rapamycin). Treatment with inhibitors alone did not have any significant effect on the branching index. ***: $p < 0.001$ when compared to all conditions, except **: $p < 0.01$ when compared to NRG1/NT-3/NGF/BDNF + Rapamycin condition.

DISCUSSION AND CONCLUSION

Treatment with NRG1, NT-3, NGF or BDNF alone produced a small and transient increase in Akt phosphorylation. Surprisingly, application of NRG1, NT-3, NGF and BDNF together resulted in a large and sustained increase in Akt phosphorylation, assessed both by the luminex assay and by standard immunoblotting. This synergistic activation of the PI3K/Akt pathway substantially enhanced morphological differentiation of OLs *in vitro*. Furthermore, we demonstrated that the synergistic action of these factors requires the activation of mTor downstream of the PI3K/Akt pathway.

It was previously shown that mTor activation is critical for early stages of differentiation in OLs, mediating differentiation from the precursor stage ($A2B5^+$ cells) to immature OL stage ($GalC^+$ cells). However, there was no significant effect on differentiation when mTor was inhibited in later stages of differentiation, at 3-4 DIV (Tyler et al., 2009). Consistent with that finding, we did not see any effect on morphological maturation when baseline mTor activity was inhibited at 4 DIV, which agrees with the findings of Tyler et al. (2009). Nonetheless, we showed that mTor activity is required for the morphological differentiation induced by collectively applied NRG1/NT-3/NGF/BDNF. This raises the possibility that the PI3K/Akt/mTor pathway plays a broader role in OL differentiation, regulating more than one stage of differentiation in response to growth factors present in the OL environment during development *in vivo*. The increase in pAkt levels during OL differentiation *in vitro* is consistent with such a role.

The mechanism regulating the synergistic effect of collectively applied neurotrophins and NRG1 is unknown. Possible redundant action of neurotrophins also needs to be addressed, to gain insight into which Trk receptors might mediate the synergy. The synergy could be mediated by the regulation of extracellular receptors, either by affecting receptor trafficking or by regulating the formation of

different receptor complexes. Alternatively, the synergy could be explained by activation of parallel signaling pathways resulting in an increased and sustained Akt activation. Likewise, the mechanism by which mTor activation regulates morphological differentiation of OLs needs to be elucidated. Given the major role of mTor in protein translation, activation of local translation of myelin proteins or cytoskeletal elements such as actin might underlie the Akt/mTor dependent increase in branching in OLs.

Sequential steps of OL differentiation can be viewed as an integration of signalling pathways regulating different aspects of maturation, downstream of the large array of factors to which OLs are exposed during their differentiation. Hopefully, understanding how different signaling pathways are activated during differentiation will provide insight into which factors can promote differentiation and myelination both during development and after demyelination, such as in MS.

CHAPTER III
DCC EXPRESSION BY OLIGODENDROCYTES REGULATES MYELIN AND PARANODAL MAINTENANCE IN VIVO

Part I: Cell autonomous requirement for DCC in paranodal organization assessment via an intravitreal transplantation approach

The first part of this chapter was published as Figure 8 in:

> Jarjour AA, Bull SJ, Almasieh M, Rajasekharan S, Baker KA, Mui J, Antel JP, Di Polo A, Kennedy TE (2008) Maintenance of axo-oligodendroglial paranodal junctions requires DCC and netrin-1. J Neurosci 28:11003-11014.

Part II: Cell autonomous requirement for DCC in paranodal organization assessment via inducible conditional knock out approach

The results included in the second part of this chapter are included in a manuscript currently in preparation for submission.

PREFACE AND RATIONALE

As described in chapter I, paranodal axoglial junctions are essential for the segregation of myelinated axons into distinct domains and for the efficient conduction of action potentials. We have previously established that netrin-1 and DCC are enriched at paranodal axo-oligodendroglial junctions both *in vivo* and *in vitro* (Jarjour et al., 2008). Myelination in the CNS of mice occurs during the first few post-natal weeks. Mice null for netrin-1 or DCC die within a few hours after birth; therefore, it is not possible to examine myelin formation in these animals *in vivo*. Studies carried out *in vitro* have provided evidence that netrin-1 and DCC are required for the maintenance of normal paranodal junctions (Jarjour et al., 2008). In myelinated cultures generated from $DCC^{-/-}$ and $netrin\text{-}1^{-/-}$ knockout pups, the paranodes formed normally, but became disorganized after two months in culture. Ultrastructural analysis indicated that the paranodal loops detached from the axon and the transverse bands were disorganized. Maintenance of the different domains integrity was also impaired, as revealed by the widening of Caspr staining at the paranode, Na^+ channel staining at the node, and the invasion of K^+ channel staining into the paranode (Jarjour et al., 2008).

In the following chapter, I have aimed to determine if DCC is required cell-autonomously in OLs to function at paranodes. Neurons express both DCC and netrin-1, but *in vitro*, netrin-1 expression appears to be primarily dendritic (Tritsch and Kennedy, unpublished observation). Furthermore, confocal imaging of DCC immunostaining at paranodes suggests glial localization (Jarjour et al., 2008). Additionally, the studies described aimed to confirm that the abnormal phenotype observed in organotypic cultures derived from conventional DCC null mice also occurs *in vivo* in mice specifically lacking DCC from mature OLs.

To determine if netrin signalling is required for the organization of paranodal junctions *in vivo*, we first used an intravitreal transplantation model and assessed the capacity of oligodendrocyte precursor cells (OPCs) derived from $DCC^{-/-}$ mice

to myelinate retinal ganglion cell axons when transplanted into the eyes of wild-type mice. The results of these experiments are presented in the first part of this chapter. We then used an inducible conditional knockout model to specifically ablate DCC expression in mature OLs. The analysis of paranodal, myelin, and the behavioural phenotypes of these mice is presented in the second part of this chapter. With these two models, we provide evidence supporting the conclusion that DCC expression by OLs is required for proper maintenance of myelin and paranodes *in vivo*.

MATERIALS AND METHODS

Animals

$DCC^{lox/lox}$ and $PLPcreER^{T}$ mice were obtained from Anton Berns (The Netherlands Cancer Institute, Amsterdam, Netherlands) and Samuel David (McGill University, Montreal, Canada) respectively. Mice heterozygous for the conventional *dcc* knockout (Fazeli et al., 1997) were obtained from Robert Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA, USA), and were extensively backcrossed into a CD-1 genetic background. CMVcre animals were kindly provided by David Colman (McGill University, Montreal, Canada). ROSA26 mice were kindly provided by Jean-François Cloutier (McGill University, Montreal, Canada). All procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

Tamoxifen induction

Tamoxifen (Sigma; T5648) was dissolved in a 10:1 mixture of sunflower oil/ethanol at a concentration of 10 mg/ml. 0.1 ml (1mg) was injected intraperitoneally twice a day for five consecutive days into the test group, as well as the control littermates. Injections were performed between 4.5 and 6 weeks of age. All animals used were males.

Mouse OPC isolation

OPCs were obtained from a mixed glial culture derived from newborn mice neocortices, as described (Armstrong, 1998). In brief, the forebrains of newborn mouse pups were removed, chopped, and incubated for 45-60 min at 37°C in a solution of 1.2 U/ml of papain, 0.24 mg/ml L-cysteine, 40 μg/ml of DNaseI in MEM/HEPES. Dissociated tissue was then triturated with syringes and plated in T75 flasks containing 10% heat-inactivated horse serum and 1% penicillin/streptomycin in DMEM. Cultures were allowed to proliferate for 10-12 days, with the media being changed every 2 days. The $DCC^{-/-}$ pups were identified based on stereotypical behaviours and cells from these pups were cultured

separately from their wild-type and heterozygote littermates. Genotypes were later confirmed by PCR. After 10-12 days *in vitro*, OPCs were obtained by an overnight shake-off of the flasks, and purified by differential adhesion.

Intravitreal transplantation and retina processing

Isolated mouse OPCs were concentrated at a density of 15 000 cells/µl in OL defined medium containing insulin and 2 µl of cell suspension was transplanted into an adult wild type CD1 mouse eye as described previously (Setzu et al., 2004). Animals were perfused transcardially 8 weeks after transplantation with 4% PFA, the eyes enucleated, postfixed, and the retinas dissected. Flat-mounted retinas were permeabilized in 2% Triton X-100, 0.5% DMSO in PBS for 4 days and blocked for 2 hrs in 10% Normal Goat Serum (NGS), 2% Triton X-100 and 0.5% DMSO in PBS. Retinas were then incubated in primary antibodies for 48 hrs, washed once for 5 min, and thrice for 20 min. Secondary antibodies were applied for 2 hrs, and retinas were finally washed and mounted on slides.

Antibodies

The following antibodies were used in this study: mouse monoclonal anti-Caspr (University of California Davis NeuroMab; catalog #75-001), rabbit polyclonal anti-Kv1.2 (Alomone Labs; catalog #APC-010), rabbit polyclonal anti-NFM (Millipore; catalog #AB1987), goat polyclonal anti-DCC (Santa Cruz; catalog #SC6535), mouse monoclonal anti-DCC (BD Biosciences Pharmingen; catalog #554223), rabbit polyclonal anti-Calnexin (Biovision; catalog #3811-100), rabbit polyclonal anti-MAG and mouse monoclonal anti-PLP (gift of Dr. David Colman, McGill University), rabbit polyclonal anti-neurofascin (Tait et al., 2000; gift of Dr. Peter Brophy, University of Edinburgh), rat polyclonal anti-MBP (Millipore; catalog #MAB386), rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz; catalog #Sc-25778), mouse monoclonal anti-CNP (Abcam; catalog #ab24566), rabbit polyclonal Olig2 (Abcam; catalog #ab81093) and mouse monoclonal anti-APC (CC1; Abcam; catalog #ab16794). Secondary antibodies used were Alexa 546-conjugated goat anti-mouse

(Invitrogen; A11003) and goat anti-rabbit (Invitrogen; A11010), Alexa 488-conjugated goat anti-rabbit (Invitrogen; A11008), HRP-conjugated donkey anti-goat (Cederlane; 705-035-147), donkey anti-rat (Cederlane; 712-035-153), donkey anti-mouse (Cederlane; 715-035-150), and donkey anti-rabbit (Cederlane; 711-035-152).

Immunohistochemistry

For the study of adult PLPcreERT^{+}DCC$^{lox/lox}$ mice, animals were deeply anesthetized and perfused with phosphate buffer saline pH 7,4 (PBS) followed by 4% paraformaldehyde (PFA). Brain and spinal cord were dissected, postfixed in 4% PFA for 1 hr and equilibrated in 30% sucrose at 4°C. After embedding in optimal cutting temperature compound (Sakura Finetek), 16 µm sections were cut on a cryostat. For the study of CMVcre^{+}DCC$^{lox/lox}$, embryos were fixed in Carnoy's solution (60% ethanol, 30% chloroform and 10% acetic acid), dehydrated and embedded in paraffin. 8 µm sections were cut and processed for staining. Briefly, after blocking for 1 hour at room temperature (RT) in 3% BSA 0.3% Triton X-100 in PBS, primary antibodies were added (diluted in blocking solution), incubated overnight at 4°C, and then rinsed three times with PBS. Cells were then incubated with secondary antibodies, along with phalloidin and/or Hoescht when needed, for 1.5 hour at RT, before washing and mounting with Fluoromount-G (Southern Biotech). Images were captured with a Magnafire CCD camera (Optronics, Goleta, CA) and an Axiovert 100 microscope (Carl Zeiss Canada, Toronto, ON). For paranode analysis, images were captured using a Zeiss LSM 510 confocal microscope and quantified by a blinded experimenter using the LSM510 software.

X-Gal staining

Ten days after tamoxifen administration, PLPcreER^{T+}ROSA26 mice were anesthetized and perfused with PBS followed by 4% PFA containing 2 mM MgSO4, and 5 mM EGTA. 16µm brain and spinal cord sections were then rapidly processed for X-Gal staining as previously described (Mombaerts et al., 1996).

Electron microscopy

Mice were deeply anesthetized and perfused with 0.1 M phosphate buffer, pH 7.4, followed by 2.5% glutaraldehyde 2% PFA, and then by 2% PFA. Tissue was dissected and post-fixed for 1 week at 4°C, rinsed with buffer and postfixed in 2% osmium tetroxide for 40 min. Fixed tissue was then dehydrated through a graded ethanol series and embedded in Epon. Ultrathin sections (70-100 nm) were prepared, placed onto 200 mesh copper grids and stained with 4% uranyl acetate for 5 min, followed by Reynold's lead citrate for 3 min. Images were observed using a transmission electron microscope at 120 kV and captured using a Tecnai 12 (FEI) Gatan Bioscan CCD camera.

g-ratio measurement

G-ratio (axon diameter/fiber diameter) was calculated with a g-ratio calculator Plug-in for ImageJ (available online at http://gratio.efil.de/) applied to electron microscopy pictures of coronal sections of optic nerves. 60 myelinated axons per animal were randomly selected for measurement (6 animals per group, for a total of 360 axons per genotype). Mice were aged between 6 and 9 months of age. No effect of age was detected.

Behavioral Testing

Open field tests were performed in a square open field (50 cm x 50 cm) surrounded by 30 cm high walls (Figure 3-1A). After a 20 min habituation period, recordings of individual mouse movements were performed for 2 hrs using VideoTrack software (ViewPoint Life Sciences, Montreal, Canada). The hanging wire grip test was performed as described (Sango et al., 1996). This test gives information on overall muscle strength. Briefly, mice were put on a cage lid held upside down and the time that each mouse was able to grip to the wires without falling was recorded (Figure 3-1B). A cut-off time was set to 60 s. The balance beam test was performed as previously described (Carter et al, 1999) to assess general balance and coordination. Briefly, mice were trained for 4 consecutive days to cross a narrow beam (0.8 cm diameter, 50 cm long) and reach an enclosed

safety platform (Figure 3-1C). On the fifth day, the time taken by each mouse to cross the beam was recorded; with a cut-off time set at 60 s. Accelerating rotarod (Rotamex; 3 cm rod diameter, grey PVC with a knurled finish) testing was performed after 3 consecutive days of training. During each training session, the mice received 5 trials on an accelerating rotarod (1 rpm to 24 rpm) for a maximum of 150 sec. On the test day, mice were tested on an accelerating rod (1 rpm/5 sec) that reached a maximum speed of 24 rpm. Mice were allowed between 3 and 5 trials with a rest of at least 20 mins between each trial. The latency to fall was recorded, with a cut-off time of 300 sec.

Brain lysates and Western blotting

E14 brains were lysed in RIPA buffer (10 mM Phosphate buffer pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Adult brain tissue was lysed in 3% Triton X-100 buffer (20mM Tris pH 8, 150 mM NaCl, 10 mM EGTA, 10 mM EDTA, 3% Triton X-100) to maximize myelin membrane solubilisation containing protease inhibitors. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were then subjected to standard western blotting. Densitometric analysis was performed using Adobe Photoshop. Values were normalized to loading control values for GAPDH. To reduce the variability due to experimental conditions (each set of control and $PLPcreER^{T+}DCC^{lox/lox}$ littermates were harvested at different times), each $PLPcreER^{T+}DCC^{lox/lox}$ was compared to its control littermate. Five mice of each genotype were used in the analysis.

Cell number count

Optic nerve and spinal cord sections from animals 9 months post-induction were immunolabelled with CC1 and Olig2, both markers of OLs. Pictures were taken randomly (3 fields of view per animal; 3 animals per genotype) and CC1/Olig2 double positive cells were counted.

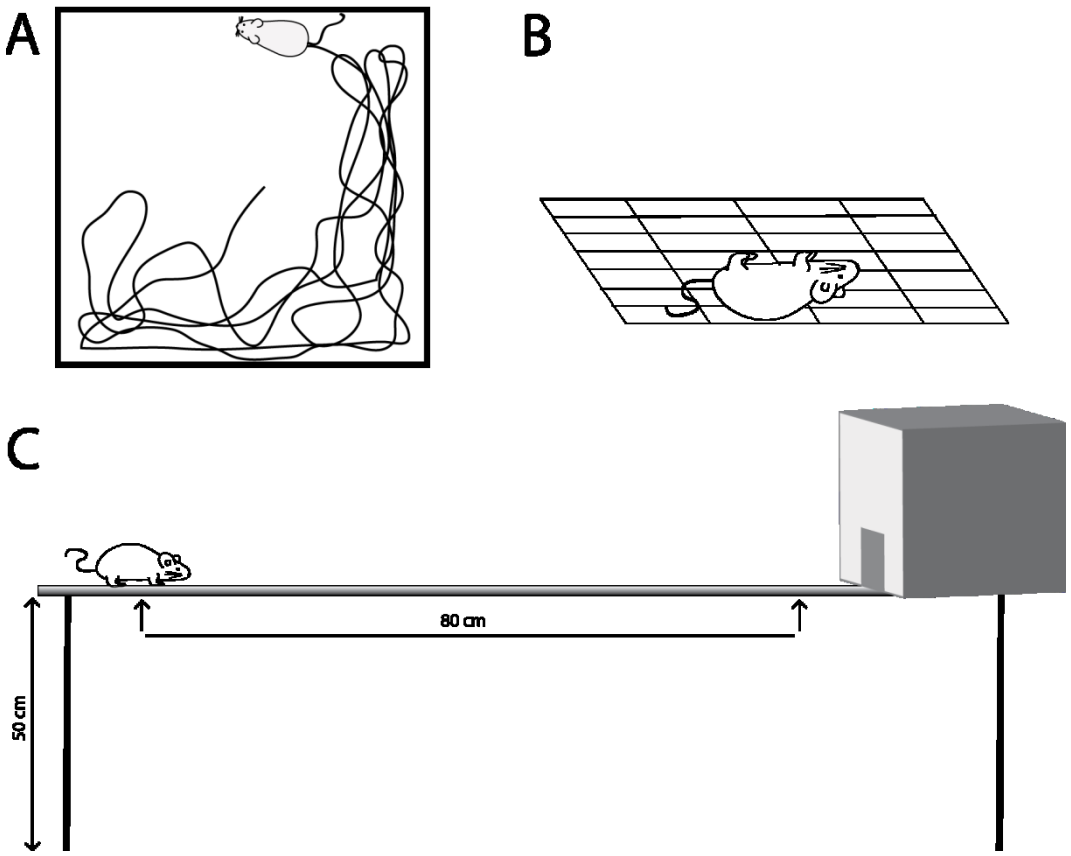


FIGURE 3-1: Behaviour tests. **A)** Open field test was performed in a square open field (50 cm x 50 cm) surrounded by 30 cm high walls. Animal's movements were recorded by a tracking device linked to a camera **B)** The hanging wire grip test gives information on muscle strength. Mice were put on a cage lid held upside down and the time that each mouse was able to grip to the wires without falling was recorded. **C)** The balance beam was used to assess general balance and coordination. After a series of training trials, the time taken by mice to cross a narrow beam (0.8 cm diameter, 50 cm long) and reach a closed safety platform was recorded.

III.1 Cell autonomous requirement for DCC in paranodal organization: Assessment via an intravitreal transplantation approach

To determine if DCC expression by OLs is required for paranode maintenance *in vivo*, we initially assessed the capacity of OPCs derived from $DCC^{-/-}$ mice to myelinate retinal ganglion cell (RGC) axons. RGCs cell bodies are located in the retina. They send their axons through the optic nerve head into the optic nerve, and synapse in the lateral geniculate body. RGCs axons are myelinated in the optic nerve; however, OPCs do not invade the retina during development and the proximal segment of the axon within the retina remains unmyelinated in rat, mouse and humans (Baumann and Pham-Dinh, 2001). The intraretinal segment of the RGC axon thus provides a unique opportunity to assess the capacity of OPCs transplanted in to the retina to myelinate, in the absence of endogenous OPCs. Transplantation of rat OPCs into adult rat retinas can generate abundant MBP positive myelin segments (Setzu et al., 2004), but this method had not been applied to the mouse. Moreover, formation of paranodal domains was never assessed in this model.

Formation of paranodal domain and clustering of Caspr in that model

To validate the model, wild type OPCs were first isolated and transplanted into two month old wildtype mice (Figure3-2A). Around 3 weeks after transplantation, the retinas were dissected and stained using anti-MBP and anti-Caspr antibodies. Abundant MBP-positive myelin segments were observed along RGC axons of eyes that received OPCs (Figure 3-2B and C), but no MBP immunoreactivity was detected in the control eye (data not shown). Paranodal specializations, visualized by labelling for the paranodal marker Caspr, were readily detectable along myelinated fibres (Figure 3-2D). We concluded that the OPC transplantation paradigm to study myelin can be applied to a mouse model. Furthermore, this system can be used to study paranodes, as myelination by exogenous OLs triggers

the proper localisation of Caspr proteins at points of paranodal loop contact on the axon, at each side of the node of Ranvier.

Disruption of paranodal domains formed by DCC$^{-/-}$ oligodendrocytes *in vivo*

Following validation of the method, OPCs were then purified from DCC-deficient mice and from their wild type and heterozygous littermates, and injected into normal CD1 adult mouse eyes. In this experimental paradigm, OLs are DCC deficient, whereas the RGCs axons are wildtype and are thus able to express DCC. Eight weeks after transplantation, quantitative analysis revealed an increased length of Caspr immunoreactivity in paranodes formed by DCC$^{-/-}$ OLs (Figure 3-3C and D) compared with paranodes formed by DCC expressing OLs (either DCC$^{+/+}$ or DCC$^{+/-}$) (Figure 3-3A, B and E). This phenotype is similar to what was observed in long term DCC$^{-/-}$ organotypic cultures (Jarjour et al., 2008). Some paranodes formed by DCC$^{-/-}$ OLs displayed leakage or ectopic clustering of Caspr immunoreactivity along the axon (Figure 3-3D, arrowhead), suggesting an ongoing disorganization of paranodal junctions. The abnormal paranodal phenotype observed provides a first indication that DCC is required cell autonomously in OLs to function at paranodes *in vivo*.

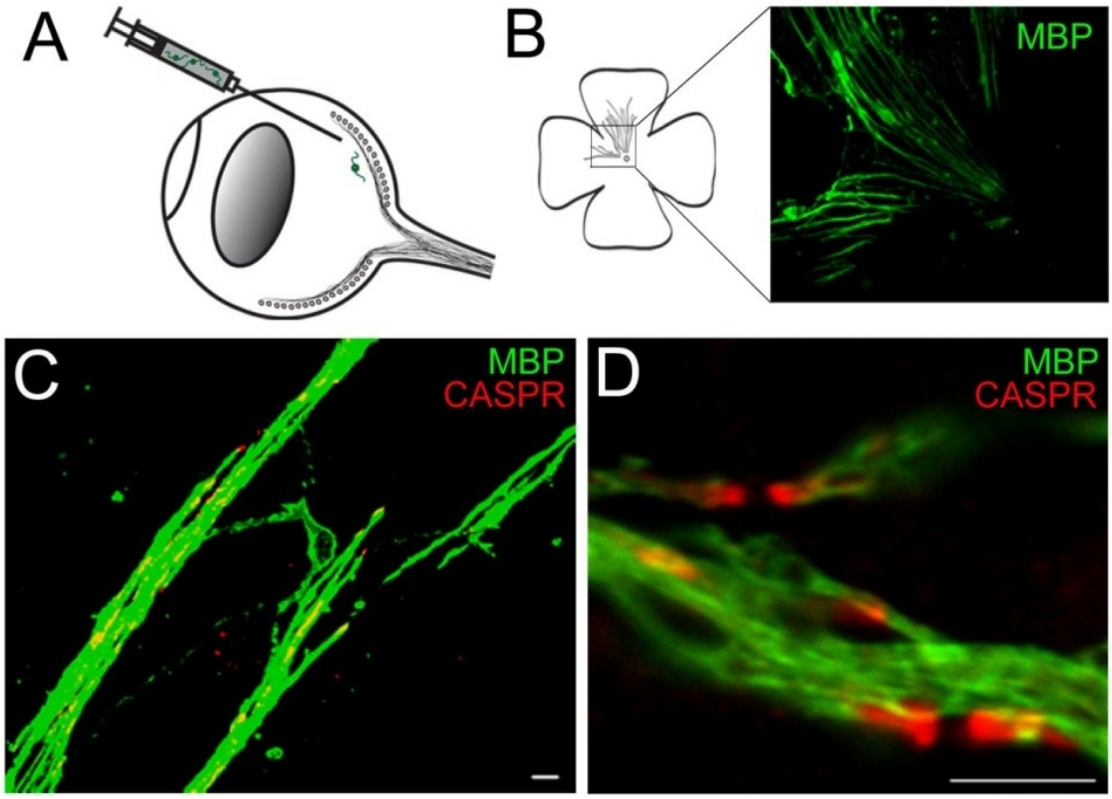


FIGURE 3-2: Formation of MBP-positive myelin and paranodal domains following intravitreal transplantation of OPCs. Injection of OPCs into mouse eyes (A) leads to formation of MBP-positive myelin segments (B,C) and formation of paranodes (D). A flat-mounted retina (B) was double-labelled with antibodies against MBP to visualize myelin and Caspr to visualize paranodes (C-D). B: 10X objective. C: 100X objective D: 100X objective, digital zoom 2. Scale bars correspond to 5 μm.

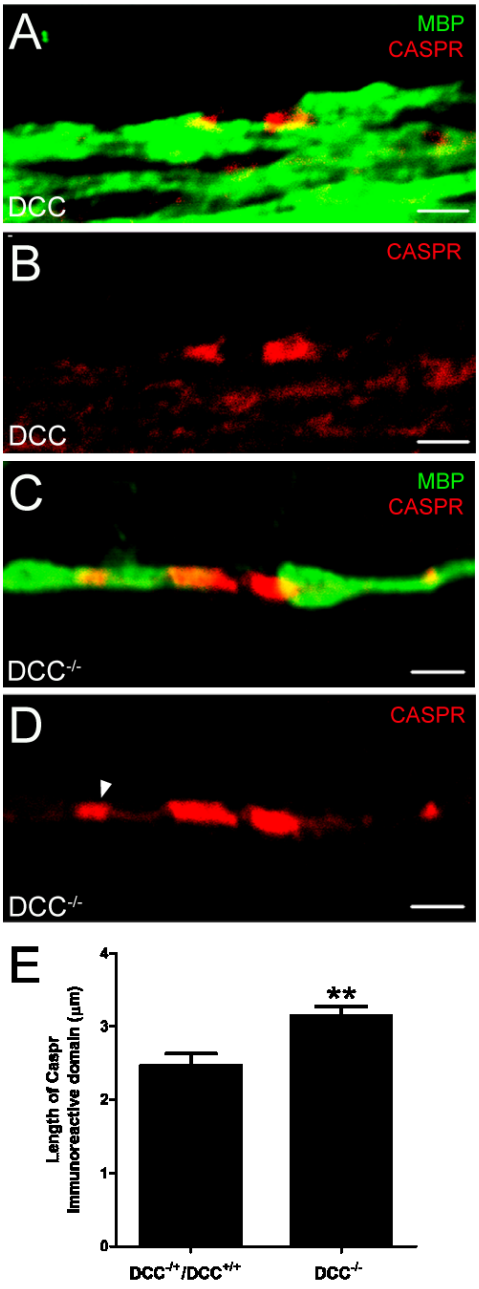


FIGURE 3-3: **Disruption of paranodal domains formed by DCC$^{-/-}$ oligodendrocytes in the intravitreal transplantation method.** Caspr immunoreactive domain length was measured in paranodes formed by DCC expressing OLs (A,B) and DCC$^{-/-}$ OLs (C,D). Some paranodes formed by DCC$^{-/-}$ OLs exhibited leakage of Caspr out of the paranode (D, arrowhead). The Caspr immunoreactive domains were lengthened in DCC$^{-/-}$ myelin group, compared with the wild-type and heterozygote group (E). A-D: 100X objective, digital zoom 4. Scale bars correspond to 2 µm. **: $p < 0.01$.

III.2 Cell autonomous requirement for DCC in paranodal organization: Assessment via inducible conditional knock out approach

To study the specific effect of the absence of DCC expression by OLs at paranodes *in vivo*, we used a conditional knockout strategy to selectively delete DCC from OLs *in vivo*. $DCC^{lox/lox}$ mice carry loxP site within their *dcc* gene. Cre-mediated recombination of the loxP sequence removes exon 23, coding for the transmembrane domain of DCC, and ablates functional DCC expression. Our goal was to trigger the recombination event in mature OLs. We thus chose the $PLPcreER^{T}$ mouse line in which Cre expression is specific to OLs, in addition to being inducible, so that recombination could be specifically triggered in mature OLs once myelination is complete in the CNS.

Validation of $DCC^{lox/lox}$ mouse line

DCC knockout mice die at birth and their nervous systems are characterized in part by impaired crossing of commissural axons during development (Fazeli et al., 1997). In order to confirm that recombination of the loxP sites by Cre yields a non-functional DCC protein, we crossed $DCC^{lox/lox}$ to the CMVcre mouse line, which expresses Cre under a human cytomegalovirus promoter. The CMV promoter drives Cre expression in a ubiquitous manner before the implantation stage (Schwenk et al., 1995). Therefore, all cells should be recombined and the $CMVcre^{+}DCC^{lox/lox}$ embryos should not express any DCC protein and phenocopy the $DCC^{-/-}$ embryos. $CMVcre^{+}DCC^{lox/lox}$ and $DCC^{-/-}$ embryos were collected at embryonic day 14 (E14). We first examined brain lysates to assess DCC protein expression in the different knockouts. DCC protein was not detected in brain lysates of either $CMVcre^{+}DCC^{lox/lox}$ or $DCC^{-/-}$ mice (Figure 3-4B).

To confirm the functional knockout of DCC by Cre-mediated recombination, the formation of the spinal cord ventral commissure was investigated in the $CMVcre^{+}DCC^{lox/lox}$ embryos. This commissure is substantially reduced in netrin-1 (Serafini et al., 1996) and conventional DCC knockout mice (Fazeli et al., 1997). At E14, the ventral commissure of the $CMVcre^{+}DCC^{lox/lox}$ embryos was thinner

than its CMVcre^{-}DCC$^{lox/lox}$ littermates (Figure 3-4A). This phenotype is similar to what was observed in E14 DCC$^{-/-}$ spinal cord. These results demonstrate the efficiency of DCC knockout upon Cre-mediated recombination.

Validation of the induction of Cre recombination in PLPcreER^{T+}

To study the role of DCC in myelin maintenance, we used the PLPcreER^{T} line, in which Cre-mediated recombination can be temporally regulated by administration of tamoxifen (Doerflinger et al., 2003). Cre expression is driven by the PLP promoter, which is activated in differentiated OLs and to a limited extent in differentiated Schwann cells (Puckett et al., 1987) and some spinal motor neurons (Jacobs et al., 2004). To confirm that the injection protocol (1 mg tamoxifen twice a day for five consecutive days) was inducing efficient recombination, we first crossed the PLPcreER^{T} with ROSA26 mice, in which the reporter gene β-galactosidase expression is turned on as a result of Cre-mediated recombination (Araki et al., 1995). In PLPcreER^{T+}ROSA26 mice, high expression of the reporter protein was obtained 10 days after the end of the tamoxifen injection regimen in cells of all white matter tracts observed, but not after injection with vehicle (Figure 3-5). Nevertheless, for all subsequent experiments, controls, also referred to as wildtype, consisted of PLPcreER^{T-}DCC$^{lox/lox}$ and PLPcreER^{T+}DCC$^{+/+}$ littermates that were submitted to the same tamoxifen regimen as the PLPcreER^{T+}DCC$^{lox/lox}$ group to control for any indirect effect of tamoxifen. In addition, to prevent any gender difference, only males were used in this analysis.

Expression of DCC and netrin-1 in PNS myelin

As indicated above, PLP is highly expressed in the CNS, whereas its expression in the PNS is minimal. Schwann cells are the myelinating cells of the PNS, and in contrast to OLs that can myelinate several axon segments, they associate with only one axon segment. Nonetheless, myelin structure is morphologically similar in the CNS and PNS. However, protein and lipid compositions differ, which renders them differentially susceptible to myelin disorders (Quarles, 2005). To assess for a possible effect of the conditional knockout on peripheral myelin, we

first determine if DCC and netrin-1 were expressed in rat sciatic nerve. The teased sciatic nerve preparation allows us to follow and study independent nerve fibres. Immunostaining of teased rat sciatic nerve revealed paranodal enrichment of DCC and netrin-1 in the PNS (Figure 3-6). In addition to the paranodal localization, DCC appeared to be enriched in the Schwann cell microvilli. DCC expression by Schwann cells and localization to noncompact myelin in the PNS was recently described by another group (Webber et al., 2011).

We conclude that DCC and netrin-1 are enriched at paranodes both in the CNS and the PNS. Using conditional knockout mice to remove DCC expression from OLs, we could thus also affect peripheral myelin. In the PLPcreER^{T} line, Cre was shown to be expressed in some Schwann cells (Doerflinger et al., 2003). However, the age at which recombination is induced was shown to be critical when using the PLPcreER^{T+} mouse line. Before P16, the PLP promoter is active in Schwann cells and some neurons; however, after 3 week of age, the PLP promoter induces Cre expression exclusively in OLs (Michalski et al., 2011). In our experiments, mice are injected between 4.5 and 6 weeks of age. In addition, examination of sciatic nerves of tamoxifen induced PLPcreER^{T+}ROSA26 did not reveal high levels of recombination in Schwann cells. Therefore, phenotypes observed in PLPcreER^{T+}DCC$^{lox/lox}$ will result from the absence of DCC in OLs, and not by the absence of DCC in Schwann cells.

DCC expression levels and turnover rate in PLPcreER^{T+}DCC$^{lox/lox}$ mice

Signaling downstream of DCC regulates OPC migration (Jarjour et al., 2003; Tsai et al., 2003) and process retraction (Rajasekharan et al., 2010) as well as process branching by mature OLs (Rajasekharan et al., 2009). To avoid disruption of these functions and to isolate DCC function in fully formed myelin, we induced recombination in mice between 4.5 and 6 weeks of age, when myelination is complete in the rodent CNS (Foran and Peterson, 1992; Hamano et al., 1998).

To confirm the knockout of DCC expression in OLs, we used western blots to assay lysates of microdissected cerebellum, a white matter rich region, two weeks after induction. At this time point, DCC protein levels did not differ between PLPcreER^{T+}DCC$^{lox/lox}$ and their control littermates. However, 2 months after induction, DCC protein levels decreased in PLPcreER^{T+}DCC$^{lox/lox}$, and remained low 8 months after induction (Figure 3-4C). Residual DCC protein detected in these samples likely resulted from protein expression by non-recombined OLs or by neurons, which express DCC in the adult CNS (Manitt et al., 2001; Manitt et al., 2004; Shatzmiller et al., 2008). Relatively slow turnover of proteins localized to paranodes has been reported previously (Hedstrom et al., 2008). In light of the apparent long half life of DCC in myelin and because we were interested in studying the effect of the absence of DCC on myelin maintenance, we chose in subsequent experiments to examine mice that were aged for at least 6 months after tamoxifen induction.

Maintenance of paranodal junctions and myelin architecture in PLPcreER^{T+}DCC$^{lox/lox}$ mice

In vitro, the absence of DCC expression was shown to cause a defect in the maintenance of paranodes, characterized by disorganization of paranodal ultrastructure (Jarjour et al., 2008). Paranodal ultrastructure was thus examined 6 months after induction of Cre expression in sagittal sections of optic nerve (Figure 3-7A). The degree of disorganization was assessed by scoring each paranode as previously described (Jarjour et al., 2008) according to the presence or absence of 4 faults: lack of transverse bands, lack of interloop densities, loops detached from the axon, or everted loops (Figure 3-7B).

Paranodes in PLPcreER^{T+}DCC$^{lox/lox}$ mice had more faults than in wildtype mice (Figure 3-7C). The majority of wildtype paranodes analyzed were normal, whereas the majority of PLPcreER^{T+}DCC$^{lox/lox}$ paranodes were scored as faulty, having one fault or more (Figure 3-7D). Of note, some wildtype control paranodes were mildly or severely disorganized, a phenomenon that occurs both randomly

and as a result of aging in normal animals (Mierzwa et al., 2010b; Shepherd et al., 2010). Strikingly, while 15% of control paranodes had detached glial loops, 52% of PLPcreER^{T+}DCC$^{lox/lox}$ paranodes had loops not contacting the axon (Figure 3-7E). The presence of glial loops not contacting the axon was the most prevalent type of fault in PLPcreER^{T+}DCC$^{lox/lox}$ paranodes (Figure 3-7A, arrowhead). In contrast, absence of DCC expression by OLs did not lead to a dramatic increase in the proportion of paranodes which displayed abnormal interloop densities or spacing between the loops (Figure 3-7E; 19% for controls vs 25% for PLPcreER^{T+}DCC$^{lox/lox}$), which suggests that DCC may regulate axo-glial interactions more than glial-glial interactions. Thus, the CNS of PLPcreER^{T+}DCC$^{lox/lox}$ mice exhibit paranodal defects 6 months after induction that can be detected by electron microscopy examination of optic nerve myelin.

Maintenance of paranodal domain organization in PLPcreER^{T+}DCC$^{lox/lox}$ mice

Caspr is an axonal protein, highly enriched at paranodes, which interacts with contactin in cis and with neurofascin-155 on the glial loop. Absence of DCC *in vitro* results in diffusion of Caspr along the axon and widening of Caspr immunoreactive domains at paranodes (Jarjour et al., 2008). This effect is hypothesized to result from disruption of axoglial contacts at the paranode, which allows unbound Caspr to diffuse along the axonal membrane.

To investigate the effect of abnormal paranodal ultrastructure on domain organization in PLPcreER^{T+}DCC$^{lox/lox}$ mice, Caspr domain length was measured in corpus callosum, cerebellum, spinal cord and optic nerve at both 6 months and 9 months (Figure 3-8A, B, C) after tamoxifen induction. Surprisingly, Caspr domain length was not affected 6 months after induction in any of the four regions analyzed (Figure 3-8D; cKO 6m). Thus, even if paranode ultrastructure was affected 6 months after induction, it did not translate into a measurable disorganization of the Caspr domain. In contrast, 9 months after induction, we detected a significant increase in the length of Caspr immunoreactivity in all

regions analyzed (Figure 3-8D; cKO 9m). In some cases, juxtaparanodal K^+ channels (Kv1.2) were found leaking into the paranode (Figure 3-8B; white arrowhead), or Caspr was seen to invade the nodal region (Figure 3-8C; white arrowhead). This phenotype is similar to what was observed *in vitro* in long term $DCC^{-/-}$ slices (Jarjour et al., 2008). These results, together with the electron microscopy observations, indicate that the absence of DCC expression in OLs leads to a disorganization of paranodal ultrastructure that precedes paranodal domain disorganization. Hence, absence of DCC expression by OLs leads to a progressive disruption of the juxtaparanodal/paranodal/nodal domain integrity.

Abnormalities in compact myelin architecture and protein content in $PLPcreER^{T+}DCC^{lox/lox}$ mice

Next, we investigated whether the paranodal defects seen in $PLPcreER^{T+}DCC^{lox/lox}$ were accompanied by defects in compact myelin. We first examined myelin architecture by looking at optic nerve cross sections by electron microscopy. In cross sections of optic nerves from $PLPcreER^{T+}DCC^{lox/lox}$ mice six months after tamoxifen induction, we observed a substantial increase in myelin outfoldings (Figure 3-9A, arrows). This observation suggests that in addition to paranodal defects, compact myelin exhibits abnormalities in $PLPcreER^{T+}DCC^{lox/lox}$ mice.

Destabilization of myelin and disruption of juxtaparanodal, paranodal and nodal domain integrity could lead to alterations in myelin protein content. Alternatively, the absence of DCC could affect other myelin protein levels. We thus looked at myelin protein content in $PLPcreER^{T+}DCC^{lox/lox}$ mice and controls. To biochemically assay myelin protein expression, we microdissected the cerebellum of mice 7-9 months after induction and looked at the expression of compact myelin proteins (MBP, PLP), proteins associated with non-compact OL membranes (CNP, MAG), and paranodal proteins (Caspr, neurofascin). Each $PLPcreER^{T+}DCC^{lox/lox}$ was compared to its control littermate and the fold change of protein expression was analyzed. MBP and MAG levels were significantly

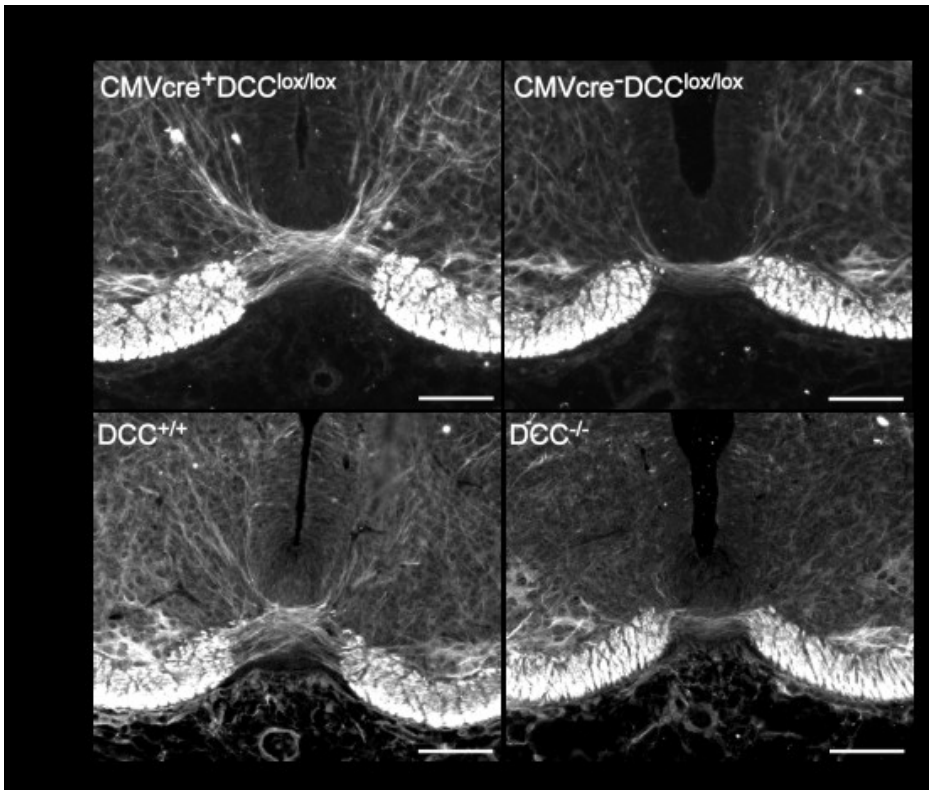
decreased in PLPcreER^{T+}DCC$^{lox/lox}$ mice, while paranodal and nodal proteins Caspr and neurofascins were not affected. The mean values of PLP levels were variable but mostly decreasing, although this change was not significant (Figure 3-9C).

Changes in the level of compact myelin proteins like MBP suggest alterations of compact myelin. Therefore, we examined myelin thickness to assess for any differences in compact myelin. However, the analysis of G-ratio (axon diameter/fiber diameter) in control and PLPcreER^{T+}DCC$^{lox/lox}$ mice did not reveal a significant change in the thickness of myelin sheaths in the absence of DCC expression by OLs (Figure 3-9B). To assess for a possible loss of OLs in PLPcreER^{T+}DCC$^{lox/lox}$ that could account for the alterations in myelin protein content, cells counts were performed in the optic nerve and spinal cord. The number of Olig2/CC1 double positive cells did not change in either region 9 months after induction (Figure 3-9D-F), suggesting that the loss of DCC expression by OLs did not affect their survival.

PLPcreER^{T+}DCC$^{lox/lox}$ mice develop a coordination deficit

Most myelin and paranodal protein mutants exhibit varying degrees of motor deficits (Bhat et al., 2001; Boyle et al., 2001; Mierzwa et al., 2010b). PLPcreER^{T+}DCC$^{lox/lox}$ mice did not exhibit an overt behavioural phenotype. Nonetheless, we tested the mice for behavioural deficits 6 months after tamoxifen induction. When assayed using the open field test, PLPcreER^{T+}DCC$^{lox/lox}$ mice exhibited normal motility. In a 2 hour test period, they walked a mean distance that was not significantly different from their control counterparts (Figure 3-10A; n=7 for the control group, n=8 for the knockout group) and no difference in speed of walking or time spent walking was detected (data not shown). Muscle strength assessed with the grip test was also normal in PLPcreER^{T+}DCC$^{lox/lox}$ mice when compared to controls (Figure 3-10B; n=20 for the control group, n=19 for the knockout group). However, at 6 months after induction, PLPcreER^{T+}DCC$^{lox/lox}$ mice exhibited balance and coordination deficits when tested with the balance

beam. This test measures the time taken by a mouse to cross a narrow beam and reach an enclosed safety platform. PLPcreER^{T+}DCC$^{lox/lox}$ mice took more time to cross the balance beam than their wild type littermates (Figure 3-10D; n=11 for both groups). Yet, when tested one month after induction, PLPcreER^{T+}DCC$^{lox/lox}$ mice performed as well as controls in this test (Figure 3-10C; n=8 for the control group, n=10 for the knockout group). This difference suggests a progressive loss of coordination due to the loss of DCC expression in OLs. To confirm the results obtained with the balance beam, PLPcreER^{T+}DCC$^{lox/lox}$ mice were then tested on the rotarod, another behavioural test sensitive to motor coordination and balance. PLPcreER^{T+}DCC$^{lox/lox}$ mice also exhibited decreased motor performance when tested on an accelerating rotarod 6 months after induction (Figure 3-10E; n=9 for the control group, n=11 for the knockout group). PLPcreER^{T+}DCC$^{lox/lox}$ mice had a significantly lower latency to fall compared to their control littermates. Together, these results show that absence of DCC expression in OLs leads to the development of motor coordination and balance deficits.


CMVcre+DCClox/lox
CMVcre-DCClox/lox
DCC+/+
DCC-/-

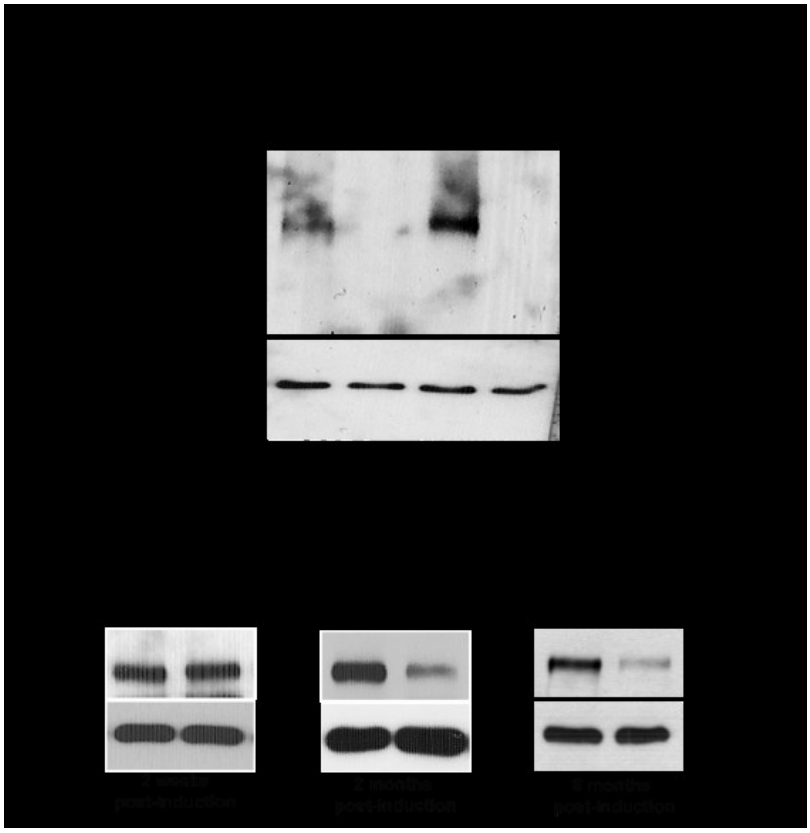


FIGURE 3-4: Validation of mouse lines. A, B) Cre-mediated recombination of the floxed DCC allele ablates expression of a functional DCC protein. $DCC^{lox/lox}$ mice were bred to CMVcre mice to induce ubiquitous recombination and embryos were collected at E14. **A)** $CMVcre^{+}DCC^{lox/lox}$ embryos have a thinner spinal cord ventral commissure compared to littermate controls at E14. A similar phenotype was observed in E14 $DCC^{-/-}$ spinal cord. The commissure is visualized by staining with NFM antibodies. Scale bar = 100 μm. **B)** No DCC protein was detected in brain lysates of E14 $CMVcre^{+}DCC^{lox/lox}$ and $DCC^{-/-}$ embryos. **C)** DCC protein has a slow turnover rate in myelinating OLs. To assess DCC protein expression after tamoxifen induction, cerebellar lysates were compared for their expression of DCC. Two weeks after induction, no differences in DCC protein levels were detected. Two months after induction, a reduction in DCC protein levels was observed, which could still be observed 8 months after induction.

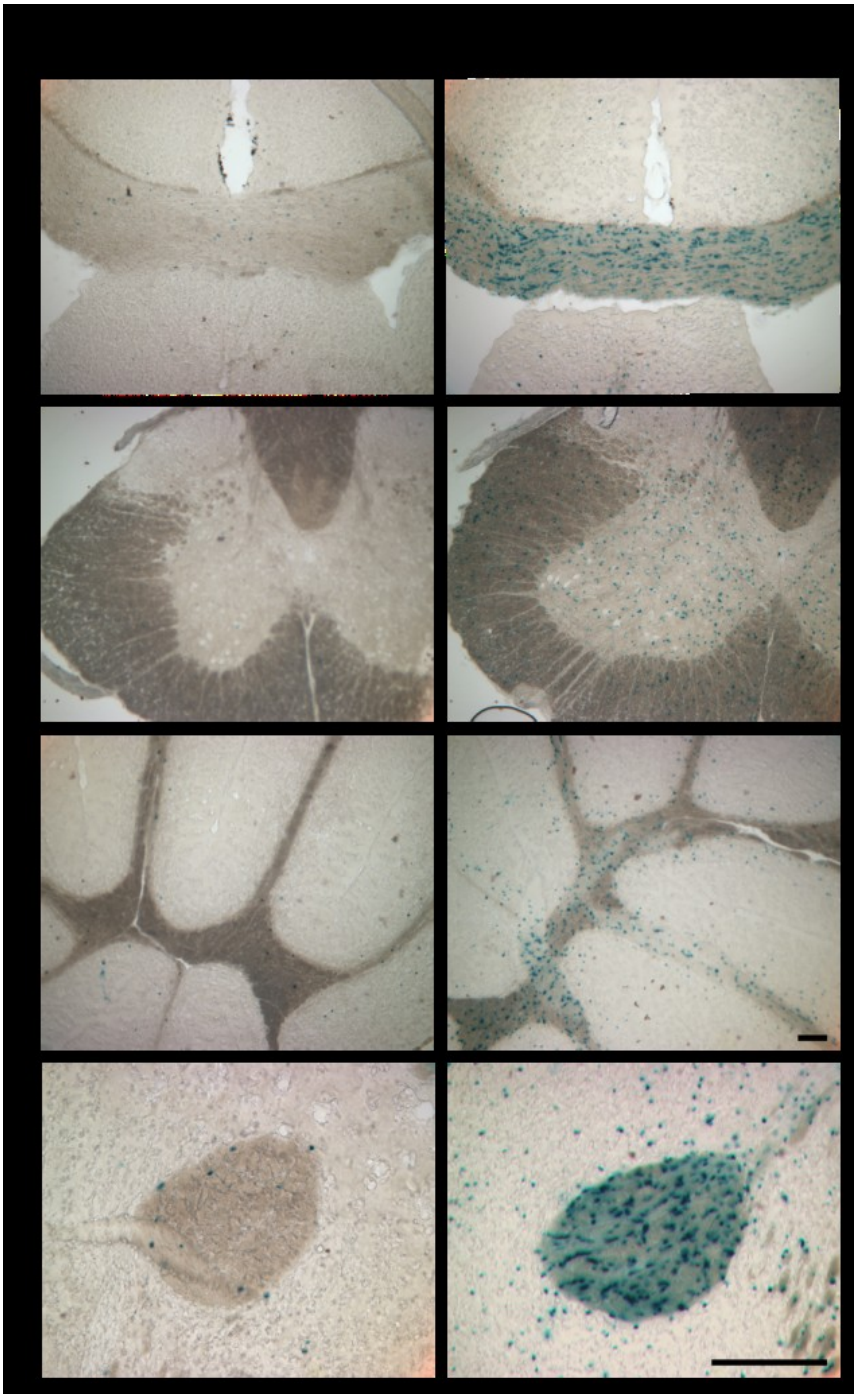


FIGURE 3-5: Validation of the tamoxifen induction protocol. Validation of our induction protocol was performed by crossing PLPcreER^{T} mice to ROSA26 mice. Injection with tamoxifen (1 mg intraperitoneal; twice a day for five consecutive days) into PLPcreER^{T+}ROSA26 mice revealed successful Cre recombination as revealed by β-galactosidase expression. 10X objective for all except anterior commissure (40X). Scale bar = 200 μm.

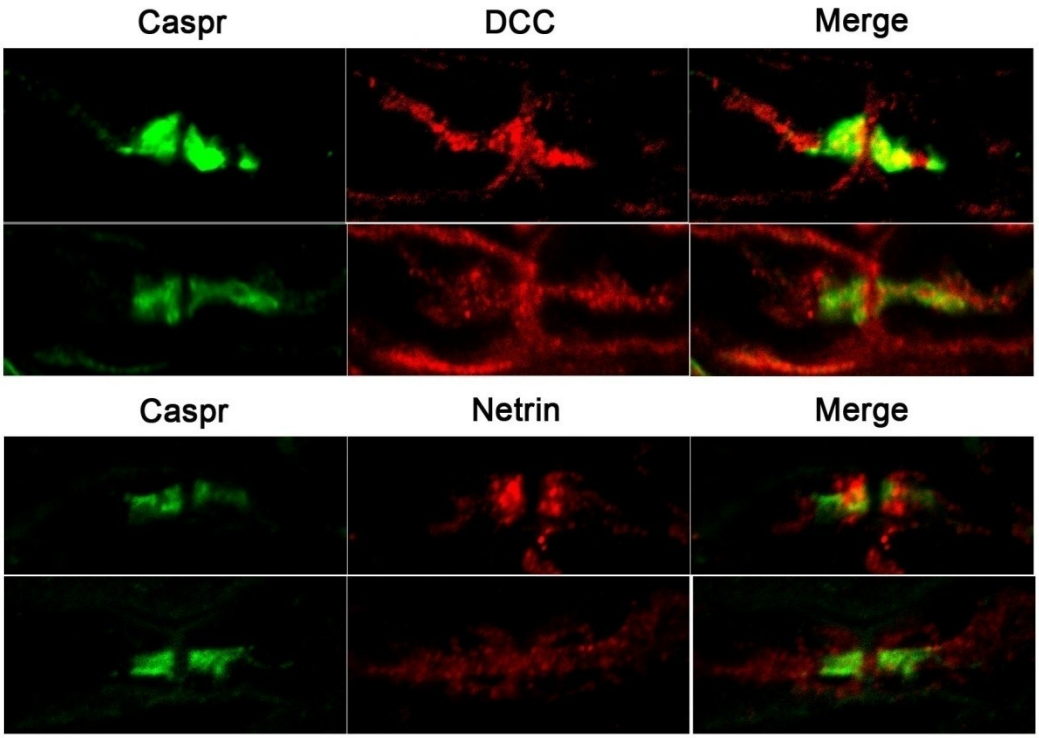


FIGURE 3-6: Expression of DCC and netrin-1 at the PNS paranode. Teased sciatic nerve fibres were immunostained with antibodies against Caspr, as well as DCC or netrin-1. Top panel: DCC expression was localized at the paranode, and possibly to the Schwann cell microvilli. Bottom panel: Netrin-1 expression was variable, but always present at the paranode. All panels: 100X objective, digital zoom 4

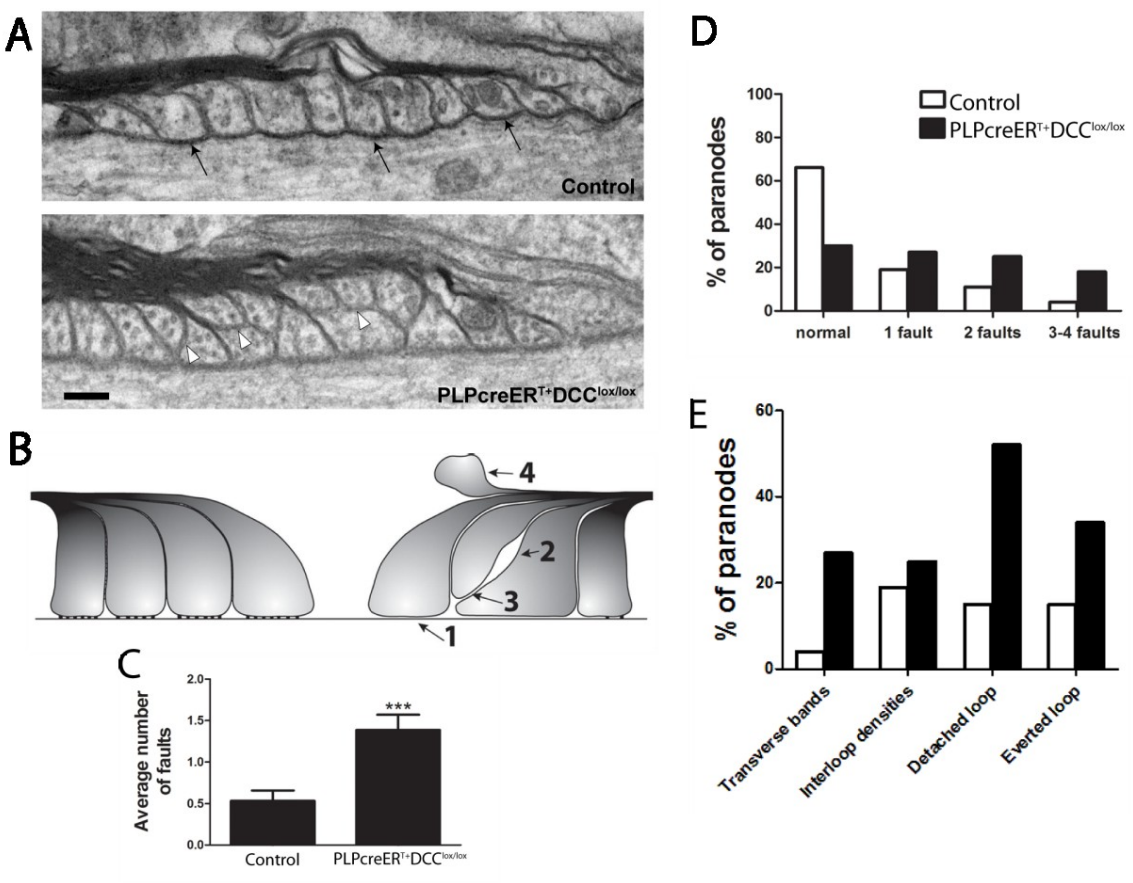
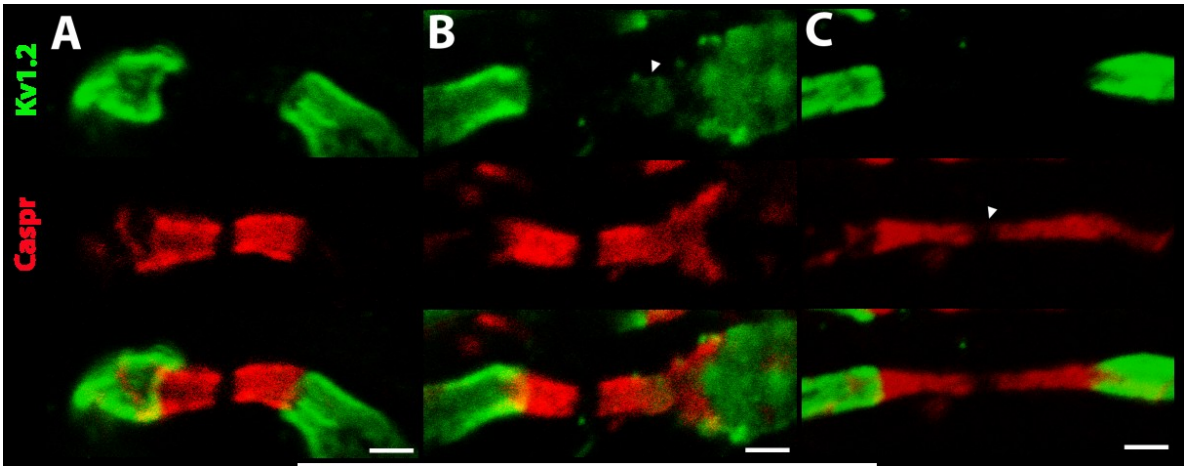
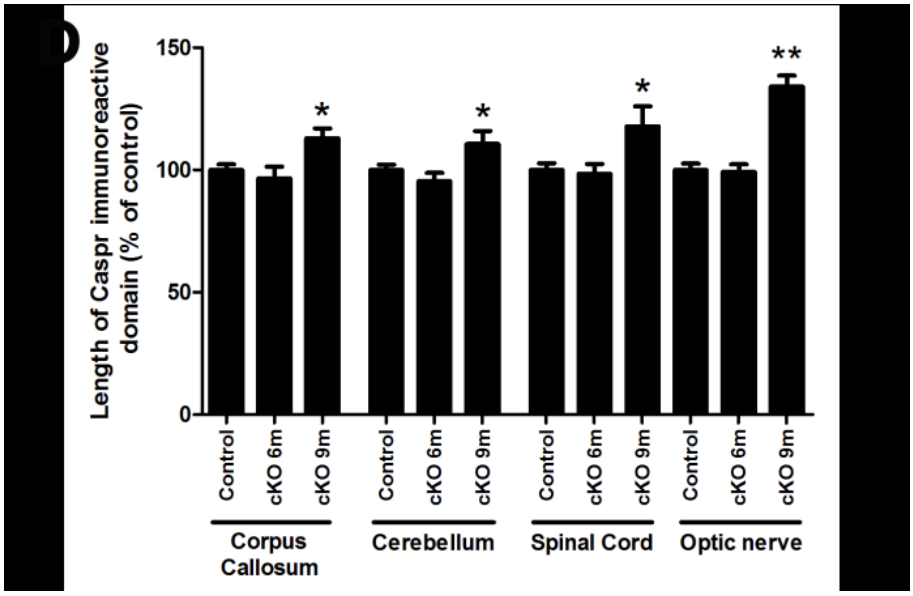


FIGURE 3-7: Paranodal architecture becomes disrupted in PLPcreER^{T+}DCC$^{lox/lox}$ mice. **A)** Example of paranodes in wildtype and PLPcreER^{T+}DCC$^{lox/lox}$ optic nerve. The majority of control paranodes exhibited normal loops contacting the axon and visible transverse bands (black arrows), whereas the majority of knockout paranodes were abnormal. The depicted knock out paranode shows loops detached from the axon (white arrowheads). Scale bar = 0,2 µm **B)** Paranodes were scored according to 4 faults: absence of transverse bands (1), absence of interloop densities (2), detached loops (3) and everted loops (4). **C)** At 6 months after induction, PLPcreER^{T+}DCC$^{lox/lox}$ paranodes display more faults per paranode than the control paranodes. **D)** The majority of wildtype paranodes are normal while the majority of PLPcreER^{T+}DCC$^{lox/lox}$ paranodes are abnormal, bearing one fault or more. **E)** Prevalence of each type of fault in PLPcreER^{T+}DCC$^{lox/lox}$ paranodes compared with controls. Three animals of each genotype were analyzed. (***: p=0,0002; two tailed Student's T test).


A
B
C
Kv1.2
Caspr


D
Length of Caspr immunoreactive domain (% of control)
150
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50
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Control
cKO 6m
cKO 9m
Corpus Callosum
Cerebellum
Spinal Cord
Optic nerve

FIGURE 3-8: Progressive paranodal domain disorganisation in PLPcreER^{T+}DCC$^{lox/lox}$ mice. Paranodal domain organization was assessed by looking at the length of the paranodal marker Caspr. Examples of spinal cord paranodes seen in control **(A)** and PLPcreER^{T+}DCC$^{lox/lox}$ **(B, C)** mice 9 months after tamoxifen induction are depicted. Paranodal domains are visualized with Caspr antibodies and juxtaparanodal domains are visualized with potassium channel (Kv1.2) antibodies. Some paranodes displayed Caspr leaking out of the paranodes, Kv1.2 leaking into the paranodes (B; white arrowhead) or widening of Caspr immunoreactive domains and Caspr diffusion into the node (C; white arrowhead). **D)** Length of Caspr immunoreactive domain was quantified in different regions of the CNS. Caspr immunoreactive domains are similar to controls 6 months after induction of PLPcreER^{T+}DCC$^{lox/lox}$ mice (cKO 6m), but disorganized 9 months after tamoxifen induction (cKO 9m) in the corpus callosum, cerebellum, spinal cord and optic nerve. Three animals were analyzed per group and compared to their littermate controls. Scale bar = 2 μm (*: $p<0,05$; **: $p<0,01$; Tukey's test).

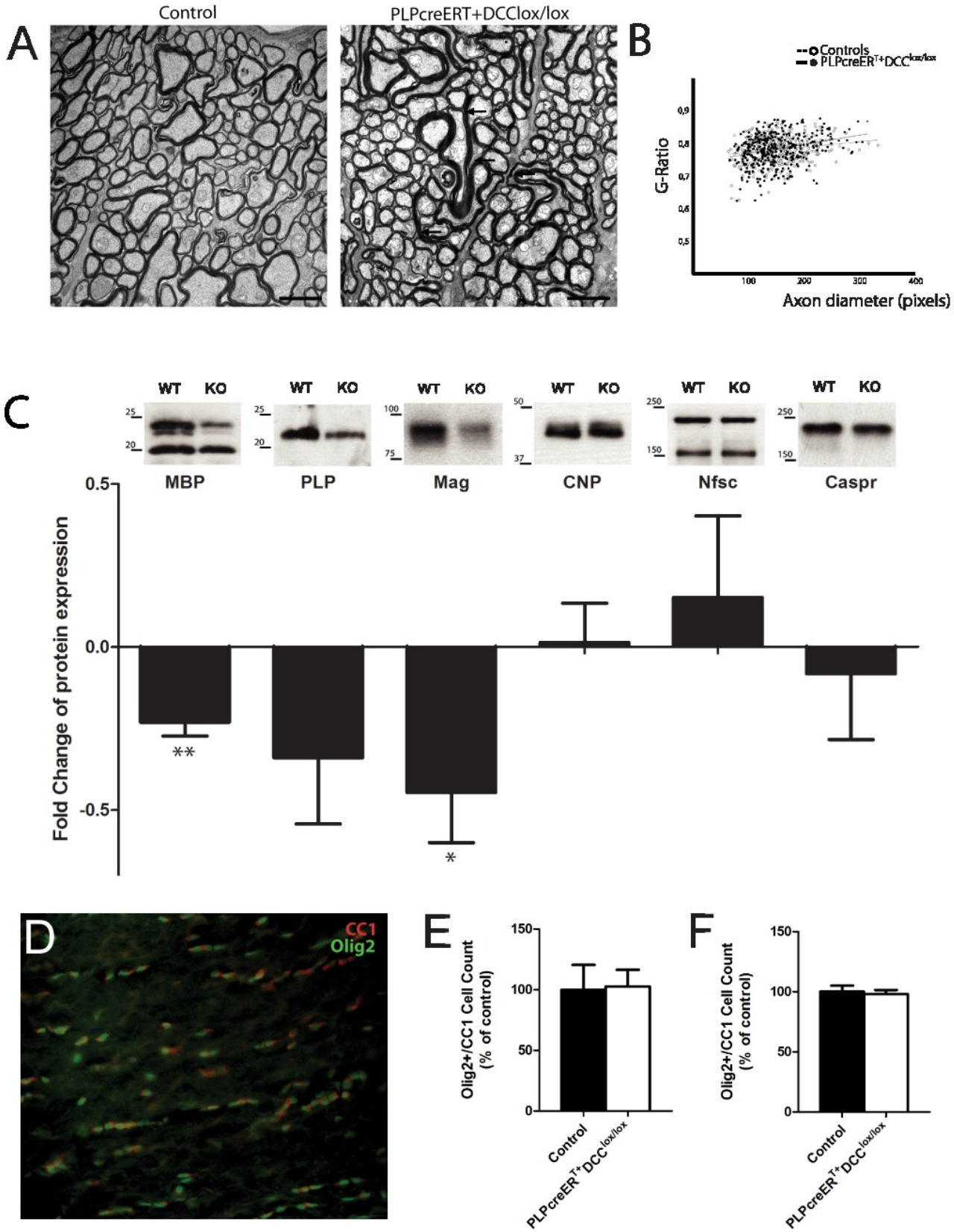
A
Control
PLPcreERT+DCClox/lox
B
Controls
PLPcreER^{T+}DCC$^{lox/lox}$
G-Ratio
Axon diameter (pixels)
C
WT KO
MBP
PLP
Mag
CNP
Nfsc
Caspr
Fold Change of protein expression
**
*
D
CC1
Olig2
E
Olig2+/CC1 Cell Count (% of control)
Control
PLPcreER^{T+}DCC$^{lox/lox}$
F
Olig2+/CC1 Cell Count (% of control)
Control
PLPcreER^{T+}DCC$^{lox/lox}$

FIGURE 3-9: Abnormalities in compact myelin architecture and protein content. **A)** Cross sections of optic nerve were examined 6 months after tamoxifen induction of control and PLPcreER^{T+}DCC$^{lox/lox}$ mice. Numerous myelin outfoldings were observed in PLPcreER^{T+}DCC$^{lox/lox}$ myelin (black arrows). Scale bar =2 μm **B)** PLPcreER^{T+}DCC$^{lox/lox}$ did not exhibit any changes in their myelin G-ratios compared to controls. G-ratios (ratio between the diameter of the axon and the thickness of its myelin sheath) were calculated in optic nerves of mice between 6 and 9 months after tamoxifen induction and are plotted against axon diameter. 6 animals of each genotype were used for this analysis. No effect of age was detected. **C)** Decreased levels of MBP and MAG were observed in PLPcreER^{T+}DCC$^{lox/lox}$ compared with their control littermates 7-9 months after induction. Levels of CNP, Neurofascin (Nfsc) and Caspr were unaffected. Examples of immunoblots for each protein are shown along with the fold change of protein expression for each protein. 5 pairs of brothers were analyzed (**: $p<0,01$, *: $p<0,05$; One sample t test). **D)** The numbers of Olig2/CC1 positive OLs was counted in different regions 9 months after induction to assess for any loss of OLs. The number of OLs was not affected either in the spinal cord **(E)** or the optic nerve **(F).** 3 pairs of brothers were analyzed.

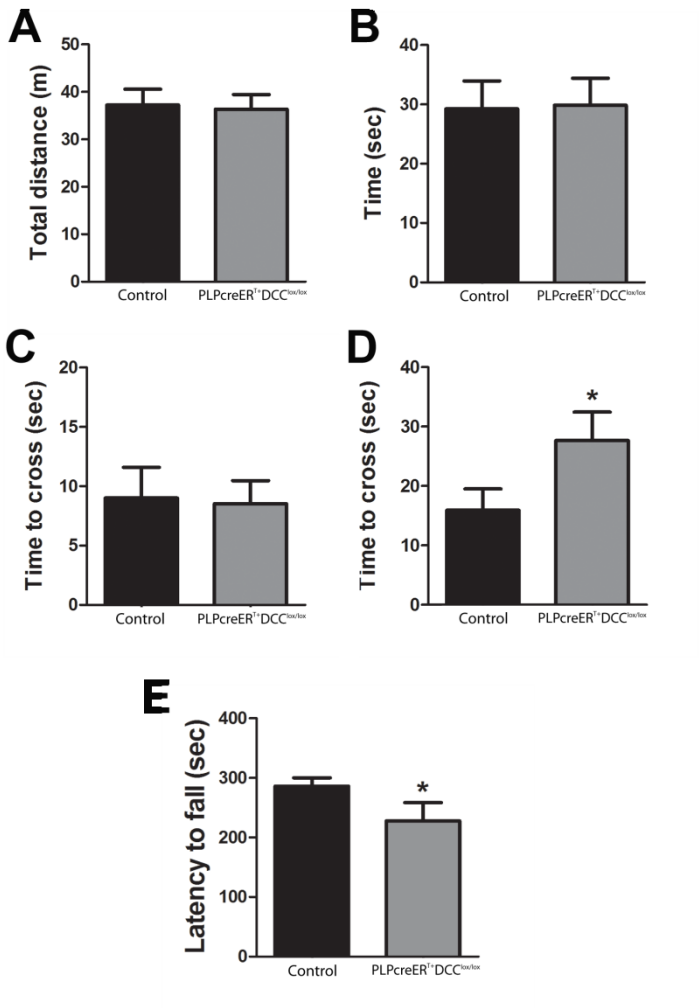

FIGURE 3-10: PLPcreER^{T+}DCC$^{lox/lox}$ mice develop a balance and coordination deficit. Different aspects of locomotion were tested 6 months after tamoxifen induction. **A)** Gross motility was normal in PLPcreER^{T+}DCC$^{lox/lox}$ mice as tested in the open field test (total distance travelled in 2 hrs testing session). **B)** Motor strength was also similar to their control littermates, as tested with the hanging wire grip test. **C)** PLPcreER^{T+}DCC$^{lox/lox}$ mice exhibited balance and motor coordination impairment 6 months after induction, as tested in the balance beam test. **D)** Motor coordination and balance are normal 1 month after induction, which indicates that the coordination phenotype develops progressively. **E)** PLPcreER^{T+}DCC$^{lox/lox}$ exhibited decreased motor performance 6 months after induction when tested on an accelerating rotarod. (*: $p<0.05$; one tail T test)

DISCUSSION AND CONCLUSION

Our findings indicate that DCC expression by OLs is required for the maintenance of axoglial junctions *in vivo*. Both by intravitreal transplantation of OPCs derived from conventional DCC knockout mice and by examining inducible conditional DCC knockout mice, I have shown that selective loss of DCC from mature OLs disrupts myelin and paranode ultrastructure and causes a progressive disorganization of paranodal domains along axons. Furthermore, loss of DCC function from OLs leads to alterations in compact myelin and defects in coordinated behaviour.

In aged PLPcreER^{T+}DCC$^{lox/lox}$, we described a decrease in MAG and MBP protein levels compared to controls. The cause of this alteration in myelin protein expression is unknown, but could be a consequence of a general instability of myelin in the absence of DCC. Myelin instability observed in PLPcreER^{T+}DCC$^{lox/lox}$ mice could be caused by an adhesion defect in absence of DCC. Netrin-1 and DCC mediate the formation of adhesive contacts in several tissues and organs (Baker et al., 2006). Loss of DCC expression by OLs could lead to adhesion defects between the OL and the axon both at the paranode and at the level of the internode. Of note, PLPcreER^{T+}DCC$^{lox/lox}$ paranodes displayed more paranodal loops detached from the axon, but the interaction between the loops did not seem to be affected. It is possible that disruption of DCC expression causes adhesion defects between the glial loops and the axon, but it would not be involved in adhesion between the glial loops themselves. Absence of DCC expression by OLs could thus lead to defective axo-glial adhesive contacts, and such defects could explain the myelin outfoldings, as well as the increased incidence of detached loops observed in the conditional DCC knockout mice.

The paranodal phenotype observed *in vivo* was not as severe as what was described *in vitro* in DCC knockout organotypic slices (Jarjour et al., 2008). This could be explained by different factors. First, the recombination efficiency

induced by tamoxifen administration is not one hundred percent (Doerflinger et al., 2003), which could dilute the phenotype observed. Second, normal ongoing remyelination could also occur. In this study, mice were induced only once with tamoxifen, so OLs that differentiated later in life would still express DCC. Not much is known about the degree of remyelination that occurs under normal conditions or if myelin turnover is frequent. Existence of newly formed OPCs in the adult nervous system has been described (Ffrench-Constant and Raff, 1986; McCarthy and Leblond, 1988), but the extent to which these cells contribute to continuous remyelination is unknown.

Deficits in the maintenance of myelin and intact axoglial junctions could lead to the development of pathological conditions. Myelin instability and damage have been proposed to underlie the age-related cognitive deficits observed in the elderly (Bartzokis, 2004). It was also proposed to constitute a predisposition to immune attacks, which could lead to the development of pathologies like MS (Mastronardi and Moscarello, 2005). Notably, paranodal disorganization was identified as an early sign of demyelination around MS lesions (Wolswijk and Balesar, 2003; Howell et al., 2006). Identifying factors that play a role in stability and maintenance of myelin is thus of tremendous importance to develop therapeutic strategies to prevent and treat these pathological conditions.

CHAPTER IV
GLIAL SCAR ASSOCIATED ASTROCYTES AND FIBROBLASTS EXPRESS NETRIN-1 IN THE INJURED MOUSE SPINAL CORD

The results included in this chapter are included in:

Baker KA, Bull SJ, David S, and Kennedy TE. Glial scar associated astrocytes and fibroblasts express netrin-1 in the injured mouse spinal cord. *Submitted to Journal of Neuroscience Research.*

PREFACE AND RATIONALE

As described in chapter I, the expression of netrin-1 and its receptors changes during development of the CNS. In the mature CNS, netrin-1 is expressed by neurons and myelinating OLs (Manitt et al., 2001; Jarjour et al., 2008; Rajasekharan et al., 2009), and has been proposed to be a myelin-associated inhibitor of axon regeneration following injury (Manitt et al., 2001; Manitt et al., 2006; Low et al., 2008). When present at lesion sites, netrin-1 was shown to inhibit axon regeneration, an effect mediated by axonal expression of UNC5 receptors (Low et al., 2008).

However, descriptions of netrin-1 expression patterns after spinal cord injury do not agree. In adult rat, netrin-1 expression decreases after dorsal myelotomy. Both netrin-1 mRNA and protein were reduced after injury and stayed below control levels for at least seven months post-injury (Manitt et al., 2006). In contrast, Wehrle et al. (2005) reported an increase in netrin-1 transcript and protein at the core of the glial scar eight days following spinal cord hemisections and cerebellar lesions in mice. By immunohistochemistry, they identified the glial scar associated microglia and macrophages as netrin-1 positive; however, they did not confirm that these cells were the source the netrin-1 at the injury site (Wehrle et al., 2005).

In this chapter, we aim to resolve these conflicting results, and to identify the source of netrin-1 after spinal cord injury, by using netrin-$1^{\beta geo/+}$ mice, which express a fusion reporter gene downstream of the netrin-1 promoter (Skarnes et al., 1995).

MATERIALS AND METHODS

Animals

Netrin-1$^{\beta geo/+}$ mice, generated by Skarnes et al., (1995) and further characterized by Serafini et al., (1996), were provided by Dr. Marc Tessier-Lavigne (Genentech, South San Francisco, CA). Sprague Dawley newborn pups were obtained from Charles River Canada (Montreal, Québec, Canada). All procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

Dorsal Hemisection Surgeries and Spinal Cord Processing

Six 8-week old male netrin-1$^{Bgeo/+}$ heterozygotes and two 8-week old male wild type littermates were anesthetized using a ketamine:xylazine:acepromazine (50:5:1 mg/kg) mixture. A laminectomy was performed at the T8 level and a dorsal hemisection made with a pair of microscissors. Animals were closely monitored after the surgery. Eight days following dorsal hemisections, mice were anaesthetized and then perfused with PBS and 4% PFA. Spinal cords were removed and post-fixed overnight in 4% PFA followed by cryoprotection in 30% sucrose in PBS. Four 5 mm segments of the spinal cords, 2 rostral and 2 caudal from the injury epicenter, were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), then rapidly frozen in liquid nitrogen-cooled isopentane and stored at -80°C prior to sectioning. Twenty micron thick sections were cut with a cryostat (Leica Microsystems; Richmond Hill, ON) and collected on Superfrost Plus electrostatic slides (Fisher Scientific Co., Ottawa, ON).

Mouse astrocyte isolation

To investigate netrin-1 expression by reactive astrocytes, mechanical injury was produced in cultures of confluent astrocytes and fibroblasts derived from a mix of netrin-1$^{Bgeo/+}$ mice and their wild type littermates (Barral-Moran et al., 2003). As netrin-1$^{Bgeo/Bgeo}$ mice die at birth (Skarnes et al., 1995), brains from both netrin-

$1^{Bgeo/+}$ and wild type littermates were pooled. Astrocyte/fibroblast cultures were obtained by removing microglia and oligodendrocyte precursors by shaking off the glial cultures on an orbital shaker overnight at 37°C (180 rpm). The cells were then trypsinized, and plated on 12 mm coverslips coated with 20 µg/ml PDL at a density of 100,000 cells/coverslip. The astrocyte enriched cultures were then grown in 10% FBS, 1% penicillin/streptomycin, 1% glutamine in DMEM. At confluence (~3 days), the media was replaced, with half of the wells receiving serum-free media. On the following day, mechanical injuries were produced by sliding a 22 gauge needle across the astrocyte monolayer twice, first vertically and then horizontally, as described (Barral-Moran et al., 2003). On the following day, cultures were fixed in 4% paraformaldehyde (PFA) for 15 min, rinsed with phosphate buffered saline (PBS) and processed for immunocytochemistry.

Immunocytochemistry

Cells were fixed with ice cold 4% PFA for 30 minutes, and rinsed with PBS thrice. After blocking for 1 hr at RT in 3% BSA, 0.1% triton X-100 in PBS, primary antibodies diluted in blocking solution were added, incubated overnight at 4°C, and then rinsed three times with PBS. Cells were then incubated with secondary antibodies for 1 hr at RT, before washing and mounting with Fluoromount-G (Southern Biotech).

Immunohistochemistry

Spinal cord sections were washed in PBS and then blocked in 3% BSA, 0.3% triton X-100 in PBS for 1 hr. Sections were incubated overnight in primary antibody diluted in blocking solution at 4°C. The next day, sections were rinsed with PBS before being incubated with the secondary antibodies diluted in 3% BSA in PBS for 1 hr at RT. Sections were then washed with PBS and coverslipped in GelMount anti-fading medium (Biomeda Corporation, Foster City, CA). Epifluorescent images were taken with a Magnafire CCD camera (Optronics, Goleta, CA) and an Axiovert 100 microscope (Carl Zeiss Canada, Toronto, ON). To assess intracellular localization of β-gal staining, z-stacks

images were captured using a Zeiss LSM 510 confocal microscope (Carl Zeiss Canada, Toronto, ON).

Antibodies

The following antibodies were used in this study: rabbit polyclonal anti-β-gal (3 Prime Inc., Boulder, CO), chicken polyclonal anti-vimentin (Encor Biotechnology, catalog #CPCA-Vim), mouse monoclonal anti-CD11b (Mac1; AbD Serotec, catalog #MCA2087), CY3-conjugated mouse anti-Glial fibrillary acidic protein (GFAP; Sigma-Aldrich, catalog #C9205), mouse monoclonal anti-CNP (Abcam; catalog #ab24566), and rat monoclonal anti-netrin-1 (R&D Systems, catalog #MAB1109).

Quantification of ß-gal expression after injury

Approximately 340 X 340 μm images of ß-gal staining were taken of the dorsal white matter (DWM) at 0.7 mm (injury site measurements) and 5.7 mm rostral and caudal to the lesion. Quantification was performed by a blinded experimenter using ImageJ software (National Institutes of Health, Bethesda, MD) to measure the relative area (in arbitrary units) of ß-gal immunopositivity within the field of interest. A non parametric one-way analysis of variance followed by a Wilcoxon test were used to compare paired values of injury site and control values of 5.7 mm distal to the site in the six netrin-$1^{\beta geo/+}$ injured mice.

RESULTS

To identify cells that express netrin-1 in the adult mouse spinal cord following injury, we utilized a mouse line carrying a gene trap vector (pGT1.8TM) encoding β-gal inserted into the netrin-1 gene. Mice carrying the inserted transgene express a chimeric netrin-1/β-gal fusion protein regulated by the endogenous netrin-1 promoter (Skarnes et al., 1995; Serafini et al., 1996). The protein chimera consists extracellularly of domain VI and the first EGF-like repeat of domain V of netrin-1, followed by a transmembrane domain encoded by the inserted transgene and an intracellular ß-gal, fused to neomycin phosphotransferase (ß-geo). Mice homozygous for this insertion, netrin-$1^{Bgeo/Bgeo}$, are severe netrin-1 hypomorphs and die within a few hours of birth. They exhibit a severe neural phenotype that is consistent with axon guidance errors produced by loss of netrin-1 function (Serafini et al., 1996). In contrast, mice heterozygous for the insertion, netrin-$1^{Bgeo/+}$, develop to adulthood and exhibit no identified abnormal phenotype (Serafini et al., 1996; Braisted et al., 2000; Jarjour et al., 2003; Shatzmiller et al., 2008).

Netrin-1 expression is upregulated at the site of injury

To investigate netrin-1 expression after spinal cord injury, dorsal hemisections were performed in netrin-$1^{\beta geo/+}$ heterozygous mice. Two wild-type and six heterozygous 8-week old male mice received dorsal hemisection injuries. To correspond to the time point used previously by Wehrle et al. (2005), we chose to examine netrin-1 expression eight days after spinal cord injury. Mice were perfused eight days after injury and β-gal immunoreactivity was used to identify netrin-1 expressing cells at the injury site.

Immunostaining revealed punctate β-gal expression by cells within the injury epicenter associated with the glial scar (Figure 4-1A-C; Figure 4-2A-D). Immunostaining for β-gal did not entirely correspond with that of netrin-1 (Figure 4-1A-C), likely because netrin-1 is secreted from netrin-producing cells whereas

β-gal expression is seen as punctuate intracellular staining (Figure 4-1A-C; G-I; 4-4A-H; 4-5A-D) or in association with the cell membrane (Figure 4-1D-F). This pattern of β-gal staining was previously described in these mice (Shatzmiller et al., 2008). β-gal expression by large cells in ventral gray matter (Figure 4-1D-F) and CNP-positive cells in the white matter (Figure 4-1G-I) neighboring the site of injury were also detected, corresponding to motor neurons and OLs, as previously described (Manitt et al., 2001; Wehrle et al., 2005; Manitt et al., 2006).

The increased β-gal expression associated with the injury area was not found in dorsal sections located more than 5 mm rostral (Figure 4-2E, F) or caudal (not shown) to the injury epicenter. In tissue sections with an intact central canal, β-gal expression was detected in the ependymal cells surrounding the central canal (Figure 4-2E), consistent with previous findings in rodents and lamprey (Petit et al., 2007; Shifman and Selzer, 2007; Low et al., 2008). β-gal immunostaining was not observed in sections of injured wild-type spinal cord, demonstrating the specificity of the β-gal antibody (Figure 4-2G, H). The area of ß-gal immunostaining in the dorsal white matter (DWM) (Figure 4-3A) was determined in transverse sections 5.7 and 0.7 mm rostral and caudal to lesion site. Very little ß-gal immunostaining was observed in the DWM 5.7 mm rostral and caudal to the lesion. However, a significant increase in the area of ß-gal immunopositive staining was seen within 0.7 mm of the lesion site (Figure 4-3B; $p<0.05$).

Netrin-1 is expressed by fibroblasts and few astrocytes in the injured spinal cord

Using *in situ* hybridization and immunohistochemistry, Wehrle et al. (2005) reported widespread netrin-1 expression by cells in the glial scar eight days following injury. Double labeling demonstrated that microglia/macrophages (identified by isolectin B4 staining) were netrin-1 immunoreactive; but it was not determined if these cells were the source of netrin-1. Here, using the netrin-1$^{\beta geo/+}$ reporter mice, we identified netrin-1 expressing cell types at the site of injury. Double immunofluorescence labelling using Mac-1, an antibody specific for

microglia/macrophages (Malhotra et al., 1986), and an antibody against β-gal was performed. Use of the Mac-1 antibody was shown to produce more consistent microglia staining than IB4 staining (Goings et al., 2006). Many Mac-1 positive microglia/macrophages were readily detected at the site of injury, and β-gal immunoreactivity was detected in close proximity to these cells; however, analysis of stacks of serial confocal images did not detect co-localization of Mac-1 and β-gal immunoreactivity, suggesting that microglia/macrophages are not a source of netrin-1 expression at an injury site in mouse spinal cord (Figure 4-4A, B).

Fibroblasts can be distinguished from astrocytes by expression of vimentin but not GFAP, whereas resting astrocytes express GFAP but not vimentin, and reactive astrocytes express both vimentin and GFAP (Conrad et al., 2005). Triple-staining for β-gal, vimentin and GFAP revealed vimentin+/GFAP- fibroblasts in the dorsal portion of the spinal cord near the injury epicenter, surrounded by a dense network of vimentin+/GFAP+ reactive astrocytes and their processes. Confocal image analysis revealed expression of β-gal by vimentin+/GFAP- fibroblasts (Figure 4-4C, D). β-gal immunoreactivity, indicating netrin-1 expression, was also associated with some vimentin+/GFAP+ reactive astrocytes near the injury site (Figure 4-4E, F). Notably, previous studies have shown that netrin-1 was not expressed by resting or reactive astrocytes in the injured rat spinal cord (Manitt et al., 2006; Löw et al., 2008). Consistent with Manitt et al., (2006), we did not detect netrin-1 expression by vimentin-/GFAP+ resting astrocytes in the ventral white matter of the mouse spinal cord, although some reactive vimentin+/GFAP+ astrocytes expressed β-gal at the injury site.

Netrin-1 expression by fibroblasts and reactive astrocytes *in vitro*

To further verify the expression of netrin-1 by astrocytes and fibroblasts, we examined mixed-glial cultures derived from the brains of newborn wild-type and heterozygous mouse pups. Cells were grown to confluence, at which point a mechanical injury was created by scratching the surface with a needle tip.

Immunostaining revealed astrocytes growing on a layer of fibroblasts. Consistent with our findings *in vivo,* β-gal immunoreactivity was detected in vimentin+/GFAP- fibroblasts (Figure 4-5A, B), and also associated with vimentin+/GFAP+ astrocytes (Figure 4-5C, D). This immunoreactivity persisted, regardless of the presence (Figure 4-5A, C) or absence (Figure 4-5B, D) of serum in the culture media. A small number of Mac-1^{+} microglia/macrophages remained in the primarily astrocyte/fibroblast-rich cultures. Consistent with our findings *in vivo*, β-gal immunoreactivity, indicative of netrin-1 expression, was not detected in these cells.

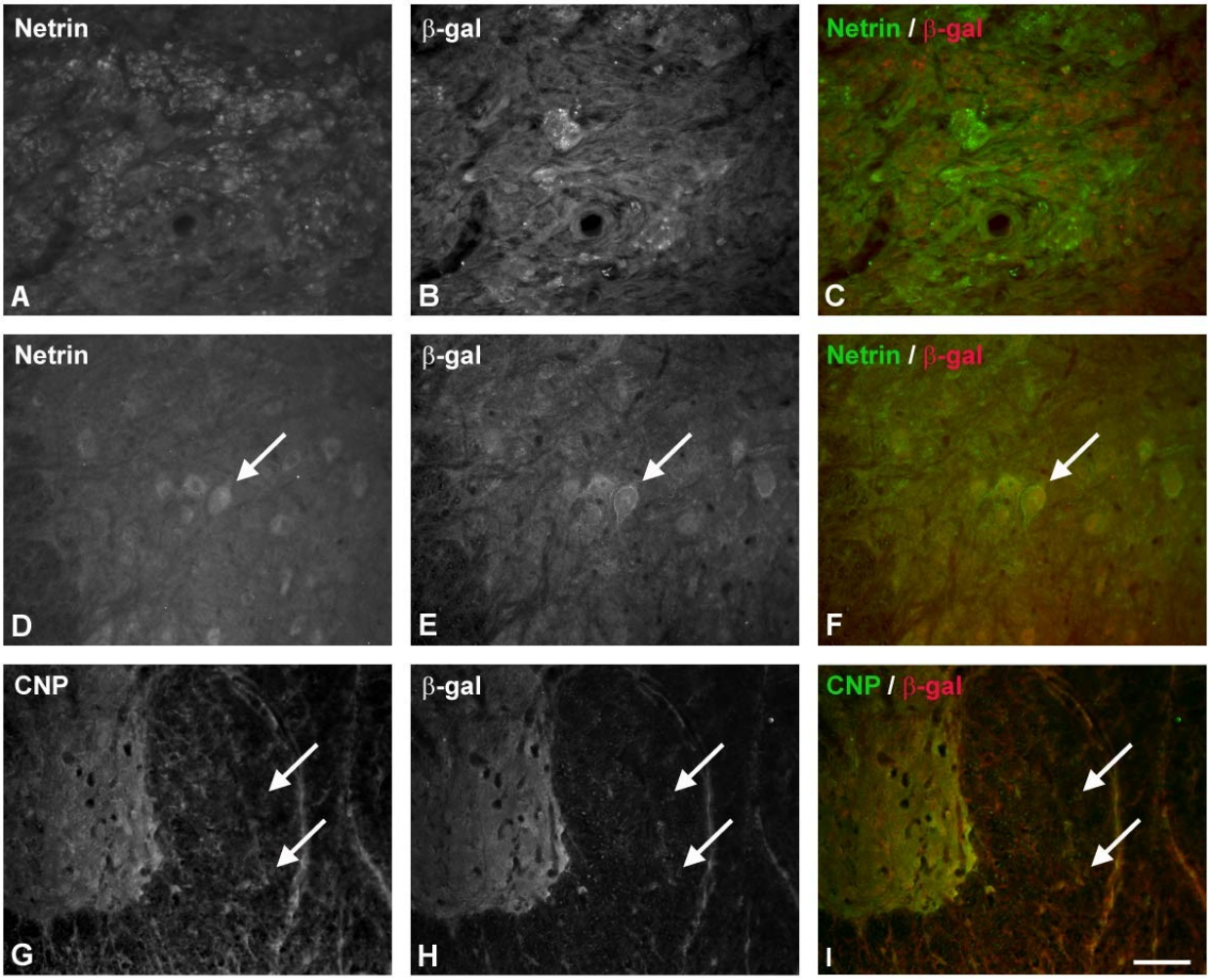

FIGURE 4-1: Examples of β-gal immunostaining in netrin-1$^{Bgeo/+}$ mouse thoracic spinal cord 8 days after dorsal hemisections. A-C) Immunostaining for netrin-1 (red) and β-gal (green) showed some overlap at the injury site; however, netrin-1 expression was more widespread than β-gal expression. **D-F)** The netrin-1-β-gal protein chimera consists of a transmembrane domain encoded by the inserted transgene and is primarily found intracellularly or associated with the cell membrane. The typical distribution of β-gal immunoreactivity within a cell is apparent in the large cells in the ventral horn, likely motor neurons **(arrow)**. **G-I)** β-gal immunostaining is also associated with CNP-positive OLs **(arrows)**. Scale bar: 50 μm

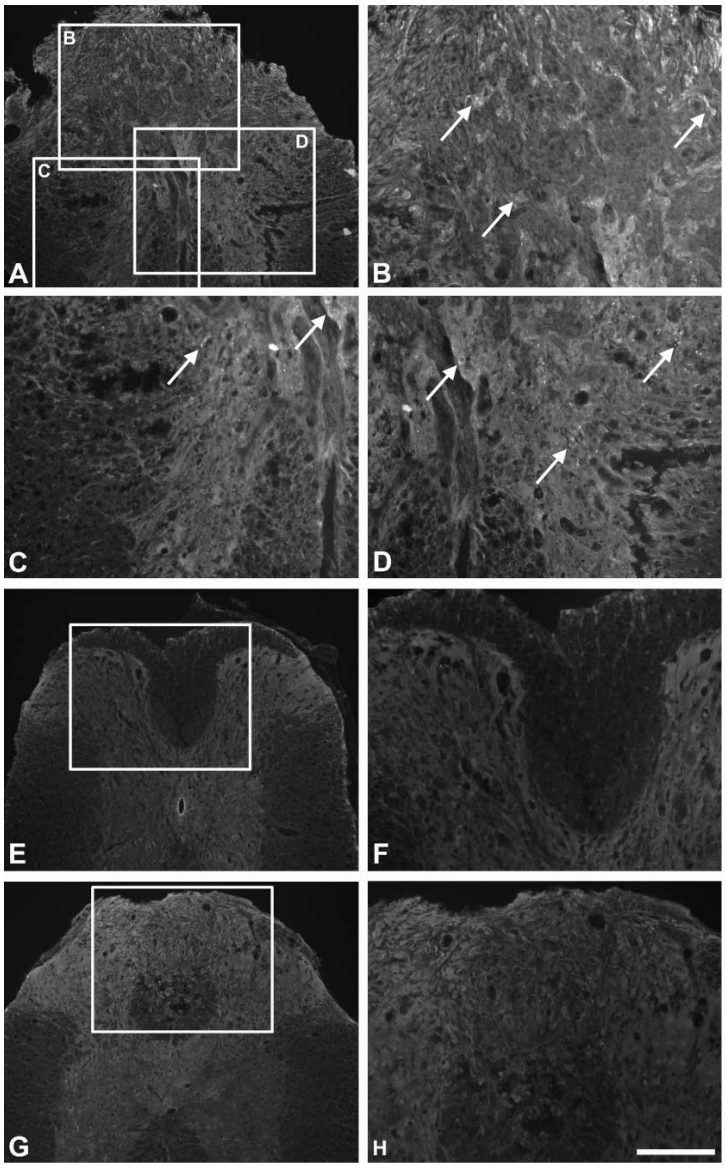

FIGURE 4-2: β-gal immunoreactivity in a transverse section of injured netrin-1$^{Bgeo/+}$ mice thoracic spinal cord. **A-D)** Intracellular β-gal immunoreactivity (arrows), labeling cells expressing netrin-1, is present at the injury site. **B-D)** Higher power photomicrographs of the boxes in A. **E,F)** β-gal immunostaining is not as extensive 5-6 mm rostral to the injury site, consistent with staining in the uninjured mouse and rat spinal cords (Manitt et al., 2001). **G,H)** In wild-type mice, no β-gal immunoreactivity was detected at the site of injury. All images are oriented similarly, with the top corresponding to the dorsal side of the spinal cord. Scale bar in B-D, F, H corresponds to 300 μm, and in A, E, G to 150 μm.

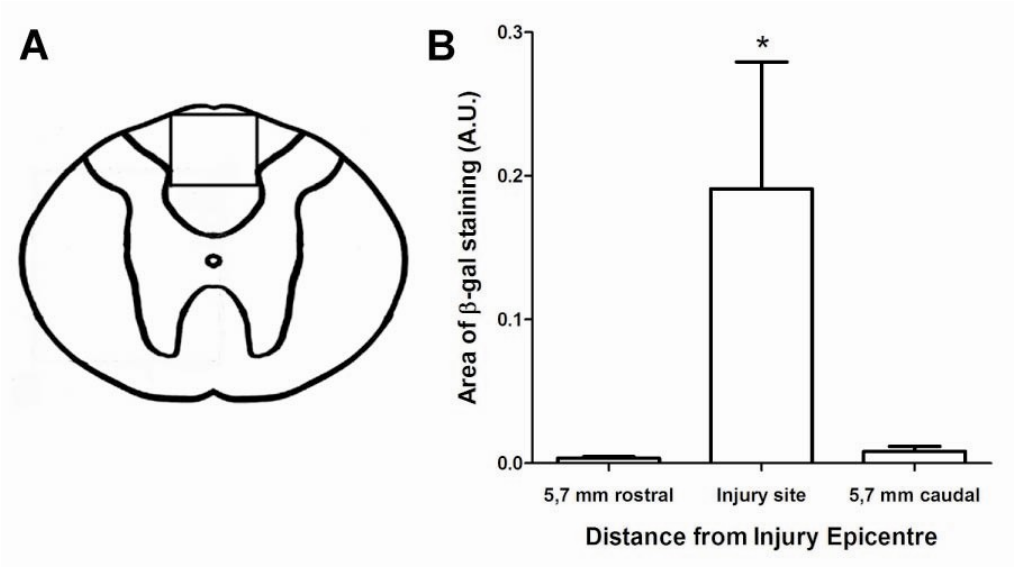

FIGURE 4-3: Quantification of β-gal immunostaining in the dorsal white matter of the injured mouse spinal cord. **A)** A schematic representation of a transverse section through the spinal cord showing the region in which the extent of β-gal staining was quantified is depicted. **B)** The area (in arbitrary units (A.U.); + standard error of the mean) of β-gal immunostaining was determined at 5.7 mm and 0.7 mm rostral and caudal of the injury epicenter. The values 0.7 mm rostral and caudal to the epicenter were averaged to provide a representative value of the area of β-gal staining surrounding the lesion area. A significant increase in β-gal staining was detected adjacent to the lesion as compared to 5.7 mm rostral or caudal to the lesion site. ($p<0.05$)

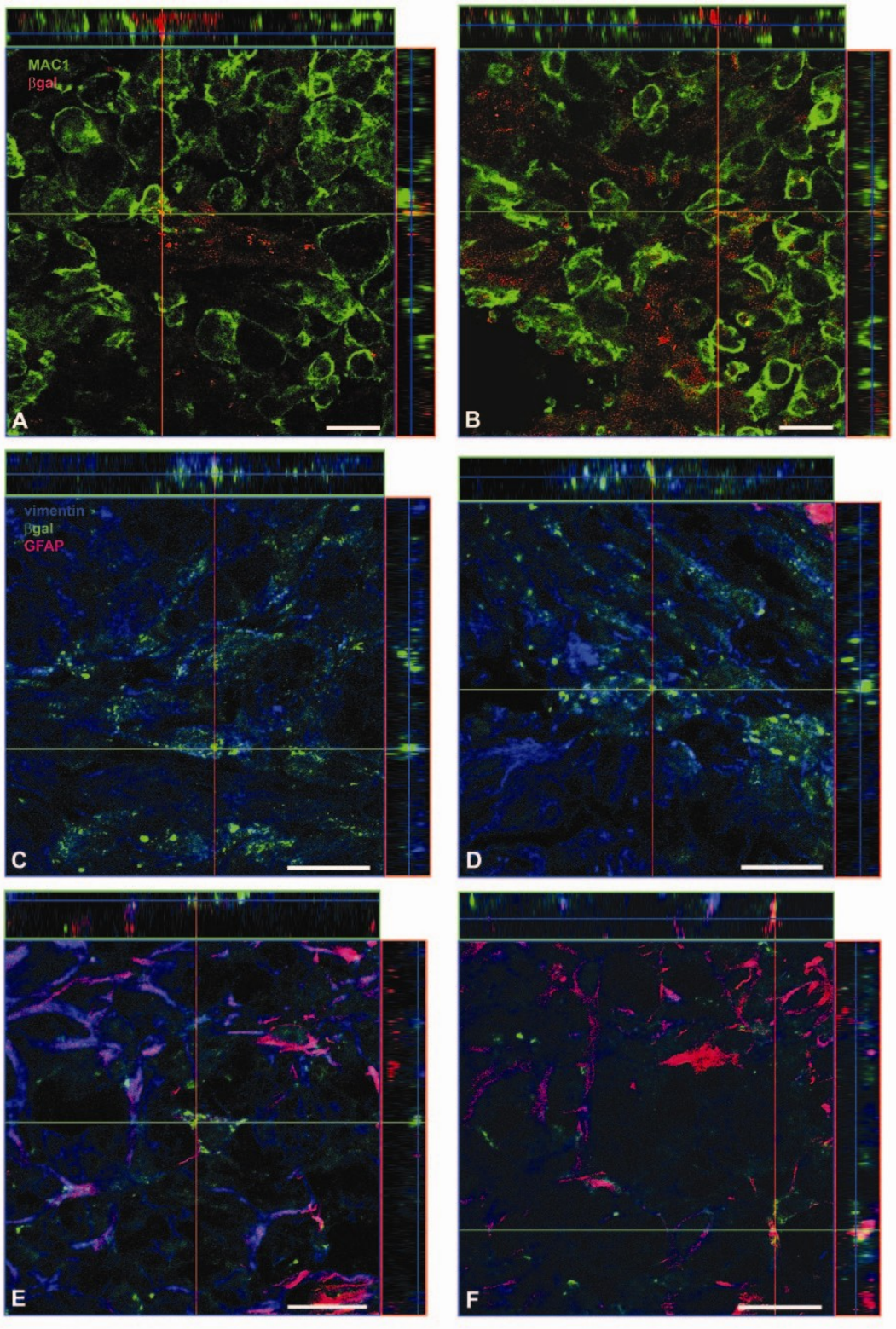
MAC1
βgal
A
B
vimentin
βgal
GFAP
C
D
E
F

FIGURE 4-4: Identification of the cell types that express netrin-1 at an injury site in adult netrin-1$^{Bgeo/+}$ mouse spinal cord. **A,B)** β-gal immunoreactivity (red) was occasionally found closely associated with microglia/macrophages (Mac1+ cells, green); however, co-localization of β-gal within the Mac1+ cell was never detected. **C,D)** β-gal+ immunoreactivity (green) was detected in fibroblasts (GFAP-, red/vimentin+, blue) in the dorsal portion of the injury site. **E,F)** β-gal+ immunoreactivity (green) could also be detected in some reactive astrocytes (GFAP+, red/vimentin+, blue). For A-F, the boxes on the top and right side of each panel present a view through the orthogonal axis of the Z-stack generated by confocal imaging. Scale bar: 20μm.

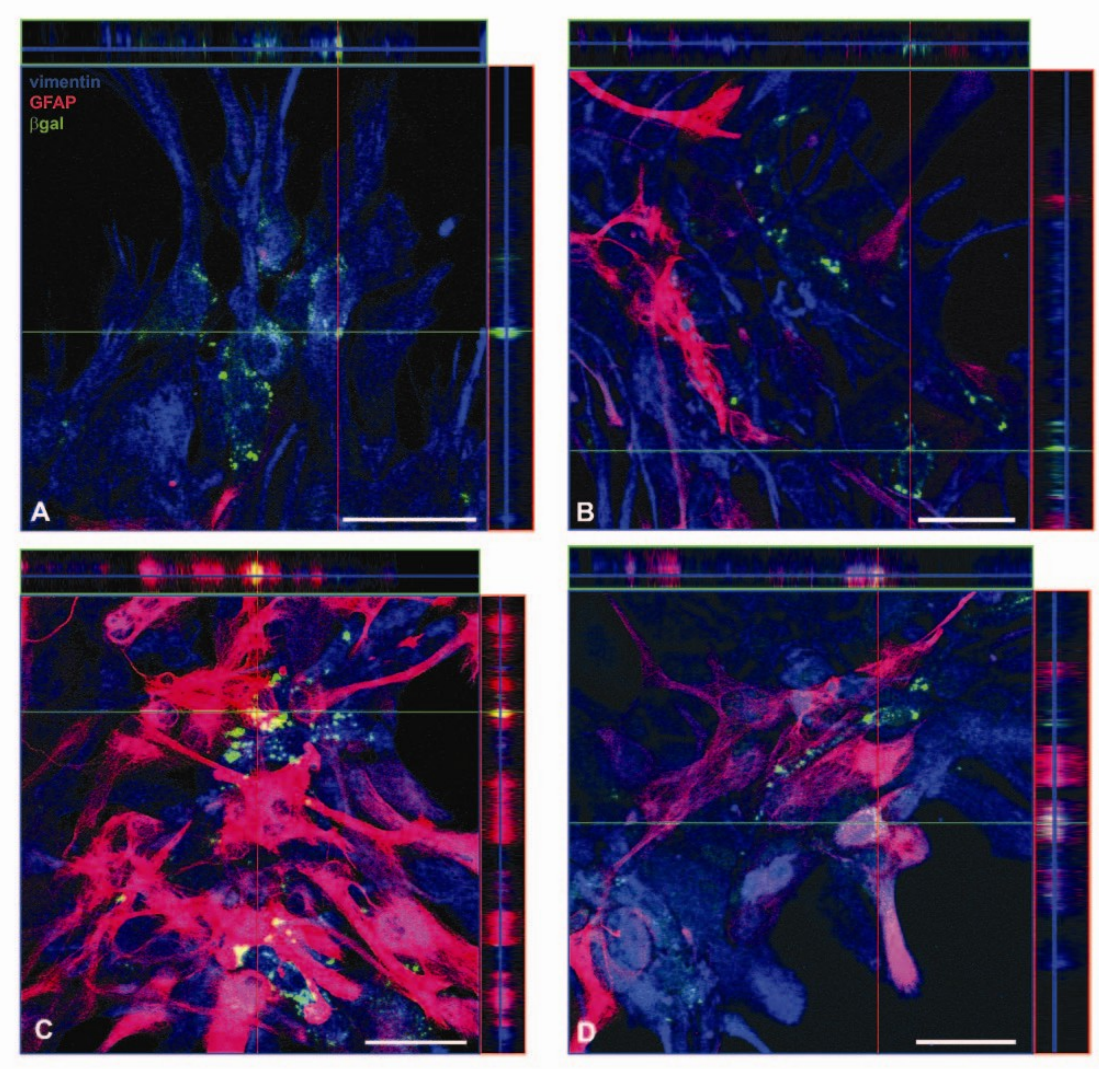

FIGURE 4-5: *In vitro* β-gal expression after scrape injury of netrin-1$^{Bgeo/+}$-derived glial cultures. A,B) Widespread expression of β-gal (green) by GFAP-(red)/vimentin+(blue) fibroblasts in glial enriched cultures following a mechanical (scratch) injury. **C,D)** GFAP+/vimentin+ reactive astrocytes also co-expressed β-gal *in vitro*. Two representative micrographs are included. A and C illustrate cultures grown in media with serum, while B and D illustrate cultures grown in serum-free media. The boxes on the top and right side of each panel present a view through the orthogonal axis of a Z-stack generated by confocal imaging. Scale bar: 50µm.

DISCUSSION AND CONCLUSION

In agreement with the findings described by Wehrle et al. (2005), we observed significantly higher expression of netrin-1 (β-gal) within the lesion. However, we did not detect expression of netrin-1 in microglia/macrophages as previously reported (Wehrle et al, 2005). In contrast, our findings provide evidence that netrin-1 is expressed in the injured mouse spinal cord by fibroblasts and some reactive astrocytes, but not by microglia/macrophages within the glial scar. Similar results were obtained examining cells expressing netrin-1 in mixed glial cultures derived from *netrin-1*$^{\beta geo/+}$ mice. The observation that netrin-1 immunoreactivity was more widely distributed than that of β-gal (Figure 4-1C) suggests that netrin-1 is secreted into the injury environment and likely binds to either its transmembrane receptors or to HSPGs on the surface of cells (Kappler et al., 2000; Shipp and Hsieh-Wilson, 2007). This possibility may partially explain the results of Wehrle et al. (2005), describing netrin-1 expression by microglia/macrophages at the injury site. It is also possible that other cells not investigated here may also express netrin-1 in the injured mouse spinal cord, such as endothelial cells (unpublished observations), or Schwann cells, which can migrate into the injured spinal cord and have been shown to express netrin-1 after peripheral nerve injury (Madison et al., 2000).

In contrast to the results obtained in mice, examination of netrin-1 expression after spinal cord injury in rat yielded opposite results (Manitt et al., 2006; Low et al., 2008). Such species-specific differences in the response to spinal cord injury have been described previously (Steward et al., 1999; Inman and Steward, 2003; Sroga et al., 2003). Even more subtle strain-specific differences exist within the same species (Basso et al., 2006; Kigerl et al., 2006). Strikingly, the most important difference in the response to spinal cord injury between mice and rats is the level of cavitation formed at the site of injury. In contrast to rats, which exhibit progressive necrosis and cavitation following injury, the cystic cavity fills with a connective tissue matrix in mice (Steward et al., 1999; Byrnes et al., 2010).

Differences in the magnitude and the onset of the inflammatory responses are proposed to underlie this distinct cavity formation response. Mice display a delayed infiltration of lymphocytes and dendritic cells into the lesion compared to rat (Sroga et al., 2003). Furthermore, fibroblasts invade the lesioned area more aggressively in mice (Sroga et al., 2003). Such differences in both the cavity formation and the inflammatory responses could underlie the discrepancy of results obtained in mice and rats.

Previous analyses detected netrin-1 immunohistochemical reactivity associated with microglia/macrophages in the injured murine CNS (Wehrle et al., 2005), macrophages in the sciatic nerve of rats with experimental allergic neuritis (Moon et al., 2006), and inflammatory cells invading the spinal cord of rats at the peak stage of experimental autoimmune encephalomyelitis (Moon et al., 2011). In contrast, our analysis using adult netrin-$1^{Bgeo/+}$ mice did not detect endogenous netrin-1 expression by microglia/macrophages. However, we detected β-gal staining in close proximity to microglia/macrophages. A possible explanation for the reports that microglia/macrophages exhibit netrin-1 immunostaining may be that these cells phagocytosed a netrin-1 expressing cell or netrin-1 containing debris from the site of injury. Netrin-1 is associated with myelin at the paranode (Jarjour et al., 2008; Low et al., 2008), and thus myelin debris produced by the breakdown of myelin following injury most likely contains netrin-1 protein. Alternatively, netrin-1 protein may be bound to heparin sulfate proteoglycans on the surface of microglia/macrophages (Kappler et al., 2000; Shipp and Hsieh-Wilson, 2007). Taken together, our results provide evidence that microglia or macrophages do not express netrin-1 following CNS injury, but support the conclusion that fibroblasts and a limited number of reactive astrocytes are the predominant source of netrin-1 at such sites of injury in the mouse CNS. Inhibitory cues such as netrin-1 secreted after CNS injury are therapeutic targets and their neutralization could promote axonal regeneration and remyelination.

CHAPTER V
DISCUSSION

In this chapter, I will briefly describe the results obtained in chapters II, III and IV, and discuss how they integrate into previous models of myelin formation, myelin maintenance, and myelin-associated inhibition following injury.

V-1. MYELIN FORMATION: MECHANISMS PROMOTING OL DIFFERENTIATION

Akt/mTor activity plays a crucial role in the early OL differentiation and myelination process (Flores et al., 2008; Narayanan et al., 2009; Tyler et al., 2009). In chapter II of this thesis, we demonstrate that at late stages of OL differentiation *in vitro*, the PI3K/Akt/mTor pathway is also involved in morphological differentiation downstream of neurotrophins and NRG1 signaling. We show that, individually applied, NT-3, BDNF, NGF, or NRG1 have limited to no effect on Akt activation and morphological maturation. However, when applied collectively, they synergize and induce a PI3K/Akt/mTor dependent morphological differentiation.

Receptor activation downstream of NGF, NT-3, BDNF and NRG1

The mechanism underlying the synergistic effect of collectively applied neurotrophins and NRG1 is unknown. The synergy could be mediated by convergence of signals downstream of classical receptor or co-receptor interactions. NGF, BDNF and NT-3 bind preferentially to TrkA, TrkB and TrkC, respectively, and they all bind to p75 (Huang and Reichardt, 2001). In addition to their classical receptors, NGF and other neurotrophins were recently shown to bind to integrins (Staniszewska et al., 2008). NRG1 binds to the ErbB class of receptors that includes members ErbB2, ErbB3 and ErbB4, and can activate several signalling pathways (Falls, 2003). Like neurotrophins, NRG1 can bind directly to integrins, and this binding was shown to modulate NRG1/ErbB signalling (Colognato et al., 2002; Ieguchi et al., 2010). Moreover, β1 integrin engagement in OLs was shown to be associated with Akt activation and myelin sheet formation downstream of NRG1 signals *in vitro* (Barros et al., 2009). Co-activation of integrin receptors and neurotrophins/ErbB receptors could thus underlie the synergistic effect on Akt signalling seen with the combination of factors. Formation of different receptor complexes could affect intracellular recruitment of signalling partners, and mediate the synergistic action of neurotrophins and NRG1.

Context-dependent effects of NGF, NT-3, BDNF and NRG1 in OLs

The effects of NGF, NT-3, BDNF and NRG1 on OPCs and OLs have been examined previously in multiple studies. *In vitro*, NRG1 was shown to promote survival and proliferation of OLs (Canoll et al., 1999), an effect dependent on Akt activation (Flores et al., 2000). However, reports describing the effect of NRG1 on OL differentiation do not agree. While some groups report that NRG1-promotes OL differentiation (Calaora et al., 2001; Park et al., 2001), others describe an inhibitory effect of NRG1 on differentiation (Canoll et al., 1999; Sussman et al., 2005). This discrepancy could be explained by the receptor complex activated by NRG1. ErbB2, even while lacking a ligand-binding activity, mediates terminal differentiation of OLs (Park et al., 2001). ErbB4 activation, in contrast, inhibits OL differentiation (Sussman et al., 2005), while ErbB3 is not required at this stage of OL maturation (Schmucker et al., 2003). Despite these effects, NRG1 signaling seems dispensable for OL differentiation and myelination *in vivo* (Brinkmann et al., 2008). However, when overexpressed during myelination, NRG1 promotes myelination independently of proliferation (Brinkmann et al., 2008). Interestingly, the authors hypothesized that this effect of NRG1 *in vivo* is mediated by the PI3K/Akt/mTor pathway, based on unpublished observations. The difference between *in vitro* and *in vivo* studies on NRG1 in the CNS highlights the complexity of OL differentiation studies, whose results might vary depending on specific factors present in OL environment.

Reported effects of neurotrophins on OLs also vary. While NGF was shown to have no effect on survival of OPCs (Barres et al., 1993), it was shown to inhibit myelination through an indirect effect on axonal TrkA receptors (Chan et al., 2004). NGF also upregulates the expression of LINGO-1 on axons, thereby inhibiting myelination (Lee et al., 2007). However, NGF increases remyelination in the mature nervous system following a lysolecithin-induced demyelination challenge (Althaus, 2004). NT-3 promotes proliferation and survival of OPCs when combined with PDGF and insulin (Barres et al., 1993; Barres et al., 1994).

In vitro, NT-3 was also shown to enhance differentiation (Lachyankar et al., 1997; Heinrich et al., 1999). Both NT-3 and BDNF promote remyelination of regenerating axons *in vivo* (McTigue et al., 1998). Unlike NT-3, no effect of BDNF on survival of OPCs was described (Barres et al., 1993). On the other hand, BDNF had an effect on myelin protein expression, which was mediated by activation of the MAPK pathway (Du et al., 2006). Interestingly, BDNF promotes differentiation and myelination of human OPCs when used in conjunction with IGF-1 (Cui et al., 2010). We could speculate that some of the effects described downstream of neurotrophins or NRG1 depend on the activation of the Akt pathway. In studies done *in vitro*, the insulin present in the media could promote Akt phosphorylation and thus prime the cells to respond positively to certain factors. Also, when looking at effects on remyelination *in vivo*, it is very challenging to isolate the action attributed to individual factors, since sites of demyelination and/or injury contain several cues either present in the myelin or secreted by the glial scar. It is therefore possible that some of the findings mentioned above are the result of the integration through the Akt pathway of multiple extracellular and intracellular signals. In addition, the effects of different factors on OL maturation are dependent on the stage of differentiation. Different downstream signalling pathways can be activated depending on the maturation stage, and a switch in the response to the same cue could occur along the differentiation from OPCs to OLs.

Signaling switches along OL differentiation

Signaling switches have been previously described in OLs. For example, the interaction of laminin with integrins has been proposed to underlie a switch in the signalling pathway promoting OL survival from being PI3K-dependent to being MAPK-dependent (Colognato et al., 2002; Colognato et al., 2004). Such switches in signalling pathways could explain why OLs respond differently to growth factors as a consequence of their differentiation state. Switching responses have also been linked to the receptors expressed by OLs. In OLs with no TrkA expression, treatment with NGF induces p75-dependent JNK signalling, which

leads to OL cell death (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998). However, when TrkA expression is induced in these OLs, the response to NGF switches to being pro-survival, an effect dependent on both decreased JNK signalling and increased ERK signalling (Yoon et al., 1998). As mentioned in chapter I, another example of how OLs modify their response to the same cue during differentiation is the different response elicited by netrin-1 in OPCs and OLs (Jarjour et al., 2003; Tsai et al., 2003; Rajasekharan et al., 2009), a molecular switch resulting in differential regulation of RhoA activity (Rajasekharan et al., 2010). Thus, an extracellular cue can have distinct effects on cells of the OL lineage depending on receptor expression and on the intracellular signalling state, both of which can vary during OL differentiation.

Understanding the different molecular switches in OL differentiation, either intrinsic or environmentally-induced, becomes important when looking at myelination outside of a developmental context. Remyelination of demyelinated plaques in MS is dependent on the reactivation of OPC migration, proliferation and differentiation within the plaques (Miron et al., 2011). Understanding why a factor inhibiting myelination during development can promote remyelination in the adult, such as described with NGF (Althaus, 2004; Lee et al., 2007), might depend on the appreciation and consideration of such molecular switches in OLs.

Candidate intracellular mediators of signalling integration

A pivotal role of Focal Adhesion Kinase (FAK) downstream of extracellular matrix (ECM) components in morphological maturation of OLs has recently been described (Lafrenaye and Fuss, 2010). FAK activation has an inhibitory effect on OL maturation in pre-myelinating OLs, while it promotes maturation in later stages of OL development. This switch was shown to be in part due to the ECM composition, and could be an indication that OLs require the integration of both the extracellular signalling cues and intracellular signalling environments for successful differentiation to proceed. Interestingly, FAK links integrins to the MAPK pathway (Schlaepfer et al., 1999), to the Rac1 pathway (Chang et al.,

2007) and to the PI3K-Akt pathway (Xia et al., 2004). Integration of signals through FAK could underlie the synergistic effect and the convergence of different signalling pathways.

The Src family tyrosine kinase fyn is implicated in several processes during OL development. Fyn knockout mice are hypomyelinated (Umemori et al., 1994; Sperber et al., 2001), and fyn influences OL process elongation and branching *in vitro* (Osterhout et al., 1999; Rajasekharan, 2008). There are multiple downstream targets of fyn that can be implicated in OL differentiation. Fyn is activated downstream of integrin α6β1 in OLs, and it mediates late stages of OL differentiation (Colognato et al., 2004; Laursen et al., 2009; Relucio et al., 2009). IGF-1 and netrin-1 failed to stimulate morphological maturation of fyn null OLs (Sperber and McMorris, 2001; Rajasekharan, 2008), highlighting its importance in signal transduction downstream of multiple extracellular cues. Fyn recruits tau and tubulin to OL lipid rafts (Klein et al., 2002), and an intact tau and microtubule network is critical for the transport of vesicles containing myelin protein (Lunn et al., 1997; Song et al., 2001). In addition, activated fyn can phosphorylate the protein PI3K enhancer (PIKE) (Tang et al., 2007), which stimulates PI3K and enhances Akt activation (Ye et al., 2000). Importantly, Trk receptors interact with fyn (Rajagopal and Chao, 2006; Pereira and Chao, 2007). Synergy of signals leading to Akt activation and to morphological differentiation could thus be in part mediated by fyn activation.

Of course, there are other mechanisms by which synergistic action of different factors could lead to increased Akt activation and morphological differentiation in OLs. For example, inhibition of an Akt phosphatase, like PHLPP (PH domain and Leucine rich repeat Protein Phosphatase), together with PI3K/Akt activation, could lead to increased and prolonged Akt activation. Also, synergy could result from different regulation of endocytosis, which has already been shown to regulate neurotrophin signalling to promote differentiation (Zhang et al., 2000),

and Akt signaling (Zheng et al., 2008), as well as signaling downstream of the ErbB receptor (Sorkin and Goh, 2009).

mTor-dependent pathways regulating morphology

mTor activation is largely associated with activation of protein synthesis; however, downstream targets of mTor complexes are still being identified and mTor's functions go beyond activation of translation. In mammalian cells, mTor can be incorporated into two different complexes: mTORC1 and mTORC2, in which mTor associates with either Raptor (Regulatory Associated Protein of mTor) or Rictor (Rapamycin Insensitive companion of mTor), respectively. These complexes have distinct downstream targets and cellular effects. Translation activation through the inhibition of 4EBP1 and activation of S6K1 occurs through mTORC1 activation. mTORC2 activation leads to phosphorylation of different kinases, including Akt and protein kinase Cα (PKCα). Rictor was first identified as conferring insensitivity to rapamycin (Sarbassov et al., 2004). During acute treatment, rapamycin inhibits mTORC1 but not mTORC2; however, chronic rapamycin treatment can inhibit mTORC2 by blocking complex assembly (Sarbassov et al., 2006). Morphological maturation induced by NT-3/BDNF/NGF/NRG1 after 24 hours was inhibited by the presence of rapamycin, which can, after one day, inhibit both mTORC1 and mTORC2. Both complexes could therefore mediate the observed morphological differentiation. Interestingly, Woods and colleagues provide evidence for the implication of both complexes in OL differentiation *in vitro* (Tyler et al., 2009).

In addition to inhibiting mTor-dependent activation of translation, rapamycin can also inhibit cytoskeletal reorganization downstream of IGF-1 in cancer cells. This effect is caused in part by inhibiting mTor-dependent phosphorylation of focal adhesion proteins like FAK, paxillin and $p130^{cas}$ (Liu et al., 2006; Liu et al., 2008). mTor activation is also linked to increased RhoGTPase expression and activation (Liu et al., 2010), thus mediating reorganization of the actin cytoskeleton. This raises the possibility that the PI3K/Akt/mTor-dependent

morphological maturation and increased branching observed when OLs are treated with NT-3/BDNF/NGF/NRG1 could be caused by the increased expression, as well as direct activation, of cytoskeletal elements by mTor.

OL differentiation is the result of the integration of different signaling pathways activated by different sets of receptors. In this thesis, I have presented evidence that NT-3/BDNF/NGF/NRG1 can converge on the PI3K/Akt/mTor pathway in OLs to promote later stages of differentiation of mature OLs. Mapping the signalling events implicated in OL differentiation could help in the understanding of the process of myelin formation and could potentially reveal therapeutic targets to promote endogenous remyelination in demyelinating diseases like MS.

V- 2. MYELIN MAINTENANCE: MECHANISMS PROMOTING MYELIN STABILITY THROUGHOUT LIFE

Following the formation of myelin, maintenance mechanisms ensure that its functions stay intact the adult nervous system. In chapter III of this thesis, I have used a temporally regulated and cell type specific conditional knockout approach to study the role of DCC expression in mature myelinating OLs. We found that DCC expression by OLs is necessary for the maintenance of paranodal and myelin cytoarchitecture. Furthermore, the disorganization of paranodal architecture leads to loss of domain integrity around the node of Ranvier. Myelin phenotypes include the development of abnormal myelin profiles and alterations of myelin protein expression. Paranodal and myelin instability caused by the loss of DCC leads to the development of balance and coordination deficits.

Comparison with other myelin mutants

Loss of DCC does not cause a dramatic paranodal phenotype, as does the loss of other paranodal components like NF155 (Pillai et al., 2009), Caspr (Bhat et al., 2001) or contactin (Boyle et al., 2001). Furthermore, there is only limited disruption of transverse bands (TBs) in the absence of DCC expression in OLs, while mutations of most of other paranodal components display complete lack of TBs. Whether DCC interacts with one or more of these proteins at the paranodes is not known. However, the absence of NF155, Caspr or contactin results in paranode formation defects, whereas the absence of DCC produces a myelin and paranode maintenance defect. Like DCC, other myelin protein mutants have been shown to exhibit paranodal maintenance defects, and some of them are described below.

Myelin and lymphocytes (MAL) protein is a proteolipid expressed by myelinating cells in the CNS and PNS. When MAL is absent from myelin, paranodal loops form normally, but start to disorganize at a time point corresponding to the onset of MAL expression. This disorganization is accompanied by compact myelin

defects, and a reduced level of expression of Caspr, Kv1.2 and NF155. Furthermore, NF155 incorporation into lipid microdomains on paranodal loops seemed to be dependent on the presence of MAL, and failure of these membrane domains to form seems to underlie the paranodal defects observed in MAL mutants (Schaeren-Wiemers et al., 2004). This phenotype is distinct from what we observed in the DCC conditional mutants, since we did not see alterations in paranodal protein levels. However, we cannot exclude an interaction between DCC and MAL at paranodes or in myelin lipid raft, since DCC can also localize to lipid raft and associate with specific signaling partners in these domains (Herincs et al., 2005; Petrie et al., 2008).

Stabilization of the paranodal components by the axonal cytoskeletal adaptor protein 4.1B was also shown to play an important role in the maintenance of paranodes. Paranodal domains appear normal at one month of age in the CNS of protein 4.1B mutants, but become progressively disorganized starting at four months of age (Buttermore et al., 2011). Protein 4.1B interactions with the cytoplasmic tail of both Caspr and Caspr2 mediate the stabilization of the protein complexes at the paranode and the juxtaparanode (Horresh et al., 2010). Such interactions with cytoskeletal components are not defined on the glial side of paranodes, and there are no known intracellular binding partners of NF155 or DCC at the paranode. However, we could hypothesize that similar stabilization mechanisms are equally important in paranodal loops, linking the paranodal adhesive complexes to the OL cytoskeleton.

CGT mutants, lacking the enzyme responsible for the formation of both GalC and sulfatide, cannot form proper lipid raft domains in myelin, leading to aberrant localization of proteins like NF155 and causing a severe paranodal and myelin phenotype (Dupree et al., 1998). When only sulfatide is absent (CST mutant), mice develop normally, but myelin and paranode maintenance defects appear as they age (Ishibashi et al., 2002; Marcus et al., 2006). Biochemical analysis revealed alterations in the expression of several proteins associated with adhesion

and cytoskeletal dynamics in these mutants (Fewou et al., 2010), which supports the hypothesis that OL paranodal proteins are located in membrane microdomains and interact with several adaptor partners and cytoskeletal elements. We could speculate that DCC, along with other membrane proteins, forms a bridge with cytoskeletal elements in these membrane microdomains, and that in its absence, similar adhesion defects as those seen in the CST mutant develop.

Mice deficient for MAG exhibit deficits in the maintenance of myelinated fibers, and they develop a myelin and an axonal degeneration phenotype when they are around six month old (Li et al., 1994; Fruttiger et al., 1995). In that regard, the MAG knockout mice closely resemble our DCC conditional knockout. Interestingly, we observed a decrease in MAG protein levels in the absence of DCC expression by OLs, but the significance of this decrease has not yet been investigated.

Comments on altered myelin protein expression

How could the absence of DCC in OLs affect MAG expression? If DCC mediates adhesion between the glial and axonal membrane, it could be present in a complex along with MAG, and promote its stabilization. Absence of DCC could thus lead to an increase in MAG degradation. Alternatively, DCC and MAG could be co-transported to the myelin membrane, and the absence of DCC might cause a defect in MAG transport. Beyond the mechanisms by which removal of DCC leads to a decrease in MAG expression, the effect of this decrease could affect MAG-related functions in the conditional DCC knockout mice.

MAG is involved in axonal neurofilament phosphorylation, which leads to a myelination-induced axonal expansion (Yin et al., 1998). Axonal diameter at the internode can be up to five-fold the diameter at the node of Ranvier, and this axonal expansion is mediated through MAG-dependent phosphorylation of NFH and NFM (Sousa and Bhat, 2007). Despite the reduction in MAG protein expression levels observed in our conditional DCC knockout mice, we did not

detect any significant alterations in axonal diameter both at the paranode, by measuring Caspr width, and at internodes, by measuring axon diameter for the G-ratio analysis. However, for G-ratio analysis, we excluded axon profiles that were too difficult to quantify, such as those presenting extensive myelin outfoldings. Those outfoldings could be in part explained by the reduction in axonal diameter caused by the decreased MAG levels. More careful examination of neurofilament spacing and axonal diameter is needed to draw any conclusion in regard to the effect of the decreased levels of MAG in the absence of DCC. In addition, the mechanism by which the absence of DCC affects MAG levels needs to be investigated further.

Suprisingly, the decreased levels of MBP in our conditional DCC knockout mice seem to be without consequences for compact myelin integrity. In fact, this can be explained by the observation that there is no effect on myelin integrity even when up to 50% of MBP protein is lost (Shine et al., 1992). However, the cause of this diminution of MBP protein in our conditional DCC knockout mice is not clear. Such a phenomenon could be the result of a general instability of myelin, which could lead to a decreased stability of MBP protein or a diminution of its localized translation to the myelin sheath. Interestingly, DCC was recently shown to be directly involved in local translation. DCC associates physically with translation initiation factors and ribosomal subunits through its P1 domain. Following netrin-1 binding to DCC, the translation machinery is released and translation can proceed (Tcherkezian et al., 2010). According to that model, removal of DCC would not necessarily result in less transcripts being translated, since there would be less inhibition of translation. However, DCC could affect MBP translation in an indirect manner. For example, the Src family tyrosine kinase fyn was shown to regulate local translation of MBP (Lu et al., 2005; White et al., 2008). Our group showed that netrin-1 binding to DCC causes fyn to be recruited to a complex with DCC in OLs (Rajasekharan et al., 2009). DCC may thus regulate MBP local translation indirectly through the activation of fyn, and the absence of DCC from myelin membranes might lead to a reduction in MBP protein.

Paranodal remodelling and aging

To our surprise, an unexpectedly high proportion of paranodes were abnormal in wild type myelin. This result could indicate normal myelin remodelling throughout life or disorganization of paranodes due to normal aging, as described in Chapter I (section I.3.1). Recent evidence has demonstrated an age-dependent reorganization of paranodes (Shepherd et al., 2010). The authors demonstrate that while TBs were gradually lost in mice older than 17 months, detachment of loops from the axon did not increase with age, but rather correlated with myelin thickness (Shepherd et al., 2010). Hence, the detached loop phenotype we observed in conditional DCC knockout mice is unlikely a result of an accelerated aging process in the absence of DCC.

The degree of myelin remodelling occurring through normal life has never been tested. Like myelin, paranodes are thought of as fixed and immobile structures. Technical limitations render the study of myelin and paranode plasticity difficult. However, our results show that even in the wild type mice, paranodes exhibit disorganization, albeit to a lesser degree than in our conditional DCC knock out mice. Another structure of the nervous system, the synapse, once thought to be fixed, was shown to be subject to a high degree of turnover and continuous remodelling. Interestingly, the Kennedy lab has also been investigating a role for DCC in the maintenance and maturation of CNS synapses (Horn and Kennedy, unpublished). It is tempting to speculate that, like the synapse, the paranode undergoes constant remodelling and that the stability of the adhesion between the axon and the glial loops is constantly challenged. In such a model, DCC could have a role in the stabilization of the paranodal and myelin entities, by mediating adhesion between axonal and OL membrane, so that in its absence, adhesive contacts and interactions would be more easily challenged, leading to myelin instability.

Beyond the paranode: evidence for a broader role of DCC in myelin

While we previously described a role of DCC and netrin-1 solely in the maintenance of paranodes (Jarjour et al., 2008), the results presented here argue for a broader role of DCC in the maintenance of myelin. In fact, while DCC might be concentrated at the paranode, we cannot exclude that it is also present in other compartments of noncompact myelin such as the adaxonal or abaxonal membrane. We have also shown here that DCC is present outside the paranodes in PNS myelin, a finding that was recently confirmed by another group (Webber et al., 2011). Visualization of independent fibers with the teased nerve preparation of sciatic nerves is an easier task in the PNS, which could explain the detection of DCC in these other noncompact myelin compartments in the PNS. Similarly, DCC might also be present outside the paranode in the CNS, and mediate functions beyond paranodal maintenance.

Signaling downstream of DCC in mature myelin

Myelin outfoldings were observed in conditional DCC knockout mice. This phenomenon is thought to reflect normal but sparse remodelling in wild type myelin. The higher prevalence of these outfoldings in the myelin of conditional DCC knockout myelin could indicate destabilization and a higher level of remodelling in the absence of DCC expression by OLs. Interestingly, loss of Cdc42 and Rac1 expression in OLs gave rise to an outfolding phenotype and a bigger inner tongue during early myelination (Thurnherr et al., 2006). These Rho GTPases act downstream of DCC in growth cone remodelling in response to netrin-1 (Shekarabi, 2005). However, in purified mature OLs grown in culture, DCC-dependent morphological response to netrin-1 is independent of cdc42 or Rac1 activation (Rajasekharan et al., 2009). A Rho GTPase signaling switch mediated by DCC has already been described in OLs along their maturation path (Rajasekharan et al., 2010) and an interaction of cdc42, Rac1 and DCC in mature myelinating OLs *in vivo* cannot be ruled out. It would of interest to examine the effect of transgenically removing cdc42 and Rac1 in fully myelinated adult mice to test for the appearance of these outfoldings.

Implication in pathological conditions

Deficits in the maintenance of myelin and intact axoglial junctions could lead to the development of pathological conditions. Myelin instability and damage have been proposed to underlie the age-related cognitive deficits observed in the elderly (Bartzokis, 2004). The instability was also suggested to be a predisposition to immune attacks, which could lead to the development of pathologies like multiple sclerosis (Mastronardi and Moscarello, 2005). In such a model, instability of myelin would be responsible for the exposure of myelin antigens triggering an auto-immune response. Also, when the CNS is damaged, myelin instability could lead to increased myelin breakdown, releasing more inhibitors into the environment and impeding both regeneration and remyelination. Unveiling the mechanisms by which myelin structure is maintained throughout life could help understand the etiology of myelin-associated pathologies.

V- 3. MYELIN BREAKDOWN: EXPRESSION OF NETRIN-1 IN THE INJURED CNS

Section I.3.3 discussed the inhibitory components present in the injured CNS. In chapter IV, using netrin-1$^{\beta geo/+}$ reporter mice, we showed that eight days after spinal cord injury, netrin-1 expression is increased at the injury site. We also demonstrated that this increase in netrin-1 expression can be attributed to fibroblasts and some reactive astrocytes. These results, together with the results presented by Dusart and colleagues (Wehrle et al., 2005), suggest that in addition to being potentially released by myelin breakdown following injury, netrin-1 is also secreted by cells forming the glial scar. The presence of netrin-1 could contribute to the inhibitory environment created after an injury in the CNS.

Effect of netrin-1 on regeneration and remyelination after spinal cord injury

During development, netrin-1 acts both as an attractant and a repellent cue for growing axons and migrating cells. In fact, DCC expression levels are high in the developing CNS, but decrease as development proceeds; and the Unc5 homologues expression predominate in the adult CNS (Manitt et al., 2004). This switch in netrin-1 receptor expression suggests that netrin-1 could act as an inhibitor of axon regeneration after injury. This hypothesis is supported by the insensitivity of CGRP nociceptive neurons, which lack UNC5 expression, to netrin-1 mediated inhibition in the injured rat spinal cord (Low et al., 2008). Moreover, changes in netrin-1 receptor expression after spinal cord injury support the idea that it acts as an inhibitor of regeneration. Two independent studies reported changes in netrin-1 receptors expression following injury. Low et al. (2008) reported no change in DCC expression, but an increase in UNC5 levels for up to two months after spinal cord injury in rat. In contrast, our group described a decrease in DCC protein levels, lasting for at least seven months, whereas no changes were detected in UNC5 levels (Manitt et al., 2006). Nonetheless, in both cases, the ratio of UNC5 to DCC proteins is shifting after injury, supporting a role for netrin-1 as an inhibitor of regeneration.

Not only do axons need to regenerate after spinal cord injury, but the myelin sheath also needs to be replaced. After spinal cord injury, OLs are subject to necrosis and apoptosis, starting within 15 minutes at sites of injury, and detected up to two to three weeks post-injury at distal sites, a phenomenon linked to Wallerian degeneration (Almad et al., 2011). OLs are very vulnerable to ischemic and oxidative stress (Thorburne and Juurlink, 1996; McAdoo et al., 1999; Almad et al., 2011), but are also affected by secondary excitotoxicity (Xu et al., 2004; Xu et al., 2008), and can be targeted by an activated immune system (Antel et al., 1994; Popovich et al., 1997; Schonberg et al., 2007). Preventing myelin breakdown far from the injury, to protect the ensheathed axons, as well as promote remyelination of regenerated axons, are both therapeutic avenues to decrease the level of functional impairment after spinal cord injury. In addition to acting as a repellent cue for regenerating axons after spinal cord injury, netrin-1 could thus also play a role in the remyelination process, given its known roles in OL biology, regulating OL process branching and myelin membrane formation (Rajasekharan et al., 2009).

Netrin-1 in other CNS pathologies: potential role in MS

Beyond spinal cord injury, netrin-1 expression at sites of CNS injury could also inhibit remyelination after a demyelinating insult. In demyelinated MS plaques, naked axons are surrounded by an astrocytic glial scar (Fawcett and Asher, 1999), which expresses markers of reactive astrocytes (Holley et al., 2003). Despite the absence of fibroblasts in MS lesions, netrin-1 is still detected within MS plaques (Rajasekharan and Kennedy, unpublished observations). In addition to being released from destructed myelin, netrin-1 could also be secreted by infiltrating activated immune cells or by reactive astrocytes. The presence of netrin-1 in MS lesions could have consequences on remyelination efficiency, as netrin-1 was shown both to repel migrating OPCs and to promote branching and membrane formation in mature OLs (Jarjour et al., 2003; Tsai et al., 2003; Rajasekharan et al., 2009). However, fetal OPCs behave differently than adult-derived OPCs, at least in humans (Ruffini et al., 2004), and preliminary evidence shows that netrin-

1 elicits different responses in these two cell types (Rajasekharan and Kennedy, unpublished observation). More detailed investigation of the effect of netrin-1 on adult-derived progenitors is needed to determine the consequence of netrin-1 presence in the injured CNS on remyelination.

The glial scar: a necessary evil after a CNS insult?

The glial scar is often considered the major barrier to regeneration following CNS injury. In addition to forming a physical barrier for growing axons, cells of the glial scar also express several inhibitory molecules (Moore et al., 2011). However, some evidence suggests that astrocytes are crucial in the immediate response to injury, and that in the first week following a CNS insult, the glial scar mediates several processes that are beneficial to regeneration. Cells of the glial scar can act as scavengers, preventing the spreading of excitotoxicity (Chen et al., 2001; Cui et al., 2001); provide trophic support to surviving neurons and OLs (Faulkner et al., 2004; Moore et al., 2011); regulate immune responses (Rolls et al., 2009); and even promote revascularization of the injury site (Stichel and Muller, 1998; Parri and Crunelli, 2003). Likewise, expression of netrin-1 by the glial scar might have beneficial effects on survival, revascularization, or recruitment of immune cells to the lesioned area, for example. Like the glial scar, both detrimental and beneficial effects on repair could thus be attributed to netrin-1. The specific effects of netrin-1 on different aspects of CNS repair after injury is an area that warrants further investigation.

GENERAL CONCLUSION AND PERSPECTIVES

Myelination is the result of evolutionary pressure to increase the velocity of signal transduction without increasing axon caliber, and while maintaining a relatively low expense of energy by neurons. Myelination is a carefully orchestrated process that leads to the development of a complex and highly organized architecture. In the course of my PhD, I explored different aspects of OL and myelin biology. In this thesis, I first describe a synergy of signals leading to Akt/mTor dependent morphological maturation of OLs. However, this work was done *in vitro*, and might not reflect the complexity of the OL environment during development. The study of OL differentiation *in vivo* is challenged by the complexity of the OL environment and the cell-cell interactions involved. Hopefully, technical advances will allow the study of signaling mechanisms and myelination process *in vivo*. Nonetheless, instead of studying the effects of single factors, mapping the signaling events leading to successful OL differentiation and myelination could provide greater insight into what is happening during myelination *in vivo.*

In the second part of this thesis, I described a role for OL expression of DCC in the maintenance of myelin and paranodes *in vivo*. The high level of complexity of myelin, both structural and metabolic, renders it particularly vulnerable and myelinated fibres are indeed affected in several dysmyelinating and demyelinating diseases. The mechanisms by which DCC promotes myelin architecture stability could thus provide insight into myelin protective mechanisms, important in the prevention of myelin breakdown in several myelin-associated pathologies. The involvement of netrin-1 in this process also needs to be addressed, as well as the source of secreted netrin-1 that accumulates in myelin. A conditional netrin-1 knockout mouse is presently being validated and characterized, and hopefully will provide another piece of information towards building a model of DCC function in OLs. The use of this mouse model could also help in dissecting the effect of netrin-1 after CNS insult, such as spinal cord injury. To what extent myelin-

derived and glial scar-secreted netrin-1 contributes to the failure of regeneration and remyelination is an obvious question that can hopefully be addressed in the near future.

Taken together, the work presented in this thesis addresses key aspects of OL and myelin biology. Global understanding of the mechanisms by which myelin is formed, maintained, and repaired after a CNS insult could unveil possible targets in the treatment of myelin-associated pathologies. Providing knowledge that will set the base for the development of tools and therapeutic agents that will improve our global quality of life is the ultimate goal of any researcher. I hope that this thesis and studies carried out during my PhD research constitutes a step further towards that goal.

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