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Helping to Start and Stop Myelination: Csk has Dual Regulatory Functions during Oligodendrocyte Development

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Abstract of the Dissertation

Helping to Start and Stop Myelination: Csk has Dual Regulatory Functions during Oligodendrocyte Development

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The ability of oligodendrocyte progenitors (OPCs) to generate sufficient numbers of myelinating oligodendrocytes is critical both for developmental myelination and for repair following demyelination. The timing and location of gliogenesis are tightly controlled but the molecular mechanisms that underlie this control are poorly understood. Here I report that the C-terminal Src Kinase (Csk) acts as a molecular switch for Src Family Kinase activity in oligodendrocytes, with distinct and opposing roles for Csk in early versus late stages of development. Early in development Csk is critical for the appropriate onset of oligodendrocyte progenitor differentiation. Csk suppresses OPC proliferation such that Csk depletion in OPCs *in vitro* and *in vivo* led to proliferation under conditions that normally promoted cell cycle exit. Hyperproliferation of Csk-deficient OPCs resulted in delayed oligodendrocyte maturation accompanied by delayed myelination onset, while survival of newly-formed oligodendrocytes was increased. These data suggest that, during myelination onset, Csk is a pro-differentiation factor that promotes timely OPC cell cycle exit. Adult Csk null mice, however, developed hypermyelination, suggesting that Csk also contributes to myelination arrest. Thus, Csk deletion caused increased levels of myelin basic protein as well as increased myelination in the cortex, cerebellum and spinal cord, although there was no change in the number of mature oligodendrocytes. Further analysis of myelin ultrastructure using transmission electron microscopy revealed increased numbers of myelin wraps such that the g-ratio was significantly decreased. To address a putative role for Csk during myelin repair I evaluated the response of Csk null mice to cuprizone-induced demyelination. Preliminary analyses reveal that during remyelination Csk mutant animals exhibit increased numbers of OPCs and mature oligodendrocytes, increased myelin content, and lower g-ratio as well as decreased axonal degeneration. I, therefore, propose that Csk is a novel regulator of oligodendrocyte development with two distinct roles: generating appropriate numbers of oligodendrocytes at the onset of myelination, and terminating wrapping during myelination.

Dedicated to Christos Nardis for his endless love and support.

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List of Abreviations

BrdU	bromo-D-uridine
Cbp	Csk binding protein
CC3	cleaved caspase 3
Cdk	cyclin-dependent kinase
Chk	Csk-homologous kinase
CGT	UDP-galactose:ceramide galactosyltransferase
CNP	2', 3'- cyclic nucleotide 3'- phosphodiesterase
CNS	central nervous system
CNTF	ciliary neurotrophic growth factor
Csk	C-terminal Src Kinase
CTB	cholera toxin subunit B
DCC	Deleted in Colorectal Cancer
DIG	detergent-insoluble glycosphingolipid-enriched membrane microdomains
EAE	experimental allergic encephalomyelitis
ECM	extracellular matrix
FGF	fibroblast growth factor
GalC	galactosylceramide
GEM	glycosphingolipid-enriched membrane microdomain
hnRNP A2	heterogeneous nuclear ribonucleoprotein A2
IGF1	inslulin-like growth factor

IRS-1	insulin receptor substrate 1
LIF	leukemia inhibitory factor
LINGO1	LRR and Ig domain containing, Nogo-receptor interacting protein
Lm2	laminin2
MAG	myelin-associated glycoprotein
MAL	myelin and lymphocyte protein
MBP	myelin basic protein
MOBP	myelin-associated oligodendrocyte basic protein
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NCAM	neuronal associated adhesion molecule
NGF	nerve growth factor
OPC	oligodendrocyte progenitor cell
PAG	phosphoprotein associated with glycosphingolipid-enriched membrane microdomains
PDGF	platelet-derived growth factor
PDGFRα	platelet-derived growth factor α-receptor
PDL	poly-D-lysine
pHisH3	phosphorylated histone H3
PLP	proteolipid protein
PMD	Palizaeus-Merzbacher disease
PNS	peripheral nervous system
PSA-NCAM	polysialylated form of neural cell adhesion molecule
QKI	Quaking homolog, KH domain RNA binding protein

SFK	Src family kinase
SH domain	Src homology domain
SHH	sonic hedgehog
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMO	smoothened
TUNEL	Terminal Transferase dUTP Nick End Labeling
WASP	Wiskott-Aldrich syndrome protein
WAVE2	WASP family verprolin-homologous protein 2
WT	wild type

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Chapter 1:

Introduction to Oligodendrocyte Biology and Myelination

Myelin is Necessary for Efficient Neuronal Communication

Myelination is the ensheathment of axons with a multilayered myelin membrane, which is produced by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the periphery. This process starts late in embryonic development and peaks in the first years of life, and to date, it is best known for its role in permitting saltatory conduction of nerve impulses. Myelin is a necessity of evolution that serves to maximize the efficiency and velocity of action potentials along an axon and is present in complex organisms ranging from fish to primates and humans. The necessity for normal myelin is illustrated by the fact that when myelin is compromised, neurological deficits ensue. Human myelinopathies range from inherited leukodystrophies, some of which are characterized by insufficient myelination during development, to demyelinating diseases that are characterized by damage of normal myelin followed by inefficient repair or remyelination (reviewed in[1]). In this document I will focus on CNS myelination both during development and following demyelination, as well the cells that myelinate the CNS, the oligodendrocytes.

Action potentials represent the principal mode of communication between neurons. Since they allow for the transmission of signals over long distances, action potentials are critical for modulating the behavior of complex organisms. During evolution organisms not only became

larger and more complex, but also encountered harsher and more dangerous environments that necessitated split-second life and death decisions. Thus, a requirement for faster conduction emerged, with action potential speed becoming a limiting factor towards permitting these more complex behaviors. To maximize the velocity of communication, longer and thicker axons evolved. Although increased axonal thickness leads to an initial increase in conduction velocity, it is eventually accompanied by increased resistance, which in turn, is inhibitory to current flow. Because of its unique composition, i.e. high lipid, but low water content, myelin allows for the electrical insulation of axons, counteracts the decrease in current caused by increased resistance, and ensures that action potentials are propagated in an efficient manner[2].

Myelin is an extension of the oligodendrocyte or Schwann cell membrane that is enriched in specialized lipids and proteins. Because of its increased lipid content, myelin has a high degree of resistance that insulates axons. As a result significant membrane depolarization does not occur within myelin-covered axonal segments termed internodes, but instead is confined to nodes of Ranvier. First described by Ranvier in late 19th century, these myelin-free axonal segments located in between the internodes that are enriched in sodium channels and other components of the machinery necessary to generate axon potentials. Axon potentials, therefore, not only arise within myelin-free axonal segments, but can be more efficiently propagated along myelinated axons as they jump from one node of Ranvier to another in a process called saltatory conduction. (reviewed by [1])

Oligodendrocyte Function is Necessary for Normal Brain Development and Function

The importance of myelination for normal brain development and function is illustrated by diseases where myelin is compromised. Loss of myelin, or failure to generate myelin in the first place, disrupts the efficiency and speed of conduction and ultimately leads to loss of function and neuronal death, which in turn leads to cognitive impairment. Hypomyelination in humans can be due to environmental insult or inherited mutations. Neonatal ischemic injury, which occurs primarily in children born before 32 weeks of gestation, results in widespread death of oligodendrocyte progenitor cells (OPCs) leading to delayed myelination, and, in turn, neurological dysfunction and seizures. The degree of hypomyelination depends on the severity of the insult and the extent of OPC death. However, even if myelin eventually appears normal by margnetic resonance imaging (MRI), neurological dysfunction may still persist, suggesting that it is not only important to myelinate but to myelinate at the right time.[3-5]

Inherited leukodystrophies are a wide variety of white matter diseases characterized by failure to generate normal myelin and hypomyelination, and/or failure to adequately maintain myelin stability and demyelination. One of the best characterized leukodystrophies is Palizaeus-Merzbacher disease (PMD), which is caused by mutations and duplications of the myelin-associated, proteolipid protein (PLP)[6]. Interestingly, disease onset can be caused by loss of PLP production, as well as by overexpression of mutant forms of PLP, which cause unfolded protein response and oligodendrocyte toxicity. Because of the severe oligodendrocyte death associated with PMD, normal numbers of myelinating oligodendrocytes are not generated, leading to hypomyelination.[7-9] Since PLP is an integral myelin membrane protein necessary for myelin

stability, loss of functional PLP also results in early onset dysmyelination and demyelination[10]. Hypomyelination, dysmyelination and demyelination associated with PMD all lead to axonal dysfunction and neurodegeneration, thus once again illustrating the importance of myelin for normal brain development and function. (reviewed in [11])

Myelin damage can also occur in adulthood as part of the aging process[12-13] or due to a general CNS insult such as stroke[14]. The most common demyelinating disease, however, is multiple sclerosis (MS). MS is an acquired autoimmune, demyelinating disease that can be identified by the presence of multifocal white matter lesions visualized by MRI. Histopathologically, MS can be identified through the presence of both white and grey matter lesions associated with demyelination, oligodendrocyte death, and reactive gliosis[15]. Although myelin damage and oligodendrocyte death represent the initial insult of MS, the associated progressive axonal loss is thought to be the cause for persistent neurological dysfunction. There are two forms of MS, relapsing-remitting characterized by recurring episodes of myelin insult and neurological deficits followed by episodes of remyelination and functional recovery, and, primary progressive, in which remyelination is inefficient from the start and is accompanied by progressive myelin and axonal loss. However, as irreversible axonal loss increases, relapsing-remitting MS can enter a progressive phase of the disease, thus illustrating the inefficiency of remyelination and the importance of myelin for neuronal survival and function (reviewed by [16-17]) Interestingly, substantial myelin abnormalities have also been reported schizophrenia[18], as well as in several neurodegenerative disorders, such as Alzheimer's disease[19].

Although the neuronal pathology associated with demyelinating diseases illustrates the importance of myelination for axonal survival, oligodendrocytes can regulate neuronal survival independent of myelination as well. Axonal pathologies in the form of axon swellings are associated with normally-myelinated transgenic animals that lack genes encoding for either one of two oligodendrocyte-specific proteins, proteolipid protein (PLP)[20] or 2', 3'- cyclic nucleotide 3'- phosphodiesterase (CNP)[21]. Both PLP1$^{-/-}$ and CNP1$^{-/-}$ mice initially are myelinated normally, but with age, develop axonal swellings that lead to progressive axonal degeneration[20-21]. On the other hand, axonal swellings are absent in *shiverer* mice that lack myelin basic protein (MBP) and are completely devoid of myelin[20], suggesting that axonal swellings do not depend on hypomyelination itself, but on the lack of, or aberrant, trophic support from dysfunctional, PLP1$^{-/-}$ or CNP1$^{-/-}$ oligodendrocytes. A possible mechanism for oligodendrocyte-mediated trophic support of axons can be provided by peroxisomes, which are ubiquitous organelles associated with lipid metabolism and elimination of reactive oxygen species. Mice lacking the essential peroxisome biosynthesis factor peroxin5 (Pex5$^{-/-}$), and thereby lacking oligodendroglial peroxisomes, also exhibit axonal swellings in adult CNS white matter tracts[22]. In addition, peroxisomal dysfunction is thought to be the causative defect associated with X-linked adrenoleukodystrophy, an inherited CNS myelinopathy affecting human males (reviewed in [23]). Taken together, these findings suggest that oligodendrocytes may be necessary for myelination-independent trophic support of axons, which may be mediated through the elimination of axonal reactive oxygen species by oligodendroglial peroxisomes. Since CNS myelinopathies can result from loss of oligodendrocyte progenitors, oligodendrocytes, or myelin, to effectively treat disease we need to expand our understanding of the formation of CNS myelin and the biology of the cells that form myelin, the oligodendrocytes.

Oligodendrocytes: The Myelin Forming Cells in the CNS

Oligodendrocyte development proceeds through stages that include lineage specification, progenitor pool expansion, differentiation to newly-formed or premyelinating oligodendrocytes, and maturation to form myelinating oligodendrocytes. Each of these steps can be identified by the unique cell morphology, as well as by the expression of stage-specific markers (Figure 1).

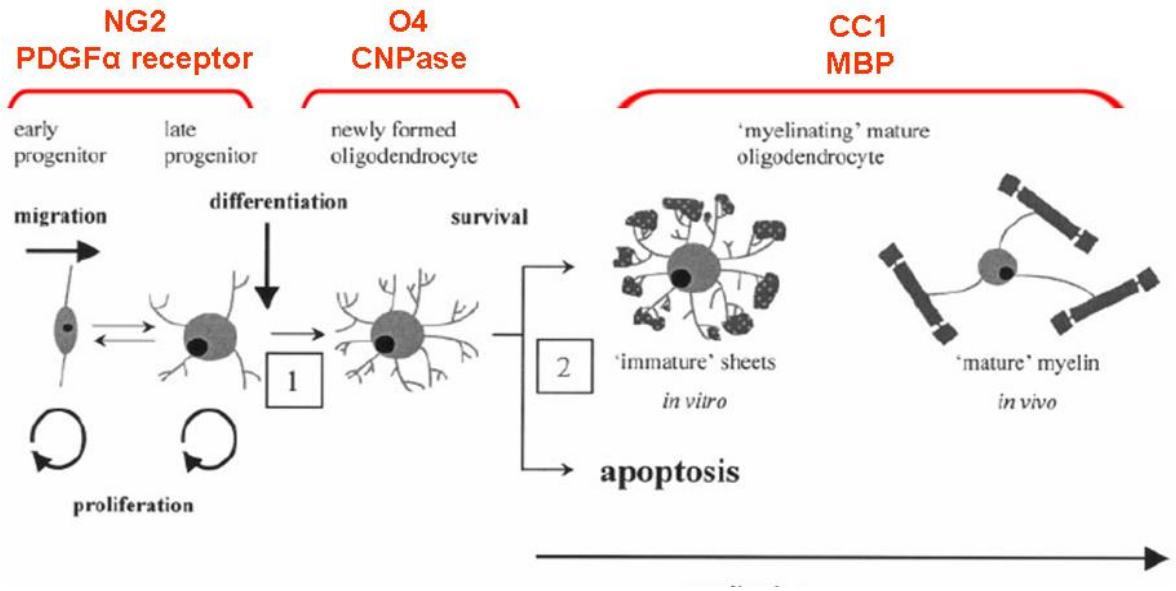


Figure 1: Schematic representation of oligodendrocyte development. Individual steps in oligodendrocyte development can be identified by the markers shown in red. (Figure adapted from [201])

Failure to complete any of the aforementioned lineage stages results in incomplete myelination or remyelination, and therefore, disease. For example, in children that have undergone neonatal hypoxia, oligodendrocyte progenitor cells (OPCs) die in large numbers leading to delayed differentiation and myelination. Even if myelination is eventually completed and gives rise to normal-appearing white matter by MRI, the initial delay may still lead to axonal dysfunction and cognitive deficits[3-5]. In addition, numerous reports have identified the presence of OPCs and newly-formed oligodendrocytes within MS plaques[24]; however, incomplete or absent differentiation of these cells leads to inefficient remyelination, axonal death, and symptomatic

onset. In view of these observations, an important goal is to identify molecules that regulate the transition points between different stages of the oligodendroglial lineage.

Lineage Specification of Oligodendrocyte Progenitor Cells

Oligodendrocytes arise from a pool of pluripotent neuroepithelial cells residing primarily in the ventricular zone of the embryonic neural tube. The classical view of oligodendrogenesis is that it takes place during late embryonic and early postnatal development, following sequential waves of neurogenesis and astrogliogenesis. As in many scientific fields, the study of oligodendrocyte specification has been under both intense investigation and fierce conflict. Here I will focus on the main findings that have sparked this debate over the years.

The conflict within the scientific community arises from the fact that oligodendrogenesis occurs in a diverse spatiotemporal manner within the subventricular zones of the developing spinal cord and brain. Following lineage specification, oligodendrocyte progenitors populate both myelinated white matter tracts and non-myelinated grey matter regions. It is therefore reasonable to predict that oligodendrogenesis takes place throughout multiple regions of the CNS. This view is supported by the finding that oligodendrocytes can arise from radial glial cells[25], which are similarly present in the entire CNS. Both dorsal and ventral sources for oligodendrocytes were also identified using a LacZ reporter mouse where LacZ was expressed under the promoter of the oligodendrocyte-specific gene proteolipid protein (PLP)[26-27]. Further support of the hypothesis that oligodendroglia arise from multiple CNS regions was lent by the observation that cultured glial-specific precursors were able to give rise to oligodendrocytes and

astrocytes regardless of which embryonic spinal cord region they were initially isolated from[28-30]. However, the numerous limitations of these studies, together with the identification of the highly oligodendrogenic pMN domain within the ventral embryonic spinal cord, have brought forth the idea that oligodendrogenesis is restricted to the ventral regions of the neural tube.

Fate mapping experiments using a series of transgenic reporter lines have helped resolve his debate and have consolidated the two opposing views of oligodendrogenesis into a unified hypothesis. These studies have found that while the majority of oligodendrocytes are born within the ventral regions of the embryonic CNS, some oligodendrocytes originate from dorsal regions as well. Support of this hypothesis is given by the finding that Nkx6.1 and Nkx6.2 double mutant mice lack ventrally-derived OPCs, but still possess OPCs in the dorsal spinal cord[28,31-33]. Importantly, Nkx 6.1 and Nkx6.2 double mutants lack expression of all Nkx6 homeodomain transcription factors and can therefore be designated as Nkx6 null. Nkx6 expression is confined within the p3, pMN, p2 and p1 domains of the ventral spinal cord (Figure 2A). Loss of Nkx6, therefore, signifies the loss of these ventral domains and thus ventral oligodendrocyte production. The dorsal OPCs present in Nkx6 null mice are exclusively of dorsal origin as they coexpress the dorsal transcription factor Pax7 (paired box gene 7).[31-32] Interestingly, Pax7 also colabels a subset of OPCs in wild type mice that are generated around embryonic day 15 (E15). The number of Pax7 positive OPCs is increased in Nkx6 null mice, suggesting that ventrally-derived OPCs, which originate earlier (E12.5), may outcompete their dorsally-derived counterparts for pro-survival and pro-proliferation factors.[31] Dorsally-derived OPCs not only originate later in development, but also contribute to, at most, 10-15% of cells of the oligodendrocyte lineage[25,31]. In addition, OPCs that originate in the dorsal column were observed in Cre recombinase-dependent reporter lines under control of embryonic transcription factor

promoters including those for Msx3 (homeobox, msh-like) and Dbx-1 (developing brain homeobox 1)[25]. These findings suggest that within the embryonic spinal cord, most but not all, OPCs arise from ventral regions.

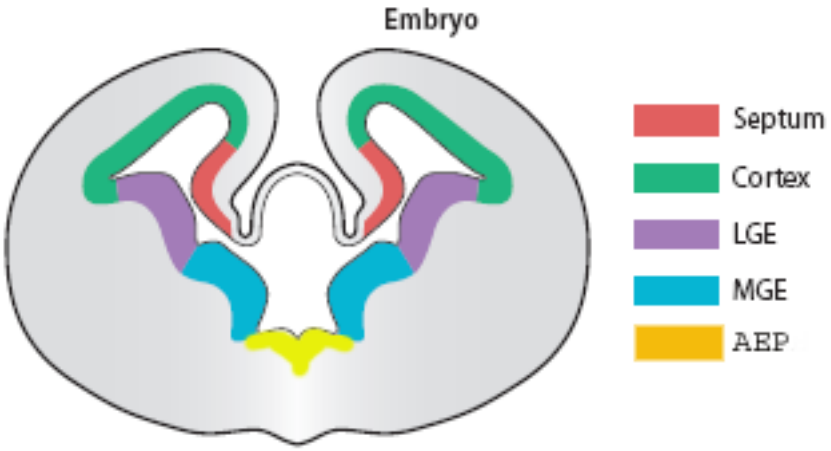


Figure 2: Origins of OPCs in the developing rodent forebrain. The septum, cortex, lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), and anterior entopendunclular area are represented. (Figure modified from [218])

Within the embryonic forebrain oligodendrogenesis proceeds in a manner similar to that observed in the spinal cord. Again fate mapping experiments have shown that oligodendrogenesis begins within ventral regions and is followed by a contribution from dorsal regions. Forebrain oligodendrocytes born before E18 arise from the medial ganglionic eminence (MGE), the anterior entopenduncular area (AEP) and the preoptic area. After E18, OPCs are specified within the lateral and caudal ganglionic eminances (LGE and CGE) and, finally, within the postnatal cortex.[34] (Figure 2) Interestingly, unlike spinal cord OPCs, ventrally-derived (MGE and AEP) forebrain OPCs die out and disappear by birth[34], suggesting the existence of active remodeling within the CNS. In the postnatal cortex, OPCs arise from neural progenitors that lie

within the postnatal ventricular zone and line the tips of the lateral ventricles. Since the postnatal ventricular zone is derived from the LGE and the lateral cortex, and not from ventral regions (MGE and AEP)[35-37], postnatal gliogenesis may contribute to the loss of ventrally-derived OPCs in the brain[38]. These findings raise the question of whether there are distinct roles for the distinct populations of oligodendrocytes.

Lineage specification is governed by a variety of transcription factors and morphogens. The characterization of a series of animal models has led to the discovery of the transcription factor Olig2 as not only necessary, but also sufficient, for oligodendrogenesis[39-41]. Olig2 is expressed throughout the oligodendrocyte lineage, and mice lacking this transcription factor fail to generate oligodendroglia[28,33,42]. It should be noted that oligodendrocytes in the ventral spinal cord arise from the same region as motor neurons, the pMN domain, and in addition to their failure of oligodendrogenesis, $Olig2^{-/-}$ animals also exhibit impaired motor neuron specification. On the other hand, ectopic expression of Olig2 induces expression of the transcription factor Sox10, which in turn causes production of oligodendrocytes at the expense of neurons[43-44].

Oligodendrocyte specification is regulated by a series of morphogens that elicit their effects through the activation of the transcription factors described above. Gradient morphogen expression provides dorso-ventral and anterior-posterior patterning of the developing neural tube. The ventrally derived morphogen Sonic Hedgehog (SHH) is necessary and sufficient to induce expression of the *Olig2* gene[39], which is absolutely necessary for oligodendrogenesis. SHH also activates the expression of *Nkx6*, which is necessary for ventral oligodendrocyte specification. Although important for oligodendrogenesis[45-48], SHH signaling is not necessary for

this process. Since SHH is expressed along the ventral floorplate, it is therefore virtually absent in the dorsal spinal column. Similarly, there is no SHH expression within the cortex. It is therefore reasonable to suggest that due to its absence, SHH cannot initiate signaling events within these regions, and therefore, cannot regulate dorsal oligodendrogenesis. This hypothesis is supported by a number of studies where SHH signaling is ablated or inhibited. SHH initiates a signaling cascade by activating the smoothened (Smo) receptor, which in turn can be inhibited by cyclopamine. Smoothened null blastocysts generated from $Smo^{-/-}$ embryonic stem cells can give rise to oligodendrocytes in culture[31], while dorsally-derived oligodendrocytes can still be generated in the presence of cyclopamine[46,49]. Taken together, these observations suggest that, although SHH signaling is necessary for ventral oligodendrogenesis, oligodendrocyte specification in the dorsal neural tube, as well as within the postnatal cortex, occurs in a SHH-independent manner. On the other hand, exogenous stimulation with fibroblast growth factor (FGF) in cyclopamine-treated cultured cells can induce SHH-independent oligodendrogenesis[49]. Since FGF is also present in the dorsal neural tube, these findings suggest that FGF may be necessary for dorsal oligodendrogenesis.

Interestingly, despite their diverse spatiotemporal origin, all oligodendrocytes seem to possess similar developmental and morphological characteristics. And, there is no compelling evidence for the existence of functionally distinct oligodendrocytes. Moreover, if OPCs arising from any region are ablated, they are eventually replaced by OPCs that have migrated in from other regions[34]. This finding not only demonstrates the redundancy of the system, but also the importance of OPC proliferation.

Oligodendrocyte Progenitor Cell Proliferation

In the late 1980s *in vitro* clonal density experiments gave rise to the hypothesis that OPC proliferation is controlled by purely cell autonomous mechanisms. The observation that OPCs dissociated from the brain develop *in vitro* on the same timeline as they would *in vivo* supports this hypothesis.[50] In addition, OPCs grown in the presence of saturating levels of platelet derived growth factor (PDGF) and thyroid hormone divide 6-8 times prior to cell cycle arrest and differentiation, while daughter cells grown in isolation, exit the cell cycle after an equivalent number of divisions[50-51], suggesting that OPCs may be able to "count" the number of cell divisions they undergo . Interestingly, although OPCs grown at 33°C divide slower, they differentiate at the same time as OPCs grown at 37°C, suggesting that OPCs not only count the cell divisions they undergo but that they also "count" time[52]. Taken together, these findings suggest that OPCs must possess a cell intrinsic mechanism, referred to as "the timer"[50], that limits the number of cell divisions and/or the time spent within the cell cycle.

Proliferation of oligodendrocyte progenitors is controlled by both extrinsic and intrinsic factors. An important cell extrinsic regulator of the OPC "timer" is the growth factor PDGF, which in the CNS is produced and secreted by astrocytes and neuronal cell bodies[53]. Addition of saturating levels of exogenous PDGF to cultured OPCs leads to proliferation in the absence of differentiation, while PDGF withdrawal triggers cell cycle arrest *in vitro*[50,54]. *In vivo*, loss of PDGF leads to premature OPC cell cycle exit[55], while overexpression of PDGF in neurons is accompanied by significant oligodendrocyte progenitor pool expansion[56]. PDGF promotes OPC proliferation by activating the PDGF α receptor (PDGFαR), which in turn leads to a rise in

cytosolic Ca^{2+} levels[57] and expression of early oligodendrocyte genes[57-59]. In the absence of ligand, PDGFα receptors are downregulated, leading to cell cycle exit and differentiation[57].

Although it is the best characterized OPC mitogen, PDGF is not the only growth factor capable of promoting OPC proliferation. Exogenous stimulation of cultured OPCs with either fibroblast growth factor 2 (FGF2)[60], insulin-like growth factor 1 (IGF1)[61], neuregulin[62], or neurotrophin 3 (NT3)[63], can also promote OPC proliferation. Furthermore, mice lacking the ciliary neurotrophic factor (CNTF) have reduced numbers of proliferating OPCs, suggesting that CNTF may be necessary for OPC proliferation *in vivo*[64]. In addition to their autonomous functions, combinations of growth factors can have additive effects on OPC proliferation. For example, IGF1, neuregulin1, NT3 and FGF2 have all been shown to potentiate PDGF-induced proliferation *in vitro*[62,65-67], while synergistic effects were also observed in cultured OPCs upon co-administration of FGF2 with either IGF1[67], or nerve growth factor (NGF)[63]. Interestingly, treatment of OPCs with FGF2 was able to stimulate PDGFαR expression[66], suggesting that FGF2 signaling can potentiate the response of OPCs to exogenous PDGF stimulation. On the other hand, PDGF-induced proliferation can be inhibited by transforming growth factor β (TGFβ) *in vitro*[68]. Taken together, these findings suggest that in the developing brain OPCs integrate signals from a variety of exogenous factors to mount a single response, proliferation.

Intracellularly, eukaryotic cell proliferation is controlled by the activity of cyclin-dependent kinases (Cdks), which are cyclically controlled to trigger the transition between the different stages of the cell cycle. Cdk activity is modulated by factors such as cyclins and cyclin-dependent inhibitors. To acquire full activity Cdks associate with cyclins, which not only

phosphorylate to activate the Cdks, but also recruit them to their ligands. The cyclin-Cdk complex is therefore critical for cell cycle progression and therefore proliferation. To ensure that cells do not enter particular cell cycle stages prematurely, and therefore interfere with the efficiency of proliferation, a system of checkpoints has been developed. Cyclin-dependent Cdk inhibitors disrupt cyclin-Cdk complex assembly and activity, and are therefore sufficient for cell cycle arrest. There are two classes of cyclin-dependent Cdk inhibitors; the Cip/Kip family including $p21^{Cip1}$, $p27^{Kip1}$ and $P57^{Kip2}$, and the Ink4 family consisting of $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$. (reviewed in[69]) Overexpression of $p27^{Kip1}$ promotes OPC cell cycle exit even in the presence of the mitogen PDGF, while loss of $p27^{Kip1}$ causes hyperproliferation in the absence of mitogen stimulation. In addition, protein levels and, $p27^{Kip1}$ expression is induced when OPCs are cultured at 33°C, a condition that promotes cell cycle arrest after fewer cell divisions[52]. Since the intracellular timer runs faster at a lower temperature[52], this observation further highlights the importance of $p27^{Kip1}$ as an effector of the cell intrinsic mechanism that controls OPC proliferation. Interestingly, even though $p27^{Kip1}$ levels progressively increase in the presence of PDGF this increase is not sufficient to induce cell cycle exit in the absence of thyroid hormone[59]. In addition to $p27^{Kip1}$, other Cdk inhibitors that have been identified as important cell intrinsic factors that control OPCs proliferation include $p57^{Kip2}$ [70] and $p18^{Ink4c}$ [71].

Oligodendrocyte Differentiation

Differentiation of OPCs to early oligodendrocytes involves several steps: cell cycle exit, process outgrowth and branching, and survival during a wave of apoptosis that eliminates many newly-formed oligodendrocytes. Then, upon expression of myelin-specific lipids and proteins newly-formed oligodendrocytes transition to mature oligodendrocytes and differentiation is

completed. This complex process is regulated by extracellular cues such as growth factors and extracellular matrix (ECM) molecules, as well as oligodendrocyte-intrinsic factors such as transcription factors, kinases and even Cdk inhibitors.

Extracellular Regulators of Oligodendrocyte Differentiation

One of the first described extracellular regulators of oligodendroglial differentiation is the thyroid hormone. As described above, oligodendrocyte progenitors grown in the presence of saturating levels of exogenous PDGF fail to differentiate[50-51]. But, if 8 days later, these same cells are also treated with thyroid hormone they differentiate within 4 days[51]. These findings suggest that thyroid hormone is sufficient to stop proliferation and initiate differentiation even in the presence of mitogen stimulation *in vitro*. In addition, thyroid hormone treatment of dissociated brain cells results in increased numbers of differentiated oligodendrocytes, suggesting that the thyroid hormone can promote differentiation in the absence of mitogen stimulation as well[51]. Furthermore, thyroid hormone treatment can induce myelin gene expression in oligodendrocytes *in vitro*[72]. On the other hand, OPCs can differentiate into newly-formed oligodendrocytes within 1-2 days in the absence of thyroid hormone and PDGF treatment[50,73-74], suggesting that, in the absence of mitogen stimulation, thyroid hormone is not necessary for differentiation. Thyroid hormone is, therefore, an extracellular factor that can promote oligodendroglial differentiation *in vitro*, although it is not required.

Examination of animals with increased or decreased thyroid gland function and therefore altered thyroid hormone levels revealed that thyroid hormone also promotes oligodendrocyte differentiation and myelination *in vivo*[75-78]. Hypothyroid rodents are hypomyelinated and have

fewer mature oligodendrocytes in the optic nerve at postnatal day 7 (P7)[78]. In addition, mice lacking thyroid receptor expression exhibit increased OPC proliferation, leading to delayed OPC-to-oligodendrocyte transition, delayed oligodendroglial differentiation and hypomyelination in the optic nerve[79]. On the other hand, oligodendrocyte differentiation and myelination were accelerated in hyperthyroid mice[77]. Taken together these findings identify thyroid hormone as an extracellular signal that promotes differentiation even in the presence of mitogen stimulation *in vivo*.

There are two thyroid hormone receptor isoforms expressed within oligodendroglia; the α isoform (TRα) and the β1 isoform (TRβ1). While TRα levels remain constant throughout development, TRβ1 levels increase as sensitivity to thyroid hormone increases, suggesting that the pro-differentiation effects of this hormone may be mediated through TRβ1. Interestingly, at 33°C, when the "timer" runs faster, TRβ1 levels increase with similar kinetics to those previously described for the cell cycle inhibitor $p27^{Kip1}$. OPCs cultured at 33°C exit the cell cycle faster than, and differentiate after fewer divisions than OPCs cultured at 37°C.[52] It is therefore likely that at lower temperatures proliferating OPCs become primed for differentiation by increasing their sensitivity to thyroid hormone. Thus, although not required for differentiation, thyroid hormone signaling coordinates the initiation of differentiation in oligodendrocyte progenitors.

Oligodendrocyte differentiation can also be regulated through activation of the Notch signaling pathway. While oligodendrocytes express the Notch1 receptor, the Notch ligands Delta1 and Jagged1 are expressed by neurons and astrocytes. Activation of Notch1 by either

ligand is inhibitory to oligodendrocyte differentiation.[80-81] Exogenous addition of the soluble ligand Delta1 inhibited differentiation *in vitro*[81]. A similar result was observed when OPCs were plated on a monolayer of cells expressing the transmembrane Notch1 ligand, Jagged1[80]. OPC proliferation was not affected in either experiment suggesting that Notch1 activation regulates differentiation specifically[80]. Since Delta1 is a secreted ligand, while Jagged1 is membrane-associated, these findings suggest that oligodendrocyte differentiation can be regulated by both paracrine and juxtaparacirine mechanisms. The role of Notch signaling in inhibiting differentiation is further supported *in vivo* through the examination of Notch1 conditional knockout animals. Genetic ablation of Notch1 within the oligodendrocyte lineage, using Cre recombinase driven by the CNP promoter, resulted in premature oligodendrocyte differentiation, as well as ectopic differentiation within the CNS grey matter[82]. Precocious oligodendrocyte differentiation was also observed in mice where Notch1 was specifically deleted in Olig1-expressing cells[83]. Furthermore, loss of Notch1 led to enhanced differentiation, which resulted in improved remyelination after lysolecithin-induced demyelination. The authors of the latter study also reported that the enhanced differentiation was at the expense of proliferation, thus for the first time, making the connection between Notch1 signaling and OPC proliferation.[84] Taken together these findings suggest that the Delta1/Jagged1-Notch signaling axis is not only inhibitory to oligodendrocyte differentiation, but is also responsible for the spatial and temporal control of oligodendrocyte differentiation. Interestingly, a number of recent studies have reported that when in a complex with the axonal adhesion molecule F3 contactin, oligodendroglial Notch activation could also promote oligodendroglial differentiation.[85-86] These latter findings provide an additional example of the importance of axon-oligodendroglial interactions in regulating oligodendrocyte development and myelination.

Components of the extracellular matrix (ECM) are also thought to regulate oligodendrocyte differentiation. For instance, the ECM component laminin has been shown to act as a molecular signaling switch to the growth factor neuregulin during oligodendrocyte survival[87]. Laminin is a secreted glycoprotein whose three subunits (α, β, and γ) are products of unique genes that assemble to form a cruciform structure (reviewed by [88]). Although laminin expression in the brain appears to be transient, its importance for myelination was uncovered by mutations in laminin α2 (a subunit of laminin2) that cause congenital muscular dystrophies accompanied by brain hypomyelination. Myelination is also impaired in laminin α2-deficient (dy/dy)[89] and laminin α2 knockout (Jenne Relucio unpublished observations) mice. The hypomyelination defects observed in these mouse models were due to delayed oligodendrocyte differentiation ([89] and Jenne Relucio – unpublished observations) suggesting that laminin is necessary for timely oligodendrocyte differentiation *in vivo*. Furthermore, OPC proliferation is increased in the corpus callosum of the laminin α2 knockout animals, revealing a role for laminin2 in promoting OPC cell cycle exit *in vivo* (Jenne Relucio – unpublished observations). *In vitro* laminin2 promotes newly-formed oligodendrocyte survival[87], as well as oligodendrocyte differentiation and myelin sheet formation[90]. The role of laminin in differentiation and myelination suggests that downstream effectors of laminin may prove to be good targets for myelination-promoting therapies. In addition to laminins, other ECM molecules such as fibronectin[91], have been implicated in promoting oligodendroglial development, however, neither of these has been shown to regulate myelination *in vivo*.

Cell-Intrinsic Regulators of Oligodendrocyte Differentiation

Laminin2 signaling is mediated in oligodendrocytes through activation of $\alpha_6\beta_1$ integrin[90] and/or dystroglycan[92]. Dystroglycan is necessary for oligodendrocyte differentiation and myelination *in vitro* as it promotes oligodendrocyte differentiation, myelin membrane formation and expression mature oligodendrocyte markers in cultured oligodendroglia, as well as myelin segment formation in oligodendrocyte-neuron co-cultures[92]. Studies using cultured oligodendrocytes have also suggested that the intracellular domain of β_1 integrin is necessary for laminin2-induced oligodendroglial process outgrowth and myelin sheet formation[93-94]. The role of integrin signaling in oligodendrocyte differentiation and myelination *in vivo*, however, is controversial. Oligodendrocyte-specific deletion of β_1 integrin (CNP-cre-mediated β_1 deletion) resulted in a small increase of oligodendrocyte apoptosis in the cerebellum, but led to no myelination abnormalities[95]. On the other hand, decreased myelination, but normal oligodendroglial differentiation, was observed within the spinal cord of mice lacking β_1 integrin expression in oligodendroglia (NG2-cre-mediated β_1 deletion), as well as in all neural progeny, i.e. neurons, astrocytes and oligodendroglia (Nestin-cre-mediated β_1 deletion)[96]. In oligodendroglia, integrin activation by fibronectin and laminin2 is mediated by the activation of Src family kinases (SFKs). The role of these intracellular signaling molecules in oligodendrocytes development will be discussed in detail in Chapter 2.

Since oligodendrocytes have to proliferate before they differentiate, factors, such as Cdk inhibitors, that promote cell cycle exit can therefore stimulate differentiation. Although the importance of $p27^{Kip1}$ in promoting OPC cell cycle exit has been discussed in detail above, it is not the only Cdk inhibitor necessary for proper oligodendroglial development. Unlike $p27^{Kip1}$,

the role of $p21^{Cip}$ is specific to oligodendrocyte differentiation. Surprisingly, proliferation is not affected in $p21^{-/-}$ OPCs; they exit the cell cycle in a timely manner. However, $p21^{-/-}$ exhibit a delay in differentiation, which *in vivo* results in a delay in cerebellar myelination[97-98]**.** Interestingly, thyroid hormone, a pro-differentiation factor described above, promotes p53-mediated p21 transcription and therefore p21 expression[98]. The Cdk inhibitor p21 may therefore serve as a cell-intrinsic effector of thyroid hormone to promote oligodendrocyte differentiation independent of proliferation.

Newly-Formed Oligodendrocyte Survival

For efficient myelination to occur, oligodendrocytes need to be generated in sufficient numbers. However, as many as 50% of newly-formed oligodendrocytes, at least in the developing rat optic nerve, undergo a wave of programmed cell death[99], suggesting that oligodendroglial apoptosis may be part of the developmental program of oligodendrogenesis. Furthermore, optic nerve transection, which resulted in neuronal apoptosis, also led to oligodendroglial apoptosis[100], while overexpression of the anti-apoptotic protein Bcl-2 (B cell lymphoma 2) in retinal gangion neurons, which increased neuronal survival and the axonal numbers, resulted in a proportional increase in the number of oligodendroglia[101]. These observations have led to the hypothesis that axonal signals are necessary to provide trophic support for oligodendrocyte survival, and that by secreting trophic factors, axons may regulate oligodendrocyte numbers. Interestingly, although overexpression of PDGF in transgenic animals led to OPC hyperproliferation and increased numbers of OPCs, upon differentiation, the numbers of mature oligodendrocytes normalized to those observed in wild type animals[56], further

suggesting the necessity for axonal-derived trophic support of oligodendroglial survival. A number of extracellular factors have been identified to promote oligodendrocyte survival.

In addition to promoting OPC proliferation as described above, growth factors increase oligodendroglial cell numbers by providing trophic support for, and thereby stimulating the survival of OPCs and newly-formed oligodendrocytes. Growth factors such as NGF[63], NT3[63], IGF1[102], and neuregulin1, have been shown to promote newly-formed oligodendrocyte survival *in vitro*. Although newly-formed oligodendrocyte survival was ultimately normalized in transgenic animals overexpressing PDGF, PDGF has been shown to promote oligodendroglial survival *in vitro*[103] and *in vivo*[56,99]. In addition, overexpression of neuregulin1 in rat optic nerves decreased apoptosis, resulting in increased numbers of mature oligodendrocytes[104], suggesting that neuregulin1 promotes oligodendroglial survival *in vivo* as well. While all of the aforementioned growth factors stimulate survival, they do so by stimulating different oligodendroglial signaling pathways. IGF1 and neuregulin1 promote survival by promoting Akt phosphorylation and activity[62,87,105-107], on the other hand, NGF and NT3 elicit their trophic effects through phosphorylation and activation of the MAP kinase pathway[63]. How can the same sets of growth factors regulate both OPC survival and proliferation?

In addition to growth factors, extracellular matrix components can also promote oligodendroglial survival. Studies in cultured oligodendrocytes have shown that the extracellular matrix molecules fibronectin and laminin2 can promote survival of OPCs and newly-formed oligodendrocytes respectively[87,91,108]. Although oligodendroglial survival is unaffected in adult laminin2-deficient mice[89], mature oligodendrocyte survival is impaired in mice completely

lacking laminin2 expression (laminin α2 knockout mice, Jenne Relucio - unpublished observations). Increased apoptosis of mature, MBP-positive oligodendrocytes were also observed in animals lacking the α_6 integrin receptor[87], suggesting that the pro-survival effects of laminin2 treatment may be mediated, at least in part, by integrin signaling. Upon examination of cultured, wild type oligodendrocytes, the authors of this study further suggested that integrin signaling may be necessary to enhance neuregulin1-induced survival by switching neuregulin1 signaling from a dependence on the PI3K/Akt pathway to the MAPK pathway[87]. Interestingly, depletion of dystroglycan, the second laminin2 receptor in oligodendrocytes, did not affect laminin2-mediated survival of newly-formed oligodendrocytes *in vitro*, suggesting that the pro-survival effects of laminin2 are mediated exclusively by integrin signaling in oligodendroglia[92]. Taken together, these findings suggest that trophic factors present in the extracellular milieu are necessary to promote oligodendroglial survival and thereby the generation of sufficient numbers of oligodendrocytes. These findings further suggest that axon-oligodendroglial contact may switch growth factor signaling from promoting oligodendroglial proliferation to promoting survival and differentiation.

Myelination

As described previously, myelin is not only necessary for the efficiency and velocity of nerve communication, but for neuronal survival and integrity as well. Since both mature and myelinating oligodendrocytes express myelin proteins and lipids, the only difference between the two stages of the lineage is that myelinating oligodendrocytes wrap their processes around an axon. What are the cues that prompt a mature oligodendrocyte to myelinate an axon? In both the

CNS and the PNS, some axons are myelinated while others are not. It is therefore reasonable to suggest that the axons themselves can regulate myelination.

Axonal Diameter and Electrical Activity Stimulate Myelination

Within myelinated regions of the nervous system, axons differ, not only by their myelination status, but also by their diameter. Examination using transmission electron microscopy reveals that the axons that are myelinated generally have a larger diameter than the ones that are not. Although the precise axon sizes differ regionally within the CNS, axons with diameters lower than 0.2µm remain unmyelinated regardless of the region. These observations lead to the question: could axon caliber dictate whether myelination takes place, or, does myelination increase axon diameter? In 1989 Voyvodic et al. were able to increase the diameter of sympathetic axons by increasing the size of their target. This increase was sufficient to induce myelination in axons that should have otherwise remained unmyelinated[109]. Although this experiment was carried out in the PNS, it is generally accepted that large axon caliber promotes myelination in the CNS as well.

In addition to size, the electrical activity of the axon promotes myelination[110] suggesting that neuronal differentiation may regulate myelination. The hypothesis that axonal electrical activity is a pro-myelinating factor originated in 1980 with the observation that premature eye opening, which stimulates activity, leads to accelerated myelination in the rabbit optic nerve[111]. This hypothesis was finally validated a decade later by Barres *et. al.*, who showed that treatment of rat optic nerves with tetradotoxin, which inhibits Na^+-dependent electrical activity, leads to impaired oligodendrocyte proliferation and myelination[110]. Myelination is also impaired in the

naturally occurring blind scape-mole rats[112] and in wild type mice that have been reared in the dark. On the other hand, induction of neuronal firing with α-scorpion toxin promotes myelination[113], suggesting that neuronal activity is not only necessary but also sufficient to promote myelination.

Since action potentials cause the release of ATP and adenosine[114-115] it is reasonable to propose that these secreted molecules may communicate neuronal electrical activity to oligodendrocytes, and thus, trigger myelination. Addition of adenosine binds to cultured oligodendrocytes promoted cell cycle exit and differentiation and myelination[116]. ATP can also regulate oligodendrocyte behavior, but unlike adenosine it does so indirectly with the help of astrocytes. ATP promotes astrocytic release of the leukemia inhibitory factor (LIF), which in turn promotes oligodendrocyte differentiation and myelination *in vitro*[117]. LIF is also necessary for myelination *in vivo* as $LIF^{-/-}$ mice are hypomyelinated[118]. Interestingly, in the PNS, where astrocytes are absent, ATP is able to bind directly to P2 receptors in Schwann cells and therefore promote differentiation and myelination[119]. The role of astrocytes as mediators of axonal signals in myelination reveals the existence of an ATP-dependent, tripartite signaling pathway of myelination in the CNS. Interestingly astrocyte abnormalities can lead to impaired CNS myelination. Examples of this are seen in $GFAP^{-/-}$ mice (glial fibrillary protein) and in patients that lack GFAP expression and therefore suffer from Alexander's disease, an inherited leukodystrophy[120]. These findings highlight the importance of axo-glial communication in regulating myelination.

Axo-Glial Interactions Regulate Myelination

To promote myelination, axons need to communicate with oligodendrocytes either directly or indirectly. Neuronally-expressed molecules, whether on the axonal surface or secreted into the ECM, have been shown to both promote and inhibit myelination. The interaction between nerve growth factor (NGF) and its receptor TrkA is an interesting example where both secreted and associated neuronal signals regulate myelination. Addition of NGF to co-cultures of oligodendrocytes and dorsal root ganglion neurons (DRG) *in vitro* inhibited myelination. Interestingly, this effect was specific to TrkA-expressing neurons and was not observed in co-cultures containing TrkB expressing neurons.[121] These findings suggest that NGF-induced TrkA activation modulates an axonal signal that controls myelination. A candidate for this axonal signal is LINGO-1 (LRR and Ig domain containing, Nogo-receptor interacting protein), since activation of TrkA induces the axonal expression of this cell surface associated protein[122]. Furthermore, LINGO-1 is inhibitory to myelination as treatment with either dominant negative LINGO-1 lentivirus or LINGO-1 Fc increased the numbers of mature oligodendrocytes as well as the number of myelinated segments in OPC-DRG co-cultures *in vitro*[123]. Since LINGO-1 is expressed on both oligodendrocytes and neurons, and since oligodendrocyte differentiation was also affected upon LINGO-1 downregulation, [123] it is not clear whether LINGO-1 is a direct effector of NGF-TrkA signaling in oligodendrocytes, and, whether LINGO-1 is indeed an inhibitor of myelination and not only differentiation.

Since oligodendrocytes wrap around and therefore directly interact with axons, molecules on the axonal surface are critical for myelination. An axon-associated signal that regulates myelination is the neuronal cell adhesion protein PSA-NCAM (polysilyslated neural cell

adhesion molecule). During neuronal development neural cell adhesion molecule (NCAM) is polysialylated to form PSA-NCAM. This posttranslational modification inhibits homophilic interactions between NCAMs, as well as for heterophilic interactions with heparin sulfate proteoglycans such as the OPC marker NG2[124]. Studies performed on dissociated cerebellar hemispheres *in vitro* showed that elimination of PSA-NCAM using blocking antibodies can promote myelination. Although necessary for myelination, PSA-NCAM is not required for oligodendrocyte differentiation, as the numbers of MBP positive cells remained the same in this experiment[125]. PSA-NCAM is therefore an axonal signal that is inhibitory to oligodendrocyte myelination. PSA-NCAM expression decreases right before the onset of myelination[126] suggesting that this axonal adhesion molecule may regulate the proper timing of myelination and that direct axo-glial contact may not be sufficient for myelination. Interestingly, PSA-NCAM is re-expressed in MS lesions but not in lesions that remyelinate[127-128]. These findings suggest that, whether during development or disease, the absence of PSA-NCAM creates an environment permissive to myelination.

The neuronal growth factor neuregulin1 is both necessary and sufficient for PNS myelination. However, the role of neuregulin1 and its associated signaling is controversial in the CNS. Early studies have shown that exogenous addition of soluble neuregulin1 promotes OPC proliferation and oligodendrocyte survival *in vitro*[62,129], while overexpression of neuregulin1 in the developing rat optic nerve promotes OPC proliferation and newly-formed oligodendrocyte survival *in vivo*[104]. On the other hand, exogenous addition of soluble neuregulin1 can both promote[130] and inhibit[62] oligodendroglial differentiation *in vitro*. In terms of myelination, however, the role of neuregulin1 signaling is controversial. CNS hypomyelination phenotypes

have been observed in the CNS of neuregulin1 deficient mice[128], as well as in transgenic animals overexpressing dominant-negative forms of the oligodendroglial neuregulin1 receptors ErbB2[131] and ErbB4[132]. However, using a variety of transgenic animal models Brinkmann *et al.* recently reported that, although neuregulin1/ErbB signaling is necessary and sufficient for PNS myelination, it may be dispensable for CNS myelination[133]. In support of previous findings that neuregulin1 type III promotes myelination in neuron-oligodendrocyte cocultures[128], Brinkmann *et al*. found that overexpression of neuregulin1 type III in neurons not only led to hypermylination in the CNS, but to more efficient remyelination following lysolecithin-induced demyelination as well. However, genetic ablation of neuronal neuregulin1 type III, as well genetic loss of oligodendrocyte ErbB receptors, had no effects on CNS myelin, suggesting that neuregulin1/ErbB signaling may be dispensable for CNS myelination.[133]

As previously described, IGF1 promotes proliferation[106] and survival[107] of OPCs, as well as survival of newly-formed oligodendrocytes[99]. $IGF1^{-/-}$ mice exhibit decreased numbers of oligodendroglia in the corpus callosum and anterior commissure[134], further illustrating the necessity of this growth factor for generating sufficient numbers of oligodendrocytes. In view of these observations, it is not surprising that this growth factor also promotes myelination. Although IGF1 null mice are hypomyelinated in the CNS[134], the degree of hypomyelination observed, is directly proportional to the extent of neuronal loss observed, suggesting that the hypomyelination phenotype may be due to decreased neuronal survival, and not necessarily due to loss of oligodendroglial myelination potential. IGF1 signaling in oligodendrocytes is inhibited by IGF binding protein 1 (IGFBP), and overexpression of IGFBP1 in oligodendroglia *in vivo*, results in hypomyelination[135], suggesting that IGF1 signaling within cells of the oligodendrocyte

lineage may be necessary for myelination. On the other hand, overexpression of IGF1 in transgenic animals results in hypermyelination in the presence of normal neuronal numbers[135-136], suggesting that IGF-1 is sufficient for myelination. Taken together these findings suggest that IGF1 signaling in oligodendroglia is necessary and sufficient for myelination.

Yet another example of the axonal control of myelination is the regulation of PLP localization. While PLP is primarily intracellular in mature oligodendrocytes grown in isolation, in the presence of axons, PLP is redistributed to the myelin membrane, where it constitutes one of the major components of myelin. PLP can be synthesized and trafficked to the plasma membrane in mature oligodendrocytes in the absence of neurons[137], however soon after, membrane PLP is endocytosed and redistributed to late endosomal/lysosomal compartments[138-139]. However, in neuron-oligodendrocyte co-cultures, clathrin-dependent endocytosis of PLP is decreased, while exocytosis is induced, resulting in the redistribution of PLP to the membrane where it colocalizes with myelin components such as MBP.[140]

Oligodendrocyte-Intrinsic Signals that Promote Myelin Wrapping and Myelination

As described previously, CNS myelination is the ensheathment of axons by spiral extensions of the oligodendroglial plasma membrane. To adequately myelinate axons, myelinating oligodendrocytes need to: 1) be generated in sufficient numbers, 2) be morphologically complex, and, 3) have generated sufficient amounts of myelin lipids and proteins. Although signals that promote the production of sufficient numbers of mature oligodendrocytes are necessary for normal myelination, only one cell-intrinsic signaling pathway sufficient to induce myelination has been identified thus far.

The oligodendroglial phosphoinositide 3-kinase (PI3K)/Akt pathway is activated by extracellular cues necessary for oligodendroglial development such as neuregulin1[62] and IGF-1[105-107]. Studies using cultured oligodendroglial cells have shown that growth factor-mediated activation of the PI3K/Akt pathway is necessary to promote OPC proliferation[106], as well as survival of both progenitor[107,141] and mature oligodendrocytes[142] *in vitro*. Interestingly, overexpression of a constitutively active form of Akt, Akt-DD, under the influence of the oligodendroglial-specific *Plp* promoter in transgenic mice (*Plp*-Akt-DD), did not result in significant changes in oligodendroglial proliferation and survival, or oligodendroglial cell numbers, but, surprisingly, caused severe CNS hypermyelination instead[143]. Subsequently, Narayanan *et al*. identified that the pro-myelination effects of constitutively-active Akt overexpression were mediated through activation of the mammalian target of rapamycin (mTOR), and that rapamycin treatment of wild type animals resulted in a hypomyelination phenotype, suggesting that mTOR activity is necessary for myelination[144]. While PI3K activity results in production of phosphatidylinsoitol 3,4,5-trisphosphate (PIP3) and therefore activation of Akt, PTEN (phosphatase and tensin homolog) activity leads to PIP3 dephosphorylation and Akt inhibition. Loss of oligodendroglial PTEN results in hyperactivation of Akt and CNS hypemyelination[145]. Taken together these findings suggest that activation of Akt/mTOR signaling in oligodendrocytes is sufficient to promote myelin wrapping and therefore myelination.

Chapter 2:

Introduction to the Role of Src Family Kinases and their Regulators during Oligodendroglial Development and CNS Myelination

Src Family Kinase Expression and Activity in Oligodendroglia

Src family kinases (SFKs) are intrinsic effectors that regulate cellular functions including proliferation, survival, process extension, migration and differentiation in a number of cell types. Since oligodendrocyte development and myelination are dependent on all of the above cellular processes, it is reasonable to predict that SFKs may similarly regulate oligodendrocyte differentiation. Of the nine family members only four; Fyn[146], Lyn[146], Src[147] and Yes[148], have been detected within the oligodendrocyte lineage. While Fyn and Lyn are highly expressed, Src is expressed only at low levels[108]. On the other hand, following its initial identification[148], Yes expression has not been confirmed by subsequent reports and is thought to be absent in oligodendroglia. Fyn and Lyn expression increases with oligodendrocyte differentiation *in vitro*[108,148], while low Src expression seems to be confined to day 1 and day 2 of differentiation, when cultures consist primarily of newly-formed oligodendrocytes positive for galactocerebroside (GalC)[108]. However, since SFKs are nonreceptor tyrosine kinases their function depends on their activity level, and not just on their protein level. For instance,

preparations of myelin membranes have shown that while both Fyn and Lyn are expressed and translocated within myelin membranes[149-150], the activity of Fyn was higher than that of Lyn[150]. Fyn activity furthermore peaks at the onset of myelination (P4-P10), suggesting that Fyn activation is timed to influence the myelination process[149-150]. Taken together, these studies conclude that SFKs are not only expressed in oligodendrocytes *in vitro* and *in vivo*, but that their activity is developmentally regulated. It is therefore reasonable to suggest that not only do SFKs play a role in oligodendrocyte development, but that mechanisms responsible for SFK activity regulation must be similarly present in oligodendroglia.

The Src Family Kinase Fyn is Necessary for Myelination

The hypothesis that Fyn is essential for normal myelination was first proposed by Umemori and colleagues who reported that genetic deletion of Fyn ($Fyn^{-/-}$) results in a 40-50% reduction in myelin within the brain and spinal cord[149,151]. Subsequent examination of $Fyn^{-/-}$ brains revealed that both myelin thickness and the number of myelinated fibers were reduced[147]. Recently, severe hypomyelination has been also reported in mice lacking Fyn on a genetically pure C57BL/6 background[152]. The hypomyelination phenotype in Fyn null mice was accompanied by decreased protein levels of a number of myelin components including MBP[147,149,152] and MAG[149]. Furthermore, loss of Fyn led to reduced mRNA levels and therefore expression of genes encoding myelin-associated proteins including MBP, PLP, myelin oligodendrocyte glycoprotein (MOG), myelin and lymphocyte protein (MAL), myelin-associated oligodendrocyte basic protein (MOBP) and the UDP-galactose:ceramide galactosyltransferase (CGT)[153]. Similarly, hypomyelination was observed in transgenic mice, in which a kinase

deficient form of Fyn is overexpressed in a Fyn null background[147], demonstrating the importance of Fyn kinase activity in myelination.

Although the hypomyelination phenotype of $Fyn^{-/-}$ mice was accompanied with decreases in the protein[147,149,152] and mRNA[153] levels of myelin components, it was not clear if these decreases were due to decreased oligodendrocyte differentiation or due to a direct role of Fyn in the expression of these myelin components. Studies using cultured oligodendrocytes have shown, however, that Fyn activity is necessary for the expression of at least one myelin component, MBP. Independent studies have shown that *in vitro* Fyn is required to promote *MBP* gene transcription[151], as well as translation of MBP mRNA through phosphorylation of two mRNA binding proteins, QKI (Quaking homolog, KH domain RNA binding protein)[154] and hnRNP-A2 (heterogeneous nuclear ribonucleoprotein A2)[155]. QKI is an mRNA binding protein that preferentially binds to, and therefore, stabilizes exon-2 MBP mRNAs that give rise to the 21.5kD and 18kD MBP isoforms, which are preferentially depleted in $Fyn^{-/-}$ animals. Furthermore, in the CG4 oligodendrocyte cell line, Fyn promotes MBP mRNA stability and translation when it phosphorylates the C-terminal residues of QKI to induce QKI-MBP mRNA binding.[154] Unlike QKI, when hnRNP-A2 binds to MBP mRNA, it represses MBP mRNA translation. Phosphorylation of hnRNP-A2 by Fyn results in release of MBP mRNA, thus allowing MBP mRNA translation *in vitro*.[155] Taken together, these findings suggest that Fyn participates in both transcriptional and post-transcriptional regulation of MBP protein expression. Since MBP is necessary for intracellular membrane adhesion, and therefore myelin stability, these observations further suggest that by promoting MBP protein expression, Fyn may serve an important function in promoting myelin stability.

Fyn Activity is Necessary for Oligodendroglial Differentiation

In addition to hypomyelination, $Fyn^{-/-}$ mice also exhibit decreased numbers of oligodendroglial cells (Olig2-positive), as well as decreased morphological differentiation of individual mature oligodendrocytes[152], suggesting that Fyn may also be necessary for oligodendrocyte differentiation. Interestingly, defects in oligodendrocyte development have also been observed in cultures of primary oligodendrocytes lacking either Fyn protein expression[108,156] or Fyn activity, as well as in wild type oligodendrocytes treated with SFK pharmacological inhibitors[148]. Specifically, loss of Fyn resulted in decreased oligodendrocyte differentiation, as the numbers of newly-formed (O4-positive and CNP-positive), as well as mature, MBP-positive oligodendrocytes were reduced by 30-50% in cultured $Fyn^{-/-}$ oligodendrocytes[156]. In addition, cultured $Fyn^{-/-}$ oligodendrocytes were unresponsive when stimulated with IGF1, which normally promotes oligodendrocyte differentiation and morphological complexity[61,157], suggesting that Fyn acts as a downstream effector of IGF-1 signaling.[156] In addition to IGF1, Fyn also acts downstream of the pro-differentiation, ECM molecule laminin2. Studies from Colognato and colleagues using primary rat oligodendrocytes treated with Fyn shRNA have shown that Fyn is necessary for laminin2-mediated newly-formed oligodendrocyte survival, differentiation to mature oligodendrocytes, and laminin2-mediated enhancement of myelin sheet formation[108]. Furthermore, treatment with the SFK inhibitor PP2 also decreased oligodendrocyte morphological complexity in wild type oligodendroglia, suggesting that SFK activity is also necessary for oligodendrocyte differentiation *in vitro*[148]. The defects in morphological oligodendrocyte differentiation associated with Fyn loss-of-function suggested that in addition to oligodendrocyte differentiation, Fyn activity may also promote oligodendrocyte process outgrowth and branching.

In order to differentiate and efficiently myelinate multiple internodes, cells of the oligodendrocyte lineage need to extend multiple processes, often several micrometers away from the cell body. Process outgrowth is therefore necessary for both oligodendrocyte differentiation and myelination. Process outgrowth is dependent on cytoskeletal rearrangements and is controlled by the Rho, Rac and Cdc42 GTPases. In oligodendrocytes, process outgrowth is dependent on activation of Cdc42 and Rac[158], and inhibition of RhoA[159]. It is therefore reasonable to suggest that molecules that modulate the activity of these GTPases, also regulate process outgrowth. Fyn activity is necessary for fibronectin-dependent[158] and independent[159] process extension in primary rat oligodendrocytes *in vitro*, and pharmacological inhibition of Fyn was able to inhibit fibronectin-induced Rac and Cdc42 activation and process outgrowth[158,159]. These findings suggest that Fyn is an intracellular effector of fibronectin, whose activity is necessary for Rac and Cdc42 activation during oligodendroglial process remodeling. In addition, Fyn also acts as a downstream effector of laminin2-mediated process outgrowth in the CG4 oligodendrocyte cell line. In this study, Hoshina *et al*. found that Fyn activity was increased when cells were plated on laminin2. This increase in Fyn activity led to phosphorylation of focal adhesion kinase (FAK), and, in turn, activation of Rac and Cdc42 and process outgrowth[160]. Taken together, these studies suggest that Fyn may act as a downstream integrator of laminin2 and fibronectin signaling to mediate activation of Rac and Cdc42 GTPases and modulate process outgrowth.

Since oligodendrocyte process outgrowth is also dependent on the inhibition of Rho, molecules such as the GTPase activating protein p190 Rho GAP that facilitate turnover of Rho-bound GTP to GDP and thus inhibit Rho, should also promote process outgrowth. Overexpression of a kinase-deficient mutant of Fyn decreased p190 Rho GAP phosphorylation.

Reduced Fyn kinase activity therefore resulted in reduced turnover of Rho-bound GTP and induced Rho activation, which in turn inhibited process outgrowth in primary rat oligodendrocytes *in vitro*.[159] These findings suggest that Fyn acts as an upstream inhibitor of Rho GTPase activity, and is therefore, necessary for process extension of oligodendrocytes *in vitro*. Furthermore, Fyn also promotes RhoA inhibition and subsequent process outgrowth downstream of the Netrin1. Netrin1 is an extracellular signal that binds and activates the DCC (Deleted in Colorectal Cancer) receptor in oligodendrocytes. In mature oligodendrocytes *in vitro*, Netrin1 binding to DCC results in recruitment of Fyn to the intracellular domain of DCC, where active Fyn is situated in close proximity to FAK. Activation and recruitment of Fyn to DCC promotes phosphorylation of FAK, which in turn results in inhibition of RhoA, thereby promoting cytoskeletal reorganization and process outgrowth. Interestingly, Netrin1 did not induce process outgrowth and branching in $Fyn^{-/-}$ oligodendrocytes *in vitro*, suggesting that Fyn is an obligate effector of Netrin1/DCC signaling in oligodendrocytes that is absolutely necessary for Netrin1-mediated process outgrowth.[161] Taken together, these findings suggest that Fyn activity is necessary for process outgrowth that is also dependent on RhoA inhibition.

Fyn can promote process outgrowth in an activity-independent manner as well. The type II polyproline helix recognition domain (SH3) of Fyn does not directly modulate Fyn activity, but instead participates in protein-protein interactions[162]. In cultured oligodendrocytes, the SH3 domain of Fyn directly associates with the microtubule stabilizing protein Tau. This Fyn SH3-Tau interaction is in turn necessary for Tau-mediated microtubule assembly and bundle formaion.[163] Thus, in addition to Fyn kinase activity, Fyn protein-protein interactions are necessary to promote oligodendrocyte process outgrowth.

In addition to newly-formed and mature oligodendrocytes, Fyn has been shown to function in OPCs to promote PDGF-mediated migration. PDGF can induce OPC migration by promoting Cdk5-mediated phosphorylation of the actin binding proteins WAVE2 and WASP. Pharmacological inhibition of Fyn activity, as well as Fyn depletion using RNAi, inhibited PDGF-dependent activation of Cdk5, demonstrating that Fyn is an important mediator of PDGF-dependent OPC migration. In addition, saturating levels of exogenous PDGF were sufficient to induce Fyn activation, suggesting that PDGF may serve as an extracellular regulator of Fyn activity in oligodendrocyte progenitors. Interestingly, Fyn depletion or inhibition did not reduce cell migration in the absence of exogenous PDGF, suggesting that Fyn is not necessary for PDGF-independent migration.[164]

For proper myelination to occur, mature oligodendrocytes not only need to be generated in sufficient numbers, but need to establish appropriate axon-oligodendrocyte interactions. Although Fyn has never been studied directly in the context of axon-oligodendroglial interactions, several independent studies suggest that oligodendrocyte Fyn may participate in axon-oligodendroglial interactions. In 1994, Umemori *et al.* identified that within myelin preparations Fyn associates with large MAG[149], a protein necessary for axon-oligodendrocyte adhesion[165]. By co-expressing MAG together with wild type or mutant Fyn constructs in COS cells, the authors of this study also concluded that the SH2 and SH3 domains of Fyn were necessary for MAG-Fyn association[165]. Interestingly, crosslinking of large MAG induced activation of Fyn, but not activation of Src or Lyn[149], suggesting that while Fyn activity may be necessary to mediate MAG-induced axon-oligodendrocyte adhesion, Src and Lyn may be dispensable to this process. In addition to MAG-mediated adhesion, axon-oligodendroglial

interactions can be mediated through association between axonal adhesion molecules such as L1 and NCAM, and oligodendrocyte cell surface molecules including F3 and the 120kD NCAM isoform. Fyn was found to be associated with axonal L1 and oligodendroglial F3 in cerebellar lysates[166], as well as with the 120kD NCAM isoform and F3 within DIG fractions of cultured oligodendrocytes[150], suggesting that Fyn may participate in L1/F3-mediated and NCAM/NCAM-mediated axon-oligodendroglial adhesion. Furthermore, F3 crosslinking increased Fyn activity[150], suggesting once again that Fyn activity may be stimulated by axon-oligodendroglial contact. Recently, studies utilizing oligodendrocyte-neuron co-cultures have shown that axon-oligodendroglial contact and subsequent myelination may be regulated by contactin, a receptor for axonal L1. Interestingly, contactin associated with $\alpha_6\beta_1$ integrin to enhance Fyn activity[167]. Taken together these observations suggest that Fyn may not only participate in axon-oligodendroglial interactions, but that through its activation, Fyn may also mediate axon contact-dependent oligodendrocyte survival and differentiation.

The Src Family Kinase Lyn and Oligodendrocyte Development

As described above, in addition to Fyn, the SFKs Lyn and Src are also expressed in oligodendrocytes[108,146,150]. Src expression in cells of the oligodendrocyte lineage is low, and is also limited to cells differentiated for 1-2 days in culture[108]. On the other hand, Lyn is expressed at high levels in both cultured oligodendrocytes and isolated myelin preparations[150]. Interestingly, although Src is expressed in oligodendrocytes, to date no distinct function for this protein has been reported in terms of oligodendrocyte development. Furthermore, although Fyn null mice are hypomyelinated[147,149,151-152], mice lacking either Lyn or Src have no obvious white

matter abnormalities[147], suggesting that both Lyn and Src may be dispensable for oligodendrocyte development and myelination. Although it may not be necessary *in vivo*, Lyn regulates two important processes during oligodendrocyte development *in vitro*, namely OPC proliferation[108] and survival[91]. Studies using primary rat oligodendrocytes have found that Lyn is required for PDGF-induced OPC proliferation in cells plated on fibronectin, but not in cells plated on either laminin2 or the control substrate poly-D-lysine (PDL). Interestingly, Lyn was not required for OPC proliferation in the absence of PDGF stimulation, suggesting that Lyn may not participate in the cell-intrinsic "timer" of proliferation in the absence of PDGF.[108] In cells cultured on fibronectin in the presence of saturating amounts of PDGF, Lyn was also necessary to suppress the activity of acid sphingomyelinase, an enzyme that catalyzes the hydrolysis of sphingomyelin to ceramide. By limiting ceramide production and associated apoptosis, Lyn was therefore required for fibronectin- and PDGF-mediated survival.[91] Recently, Lyn was also found to promote survival downstream of human remyelination-promoting antibodies and fibronectin/integrin $\alpha_v\beta_3$ signaling[168]. On the other hand, Lyn is not necessary for laminin2-mediated oligodendrocyte survival, differentiation and myelin sheet formation *in vitro*[108]. Furthermore, in cultured oligodendrocytes, Lyn associates with PDGFαR and the fibronectin receptor integrin $\alpha_v\beta_3$, but not with the laminin receptor, integrin $\alpha_1\beta_6$.[108] It should be noted that although Lyn null mice have no reported myelin phenotype, they do exhibit increased demyelination and higher clinical scores during experimental autoimmune encephalomyelitis (EAE), an experimental model for MS. However, the authors of this study suggested that the increased clinical scores observed in $Lyn^{-/-}$ mice were as a result of the importance of Lyn in B and T cell function, and not due to any role of this protein in oligodendrocyte biology and remyelination.[169] Taken together, these findings suggest that although it modulates OPC

proliferation and survival downstream of PDGF and fibronectin, Lyn may be dispensable for oligodendrocyte development in the absence of these two extracellular cues.

Regulation of Src Family Kinase Activity – Lessons from Lymphocyte and Cancer Biology

The SFK family of non-receptor tyrosine kinases share common domain structure, function and regulation (Reviewed in [162,170]). All SFKs contain six conserved domains: a tyrosine kinase domain (Src homology (SH) domain 1); a phosphotyrosine recognition domain (SH2); a type II polyproline helix recognition domain (SH3); a membrane-targeting N-terminal domain (SH4); and a short, flexible C-terminal tail. Two modifications in the SH4 domain, myristoylation and farnesylation, target SFKs to glycosphingolipid-enriched membrane microdomains (GEMs) also known as lipid rafts[162]. Fyn, for example, is myristoylated at glycine 2 and palmitoylated at cysteines 3 and 6[171-172].

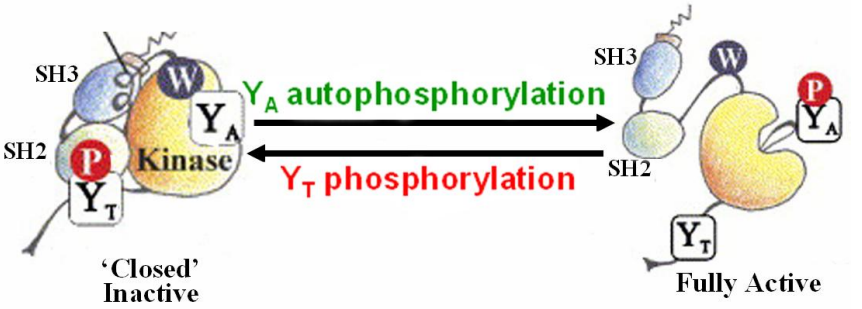


Figure 3: Model for SFK activity regulation. Phosphorylation at Y529 (Y_T) by Csk or Chk leads to folding of the enzyme to an inactive conformation. SFKs are activated after dephosphorylation at Y529 (Y_T) and autophosphorylation at Y418 (Y_A). (Cartoon modified from [170])

SFK activity is modulated largely by phosphorylation and dephosphorylation events at two consensus tyrosine (Y) residues, Y418 and Y529. SFK kinase activity is inhibited when the

negative regulatory tyrosine (Y529) is phosphorylated by either of two non-receptor tyrosine kinases: C-terminal Src kinase (Csk)[173] or Csk-homologous kinase (Chk)[174]. Csk or Chk phosphorylation of the SFK negative regulatory Y529 creates a binding site for the SFK SH2 domain. This promotes SFK folding into a closed, inactive conformation that is stabilized by phosphorylated Y529-to-SH2 domain, as well as, polyproline II linker-to-SH3 domain interactions. Conversely, SFKs are activated by displacement of the SH2 and SH3 domains from their aforementioned binding partners. Dephosphorylation of the inhibitory Y528 by a variety of tyrosine phosphatases causes the displacement of the SH2 domain, which triggers SFK unfolding to restore partial kinase activity. However, full SFK activity is only attained upon autophosphorylation of a conserved tyrosine in the catalytic SH1 domain (Y417) (Figure 3). Finally, SFK activity can also be modulated by protein-protein interactions. For example, binding of the membrane protein caveolin is inhibitory, while interaction of the adaptor protein $p130^{CAS}$ with the SH3 domain results in SFK unfolding and activation (Reviewed in [170]). Since Fyn enzymatic activity is critical for normal myelination[147], an understanding of the regulatory processes that dictate Fyn activity will be important for understanding myelination.

Because full SFK activity is achieved upon autophosphorylation of the autocatalytic Y418, the principle mode regulation of SFK activity is thought to be through phosphorylation of the C-terminal Y529, which is mediated by either of two non-receptor tyrosine kinases, Csk or Chk. However, Csk is considered to be the primary catalytic SFK inhibitor as it is expressed ubiquitously[173], while Chk expression is restricted predominantly to neuronal and hematopoietic cells[174]. Evidence of the functional importance of Csk comes from gene-knockout studies, where Csk deletion leads to constitutive SFK activation[175], early embryonic lethality (E9-E10), and

severe neural tube defects[176]. In contrast, deletion of Chk leads to no overt phenotypic abnormalities[174,177].

Structurally, Csk is similar to its substrates, the SFKs, as it contains an SH1, an SH2 and an SH3 domain, but it lacks N-terminal fatty acid acylation sequence and thus, unlike SFKs, is localized predominantly in the cytosol[173]. Interestingly, Csk exhibits remarkable substrate specificity for SFKs, which depends on the tertiary structure of SFKs and is conferred by 1) recognition of two consensus residues (glutamate 525 and glutamine 527) near the negative regulatory Y528, 2) docking-based substrate recognition by the catalytic domain, and 3) a transient, phosphorylation-dependent interaction between the SH2 domain of Csk and the autocatalytic Y418. Furthermore, although Csk is structurally similar to the SFKs as it contains an SH1, an SH2 and an SH3 domain, it lacks N-terminal fatty acid acylation sequence and thus, unlike SFKs, is localized predominantly in the cytosol[173].

While Csk is cytosolic[173], its substrates, the SFKs, are tethered to the membrane and are often associated with lipid rafts[171-172,178], indicating the necessity for spatial regulation of Csk. In addition, all three Src homology domains of Csk, including the SH2 and the SH3, which participate in protein-protein interactions, are required for Csk function and membrane recruitment[173]. These observations suggest that adaptor proteins may be necessary to recruit Csk to the membrane. The first adaptor protein identified to perform this function was Csk-binding protein (Cbp)[179], also called phosphoprotein associated with GEMs (PAG) (from hereon referred to as Cbp)[180], which was isolated independently from Csk- and SFK-enriched brain lysates[179] and

from GEM fractions of leukocytes[180]. Cbp-dependent recruitment of Csk to the membrane is dependent of phosphorylation of a tyrosine in the cytoplasmic domain of Cbp (Y317). Interestingly, SFKs can phosphorylate Cbp to facilitate Csk recruitment, thereby creating a negative feedback loop mechanism of SFK activity regulation. Finally, dephosphorylation of Cbp Y317 by the tyrosine phosphatases CD45 and SHP1 can disrupt Csk/Cbp association and thus increase SFK activity.[181] Subsequent studies have identified that insulin receptor substrate-1 (IRS-1)[182], linker for activation of T cells (LAT), and paxillin[183], can also recruit Csk to the membrane, and therefore, regulate SFK activity. Although the presence of Csk in cultured oligodendrocytes has previously been identified[108,184-185], the role of Csk in SFK regulation and oligodendroglial development is unknown.

While the necessity for Fyn activity to promote normal myelination is established, the regulatory elements that modulate oligodendrocyte Fyn activation are not known. Csk has been characterized as a critical Fyn regulator in systems such as T lymphocyte activation[186], where Fyn activity is also crucial. Although not studied to date, Csk represents a good candidate as a Fyn-regulatory protein in the oligodendrocyte lineage. My work was designed to uncover the mechanisms that regulate Fyn function during myelination and to test the hypothesis that **Csk regulates oligodendrocyte Fyn activity and is essential for oligodendrocyte development and myelination.**

Chapter 3:

C-Terminal Src Kinase Inhibits Fyn Activity in Oligodendrocytes

Rationale

Generating and maintaining normal oligodendrocyte numbers is essential for normal brain development and function, since hypomyelinated regions and demyelinated regions of the CNS lead to pathologies ranging from inherited leukodystrophies to multilple sclerosis. Thus elucidating the regulatory mechanisms that govern oligodendrocyte development, and therefore, myelination and remyelination is critical for the development of therapeutic strategies to combat myelin-associated pathologies. As I have described previously, the importance of Fyn for oligodendrocyte development and function is well established. Mice lacking Fyn[147,149,151-152] or Fyn activity[147] are hypomyelinated in the CNS, suggesting that Fyn activity is necessary for oligodendrocyte-mediated myelination. Fyn activity promotes both transcriptional[151] and post-transcriptional[154-155] regulation of the myelin-associated protein MBP, suggesting that it may be necessary for not only myelination, but for myelin stability as well. In addition to myelination, Fyn activity is also necessary for proper oligodendrocyte development. $Fyn^{-/-}$ mice generated recently on a pure C57BL/6 background, exhibit decreased oligodendroglial cell numbers even at stages prior to myelination[152], suggesting that Fyn may be necessary for normal

oligodendrogenesis *in vivo*. Similarly, Fyn activity is necessary for proper oligodendrocyte morphological differentiation *in vitro,* as it promotes process outgrowth and branching of newly-formed oligodendrocytes[158-159,161,163], and myelin sheet formation in mature oligodendrocytes, where it acts downstream of the pro-differentiation factor laminin2[108]. Fyn activity is also necessary for the generation of appropriate numbers of mature oligodendrocytes, as it promotes oligodendrocyte survival[108,148] and differentiation[108,156]. Fyn activity is not only necessary for myelination, but is also developmentally regulated within myelin preparations, such that it peaks at the onset of myelination[149-150], suggesting that regulatory elements that modulate Fyn activity and function must be present in oligodendrocytes. And, while Fyn tyrosine kinase activity is essential for oligodendrocyte development and myelination[147], the regulatory mechanisms that are responsible for turning Fyn "on" and "off" in oligodendrocytes remain unknown.

All SFKs have two principle conformation states that are controlled by phosphorylation: an open, catalytically-active form characterized by autophosphorylation, and a closed, catalytically-inactive form following phosphorylation of the C-terminal tyrosine[187]. The balance between these two conformations in some cell types has been found to be largely dictated by phosphorylation of the C-terminal tyrosine, which can occur via C-terminal Src Kinase (Csk) [173,188-189], or its homolog Csk homologous kinase (Chk)[174]. Unlike Chk[174], Csk is ubiquitously expressed[173] and is thus proposed to be the principle SFK regulator. While genetic deletion of Csk leads to constitutive SFK activation[175] and severe neural tube defects leading to early embryonic lethality (E9-E10)[176], genetic loss of Chk leads to no overt phenotypic abnormalities[174,177]. Although Csk has been previously identified in oligodendrocyte cultures *in vitro*[108,184-185], its role in oligodendroglial development is unknown. In view of the importance of

Fyn activity for oligodendrocyte differentiation and function, I designed this work to test the hypothesis that **Csk regulates Fyn activity and is essential for oligodendrocyte development and myelination**. Before I could test this hypothesis, however, I needed to establish whether Csk is expressed in oligodendrocytes *in vivo*, whether Csk expression is regulated developmentally in oligodendrocytes, and finally, whether Csk is indeed a negative regulator of Fyn function in oligodendroglia. Findings that address these questions are described in this chapter.

Results

Fyn Activity and Inhibition are Developmentally Regulated in Oligodendrocytes

Although Fyn activity is required for myelination[147] the mechanisms that regulate oligodendrocyte Fyn remain poorly understood. Previous studies have shown that, while Fyn protein levels increase with oligodendrocyte differentiation, Fyn activity peaks at the onset of myelination *in vivo* [149-150,154] and, *in vitro*, is higher in mature oligodendrocytes than in oligodendrocyte progenitor cells (OPCs)[108,148]. To determine whether Fyn phosphorylation at its C-terminal negative regulatory tyrosine (Y529) changes during oligodendrocyte development, I differentiated OPCs on poly-D-lysine (PDL) or the extracellular matrix protein laminin2 (Lm2) for up to 6 days (Fig. 4). Laminin was chosen as a substrate because previous studies have found that laminin promotes oligodendrocyte maturation and alters Fyn activity[89,108]. I confirmed that Fyn protein levels increased with differentiation, with Fyn protein levels increasing by approximately 4-fold during the differentiation time course (Fig. 4A; 395.2±88.4% on PDL at day 6, P=0.0494, n=5; 397.3±86.9% on Lm2 at day 6, P=0.132, n=6). Using relative

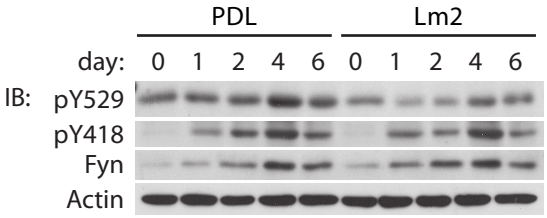
phosphorylation at the autocatalytic site (Y418) as a readout for Fyn activity, I also confirmed that Fyn activity increased substantially as OPCs differentiated into newly-formed oligodendrocytes at day 1 (Fig. 4B; 327.5±92.3% on PDL, P=0.056, n=6; 278.3±88.4% on Lm2, P=0.219, n=6). Relative phosphorylation of Fyn Y418 remained high through day 4 of differentiation (Fig. 4B; at day 2, 251.4±54.8% on PDL, P=0.0294, n=6; 246.8±73.0% on Lm2, P=0.179, n=6; at day 4, 318.7±79.2% on PDL, P=0.0294, n=6; 366.1±119.8% on Lm2, P=0.195, n=6), but by day 6 returned to levels comparable to those observed at differentiation onset (Fig. 4B; day 6; 162.8±44.5% on PDL, P=0.585, n=5; 175.4±58.8% on Lm2, P=0.314, n=5).

In contrast, relative phosphorylation at the C-terminal Y529, a readout for Fyn inhibition, declined substantially by day 1 in OPCs plated on PDL, dropping to 45±13.2% of levels observed at day 0 (Fig. 4C; P=0.0032, n=6), yet by day 6 returned to levels similar to those observed at differentiation onset (Fig. 4C; day 6, 72±17.4%, P=0.889, n=5). OPCs plated on the pro-differentiation substrate Lm2, however, showed an acute decrease in Fyn inhibition such that the relative phosphorylation of Fyn Y529 was 37.4±5.8% of levels observed on PDL (Fig. 4C; day 0; P=0.0045, n=6). And, while not statistically significant, relative Y529 phosphorylation remained lower in cells differentiated on Lm2 than on PDL for the duration of the time course. (Fig. 4C). Together these findings suggested that Fyn regulatory proteins were likely to be expressed in oligodendrocytes and that their expression and/or activity changed during OPC differentiation.

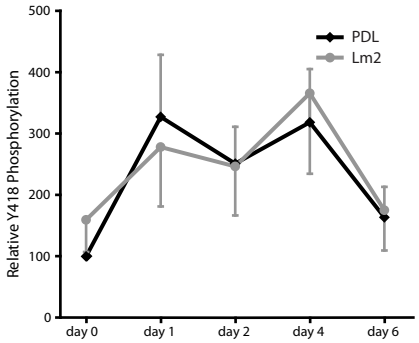
Figure 4. Fyn activity is developmentally regulated in oligodendroglia. (a) Western blots analysis of Fyn and Fyn phosphorylation in oligodendrocytes differentiated on PDL and Lm2 for 0, 1, 2, 4 and 6 days after growth factor withdrawal. Actin blots are included as a loading control. **(b)** Densitometry to determine relative phosphorylation of the autocatalytic Y418 (pY418/Fyn). The average percent change ± sem relative to phosphorylation at day 0 on PDL is shown on both PDL (black squares) and Lm2 (grey circles). **(c)** Densitometry to determine relative phosphorylation at the negative regulatory Y529 (pY529/Fyn). The average percent change ± sem relative to phosphorylation at day 0 on PDL is shown on both PDL (black squares) and Lm2 (grey circles).

Figure 4

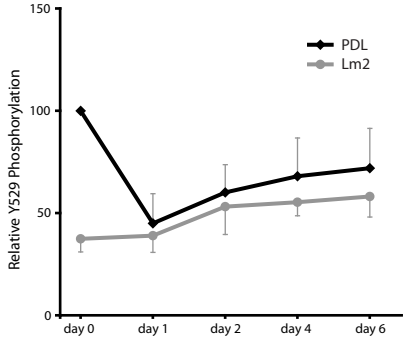
a)


PDL
Lm2
day: 0 1 2 4 6 0 1 2 4 6
IB: pY529
pY418
Fyn
Actin

b)


Relative Y418 Phosphorylation
500
400
300
200
100
0
PDL
Lm2
day 0
day 1
day 2
day 4
day 6

c)

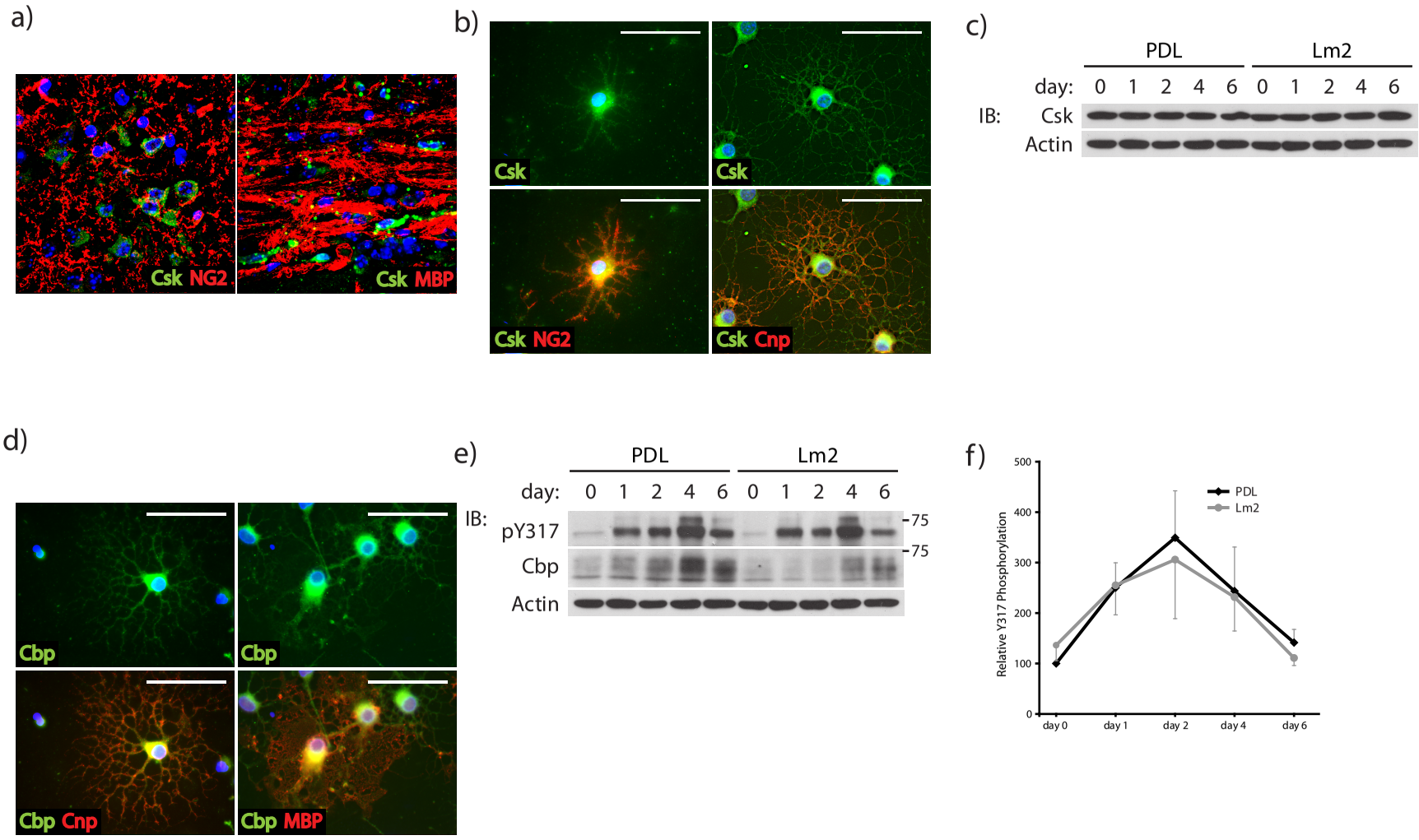

Relative Y529 Phosphorylation
150
100
50
0
PDL
Lm2
day 0
day 1
day 2
day 4
day 6

Csk is Expressed within Cells at the Oligodendrocyte Lineage *in vitro* and *in vivo*

In T lymphocytes, where Fyn activity is important for T-cell activation and thus the generation of an appropriate immune response, Fyn is inhibited by Csk-mediated Y529 phosphorylation[186]. Although Csk has been previously identified in cultured oligodendrocytes[108,184-185], its expression *in vivo*, as well as temporal expression during OPC differentiation, have not been characterized. Here I report that Csk is expressed within OPCs, identified by the presence of chondroitin sulfate proteoglycan NG2 immunoreactivity, as well as within CC1-positive mature oligodendrocytes, within the developing mouse corpus callosum (Fig5A). I also identified Csk immunoreactivity within NG2-positive OPCs *in vitro*, and confirmed that Csk is expressed in newly-formed oligodendrocytes immunoreactive for CNP, as well in mature, MBP-positive oligodendrocytes (Fig. 5B, and data not shown).

Next, I asked whether changes in Csk protein levels during differentiation could account for the altered Fyn Y529 phosphorylation seen during the time course described in Fig. 4. I predicted that Csk protein levels would mimic levels of its putative substrate, phosphorylated Y529 (Fig. 4C) such that Csk would be highly expressed at the onset of differentiation, but its expression would decrease during differentiation. I was surprised, therefore, that no significant change in Csk protein levels occurred either during OPC differentiation or in response to Lm2 (Fig. 5C). Previous reports have shown, however, that Fyn, which is myristoylated and palmitoylated[171-172], resides in specialized membrane microdomains termed lipid rafts, while Csk, which lacks these post-translational modifications, is found primarily in the cytoplasm, at least in T-cells[189]. Thus, I speculated whether spatial regulation of Csk could account for the altered phosphorylation of Fyn observed during oligodendrocyte differentiation (Fig. 4).

Figure 5. Csk and Cbp are expressed in oligodendroglia. (a) Indirect immunofluorescence to visualize cells positive for Csk (green), NG2 (red) or MBP (red), and nuclei (bleu) in the corpus callosum of postnatal day 21-old wild type mice. Scale bars: 50μm. **(b)** Indirect immunofluorescence to visualize Csk (green) in OPCs (NG2(+), red) at day 1 of differentiation, and in newly-formed oligodendrocytes (CNP(+), red) at day 2 of differentiation. Scale bars: 50μm. **(c)** Western blot analysis of Csk in oligodendrocytes differentiated for indicated times. Actin blots are included as a loading control. **(d)** Indirect immunofluorescence to visualize Cbp (green) in newly-formed oligodendrocytes (CNP(+), red) differentiated for 2 days, and in mature oligodendrocytes (MBP(+), red) differentiated for 4 days. Scale bars: 50μm. **(e)** Western blot analysis of Cbp and Cbp Y317 phosphorylation in oligodendrocytes differentiated for indicated times. Actin blots are included as a loading control. **(f)** Densitometry to determine relative phosphorylation at Cbp Y317 (pY317/Cbp). The average percent change ±sem relative to phosphorylation at day 0 on PDL is shown on both PDL (black squares) and Lm2 (grey circles). **(g)** Western blots of immunoprecipitated complexes isolated with Csk antibodies to detect Cbp and phosphorylated Cbp (pY317). **(h)** Western blots of immunoprecipitated complexes isolated with GFP antibodies to detect transfected Cbp constructs and associated Csk in cells transfected with Cbp-EYFP (Cbp), CbpY314F-EYFP (CbpY314F) and empty vector (ctrl).


a)
Csk NG2
Csk MBP
b)
Csk
Csk
Csk NG2
Csk Cnp
c)
PDL
Lm2
day: 0 1 2 4 6 0 1 2 4 6
IB: Csk
Actin
d)
Cbp
Cbp
Cbp Cnp
Cbp MBP
e)
PDL
Lm2
day: 0 1 2 4 6 0 1 2 4 6
IB: pY317
Cbp
Actin
75
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Relative Y317 Phosphorylation
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day 1
day 2
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g)
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IP
Input
Csk
IgG
Input
Csk
IgG
IB: pY317
Cbp
h)
Ctrl
Cbp
CbpY317F
GFP
Csk

Figure 5

Csk and the adaptor protein Cbp associate in oligodendroglia

A small amount of oligodendroglial Csk was previously reported to be insoluble following lysis with cold Triton X-100, suggesting that a subset of Csk may reside within lipid rafts[108]. On the other hand, Csk binding protein (Cbp, also called phosphoprotein associated with glycosphingolipid-enriched membrane microdomains, or PAG), an adaptor for Csk in T lymphocytes, is palmitoylated and recruited to T-cell lipid rafts [179-180]. I therefore investigated whether oligodendroglia employed strategies to alter the proximity of Csk to Fyn e.g. recruitment of Csk to the plasma membrane via Cbp. I found that Cbp was expressed in cultured oligodendrocytes (Fig. 5D), and that, similar to Fyn, Cbp protein levels increased roughly 3-fold during oligodendrocyte differentiation (Fig. 5E) at day 6; 306.6±81.2% on PDL, P=0.0162, n=7; 345.5±85.6% on Lm2, P=0.0193, n=5). In T lymphocytes, Cbp phosphorylation at Y317 creates a binding site for the SH2 domain of Csk, and is thus necessary for Csk recruitment[179]. I therefore examined phosphorylation of Cbp Y317 during oligodendrocyte differentiation and found that it peaked at day 2 (Fig. 5F; 349.2±87.1% on PDL, P=0.0120, n=8; 305.9±106.8 on Lm2, P=0.730, n=6). These data suggested that, even if Csk protein levels remain constant, Csk location at the plasma membrane via Cbp may be regulated temporally during oligodendrocyte development.

To ascertain whether Csk and Cbp could be found associated in a protein complex, I first used Csk-specific antibodies to co-immunoprecipitate Csk interacting proteins within oligodendroglial cell lysates (Fig. 5G). Cbp co-immunoprecipitated with Csk in lysates obtained from OPCs differentiated on either PDL or Lm2, however, cells grown on Lm2 had more Cbp in

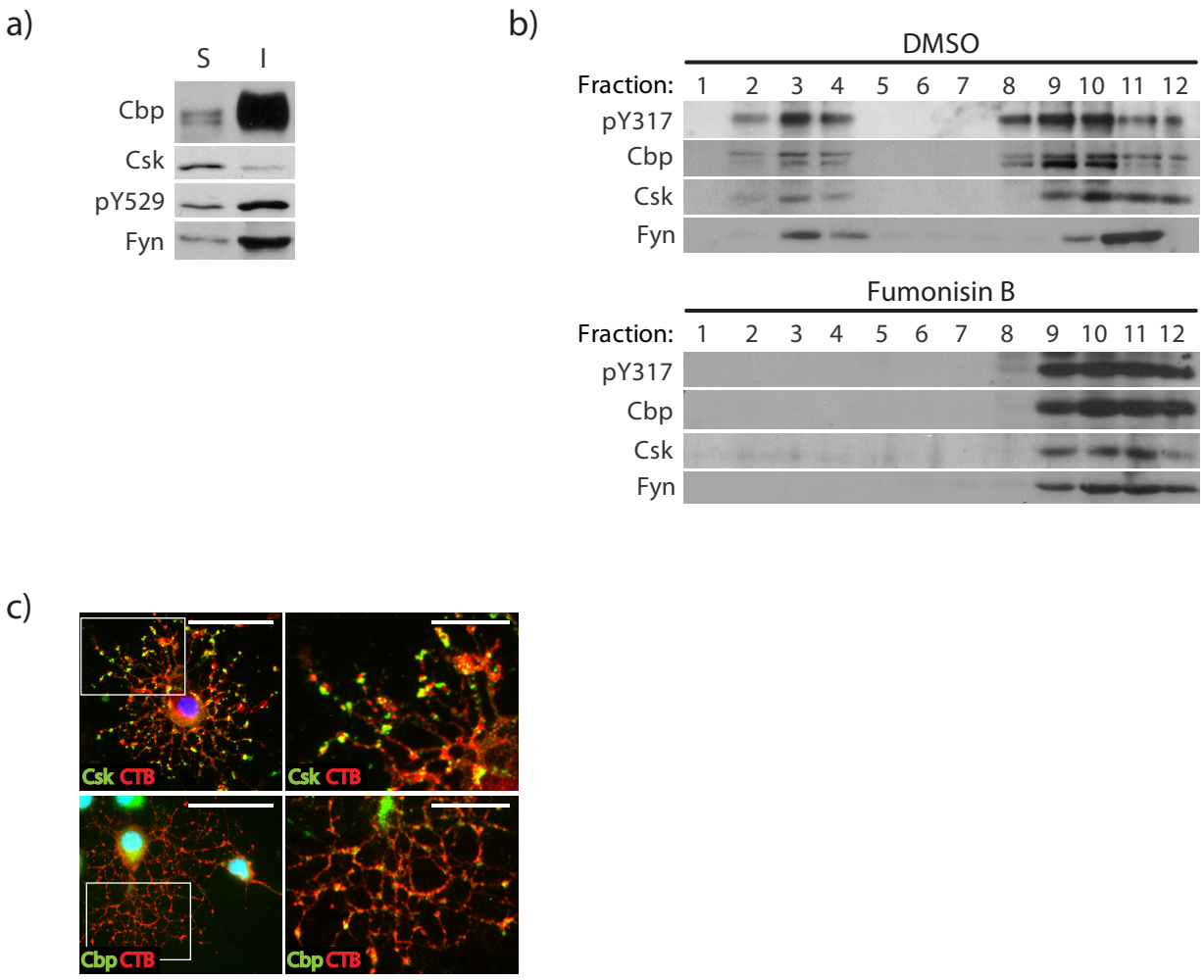
Csk pulldowns (that appeared less phosphorylated), suggesting that Lm2 may regulate the Csk-Cbp association (Fig. 5G). Second, I transfected OPCs with contructs designed to express GFP fusion proteins that contained either wild type Cbp, or Cbp-Y317F, a mutant protein previously shown to lack the ability to bind Csk[179]. Using agarose beads conjugated to GFP-antibodies, I isolated either Cbp or Cbp-Y317F from oligodendrocyte lysates, then used Western Blot analysis to detect the presence or absence of Csk in the isolated protein complexes (Fig. 5H); while WT Cbp protein complexes contained Csk, Cbp-Y317F mutant complexes did not. Together these data suggested that Csk and Cbp associations occur in oligodendroglia, and that this association relies on phosphorylated Cbp Y317.

Csk and Cbp are found in oligodendroglial lipid rafts

Given that Csk and Cbp associations occurred in oligodendroglia, I next sought to determine whether membrane location and/or association played a role. The insolubility of proteins in cold Triton-X-100 can indicate the possibility of a lipid raft association; I therefore lysed oligodendrocytes following 4 days of differentiation with 1% Triton X-100 at 4ºC, and analyzed the soluble and insoluble fractions by Western blot (Fig. 6A). Total Fyn (76.5±4.0% insoluble, P=0.0220, n=3), catalytically-active Fyn (pY418; 65.7±5.2% insoluble, P=0.0948, n=3), and inactive Fyn (pY529; 67.4±8.0% insoluble, P=0.160, n=3), were predominantly localized in the insoluble pool, whereas Csk was primarily found in the soluble pool (65.6±3.5% soluble, P=0.0460, n=3). I also found that Cbp (67.4±2.9% insoluble, P=0.0262, n=3) and phosphorylated Cbp (pY317) (64.4±1.8% insoluble, P=0.0149, n=3) were significantly enriched in the insoluble pool. Similar results were observed in cells grown on Lm2 (data not shown).

Figure 6. Csk and Cbp reside in GM1-containing lipid rafts in oligodendroglia. (a) Western blot analysis of Csk, Cbp, Fyn, and phosphorylated Fyn (pY529) in Triton X-100 soluble (S) and insoluble (I) fractions in oligodendrocytes differentiated for 4 days. **(b)** Lysates from oligodendrocytes differentiated for 2 days were subjected to sucrose gradient ultracentrifugation and fractionated to isolate detergent-insoluble glycosphingolipid-enriched membrane microdomains (DIGs). Western blot analysis to determine whether Csk, Cbp, phosphorylated Cbp (pY314), and Fyn reside in DIGs (fractions 2-4, upper panel). Treatment with FumonisinB caused DIG disruption and redistribution of Csk, Cbp, pY314, and Fyn to non-DIG, higher density fractions (fractions 8-12, lower panel). **(c)** Indirect immunofluorescence to visualize Csk (green, upper panels) and Cbp (green, lower panels) in conjunction with cholera toxin subunit B (CTB, red), a marker for GM1-positive lipid rafts.

Figure 6


a)
S
I
Cbp
Csk
pY529
Fyn
b)
DMSO
Fraction: 1 2 3 4 5 6 7 8 9 10 11 12
pY317
Cbp
Csk
Fyn
Fumonisin B
Fraction: 1 2 3 4 5 6 7 8 9 10 11 12
pY317
Cbp
Csk
Fyn
c)
Csk CTB
Csk CTB
Cbp CTB
Cbp CTB

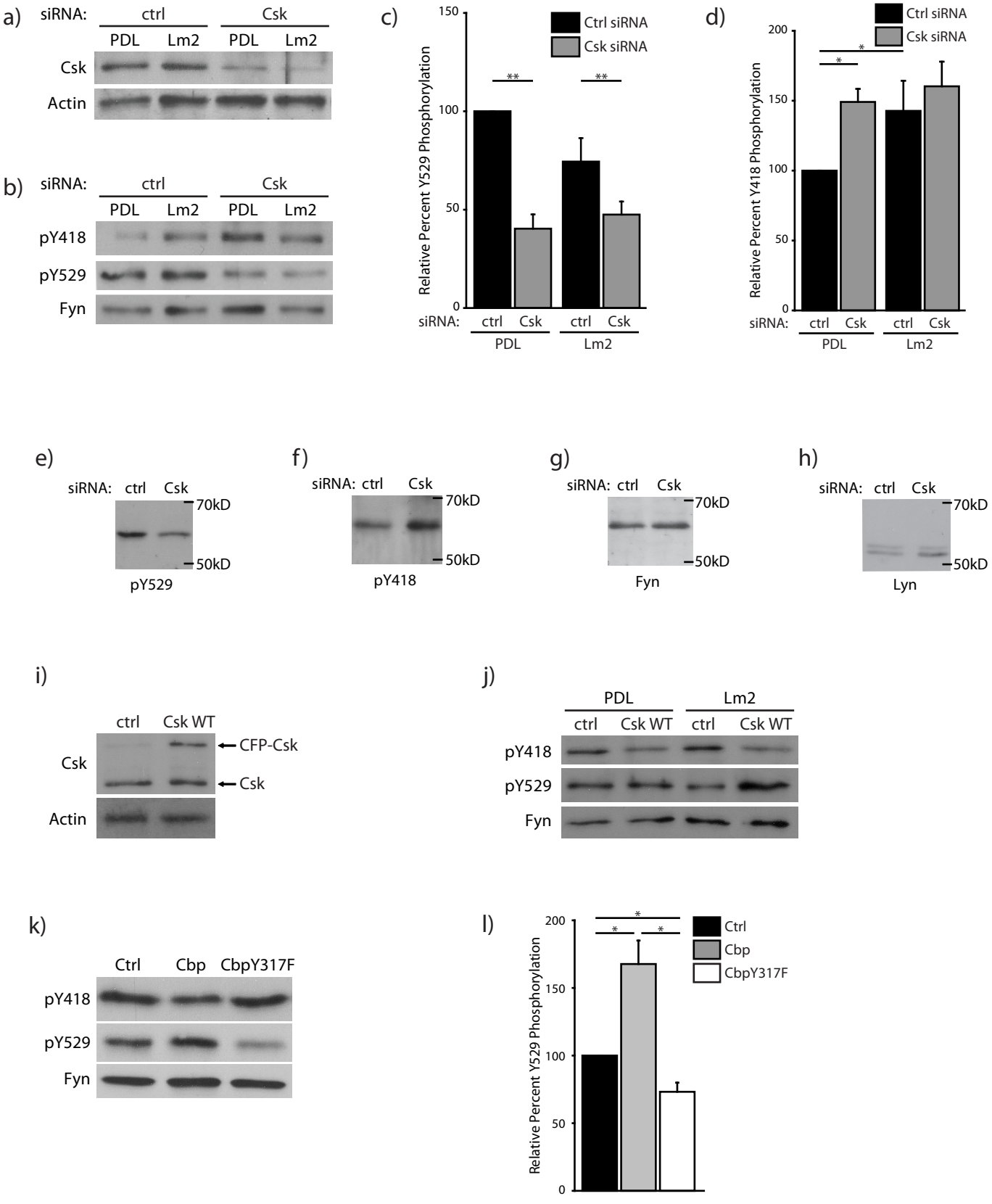
Solubility in cold Triton-X-100, however, provides only a rough assessment of potential membrane-association, as cytoskeleton-associated proteins can also be insoluble in cold Triton X-100. To test whether Cbp and Csk can indeed distribute to oligodendrocyte lipid rafts, I isolated detergent-insoluble glycosphingolipid-enriched membrane microdomains (DIGs) by sucrose-gradient ultracentrifugation. I confirmed that Fyn was found within DIG fractions (fractions 2-4) in oligodendrocytes differentiated for 2 and 4 days (Fig. 6B, and data not shown). In addition, I found Csk, Cbp, and phosphorylated Cbp (pY317) in DIG fractions (Fig. 6B). Treatment of oligodendrocytes with the sphingolipid biosynthesis inhibitor, fumonisin B1[163], completely redistributed Fyn, Csk, and Cbp into the non-DIG fractions (Fig. 6B). To further characterize Csk and Cbp location I used fluorescently-labeled cholera toxin subunit B (CTB), which binds to clustered GM1 gansglioside[190] to identify GM1-containing lipid rafts in live cells (Fig. 6C). Cells were subsequently fixed and co-labeled with Csk or Cbp antibodies; both proteins were found to partially co-localize with CTB, suggesting that both Csk and Cbp, at least in part, reside in GM1-containing lipid rafts.

Csk negatively regulates Fyn activity in Oligodendrocytes *in vitro*

To test whether Csk regulates oligodendroglial SFKs I used small interfering RNA (siRNA) to specifically deplete Csk mRNA. OPCs transfected with Csk-specific siRNAs, or non-targeting control siRNAs, were differentiated for 1, 2, or 4 days on PDL or Lm2. Western blot analysis confirmed substantially decreased Csk protein in oligodendrocytes transfected with Csk siRNA (Fig. 7A). In Csk-depleted cells, Fyn phosphorylation at its negative regulatory Y529 was found to be decreased by approximately 50% relative to that in control cells (Fig. 7B,C; 40.4±7.8% on PDL, P=0.0059, n=7; 47.5±7.2% on Lm2, P=0.0071, n=7). In contrast,

Figure 7. Csk and Cbp negatively regulate oligodendroglial Fyn. OPCs were transfected with control (ctrl, black bars) or Csk-specific (Csk, grey bars) siRNA and differentiated for 2 days on PDL or Lm2 **(a-g)**. **(a)** Western blot analysis of Csk and actin (loading control). **(b)** Western blot analysis of Fyn and Fyn phosphorylation (pY418, pY529). **(c)** Densitometry to determine relative phosphorylation at Fyn Y529 (pY529/Fyn). The average percent change ±sem relative to control (ctrl) siRNA-transfected cells on PDL is shown (***P*<0.01). **(d)** Densitometry to determine relative phosphorylation at Fyn Y418 (pY418/Fyn). The average percent change ±sem relative to control (ctrl) siRNA-transfected cells on PDL is shown (**P*<0.05). **(e-h)** OPCs were transfected with control (ctrl) or Csk-specific (Csk) siRNA and differentiated for 2 days on PDL. Low percentage SDS-PAGE was performed to separate Fyn and Lyn, followed by Western blot analysis of: **(e)** Fyn, **(f)** Lyn, **(g)** phosphorylated Y418 (Y418), and **(h)** phosphorylated Y529 (pY529). **(i)** Western blot analysis to detect Csk and actin (loading control) in cells transfected with CFP-Csk (Csk WT) or empty vector (ctrl). **(j)** Western blot analysis of Fyn and Fyn phosphorylation (pY418. pY529) in cells transfected with Csk-ECFP (Csk WT) or empty vector (ctrl) differentiated for 2 days on PDL or Lm2. **(k)** Western blot analysis of Fyn and Fyn phosphorylation (pY418, pY529) in cells transfected with Cbp-EYFP (Cbp), CbpY317F-EYFP (CbpY317F) or empty vector (ctrl) differentiated for 2 days. **(l)** Densitometry to determine relative phosphorylation at Fyn Y529 (pY529/Fyn). The average percent change ±sem relative to empty vector (ctrl) transfected cells is shown (**P*<0.05).

Figure 7


a)
siRNA:
ctrl
Csk
PDL
Lm2
PDL
Lm2
Csk
Actin
b)
siRNA:
ctrl
Csk
PDL
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PDL
Lm2
pY418
pY529
Fyn
c)
Ctrl siRNA
Csk siRNA
Relative Percent Y529 Phosphorylation
150
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50
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siRNA:
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Relative Percent Y418 Phosphorylation
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Fyn
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siRNA:
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Csk
70kD
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Csk WT
Csk
CFP-Csk
Csk
Actin
j)
PDL
Lm2
ctrl
Csk WT
ctrl
Csk WT
pY418
pY529
Fyn
k)
Ctrl
Cbp
CbpY317F
pY418
pY529
Fyn
l)
Relative Percent Y529 Phosphorylation
200
150
100
50
0
*
*
*
Ctrl
Cbp
CbpY317F

autophosphorylation at Fyn Y418 was significantly increased in Csk-depleted cells relative to that in control cells (Fig. 7D; 149.1±10.0%, P=0.0210, n=7, on PDL). Control OPCs plated on Lm2 had increased Fyn Y418 phosphorylation relative to control cells on PDL (Fig. 7D; 142.7±23.2%, P=0.0305, n=7), whereas Csk-depleted cells on Lm2 displayed Fyn Y418 phosphorylation similar to control cells on Lm2 (Fig. 7D; 160.3±19.0% in Csk siRNA-cells versus 142.7±23.2% in control cells, P=0.568, n=7). To determine whether Lyn phosphorylation status also changed following Csk depletion, I separated Lyn (53/56 kDa) from Fyn (59 kDa) using low percentage SDS-PAGE. Virtually no Lyn phosphorylation was detected at either Y418 or Y529 (Fig. 7E-H). Using this method I was not able to similarly examine Src (60 kDa) phosphorylation. It should be noted, however, that Src levels are extremely low in oligodendrocytes[108,148]. Together these observations suggested that Csk may preferentially negatively-regulate Fyn relative to other oligodendrocyte SFKs, possibly by virtue of differential spatial associations.

To confirm Csk's role in regulating Fyn phosphorylation, I next transfected oligodendroglia with either a Csk-CFP fusion protein or with CFP alone, and examined Fyn phosphorylation following differentiation on Lm2 (Fig. 7I). I found that Csk overexpression led to increased Fyn Y529 phosphorylation, whereas Fyn Y418 phosphorylation was decreased (Fig. 7J). Finally, to determine the role of the Csk adaptor protein Cbp, I examined Fyn phosphorylation in cells that expressed either WT Cbp or Cbp-Y317F mutant protein (Fig. 7K). Relative to control transfected cells, I detected a significant increase in Fyn Y529 phosphorylation in the presence of WT Cbp overexpression (Fig. L; n=3; 167.7±17.3%, P=0.042, compared to empty vector control) and a significant decrease in Fyn Y529

phosphorylation in the presence of mutant Cbp-Y317F overexpression (Fig. L; n=3, 73.2±6.7%, *P*=0.028). Thus, loss of Csk leads to reduced Y529 phosphorylation and overexpression of the Csk adaptor, Cbp, leads to increased Y529 phosphorylation. Given that Cbp effects are dependent on the Csk binding site (Y317) this suggests that Csk, by virtue of its ability to bind Cbp, negatively regulates oligodendroglial Fyn.

Discussion

The Src family kinase (SFK) Fyn is necessary for normal CNS myelination such that Fyn gene deletion[147,149,151-152], or transgenic knock-in of kinase-dead Fyn[147], both result in hypomyelination. Although within myelin preparations Fyn activity is regulated developmentally[149-150], what has remained unclear is how Fyn activity is regulated during oligodendroglial differentiation. In a differentiation time course of primary rat oligodendrocytes I have identified that Fyn activity is temporally-regulated during oligodendroglial differentiation, suggesting that molecules that modulate Fyn function must not only be present in oligodendrocytes, but may be similarly developmentally-regulated. I have therefore identified that C-terminal Src Kinase (Csk) and its adaptor protein Cbp are not only expressed within oligodendroglial GM1-containing lipid rafts, but also associate in oligodendrocytes. Although Csk expression within a differentiation time course of cultured oligodendrocytes did not mimic Fyn inhibition, phosphorylation of the Csk binding site within lipid rafts, Cbp Y317, was indeed regulated developmentally. These observations suggest that Csk location at the plasma membrane, as well Csk function, may be regulated temporally via Cbp during oligodendrocyte development. In addition, I identify Csk as the principle negative regulator of Fyn in oligodendroglia, as Csk depletion using small interfering RNAs, as well as overexpression of

Csk constructs, induced significant changes in Fyn phosphorylation and activity. Furthermore, inhibition of Fyn required Cbp Y317, and therefore Csk/Cbp association, suggesting that Cbp participates in Fyn regulation likely by recruiting Csk to GM1-containing rafts. Taken together these observations suggest that Csk and Cbp may be two regulators of Fyn function during oligodendroglial development and myelination.

Fyn Activity is Regulated During Oligodendroglial Differentiation *in vitro*

Fyn is necessary for normal CNS myelination such that Fyn gene deletion, or transgenic knock-in of kinase-dead Fyn, both result in hypomyelination[147,149]. What has remained unclear is the role of SFKs during OPC development. Although oligodendrocytes express three SFKs, Fyn, Lyn, and, to a lesser degree, Src[108,148,150,156], initial reports indicated that Fyn was the sole active SFK[148]. Kramer and colleagues subsequently identified oligodendroglial Lyn activity, but determined it to be 3-fold lower than that of Fyn[150]. By separating proteins by low percentage SDS-PAGE and detecting phosphorylated Y418, which is autophosphorylated in catalytically-active SFKs[187], I confirmed that Fyn, not Lyn, was the major active SFK during oligodendrocyte development(Fig. 7E-H). Our Fyn activation time course agreed with previous reports[148], i.e. activity was low in OPCs but increased during oligodendrocyte differentiation (Fig. 4). While Src protein levels were low, however, I cannot rule out potential contributions from Src given that Fyn (59 kDa) and Src (60 kDa) could not be separated adequately by SDS-PAGE.

C-terminal Src kinase (Csk) and its Binding Partner, Cbp, Negatively Regulate Oligodendroglial Fyn

SFKs are regulated through two phosphorylation events: autophosphorylation at a catalytic subunit tyrosine and phosphorylation at the C-terminal regulatory tyrosine[187]. Here I report that Fyn was largely inactive i.e. phosphorylated at its regulatory tyrosine (Y529), at the onset and tail-end of differentiation, whereas Y529 phosphorylation was significantly *decreased* as newly-formed oligodendrocytes were generated i.e. day 1 of differentiation (Fig. 4C). Thus, phosphorylation of the Fyn regulatory, C-terminal tyrosine was regulated developmentally. Although the C-terminal Src Kinase (Csk) has been identified in oligodendrocytes[108,184-185], Csk function during oligodendrogenesis has remained unknown. Here I report that Csk is the principle negative regulator of Fyn in oligodendrocytes (Fig. 7). However, since Fyn inhibition changed during oligodendrocyte differentiation, I was surprised to find that Csk protein levels remained constant (Fig. 5). Csk is a constitutively-active, cytosolic kinase in other cells, while its SFK targets are post-translationally modified and largely reside at the membrane in glycosphingolipid-enriched membrane microdomains (GEMs), or lipid rafts[187]. Thus oligodendrocyte Csk function is likely to be regulated spatially through association with putative adaptor proteins including insulin receptor substrate-1 (IRS-1)[182], paxillin[183], and/or Csk binding protein (Cbp/PAG)[179-180]. Here I report 1) that Cbp is expressed in oligodendrocytes (Fig. 5) and, like Fyn, resides in GEMs (Fig. 6), 2) that Cbp interacts with Csk via a Cbp Y317 phosphorylation site (Fig. 5), and 3) that Fyn inhibition at Fyn Y529 requires Cbp Y317 (Fig. 5). Thus, Cbp participates in Fyn regulation, likely by recruiting Csk to oligodendrocyte GEMs.

While Csk was found to mediate phosphorylation of Fyn's regulatory tyrosine, I cannot rule out other potential mechanisms to regulate this site. For instance, Csk homologous kinase (Chk) has been reported to phosphorylate SFK regulatory tyrosines in several cell types[191-194]. *Chk*$^{-/-}$ animals develop normally[174,177], apart from hematopoietic lineage cell defects[195], unlike *Csk*$^{-/-}$ animals, which die *in utero* due to impaired neural tube formation[176], suggesting that Chk may be dispensable for development and/or is redundant to Csk. Chk is expressed in oligodendrocytes[184], however, *Chk*$^{-/-}$ animals show no apparent myelin deficits[174,177,195] suggesting that Chk may also be dispensable for oligodendrogenesis. Several phosphatases, including PTPα, PTPε, SHP-1, SHP-2, and CD45, have also been suggested to relieve Csk-mediated SFK inhibition[170,187]. These phosphatases are expressed in oligodendrocytes[196], however, it is unknown if they regulate oligodendrocyte SFKs. Fyn has been reported to physically associate with CD45 in oligodendrocytes[185], but the functional significance of this interaction remains unknown. Interestingly, *SHP-1*$^{-/-}$ and *CD45*$^{-/-}$ animals are hypomyelinated[185,197-198], while transgenic mice that overexpress catalytically-inactive PTPε exhibit delayed optic nerve myelination[199]. It remains unclear, however, whether SFKs are dysregulated in these animals.

Chapter 4:

Csk Promotes Cell Cycle Exit of Oligodendroglial Progenitor Cells

Rationale

Brain development and homeostasis relies on the ensheathment of axons by myelin to ensure the efficient propagation of nerve impulses. In the CNS, myelination is carried out by oligodendrocytes, which arise from a pool of neural precursor cells that differentiate into oligodendroglial progenitor cells (OPCs). OPCs proliferate and migrate to populate future white matter regions, and, upon cell-cycle exit, differentiate into myelinating oligodendrocytes that are capable of ensheathing axons. Generating sufficient numbers of mature oligodendrocytes from OPCs is essential for myelination, both in the developing brain and following demyelination. For example, seemingly sufficient numbers of OPCs have been observed associated with demyelinated lesions in multiple sclerosis, but these stalled cells have failed to differentiate into myelinating oligodendrocytes[24]. To understand this remyelination failure, the molecular mechanisms that control the ability of proliferative OPCs to transition to myelination-competent oligodendrocytes need to be elucidated.

In many cells, Src Family Kinases (SFKs) regulate the transition between proliferation and differentiation[187]. Indeed Src was first isolated from the Rous sarcoma virus as an oncogene

that, when inappropriately activated, drove cells to hyperproliferate[200]. During oligodendrogenesis, the SFK Fyn is essential for normal brain myelination[147,149,152], and subsequent *in vitro* studies have established that Fyn activity regulates critical steps during oligodendrocyte maturation: process outgrowth[158-159,161,163], survival[108], and expression of myelin basic protein[151,154-155]. It remains unknown, however, if Fyn activity and/or Fyn regulatory mechanisms participate in the control OPC proliferation and/or development.

In Chapter 3 Csk was identified as a negative regulator of oligodendroglial Fyn activity with a critical role in dictating the balance between Fyn "on" and "off" states. Furthermore, it was found that Fyn was largely inhibited in differentiating OPCs, but whether this inhibition has a functional significance remains to be elucidated. While Csk has been shown to act as a tumor suppressor in epithelial-derived cancers[189], its role in oligodendroglial development is unknown. In this chapter I will describe experiments designed to elucidate the potential role of Csk, in regulating OPC proliferation, a process necessary for generating appropriate oligodendrglial numbers and hence myelination.

Results

Csk Promotes Oligodendrocyte Progenitor Cell Cycle Exit *in vitro*

To identify a potential role for Csk in oligodendrogenesis, I transfected primary rat OPCs with either siRNAs specific for Csk mRNA, or control, non-targeting siRNAs, and evaluated cell phenotype. Following Csk depletion in OPCs I noted a striking increase in OPC numbers, suggesting that Csk regulated proliferation and/or survival. To investigate OPC proliferation,

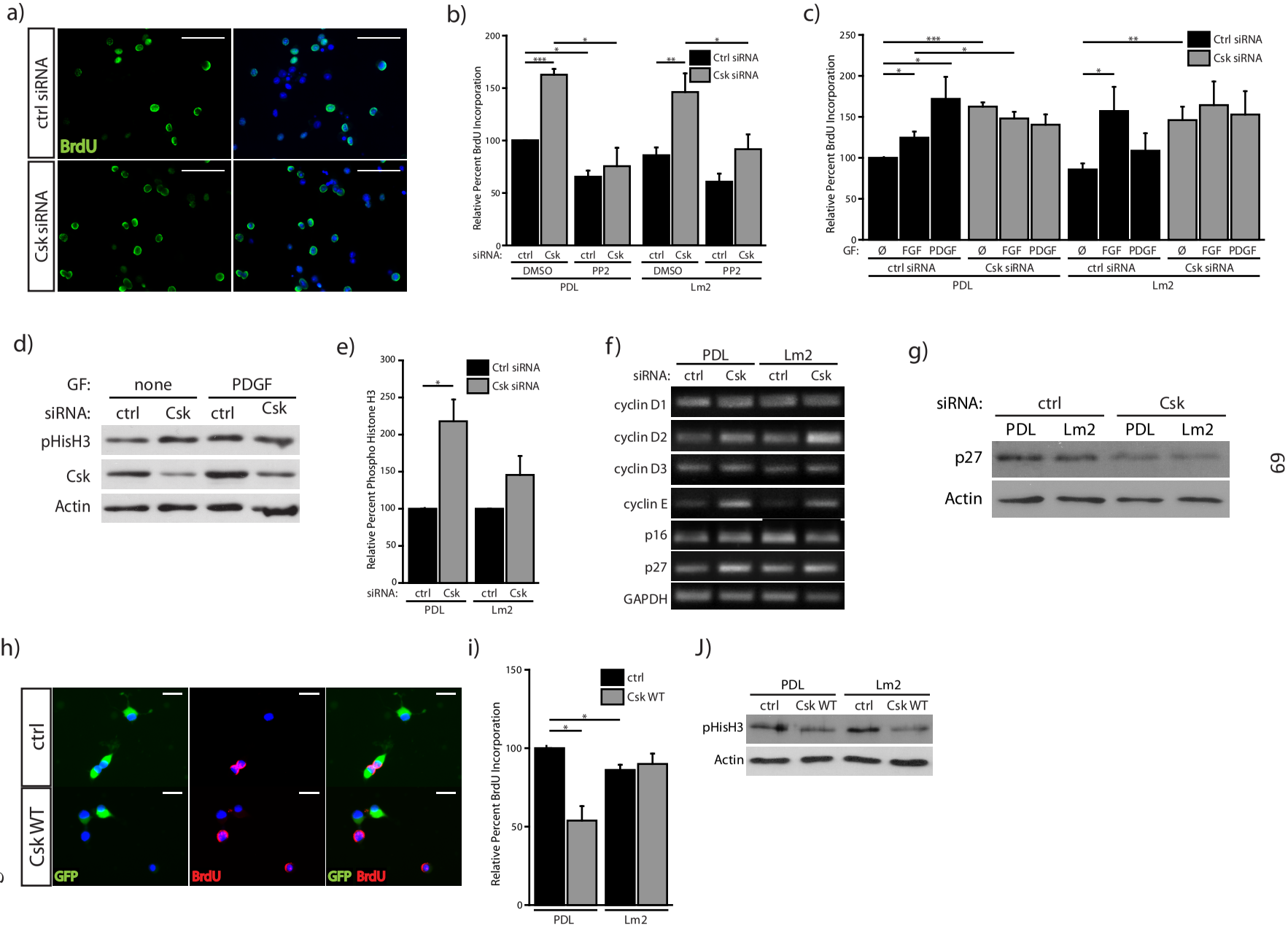
OPCs were transfected with either control or Csk-specific siRNA, and plated on either poly-D-lysine (PDL) or laminin2 (Lm2), and, after a 16-hour recovery period, switched to serum-free Sato's medium for 8 hours (with bromo-D-uridine (BrdU)) to trigger cell cycle exit (Fig. 8A). Laminin2 was chosen as a substrate because previous studies have found that laminin promotes oligodendrocyte differentiation and alters Fyn activity[89,108]. Despite being placed in medium designed to trigger cell cycle exit, Csk depletion led to a 162.5±5.1% increase in BrdU incorporation relative to that in control cells (Fig. 8B; P=0.0001, n=7, on PDL). Csk-depleted cells on Lm2 similarly showed an ~70% increase in BrdU incorporation compared to control cells on Lm2 (Fig. 8B; 146±16.5% of control cells on PDL, P=0.0094, n=6), a change similar to that observed on PDL. To verify that the increase in proliferation was due to increased SFK activity, I attempted to reverse the effect with PP2, a SFK inhibitor. PP2 treatment of control-transfected OPCs resulted in a significant decrease in BrdU incorporation (Fig. 8B; 63.3±5%, P=0.0316, n=4, on PDL) revealing a novel role for SFK activity in normally promoting OPC proliferation. Furthermore, PP2 treatment of Csk siRNA-transfected cells prevented the pro-proliferation effect of Csk depletion, such that BrdU incorporation levels were similar to those observed in untreated, control-transfected cells (Fig. 8B; 75.3±15%, P=0.421, n=4, on PDL; 91.5±12.2%, P=0.1300, n=4, on Lm2). Together these data indicated that Csk depletion caused inappropriate Fyn activation, which in turn delayed OPC cell cycle exit.

Next, I examined whether Csk depletion caused enhanced OPC proliferation under conditions designed to trigger OPC proliferation. FGF and PDGF are well-established OPC mitogens[201] and therefore, as expected, treatment with these factors led to increased BrdU incorporation (Fig. 8C). PDGF caused the strongest mitogenic response in OPCs plated on PDL

(Fig. 8C; 171.9±26.9%, P=0.0459, n=5), whereas FGF, interestingly, caused the strongest mitogenic response in OPCs plated on Lm2 (Fig. 8C; 157.1±29.5%, P=0.0484, n=5), suggesting a Lm2-mediated switch in growth factor responsiveness. Csk-depleted cells, however, were unresponsive to either growth factor, since neither PDGF nor FGF caused significant changes in BrdU incorporation relative to untreated, Csk-depleted cells on PDL (Fig. 8C, n=5; 148.1±8.1% with FGF, P=0.401; 140.4±12.6% with PDGF, P=0.245; compared to 162.5±5.1% with no growth factor) or on Lm2 (Fig. 8C, n=5; 164.4±29.5% with FGF, P=0.736; 152.8±28.5% with PDGF, P=0.854; compared to 146.0±16.5% with no growth factor). These results suggested that, once SFKs are maximally activated via Csk loss, additional stimulation from mitogens cannot further promote OPC proliferation.

To additionally evaluate OPC proliferation, phosphorylated histoneH3 levels were evaluated by Western Blot in siRNA-transfected cells differentiated for 1 day in serum-free Sato's medium (Fig. 8D,E). Csk-depleted cells, grown in medium that typically promotes cell cycle withdrawal, showed increased levels of phosphorylated histoneH3 compared to control cells (Fig. 8E, n=7; 218±29.3% on PDL, P=0.0427; 145.6±25.7% on Lm2, P=0.0607). As expected, PDGF caused an increase in histoneH3 phosphorylation in control siRNA-transfected cells (Fig. 8E, n=5; 166±43.8% on PDL, P=0.213; 146.8±11.9% on Lm2, P=0.0279). Again, Csk-depleted cells were unresponsive to PDGF, as phosphorylated histoneH3 levels were similar in Csk-depleted cells either with, or without, PDGF (Fig. 8E, n=5; 237.4±75.8% on PDL versus 218±29.3% in untreated cells, P=0.900; 151±26.2% on Lm2 versus 145.6±25.7% in untreated cells, P=0.0234). Similar results were observed with FGF (data not shown).

Figure 8. Csk promotes cell cycle exit of oligodendrocyte progenitors *in vitro*. (a-c) OPCs were transfected with control (ctrl) or Csk-specific (Csk) siRNA and grown in serum-free medium with or without mitogens (as indicated) to stimulate cell cycle withdrawal for 8 hours of BrdU incorporation. **(a)** Representative images of immunocytochemistry to detect BrdU (green) and DAPI (blue). Scale bars: 50μm. **(b)** Relative percent BrdU-positive control (ctrl, black bars) or Csk-specific siRNA (Csk, grey bars) transfected cells. The average percent change ±sem in BrdU-positive cells relative to control-transfected cells grown on PDL in DMSO is depicted (**P*<0.05, ***P*<0.01, ****P*<0.001). **(c)** Relative percent BrdU-positive control (ctrl, black bars) or Csk-specific siRNA (Csk, grey bars) transfected cells in the presence of 10 μg/ml FGF or PDGF. The average percent change ±sem in BrdU-positive cells relative to control siRNA-transfected cells (ctrl) grown on PDL in DMSO is depicted (**P*<0.05, ***P*<0.01, ****P*<0.001). **(d)** Western blot analysis of Csk, phosphorylated histoneH3 (pHisH3) and actin (loading control) in control (ctrl) or Csk-specific (Csk) siRNA transfected cells grown in Sato's medium +/- 10μg/ml PDGF for 24h. **(e)** Densitometry to determine relative percent phosphorylated histoneH3 in control (ctrl, black bars) or Csk-specific (Csk, grey bars) siRNA transfected cells treated as in (d). The average percent change ±sem in phosphorylated histoneH3 relative to control-transfected cells grown on PDL is depicted (**P*<0.05). **(f)** Semi-quantitative reverse transcriptase PCR (RT-PCR) analysis for cell cycle regulators was performed on mRNA isolated from control (ctrl) or Csk-specific (Csk) siRNA transfected cells grown for 24h in serum-free medium on PDL or Lm2. RT-PCR for GAPDH was performed as a loading control. **(g)** Western blot analysis of $p27^{Kip}$ and actin (loading control) in control (ctrl) or Csk-specific siRNA (Csk) transfected cells differentiated for 1 day on PDL or Lm2.**(h, i)** BrdU incorporation assay (8h in Sato's medium) on OPCs transfected with Csk-ECFP (Csk WT) or vector control (ctrl). **(h)** Representative images of immunocytochemistry to detect BrdU (red), GFP (green, to detect transfected cells), and DAPI (blue) are shown. Scale bar: 20μm. **(i)** Graphical representation of relative percent BrdU incorporation in cells transfected with Csk-ECFP (Csk WT, grey bars) or vector control (ctrl, black bars) grown in Sato's medium with BrdU for 8 hours on PDL and Lm2. The average percent change ±sem in BrdU-positive cells relative to control-transfected cells on PDL is depicted (**P*<0.05). **(j)** Western blot analysis of phosphorylated histoneH3 (pHisH3) and actin (loading control) in cells transfected with Csk-ECFP (Csk WT) or control vector (ctrl) and differentiated for 1 day.


a)
ctrl siRNA
Csk siRNA
BrdU
b)
Relative Percent BrdU Incorporation
Ctrl siRNA
Csk siRNA
siRNA: ctrl Csk ctrl Csk ctrl Csk ctrl Csk
DMSO PP2 DMSO PP2
PDL Lm2
c)
Relative Percent BrdU Incorporation
Ctrl siRNA
Csk siRNA
GF: Ø FGF PDGF Ø FGF PDGF Ø FGF PDGF Ø FGF PDGF
ctrl siRNA Csk siRNA ctrl siRNA Csk siRNA
PDL Lm2
d)
GF: none PDGF
siRNA: ctrl Csk ctrl Csk
pHisH3
Csk
Actin
e)
Relative Percent Phospho Histone H3
Ctrl siRNA
Csk siRNA
siRNA: ctrl Csk ctrl Csk
PDL Lm2
f)
PDL Lm2
siRNA: ctrl Csk ctrl Csk
cyclin D1
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cyclin D3
cyclin E
p16
p27
GAPDH
g)
siRNA: ctrl Csk
PDL Lm2 PDL Lm2
p27
Actin
h)
ctrl
Csk WT
GFP
BrdU
GFP BrdU
i)
Relative Percent BrdU Incorporation
ctrl
Csk WT
PDL Lm2
J)
PDL Lm2
ctrl Csk WT ctrl Csk WT
pHisH3
Actin

Figure 8

Using semi-quantitative reverse transcriptase PCR (RT-PCR), I evaluated mRNA levels of cell cycle regulators in Csk- or control-siRNA-transfected OPCs (Fig. 8F, n=4). Although Csk depletion caused no change in cyclin D2 mRNA levels (101.5±17.2%, P=0.957), other cell cycle regulators including cyclin D1 (149.9±8.6%, P=0.086), cyclin D3 (144.3±6.4%, P=0.109), cyclin E (155.4±7.9%, P=0.0383) and $p16^{INK}$ (133.3±6.0%, P=0.093) showed increased mRNA levels. $p27^{Kip1}$ mRNA levels, which in OPCs have been shown to increase with each cell cycle, were also increased in Csk-depleted cells (133.3±15.1, P=0.180). These data confirmed that Csk loss causes dysregulation of factors known to regulate cell cycle progression. Interestingly, while levels of $p27^{Kip1}$ mRNA were increased with Csk depletion, levels of $p27^{Kip1}$ protein were *decreased* (Fig. 8G), suggesting that Csk may be involved in post-transcriptional regulation of $p27^{Kip1}$ expression.

To verify that the *presence* of Csk indeed decreases OPC proliferation and thus promotes timely cell cycle exit, I performed BrdU incorporation assays in cells transfected with either Csk or control vector (Fig. 8H,J). OPCs with Csk overexpression showed an ~50% reduction in BrdU incorporation (Fig. 8I; 53.8±8%, P=0.0244, n=4, on PDL) compared to that observed in control OPCs. Interestingly, although Lm2 caused a small reduction in BrdU incorporation in control cells (Fig. 8I, n=4; 86.1±2.84%, P=0.0192), Csk overexpression in OPCs plated on Lm2 did not cause a significant decrease in BrdU incorporation (Fig. 8I, n=4; 75.5±5.7% versus 86.1±2.84% in control cells on Lm2, P=0.09), suggesting that laminins could alter OPC responsiveness to excess Csk. Phosphorylated histoneH3 levels were also decreased in Csk-overexpressing cells (Fig. 8J, n=3; 77.8±8.2% on PDL, P=0.129; 82.18±14.0% versus 77.8±13.6% in control cells on Lm2, P=0.669), although this trend was not significant.

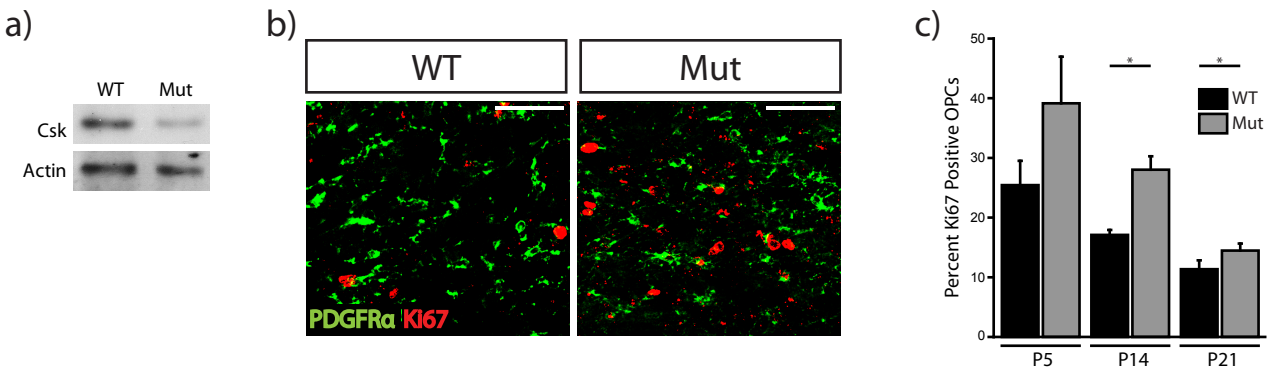
Csk Promotes Oligodendrocyte Progenitor Cell Cycle Exit *in vivo*

To address whether Csk loss-of-function altered OPC proliferation in the developing brain, I utilized a Cre-Lox approach to generate an oligodendroglial-specific *Csk* gene deletion mouse (Csk Mut – $Csk^{fl/fl}$; CNP-$cre^{+/-}$) using Cre recombinase expressed downstream of the promoter of the oligodendrocyte-specific *CNP* gene (see Methods). Csk protein was observed to be efficiently depleted in cultured OPCs isolated from P2 Csk mutant animals compared to their wild type littermates (Fig. 9A), thus confirming efficient recombination of oligodendroglial *Csk*. To determine whether deletion of Csk in OPCs leads to hyperproliferation similar to that observed using RNA interference *in vitro*, immunocytochemistry was used to detect the presence or absence of the proliferation marker Ki67 within PDGFRα-positive OPCs of mutant (Mut) corpus callosa or those of their wild type (WT) littermates. The percentage of Ki67-positive OPCs was increased in the Mut animals at all time points examined (Fig. 9B,C n=3; 153.6±17.4% of WT at P5, P=0.127; 164.4±13.8% of WT at P14, P=0.041; 129.1±6.2% of WT at P21, P=0.016) suggesting that gene deletion of Csk results in increased OPC proliferation. However, the magnitude of this increase in proliferation appeared to decrease with development, indicating that, eventually, Csk-independent mechanisms drive OPCs to exit the cell cycle.

Because OPC proliferation versus cell cycle exit can be highly influenced by OPC cell density[202], I sought to determine whether Csk loss-of-function in a small subset of OPCs would also cause a dysregulation in proliferative capacity. In other words, would individual Csk-deficient OPCs also proliferate more in an environment with normal numbers of OPCs? To test this I depleted Csk levels in a small subset of OPCs and evaluated proliferation of infected cells in the developing postnatal brain. Lentiviral particles encoding either short hairpin RNA

Figure 9. Csk promotes cell cycle exit of oligodendrocyte progenitor cells *in vivo*. (a) Western blot analysis of Csk and actin (loading control) in lysates from OPCs isolated from P2 wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals. **(b)** Indirect immunofluorescence to visualize cells positive for PDGFαR (green), Ki67 (red) or CC1 (red), and nuclei (blue) in the corpus callosum of postnatal day 21 (P21) wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals.Scale bars: 50μm. **(c)** Percentage ±sem of Ki67-positive OPCs that were PDGFαR-positive in the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) at P5, P14, and P21 (**P*<0.05).

Figure 9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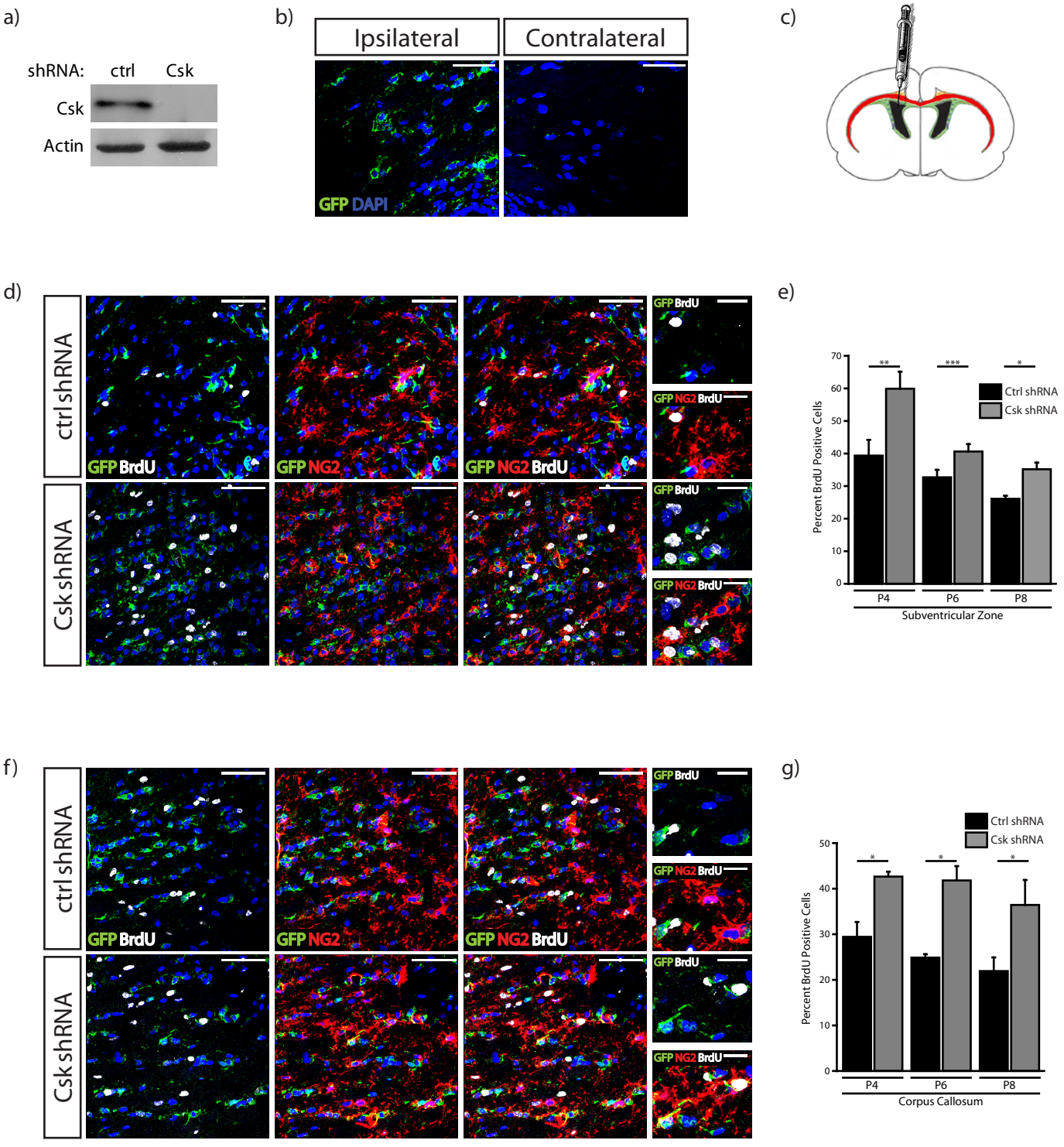
WT
Mut
Csk
Actin
b)
WT
Mut
PDGFRα
Ki67
c)
Percent Ki67 Positive OPCs
50
40
30
20
10
0
WT
Mut
*
*
P5
P14
P21

Figure 10. Csk depletion using short hairpin RNA promotes oligodendrocyte progenitor cell proliferation *in vivo.* (a) Western blot analysis of Csk and actin (loading control) in neurospheres infected with lentiviral particles encoding for control (ctrl) or Csk-specific (Csk) shRNA. **(b)** Indirect immunofluorescence to detect infected (GFP+, green) cells in postnatal day 6 coronal sections from rats injected into the right lateral ventricle with lentiviral particles encoding for control (ctrl) shRNA and turboGFP at postnatal day 2. GFP immunoreactivity is detectable in the ipsilateral, but not in the contralateral subventricular zone. **(c)** Cartoon representation of a coronal section of a rat brain depicting the injection site, i.e. the lateral ventricle, and the two regions analyzed i.e. the subventricular zone (green) and the corpus callosum (red). **(d)** Indirect immunofluorescence to visualize infected (GFP+, green) OPCs (NG2+, red) and BrdU-positive cells (white) in the subventricular zone at postnatal day 6 following 24h BrdU incorporation. Scale bars: 50μm in overview images, 20μm in higher magnification insets. **(e)** Percent BrdU-positive cells ±sem in the infected OPC population (GFP/NG2 double positive) in the subventricular zone of rats injected with lentiviral particles encoding for control (ctrl, grey bars) and Csk-specific (Csk, grey bars) shRNA at postnatal day 2, and sacrificed at postnatal day 4 (P4), 6 (P6) and 8 (P8). BrdU was injected 24h prior to sacrifice (**P*<0.05, ***P*<0.01, ****P*<0.001). **(f)** Indirect immunofluorescence to visualize infected (GFP+, green) OPCs (NG2+, red) and BrdU-positive cells (white) in the corpus callosum at postnatal day 6 following 24h BrdU incorporation. Scale bars: 50μm in overview images, 20μm in higher magnification insets. **(g)** Percent BrdU-positive cells ±sem in the infected OPC population (GFP/NG2 double positive) in the corpus callosum of rats injected with lentiviral particles encoding for control (ctrl, grey bars) and Csk-specific (Csk, grey bars) shRNA at postnatal day 2, and sacrificed at postnatal day 4 (P4), 6 (P6) and 8 (P8). BrdU was injected 24h prior to sacrifice (**P*<0.05).

Figure 10


a)
shRNA: ctrl Csk
Csk
Actin
b)
Ipsilateral
Contralateral
GFP DAPI
c)
d)
ctrl shRNA
Csk shRNA
GFP BrdU
GFP NG2
GFP NG2 BrdU
e)
Percent BrdU Positive Cells
Ctrl shRNA
Csk shRNA
P4
P6
P8
Subventricular Zone
f)
g)
Corpus Callosum

(shRNA) targeted to rat Csk, or control shRNA, as well as a GFP variant to track cells were injected, into the lateral ventricles of postnatal day 2 rats (Fig. 10). Subsequently, infected cells were identified in the subventricular zone (SVZ) and corpus callosum using GFP immunocytochemistry on fixed tissue (Fig. 10B). I confirmed shRNA efficiency in rat neural precursors by infecting rat neurosphere cultures with control or Csk shRNA lentiviral particles and analyzing Csk protein levels by Western blot (Fig. 10A). To evaluate the effect of Csk depletion on OPC proliferation *in vivo* I injected BrdU intraperitoneally 24 hours prior to sacrificing the animals and scored the number of BrdU-positive cells within the infected (GFP-positive), NG2-positive OPC population. (Fig. 10D-F) NG2-positive OPCs that expressed Csk shRNA had increased BrdU incorporation (relative to control cells) at all time points examined in both the SVZ (Fig. 10D,E n=3: 153.7±6.2% at P4, P=0.0017; 124.5±2.1% at P6, P=0.0007; and 134.7±4.1% at P8, P=0.0187) and the corpus callosum (Fig. 10F,G n=3: 148.1±14.6% at P4, P=0.0327; 167.9±9.6% at P6, P=0.0241; and 165.7±8.2% at P8, P=0.0368). Together, these findings suggested that the normal function of Csk is to promote cell cycle *exit* of OPCs, and in doing so, may indirectly promote OPC differentiation into newly-formed oligodendrocytes.

Discussion

The ability to transition from proliferative progenitor cells to mature cells is a critical process that, when dysregulated can contribute to disease. A developmental stall, for example is seen in multiple sclerosis, where OPCs are able to repopulate demyelinated lesions, but often fail to myelinate axons[24]. However, the molecular mechanisms that control whether or not OPCs exit the cell cycle and become myelin-competent oligodendrocytes remain unclear. In this chapter, I identify Csk as a molecular switch in OPCs that is critical for the appropriate timing of OPC cell

cycle exit *in vitro* and *in vivo*. Csk suppresses Fyn and thus suppresses OPC proliferation, as Csk-deficient OPCs had enhanced Fyn activity (See Chapter 3) and proliferated under conditions that normally promoted cell cycle exit. Furthermore, treatment with the SFK inhibitor PP2 was able to effectively rescue the hyperproliferation phenotype of Csk-deficient OPCs, suggesting that the pro-proliferation effect of Csk loss-of-function was mediated by increased SFK (like Fyn) activity. On the other hand, overexpression of Csk in cultured OPCs, which suppressed Fyn activity (See Chapter 3), in turn led to premature cell cycle exit even in the presence of exogenous PDGF. Taken together these findings not only suggest that Csk is a novel regulator of the OPC-to-oligodendrocyte transition with a key role in generating appropriate numbers of oligodendroglia, but also reveal a previously unidentified role for oligodendroglial Fyn in OPC proliferation.

Csk Promotes Timely Cell Cycle Exit of Oligodendrocyte Progenitor Cells

Csk has been shown to promote cell cycle exit in a variety of tissues and organisms. Thus Csk loss in *Drosophila melanogaster* causes hyperproliferation and delays organ growth[203], while immune-specific deletion of Csk in mice expands the progenitor pool and delays differentiation[204]. In the current study I found that Csk regulates OPC proliferation, a finding that revealed a previously unknown role for SFKs (like Fyn) in OPC cell division. Furthermore I observed that the highest degree of Fyn inhibition (Y529 phosphorylation) was at the very onset of OPC differentiation, 2 hours post-plating in low serum medium (Fig. 4), consistent with a model in which Csk suppression of Fyn contributes to the ability of OPCs to stop dividing and thus proceed to differentiation. Oligodendrocyte Lyn was shown previously to modulate OPC

proliferation following exposure to both PDGF and fibronectin, but not on other substrates[108]. Interestingly, I did not detect Lyn phosphorylation at either the autocatalytic Y418, nor the C-terminal Y529, in primary rat oligodendrocytes (See Chapter 3). Furthermore, although I could not distinguish Fyn from Src, the latter is expressed at very low levels in oligodendrocytes[108] and has no previously reported function in this system. I therefore, conclude that the pro-proliferation effect of Csk loss-of-function may be mediated exclusively through Fyn. Here I report that SFK (likely Fyn) activity in general may be a key factor in timing progenitor cell cycle arrest, whereas aberrant Fyn activity, such as that observed following Csk depletion (Fig. 7), may prolong the time spent by OPCs in the cell cycle and thus delay differentiation. Indeed I found increased proliferation in Csk-depleted OPCs *in vitro*, even in the absence of growth factors, or, in the presence of laminin2, a pro-differentiation ECM protein (Fig. 8). The hyperproliferation of Csk-depleted OPCs was effectively reversed through SFK pharmacological inhibition (Fig. 8), further demonstrating that excess SFK activity was sufficient to drive proliferation. Similarly, increased proliferation was observed in Csk-depleted OPCs in neonatal rodent brains (Figs.9, 10). Since Csk depletion rendered cells unresponsive to mitogen stimulation (Fig. 8), it can be furthermore surmised that Fyn is an obligate mitogen effector that, when activated, may be sufficient to maximize OPC proliferation. Taken together these findings suggest a novel role for SFKs in promoting OPC proliferation and reveal that Csk normally promotes timely OPC cell cycle exit via Fyn inhibition.

Laminin has been found to promote oligodendrocyte differentiation e.g. adult laminin-deficient mice display increased numbers of OPCs and decreased numbers of oligodendrocytes[89,205]. OPCs plated on laminin2, however, showed decreased proliferation (Fig.

8), suggesting that laminin promotes differentiation, at least in part, by stimulating cell cycle exit. Csk-depleted cells plated on laminin2, however, remained highly proliferative, suggesting that dysregulated Fyn signaling seen in the absence of Csk acts downstream of laminin2. However, laminin2 prevented Csk overexpression from *suppressing* proliferation, suggesting that laminin2 may be able to modulate SFKs in a Csk-independent manner. For instance, laminin2 may recruit or activate phosphatases and thus relieve, or by-pass, Csk-mediated inhibition. Another possibility is that laminins regulate the spatial association of integrins and receptor tyrosine kinases, as has been shown for laminin-mediated oligodendrocyte survival[201]. In addition, Laursen and colleagues reported recently that laminin2 promotes association of $\alpha_6\beta_1$-integrin and contactin within oligodendrocyte rafts to modulate Fyn activity[167], raising the possibility that integrin-contactin complexes may also regulate Csk-driven cell cycle exit. Similar integrin switches occur in other cells; in keratinocytes, for example, raft-associated $\alpha_6\beta_4$-integrin promotes EGF-dependent proliferation, while non-raft-associated $\alpha_6\beta_4$ enhances adhesion and hemidesmosome formation[206].

Csk-depleted cells had increased levels of $p27^{Kip1}$ mRNA, while $p27^{Kip1}$ protein was *decreased* (Fig. 8), suggesting that aberrant SFK activity may also post-transriptionally inhibit $p27^{Kip1}$ expression. Previous studies have identified $p27^{Kip1}$ as an important cell intrinsic regulator of OPC cell cycle exit[59,207-209]. $p27^{Kip1}$ protein levels increase with each cell cycle, eventually triggering OPC cell cycle exit[52]. Thus, $p27^{Kip1}$ overexpression promotes OPC cell cycle exit even in the presence of saturating levels of exogenous PDGF[98], while $p27^{Kip1}$ loss causes hyperproliferation in the absence of mitogens[207,210]. In addition, QKI, an mRNA binding protein, binds to and stabilizes $p27^{Kip1}$ mRNA[211] when phosphorylated by Fyn[154], but can also

repress translation. Suppressed $p27^{Kip1}$ mRNA translation may delay $p27^{Kip1}$ protein accumulation and therefore delay cell cycle exit and differentiation. Thus an interesting possibility is that Csk depletion delays cell cycle exit in part due to decreased $p27^{Kip1}$ mRNA translation.

Chapter 5:

Csk Loss-of-Function Leads to Delayed Oligodendrocyte Differentiation

Rationale

Oligodendrocyte differentiation is a complex process associated with extensive morphological changes, as well as alterations of the cellular proteome and behavior. Oligodendroglial differentiation requires timely OPC cell cycle exit, process outgrowth and branching, and finally, the expression of myelin-associated genes. The importance of oligodendroglial differentiation for normal brain development and function is illustrated by inherited myelinopathies, as well as by animal models with CNS hypomyelination defects. For example, hypothyroid mice[78], as well as mice lacking thyroid hormone receptors[79], exhibit hypomyelination phenotypes as a result of prolonged OPC proliferation, which in turn leads to delayed oligodendroglial differentiation. Taken together, these findings illustrate the necessity of timely cell cycle exit for proper oligodendrocyte differentiation and myelination.

As described in detail in Chapter 2, Fyn activity is essential for normal brain myelination[147], and subsequent *in vitro* studies have established that Fyn activity promotes critical steps during oligodendrocyte differentiation: process outgrowth[158-159,161,163], survival[108], and expression of myelin basic protein[151,154-155]. In this study, I have identified Csk as an

inhibitor of Fyn function in oligodendrocytes (See Chapter 3). In view of these observations, I initially formulated the hypothesis that **by inhibiting Fyn activity, Csk should also inhibit oligodendrocyte differentiation.** However, when examining the role of Csk in OPC proliferation, I found that Csk loss-of-function resulted in increased OPC proliferation *in vitro* and *in vivo*, suggesting a previously unidentified role for Fyn activity in promoting OPC proliferation. In view of these observations I concluded that Csk has a distinct function in promoting timely cell cycle exit of oligodendroglial progenitor cells *in vitro* and *in vivo* (see Chapter 4). Since timely OPC cell cycle exit is necessary for OPC-to-oligodendrocyte transition, and therefore, oligodendrocyte differentiation, Csk may actually *promote* oligodendrocyte differentiation, and Csk loss-of-function may result in a developmental delay of oligodendrocyte lineage progression. In this chapter, I will describe experiments designed to test these possibilities and to better understand the role of Csk in oligodendrocyte differentiation.

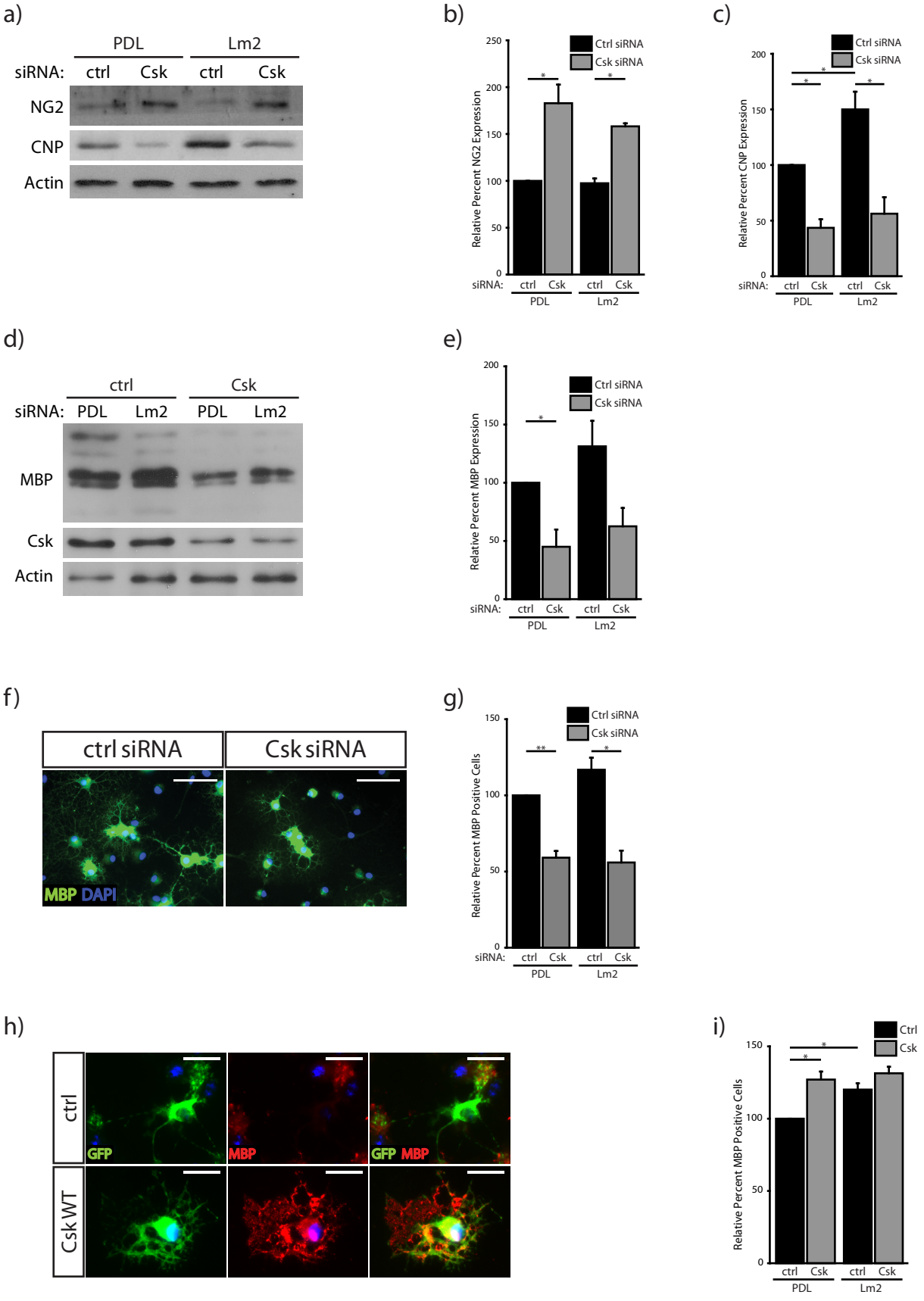
Results

Csk Regulates Oligodendroglial Differentiation *in vitro*

To characterize the function of Csk during oligodendroglial differentiation *in vitro* I transfected rat OPCs with control or Csk-specific siRNA and, after a 16-hour recovery period, differentiated the cells for 1, 2, or 4 days, and examined the levels of oligodendroglial stage-specific proteins (Fig. 11A). Csk-depleted cells, relative to control cells, showed higher levels of NG2 (an OPC marker) in cells differentiated for either 1 day (Fig. 11B, n=4; 182.2±19.9% on PDL, P=0.0250; 158.2±3.2% on Lm2, P=0.0220) or 2 days (data not shown), irrespective of

Figure 11. Csk regulates oligodendroglial differentiation *in vitro*. OPCs transfected with control (ctrl) or Csk-specific (Csk) siRNA were differentiated for 1 day **(a-c)** or 4 days **(d-g)** on PDL or Lm2. **(a)** Western blot analysis of NG2, CNP, and actin (loading control). **(b)** Densitometry to determine relative percent NG2 levels in control (ctrl, black bars) and Csk-specific (Csk, grey bars) siRNA transfected cells. Percent change ±sem in NG2 levels (NG2/actin) relative to that in control (ctrl) siRNA transfected cells on PDL is shown (**P*<0.05). **(c)** Densitometry to determine relative percent CNP levels in control (ctrl, black bars) and Csk-specific (Csk, grey bars) siRNA transfected cells. Percent change ±sem in CNP levels (CNP/actin) relative to that in control (ctrl) siRNA transfected cells grown on PDL is shown (**P*<0.05). **(d)** Western blot analysis of MBP, Csk, and actin (loading control). **(e)** Densitometry to determine relative percent MBP levels in control (ctrl, black bars) and Csk-specific (Csk, grey bars) siRNA transfected cells. Percent change ±sem in MBP levels (MBP/actin) relative to that in control (ctrl) siRNA transfected cells grown on PDL is shown (**P*<0.05). **(f)** Indirect immunofluorescence to visualize MBP-positive cells (green) and nuclei (DAPI, blue). Scale bar: 50μm. **(g)** Relative percent MBP-positive control (ctrl, black bars) or Csk-specific (Csk, grey bars) siRNA transfected cells. Percent change ±sem in the number of MBP positive cells relative to control (ctrl) transfected cells grown on PDL is shown (**P*<0.05, * *P*<0.01). **(h, i)** OPCs were transfected with either Csk-ECFP (Csk WT) or vector control (ctrl) and differentiated for 4 days. **(h)** Representative images using immunocytochemistry to visualize MBP-positive (red), transfected (GFP+, green) cells are shown. Scale bars: 50μm. **(i)** Relative percent MBP-positive cells that were transfected with Csk-ECFP (Csk WT, grey bars) or vector control (ctrl, black bars). Percent change ±sem in the percent MBP-positive cells relative to that in control vector (ctrl) transfected cells on PDL is shown (**P*<0.05).

Figure 11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PDL
Lm2
siRNA: ctrl Csk ctrl Csk
NG2
CNP
Actin
b)
Ctrl siRNA
Csk siRNA
Relative Percent NG2 Expression
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PDL
Lm2
c)
Ctrl siRNA
Csk siRNA
Relative Percent CNP Expression
siRNA: ctrl Csk ctrl Csk
PDL
Lm2
d)
ctrl
Csk
siRNA: PDL Lm2 PDL Lm2
MBP
Csk
Actin
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Csk siRNA
Relative Percent MBP Expression
siRNA: ctrl Csk ctrl Csk
PDL
Lm2
f)
ctrl siRNA
Csk siRNA
MBP DAPI
g)
Ctrl siRNA
Csk siRNA
Relative Percent MBP Positive Cells
siRNA: ctrl Csk ctrl Csk
PDL
Lm2
h)
ctrl
Csk WT
GFP
MBP
GFP MBP
i)
Ctrl
Csk
Relative Percent MBP Positive Cells
ctrl Csk ctrl Csk
PDL
Lm2

substrate. Csk-depleted cells also retained low levels of NG2 after 4 days in differentiation conditions, a point at which virtually all normal OPCs have differentiated into oligodendrocytes and have no detectable NG2 expression (data not shown). Conversely, Csk-depleted cells maintained in differentiation conditions had significantly less CNP than normal cells, a protein expressed in newly-formed oligodendrocytes but not in OPCs, at both day 1 (Fig. 11C, n=4; 43.6±7.7% on PDL, P=0.0245; 56.2±14.8% on Lm2, P=0.0265) and day 2 (data not shown). While control cells grown on the pro-differentiation substrate Lm2 showed no change in NG2 protein levels by day 1 (Fig. 11B, n=4; 97.4±5.1%, versus cells grown on PDL, P=0.471), control cells cultured on Lm2 had significantly increased levels of CNP protein (Fig. 11C, n=4; 150.0±15.9% of cells on PDL, P=0.0285). Together these data suggested that Csk depletion resulted in prolonged manifestation of progenitor characteristics, i.e. proliferation (See Chapter 4) and the presence of NG2 (Fig. 11B), which was accompanied by delayed differentiation (Fig. 11C).

To evaluate whether delayed differentiation of Csk-depleted OPCs also led to delayed oligodendrocyte maturation, I examined MBP levels in siRNA-transfected OPCs differentiated for 4 days (Fig. 11D). I found that Csk-depleted cells showed lower MBP levels (Fig. 11E; 45.0±16.5% of control cells when on PDL, P=0.0275, n=5; 52.7±17.9% of control cells when on Lm2, P=0.140, n=4). To address whether the decrease in MBP protein was due to an overall decrease in MBP expression, or due to decreased numbers of mature oligodendrocytes, I used immunocytochemistry to assess the percentage of MBP-positive, i.e. mature oligodendrocytes (Fig. 11F,G). I found that, compared to control cells, Csk-depleted cells showed a significantly decreased percentage of MBP-positive cells at both day 2 (data not shown) and day 4 (Fig. 11G,

n=3; 59.1±4.4% on PDL, P=0.0037; 55.9±7.8% on Lm2, P=0.048), suggesting that Csk depletion led to delayed oligodendrocyte maturation. To evaluate the effect of Csk overexpression on oligodendrocyte maturation, I transfected OPCs with wildtype Csk or control vector, differentiated for 2 (data not shown) or 4 days, then assessed the percentage of MBP-positive cells within the population of transfected cells (Fig. 11H). Csk-overexpressing cells showed a significantly increased percentage of MBP-positive cells relative to control vector-transfected cells (Fig. 11I, n=4; 144.4±4.8% at day 2, P=0.0031; 127±5.5% at day 4, P=0.0037). Lm2, compared to PDL, caused a small but significant increase in the percentage of MBP-positive cells in the control vector-transfected cells at the equivalent time points (Fig. 11I, n=4; 119.9±6.2% at day 2, P=0.026; 120.1±4.4% at day 4, P=0.012), confirming previous reports that Lm2 promotes OPC differentiation[89,108]. Although Csk overexpression further stimulated oligodendrocyte differentiation on Lm2 at day 2 (122.3±3.9% of control cells grown on Lm2, P=0.01, n=4), by day 4 this effect was no longer significant (109.5±3.6% of control cells grown on Lm2, P=0.09, n=4), indicating that laminins can speed up OPC differentiation via mechanisms that are not regulated by Csk. Overall, these findings suggested that Csk normally helps to generate appropriate numbers of mature oligodendrocytes, as Csk depletion perturbed timely oligodendroglial differentiation *in vitro*.

Csk Regulates Oligodendroglial Differentiation *in vivo*

To further characterize the role of Csk in oligodendroglial differentiation *in vivo*, and to assess whether the increase in progenitor proliferation observed in oligodendrocyte-specific Csk null (Csk mutant) animals resulted in changes in oligodendroglial differentiation, I evaluated cells in the corpus callosum of P5, P14 and P21 Csk mutant and WT littermates for the presence

Figure 12. Csk regulates oligodendroglial differentiation *in vivo*. (a) Indirect immunofluorescence to visualize positive for PDGFαR (green) or CC1 (red), and nuclei (blue) in the corpus callosum of postnatal day 21 (P21) wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals.Scale bars: 50μm. **(b)** Percentage ±sem of PDGFαR-positive OPCs in the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) at P5, P14, and P21. **(c)** Percentage ±sem of CC1-positive oligodendrocytes in the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) at P5, P14, and P21 (**P*<0.05, ***P*<0.01). **(d)** Western blot analysis of the OPC protein NG2 and actin (loading control) in cortical lysates from P5 wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals. **(e)** Western blot analysis of the mature oligodendrocyte protein MBP and actin (loading control) in cortical lysates from P14 and P21 wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals.

Figure 12

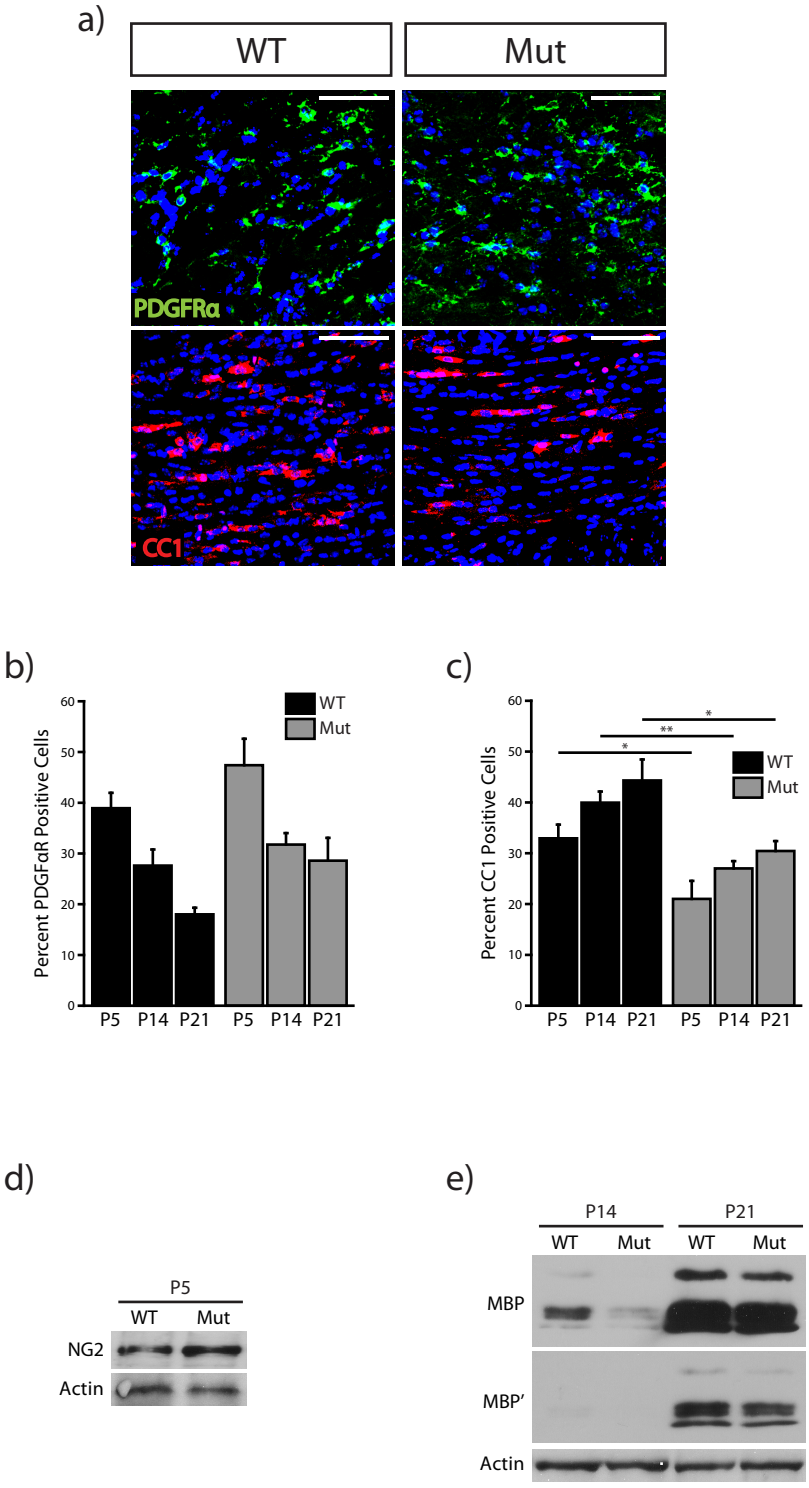
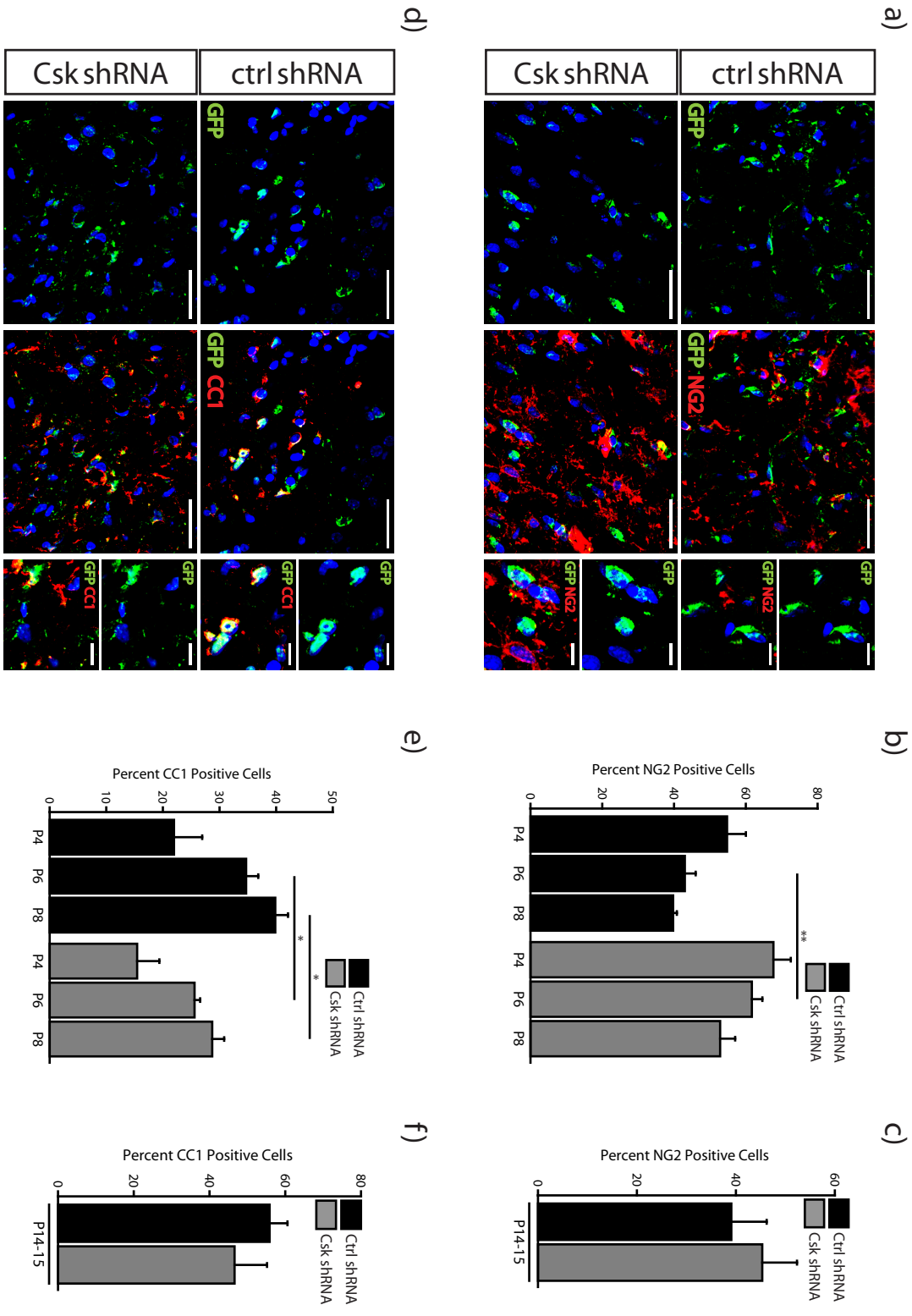

a)
WT
Mut
PDGFRα
CC1
b)
Percent PDGFαR Positive Cells
WT
Mut
P5 P14 P21 P5 P14 P21
c)
Percent CC1 Positive Cells
WT
Mut
P5 P14 P21 P5 P14 P21
d)
P5
WT Mut
NG2
Actin
e)
P14 P21
WT Mut WT Mut
MBP
MBP'
Actin

Figure 13. Csk depletion using short hairpin RNA results in delayed oligodendroglial differentiation *in vivo*. Postnatal day 2 (P2) rats were injected into the lateral ventricle with lentiviral particles encoding for turboGFP, as well as control (ctrl) or Csk-specific (Csk) shRNA, and sacrificed at P4, P6, P8 or P14/15 **(a-f)**. **(a)** Indirect immunofluorescence to visualize control (ctrl) or Csk-specific (Csk) infected (GFP+, green) cells that were also positive for the OPC marker NG2 (red) in the P6 corpus callosum. Scale bars: 50μm in overview images, 20μm in inset high magnification images. **(b)** Percentage ±sem of infected cells that were NG2-positive in the corpus callosum at P4, P6, and P8, following P2 injection of lentiviral particles encoding for control (ctrl, black bars) and Csk-specific (Csk, grey bars) shRNA (***P*<0.01.). **(c)** Percentage ±sem of infected cells that were NG2-positive in the corpus callosum at P14-P15, following P2 injection of lentiviral particles encoding for control (ctrl, black bars) and Csk-specific (Csk, grey bars) shRNA. **(d)** Indirect immunofluorescence to visualize control (ctrl) or Csk-specific (Csk) infected (GFP+, green) cells that were also positive for the mature oligodendrocyte marker CC1 (red) in the P6 corpus callosum. Scale bars: 50μm in overview images, 20μm in inset high magnification images. **(e)** Percentage ±sem of infected cells that were CC1-positive in the corpus callosum at P4, P6, and P8, following P2 injection of lentiviral particles encoding for control (ctrl, black bars) and Csk-specific (Csk, grey bars) shRNA (***P*<0.01.). **(f)** Percentage ±sem of infected cells that were CC1-positive in the corpus callosum at P14-P15, following P2 injection of lentiviral particles encoding for control (ctrl, black bars) and Csk-specific (Csk, grey bars) shRNA.

Figure 13


a)
Csk shRNA
ctrl shRNA
GFP
GFP NG2
b)
Percent NG2 Positive Cells
P4
P6
P8
Csk shRNA
Ctrl shRNA
c)
Percent NG2 Positive Cells
P14-15
d)
GFP CC1
e)
Percent CC1 Positive Cells
f)
Percent CC1 Positive Cells

or absence of oligodendroglial lineage markers. Across all three time points I observed increased percentages of PDGFRα-positive cells (OPCs) in Csk-deficient animals compared to their WT littermates (Fig. 12A,B n=3; 121.3±6.0% of WT at P5, P=0.094; 118.8±18.5% of WT at P14, P=0.473; 156.7±14.9% of WT at P21, P=0.081); although these changes were not statistically significant. However, I found that the percentage of CC1-positive cells, indicative of mature oligodendrocytes, was significantly *decreased* within Csk mutant corpus callosa at all time points examined (Fig. 12A,C, n=3; 63.3±7.0% of WT at P5, P=0.033; 67.7±1.1% of WT at P14, P=0.00499; 69.2±3.4% of WT at P21, P=0.036). In addition, I evaluated the expression of oligodendrocyte lineage markers in cortical lysates from oligodendrocyte-specific Csk null and WT littermates using western blot analysis and found that Csk mutant cortices showed increased levels of the progenitor marker NG2 (Fig. 12D, n=3; 199.1±68.8% of WT at P5, P=0.212; 208.6±27.4% of WT at P14, P=0.003), while expression of the myelin component MBP was *decreased* (Fig. 12E, n=3; 42.7±8.8% of WT at P14, P=0.077; 49.5±6.5% of WT at P21, P=0.034). Taken together these findings reveal that genetic deletion of Csk in oligodendroglia significantly perturbs oligodendrocyte differentiation.

To evaluate whether the delayed oligodendroglial differentiation observed in oligodendrocyte-specific Csk null mice was due to a cell-autonomous dysfunction of Csk deficient oligodendrocytes, or due to global changes in the extracellular milieu of Csk mutant animals, I injected lentiviral particles encoding for control or Csk-specific shRNA into the lateral ventricles of P2 rats as described for Fig. 10. Using immunocytochemistry I identified infected (GFP-positive) cells in the corpus callosum of P4, P6, P8 and P14 animals and scored them for

the presence or absence of oligodendroglial lineage markers (Fig. 13). As expected, I found that the percentage of NG2-positive cells within each GFP-positive population decreased as time post-injection increased (Fig. 13B). Csk-depleted cells, however, showed an increased percentage of cells that were NG2-positive, compared to control shRNA-infected cells at the same time points (Fig. 13A,B n=3; 124.8±8.8% at P4, P=0.08; 143.9±4.0% at P6, P=0.01; 135.3±9.2% at P8, P=0.05). Conversely, the percentage of CC1-positive oligodendrocytes was *decreased* within Csk-depleted cell populations over time (Fig. 13D,E n=3; 70.3±5.0% of control at P4, P=0.06; 74.2±4.4% of control at P6, P=0.0486; 72.1±3.3% of control at P8, P=0.0178). By P14, however, the numbers of both NG2-positive and CC1-positive cells (Fig. 13C,F) were comparable in both control and Csk-depleted shRNA-infected cell populations, suggesting that Csk depletion delays, but does not prevent, oligodendroglial maturation.

Discussion

Previous studies have shown that Fyn function is necessary for two critical aspects of oligodendroglial differentiation *in vitro*: 1) morphological differentiation of oligodendrocytes by promoting process outgrowth and branching[158-159,161], as well as laminin2-induced myelin sheet formation[108], and 2) oligodendroglial maturation by promoting expression of the myelin-associated protein MBP[151,154-155]. In view of these observations, I initially hypothesized that by inhibiting Fyn activity, Csk should in turn inhibit oligodendroglial differentiation, and conversely, that Csk loss-of-function should *promote* oligodendroglial differentiation. To test this hypothesis, I used RNA interference to deplete oligodendroglial Csk *in vitro* and *in vivo*, as well as a Cre-Lox approach to specifically delete Csk in oligodendroglia in transgenic mice, and analyzed the expression of lineage-specific markers. Interestingly, I found that Csk depletion in

cultured oligodendroglia resulted in increased levels of the OPC-specific protein NG2, but *decreased* levels of the newly-formed oligodendrocyte marker CNP, as well as the mature oligodendrocyte protein MBP. Similarly, Csk loss-of-function *in vivo* led to increased numbers of NG2-positive OPCs, but decreased numbers of CC1-positive, mature oligodendrocytes. These findings suggest that Csk loss-of-function did not lead to the predicted increase in oligodendroglial differentiation, but resulted in a developmental *delay* instead. On the other hand, Csk overexpression resulted in increased oligodendroglial differentiation as the numbers of NG2-positive OPCs were decreased, while the numbers of MBP-positive, mature oligodendrocytes were *increased*. Taken together, these observations suggest that the normal function of oligodendroglial Csk is to promote oligodendrocyte differentiation. Why do loss of Fyn activity[156], as well as loss of Fyn inhibitor Csk, both result in decreased oligodendroglial differentiation?

In Chapter 4, I found that loss of Csk resulted in increased OPC proliferation and concluded that the normal role of Csk is to promote OPC cell cycle exit. In addition, I reported a previously unidentified role for Fyn activity in promoting OPC proliferation. Since OPC cell cycle exit occurs prior to differentiation, the two processes cannot always be uncoupled. Furthermore, conditions that delay OPC cell cycle exit lead to delayed oligodendrocyte differentiation and maturation. Notable examples of this phenomenon include: transgenic animals overexpressing PDGF, hypothyroid rodents, as well as mice lacking expression of thyroid hormone receptors[79] or of the Cdk inhibitor $p27^{Kip1}$ [59]. In these animal models OPC hyperproliferation and delayed cell cycle exit resulted in delayed oligodendroglial differentiation, which was sometimes accompanied by hypomyelination defects[59,79]. It is,

therefore, reasonable to suggest that the delayed oligodendroglial differentiation observed with Csk loss-of-function was a direct result from delayed OPC cell cycle exit (See Chapter 4).

As described above, a substantial body of work has implicated Fyn activation as a necessary step during late oligodendrocyte development i.e. differentiation and myelination. I was surprised therefore that Csk depletion, which *elevated* Fyn activity, led to delayed oligodendrocyte differentiation. However our finding that Csk was necessary for appropriate transition of OPCs to newly-formed oligodendrocytes revealed that SFK suppression also was important for OPC differentiation. Csk depletion led, therefore, to stalled, or delayed, OPC differentiation in Csk-deficient OPCs compared to normal OPCs. But Csk-deficient OPCs *in vivo* eventually differentiated such that by 12 days post-infection, Csk-deficient cells were as likely as control cells to have differentiated into mature oligodendrocytes (Fig. 13). Together, these findings suggested that Fyn has distinct and opposing roles during oligodendrocyte development such that, at the onset of oligodendrogenesis, Fyn promotes progenitor cell division and hence progenitor expansion, while later, Fyn promotes oligodendrocyte differentiation. One might predict, therefore, that once Csk-deficient OPCs exit the cell cycle, increased Fyn activity in Csk-deficient oligodendrocytes may drive *increased* differentiation. To fully address Csk's role in differeniation, however, novel experimental tools need to be developed that allow us to modulate oligodendroglial Csk function in post-mitotic cells.

Chapter 6:

Csk Loss-of-Function Promotes Oligodendrocyte Survival

Rationale

Developing oligodendroglia undergo a massive wave of programmed cell death to ensure that mature oligodendrocytes are not generated in excess. However, excess apoptosis can result in human disease both during development and in adulthood. For example, neonatal cerebral ischemia causes massive oligodendroglial apoptosis, which in turn results in delayed myelination onset and neurological dysfunction[3-5]. Similarly, mutations in the *Plp* gene, associated with the inherited leukodystrophy PMD, lead to oligodendrocyte apoptosis and dysmyelination[6]. In addition, demyelinating diseases such as multiple sclerosis show damage and death of mature, myelinating oligodendrocytes, which is presumed to contribute to the pathology. Factors that promote oligodendrocyte survival are therefore critical for generating and maintaining sufficient numbers of myelinating oligodendrocytes, and in turn, are necessary for normal brain homeostasis.

Studies in cultured oligodendrocytes have identified the SFKs Fyn and Lyn as cell-intrinsic effectors of extracellular pro-survival cues. While Fyn expression is necessary for laminin2-induced[108], as well as L1-induced[167], survival of newly-formed oligodendrocytes, Lyn activity downstream of PDGF and fibronectin signaling is necessary to suppress ceramide

production, and therefore, apoptosis of OPCs[91]. It remains unknown, however, if SFK activity and/or SFK regulatory mechanisms similarly regulate oligodendroglial survival *in vivo*. Interestingly, although $Fyn^{-/-}$ mice have decreased numbers of oligodendroglial cells[152], it remains unclear whether this loss of oligodendroglia is due decreased proliferation and/or decreased survival. In chapter 3, I identified that depletion of the SFK inhibitor Csk, resulted in increased Fyn activity. I therefore, hypothesized that **Csk loss-of-function may promote oligodendrocyte survival.** Furthermore, loss of Csk led to OPC hyperproliferation (See Chapter 4) and increased numbers of NG2-positive progenitors, but *decreased* numbers of CC1-positive, mature oligodendrocytes and delayed differentiation (See Chapter 5). In view of these observations I concluded that timely OPC cell cycle exit, and therefore OPC-to-oligodendrocyte transition were necessary for timely oligodendroglial differentiation. An alternative explanation for the decreased numbers of mature oligodendrocytes observed in the absence of Csk, however, is that Csk loss-of-function could lead to impaired survival.

Results

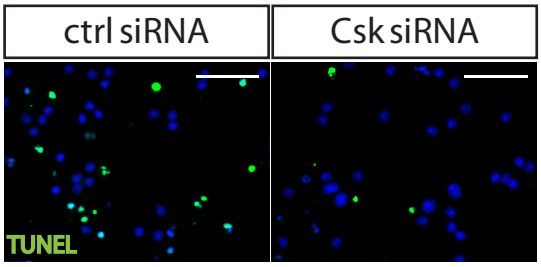
Csk Promotes Apoptosis in Newly-Formed Oligodendrocytes *in vitro*

To test the hypothesis that Csk loss-of-function decreases oligodendroglial survival and to address whether the decreased percentage of mature oligodendrocytes observed in Csk-depleted cells was due to impaired survival, I evaluated apoptosis in newly-formed oligodendrocytes (Fig. 14). Csk-depleted cells showed a significant decrease in TUNEL-positive cells compared to control cells (Fig. 14A,B n=4; 50±7.3% on PDL, P=0.00033; 43.2±8% on Lm2, P=0.0252). To test whether the anti-apoptotic effect was indeed due to increased SFK

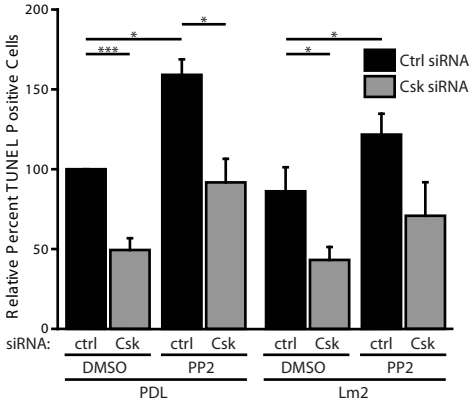
Figure 14. Csk promotes oligodendroglial apoptosis *in vitro*. OPCs were transfected with control (ctrl) or Csk-specific (Csk) siRNA and differentiated for 1 day on PDL or Lm2 **(a-d)**. **(a)** Immunocytochemistry to detect TUNEL (green) and DAPI (blue) in control (ctrl) or Csk-specific (Csk) siRNA transfected cells. Scale bars; 50μm. **(b)** Relative percent TUNEL-positive control (ctrl, black bars) or Csk-specific (Csk, grey bars) siRNA transfected cells. Relative percent change ±sem in TUNEL-positive cells relative to that in control (ctrl) siRNA transfected cells treated with DMSO on PDL is shown (**P*<0.05, ****P*<0.001). **(c)** Western blot analysis of cleaved caspase3 (CC3) and actin (loading control). **(d)** Densitometry to determine relative percent cleaved caspase3 (CC3) in control (ctrl, black bars) and Csk-specific (Csk, grey bars) siRNA transfected cells. Percent change ± sem in CC3 protein (CC3/actin) relative to that in control siRNA transfected cells on PDL is shown (**P*<0.05). **(e)** Immunocytochemistry to detect TUNEL (red) in cells that were transfected (GFP(+), green) with either Csk (CskWT) or control vector (ctrl) and differentiated for 1 day. Scale bars: 50μm. **(f)** Relative percent TUNEL-positive control vector (ctrl, black bars) or Csk (CskWT, grey bars) transfected cells. Relative percent change ± sem in TUNEL-positive cells relative to that in control vector (ctrl) transfected cells on PDL is shown (**P*<0.05, ***P*<0.01, ****P*<0.001).

Figure 14

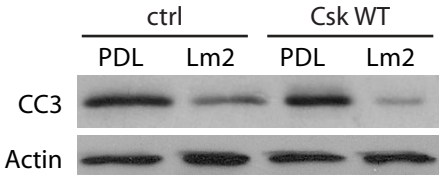
a)



b)



c)



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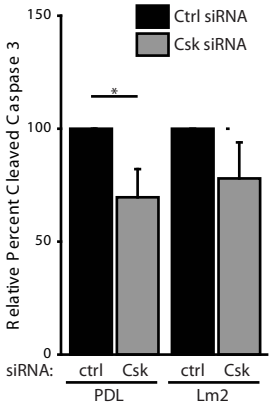

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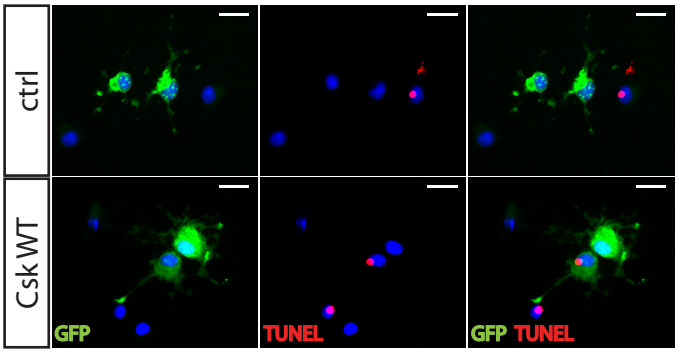
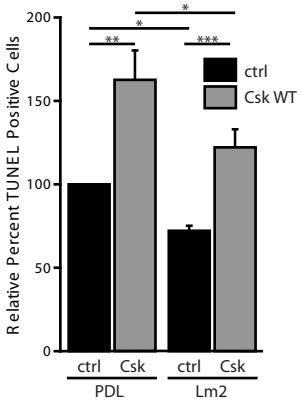


f)



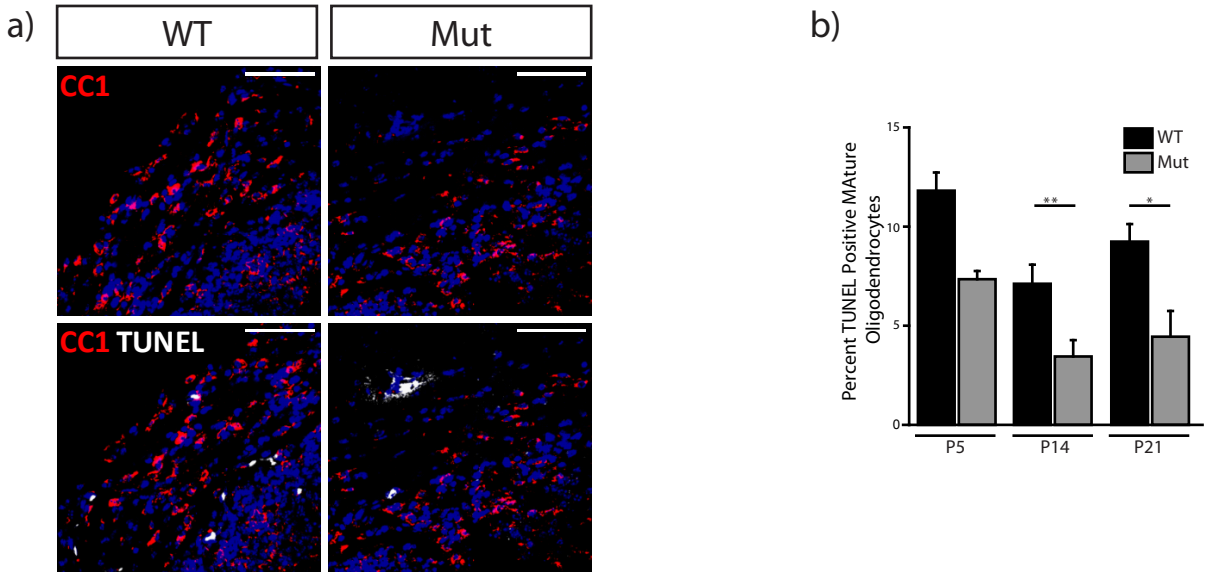
activity, I attempted to block this phenotype using the SFK inhibitor PP2. While control cells treated with PP2 already showed significantly increased percentages of TUNEL-positive oligodendrocytes (Fig. 14B, n=3; 159.1±9.6% on PDL, P=0.0390; 121.7±13.1% on Lm2, P=0.0375), PP2 treatment of Csk-depleted cells ameliorated the effect of Csk depletion such that the percentage of TUNEL-positive cells were similar to those of vehicle-treated, control-transfected cells (Fig. 14B, n=3; 91.7±14.8% on PDL, P=0.519; 70.8±24.2% on Lm2, P=0.967, n=3); these data indicated that the pro-survival effects of Csk depletion were due to excess SFK activity. As an additional readout for apoptosis, I monitored levels of cleaved caspase-3 protein (CC3) in control and Csk-depleted OPCs differentiated for 1 day. Csk-depletion significantly decreased CC3 levels in newly-formed oligodendrocytes relative to that in control cells (Fig. 14C,D n=6; 69.7±12.4% on PDL, P=0.0414; 278±16% on Lm2, P=0.157). Csk overexpression, however, had the opposite effect i.e. it significantly increased the percentage of TUNEL-positive oligodendrocytes (Fig. 14E,F, n=4; 162.6±16.3% on PDL, P=0.0037; 122.2±10% on Lm2, P=0.0004). In agreement with prior studies[108], I observed that Lm2 provided a significant protection from apoptosis as the percentage of TUNEL-positive cells was decreased to 72.2±2.8% on Lm2 substrate (Fig. 13F, n=4; P=0.0135). These findings suggested that Csk promotes apoptosis in newly-formed oligodendrocytes, providing an additional means for Csk to regulate oligodendrocyte numbers.

Csk Promotes Mature Oligodendrocyte Apoptosis *in vivo*

To test whether Csk regulates oligodendroglial survival *in vivo*, and to determine whether the decreased numbers of CC1-positive, mature oligodendrocytes observed in oligodendrocyte-

Figure 15. Csk promotes oligodendroglial apoptosis *in vivo*. (a) Indirect immunofluorescence to detect TUNEL (white), CC1+ (red) cells, and nuclei (blue) in the corpus callosum of postnatal day 14 (P14) wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals. Scale bars: 50μm. **(b)** Percentage ±sem of TUNEL-positive mature oligodendrocytes (CC1+) in corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) at P5, P14, and P21 (**P*<0.05, ***P*<0.01).

Figure 15


a)
WT
Mut
CC1
CC1 TUNEL
b)
Percent TUNEL Positive MAture Oligodendrocytes
15
10
5
0
WT
Mut
**
*
P5
P14
P21

specific Csk null corpus callosa were as a result of increased apoptosis, I evaluated oligodendroglial apoptosis in the corpus callosum of Csk mutant (Mut) and wild type mice at P5, P14, and P21 (Fig. 14). Using immunocytochemistry, I identified the presence or absence of apoptotic, TUNEL-positive cells within CC1-positive, mature oligodendrocytes within mutant (Mut) and wild type corpus callosum. The percentage of apoptotic CC1-positive mature oligodendrocytes was decreased in the Mut animals at all time points examined (Fig. 15A, B; n=3; 63.4±8.1% of WT at P5, P=0.069; 47.02±5.4% of WT at P14, P=0.0029; 46.25±10.3% of WT at P21, P=0.012), suggesting that gene deletion of Csk results in decreased apoptosis. These findings suggest in the developing brain Csk promotes apoptosis of mature oligodendrocytes.

Discussion

Since decreased numbers of mature oligodendrocytes were observed following Csk depletion, I considered the possibility that Csk loss triggered apoptosis. Instead, Csk depletion led to a significant *increase* in survival, suggesting that, since Csk depleted cells survive better than their wild types counterparts, increased death cannot be responsible for a decrease in oligodendrocyte numbers. Pharmacological inhibition of SFKs effectively reversed the pro-survival phenotype in Csk-depleted cells, again revealing that inappropriate SFK activation was responsible. Indeed, previous studies reported that Fyn was necessary for laminin-mediated[108] and L1-mediated-[167] oligodendroglial survival, however in the absence of these ligands, Fyn has not previously been implicated in oligodendrocyte survival. Furthermore, although Lyn activity downstream of a combination of PDGF and fibronectin signaling suppressed ceramide-mediated apoptosis of cultured OPCs[91], Lyn activity has never been implicated in oligodendroglial survival in the absence of these extracellular cues. Here I report that SFK activity contributed to

oligodendrocyte survival in the absence of laminin, L1, PDGF or fibronectin, as treatment with SFK inhibitors led to increased apoptosis (Fig. 14). A large fraction of newly-formed oligodendrocytes, if they fail to establish axon contact, undergo apoptosis at the onset of myelination[212], yet very little is known regarding why some OPCs avoid this scenario i.e do not myelinate *or* die, but instead become adult OPCs. It may be that differential dependence on exogenous factors versus intrinsic survival signaling contributes to cell outcomes during this critical window. Thus SFK activity may be an intrinsic regulator of oligodendrocyte survival that promotes survival, particularly in the presence of external stimuli i.e. laminin, L1, fibronectin or PDGF. In its role as a Fyn inhibitor, therefore, Csk may act as a "gatekeeper" to determine sensitivity to survival cues in newly-formed oligodendrocytes.

Chapter 7:

Csk Promotes Termination of Myelin Wrapping

Rationale

Myelination is the ensheathment of axons with concentric spirals of a specialized plasma membrane produced by oligodendrocytes in the CNS, and by Schwann cells in the PNS. Since myelination is the final step of oligodendrocyte differentiation it cannot necessarily be uncoupled experimentally from oligodendrocyte development. As discussed in Chapter 1, the molecules that regulate myelination specifically are very few, however, a vast array of signaling pathways regulate CNS myelination indirectly by influencing oligodendrocyte biology. For example, *in vitro* studies have suggested that Fyn activity is necessary for OPC migration, process outgrowth and branching, newly-formed oligodendrocyte survival, expression of myelin proteins such as MBP, and overall oligodendrocyte differentiation. *In vivo*, mice lacking either Fyn or Fyn activity are hypomyelinated[158-159,161,163]. Although it is unclear whether Fyn activity directly regulates myelination, the phenotype of Fyn mutant mice does suggest that Fyn activity can regulate myelination, either directly or indirectly. It is therefore reasonable to hypothesize that molecules that modulate Fyn activity, such as Csk, also regulate myelination.

To the test this hypothesis I generated and evaluated the myelin content of oligodendrocyte-specific Csk null mice ($Csk^{fl/fl}$:CNP-$cre^{+/-}$). Since Fyn activity is necessary for normal myelination, I originally formulated the hypothesis that **Csk in its role as a SFK**

inhibitor should inhibit myelination. Thus I hypothesized that **Csk loss-of-function was likely to promote Fyn activity and thus promote myelination.** I therefore expected Csk mutant mice to have normal or, more likely, increased or precocious myelin content. Interestingly, loss of Csk led to increased proliferation of oligodendrocyte progenitors, which translated into decreased oligodendrocyte differentiation during early myelination events both *in vitro* and *in vivo* (See Chapters 4 and 5). In the following chapter I evaluated the role of Csk during myelination using a Csk loss-of-function mouse in which Csk gene expression was compromised specifically in oligodendroglia.

Results

Oligodendrocyte-Specific Csk Null Mice are Hypomyelinated within the Corpus Callosum at P21

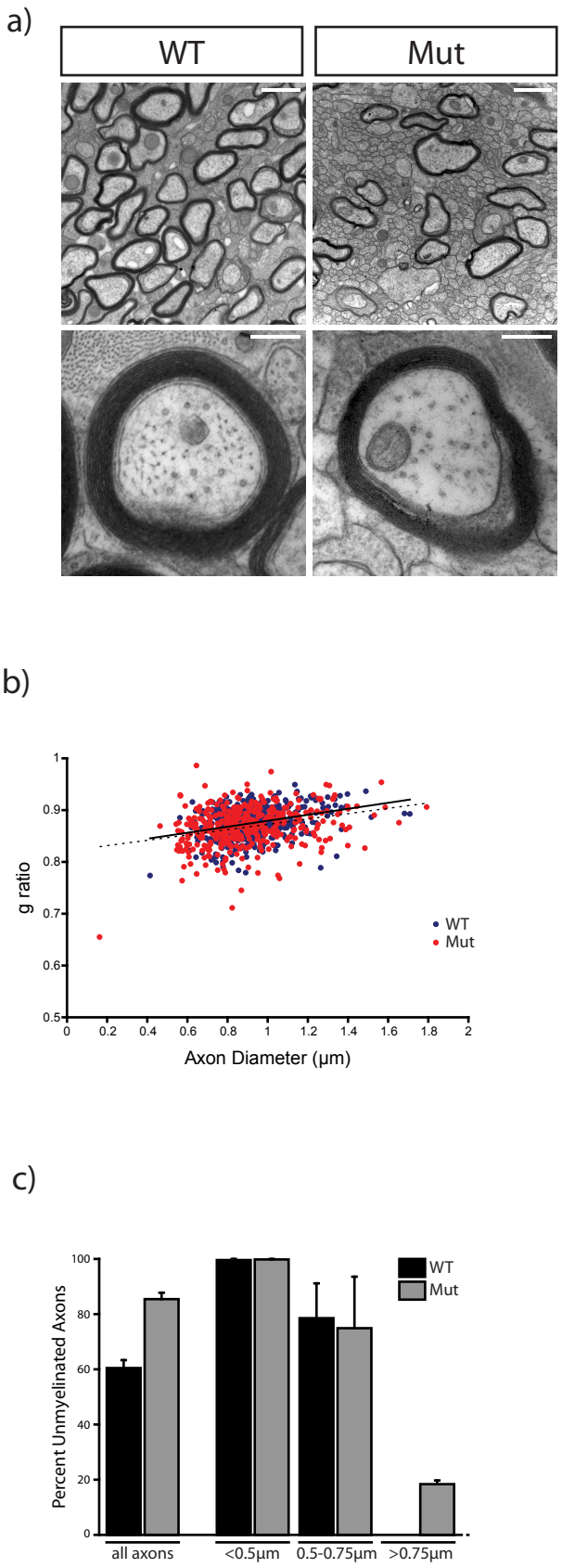
To evaluate the role of Csk in oligodendrocyte development and myelination *in vivo*, I specifically deleted Csk within oligodendroglia using mice expressing Cre recombinase downstream of the promoter for an oligodendrocyte-specific gene, CNP (CNP-cre$^{+/}$). In order to identify whether Csk plays a role in myelination I used transmission electron microscopy to evaluate the myelin content and ultrastructure within the corpus callosa of oligodendrocyte-specific Csk null (Mut) and WT littermates at P21. I found that the removal of Csk led to a substantial increase in the percentage of unmyelinated axons (Fig. 16A,C n=2; 141.4±2.3% of WT). Furthermore, I observed a significant number (18.4±1.3% for Mut, versus 0 for WT, n=2) of unmyelinated axons within the medium-to-large caliber axon group (diameter>0.75μm). It

should be noted that within the WT corpus callosum at P21 the vast majority of larger caliber axons are typically myelinated, and indeed I found none that were unmyelinated in WT animals. These data suggest that the presence of Csk may normally be necessary for the correct timing of myelination onset, likely as a direct result of Csk's ability to promote the OPC-to-oligodendrocyte transition (See Chapter 4). Indeed, I observed no apparent difference in myelin thickness or *g ratio* (diameter of axon alone/diameter of axon including the myelin) between Mut and WT corpus callosal axons (Fig. 16A,B n=2; mean *g ratio* was 0.876±0.0015 for WT and 0.867±0.0018 for Mut) suggesting that once myelination is initiated, Csk may be dispensible to the ability of the mature oligodendrocyte to undergo normal myelin wrapping.

The corpus callosum is a major white matter tract in the cortex that consists of parallel axons connecting the two cortical hemispheres. Because in the absence of inflammation this region is only sparsely populated by microglia and astrocytes, corpus callosal thickness can be used as another measure of myelination. I utilized MBP and neurofilament immunoreactivity to visualize and measure the thickness of the corpus callosum and found that at P21 oligodendrocyte-specific Csk null (Mut) mice had thinner corpus callosa compared to their wild type (WT) littermates (Fig. 5D,E). It should be noted that there was no apparent change in the number of corpus callosal axons visualized by neurofilament immunostaining. Taken together the above findings suggest that loss of oligodendrocyte Csk leads to hypomyelination of, and thinning of, the corpus callosum during the period of active myelination (P21). On the other hand, Csk function does not affect myelin wrapping at this stage as myelin thickness measured by *g* ratio was unaffected in the absence of Csk.

Figure 16. Loss of Csk leads to hypomyelination in the corpus callosum at postnatal day 21. **(a)** Transmission electron microscopy images of sagital sections through the corpus callosum of wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals at P21. Scale bars: 1μm in overview images, 200nm in high magnification images. **(b)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the corpus callosum of wild type (WT, blue circles) and oligodendrocyte-specific Csk null (Mut, red circles) at P21 (n=2) **(c)** Percentage ±sem of unmyelinated axons within the corpus callosum of P21 wild type (WT, black bars) and oligodendrocyte-specific Csk null (Mut, grey bars) animals.

Figure 16


a)
WT
Mut
b)
g ratio
Axon Diameter (μm)
WT
Mut
c)
Percent Unmyelinated Axons
WT
Mut
all axons
<0.5μm
0.5-0.75μm
>0.75μm

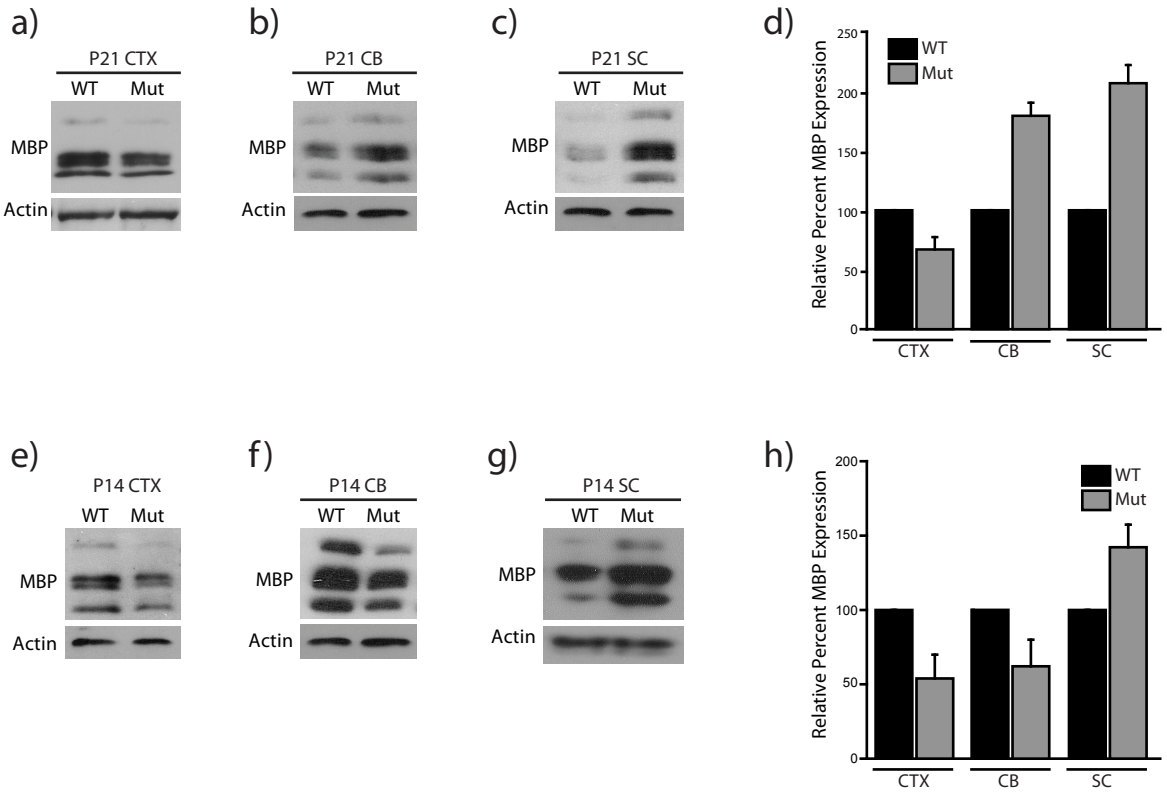
Loss of Csk Causes Regional Differences in MBP Protein Levels

Thus far I have observed that decreased mature oligodendrocyte numbers (See Chapter 4) giving rise to fewer myelinated axons. Interestingly, mice lacking either Fyn[151-152,156] or Fyn activity[147] are similarly hypomyelinated within the corpus callosum. How can both increased Fyn activity, as in Csk mutant mice, and loss of Fyn activity, as in $Fyn^{-/-}$ mice, lead to similar phenotypes? To help answer this question I characterized myelination in CNS regions that are myelinated earlier than the corpus callosum, such as the cerebellum and the spinal cord.

First, I utilized Western blot analysis to evaluate MBP protein levels within cortical, cerebellar and spinal cord lysates. I found that MBP protein levels were decreased in cortical lysates isolated from 21 day-old oligodendrocyte-specific Csk null (Mut) animals compared to their wild type (WT) littermates (Fig. 17A,D n=3; 67.0±10.3% of WT) thus confirming that loss of Csk results in decreased oligodendroglial maturation and/or myelination. On the other hand, loss of Csk led to an increase in MBP protein in the cerebellum (Fig. 17B,D n=3; 279.4±11.0% of WT) and spinal cord (Fig. 17C,D n=3; 206.8±15.2 of WT). This regional discrepancy in the Csk mutant phenotype could be due to regional differences in the role of Csk in oligodendrocyte development and myelination. Alternatively, the regional differences observed within Csk mutant animals may reflect the timing of myelination, since myelination in the CNS proceeds in a caudal to rostral manner, such that it is completed first in the spinal cord, then in the cerebellum and finally the cortex and corpus callosum. Thus, the Csk loss-of-function phenotype may have progressed to a later stage in early myelinating structures.

Figure 17. Genetic deletion of Csk in oligodendroglia leads to regional differences in myelin basic protein (MBP). (a-c) Western blot analysis of MBP and actin (loading control) in lysates isolated from **(a)** cortex (CTX), **(b)** cerebellum (**d)** spinal cord (SC) from (WT) or oligodendrocyte-specific Csk null (Mut) animals at postnatal day 21. **(d)** Densitometry to determine relative percent MBP levels in cortical (CTX), cerebellar (CB), and spinal cord (SC) lysates isolated from postnatal day 21-old control (ctrl, black bars) and oligodendrocyte-specific Csk null (Mut) animals Csk-specific (Csk, grey bars). Percent change ±sem in MBP levels (MBP/actin) relative to that in wild type animals (WT). **(e-g)** Western blot analysis of MBP and actin (loading control) in lysates isolated from **(e)** cortex (CTX), **(f)** cerebellum **(g)** spinal cord (SC) from (WT) or oligodendrocyte-specific Csk null (Mut) animals at postnatal day 14. **(d)** Densitometry to determine relative percent MBP levels in cortical (CTX), cerebellar (CB), and spinal cord (SC) lysates isolated from postnatal day 14-old control (ctrl, black bars) and oligodendrocyte-specific Csk null (Mut) animals Csk-specific (Csk, grey bars). Percent change ±sem in MBP levels (MBP/actin) relative to that in wild type animals (WT)

Figure 17


a)
P21 CTX
WT
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MBP
Actin
b)
P21 CB
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Mut
MBP
Actin
c)
P21 SC
WT
Mut
MBP
Actin
d)
Relative Percent MBP Expression
250
200
150
100
50
0
WT
Mut
CTX
CB
SC
e)
P14 CTX
WT
Mut
MBP
Actin
f)
P14 CB
WT
Mut
MBP
Actin
g)
P14 SC
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CB
SC

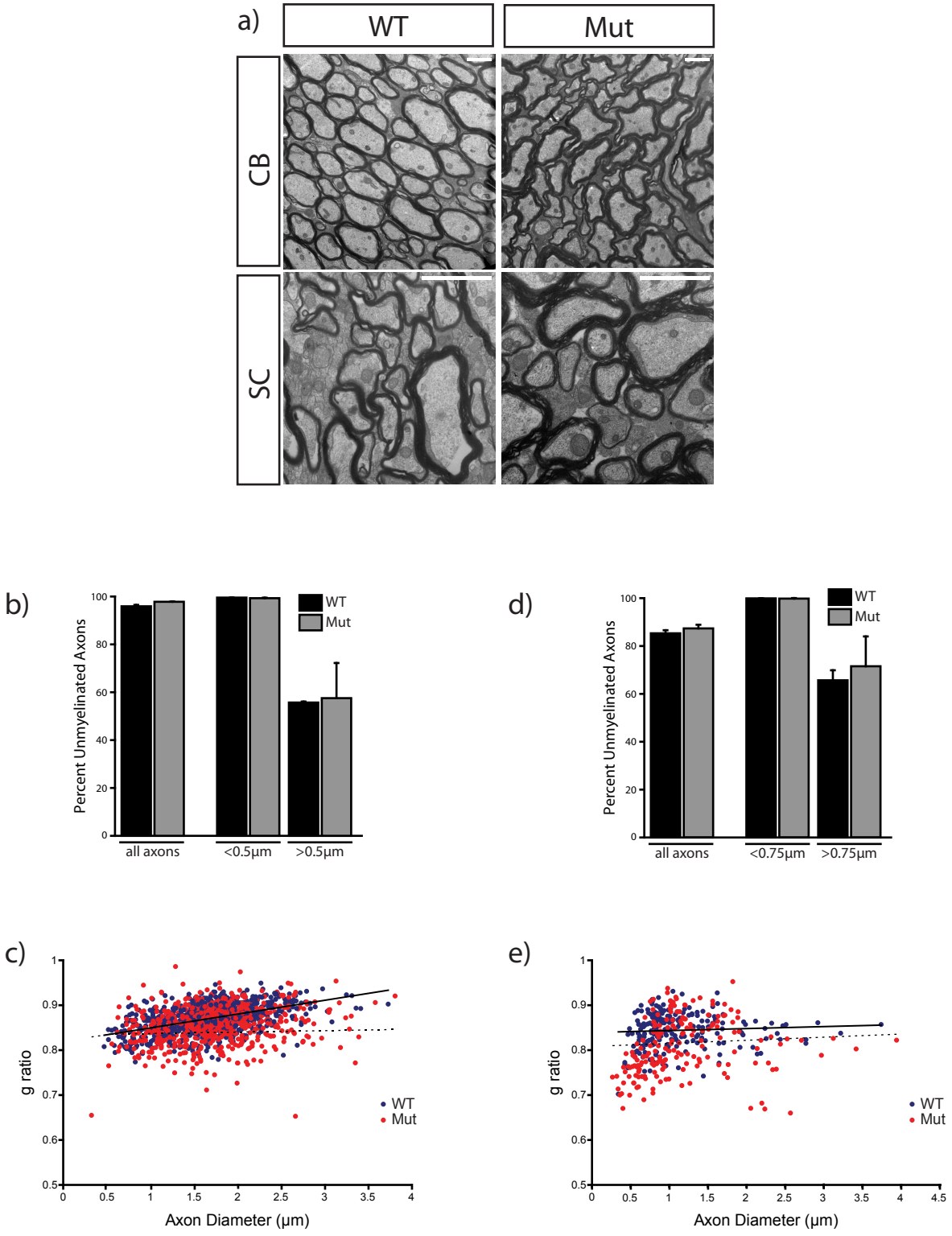
To test whether the regional variability in myelination observed within Csk mutant animals reflected the regional differences in the timing of myelination I evaluated MBP protein expression earlier, at P14. At this developmental time point the myelination process in wild type animals is almost complete in the spinal cord, actively-ongoing in the cerebellum, and just beginning in the cortex. Using Western blot analysis I found that MBP protein levels in Csk mutant spinal cord lysates were again increased (Fig. 16G,H n=3; 142.1±15.2 of WT) at P14. On the other hand, loss of Csk led to a decrease in MBP protein in cerebellar (Fig. 16F,H n=3; 62.08±17.9% of WT) and cortical lysates (Fig.E,H n=3; 53.9±16.9% of WT). These findings suggest that the Csk mutant phenotype cannot be explained by regional differences in the role of Csk in oligodendrocyte development and myelination. Instead, since MBP protein levels are lower within Csk null cerebella at P14 and higher at P21, it is therefore reasonable to suggest that, whether in the cerebellum or the cortex, low MBP protein levels can be attributed to delayed oligodendrocyte differentiation. However, since loss of Csk leads to OPC hyperproliferation, resulting in delayed OPC-to-oligodendrocyte transition and differentiation, I have not yet established a direct role of Csk in regulating myelination.

Loss of Csk Leads to Increased Myelination in the Cerebellum and Spinal Cord at P21

Although loss of Csk led to decreased myelin content within the corpus callosum at P21 (Fig. 16), it also resulted in increased MBP protein expression in two other CNS regions, the cerebellum and spinal cord (Fig. 17). To determine if increased MBP protein levels translated to increased myelination, I utilized transmission electron microscopy to examine cerebellar and

Figure 18. Loss of Csk leads to hypermyelination in the cerebellum and spinal cord at postnatal day 21. (a) Transmission electron microscopy images of coronal sections through the cerebellum (CB) and spinal cord (SC) of wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals at P21. Scale bars: 2μm. **(b)** Percentage ±sem of unmyelinated axons within the cerebellum of P21 wild type (WT, black bars) and oligodendrocyte-specific Csk null (Mut, grey bars) animals. **(c)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the cerebellum of wild type (WT, blue circles) and oligodendrocyte-specific Csk null (Mut, red circles) at P21 (n=2). **(d)** Percentage ±sem of unmyelinated axons within the spinal cord of P21 wild type (WT, black bars) and oligodendrocyte-specific Csk null (Mut, grey bars) animals. **(c)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the spinal cord of wild type (WT, black triangles) and oligodendrocyte-specific Csk null (Mut, grey circles) at P21 (n=2).

Figure 18


a)
WT
Mut
CB
SC
b)
Percent Unmyelinated Axons
WT
Mut
all axons
<0.5µm
>0.5µm
d)
Percent Unmyelinated Axons
WT
Mut
all axons
<0.75µm
>0.75µm
c)
g ratio
Axon Diameter (µm)
WT
Mut
e)
g ratio
Axon Diameter (µm)
WT
Mut

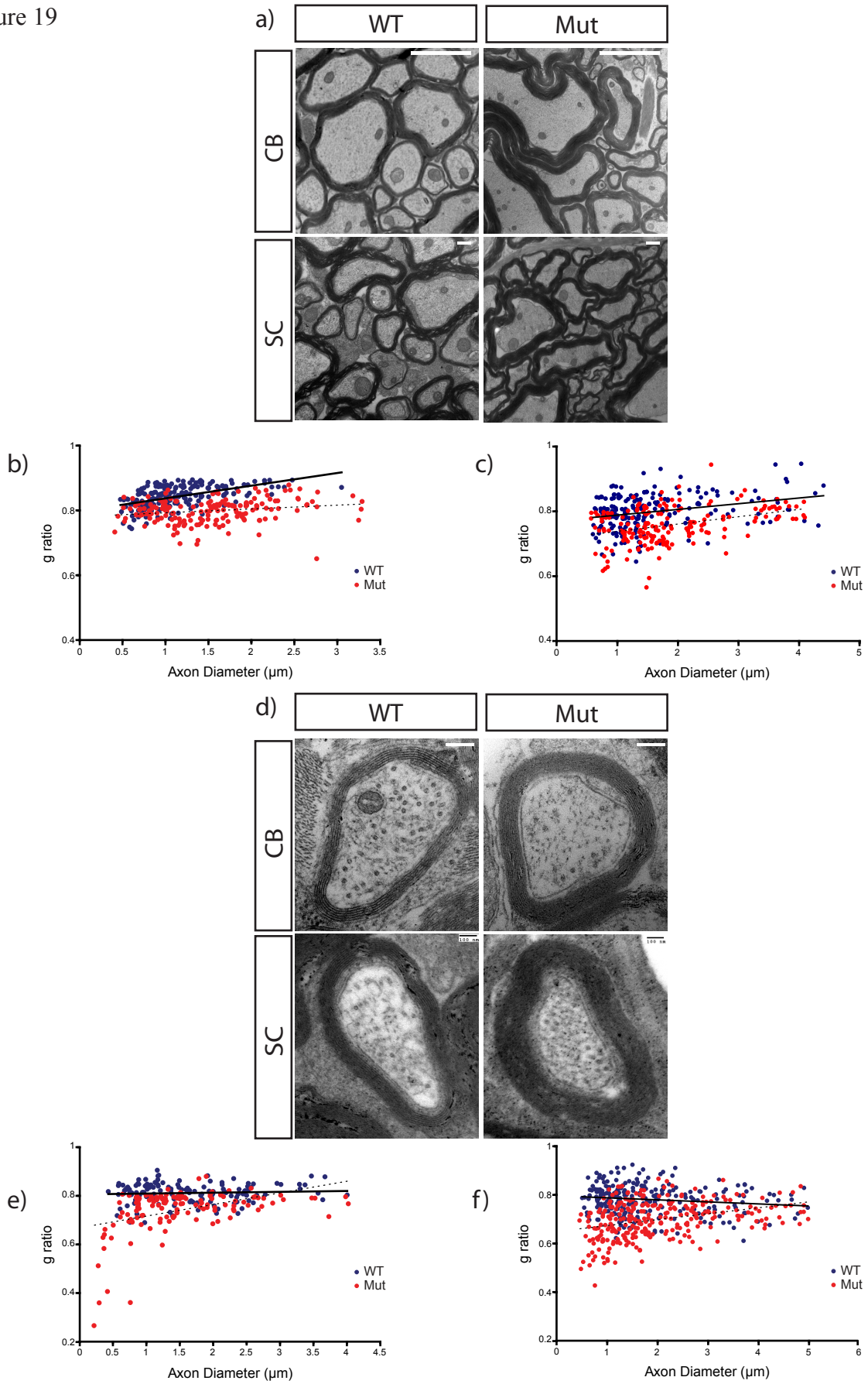
spinal cord myelin. Gross observations revealed that unlike the corpus callosum (Fig. 18), in both the cerebellum and the spinal cord, there was no significant difference between the two genotypes in the number of myelinated axons (Fig, 18A,B). On the other hand, quantification of the myelin content using *g* ratio revealed that oligodendrocyte-specific Csk null (Mut) mice had smaller *g* ratios (Fig. 18D,E) and thus thicker myelin. These data suggest that, once Csk null oligodendrocytes eventually differentiate, they wrap around axons more times and produce more myelin. Therefore, Csk may normally function to terminate myelin wrapping.

Adult Oligodendrocyte-Specific Csk Null Mice are Hypermyelinated in the CNS

The findings that oligodendrocyte-specific Csk null mice display a hypermyelination phenotype in the spinal cord and cerebellum at P21, (Fig. 18) suggest that the normal function of Csk may be to terminate myelin wrapping. To further test this hypothesis, I examined the myelin content in adult mice (3 month and 1 year old). Similar to our observations of P21 spinal cords and cerebella (Fig. 18), I found that Csk loss resulted in smaller *g* ratios and thicker myelin in these regions at both 3 months (Fig. 19A-C) and 1 year (Fig. 19D-F) of age. Interestingly, Csk mutant animals also exhibited a hypermyelination phenotype in the adult corpus callosum (Fig. 20A-C). In addition, immunohistochemistry with antibodies against MBP revealed that the hypermyelination phenotype of Csk null mice was accompanied by a progressive thickening of the corpus callosum (Fig19E-H) Taken together, these data suggest that, once Csk null oligodendrocytes fully differentiate and initiate myelination, they overcompensate and hypermyelinate their axonal targets. In addition, the differences in the myelin thickness between

Figure 19. Loss of Csk leads to hypermyelination in the adult cerebellum and spinal cord. **(a)** Transmission electron microscopy images of coronal sections through the cerebellum (CB) and spinal cord (SC) of wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals 3 months. Scale bars: 2μm. **(b)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the cerebellum of wild type (WT, blue circles) and oligodendrocyte-specific Csk null (Mut, red circles) at 3months (n=3). **(c)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the spinal cord of wild type (WT, blue circles) and oligodendrocyte-specific Csk null (Mut, red circles) at 3months (n=2). **(d)** Transmission electron microscopy images of coronal sections through the cerebellum (CB) and spinal cord (SC) of wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals 1 year. Scale bars: 100nm. **(e)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the cerebellum of wild type (WT, blue circles) and oligodendrocyte-specific Csk null (Mut, red circles) at 1year (n=2). **(f)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the spinal cord of wild type (WT, blue circles) and oligodendrocyte-specific Csk null (Mut, red circles) at 1year (n=2).

Figure 19


a)
WT
Mut
CB
SC
b)
g ratio
Axon Diameter (μm)
WT
Mut
c)
g ratio
Axon Diameter (μm)
WT
Mut
d)
WT
Mut
CB
SC
e)
g ratio
Axon Diameter (μm)
WT
Mut
f)
g ratio
Axon Diameter (μm)
WT
Mut

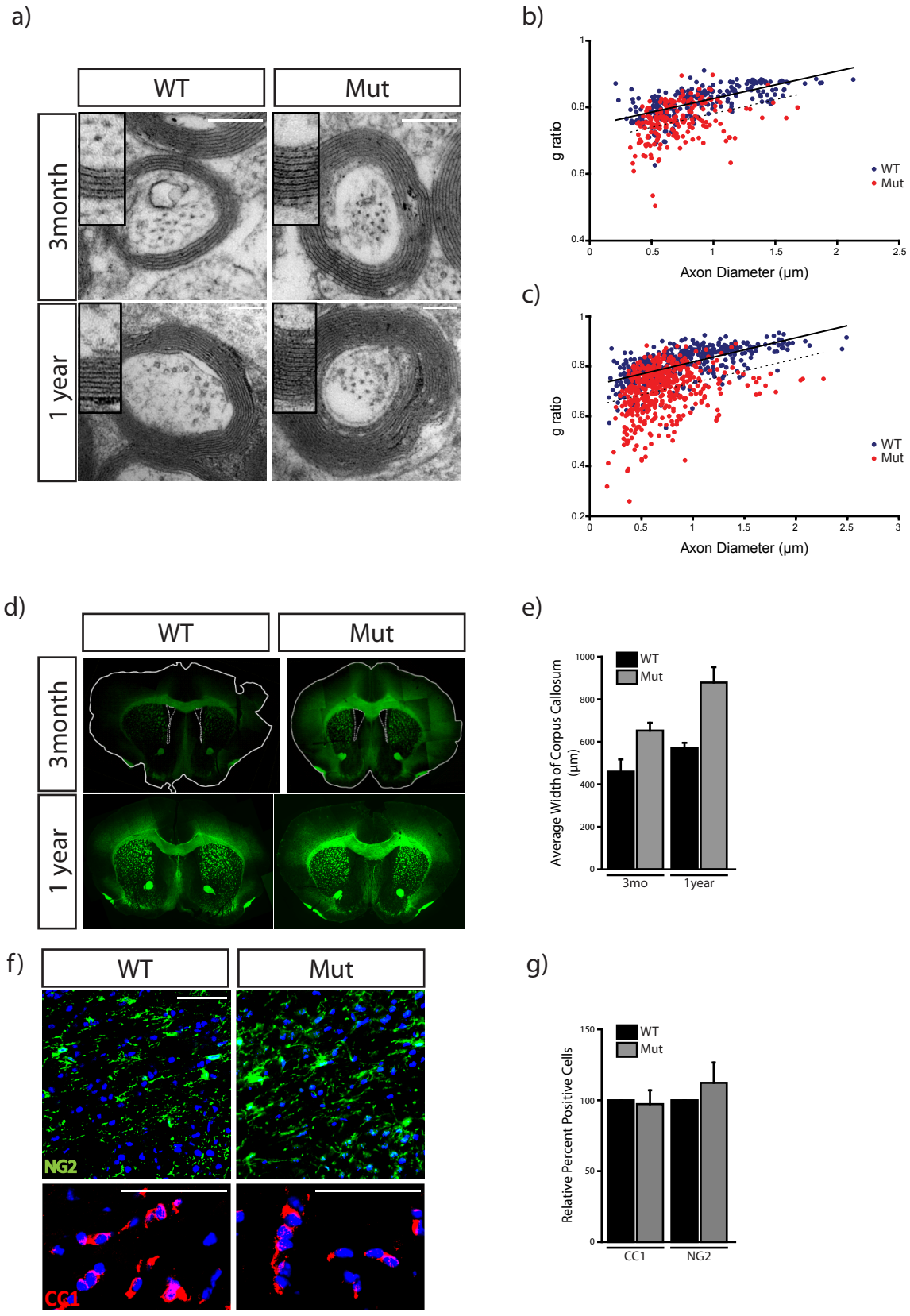
oligodendrocyte-specific Csk null and wild type mice increase with age in all regions suggesting that the myelin defect of Csk mutant mice is progressive.

Increased myelination could be due to an increase in the myelin produced by the same number of oligodendrocytes, or, it could also be due to increased numbers of mature oligodendrocytes. To investigate these two possibilities in relationship to the hypermyelination phenotype of oligodendrocyte-specific Csk null animals, I identified CC1-positive, mature oligodendrocytes by immunohistochemistry. I found that the numbers of CC1-positive oligodendrocytes within the Csk mutant corpus callosum were similar to those of their wild type littermates (Fig. 20A,B n=3; 95.3±13.2% of WT at 3months). In addition to more myelin, Csk null oligodendrocytes produce more MBP, as the overall MBP immunoreactivity is increased in the adult CNS (Fig. 20D). Thus, in the absence of Csk, individual oligodendrocytes produce more myelin, suggesting that, in addition to its roles in oligodendroglial development, Csk has a role specific to the myelination process in that it promotes the timely termination of myelin wrapping. Csk loss-of-function therefore leads to exuberant myelin wrapping and a hypermyelination phenotype.

Csk has distinct and opposing functions during oligodendrocyte development and myelination, such that early in development Csk promotes OPC cell cycle exit, while at the tail end of development, Csk promotes the termination of myelin wrapping. As discussed in Chapter 4, Csk null oligodendrocytes hyperproliferate and populate the brains of Csk mutant animals in larger numbers. On the other hand, adult Csk mutant animals contain the same number of mature

Figure 20. Loss of Csk leads to hypermyelination in the adult corpus callossum. (a) Transmission electron microscopy images of sagittal sections through the corpus callosum of wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals at 3 months and 1 year. Scale bars: 100nm. **(b)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the corpus callossum of wild type (WT, blue circles) and oligodendrocyte-specific Csk null (Mut, red circles) at 3months (n=3). **(c)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the corpus callosum of wild type (WT, blue circles) and oligodendrocyte-specific Csk null (Mut, red circles) at 1year (n=2). **(d)** Indirect immunofluorescence to detect MBP (green) in the cortex of wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals at 3 months and 1 year. **(e)** Average width of the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) at 3 months and 1 year. **(f)** Indirect immunofluorescence to detect Ki67+ (red) and NG2+ (green) or CC1+ (red) cells, and nuclei (blue) in the corpus callosum of 3 month-old wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals. Scale bars: 50μm. **(g)** Percentage ±sem of NG2-positive OPCs and CC-positive mature oligodendrocytes in corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) at 3 months.

Figure 20


a)
WT
Mut
3month
1 year
b)
g ratio
Axon Diameter (μm)
WT
Mut
c)
g ratio
Axon Diameter (μm)
WT
Mut
d)
WT
Mut
3month
1 year
e)
Average Width of Corpus Callosum (μm)
WT
Mut
3mo
1year
f)
WT
Mut
NG2
CC1
g)
Relative Percent Positive Cells
WT
Mut
CC1
NG2

oligodendrocyte as their wild type counterparts. To test whether increased numbers of OPCs persist in oligodendrocyte-specific Csk null white matter tracts, I identified NG2-positive progenitors by immunocytochemistry, and surprisingly found no significant differences in the numbers of oligodendroglial progenitors within the white matter tracts of Csk mutant and wild type mice (Fig. 20F n=3; 115.7±23.2% of WT at 3months) suggesting that as Csk mutant animals age the numbers of OPCs normalize to wild type levels. In addition, using immunocytochemistry against the proliferation marker PCNA, I identified the number of proliferating, PCNA-positive cells within the NG2-positive, progenitor cell population (Fig. 20F n=3; 17.3±4.7% in WT versus 25.8±8% in Mut). Therefore, Csk loss-of-function did not alter the degree of OPC proliferation in adult animals, as I found no significant differences in the percentage of proliferating OPCs between oligodendrocyte-specific Csk null animals and their wild type littermates (Fig. 20F). Taken together, these findings suggest that Csk may not only be dispensable for OPC proliferation in the adult, but also suggest that the hypermyelination phenotype observed in oligodendrocyte-specific Csk null mice is due to exuberant myelination that is independent of oligodendroglial production.

Discussion

Factors that control the degree of myelin wrapping may be prove to be useful pharmacological targets to enhance myelin wrapping during myelin repair. In this chapter I have described experiments that test the hypothesis that the SFK inhibitor Csk inhibits myelination, and thus, as a consequence, that Csk loss-of-function enhances myelin wrapping. By examining the myelin content within the corpus callosum of oligodendrocyte-specific Csk null and wild

type mice at a period of active myelination, P21, I found that Csk loss led to a decrease in the number of myelinated, large caliber axons, suggestive of hypomyelination. However, loss of Csk did not affect the number of myelinated axons in the cerebellum and spinal cord, two CNS regions that are myelinated earlier than the corpus callosum. Western blot analysis furthermore revealed that MBP protein levels were reduced in Csk mutant cortical lysates, but increased within cerebellar and spinal cord Csk mutant lysates at P21. At P14, when myelination is just beginning in the cortex, actively-ongoing in the cerebellum, and almost complete in the spinal cord, I found that MBP protein levels were decreased in the cortex and cerebellum of Csk mutant animals, but increased in the spinal cord. In view of these findings I concluded that during the early stages of myelination, loss of Csk leads to a delay in myelination, which is a direct result of Csk loss-of-function-mediated delay in progenitor-to-oligodendrocyte transition, and therefore, a delay oligodendrocyte differentiation (See Chapters 4 and 5). Furthermore, I concluded that, after Csk null oligodendrocytes catch up developmentally to their wild type counterparts, as in the P21 cerebellum and spinal cord, they myelinate an equivalent number of axons. Further examination of the myelin ultrastructure using transmission electron microscopy, and subsequent quantification of myelin thickness by *g* ratio, revealed thicker myelin in the cerebellum and spinal cord of 21 day-old oligodendrocyte-specific Csk null mice. Interestingly, Csk loss did not affect the myelin thickness within the P21 corpus callosum, presumably because myelination is delayed at this stage of development. With age, however, Csk mutant animals exhibited progressively thicker myelin than their wild type littermates in all of the CNS regions examined including the corpus callosum (3 month and 1year old mice). In addition, I observed no difference in mature oligodendrocyte numbers between adult oligodendrocyte-specific Csk mutant mice and their wild type littermates. These findings suggested that Csk loss-of-function

promoted myelin wrapping directly, and that the same number of Csk null mature oligodendrocytes were responsible for the hypermyelination phenotype attributed to loss of Csk. I, therefore, identified Csk as a novel regulator of myelination with a distinct function in promoting the termination of myelin wrapping, such that, when Csk is removed, myelin wrapping continues inappropriately, leading to enlarged myelin sheaths.

Oligodendrocyte-Specific Csk Null Corpus Callosa are Hypomyelinated at P21

A substantial body of work has implicated Fyn activation as a necessary step during CNS myelination. I was surprised, therefore, that Csk depletion, which *elevated* Fyn activity (See Chapter 3), led to decreased myelination of corpus callosal axons at P21 (Fig. 16). However, as described and discussed in Chapters 4 and 5, our finding that Csk was necessary for the timely transition of OPCs to newly-formed oligodendrocytes revealed a novel role for SFK *suppression* in OPC cycle exit, one that was a critical prerequisite to OPC differentiation. Csk depletion led, therefore, to stalled, or delayed, OPC differentiation in Csk-deficient OPCs compared to wild type OPCs. Specifically, within the P21 corpus callosum, Csk deficient OPCs displayed a modest increase in proliferation and overall numbers, while Csk null oligodendrocytes populated this region in reduced numbers (See Chapter 5). It is therefore reasonable to suggest that a reduced number of mature oligodendrocytes can only myelinate a more limited number of axons, thus explaining the reduced number of myelinated corpus callosal axons of 21 day-old oligodendrocyte-specific Csk null animals.

Furthermore, Western blot analysis revealed that MBP protein levels were reduced within cortical lysates isolated from Csk mutant animals at P14 and P21 (Fig. 17). Although MBP is expressed in mature oligodendrocytes before they initiate myelination, this protein is also a structural component of myelin that is expressed by myelinating oligodendrocytes. MBP protein levels can, therefore, can be used as a myelination readout that is complimentary to myelin ultrastrcuture. Thus reduced MBP protein levels in Csk mutant lysates can reflect reduced myelin content within the cortex, that is accompanied by decreased differentiation of Csk deficient oligodendroglia.

Loss of Csk Causes Regional Differences in MBP Protein Levels at P14 and P21

Although loss of Csk leads to delayed oligodendroglial differentiation, Csk null oligodendrocytes eventually do differentiate and catch up to their wild type counterparts (See Fig. 12, Chapter 5). Suggesting that although initially delayed, differentiation is eventually completed in Csk null oligodendrocytes. I therefore hypothesized that Csk null oligodendrocytes would similarly catch up to their wild type counterparts in regions that are myelinated earlier than the cortex, thus allowing us to investigate the role of Csk in myelination. To test this hypothesis I examined MBP protein expression using Western blot analysis of lysates obtained from wild type and Csk mutant cerebella and spinal cords at P14 and P21 (Fig. 17).

CNS myelination proceeds in a caudal to rostral manner such that the spinal cord is myelinated first, followed by the cerebellum and finally the cortex. In 14 day-old wild type mice, myelination is just beginning in the cortex, actively-ongoing in the cerebellum and almost complete in spinal cord. Western blot analysis revealed that loss of Csk led to decreased levels of

MBP protein in the cortex and cerebellum, while in spinal cord, MBP protein levels were *increased* in Csk mutant animals (Fig. 17). In 21 day-old wild type mice, myelination is almost completed in the spinal cord and the cerebellum, but actively ongoing in the cortex. Interestingly, loss of Csk led to an *increase* in MBP protein levels in cerebellar and spinal cord lysates, while in the Csk mutant cortical lysates, MBP protein levels remained decreased (Fig. 17). Since MBP protein levels within cerebellar lysates isolated from oligodendrocyte-specific Csk null mice were decreased at P14, but *increased* only 7 days later, at P21, I concluded that Csk in oligodendrocytes does not function in a regional-specific manner, but instead the regional differences of MBP protein were as a direct result of regionally-specific differences in the timing of oligodendrocyte differentiation and myelination. Taken together these findings suggest that Csk loss-of-function has distinct and opposing effects during oligodendrocyte development such that, at the onset of oligodendrogenesis it leads to progenitor expansion causing a delay in differentiation and MBP expression (See Chapter 5), while later it may promote oligodendrocyte differentiation and even myelination. This later role of Csk loss-of-function is also consistent with the wealth of data suggesting that Fyn promotes oligodendroglial differentiation and myelination[158-159,161,163]. In addition, Fyn promotes *MBP* gene transcription[151] and MBP mRNA translation[154-155]. Thus loss of Csk, which promotes Fyn activity, may lead to increased MBP transcription and translation, thereby explaining the increased levels of MBP protein observed in cerebellar (P21) and spinal cord (P14 and P21) lysates isolated from oligodendrocyte-specific Csk null mice. Furthermore, differences in MBP protein expression reflect regional differences in the progression of differentiation and myelination, and are not likely due to distinct regionally-specific roles for Csk function.

Loss of Csk Leads to Progressive Hypermyelination in the CNS

The findings described in Chapters 4 and 5 of this document, together with the findings published by other groups (See Chapter 2), suggest that Fyn has distinct and opposing roles during oligodendrocyte development such that, at the onset of oligodendrogenesis, Fyn promotes progenitor cell division and hence progenitor expansion, while later, Fyn promotes oligodendroglial differentiation and myelination onset. Because of the duality of Fyn/Csk function, and the initial delay in oligodendroglial differentiation caused by Csk loss-of-function and premature Fyn activation, I needed to examine the role of Csk in myelination within regions, and at developmental time points, where oligodendrocyte differentiation in Csk mutant animals has normalized to that observed in wild type animals.

At P21, MBP protein levels within Csk mutant cerebellum and spinal cord are higher than those observed in the respective regions in wild type animals (Fig. 17), suggesting that Csk mutant oligodendrocytes have overcome their initial delay in differentiation and have either caught up to their wild type counterparts, or exceeded them. I, therefore, proposed that P21 cerebella and spinal cords could be used to evaluate the role of Csk in myelination. Upon examination of the myelin ultrastructure using transmission electron microscopy I found that loss of Csk resulted in thicker myelin and smaller *g* ratios in both the cerebellum and the spinal cord at P21 (Fig. 18). These observations suggested a novel role for Csk function in myelination. I, therefore, proposed the following roles for Csk in regulating myelination; 1) Csk inhibits oligodendrocyte differentiation at developmental time points following OPC-to-oligodendrocyte transition, such as the expression of myelin components and oligodendroglial maturation; 2) Csk

inhibits myelin wrapping by inhibiting oligodendrocyte process outgrowth and branching; 3) Csk acts as a stop signal to promote the timely termination of myelin wrapping.

How might altered SFK activity lead to altered myelin wrapping? Numerous lines of evidence suggest that, at least *in vitro*, Fyn promotes oligodendrocyte process outgrowth, which may reflect the ability of oligodendrocytes *in vivo* to extend and wrap processes around axons. Resh and colleagues utilized overexpression of a kinase dead form of Fyn in primary rat oligodendrocytes to suggest that Fyn activity is necessarily for p190 Rho GAP-dependent inhibition of Rho, which in turn facilitates and promotes process outgrowth[159]. In addition, Fyn activity was found to mediate fibronectin-dependent activation of Cdc42 and Rac, which in turn promote oligodendrocyte process outgrowth[158]. Fyn activity is also necessary to promote FAK-dependent activation of Cdc42 and Rac, as well as process outgrowth, downstream of laminin2 in the CG4 oligodendrocyte cell line[154]. Furthermore, interaction of the microtubule stabilizing protein Tau with the SH3 domain of Fyn was also found necessary to promote microtubule assembly and stimulate process outgrowth in oligodendrocytes *in vitro*[163]. Finally, Fyn was shown to be a downstream effector of Netrin-DCC signaling necessary for FAK-mediated inhibition of RhoA, thereby promoting cytoskeletal remodeling, process outgrowth and branching[161]. Taken together, these studies suggest that Fyn integrates extracellular cues to regulate cytoskeletal dynamics and promote process outgrowth. It is therefore reasonable to suggest that in its role as a Fyn inhibitor, Csk can inhibit process outgrowth. Loss of Csk can, therefore, lead to increased process outgrowth that can in turn translate to increased myelin wrapping *in vivo*. Interestingly, although I did not test and quantify process outgrowth directly, I observed that, surpisingly, *both* Csk deficient (siRNA-treated) and Csk overexpressing primary

rat oligodendrocytes contained shorter processes (See Chapter 5, Fig 11). If these observations indeed prove to be correct, they suggest that for appropriate process outgrowth to occur, Fyn may need to cycle rapidly between its active and inactive states. Alternatively, shorter processes could simply be attributed to dysregulation of the OPC-to-oligodendrocyte transition, which occurs in both Csk deficient and Csk overexpressing cells. Nonetheless, it will be critical to establish whether Csk can directly regulate process outgrowth once tools and techniques are utilized that allow for Csk levels to be modulated after the transition of OPCs to oligodendrocytes.

Fyn is not only necessary for process outgrowth but for expression of MBP as well Fyn promotes *MBP* gene transcription[151] as well as MBP mRNA stability by phosphorylating the mRNA binding protein QKI (Ref) and thus preventing premature MBP mRNA degradation. In addition, Fyn phosphorylates the mRNA stabilizing protein hnRNP A2, which in turn leads disruption of the interaction between hnRNP A2 and MBP mRNA and facilitates MBP mRNA translation within oligodendrocyte processes *in vitro*[155]. Taken together, these findings suggest that Csk loss-of-function may similarly lead to increased MBP expression, thus explaining the increase MBP protein levels within the cerebellum (P21) and spinal cord (P14 and P21 Fig. 17) of oligodendrocyte-specific Csk null mice. In addition, to being a component of myelin, MBP promotes myelin stability by promoting the interaction between adjacent myelin wraps. It is therefore reasonable to propose that the increased levels of MBP protein observed in Csk mutant cerebella and spinal cords serve to increase myelin stability and therefore allow for the presence of thicker myelin within these regions.

Oligodendroglial Cell Numbers Normalize in Adult Csk Mutant Animals

Oligodendrocyte-specific Csk null mice exhibit a biphasic phenotype in terms of oligodendroglial development, such that early in development they contain increased numbers of proliferating OPCs and *reduced* numbers of mature, CC1-positive oligodendrocytes leading to delayed myelination onset and hypomyelination of the corpus callosum at P21 (See Chapter 4, Chapter 5), while late in development, loss of Csk gives rise to CNS hypermyelination (Fig. 18-20). Interestingly, the increase in myelin content of Csk mutant mice did not correspond to an increase in mature oligodendrocyte numbers, instead the numbers of CC1-positive mature oligodendrocytes present in Csk mutant mice were comparable to those present in their wild type littermates (Fig. 20). The presence of equivalent numbers of CC1-positive mature oligodendrocytes in both Csk mutant animals and their wild type littermates, suggested that in the absence of Csk the same number of mature oligodendrocytes were able to make more myelin. In addition, since in young animals loss of oligodendrocyte Csk led to a delay in differentiation and *decreased* numbers of mature oligodendrocytes, while in adult animals the numbers of mature oligodendrocytes was equivalent regardless of genotype, then Csk null oligodendrocytes must eventually catch up to their wild type counterparts in their differentiation program.

Interestingly, while I found increased numbers of proliferating OPCs within the Csk mutant corpus callosum at P21 (See Chapter 4), I found no difference in proliferation or OPC numbers between adult oligodendrocyte-specific Csk null and wild type corpus callosa (Fig. 20). These observations suggested that while Csk loss-of-function is necessary for progenitor proliferation early in development, it is dispensible for OPC proliferation late in development.

As discussed in Chapter 1 proliferation is controlled by a combination of extracellular cues such as the mitogen PDGF, and intracellular components of a cell-intrinsic "timer". PDGF is critical for OPC proliferation *in vitro*[51] and *in vivo*[56], such that in the presence of saturating levels of PDGF OPCs hyperproliferate. However, with age, the availability of PDGF declines and so does OPC proliferation, suggesting that PDGF is a limiting factor for proliferation[56]. Since PDGF is an extracellular factor produced by astrocytes[53] and neuronal cell bodies[53], which remain wild type in oligodendrocyte-specific Csk null mice, it is reasonable to suggest that PDGF availability declines in Csk mutant animals at a rate similar to that observed in wild type animals. Thus proliferation of Csk null OPCs may decline and proceed at similar levels to that of wild type OPCs in the adult, due to limited supply of available mitogens such as PDGF. It is therefore likely that early in development Csk null OPCs encounter factors in the extracellular milieu that are permissive to proliferation, while in the adult these factors are absent thus making Csk loss-of-function dispensible for proliferation.

Chapter 8:

Loss of Csk Promotes Remyelination in the Corpus Callosum

Introduction

Demyelinating diseases occur when efficient repair of damaged myelin fails. Normal myelin can be damaged as a result of trauma, immune insult, genetic mutations in molecules that regulate oligodendrocyte stability, or mature oligodendrocyte survival. The hallmark demyelinating disease is multiple sclerosis (MS). In MS, autoimmune insult leads to demyelination and mature oligodendrocyte death. In Relapsing-Remitting MS, episodes of demyelination are followed by remyelination, which although not efficient, restores normal neurological function. On the other hand, in Primary Progressive MS, remyelination fails leading to progressive loss of myelin and to neurological deficits. With time, however, as remyelination becomes less efficient, the Relapsing-Remitting form of the disease can escalate to progressive disease. It is believed that this transition reflects a decreasing capacity for myelin repair, either occurring through aging, or through the progression of the disease itself (or a combination thereof). In addition, demyelination is also a hallmark of certain leukodystrophies such as Palizaeus-Merzbacher disease (PMD), which is caused by mutations in the *Plp* gene. In PMD, oligodendrocyte differentiation can occur in a somewhat normal fashion and some myelination does occur. However, in certain forms of the disease, loss of function mutations and duplications

in the *Plp* gene can lead to premature oligodendrocyte death and early-onset demyelination (Reviewed in [16]). Interestingly, loss of myelin or altered myelination, also occur in neurological disorders such as Alzheimer's disease and schizophrenia[18]. However, it remains unknown whether abnormal myelin observed in these, and other diseases that are typically studied in the context of neuronal dysfunction, is an underlying cause of pathology, versus a secondary effect downstream of neuronal changes. Overall, however, it remains clear that loss of myelin, or inappropriate myelination, can contribute either directly or indirectly to a vast array of neurological diseases. Thus in order to develop novel therapeutic strategies it is imperative to identify molecules that either promote efficient remyelination, or limit the extent of demyelination.

I have already identified Csk as a novel regulator of oligodendrocyte biology and myelination. I have found that while early in development Csk promotes oligodendrocyte progenitor cell cycle exit (See Chapter 4), later in development Csk promotes the timely termination of myelin wrapping (See Chapter 7). Although oligodendrocyte-specific Csk null mice displayed a progressive hypermyelination phenotype, the numbers of mature oligodendrocytes were unchanged, suggesting that Csk loss promotes myelin wrapping. Since wrapping is necessary for remyelination, I hypothesized that the loss of **Csk would similarly enhance remyelination.** In addition, to remyelinate efficiently, OPCs need to be recruited to the demyelinated lesion and need to proliferate in order to generate sufficient numbers of mature oligodendrocytes. Since loss of Csk promoted OPC proliferation in young animals (See Chapter 4), I further hypothesized that in **response to demyelination Csk null OPCs would also hyperproliferate and thus more quickly generate the OPC numbers necessary for efficient**

remyelination. This latter hypothesis, however, has an important caveat in that increased progenitor proliferation should not significantly slow OPC differentiation. Since this was *not* the case early in development, however, one possibility is that oligodendrocyte-specific Csk null animals might have a phenotype similar to that observed in MS lesions in that sufficient numbers of progenitor and newly-formed oligodendrocytes are present but they fail to mature and remyelinate the lesion[24].

Remyelination can be tested experimentally using a variety of animal models. Experimental allergic encephalomyelitis (EAE) is an autoimmune disease that can be induced when animals are immunized with myelin or myelin components such as MOG peptide. Because immunization leads to an autoimmune response that is similar to that observed in MS, EAE is considered the best animal model to mimic the concurrent demyelination and remyelination that is observed in MS patients. To test demyelination and remyelination independent of major levels of inflammation, however, animal models utilizing toxins that promote oligodendroglial death and demyelination have been developed. These include; 1) stereotactic injection of ethidium bromide into the caudal cerebellar peduncle, a large white matter tract in the cerebellum, 2) stereotactic injection of lysolecithin into the corpus callosum or the spinal cord, and 3) feeding animals with the copper chelator cuprizone, which induces demyelination primarily in the corpus callosum. Treatment with ethidium bromide, lysolecithin or cuprizone induces toxic oligodendrocyte cell death leading to acute and focal demyelination generally in the absence of, or with little, inflammation. After toxic demyelination is achieved, remyelination occurs in a well characterized and predictable manner, thus allowing researchers to identify mechanisms that specifically regulate demyelination and remyelination (Reviewed in [16]). In this chapter, I

evaluated the role of Csk in demyelination and remyelination using the cuprizone model of demyelination.

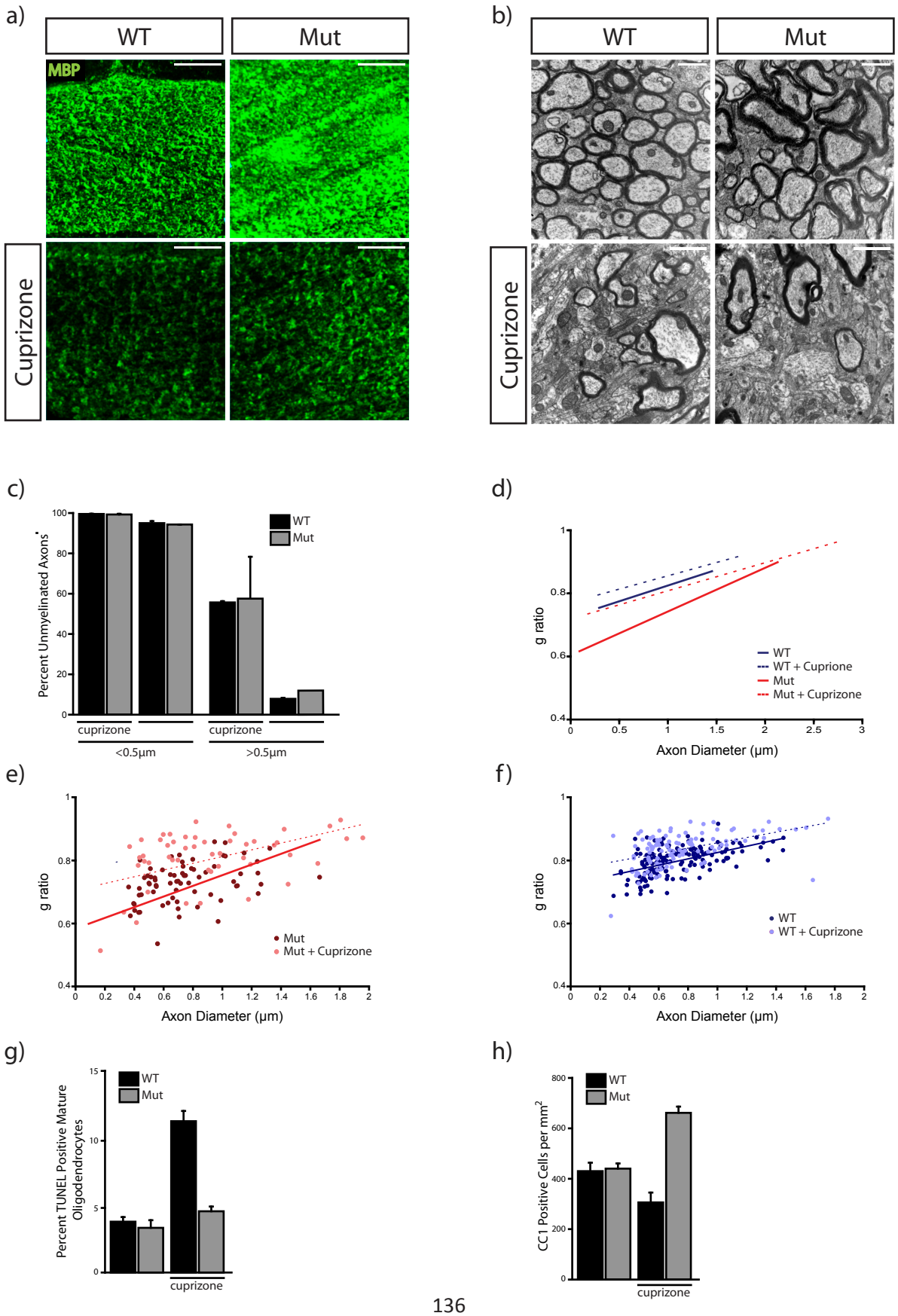
Results

Oligodendrocyte-Specific Csk Null Mice can be Demyelinated by Cuprizone

Before I could test the hypothesis that loss of Csk promotes CNS remyelination, I needed to perform the proof of principle experiment and ascertain that I could achieve efficient demyelination. This was especially critical for oligodendrocyte-specific Csk null animals, which are hypermyelinated in the adult. Although 4 weeks of cuprizone treatment can be sufficient to achieve efficient demyelination in wild type mice[213], I chose to treat animals for 6 weeks to ensure that the excess myelin present in Csk mutant corpus callosa can be removed. To measure the degree of demyelination, I compared the MBP expression in the corpus callosum of cuprizone-treated, Csk mutant and wild type mice to that of untreated age-matched animals of both genotypes. Although cuprizone treatment caused a decrease in MBP immunoreactivity in animals of both genotypes, this decrease was less pronounced in oligodendrocyte-specific Csk knockouts (Fig. 21A). This finding could reflect that Csk mutant corpus callosal axons were either demyelinated to a lesser extent than their wild type counterparts, or that they were demyelinated equally well, but the excess MBP immunoreactivity was due to residual myelin debris.

Figure 21. Cuprizone-induced demyelination in adult oligodendrocyte-specific Csk null and wild type mice. (a) Indirect immunofluorescence to detect MBP (green) in the corpus callosum of wild type (WT) or oligodendrocyte-specific Csk null (Mut). **(b)** Transmission electron microscopy images of sagittal sections through the corpus callosum of wild type (WT) or oligodendrocyte-specific Csk null (Mut). Scale bars: 2μm. **(c)** Percentage ±sem of unmyelinated axons within the corpus callosum of wild type (WT, black bars) and oligodendrocyte-specific Csk null (Mut, grey bars) animals (n=2). **(d)** Best fit lines of quantitation of myelin sheath thickness (g ratio) as a function of axon diameter in the corpus callossum of wild type (WT, blue) and oligodendrocyte-specific Csk null (Mut, red) (n=2). **(e)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the corpus callossum of cuprizone-treated (light red circles) or untreated (dark red circles) oligodendrocyte-specific Csk null (Mut) milce (n=2). **(f)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the corpus callossum of cuprizone-treated (light blue circles) or untreated (dark blue circles) wild type mice (n=2). **(g)** Percentage ±sem of TUNEL-positive mature oligodendrocytes (CC+) in corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars). **(h)** CC1-positive mature oligodendrocytes per mm^2 in the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars).

Figure 21


a)
WT
Mut
MBP
Cuprizone
b)
WT
Mut
Cuprizone
c)
Percent Unmyelinated Axons
WT
Mut
cuprizone
cuprizone
<0.5µm
>0.5µm
d)
g ratio
WT
WT + Cuprione
Mut
Mut + Cuprizone
Axon Diameter (µm)
e)
g ratio
Mut
Mut + Cuprizone
Axon Diameter (µm)
f)
g ratio
WT
WT + Cuprizone
Axon Diameter (µm)
g)
Percent TUNEL Positive Mature Oligodendrocytes
WT
Mut
cuprizone
h)
CC1 Positive Cells per mm2
WT
Mut
cuprizone

To conclusively ascertain the degree of cuprizone-mediated demyelination, I next evaluated myelin ultrastructure using transmission electron microscopy (Fig. 21B). Gross examination of cuprizone-treated corpus callosa revealed the presence of a large number of unmyelinated axons in both genotypes, revealing that some demyelination was achieved (Fig. 21B). Furthermore, the percentage of unmyelinated axons were comparable in both genotypes, suggesting that 6 weeks of cuprizone treatment was sufficient to remove even the excess myelin found in Csk mutant mice (Fig. 21C). And, although most axons were demyelinated after cuprizone treatment, some were not. One possibility is that these myelinated axons reflect ones that were "spared" the demyelination event; another possibility is that these myelinated axons are ones that have already remyelinated. These two possibilities can be partially distinguished by evaluating the *g* ratio, where the presence the thicker "mutant" myelin sheaths would suggest that axons are being protected from demyelination. On the other hand, thinner myelin (compared to age-matched animals that were not treated with cuprizone) would indicate that axons are likely to be those that have already undergone remyelination. Compared to untreated age-matched animals, cuprizone treatment led to thinner myelin and higher *g* ratios regardless of genotype (Fig. 21E,F). Interestingly, however, *g* ratios of cuprizone-treated Csk mutant corpus callossal axons were comparable to those of *untreated* wild type controls (Fig. 21D) suggesting that the rate of remyelination may be comparable between the two genotypes.

Loss of Csk Promotes Mature Oligodendrocyte Survival in the Presence of Cuprizone

Cuprizone is a copper chelator that promotes demyelination by causing apoptosis of mature oligodendrocytes. Although cuprizone did induce demyelination of corpus callosal axons in Csk mutant animals, it was unclear if this process was complete, as not only were some myelinated axons present in Csk mutant corpus callosa, but they were also ensheathed by thicker myelin relative to those in wild type cuprizone-treated animals. To determine if loss of Csk lent protection from cuprizone-mediated cell death, I evaluated mature oligodendrocyte apoptosis using immunocytochemistry to detect the mature oligodendrocyte marker CC1, in conjunction with a TUNEL assay (Fig. 21G). I found that, while Csk null and wild type oligodendrocytes underwent apoptosis to a similar degree in the absence of cuprizone treatment (Fig. 21G, n=4; 3.8±0.3% in WT versus 3.2±0.6% in Mut), in the presence of cuprizone, Csk loss promoted mature oligodendrocyte survival (Fig. 21G, n=4; 4.6±0.4%, 11.3±0.8 in WT) and thus led to increased numbers of mature oligodendrocytes in the corpus callosum. These findings suggest that the loss of Csk provides protection to cuprizone-induced apoptosis, which in turn may result in decreased demyelination.

Loss of Csk Promotes Remyelination Following Cuprizone-Induced Demyelination

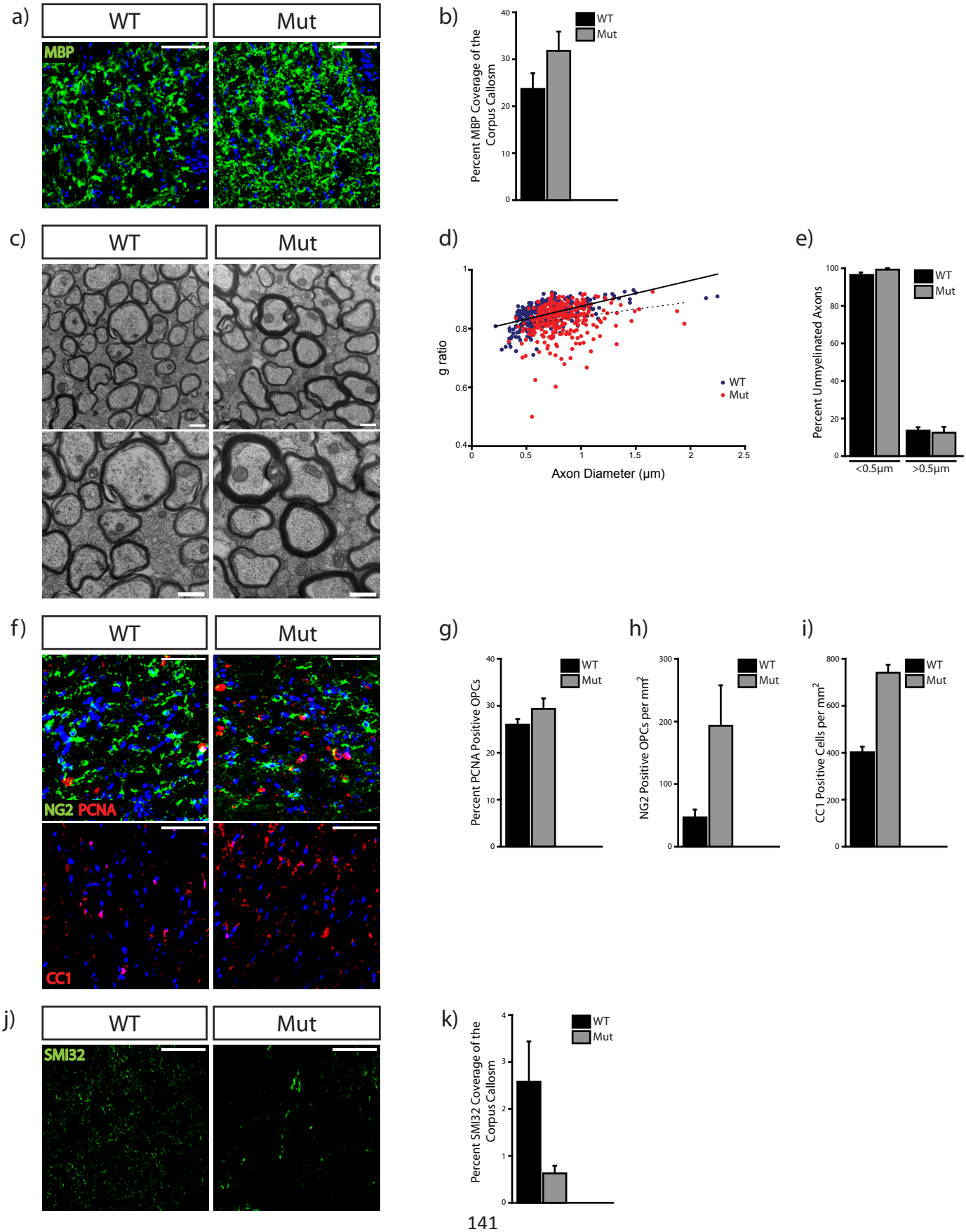
To test the hypothesis that loss of Csk promotes remyelination, I allowed cuprizone-treated Csk mutant and wild type mice to survive for another 6 weeks in the absence of

cuprizone. At the end of the experiment, I evaluated MBP expression using immunohistochemistry, and myelin ultrastructure using transmission electron microscopy, within the corpus callosum of animals from both genotypes (Fig. 22). I found that the oligodendrocyte-specific Csk null animals exhibited increased MBP immunoreactivity (Fig. 23A), as well as increased area of MBP coverage within the corpus callosum (Fig. 22A,B n=4; 31.8±4.1% in Mut versus 23.7±3.3% in WT), suggesting that Csk loss may promote remyelination following cuprizone treatment.

Examination of myelin ultrastructure using transmission electron microscopy in animals that had undergone both demyelination and remyelination revealed thicker myelin, i.e. smaller *g* ratios, within the corpus callosum of cuprizone-treated Csk mutant mice, compared to their wild type littermates (Fig. 22C,D). While the majority of large caliber axons are remyelinated, some unmyelinated axons are still present in animals of both genotypes (Fig. 22C). To test whether in the absence of Csk remyelinating oligodendrocytes ensheath more axons overall, I quantified the percent unmyelinated axons. I found fewer unmyelinated axons in oligodendrocyte-specific Csk null mice compared to their wild type littermates, further confirming that loss of Csk promotes remyelination (Fig. 22E). Taken together, the above observations suggest that the loss of Csk promotes remyelination by two distinct mechanisms; first by increasing the number remyelinated axons, and second, by increasing the myelin thickness of individual myelin internodes.

Figure 22. Loss of Csk leads to enhanced remyelination following cuprizone-induced demyelination. (a) Indirect immunofluorescence to detect MBP (green) in the corpus callosum of wild type (WT) or oligodendrocyte-specific Csk null (Mut) undergoing remyelination. **(b)** Percent ±sem MBP-covered are of corpus of wild type (WT, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) undergoing remyelination. **(c)** Transmission electron microscopy images of sagittal sections through the corpus callosum of wild type (WT) or oligodendrocyte-specific Csk null (Mut) undergoing remyelination. Scale bars: 1μm. **(d)** Quantification of myelin sheath thickness (g ratio) as a function of axon diameter in the corpus callossum of wild type (blue circles) or oligodendrocyte-specific Csk null (Mut, red circles) mice (n=2) undergoing remyelination. **(e)** Percentage ±sem of unmyelinated axons in the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) mice undergoing remyelination. **(f)** Indirect immunofluorescence to detect PCNA+ (red) and NG2+ (green) or CC1+ (red) cells, and nuclei (blue) in the corpus callosum wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals undergoing remyea,ination. Scale bars: 50μm. **(g)** Percentage ±sem of PCNA-positive OPCs (NG2+) in the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) undergoing remyelination. **(h)** NG2-positive OPCs per mm^2 in the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) undergoing remyelination ±sem. **(i)** CC1-positive mature oligodendrocytes per mm^2 in the corpus callosum of wild type (wt, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) undergoing remyelination ±sem. **(j)** Indirect immunofluorescence to detect SMI32+ (green) apoptotic axons in the corpus callosum wild type (WT) or oligodendrocyte-specific Csk null (Mut) animals undergoing remyeatination. Scale bars: 50μm. **(k)** Percent ±sem MBP-covered are of corpus of wild type (WT, black bars) or oligodendrocyte-specific Csk null (Mut, grey bars) undergoing remyelination.

Figure 22


a)
WT
Mut
MBP
b)
Percent MBP Coverage of the Corpus Callosm
WT
Mut
c)
WT
Mut
d)
g ratio
Axon Diameter (μm)
WT
Mut
e)
Percent Unmyelinated Axons
WT
Mut
<0.5μm
>0.5μm
f)
WT
Mut
NG2 PCNA
CC1
g)
Percent PCNA Positive OPCs
WT
Mut
h)
NG2 Positive OPCs per mm2
WT
Mut
i)
CC1 Positive Cells per mm2
WT
Mut
j)
WT
Mut
SMI32
k)
Percent SMI32 Coverage of the Corpus Callosm
WT
Mut

More Oligodendroglia are Present during Remyelination within Oligodendrocyte-Specific Csk Null Corpus Callosa

The observation that more axons were remyelinated in Csk mutant mice compared to their wild type littermates suggests that loss of Csk leads to increased numbers of remyelinating oligodendrocytes. Alternatively, loss of Csk can promote remyelination of more axons by an equivalent number of more complex, mature oligodendrocytes capable of remyelinating more internodes. To distinguish between these two possibilities, I performed immunohistochemistry with antibodies against CC1 and quantified the number of CC1-positive, mature olidendrocytes within Csk mutant and wild type mice that had been subjected to cuprizone-induced demyelination and subsequent remyelination in the absence of cuprizone (Fig. 22). The number of CC1-positive, mature oligodendrocytes within the corpus callosum was increased in oligodendrocyte-specific Csk null mice (Fig. 22F,I), suggesting that loss of Csk promotes remyelination of more axons, at least in part, by increasing the number of cells capable of remyelination.

Loss of Oligodendroglial Csk promotes neuronal survival during remyelination

Loss of myelin leads to neuronal dysfunction and symptomatic onset in demyelinating human diseases, as well as in animal models of demyelination. Thus efficient remyelination strategies need to not only promote remyelination, but also to promote survival of the underlying axons. Using immunohistochemistry to detect SMI32 (dephosphorylated neurofilament) I identified dying neurons in Csk mutant and wild type animals that had been subjected to

cuprizone-mediated demyelination and subsequent remyelination (Fig. 22J). Interestingly, I found that the loss of oligodendroglial Csk resulted in decreased SMI32 immunoreactivity (Fig. 22J,K), thus suggesting that compared to their wild type counterparts, Csk null oligodendroglia were able to provide increased trophic support to the underlying axons.

Discussion

The importance of efficient remyelination for normal brain development and function is illustrated by demyelinating diseases, where inefficient or complete lack of remyelination of damaged myelin results in axonal dysfunction, neuronal death and symptomatic onset. Little is known, however, about the mechanisms that promote remyelination following myelin loss. Furthermore, although the necessity of Fyn activity for normal myelination has been previously elucidated[147], a possible function for Fyn and/or Fyn regulatory molecules such as Csk has never been addressed in the context of myelin repair. Since loss of oligodendroglial Csk promoted myelination in adult animals (See Chapter 8), I hypothesized that loss of Csk may promote remyelination as well. To test this hypothesis I first needed to establish that I could effectively demyelinate hypermyelinated oligodendrocyte-specific Csk mutant mice. I found that while MBP immunoreactivity within the corpus callosum was decreased, the number of unmyelinated, corpus callosal axons was *increased* in cuprizone-treated animals of both genotypes compared to untreated animals, suggesting that 6 weeks of cuprizone treatment was sufficient to induce significant demyelination. Moreover, there was no significant difference in the number of unmyelinated axons between cuprizone-treated Csk mutant and wild type animals, suggesting that loss of Csk did not have a protective role against demyelination. To evaluate remyelination,

I allowed cuprizone-treated animals to recover for another 6 weeks in the absence of cuprizone. I found that loss of Csk resulted in thicker myelin and lower *g* ratios following remyelination, suggesting that Csk loss-of-function promoted remyelination following myelin damage. Interestingly, compared to their wild type counterparts, Csk mutant animals exhibited increased numbers of proliferating OPCs, NG2-positive oligodendrocytes, as well as CC1-positive mature oligodendrocytes, suggesting that loss of Csk, and therefore, increased SFK activity, promote the generation of sufficient numbers of myelin-producing cells. Furthermore, loss of oligodendroglial Csk was sufficient to enhance neuronal survival, as the area of coverage of SMI32, a marker for neuronal dysfunction, was decreased in Csk mutant animals. Taken together, these findings suggest that a previously unidentified function for SFK activity in promoting remyelination following demyelination, and that the SFK inhibitor Csk, is inhibitory to this process.

Oligodendrocyte-Specific Csk Null Mice can be Demyelinated by Cuprizone.

Myelin damage and demyelination are usually caused by increased cell death of mature, myelinating oligodendrocytes. Although the SFKs Fyn and Lyn have been previously implicated in promoting oligodendroglial survival downstream of prosurvival factors such as laminin2, L1, and a combination of PDGF and finbronectin *in vitro*[91,108], the role of SFK activity in oligodendroglial survival *in vivo*, has never been analyzed. In this work, I have identified that SFK activity is not only necessary for oligodendrocyte survival independent of laminin2, L1, PDGF or fibronectin *in vitro* (See Chapter 6*)*, but that enhanced SFK (likely Fyn) activity is necessary for mature oligodendrocyte survival *in vivo* as well. Furthermore, genetic loss of the

oligodendroglial SFK inhibitor Csk was sufficient to promote mature oligodendrocyte survival during development *in vivo* (See Chapter 6*)*. I therefore hypothesized that loss of oligodendroglial Csk would lead to increased survival, and therefore, *decreased* demyelination following cuprizone treatment. As expected, I found that cuprizone-treated Csk mutant animals had a lower percentage of TUNEL-positive (Fig. 21G), and therefore apoptotic, CC1-positive mature oligodendrocytes than their wild type counterparts, suggesting that loss of Csk led to enhanced survival of mature oligodendrocyte following cuprizone treatment. However, the pro-survival effect of loss of Csk did not translate into decreased demyelination following cuprizone-treatment, as the numbers of unmyelinated, corpus callosal axons were similar Csk mutant and wild type mice (Fig. 21C). These observations suggest that although loss of Csk is sufficient to promote mature oligodendrocyte survival, these pro-survival effects are not sufficient to provide protection from cuprizone-induced corpus callosal demyelination. However, by virtue of having achieved efficient demyelination, I could then evaluate the role of Csk in remyelination.

Loss of Csk Promotes Remyelination following Cuprizone-Induced Demyelination

For efficient remyelination to occur, sufficient numbers of myelinating oligodendrocytes need to be generated by recruitment of OPCs into demyelinated lesions, progenitor pool expansion through increased proliferation and decreased apoptosis, efficient oligodendroglial differentiation and maturation, and finally, ensheathment of demyelinated axons. *In vitro* studies have established the role of Fyn in promoting oligodendroglial differentiation, newly-formed oligodendrocyte survival and maturation through expression of MBP and myelin sheet formation. Furthermore, characterization of the myelin content in mice lacking Fyn, or Fyn

activity, have established the necessity of Fyn for myelination *in vivo*[147,151]. In this work, I have established that correctly regulated Fyn activity is also necessary for OPC proliferation and timely OPC-to-oligodendrocyte transition *in vitro* and *in vivo* (See Chapter 5*)*. Taken together, these observations suggest that Csk regulation of Fyn activity may be necessary for remyelination as well. By allowing animals to recover for 6 weeks following cuprizone-induced demyelination I examined remyelination in oligodendrocyte-specific Csk null and wild type animals. I found that loss of Csk resulted in increased myelin thickness following remyelination (Fig 22C,D), presumably due to exuberant myelin wrapping, suggesting that SFK activity is necessary for remyelination. Thus, by promoting the termination of myelin wrapping Csk is inhibitory to remyelination, and molecules designed to inhibit Csk function may prove promising therapeutic agents for demyelinating diseases.

Loss of Csk Promotes the Generation of Sufficient Numbers of Myelinating Oligodendrocytes

In this study I have found two distinct and opposing roles for Csk in oligodendroglial development such that early in development, Csk promotes timely OPC cell cycle exit, but late in development, Csk is inhibitory to myelination as it promotes the termination of myelin wrapping. In young animals, loss of oligodendroglial Csk resulted in increased OPC proliferation, which led to delayed OPC-to-oligodendrocyte transition and delayed oligodendroglial differentiation (See Chapters 4-5). During remyelination, loss of Csk similarly promoted OPC proliferation (Fig. 22G), and increased the numbers of NG2-positive OPCs (Fig. 22H). However, the retention of progenitor characteristics that was observed developmentally

with Csk loss-of-function did not occur during remyelination, as the numbers of CC1-positive, mature oligodendrocytes were increased in Csk mutant brains during remyelination (Fig. 22I). These findings suggest that whether or not OPC hyperproliferation results in a differentiation delay, may be Csk-independent, but instead dependent on the environmental cues. Thus during remyelination, factors in the brain environment may favor OPC differentiation despite the absence of Csk and the resulting increase in OPC proliferation.

Chapter 9:

General Methods

Animals

Conditional deletion of Csk in the oligodendrocyte lineage was achieved using CNP-Cre transgenic mice[21]. Briefly, homozygous Csk floxed mice[204] were crossed with animals heterozygous for both the Csk floxed and CNP-Cre alleles. Animals carrying two Csk floxed and one CNP-Cre alleles were considered oligodendrocyte-specific Csk null or mutant (Mut), while their littermates lacking a CNP-cre allele were used as wild type controls (WT). Genomic DNA was extracted from tail biopsies using a DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions. All animals were genotyped by PCR using previously described primers [21,204]. All animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Animals and were approved by the Stony Brook University Institutional Animal Care and Use Committee.

Cell culture

Primary rat oligodendrocyte progenitor cells (OPCs) were isolated as described [92,214]. Briefly, cerebral cortices dissected from neonatal Sprague-Dawley rats (P0-P2) were digested with papain (Worthington) and plated onto PDL-coated 75cm^2 flasks. Cells were grown in

Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% FBS at 37°C and 7.5% CO_2 for 10-14 days to obtain mixed glial cultures. Rat OPCs were isolated by mechanical dissociation and purified by differential adhesion. For differentiation experiments OPCs were seeded onto dishes or slides coated with poly-D-lysine (PDL) or Lm2 and grown, unless otherwise indicated, in Sato's medium supplemented with 0.5% fetal bovine serum (FBS; HyClone). Although mouse OPCs were isolated in a similar manner, they were purified using immunopanning similar to described methods [215]. Briefly, OPCs isolated from mixed glial cultures by mechanical dissociation were subjected to two rounds of panning on dishes coated with 2.3μg/ml BS-lectin-1 to remove microglia. Unattached cells were subsequestly incubated onto dishes coated with rat anti-PDGFRα antibody (BD Pharmigen) for 30min at 37°C to positively select for OPCs. Adherent cells were detached using 0.5X Trypsin-EDTA and were subsequently used for differentiation experiments. BS-lectin-1-coated dishes were coated for a minimum of 4 hours at RT and washed three times with Dulbecco's PBS (D-PBS) immediately before use. To coat dishes with rat anti-PDGFRα antibody, dished were pre-coated at 4°C overnight with 7μg/ml goat anti-rat IgG antibody in 50mM Tris pH9.5, washed three times with D-PBS and then coated with 0.85μg/ml rat anti-PDGFRα antibody in 0.2% endotoxin-free, heat-inactivated BSA in D-PBS for a minimum of 1hr at RT.

Transfection

OPCs were transfected as described[92]. Briefly, freshly-isolated OPCs were plated onto Petri dishes and maintained in OPC medium (Sato with 20ng/ml FGF and 20ng/ml PDGF) at 37°C overnight. Cells were collected with 0.5X Trypsin-EDTA (Sigma) and allowed to rest for

60-90 minutes at 37°C. OPCs were then pelleted, resuspended in rat oligodendrocyte nucleofection reagent (Amaxa) as per manufacturer's directions, nucleofected, plated and allowed to recover in OPC medium overnight. Rat Csk siRNA pools and non-targeting control pools (Dharmacon) were used at 30pmol/million cells, while pECFP-Csk WT, pEYFP-Cbp, and pEYFP-CbpY317F, and pECFP control constructs were used at 0.1µg/million cells. The next day, termed day 0, media was switched to differentiation media unless otherwise specified. In experiments where the effects of Csk overexpression were examined using visual assays, DNA was delivered using the lipid carrier Fugene HD (Roche) as described [108]. Briefly, day 7 mixed glial cultures were transfected overnight with 5µg DNA and 15µl Fugene HD (Roche). OPCs were isolated as usual and plated onto 8-well chamber slides at a density of 25,000 cells/well. Cells were grown in OPC medium overnight, then switched to differentiation medium for up to 4 days and fixed for immunocytochemistry. Transfected cells were identified using immunocytochemistry to detect CFP/YFP fusion proteins.

Protein analysis

Unless otherwise specified, cells were lysed in boiling 20mM Tris (pH7.4), 1% SDS buffer with added protease and phosphatase inhibitor cocktails (Calbiochem). Cell lysates were transferred to microfuge tubes and boiled at 95°C for 10 min and centrifuged at 16,000x g to remove insoluble material. Protein concentrations were determined using detergent-compatible protein assay (BioRad). Protein lysates were boiled in Laemmli solubilization buffer and 3% β-mercaptoethanol (BioRad) for 10 min, separated by SDS-PAGE, and blotted onto 0.45µm nitrocellulose membranes. Proteins of interest were detected using appropriate primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies (GE Healthcare)

for 1h at room temperature (RT), and visualized using enhanced chemiluminescence (GE Healthcare). Membranes were blocked in 4% BSA in TBS with 0.1% Tween20 (TBST) and washed in TBST. Antibody incubations were performed in blocking solution. Relative protein level was quantified using Image J software. Experiments were carried out a minimum of three times and representative blots are depicted.

Immunoprecipitation

Cells differentiated for 2 days were lysed on ice using 1% Triton X-100, 0.2% SDS, 10mM Tris (pH7.4), 5mM EDTA, and 150mM NaCl with added protease and phosphatase inhibitor cocktails (Calbiochem). Cell lysates were transferred to microfuge tubes, incubated on ice for an additional 15 min, and centrifuged at 16,000x g to remove insoluble debris. 100µg of lysate was incubated with 30µl ProteinA/G beads (Pierce) for 30 min at RT. Lysates were then centrifuged at 100xg for 2 min to pellet beads, and pre-cleared lysate was then incubated at 4°C overnight with either 2µg/ml rabbit polyclonal Csk antibody (Santa Cruz) or 2µg/ml control rabbit IgG, (Jackson Immunoresearch) along with fresh ProteinA/G beads. To immunoprecipitate YFP-Cbp and YFP-CbpY317F, agarose-conjugated mouse anti-GFP antibody (MBL) was used instead of ProteinA/G beads. All bead-protein complexes were isolated by centrifugation and washed three times with 50mM Tris pH7.5, 500mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$ and 1% Triton X-100. To release immunoprecipitated material, beads were boiled for 10 min in Laemmli solubilization buffer and 12% β-mercaptoethanol. Proteins were resolved using SDS-PAGE and immunoblotted to detect phosphorylated Cbp Y317, total Cbp, or Csk. To minimize detection of denatured IgG from the pull-down antibody, HRP-conjugated secondary antibodies specific for non-denatured IgG were used (eBioscience).

Viral particle production

To generate concentrated stocks of control or Csk shRNA-containing lentiviral particles, pGIPZ-shRNAmir vectors encoding for TurboGFP and control or Csk shRNA (OpenByosystems) were transfected into HEK293T cells together with the packaging plasmids Tat2b, Rev, Gag-Pol and VSV-G using Fugene HD transfection reagent (Roche). Transfected cells were grown in DMEM supplemented with 10% FBS (37°C, 5% CO_2). Conditioned medium was collected each day, centrifuged at 1,400 xg, passed through a 0.45µm filter to remove cellular debris, and stored on ice. Conditioned medium from 5 collection days was pooled and centrifuged at 77,000x g for 2 hours at 4°C. Viral particles were resuspended in 100µl serum-reduced medium (Opti-MEM; Gibco) and stored at -80°C. To test the effectiveness of the generated lentiviral particles, freshly-dissociated rat cerebral cortices were incubated with 25µl concentrated lentivirus (per brain) for 2 hours at 37°C. Cells were maintained as neurospheres in DMEM/F12 (50%/50%; Sigma) supplemented with B-27 (Gibco), 20ng/ml FGF, and 20ng/ml EGF for 8 days, selected with 1µg/ml puromycin (InvivoGen) for 4 days, then lysed. Lysates were separated using SDS-PAGE and immunoblotted to detect Csk.

Stereotactic viral injections in neonatal rats

Postnatal day 2 Sprague-Dawley rats were deeply anesthetized by hypothermia, positioned in a stereotactic device, and injected with 1µl of concentrated control or Csk shRNA-containing lentiviral particles into the right ventricle. The coordinates used were 1mm posterior and 1.5mm lateral to bregma at depth of 1.5mm. At P4, P6, P8 and P14 pups were deeply anesthetized by 2,2,2-tribromoethanol (0.4mg/g body weight; Aldrich) and perfused

transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (Sigma). Brains were removed, postfixed for 1hr, and cryoprotected in 30% sucrose. Serial coronal sections of 20μM thickness were obtained using a Leica CM1900 cryostat.

Immunocytochemistry

Unless otherwise specified cultured cells were fixed with 4% paraformaldehyde (PFA) for 10min at RT, permeabilized with either 100% methanol for 5 min at -20°C or 0.02% Triton X-100, and blocked in 10% donkey serum in PBS, with or without 0.02% Triton X-100, for 1 hour at RT. Proteins of interest were detected using primary antibodies overnight at 4°C, followed by fluorescent-conjugated secondary antibodies for 1 hour at RT. Nuclei were labeled with Dapi and indirect immunofluorescence was visualized using a Zeiss Axioplan fluorescence microscope. All antibody incubations were performed in 10% donkey serum in PBS and all washes were in PBS. Immunocytochemistry of 20μM perfused tissue sections was performed similarly. Briefly, perfusions were performed in 4% PFA followed by post-fixing for 2 hours. Tissue sections were then blocked in 10% donkey serum containing 0.1% Triton-X-100. In some cases, fresh frozen sections were obtained and post-fixed on the slide using 4% PFA for 10 minutes.

***In vitro* proliferation assays**

Oligodendrocyte proliferation *in vitro* was analyzed by Western Blot to detect phosphorylated histoneH3 or by indirect immunocytochemistry to visualize 5-bromo-2'-deoxy-uridine (BrdU) incorporation. At 16hrs post-transfection with siRNA, OPCs were incubated for

8h with Sato's medium supplemented with 10μM BrdU (Roche) and one or more of the indicated reagents: 10ng/ml FGF, 10ng/ml PDGF, 2.5μM PP2, or PP2 vehicle dimethyl sulfoxide (DMSO). Cells were washed, then fixed with ethanol:acetic acid (2:1) for 30 min at -20°C; BrdU incorporation was subsequently detected using 5-Bromo-2'-Deoxy-Uridine Labeling and Detection Kit I (Roche). A minimum of 1000 nuclei per condition were scored as either BrdU positive or negative. Proliferation was evaluated similarly in cells transfected with pECFP-Csk WT or pECFP control. In brief, transfected cells were fixed with 4%PFA for 10 min followed by 0.02% Triton-X-100 in blocking buffer to permeablize, then subjected to immunocytochemistry with anti-GFP antibodies, then finally post-fixed with ethanol-acetic acid prior to immunocytochemistry with anti-BrdU antibodies (as above) to detect BrdU incorporation. A minimum of 100 GFP-positive cells per condition were scored as BrdU positive or negative. All results were expressed as percent BrdU-positive cells and normalized to control.

***In vivo* proliferation assay**

To assay proliferation *in vivo*, lentiviral-injected animals were subsequently injected intraperitoneally with BrdU (20μl/g body weight) 24h prior to sacrificing. Coronal sections of 20μm thickness were postfixed with 4% parafolmaldehyde for 10 min and blocked for 1h using 10% donkey serum (Sigma) with 0.05% Triton X-100 in PBS. Rabbit anti-NG2 and chicken anti-GFP antibodies were applied in blocking solution overnight at 4°C. After incubation with appropriate secondary antibodies, sections were treated with 2N HCl for 30 min, washed thoroughly in TBS then PBS, blocked in 10% donkey serum and incubated with sheep anti-BrdU antibodies in BrdU incubation buffer (Roche) overnight at 4°C. Sections were washed in PBS, incubated with the appropriate Cy3-, Cy5- and FITC-conjugated secondary antibodies for 2h at

room temperature and followed by DAPI to visualize nuclei. Slides were mounted with SlowFade Gold reagent (Invitrogen) and analyzed on a confocal microscope (Zeiss LSM510 Meta). A minimum of 100 NG2+/GFP+ double-positive cells in the SVZ or corpus callosum were scored per animal for the presence or absence of BrdU immunoreactivity.

Reverse transcriptase polymerase chain reaction (RT-PCR)

To analyze the expression of cell cycle regulators following 1 day in Sato's medium, mRNA was extracted using a Qiagen RNeasy kit and cDNA was synthesized using First-Strand Synthesis Super-Script III kit (Invitrogen). Semi-quantitative RT-PCR was performed using primers, sequences as previously described [216], to measure relative mRNA expression of GAPDH (Tm 61.7°C, 23 cycles), cyclinD1 (Tm 56.5°C, 30 cycles), cyclinD2 (Tm 62°C, 35 cycles), cyclinD3 (Tm 63°C, 35 cycles), cyclinE (Tm 63°C, 35 cycles), $p27^{Kip1}$ (Tm 54.5°C, 35 cycles) and $p16^{Ink}$ (Tm 58.8°C, 35 cycles). Products were resolved on a 2% agarose gel and bands were quantified using ImageJ software, normalized to GAPDH levels.

Survival assays

Oligodendrocyte survival was analyzed either by Western blots to detect cleaved caspase-3 or by indirect immunocytochemistry using Terminal Transferase dUTP Nick End Labeling (TUNEL) assay (Chemicon). Cells transfected with siRNA were differentiated for 1 day in Sato's medium in the presence of either 2.5μM PP2 inhibitor or vehicle control (DMSO) then either lysed, as described above for Protein Analysis, or fixed with ethanol:acetic acid (2:1) to prepare for TUNEL. Alternatively, cells transfected with either pECFP-Csk WT or pECFP were

first identified by immunocytochemistry with anti-GFP antibodies prior to TUNEL assay; a minimum of 100 GFP-positive cells per condition were scored as either TUNEL positive or negative.

Electron microscopy and morphometric analysis

Mice were perfused transcardially with 4% paraformaldehyde/2.5% gluteraldehyde in 0.1M PBS, brains were dissected and postfixed overnight in the same fixative. Sagital sections through the medial corpus callosum were vibratomed at 100μm thickness using a Leica VT-100 Viratome. Sections were then contrasted with 0.2% osmium tetroxide in 0.1M PBS, washed in PBS and dehydrated in a graded series of ethanols. Sections were then vacuum infiltrated with Durcupan ACM Epoxy (Electon Microscopy Sciences) overnight, flat embedded between two pieces of ACLAR film (Ted Pella) and incubated at 60°C for 48-72hrs. Ultrathin (70-80nm) sections were cut using a Rechert-Jung Ultracut E ultra-microtome, placed on formvar-coated copper grids and counterstained with uranyl and lead acetates, visualized under a FEI Tecnai BioTwinG^2 transmission electron microscope and an AMT XR-60 digital camera. The axon diameter was calculated from the axon permiter measured using ImageJ software (NIH) and the *g ratio* of myelinated axons was calculated by dividing the diameter of the axon (without myelin) by the diameter of the axon including the myelin. A minimum of 300 axons per animal were analyzed using two animals per genotype.

Antibodies

The following primary antibodies were used: rabbit anti-Csk (C-20, Santa Cruz Biotechnology); mouse anti-Csk (BD Biosciences); mouse anti-Cbp (Abcam); rabbit anti-phosphorylated Src Y418; rabbit anti-phosphorylated Src Y529; rabbit anti-Fyn (FYN3, Santa Cruz Biotechnology); mouse anti-Fyn (BD Biosciences); rabbit anti-phosphorylated Cbp Y317 (which recognizes rat phosphorylated Cbp Y317; a kind gift from Drs. Jon and Sabine Lindquist); rabbit anti-NG2 (Chemicon); mouse anti-NG2 (Chemicon); rat anti-PDGFαR (CD140a, BD Pharmigen); mouse anti-CNPase (Sigma); rat anti-MBP (AbD Serotec); mouse anti-APC (CC1, Calbiochem); rabbit anti-$p27^{Kip1}$ (Santa Cruz Biotechnology); rabbit anti-phosphorylated histoneH3 (Upstate); mouse anti-BrdU (Roche); sheep anti-BrdU (Abcam); rabbit anti-cleaved caspase3 (Cell Signaling); rabbit anti-caveolin-1 (Cell Signaling); mouse anti-β-actin (Sigma); rabbit anti-GFP and mouse anti-GFP (Molecular Probes); agarose-conjugated mouse anti-GFP (MBL), and chicken anti-GFP (Aves Labs).

Reagents

The following growth factors were obtained from PeproTech Inc: recombinant human FGF-basic; recombinant human PDGF-AA; and recombinant murine EGF. B-27 supplement was obtained from Gibco. Substrate ligands were used at 10 µg/ml: PDL (Sigma) and Lm2 (Chemicon). Pharmacological inhibitors: PP2 (Calbiochem); fumonisinB1 (Sigma); puromycin (InvivoGen); protease inhibitor cocktail set III (Calbiochem); and phosphatase inhibitor cocktail set II (Calbiochem).

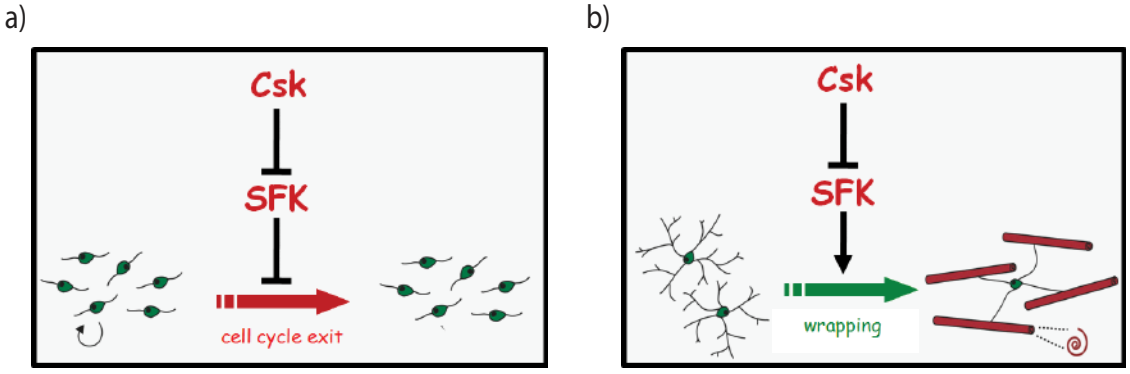
Chapter 10:

Conclusions and Future Directions

The ability of OPCs to generate sufficient numbers of myelinating oligodendrocytes is critical both for developmental myelination and for repair following demyelination. The timing and location of oligodendrocyte development and myelination are tightly controlled but the molecular mechanisms that underlie this control are poorly understood. Numerous studies have suggested that Fyn activity promotes oligodendrocyte differentiation[75-78], and that it is not only regulated during myelination[150-151], but that it is necessary for this process as well[147]. However, the mechanisms that regulate Fyn activity in oligodendroglia were previously unknown. In this study, I have identified the C-terminal Src Kinase (Csk) as molecular switch for oligodendrocyte Fyn activity, with distinct and opposing roles for Csk in early versus late stages of development. Early in development Csk is critical for the appropriate onset of oligodendrocyte progenitor differentiation and the OPC-to-oligodendrocyte transition. Csk depletion in OPCs *in vitro* and *in vivo* led to proliferation under conditions that normally promoted cell cycle exit, suggesting that Csk normally suppresses OPC proliferation and promotes cell cycle exit. Interestingly, treatment of OPCs with the SFK inhibitor PP2 not only effectively rescued the hyperproliferation phenotype of Csk-depleted OPCs, but also induced cell cycle exit of wild type OPCs, suggesting a previously-unidentified role for SFK activity in promoting proliferation in the absence of growth factor stimulation. Furthermore, hyperproliferation of Csk-deficient OPCs resulted in delayed oligodendrocyte maturation accompanied by delayed myelination onset, while also

Figure 23: Model for Csk function during oligodendrocyte development and myelination. **(a)** Oligodendrocyte progenitor differentiation requires cell cycle exit. Csk suppresses Fyn activity in oligodendrocyte progenitor cells to promote timely cell cycle exit. In the absence of Csk, oligodendrocyte progenitor differentiation is inappropriately delayed such that cells proliferate more and differentiate more slowly. **(b)** Csk promotes the termination of myelin wrapping. Fyn activity is necessary for myelination[147]. Csk suppresses Fyn activity to help terminate myelin wrapping. Hypermyelination results in the absence of Csk.

Figure 23



causing increased survival of newly-formed oligodendrocytes. Taken together, these data suggest that during myelination onset, Csk is a pro-differentiation factor that promotes timely cell cycle exit. On the other hand, later in oligodendrocyte development, Csk deletion caused increased levels of MBP, as well as increased numbers of myelin wraps in the cortex, cerebellum and spinal cord of adult Csk mutant mice. Interestingly, Csk loss led to hypermyelination in the presence of normal numbers of myelinating oligodendrocytes, suggesting that Csk also contributes to myelination arrest. Finally, to address a putative role for Csk during myelin repair I evaluated the response of Csk null mice to cuprizone-induced demyelination. Although cuprizone treatment led to demyelination, mice with Csk loss showed increased survival of mature oligodendrocytes, leading to increased numbers of myelinating oligodendroglia. During remyelination, mice with Csk loss showed not only OPC hyperproliferation and increased numbers of OPCs, but also had increased numbers of myelinating oligodendrocytes, thicker myelin and increased neuronal survival. I, therefore, propose that Csk is a novel regulator of oligodendrocyte development with two distinct roles: to generate appropriate numbers of oligodendrocytes at the onset of myelination, and to terminate wrapping during myelination and remyelination.

C-Terminal Src Kinase Inhibits Fyn Activity in Oligodendrocytes

Fyn activity within isolated myelin membranes is regulated developmentally such that it peaks at the onset of myelination. What remained unknown however were the molecular mechanisms that modulate Fyn activity during oligodendrocyte development and myelination. In this study, I found that Csk depletion resulted in increased Fyn activity, as phosphorylation at the

autocatalytic Y418 on Fyn was increased, while phosphorylation of the negative regulatory Y529 on Fyn was decreased (Fig. 7). These findings suggested that Csk is a negative regulator of oligodendroglial Fyn activity. However, I cannot exclude the possibility of other negative regulators of oligodendroglial Fyn activity. Using Western blot analysis I have also confirmed the expression of oligodendroglial Chk (data not shown), a homolog of Csk that can mediate phosphorylation of SFKs at the negative regulatory tyrosine in other systems[191-194]. Whether Chk is also a negative regulator of oligodendroglial Fyn activity, however, remains to be determined. Interestingly, Chk null animals showed no apparent myelin deficits[174,177], suggesting that Chk may either be dispensable for normal oligodendrocyte development, or that its function may redundant to that of Csk.

Surprisingly, while phosphorylation of the Fyn regulatory, C-terminal tyrosine was regulated developmentally i.e. it was *decreased* as newly-formed oligodendrocytes were generated, expression of Csk remained constant during oligodendrocyte differentiation (Fig. 4,5). In addition, while Csk is cytosolic, Fyn is post-translationally modified such that it is enriched in glycosphingolipid-enriched membrane microdomains (GEMs), suggesting the Csk function may be regulated spatially (Fig. 6). I identified that Csk binding protein, Cbp, was expressed in oligodendroglial GM1-containing rafts, and that it associated with Csk in oligodendrocytes (Fig. 5,6). The Csk association was necessary for Csk function, suggesting that Cbp may negatively regulate Fyn activity, likely by recruiting Csk to oligodendrocyte GEMs. However, whether Cbp regulates oligodendrocyte development and/or myelination remains to be examined. Interestingly, Cbp/Csk association was also dependent on phosphorylation of Cbp Y317 (Fig. 5). In other systems, SFKs can phosphorylate Cbp to facilitate Csk recruitment, thereby creating a

negative feedback loop mechanism of SFK activity regulation. Preliminary observations suggest that such a negative feedback loop mechanism may also be present in oligodendrocytes, as treatment with the SFK inhibitor PP2, which inhibited oligodendroglial Fyn activity, also resulted in decreased phosphorylation of Cbp Y317 (data not shown). The functional significance of this putative feedback loop mechanism of in terms of oligodendroglial development remains to be determined. It could be speculated, however, that such a feedback loop could ensure that some of the signal transduction controls on myelination have a "self-destruct" feature in that they ultimately turn themselves off; a challenge will be to understand if these control mechanisms can be, after myelin injury, restarted to ensure efficient remyelination.

Oligodendrocyte SFKs may also be regulated through dephosphorylation. A number of phosphatases including PTPα, PTPε, SHP-1, SHP-2 and CD45 have been suggested to relieve Csk-mediated inhibition by dephosphorylating the negative regulatory tyrosines of multiple SFKs in other systems. Although these phosphatases are expressed in oligodendrocytes[196], it remains unclear if they can regulate SFK activity. Studies from our lab have identified that while SHP-1 is necessary for oligodendrocyte differentiation, SHP-2 is necessary for OPC proliferation[217]. Given that Fyn activity is required for both OPC proliferation (See Chapter 4) and oligodendrocyte differentiation[150-162], it is reasonable to suggest that SHP-2 may promote Fyn activity in OPCs to promote proliferation, while SHP-1 may promote Fyn activity in newly-formed oligodendrocyte to promote differentiation. Furthermore, while mice lacking SHP-1 and CD45 are hypomyelinatied in the CNS, mice lacking PTPε activity are hypermyelinated. Whether SFK activity is dysregulated in these animals, however, remains to be seen. Overall, however, it appears that multiple mechanisms could be in play to regulate oligodendrocyte SFK

activity at multiple time points during development. A future goal will be to determine how Csk interacts with other putative SFK regulatory mechanisms.

Csk Promotes Cell Cycle Exit of Oligodendroglial Progenitor Cells

In this study, I have identified that Csk promotes timely OPC cell cycle exit early in development, as well as during remyelination after cuprizone-induced demyelination. Interestingly, while loss of Csk led to OPC hyperproliferation during the early stages of myelination (Fig. 9), in adult Csk mutant animals OPC proliferation was similar to that observed in their wild type littermates (Fig. 20), suggesting that the hyperproliferation observed in Csk null OPCs could be dependent on the availability of external stimuli. In wild type animals, for instance, OPC proliferation decreases with age as a result of decreased expression of the growth factor PDGF. I therefore hypothesized that PDGF may be a factor permissive to proliferation that is present in the extracellular milieu in developing Csk mutant animals, but absent, or low, in adult animals. However, PDGF levels are also low in demyelinated lesions, and likely after cuprizone-mediated demyelination, suggesting that availability of PDGF may not be sufficient to explain the hyperproliferation phenotype of Csk null OPCs during remyelination. An alternative explanation for the OPC hyperproliferation observed in Csk mutant animals during remyelination may be that, Csk loss-of-function sensitizes OPCs to limiting amounts of available mitogens, presumably through the upregulation of growth factor receptors. It will therefore be crucial to test whether Csk regulates growth factor receptor expression or degradation. Interestingly, transgenic animals overexpressing PDGFαR[56] exhibit a phenotype similar to the

one observed in Csk mutant animals in this study i.e. increased OPC proliferation and delayed oligodendrocyte differentiation during early development.

Although the goal of my thesis work was to identify a role for Csk in oligodendrocyte development, I was intrigued to discover a previously unknown function for Fyn activity in promoting OPC proliferation. I found that treatment of cultured OPCs with the SFK inhibitor PP2 led to decreased BrdU incorporation (Fig. 8), suggesting that SFK activity may be an intrinsic factor controlling the timing of progenitor cell cycle arrest. On the other hand, aberrant Fyn activity such as that observed here with Csk depletion (Fig. 8) may cause OPCs to remain in the cell cycle and delay oligodendrocyte differentiation. What are the signaling effectors that mediate Csk/Fyn signaling during proliferation? Studies using cultured oligodendroglial cells have shown that growth factor-mediated activation of the PI3K/Akt pathway is necessary to promote OPC proliferation[106], while Fyn-mediated activation of Akt was necessary to promote laminin2-induced survival[108]. Taken together, these observations suggest that increased Fyn activity in OPCs may lead to activation of the PI3K/Akt pathway to promote proliferation. Whether Csk loss-of-function results in dysregulation of the PI3K/Akt pathway *in vitro* or *in vivo* remains to be seen, but will be an interesting avenue of further investigation, particularly given that aberrant PI3K/Akt signaling has been shown to alter myelination outcome[144-145].

Previous studies have identified $p27^{Kip1}$ as an important cell intrinsic regulator of OPC cell cycle exit[59,207]. Overexpression of $p27^{Kip1}$ promotes OPC cell cycle exit even in the presence of saturating levels of PDGF, while loss of $p27^{Kip1}$ causes hyperproliferation in the absence of

mitogen stimulation.[59] In addition, protein levels of $p27^{Kip1}$ increase with every OPC cell cycle and eventually, when high enough, trigger OPC cell cycle exit.[52] Interestingly, I found that Csk depletion in cultured OPCs led to increased $p27^{Kip1}$ mRNA, while $p27^{Kip1}$ protein was *decreased*, suggesting that aberrant SFK activity may inhibit translation of $p27^{Kip1}$ mRNA. Previous studies have shown that, when phosphorylated downstream of Fyn, the mRNA binding protein QKI binds and stabilizes $p27^{Kip1}$ mRNA, thus inhibiting $p27^{Kip1}$ mRNA translation[154]. It remains to be seen whether QKI activity and binding to $p27^{Kip1}$ mRNA are increased in the absence of Csk. Delayed $p27^{Kip1}$ mRNA translation may lead to a delay in $p27^{Kip1}$ protein build up and, therefore, delayed cell cycle exit and differentiation.

Csk Loss-of-Function Leads to Delayed Oligodendrocyte Differentiation

A number of *in vitro* studies have established that Fyn activity promotes critical steps during oligodendrocyte differentiation: process outgrowth[158-159,161,163], survival[108], and expression of myelin basic protein[151,154-155]. Surprisingly, I identified that Csk loss-of-function, which led to increased Fyn activity, resulted in *delayed* oligodendrocyte differentiation *in vitro* and *in vivo*. However, Csk loss-of-function also led to OPC hyperproliferation and *delayed* cell cycle exit. Since in order to differentiate, OPCs need to first exit the cell cycle, I proposed that the differentiation *delay* observed in Csk depleted cells was a direct result of prolonged proliferation. Since Fyn activity promotes differentiation[108,151,154-155,158-159,161,163], I propose that once Csk-deficient OPCs exit the cell cycle, increased Fyn activity in Csk-deficient oligodendrocytes may therefore drive *increased* differentiation. To fully address whether Csk regulates oligodendrocyte

differentiation, however, new experimental tools need to be developed that allow us to modulate oligodendroglial Csk function exclusively in post-mitotic cells.

As described above Fyn activity promotes oligodendrocyte process outgrowth and branching[158-159,161,163]. Although I did not directly examine process outgrowth, I noted that both Csk depleted and Csk overexpressing cells exhibited shorter processes *in vitro*. Does Csk regulate process outgrowth? Laursen *et al.* proposed that process outgrowth requires dynamic regulation between Fyn "on" and "off" states. In fact contactin-mediated process outgrowth led to increased phosphorylation of Fyn at both the autocatalytic and negative regulatory tyrosines.[167] Since both Csk depletion and overexpression slow down the turnover between Fyn "on" and "off" states, one might expect that this decrease in dynamic regulation may result in impaired process outgrowth.

Csk Loss-of-Function Promotes Oligodendrocyte Survival

Oligodendroglial survival is critical for both myelination and myelin stability. Ischemic injury often leads to excess oligodendroglial apoptosis that can lead to human disease. For example, neonatal cerebral ischemia can lead to massive apoptosis of developing oligodendroglia, which in turn leads to delayed myelination often associated with neurological dysfunction[2-5]. Similarly, ischemic injury in adults in the form of stroke can lead to loss of myelin, which in turn may contribute to neurological dysfunction[14]. In this study, I found that Csk loss-of-function promotes newly-formed oligodendrocyte survival *in vitro* (Fig. 14), as well as mature oligodendrocyte survival *in vivo* (Fig. 15), suggesting that the molecules inhibiting

oligodendroglial Csk function may prove beneficial in promoting oligodendrocyte survival in patients with ischemic injury. It would also be interesting to test whether oligodendrocyte survival and myelination are improved in oligodendrocyte-specific Csk null animals subjected to neonatal hypoxia.

Csk Promotes Termination of Myelin Wrapping

In this study, I identified two distinct and opposing roles for Csk function in oligodendrocyte development and myelination such that, early in development, Csk promotes timely OPC cell cycle exit, while later in development, Csk promotes the termination of myelin wrapping. As a result, during early stages of myelination (P21 corpus callosum), oligodendrocyte-specific Csk null animals were *hypomyelinated* (Fig. 17) as a result of OPCs hyperproliferation and delayed differentiation, while during late stages of myelination (adult CNS), Csk mutant mice were *hypermyelinated* (Fig. 18-20). Since myelin is necessary for saltatory conduction and efficient neuronal communication, it will be critical to identify how the aforementioned myelin abnormalities affect electrophysiology and behavior. Myelin is necessary for brain function, but a second important question to ask is the following: is *too* much myelin detrimental to brain homeostasis?

Although a plethora of molecules are necessary for normal myelination, only a few molecules can stimulate myelination and thus only a few transgenic animal models are hypermyelinated. Overexpression of IGF1 in transgenic animals results in hypermyelination[136]. Is Csk/Fyn signaling downstream of IGF1, and does IGF1 stimulation lead to inhibition of Csk function? Studies in cultured OPCs have shown that IGF1 promotes SFK activity and that

treatment of cultured OPCs with the SFK inhibitor PP2 inhibits both IGF-stimulated proliferation and IGF-stimulated OPC survival, suggesting that SFKs may be downstream effectors of IGF1 signaling *in vitro*[106-107]. Given that both IGF1 overexpression and Csk loss-of-function result in hypermyelination, one might expect that, during myelination, IGF1 may function upstream of Fyn activity to promote myelin wrapping.

In addition, hypermyelination is also associated with hyperactivation of the Akt/mTOR pathway either via overexpression of constitutively-active Akt[144], or via genetic deletion of PTEN[145]. While Fyn can lead to activation of Akt signaling *in vitro*[108], it is unknown whether it can do so *in vivo* as well. Given that both Csk loss-of-function and Akt/mTOR gain-of-function result in hypermyelination, one might expect that, Akt/mTOR signaling may be increased in oligodendrocyte-specific Csk null mice during myelination. Furthermore, can treatment with rapamycin, which blocks mTOR, rescue the hypermyelination phenotype of Csk mutant mice?

Loss of Csk Promotes Remyelination in the Corpus Callosum

To test the role of Csk during remyelination I utilized the cuprizone model of demyelination and found that, while loss of Csk did not prevent demyelination (Fig. 21), it led to improved remyelination (Fig. 22), suggesting the Csk activity may inhibit remyelination. Interestingly, I found that loss of oligodendroglial Csk led to increased neuronal survival, however, whether this increase in neuronal survival led to increased functional recovery remains to be determined. These findings do suggest, however, that molecules designed to inhibit Csk function may prove to be promising therapeutic agents in demyelinating diseases. Interestingly, in aged wild type animals (10 months old) remyelination is impaired, suggesting that factors in

the extracellular milieu of the aging brain may be inhibitory to myelination. Since Csk loss led to increased remyelination in young animals, it would be interesting to evaluate whether remyelination is similarly improved in aged Csk mutant animals. Furthermore, prolonged demyelination in relapsing-remitting MS eventually may lead to a progressive form of the disease, in which remyelination in minimal. Similarly, prolonged cuprizone treatment (8-12 weeks) results in decreased remyelination in wild type animals. Therefore, if remyelination in Csk mutant mice after prolonged cuprizone-demyelination is improved, Csk inhibitors may prove to be promising therapeutics for primary progressive MS.

To conclude, in my thesis work I have identified that C-terminal Src kinase (Csk) is a novel inhibitor of oligodendroglial Fyn activity with two distinct and opposing roles in oligodendrocyte development: generating appropriate numbers of oligodendrocytes at the onset of myelination, and terminating wrapping during myelination and remyelination.

Bibliography

1 Sherman, D. L. & Brophy, P. J. Mechanisms of axon ensheathment and myelin growth. Nat. Rev. Neurosci. **6**, 683-690 (2005).

2 Hartline, D. K. & Colman, D. R. Rapid conduction and the evolution of giant axons and myelinated fibers. Curr. Biol. **17**, R29-35(2007).

3 Rees, S. & Inder, T. Fetal and neonatal origins of altered brain development. Early Hum. Dev. **81**, 753-761 (2005).

4 Inder, T., Neil, J., Kroenke, C., Dieni, S., Yoder, B. & Rees, S. Investigation of cerebral development and injury in the prematurely born primate by magnetic resonance imaging and histopathology. Dev. Neurosci. **27**, 100-111 (2005).

5 van der Knaap, M. S. Magnetic resonance in childhood white-matter disorders. Dev. Med. Child Neurol. **43**, 705-712 (2001).

6 Saugier-Veber, P., Munnich, A., Bonneau, D., Rozet, J.M., Le Merrer, M., Gil, R. & Boespflug-Tanguy, O. X-linked spastic paraplegia and Pelizaeus-Merzbacher disease are allelic disorders at the proteolipid protein locus. Nat. Genet. **6**, 257-262 (1994).

7 Rosenbluth, J., Stoffel, W. & Schiff, R. Myelin structure in proteolipid protein (PLP)-null mouse spinal cord. J. Comp. Neurol. **371**, 336-344 (1996).

8 Rosenbluth, J., Nave, K. A., Mierzwa, A. & Schiff, R. Subtle myelin defects in PLP-null mice. Glia **54**, 172-182 (2006).

9 Karim, S. A., Barrie, J. A., McCulloch, M. C., Montague, P., Edgar, J. M., Kirkham, D., Anderson, T. J., Nave, K. A., Griffiths, I. R. & McLaughlin, M. PLP overexpression perturbs myelin protein composition and myelination in a mouse model of Pelizaeus-Merzbacher disease. Glia **55**, 341-351 (2007).

10 Rosenbluth, J., Schiff, R. & Lam, P. Effects of osmolality on PLP-null myelin structure: implications re axon damage. Brain Res. **1253**, 191-197 (2009).

11 Edgar, J. M., McCulloch, M. C., Montague, P., Brown, A. M., Thilemann, S., Pratola, L., Gruenenfelder, F. I., Griffiths, I. R. & Nave, K. A. Demyelination and axonal preservation in a transgenic mouse model of Pelizaeus-Merzbacher disease. EMBO Mol. Med. **2**, 42-50 (2010).

12 Jack, C. R. Jr, Dickson, D. W., Parisi, J. E., Xu, Y. C., Cha, R. H., O'Brien, P. C., Edland, S. D., Smith, G. E., Boeve, B. F., Tangalos, E. G., Kokmen, E. & Petersen, R. C. Antemortem MRI findings correlate with hippocampal neuropathology in typical aging and dementia. Neurology **58**, 750-757 (2002).

13 Peters, A. The effects of normal aging on myelin and nerve fibers: a review. J. Neurocytol. **31**, 581-593 (2002).

14 Aboul-Enein, F., Rauschka, H., Kornek, B., Stadelmann, C., Stefferl, A., Brück, W., Lucchinetti, C., Schmidbauer, M., Jellinger, K. & Lassmann, H. Preferential loss of myelin-associated glycoprotein reflects hypoxia-like white matter damage in stroke and inflammatory brain diseases. J. Neuropathol. Exp. Neurol. **62**, 25-33 (2003).

15 Di Stefano, D., Scucchi, L. F., Cosentino, L., Bosman, C. & Vecchione, A. Intraoperative diagnosis of nervous system lesions. Acta Cytol. **42**, 346-356 (1998).

16 Trapp, B. D. & Nave, K. A. Multiple sclerosis: an immune or neurodegenerative disorder? Annu. Rev. Neurosci. 31, 247-269 (2008).

17 Martino, G., Franklin, R. J., Van Evercooren, A. B. & Kerr, D. A. Stem cell transplantation in multiple sclerosis: current status and future prospects. Nat. Rev. Neurol. **6**, 247-255 (2010).

18 Dwork, A. J., Mancevski, B. & Rosoklija, G. White matter and cognitive function in schizophrenia. Int. J. Neuropsychopharmacol. **10**, 513-536 (2007).

19 Ihara, M., Polvikoski T. M., Hall R., Slade J. Y., Perry R. H., Oakley A. E., Englund E., O'Brien J. T., Ince P. G. & Kalaria R.N. Quantification of myelin loss in frontal lobe white matter in vascular dementia, Alzheimer's disease, and dementia with Lewy bodies. Acta Neuropathol. **119**, 579-589 (2010).

20 Griffiths, I., Klugmann, M., Anderson, T., Yool, D., Thomson, C., Schwab, M. H., Schneider, A., Zimmermann, F., McCulloch, M., Nadon, N. & Nave, K. A. Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. Science **280**, 1610-1613 (1998).

21 Lappe-Siefke, C., Goebbels, S., Gravel, M., Nicksch, E., Lee, J., Braun, P. E., Griffiths, I. R. & Nave K. A. Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. Nat. Genet. **33**, 366-374 (2003).

22 Kassmann, C. M., Lappe-Siefke, C., Baes, M., Brügger, B., Mildner, A., Werner, H. B., Natt, O., Michaelis, T., Prinz, M., Frahm, J. & Nave, K. A. Axonal loss and neuroinflammation caused by peroxisome-deficient oligodendrocytes. Nat. Genet. **39**, 969-976 (2007).

23 Moser, H. W., Mahmood, A. & Raymond, G. V. X-linked adrenoleukodystrophy. Nat. Clin. Pract. Neurol. **3**, 140-151(2007).

24 Franklin, R. J. & Ffrench-Constant, C. Remyelination in the CNS: from biology to therapy. Nat. Rev. Neurosci. **9**, 839-855 (2008).

25 Fogarty, M., Richardson, W. D. & Kessaris, N. A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. Development **132**, 1951-1959 (2005).

26 Spassky, N., Goujet-Zalc, C., Parmantier, E., Olivier, C., Martinez, S., Ivanova, A., Ikenaka, K., Macklin, W., Cerruti, I., Zalc, B. & Thomas, J.L. Multiple restricted origin of oligodendrocytes. J. Neurosci. **18**, 8331-8343 (1998).

27 Spassky, N., Olivier, C., Perez-Villegas, E., Goujet-Zalc, C., Martinez, S., Thomas, J. & Zalc, B. Single or multiple oligodendroglial lineages: a controversy. Glia **29**, 143-148 (2000).

28 Takebayashi, H., Nabeshima, Y., Yoshida, S., Chisaka, O. & Ikenaka, K. The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. Curr. Biol. **12**, 1157-1163 (2002).

29 Sun, T., Pringle, N. P., Hardy, A. P., Richardson, W. D. & Smith, H. K. Pax6 influences the time and site of origin of glial precursors in the ventral neural tube. Mol. Cell. Neurosci. **12**, 228-239 (1998).

30 Richardson, W. D., Pringle, N. P., Yu, W. P. & Hall, A. C. Origins of spinal cord oligodendrocytes: possible developmental and evolutionary relationships with motor neurons. Dev. Neurosci. **19**, 58-68 (1997).

31 Cai, J., Qi, Y., Hu, X., Tan, M., Liu, Z., Zhang, J., Li, Q., Sander, M, & Qiu, M. Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. Neuron **45**, 41-53 (2005).

32 Vallstedt, A., Klos, J. M. & Ericson, J. Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. Neuron **45**, 55-67 (2005).

33 Lu, Q. R., Sun, T., Zhu, Z., Ma, N., Garcia, M., Stiles, S. D. & Rowitch, D. H. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell **109**, 75-86 (2002).

34 Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M. & Richardson, W. D. Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. Nat. Neurosci. **9**, 173-179 (2006).

35 Levison, S. W. & Goldman, J. E. Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. Neuron **10**, 201-212 (1993).

36 Levison, S. W. & Goldman, J. E. Multipotential and lineage restricted precursors coexist in the mammalian perinatal subventricular zone. J. Neurosci. Res. **48**, 83-94 (1997).

37 Luskin, M. B. & McDermott, K. Divergent lineages for oligodendrocytes and astrocytes originating in the neonatal forebrain subventricular zone. Glia **11**, 211-226 (1994).

38 Young, K. M., Fogarty, M., Kessaris, N. & Richardson, W. D. Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. J. Neurosci. **27**, 8286-8296 (2007).

39 Lu, Q. R., Yuk, D., Alberta, J. A., Zhu, Z., Pawlitzky, I., Chan, J., McMahon, A. P., Stiles, C. D. & Rowitch, D. H. Sonic hedgehog--regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. Neuron **25**, 317-329 (2000).

40 Takebayashi, H.,Yoshida, S., Sugimori, M., Kosako, H., Kominami, R., Nakafuku, M. & Nabeshima, Y. Dynamic expression of basic helix-loop-helix Olig family members: implication of Olig2 in neuron and oligodendrocyte differentiation and identification of a new member, Olig3. Mech. Dev. **99**, 143-148 (2000).

41 Zhou, Q., Wang, S. & Anderson, D. J. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron **25**, 331-343 (2000).

42 Zhou, Q. & Anderson, D. J. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell **109**, 61-73 (2002).

43 Maire, C. L., Buchet, D., Kerninon, C., Deboux, C., Baron-Van Evercooren, A. & Nait-Oumesmar, B. Directing human neural stem/precursor cells into oligodendrocytes by overexpression of Olig2 transcription factor. J. Neurosci. Res. **87**, 3438-3446 (2009).

44 Maire, C. L., Wegener, A., Kerninon, C. & Nait-Oumesmar, B. Gain-of-Function of Olig Transcription Factors Enhances Oligodendrogenesis and Myelination. Stem Cells **9**, 1611-22 (2010).

45 Tekki-Kessaris, N., Woodruff, R., Hall, A. C., Gaffield, W., Kimura, S., Stiles, C. D., Rowitch, D. H. & Richardson, W. D. Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon. Development **128**, 2545-2554 (2001).

46 Kessaris, N., Jamen, F., Rubin, L. L. & Richardson, W. D. Cooperation between sonic hedgehog and fibroblast growth factor/MAPK signalling pathways in neocortical precursors. Development **131**, 1289-1298 (2004).

47 Orentas, D. M., Hayes, J. E., Dyer, K. L. & Miller, R. H. Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors. Development **126**, 2419-2429 (1999).

48 Nery, S., Wichterle, H. & Fishell, G. Sonic hedgehog contributes to oligodendrocyte specification in the mammalian forebrain. Development **128**, 527-540 (2001).

49 Chandran, S., Kato, H., Gerreli, D., Compston, A., Svendsen, C. N. & Allen, N. D. FGF-dependent generation of oligodendrocytes by a hedgehog-independent pathway. Development **130**, 6599-6609 (2003).

50 Temple, S. & Raff, M. C. Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions. Cell **44**, 773-779 (1986).

51 Barres, B. A., Lazar, M. A. & Raff, M. C. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development **120**, 1097-1108 (1994).

52 Gao, F. B., Durand, B. & Raff, M. Oligodendrocyte precursor cells count time but not cell divisions before differentiation. Curr. Biol. **7**, 152-155 (1997).

53 Fruttiger, M., Calver, A. R. & Richardson, W. D. Platelet-derived growth factor is constitutively secreted from neuronal cell bodies but not from axons. Curr. Biol. **10**, 1283-1286 (2000).

54 Raff, M. C., Lillien, L. E., Richardson, W. D., Burne, J. F. & Noble, M. D. Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. Nature **333**, 562-565 (1988).

55 Fruttiger, M., Karlsson, L., Hall, A. C., Abramsson, A., Calver, A. R., Bostrom, H., Willetts, K., Bertold, C. H., Heath, J. K., Betsholtz, C. & Richardson, W. D. Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. Development **126**, 457-467 (1999).

56 Calver, A. R., Hall, A. C., Yu, W. P., Walsh, F. S., Heath, J. K., Betsholtz, C. & Richardson, W. D. Oligodendrocyte population dynamics and the role of PDGF in vivo. Neuron **20**, 869-882 (1998).

57 Hart, I. K., Richardson, W. D., Bolsover, S. R. & Raff, M. C. PDGF and intracellular signaling in the timing of oligodendrocyte differentiation. J. Cell. Biol. **109**, 3411-3417 (1989).

58 Hart, I. K., Richardson, W. D. & Raff, M. C. PDGF increases the expression of Fos and Jun in newly formed oligodendrocytes that have become resistant to the mitogenic effect of PDGF. Glia **6**, 310-313 (1992).

59 Durand, B., Fero, M. L., Roberts, J. M. & Raff, M. C. p27Kip1 alters the response of cells to mitogen and is part of a cell-intrinsic timer that arrests the cell cycle and initiates differentiation. Curr. Biol. **8**, 431-440 (1998).

60 Eccleston, P. A. & Silberberg, D. H. The differentiation of oligodendrocytes in a serum-free hormone-supplemented medium. Brain. Res. **318**, 1-9 (1984).

61 McMorris, F. A. & Dubois-Dalcq, M. Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. J. Neurosci. Res. **21**, 199-209 (1988).

62 Canoll, P. D., Musacchio, J. M., Hardy, R., Reynolds, R., Marchionni, M. A. & Salzer, J. L. GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. Neuron **17**, 229-243 (1996).

63 Cohen, R. I., Marmur, R., Norton, W. T., Mehler, M. F. & Kessler, J. A. Nerve growth factor and neurotrophin-3 differentially regulate the proliferation and survival of developing rat brain oligodendrocytes. J. Neurosci. **16**, 6433-6442 (1996).

64 Barres, B. A., Burne, J. F., Holtmann, B., Thoenen, H., Sendtner, M. & Raff, M. C. Ciliary neurotrophic factor enhances the rate of oligodendrocyte generation. Mol. Cell. Neurosci. **8**, 146-156 (1996).

65 Bogler, O., Wren, D., Barnett, S. C., Land, H. & Noble, M. Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Proc. Nat. Aca. Sci. **87**, 6368-6372 (1990).

66 McKinnon, R. D., Matsui, T., Dubois-Dalcq, M. & Aaronson, S. A. FGF modulates the PDGF-driven pathway of oligodendrocyte development. Neuron **5**, 603-614 (1990).

67 Barres, B. A. & Raff, M. C. Control of oligodendrocyte number in the developing rat optic nerve. Neuron **12**, 935-942 (1994).

68 McKinnon, R. D., Piras, G., Ida, J. A., Jr. & Dubois-Dalcq, M. A role for TGF-beta in oligodendrocyte differentiation. J. Cell Biol. **121**, 1397-1407 (1993).

69 Morgan, D. O. Principles of CDK regulation. Nature **374**, 131-134 (1995).

70 Dugas, J. C., Ibrahim, A. & Barres, B. A. A crucial role for p57(Kip2) in the intracellular timer that controls oligodendrocyte differentiation. J. Neurosci. **27**, 6185-6196 (2007).

71 Tokumoto, Y. M., Apperly, J. A., Gao, F. B. & Raff, M. C. Posttranscriptional regulation of p18 and p27 Cdk inhibitor proteins and the timing of oligodendrocyte differentiation. Dev. Biol. **245**, 224-234 (2002).

72 Jeannin, E., Robyr, D. & Desvergne, B. Transcriptional regulatory patterns of the myelin basic protein and malic enzyme genes by the thyroid hormone receptors alpha1 and beta1. J. Biol. Chem. **273**, 24239-24248 (1998).

73 Noble, M. & Murray, K. Purified astrocytes promote the in vitro division of a bipotential glial progenitor cell. EMBO J. **3**, 2243-2247 (1984).

74 Temple, S. & Raff, M. C. Differentiation of a bipotential glial progenitor cell in a single cell microculture. Nature **313**, 223-225 (1985).

75 Ibarrola, N., Mayer-Proschel, M., Rodriguez-Pena, A. & Noble, M. Evidence for the existence of at least two timing mechanisms that contribute to oligodendrocyte generation in vitro. Dev. Biol. **180** (1996).

76 Rodriguez-Pena, A., Ibarrola, N., Iniguez, M. A., Munoz, A. & Bernal, J. Neonatal hypothyroidism affects the timely expression of myelin-associated glycoprotein in the rat brain. J. Clin. Invest. **91**, 812-818 (1993).

77 Walters, S. N. & Morell, P. Effects of altered thyroid states on myelinogenesis. J. Neurochem. **36**, 1792-1801 (1981).

78 Ahlgren, S. C., Wallace, H., Bishop, J., Neophytou, C. & Raff, M. C. Effects of thyroid hormone on embryonic oligodendrocyte precursor cell development in vivo and in vitro. Mol. Cell. Neurosci. **9**, 420-432 (1997).

79 Baas, D., Legrand, C., Samarut, J. & Flamant, F. Persistence of oligodendrocyte precursor cells and altered myelination in optic nerve associated to retina degeneration in mice devoid of all thyroid hormone receptors. Proc. Nat. Aca. Sci. **99**, 2907-2911 (2002).

80 Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G. & Barres, B. A. Notch receptor activation inhibits oligodendrocyte differentiation. Neuron **21**, 63-75 (1998).

81 Grandbarbe, L., Bouissac, J., Rand, M., Hrabe de Angelis, M., Artavanis-Tsakonas, S. & Mohier, E. Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. Development **130**, 1391-1402 (2003).

82 Genoud, S., Lappe-Siefke, C., Goebbels, S., Radtke, F., Aguet, M., Scherer, S. S., Suter, U., Nave, K. A. & Mantei, N. Notch1 control of oligodendrocyte differentiation in the spinal cord. J. Cell Biol. **158**, 709-718 (2002).

83 Givogri, M. I., Costa, R. M., Schonmann, V., Silva, A. J., Campagnoni, A. T. & Bongarzone, E. R. Central nervous system myelination in mice with deficient expression of Notch1 receptor. J. Neurosci. Res. **67**, 309-320 (2002).

84 Zhang, Y., Argaw, A. T., Gurfein, B. T., Zameer, A., Snyder, B. J., Ge, C., Lu, Q. R., Rowitch, D. H., Raine, C. S., Brosnan, C. F. & John, G. R. Notch1 signaling plays a role in regulating precursor differentiation during CNS remyelination. Proc. Nat. Aca. Sci. **106**, 19162-19167 (2009).

85 Hu, Q. D., Ang, B. T., Karsak, M., Hu, W. P., Cui, X. Y., Duka, T., Takeda, Y., Chia, W., Sankar, N., Ng, Y. K., Ling, E. A., Maciag, T., Small, D., Trifonova, R., Kopan, R., Okano, H., Nakafuku, M., Chiba, S., Hirai, H., Aster, J. C., Schachner, M., Pallen, C. J., Watanabe, K. & Xiao, Z. C. F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. Cell **115**, 163-175 (2003).

86 Hu, Q. D., Ma, Q. H., Gennarini, G. & Xiao, Z. C. Cross-talk between F3/contactin and Notch at axoglial interface: a role in oligodendrocyte development. Dev. Neurosci. **28**, 25-33 (2006).

87 Colognato, H., Baron, W., Avellana-Adalid, V., Relvas, J. B., Baron-Van Evercooren, A., Georges-Labouesse, E. & ffrench-Constant, C. CNS integrins switch growth factor signalling to promote target-dependent survival. Nat. Cell Biol. **4**, 833-841 (2002).

88 Colognato, H. & Yurchenco, P. D. Form and function: the laminin family of heterotrimers. Dev. Dyn. **218**, 213-234 (2000).

89 Relucio, J., Tzvetanova, I. D., Ao, W., Lindquist, S. & Colognato, H. Laminin alters fyn regulatory mechanisms and promotes oligodendrocyte development. J. Neurosci. **29**, 11794-11806 (2009).

90 Buttery, P. C. & ffrench-Constant, C. Laminin-2/integrin interactions enhance myelin membrane formation by oligodendrocytes. Mol. Cell. Neurosci. **14**, 199-212 (1999).

91 Chudakova, D. A., Zeidan, Y. H., Wheeler, B. W., Yu, J., Novgorodov, S. A., Kindy, M. S., Hannun, Y. A. & Gudz, T. I. Integrin-associated Lyn kinase promotes cell survival by suppressing acid sphingomyelinase activity. J. Biol. Chem. **283**, 28806-28816 (2008).

92 Colognato, H., Galvin, J., Wang, Z., Relucio, J., Nguyen, T., Harrison, D., Yurchenco, P. D. & ffrench-Constant, C. Identification of dystroglycan as a second laminin receptor in oligodendrocytes, with a role in myelination. Development **134**, 1723-1736 (2007).

93 Relvas, J. B., Setzu, A., Baron, W., Buttery, P. C., LaFlamme, S. E., Franklin, R. J. & ffrench-Constant, C. Expression of dominant-negative and chimeric subunits reveals an essential role for beta1 integrin during myelination. Curr. Biol. **11**, 1039-1043 (2001).

94 Buttery, P. C. & ffrench-Constant, C. Process extension and myelin sheet formation in maturing oligodendrocytes. Prog. Brain Res. **132**, 115-130 (2001).

95 Benninger, Y., Colognato, H., Thurnherr, T., Franklin, R. J., Leone, D. P., Atanasoski, S., Nave, K. A., ffrench-Constant, C., Suter, U. & Relvas, J. B. Beta1-integrin signaling

mediates premyelinating oligodendrocyte survival but is not required for CNS myelination and remyelination. J. Neurosci. **26**, 7665-7673 (2006).

96 Barros, C. S., Nguyen, T., Spencer, K. S., Nishiyama, A., Colognato, H. & Muller, U. Beta1 integrins are required for normal CNS myelination and promote AKT-dependent myelin outgrowth. Development **136**, 2717-2724 (2009).

97 Zezula, J., Casaccia-Bonnefil, P., Ezhevsky, S. A., Osterhout, D. J., Levine, J. M., Dowdy, S. F., Chao, M. V. & Koff, A. p21cip1 is required for the differentiation of oligodendrocytes independently of cell cycle withdrawal. EMBO Rep. **2**, 27-34 (2001).

98 Tokumoto, Y. M., Tang, D. G. & Raff, M. C. Two molecularly distinct intracellular pathways to oligodendrocyte differentiation: role of a p53 family protein. EMBO J. **20**, 5261-5268 (2001).

99 Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D. & Raff, M. C. Cell death and control of cell survival in the oligodendrocyte lineage. Cell **70**, 31-46 (1992).

100 David, S., Miller, R. H., Patel, R. & Raff, M. C. Effects of neonatal transection on glial cell development in the rat optic nerve: evidence that the oligodendrocyte-type 2 astrocyte cell lineage depends on axons for its survival. J. Neurocytol. **13**, 961-974 (1984).

101 Burne, J. F., Staple, J. K. & Raff, M. C. Glial cells are increased proportionally in transgenic optic nerves with increased numbers of axons. J. Neurosci. **16**, 2064-2073 (1996).

102 Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D. & Raff, M. C. Cell death in the oligodendrocyte lineage. J. Neurobio. **23**, 1221-1230 (1992).

103 Barres, B. A., Schmid, R., Sendnter, M. & Raff, M. C. Multiple extracellular signals are required for long-term oligodendrocyte survival. Development **118**, 283-295 (1993).

104 Fernandez, P. A., Tang, D. G., Cheng, L., Prochiantz, A., Mudge, A. W. & Raff, M. C. Evidence that axon-derived neuregulin promotes oligodendrocyte survival in the developing rat optic nerve. Neuron **28**, 81-90 (2000).

105 Frederick, T. J., Min, J., Altieri, S. C., Mitchell, N. E. & Wood, T. L. Synergistic induction of cyclin D1 in oligodendrocyte progenitor cells by IGF-I and FGF-2 requires differential stimulation of multiple signaling pathways. Glia **55**, 1011-1022 (2007).

106 Cui, Q. L. & Almazan, G. IGF-I-induced oligodendrocyte progenitor proliferation requires PI3K/Akt, MEK/ERK, and Src-like tyrosine kinases. J. Neurochem. **100**, 1480-1493 (2007).

107 Cui, Q. L., Zheng, W. H., Quirion, R. & Almazan, G. Inhibition of Src-like kinases reveals Akt-dependent and -independent pathways in insulin-like growth factor I-mediated oligodendrocyte progenitor survival. J. Biol. Chem. **280**, 8918-8928 (2005).

108 Colognato, H., Ramachandrappa, S., Olsen, I. M. & ffrench-Constant, C. Integrins direct Src family kinases to regulate distinct phases of oligodendrocyte development. J. Cell Biol. **167**, 365-375 (2004).

109 Voyvodic, J. T. Target size regulates calibre and myelination of sympathetic axons. Nature **342**, 430-433 (1989).

110 Barres, B. A. & Raff, M. C. Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. Nature **361**, 258-260 (1993).

111 Tauber, H., Waehneldt, T. V. & Neuhoff, V. Myelination in rabbit optic nerves is accelerated by artificial eye opening. Neurosci. Lett. **16**, 235-238 (1980).

112 Omlin, F. X. Optic disc and optic nerve of the blind cape mole-rat (Georychus capensis): a proposed model for naturally occurring reactive gliosis. Brain Res. Bull. **44**, 627-632 (1997).

113 Carre, J. L., Demerens, C., Rodriguez-Pena, A., Floch, H. H., Vincendon, G. & Sarlieve, L. L. Thyroid hormone receptor isoforms are sequentially expressed in oligodendrocyte lineage cells during rat cerebral development. J. Neurosci. Res. **54**, 584-594 (1998).

114 Maire, J. C., Medilanski, J. & Straub, R. W. Release of adenosine, inosine and hypoxanthine from rabbit non-myelinated nerve fibres at rest and during activity. J. Physiol. **357**, 67-77 (1984).

115 Kuperman, A. S., Volpert, W. A. & Okamoto, M. Release of Adenine Nucleotide from Nerve Axons. Nature **204**, 1000-1001 (1964).

116 Stevens, B., Porta, S., Haak, L. L., Gallo, V. & Fields, R. D. Adenosine: a neuron-glial transmitter promoting myelination in the CNS in response to action potentials. Neuron **36**, 855-868 (2002).

117 Ishibashi, T., Dakin, K. A., Stevens, B., Lee, P. R., Kozlov, S. V., Stewart, C. L. & Fields, R. D. Astrocytes promote myelination in response to electrical impulses. Neuron **49**, 823-832 (2006).

118 Bugga, L., Gadient, R. A., Kwan, K., Stewart, C. L. & Patterson, P. H. Analysis of neuronal and glial phenotypes in brains of mice deficient in leukemia inhibitory factor. J. Neurobio. **36**, 509-524 (1998).

119 Stevens, B. & Fields, R. D. Response of Schwann cells to action potentials in development. Science **287**, 2267-2271 (2000).

120 Mignot, C., Boespflug-Tanguy, O., Gelot, A., Dautigny, A., Pham-Dinh, D. & Rodriguez, D. Alexander disease: putative mechanisms of an astrocytic encephalopathy. Cell. Mol. Life Sci. **61**, 369-385 (2004).

121 Chan, J. R., Watkins, T. A., Cosgaya, J. M., Zhang, C., Chen, L., Reichardt, L. F., Shooter, E. M. & Barres, B. A. NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes. Neuron **43**, 183-191 (2004).

122 Lee, X., Yang, Z., Shao, Z., Rosenberg, S. S., Levesque, M., Pepinsky, R. B., Qiu, M., Miller, R. H., Chan, J. R. & Mi, S. NGF regulates the expression of axonal LINGO-1 to inhibit oligodendrocyte differentiation and myelination. J. Neurosci. **27**, 220-225 (2007).

123 Mi, S., Miller, R. H., Lee, X., Scott, M. L., Shulag-Morskaya, S., Shao, Z., Chang, J., Thill, G., Levesque, M., Zhang, M., Hession, C., Sah, D., Trapp, B., He, Z., Jung, V., McCoy, J. M. & Pepinsky, R. B. LINGO-1 negatively regulates myelination by oligodendrocytes. Nat. Neurosci. **8**, 745-751 (2005).

124 Jakovcevski, I., Mo, Z. & Zecevic, N. Down-regulation of the axonal polysialic acid-neural cell adhesion molecule expression coincides with the onset of myelination in the human fetal forebrain. Neuroscience **149**, 328-337 (2007).

125 Lubetzki, C., Charles, P., Stankoff, B., Hernandez, P. & Zalc, B. Pivotal role of axonal adhesion molecules in central nervous system myelination. Neurol. Neurochir. Pol. **34**, 41-44 (2000).

126 Winkler, S., Stahl, R. C., Carey, D. J. & Bansal, R. Syndecan-3 and perlecan are differentially expressed by progenitors and mature oligodendrocytes and accumulate in the extracellular matrix. J. Neurosci. Res. **69**, 477-487 (2002).

127 Charles, P., Reynolds, R., Seilhean, D., Rougon, G., Aigrot, M. S., Niezgoda, A., Zalc, B. & Lubetzki, C. Re-expression of PSA-NCAM by demyelinated axons: an inhibitor of remyelination in multiple sclerosis? Brain **125**, 1972-1979 (2002).

128 Taveggia, C., Thaker, P., Petrylak, A., Caporaso, G. L., Toews, A., Falls, D. L., Einheber, S. & Salzer, J. L. Type III neuregulin-1 promotes oligodendrocyte myelination. Glia **56**, 284-293 (2008).

129 Vartanian, T., Fischbach, G. & Miller, R. Failure of spinal cord oligodendrocyte development in mice lacking neuregulin. Proc. Nat. Aca. Sci. **96**, 731-735 (1999).

130 Vartanian, T., Corfas, G., Li, Y., Fischbach, G. D. & Stefansson, K. A role for the acetylcholine receptor-inducing protein ARIA in oligodendrocyte development. Proc. Nat. Aca. Sci. **91**, 11626-11630 (1994).

131 Kim, J. Y., Sun, Q., Oglesbee, M. & Yoon, S. O. The role of ErbB2 signaling in the onset of terminal differentiation of oligodendrocytes in vivo. J. Neurosci. **23**, 5561-5571 (2003).

132 Roy, K., Murtie, J. C., El-Khodor, B. F., Edgar, N., Sardi, S. P., Hooks, B. M., Benoit-Marand, M., Chen, C., Moore, H., O'Donnell, P., Brunner, D. & Corfas, G. Loss of erbB signaling in oligodendrocytes alters myelin and dopaminergic function, a potential mechanism for neuropsychiatric disorders. Proc. Nat. Aca. Sci. **104**, 8131-8136 (2007).

133 Brinkmann, B. G., Agarwal, A., Sereda, M. W., Garratt, A. N., Muller, T., Wende, H., Stassart, R. M., Nawaz, S., Humml, C., Velanac, V., Radyushkin, K., Goebbels, S., Fischer, T. M., Franklin, R. J., Lai, C., Ehrenreich, H., Birchmeier, C., Schwab, M. H. & Nave, K. A. Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. Neuron **59**, 581-595 (2008).

134 Beck, K. D., Powell-Braxton, L., Widmer, H. R., Valverde, J. & Hefti, F. Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. Neuron **14**, 717-730 (1995).

135 Ye, P., Carson, J. & D'Ercole, A. J. In vivo actions of insulin-like growth factor-I (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice. J. Neurosci. **15**, 7344-7356 (1995).

136 Carson, M. J., Behringer, R. R., Brinster, R. L. & McMorris, F. A. Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. Neuron **10**, 729-740 (1993).

137 Hudson, L. D., Friedrich, V. L., Jr., Behar, T., Dubois-Dalcq, M. & Lazzarini, R. A. The initial events in myelin synthesis: orientation of proteolipid protein in the plasma membrane of cultured oligodendrocytes. J. Cell Biol. **109**, 717-727 (1989).

138 Simons, M., Kramer, E. M., Macchi, P., Rathke-Hartlieb, S., Trotter, J., Nave, K. A. & Schulz, J. B. Overexpression of the myelin proteolipid protein leads to accumulation of cholesterol and proteolipid protein in endosomes/lysosomes: implications for Pelizaeus-Merzbacher disease. J. Cell Biol. **157**, 327-336 (2002).

139 Kramer, E. M., Schardt, A. & Nave, K. A. Membrane traffic in myelinating oligodendrocytes. Microsc. Res. Tech. **52**, 656-671 (2001).

140 Trajkovic, K., Dhaunchak, A. S., Goncalves, J. T., Wenzel, D., Schneider, A., Bunt, G., Nave, K. A. & Simons, M. Neuron to glia signaling triggers myelin membrane exocytosis from endosomal storage sites. J. Cell Biol. **172**, 937-948 (2006).

141 Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. & Tsichlis, P. N.The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell **81**, 727-736 (1995).

142 Jaillard, C., Harrison, S., Stankoff, B., Aigrot, M. S., Calver, A. R., Duddy, G., Walsh, F. S., Pangalos, M. N., Arimura, N., Kaibuchi, K., Zalc, B. & Lubetzki, C. Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. J. Neurosci. **25**, 1459-1469 (2005).

143 Flores, A. I., Narayanan, S. P., Morse, E. N., Shick, H. E., Yin, X., Kidd, G., Avila, R. L., Kirschner, D. A. & Macklin, W. B. Constitutively active Akt induces enhanced myelination in the CNS. J. Neurosci. **28**, 7174-7183 (2008).

144 Narayanan, S. P., Flores, A. I., Wang, F. & Macklin, W. B. Akt signals through the mammalian target of rapamycin pathway to regulate CNS myelination. J. Neurosci. **29**, 6860-6870 (2009).

145 Goebbels, S., Oltrogge, J. H., Kemper, R., Heilmann, I., Bormuth, I., Wolfer, S., Wichert, S. P., Mobius, W., Liu, X., Lappe-Siefke, C., Rossner, M. J., Groszer, M., Suter, U., Frahm, J., Boretius, S. & Nave, K. A. Elevated phosphatidylinositol 3,4,5-trisphosphate in glia triggers cell-autonomous membrane wrapping and myelination. J. Neurosci. **30**, 8953-8964 (2010).

146 Umemori, H., Wanaka, A., Kato, H., Takeuchi, M., Tohyama, M. & Yamamoto, T. Specific expressions of Fyn and Lyn, lymphocyte antigen receptor-associated tyrosine kinases, in the central nervous system. Brain Res. Mol. Brain Res. **16**, 303-310 (1992).

147 Sperber, B. R., Boyle-Walsh, E. A., Engleka, M. J., Gadue, P., Peterson, A. C., Stein, P. L., Scherer, S. S. & McMorris, F. A. A unique role for Fyn in CNS myelination. J. Neurosci. **21**, 2039-2047 (2001).

148 Osterhout, D. J., Wolven, A., Wolf, R. M., Resh, M. D. & Chao, M. V. Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. J Cel.l Biol. **145**, 1209-1218 (1999).

149 Umemori, H., Sato, S., Yagi, T., Aizawa, S. & Yamamoto, T. Initial events of myelination involve Fyn tyrosine kinase signalling. Nature **367**, 572-576 (1994).

150 Kramer, E. M., Klein, C., Koch, T., Boytinck, M. & Trotter, J. Compartmentation of Fyn kinase with glycosylphosphatidylinositol-anchored molecules in oligodendrocytes facilitates kinase activation during myelination. J. Biol. Chem. **274**, 29042-29049 (1999).

151 Umemori, H., Kadowaki, Y., Hirosawa, K., Yoshida, Y., Hironaka, K., Okano, H. & Yamamoto, T. Stimulation of myelin basic protein gene transcription by Fyn tyrosine kinase for myelination. J. Neurosci. **19**, 1393-1397 (1999).

152 Goto, J., Tezuka, T., Nakazawa, T., Sagara, H. & Yamamoto, T. Loss of Fyn tyrosine kinase on the C57BL/6 genetic background causes hydrocephalus with defects in oligodendrocyte development. Mol. Cell. Neurosci. **38**, 203-212 (2008).

153 Goto, J., Tezuka, T., Nakazawa, T., Tsukamoto, N., Nakamura, T., Ajima, R., Yokoyama, K., Ohta, T., Ohki, M. & Yamamoto, T. Altered gene expression in the adult brain of fyn-deficient mice. Cell. Mol. Neurobiol. **24**, 149-159 (2004).

154 Lu, Z., Ku, L., Chen, Y. & Feng, Y. Developmental abnormalities of myelin basic protein expression in fyn knock-out brain reveal a role of Fyn in posttranscriptional regulation. J. Biol. Chem. **280**, 389-395 (2005).

155 White, R., Gonsior, C., Kramer-Albers, E. M., Stohr, N., Huttelmaier, S. & Trotter, J. Activation of oligodendroglial Fyn kinase enhances translation of mRNAs transported in hnRNP A2-dependent RNA granules. J. Cell Biol. **181**, 579-586 (2008).

156 Sperber, B. R. & McMorris, F. A. Fyn tyrosine kinase regulates oligodendroglial cell development but is not required for morphological differentiation of oligodendrocytes. J. Neurosci. Res. **63**, 303-312 (2001).

157 McMorris, F. A., Smith, T. M., DeSalvo, S. & Furlanetto, R. W. Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development. Proc. Nat. Aca. Sci. **83**, 822-826 (1986).

158 Liang, X., Draghi, N. A. & Resh, M. D. Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. J. Neurosci. **24**, 7140-7149 (2004).

159 Wolf, R. M., Wilkes, J. J., Chao, M. V. & Resh, M. D. Tyrosine phosphorylation of p190 RhoGAP by Fyn regulates oligodendrocyte differentiation. J. Neurobio. **49**, 62-78 (2001).

160 Hoshina, N., Tezuka, T., Yokoyama, K., Kozuka-Hata, H., Oyama, M. & Yamamoto, T. Focal adhesion kinase regulates laminin-induced oligodendroglial process outgrowth. Genes Cells **12**, 1245-1254 (2007).

161 Rajasekharan, S., Baker, K. A., Horn, K. E., Jarjour, A. A., Antel, J. P. & Kennedy, T. E. Netrin 1 and Dcc regulate oligodendrocyte process branching and membrane extension via Fyn and RhoA. Development **136**, 415-426 (2009).

162 Boggon, T. J. & Eck, M. J. Structure and regulation of Src family kinases. Oncogene **23**, 7918-7927 (2004).

163 Klein, C., Kramer, E. M., Cardine, A. M., Schraven, B., Brandt, R. & Trotter, J. Process outgrowth of oligodendrocytes is promoted by interaction of fyn kinase with the cytoskeletal protein tau. J. Neurosci. **22**, 698-707 (2002).

164 Miyamoto, Y., Yamauchi, J. & Tanoue, A. Cdk5 phosphorylation of WAVE2 regulates oligodendrocyte precursor cell migration through nonreceptor tyrosine kinase Fyn. J. Neurosci. **28**, 8326-8337 (2008).

165 Poltorak, M., Sadoul, R., Keilhauer, G., Landa, C., Fahrig, T. & Schachner, M. Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interaction. J. Cell Biol. **105**, 1893-1899 (1987).

166 Olive, S., Dubois, C., Schachner, M. & Rougon, G. The F3 neuronal glycosylphosphatidylinositol-linked molecule is localized to glycolipid-enriched membrane subdomains and interacts with L1 and fyn kinase in cerebellum. J. Neurochem. **65**, 2307-2317 (1995).

167 Laursen, L. S., Chan, C. W. & ffrench-Constant, C. An integrin-contactin complex regulates CNS myelination by differential Fyn phosphorylation. J. Neurosci. **29**, 9174-9185 (2009).

168 Watzlawik, J., Holicky, E., Edberg, D. D., Marks, D. L., Warrington, A. E., Wright, B. R., Pagano, R. E. & Rodriguez, M. Human remyelination promoting antibody inhibits apoptotic signaling and differentiation through Lyn kinase in primary rat oligodendrocytes. Glia, **58**, 1782-93 (2010).

169 Du, C. & Sriram, S. Increased severity of experimental allergic encephalomyelitis in lyn-/- mice in the absence of elevated proinflammatory cytokine response in the central nervous system. J. Immunol. **168**, 3105-3112 (2002).

170 Chong, Y. P., Ia, K. K., Mulhern, T. D. & Cheng, H. C. Endogenous and synthetic inhibitors of the Src-family protein tyrosine kinases. Biochim. Biophys. Acta **1754**, 210-220 (2005).

171 Resh, M. D. Interaction of tyrosine kinase oncoproteins with cellular membranes. Biochim. Biophys. Acta **1155**, 307-322 (1993).

172 Shenoy-Scaria, A. M., Gauen, L. K., Kwong, J., Shaw, A. S. & Lublin, D. M. Palmitylation of an amino-terminal cysteine motif of protein tyrosine kinases p56lck and p59fyn mediates interaction with glycosyl-phosphatidylinositol-anchored proteins. Mol. Cell. Biol. **13**, 6385-6392 (1993).

173 Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T. & Nakagawa, H. CSK: a protein-tyrosine kinase involved in regulation of src family kinases. J. Biol. Chem. **266**, 24249-24252 (1991).

174 Hamaguchi, I., Yamaguchi, N., Suda, J., Iwama, A., Hirao, A., Hashiyama, M., Aizawa, S. & Suda, T. Analysis of CSK homologous kinase (CHK/HYL) in hematopoiesis by utilizing gene knockout mice. Biochem. Biophys. Res. Commun. **224**, 172-179 (1996).

175 Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M. & Aizawa, S. Constitutive activation of Src family kinases in mouse embryos that lack Csk. Cell **73**, 1125-1135 (1993).

176 Imamoto, A. & Soriano, P. Disruption of the csk gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. Cell **73**, 1117-1124 (1993).

177 Samokhvalov, I., Hendrikx, J., Visser, J., Belyavsky, A., Sotiropolous, D. & Gu, H. Mice lacking a functional chk gene have no apparent defects in the hematopoietic system. Biochem. Mol. Biol. Int. **43**, 115-122 (1997).

178 Alland, L., Peseckis, S. M., Atherton, R. E., Berthiaume, L. & Resh, M. D. Dual myristylation and palmitylation of Src family member p59fyn affects subcellular localization. J. Biol. Chem. **269**, 16701-16705 (1994).

179 Kawabuchi, M., Satomi, Y., Takao, T., Shimonishi, Y., Nada, S., Nagai, K., Tarakhovsky, A. & Okada, M. Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature **404**, 999-1003 (2000).

180 Brdicka, T., Pavlistova, D., Leo, A., Bruyns, E., Korinek, V., Angelisova, P., Scherer, J., Shevchenko, A., Hilgert, I., Cerny, J., Drbal, K., Kuramitsu, Y., Kornacker, B., Horejsi, V. & Schraven, B. Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. J. Exp. Med, **191**, 1591-1604 (2000).

181 Lindquist, J. A., Simeoni, L. & Schraven, B. Transmembrane adapters: attractants for cytoplasmic effectors. Immunol. Rev. **191**, 165-182 (2003).

182 Tobe, K., Sabe, H., Yamamoto, T., Yamauchi, T., Asai, S., Kaburagi, Y., Tamemoto, H., Ueki, K., Kimura, H., Akanuma, Y., Yazaki, Y., Hanafusa, H. & Kadowaki, T. Csk enhances insulin-stimulated dephosphorylation of focal adhesion proteins. Mol. Cell. Biol. **16**, 4765-4772 (1996).

183 Sabe, H., Hata, A., Okada, M., Nakagawa, H. & Hanafusa, H. Analysis of the binding of the Src homology 2 domain of Csk to tyrosine-phosphorylated proteins in the suppression and mitotic activation of c-Src. Proc. Nat. Aca. Sci. **91**, 3984-3988 (1994).

184 Kim, S. O., Avraham, S., Jiang, S., Zagozdzon, R., Fu, Y. & Avraham, H. K. Differential expression of Csk homologous kinase (CHK) in normal brain and brain tumors. Cancer **101**, 1018-1027 (2004).

185 Nakahara, J., Seiwa, C., Tan-Takeuchi, K., Gotoh, M., Kishihara, K., Ogawa, M., Asou, H. & Aiso, S. Involvement of CD45 in central nervous system myelination. Neurosci. Lett. **379**, 116-121 (2005).

186 Chow, L. M., Fournel, M., Davidson, D. & Veillette, A. Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50csk. Nature **365**, 156-160 (1993).

187 Ingley, E. Src family kinases: regulation of their activities, levels and identification of new pathways. Biochim. Biophys. Acta **1784**, 56-65 (2008).

188 Okada, M. & Nakagawa, H. A protein tyrosine kinase involved in regulation of pp60c-src function. J. Biol. Chem. **264**, 20886-20893 (1989).

189 Armstrong, E., Cannizzaro, L., Bergman, M., Huebner, K. & Alitalo, K. The c-src tyrosine kinase (CSK) gene, a potential antioncogene, localizes to human chromosome region 15q23----q25. Cytogenet. Cell. Genet. **60**, 119-120 (1992).

190 Baron, W., Decker, L., Colognato, H. & ffrench-Constant, C. Regulation of integrin growth factor interactions in oligodendrocytes by lipid raft microdomains. Curr. Biol. **13**, 151-155 (2003).

191 Sakano, S., Iwama, A., Inazawa, J., Ariyama, T., Ohno, M. & Suda, T. Molecular cloning of a novel non-receptor tyrosine kinase, HYL (hematopoietic consensus tyrosine-lacking kinase). Oncogene **9**, 1155-1161 (1994).

192 Klages, S., Adam, D., Class, K., Fargnoli, J., Bolen, J. B. & Penhallow, R. C. Ctk: a protein-tyrosine kinase related to Csk that defines an enzyme family. Proc. Nat. Aca. Sci. **91**, 2597-2601 (1994).

193 Bennett, B. D., Cowley, S., Jiang, S., London, R., Deng, B., Grabarek, J., Groopman, J. E., Goeddel, D. V. & Avraham, H. Identification and characterization of a novel tyrosine kinase from megakaryocytes. J. Biol. Chem. **269**, 1068-1074 (1994).

194 Avraham, S., Jiang, S., Ota, S., Fu, Y., Deng, B., Dowler, L. L., White, R. A. & Avraham, H. Structural and functional studies of the intracellular tyrosine kinase MATK gene and its translated product. J. Biol. Chem. **270**, 1833-1842 (1995).

195 Lee, B. C., Avraham, S., Imamoto, A. & Avraham, H. K. Identification of the nonreceptor tyrosine kinase MATK/CHK as an essential regulator of immune cells using Matk/CHK-deficient mice. Blood **108**, 904-907 (2006).

196 Ranjan, M. & Hudson, L. D. Regulation of tyrosine phosphorylation and protein tyrosine phosphatases during oligodendrocyte differentiation. Mol. Cell. Neurosci. **7**, 404-418 (1996).

197 Massa, P. T., Wu, C. & Fecenko-Tacka, K. Dysmyelination and reduced myelin basic protein gene expression by oligodendrocytes of SHP-1-deficient mice. J. Neurosci. Res. **77**, 15-25 (2004).

198 Wishcamper, C. A., Coffin, J. D. & Lurie, D. I. Lack of the protein tyrosine phosphatase SHP-1 results in decreased numbers of glia within the motheaten (me/me) mouse brain. J. Comp. Neurol. **441**, 118-133 (2001).

199 Muja, N., Lovas, G., Romm, E., Machleder, D., Ranjan, M., Gallo, V. & Hudson, L. D. Expression of a catalytically inactive transmembrane protein tyrosine phosphatase epsilon (tm-PTP epsilon) delays optic nerve myelination. Glia **48**, 278-297 (2004).

200 Brugge, J. S. & Erikson, R. L. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature **269**, 346-348 (1977).

201 Baron, W., Colognato, H. & ffrench-Constant, C. Integrin-growth factor interactions as regulators of oligodendroglial development and function. Glia **49**, 467-479 (2005).

202 Rosenberg, S. S., Kelland, E. E., Tokar, E., De la Torre, A. R. & Chan, J. R. The geometric and spatial constraints of the microenvironment induce oligodendrocyte differentiation. Proc. Nat. Aca. Sci. **105**, 14662-14667 (2008).

203 Read, R. D., Bach, E. A. & Cagan, R. L. Drosophila C-terminal Src kinase negatively regulates organ growth and cell proliferation through inhibition of the Src, Jun N-terminal kinase, and STAT pathways. Mol. Cell. Biol. **24**, 6676-6689 (2004).

204 Schmedt, C., Saijo, K., Niidome, T., Kuhn, R., Aizawa, S. & Tarakhovsky, A. Csk controls antigen receptor-mediated development and selection of T-lineage cells. Nature **394**, 901-904 (1998).

205 Chun, S. J., Rasband, M. N., Sidman, R. L., Habib, A. A. & Vartanian, T. Integrin-linked kinase is required for laminin-2-induced oligodendrocyte cell spreading and CNS myelination. J. Cell Biol. **163**, 397-408 (2003).

206 Gagnoux-Palacios, L., Dans, M., van't Hof, W., Mariotti, A., Pepe, A., Meneguzzi, G., Resh, M. D. & Giancotti, F. G. Compartmentalization of integrin alpha6beta4 signaling in lipid rafts. J. Cell Biol. **162**, 1189-1196 (2003).

207 Casaccia-Bonnefil, P., Tikoo, R., Kiyokawa, H., Friedrich, V., Jr., Chao, M. V. & Koff, A. Oligodendrocyte precursor differentiation is perturbed in the absence of the cyclin-dependent kinase inhibitor p27Kip1. Genes Dev. **11**, 2335-2346 (1997).

208 Tikoo, R., Osterhout, D. J., Casaccia-Bonnefil, P., Seth, P., Koff, A. & Chao, M. V. Ectopic expression of p27Kip1 in oligodendrocyte progenitor cells results in cell-cycle growth arrest. J. Neurobiol. **36**, 431-440 (1998).

209 Ghiani, C. A., Eisen, A. M., Yuan, X., DePinho, R. A., McBain, C. J. & Gallo, V. Neurotransmitter receptor activation triggers p27(Kip1)and p21(CIP1) accumulation and G1 cell cycle arrest in oligodendrocyte progenitors. Development **126**, 1077-1090 (1999).

210 Casaccia-Bonnefil, P., Hardy, R. J., Teng, K. K., Levine, J. M., Koff, A. & Chao, M. V. Loss of p27Kip1 function results in increased proliferative capacity of oligodendrocyte progenitors but unaltered timing of differentiation. Development **126**, 4027-4037 (1999).

211 Larocque, D., Galarneau, A., Liu, H. N., Scott, M., Almazan, G. & Richard, S. Protection of p27(Kip1) mRNA by quaking RNA binding proteins promotes oligodendrocyte differentiation. Nat. Neurosci. **8**, 27-33 (2005).

212 Barres, B. A. & Raff, M. C. Axonal control of oligodendrocyte development. J. Cell Biol. **147**, 1123-1128 (1999).

213 Shen, S., Sandoval, J., Swiss, V. A., Li, J., Dupree, J., Franklin, R. J. & Casaccia-Bonnefil, P. Age-dependent epigenetic control of differentiation inhibitors is critical for remyelination efficiency. Nat. Neurosci. **11**, 1024-1034 (2008).

214 McCarthy, K. D. & de Vellis, J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Cell Biol. **85**, 890-902 (1980).

215 Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., Xing, Y., Lubischer, J. L., Krieg, P. A., Krupenko, S. A., Thompson, W. J. & Barres, B. A. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J. Neurosci. **28**, 264-278 (2008).

216 Tokumoto, Y. M., Durand, B. & Raff, M. C. An analysis of the early events when oligodendrocyte precursor cells are triggered to differentiate by thyroid hormone, retinoic acid, or PDGF withdrawal. Dev. Biol. **213**, 327-339 (1999).

217 Kuo, E., Park, D. K., Tzvetanova, I. D., Leiton, C. V., Cho, B. S. & Colognato, H. Tyrosine phosphatases Shp1 and Shp2 have unique and opposing roles in oligodendrocyte development. J. Neurochem. **113**, 200-212 (2010).

218 Kriegstein, A. & Alvarez-Buylla, A. The Glial Nature of Embryonic and Adult Neural Stem Cells. Ann. Rev. Neurosci. **32**, 149-84 (2009)