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**ACTIVATION OF THE MAMMALIAN TARGET OF RAPAMYCIN (MTOR)
IS ESSENTIAL FOR OLIGODENDROCYTE DIFFERENTIATION**

A Thesis in

Cell and Molecular Biology

by

William Andrew Tyler

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The thesis of William Andrew Tyler was reviewed and approved* by the following:

Teresa L. Wood
Adjunct Professor of Neural and Behavioral Sciences, PSU COM
University Professor and Rena Warshow Chair in Multiple Sclerosis
Vice Chair for Basic Science Research
Department of Neurology and Neurosciences, UMDNJ
Thesis Advisor
Co-Chair of Committee

Sarah K. Bronson
Associate Professor of Cell and Molecular Physiology
Co-Chair of Committee

David A. Antonetti
Assistant Professor of Cell and Molecular Physiology

Steven W. Levison
Professor of Neurology & Neurosciences, New Jersey Medical School,
UMDNJ

David J. Carey
Associate Chief Research Officer
Director, Weis Center for Research

Henry J. Donahue
Michael and Myrtle Baker Professor of Orthopaedics
Director of Cell and Molecular Biology Graduate Program

*Signatures are on file in the Graduate School

ABSTRACT

Oligodendrocytes, the myelin producing glial cells of the central nervous system, arise from progenitor cells generated in the developing neural tube. The differentiation of oligodendrocyte progenitors into mature oligodendrocytes is a complex process that involves cell cycle exit and the coordinated expression of genes required for myelination. While both extrinsic and intrinsic factors have been identified that orchestrate the differentiation and maturation of oligodendrocytes, little is known about the intracellular signaling pathways that control the overall commitment to differentiate.

Here, we provide evidence that activation of the mammalian target of rapamycin (mTOR) is essential for oligodendrocyte differentiation. Specifically, mTOR regulates oligodendrocyte differentiation at the late progenitor to immature oligodendrocyte transition as assessed by the expression of stage specific antigens and myelin proteins including myelin basic protein (MBP) and proteolipid protein (PLP). Furthermore, phosphorylation of mTOR at Ser 2448 correlates with myelination in the subcortical white matter of the developing brain.

We further demonstrate that mTOR exerts its effects on oligodendrocyte differentiation through two distinct signaling complexes, mTORC1 and mTORC2, defined by the presence of the adaptor proteins raptor and rictor respectively. Disruption of TOR complex formation via siRNA mediated knockdown of raptor or rictor significantly reduced myelin protein expression *in vitro*. Investigation of mTORC1 and mTORC2 targets revealed differential phosphorylation during oligodendrocyte differentiation. Specifically, mTORC1 targets p70S6K1 and 4E-BP are phosphorylated

during the initial phase of differentiation, while phosphorylation of the mTORC2 target Akt appears early but is sustained throughout differentiation. Finally, disruption of p70S6K1 reduces myelin protein expression suggesting that mTORC1 functions are mediated at least in part by p70S6K1. Taken together these data define mTOR signaling as an essential mediator of oligodendrocyte differentiation and myelination.

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LIST OF ABBREVIATIONS

- β -Gal: beta-galactosidase
- Δ N: truncated amino-terminus
- 4E-BP: eIF4E-binding protein
- 5'TOP: 5'oligopyrimidine tract
- bHLH: basic helix-loop-helix
- BMP: bone morphogenetic protein
- C/EBP: CCAAT/enhancer binding protein
- cdk: cyclin dependent kinase
- cdki: cyclin dependent kinase inhibitor
- CNP: 2',3'-cyclic 3'-nucleotide phosphodiesterase
- CNS: central nervous system
- dox: doxycycline
- ECM: extracellular matrix
- eIF: eukaryotic initiation factor
- FGF: fibroblast growth factor
- GalC: galactocerebroside
- GSK: glycogen synthase kinase
- HAT: histone acetyltransferase
- HDAC: histone deacetylase
- Hox: Homeobox
- Id: inhibitor of DNA binding

IGF: insulin-like growth factor

IGF-IR: insulin-like growth factor type I receptor

Lama2^{dy}: laminin-2-deficient

LFB: luxol fast blue

MAG: myelin-associated glycoprotein

MOG: myelin oligodendrocyte glycoprotein

mTOR: mammalian target of rapamycin

NRD: negative regulatory domain

O2-A: oligodendrocyte type 2-astrocyte

OPC: oligodendrocyte progenitor cell

PDGF: platelet derived growth factor

PDGFR: platelet derived growth factor receptor

PI3K: phosphatidylinositol 3-kinase

PIKK: phosphatidylinositol 3-kinase related kinase

PKC: protein kinase-C

PLP: proteolipid protein

Rheb: Ras homolog enriched in brain

rpS6: ribosomal protein S6

rtTA: reverse tet-activator

S6K: ribosomal protein S6 kinase

Ser-Thr: serine-threonine

Shh: sonic hedgehog

Sox: SRY-box containing

T3: triiodothyronine

TA: transactivation domain

TR: thyroid hormone receptor

TSC: tuberous sclerosis complex

WM: white matter

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Chapter 1

Introduction

1.1 Oligodendrocytes and myelination

Oligodendrocytes, macroglial cells of the central nervous system (CNS), synthesize and maintain myelin sheaths which facilitate the rapid conduction of nerve impulses through saltatory conduction (Baumann and Pham-Dinh, 2001). Myelin is an extension of the oligodendrocyte plasma membrane, rich in glycolipids and specialized myelin proteins, that wraps and compacts around axon segments to provide electrical insulation. Insulation provided by myelin is essential for proper neurological function in the vertebrate CNS.

In contrast to the myelin producing Schwann cells of the peripheral nervous system which establish a one to one relationship with the axons they ensheath, one oligodendrocyte is capable of extending processes that wrap up to 60 independent axon segments (Butt and Ransom, 1989). Therefore, the loss of a single oligodendrocyte can alter the transmission of multiple neurons, leading to profound neurological deficits (Waxman, 1992).

Oligodendrocytes arise from progenitor cells generated in germinal ventricular zones of the central nervous system during late embryonic and early postnatal development after the majority of neurons and astrocytes are formed (Goldman and Vaysse, 1991; Skoff et al., 1976). To ensure proper myelination, oligodendrocyte

progenitors proliferate extensively as they migrate away from germinal foci towards their final destination, settling along axon tracts in the developing brain and spinal cord (Miller et al., 1997). Upon reaching their destination, oligodendrocyte progenitor cells exit the cell cycle and differentiate into mature myelinating oligodendrocytes (Warrington and Pfeiffer, 1992). While several extrinsic and intrinsic factors have been identified that influence the generation of mature oligodendrocytes, little is known about the molecular mechanisms that integrate these signals to regulate differentiation and myelination. The following sections will review the literature concerning the generation and differentiation of the cells of the oligodendrocyte lineage.

1.2 Oligodendrocyte progenitor specification

Oligodendrocyte progenitors arise in discrete foci during vertebrate CNS development. Specifically, oligodendrocyte progenitors are born from progenitor domains along the dorso-ventral axis of the embryonic spinal cord (Richardson et al., 2006; Rowitch, 2004; Vallstedt et al., 2005), and from the ganglionic eminences of the embryonic forebrain (Kessar et al., 2006). In addition, retroviral lineage tracing studies reveal that oligodendrocyte generation continues in the postnatal forebrain from the subventricular zones adjacent to the lateral ventricles (Levison and Goldman, 1993; Levison and Goldman, 1997; Levison et al., 1999).

Studies of the embryonic spinal cord demonstrate that the specification of oligodendrocyte progenitors is governed by local signals. Specifically, sonic hedgehog (Shh) secreted ventrally by the notochord and floor plate, and bone morphogenetic

proteins (BMPs) derived dorsally from the roof plate act to define distinct progenitor domains along the dorso-ventral axis of the spinal cord (Liem et al., 2000; Orentas et al., 1999; Rowitch, 2004). The gradient of Shh-BMP activity influences the expression of key transcription factors, including members of the basic helix-loop-helix (bHLH), Olig1 and Olig2, and the homeobox containing (Hox) transcription factor Nkx.2.2, which define the identity and plasticity of progenitors that give rise to both neurons and glia (Rowitch, 2004). In the forebrain, oligodendrocyte specification is also dependent on Shh signaling and Olig transcription factor function (Lu et al., 2002; Nery et al., 2001; Tekki-Kessarlis et al., 2001). As development proceeds, oligodendrocyte progenitors proliferate as they migrate away from these germinal foci, generating the wide distribution of oligodendrocytes in the CNS (Miller, 2002).

1.3 Oligodendrocyte lineage progression

Oligodendrocyte progenitor cells (OPCs) progress through several intermediate stages of differentiation, which culminate in the generation of a mature myelinating phenotype. The stages of oligodendrocyte differentiation are characterized by the following criteria 1) morphology, 2) the expression of stage specific antigens, 3) sensitivity to mitogens, and 4) the ability to migrate. Figure 1.1 illustrates the major stages of oligodendrocyte lineage progression, as well as key stage specific antigens.

Early OPCs exhibit a characteristic bipolar morphology and express cell surface glycolipids recognized by the A2B5 monoclonal antibody (Raff et al., 1984a), the platelet derived growth factor receptor- alpha (PDGFR- α) (Hart et al., 1989), and the

NG2 chondroitin sulfate proteoglycan (Nishiyama et al., 1996). Early progenitors were first described as a bipotential progenitor isolated from the rat optic nerve, termed the O2-A, capable of generating both oligodendrocytes and type-2 astrocytes *in vitro* (Raff et al., 1983; Raff et al., 1984b). However, the O2-A moniker has been replaced in recent years by the general term oligodendrocyte progenitor cells (OPCs), primarily due to a lack of evidence that OPCs generate type-2 astrocytes *in vivo* (Espinosa de los Monteros et al., 1993). Early progenitors are highly proliferative, migratory cells which maintain an undifferentiated state in the presence of PDGF and FGF-2 *in vitro* (Tang et al., 2001).

Mitogen withdrawal initiates oligodendrocyte differentiation *in vitro* (Barres et al., 1994). As they differentiate, early OPCs transition to a simple multipolar morphology termed the late progenitor stage, characterized by the expression of a sulfated surface antigen termed POA (pro-oligodendroblast antigen) recognized by the O4 and A007 antibodies (Bansal et al., 1992). Late progenitors proliferate in response to mitogens, but no longer migrate in response to chemotaxic factors (Bansal et al., 1989; Pfeiffer et al., 1993).

As late progenitors withdraw from the cell cycle and commit to terminal differentiation, they progress to the immature oligodendrocyte stage (Bansal et al., 1989). Immature oligodendrocytes are characterized by a complex multipolar morphology and immunoreactivity to galactocerebroside (GalC) detected by the O1 and Ranscht antibodies (Bansal and Pfeiffer, 1992; Raff et al., 1978).

The terminal differentiation of mature oligodendrocytes is marked by the expression of major myelin proteins, including myelin basic protein (MBP) and proteolipid protein (PLP), as well as the minor myelin components, myelin-associated

glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG). Recently, the genomic analysis of oligodendrocyte differentiation provided evidence that terminal differentiation is further divided into two stages based on the timely expression of early myelin (MBP, PLP) and late myelin (MAG, MOG) proteins, governed by the activity of distinct transcription factors (Dugas et al., 2006).

Oligodendrocyte differentiation is thought to proceed via a constitutive, intrinsic program that is closely linked to cell cycle withdrawal (Barres et al., 1994; Raff, 2006; Tokumoto et al., 1999). However, several extrinsic factors have been identified that can influence the timing of oligodendrocyte differentiation *in vitro* and *in vivo*. The following sections will review the literature on the intrinsic and extrinsic factors that influence oligodendrocyte differentiation. While many of the extrinsic factors identified also affect the proliferation and survival of oligodendrocyte progenitors, only studies that demonstrate a defined role during differentiation will be discussed.

1.4 Intrinsic factors

1.4.1 The intrinsic timing model of OPC differentiation

Over the past 20 years since the initial characterization of the O2-A progenitor (Raff et al., 1983; Raff et al., 1984b), Martin Raff and his colleagues developed a model of oligodendrocyte differentiation that occurs via a constitutive, intrinsic timing mechanism (Raff, 2006). Interest in the developmental timing of cell fate began based on a series of experiments that showed that dissociated cultures from embryonic rat brain

gave rise to astrocytes, ependymal cells, and oligodendrocytes *in vitro* on the day corresponding to their development *in vivo* (Abney et al., 1981). To study the mechanism responsible for this phenomenon, Raff focused on a simpler model system, the timing of oligodendrocyte development in embryonic rat optic nerve cultures.

Early studies showed, that oligodendrocytes developed on schedule in dissociated cultures of the embryonic rat optic nerve, and that the proper timing of differentiation was dependent on the presence of platelet derived growth factor (PDGF) produced by astrocytes to stimulate OPC division (Raff et al., 1985; Raff et al., 1988). Additional studies showed that individual OPCs isolated from postnatal day 7 optic nerves and cultured in the presence of PDGF, or on monolayers of astrocytes, divide up to 8 times before they exit the cell cycle and differentiate (Barres et al., 1994; Temple and Raff, 1986). Furthermore, the progeny of an individual progenitor cultured in separate microwells divided the same number of times before they differentiated, suggesting that the decision to differentiate is intrinsically linked to the cell cycle through a mechanism that counts cell division or elapsed time (Barres et al., 1994; Temple and Raff, 1986). This discrepancy was postulated based on the observation that OPCs cultured at 33°C divide slower, and differentiate after fewer divisions compared to cells cultured at 37°C, suggesting that the cells measure elapsed time rather than counting cell divisions (Gao et al., 1997).

Studies performed by Ben Barres demonstrated that triiodothyronine (thyroid hormone, T3), a component of the medium used in initial experiments, was required in combination with PDGF as an effector component of the intracellular timing mechanism (Barres et al., 1994). A summary of the observations from Barres's studies is as follows:

1) In the presence of PDGF alone, OPCs continue to divide and fail to differentiate; 2) OPCs cultured in the presence of PDGF plus T3 differentiate after a limited number of cell divisions; and 3) T3 activates an effector mechanism required for the initiation of cell cycle exit in the presence of mitogens; 4) T3 is not required for differentiation per se, as OPCs grown in the absence of PDGF and T3 prematurely withdraw from the cell cycle and differentiate (Barres et al., 1994). Finally, OPCs isolated from different regions of the CNS, including the optic chiasm and cortex, exhibit cell intrinsic mechanisms similar to those observed for optic nerve derived OPCs. However, cells isolated from the optic chiasm and cortex display differences in their ability to undergo prolonged periods of self-renewal divisions and altered sensitivity to T3, which may reflect the different time courses of myelination in these CNS regions (Power et al., 2002)

Taken together, these studies led to a model of oligodendrocyte differentiation that occurs via a constitutive pathway, that is coupled to withdrawal from the cycle, and is regulated by an intracellular timing mechanism (Durand and Raff, 2000). Several putative components of the intracellular timer have been identified, which include cyclin dependent kinase inhibitors (CDKis), members of the p53 family of proteins, and the inhibitor of DNA binding proteins (Ids). The following sections will detail the roles that each of these factors plays in the regulation of OPC differentiation and their relationship to the validity of the intrinsic timing model.

1.4.2 Cell cycle components

The decision to proliferate or exit the cell cycle is regulated during the G1 phase of the cell cycle by the coordinate balance of the cyclin dependent kinases (cdks), the G1 cyclins, and cyclin dependent kinase inhibitors (cdkis) (Casaccia-Bonnet and Liu, 2003). Several studies have examined the relationship between components of the cell cycle machinery and their relationship to cell cycle exit and differentiation of OPCs (Nguyen et al., 2006). Interestingly, data from these studies support the hypothesis that cell cycle exit alone is not sufficient to promote OPC differentiation.

The decision of OPCs to exit the cell cycle appears to be regulated primarily at the level of cyclinE/cyclin-dependent kinase 2 (cdk2) complex activation. First, cdk2 kinase activity decreases sharply during OPC differentiation relative to proliferating cultures (Ghiani and Gallo, 2001; Tang et al., 1998). The reduction in cdk2 kinase activity during oligodendrocyte differentiation is due to a decrease in the expression of cdk2 and cyclin E as well as a concomitant increase in the expression of the cdkis, p27 and p21 (Casaccia-Bonnet et al., 1997; Ghiani and Gallo, 2001; Ghiani et al., 1999). Furthermore, the expression of a dominant negative form of cdk2 significantly decreases the ability of OPCs to proliferate in the presence of mitogens, effectively inducing cell cycle arrest (Belachew et al., 2002). However, the growth arrest caused by the expression of a dominant negative cdk2 alone does not induce OPC differentiation in the presence of PDGF, nor does it accelerate the rate of OPC differentiation in the absence of mitogens (Belachew et al., 2002).

The robust increase of p27 expression during oligodendrocyte differentiation observed in the previous experiments suggests that it may play a direct role in establishment or maintenance of growth arrest. In support of the intrinsic timing mechanism proposed by Raff, the levels of p27 accumulate during oligodendrocyte proliferation in the presence of PDGF and peak following 8 cell divisions (Durand et al., 1997). In addition, p27 levels increase more rapidly in OPCs grown at 33°C, suggesting that p27 is indeed a component of the intrinsic timing mechanism (Gao et al., 1997). Furthermore, the expression of p27 is upregulated during myelination *in vivo* (Friessen et al., 1997). Conversely, OPCs isolated from p27 ^{-/-} mice exhibit an enhanced ability to proliferate at the expense of differentiation (Casaccia-Bonnet et al., 1997). However, the loss of p27 does not inhibit myelination *in vivo*. In fact, p27 null mice exhibit an increase in myelin content possibly due to a coordinate increase in the numbers of OPCs generated during development (Casaccia-Bonnet et al., 1997). Finally, the ectopic overexpression of p27 *in vitro* is sufficient to induce growth arrest in cultured OPCs (Tang et al., 1999; Tikoo et al., 1998). However, in agreement with cell cycle arrest caused by the expression of a dominant negative cdk2, the overexpression of p27 is not sufficient to induce OPC differentiation (Tang et al., 1999; Tikoo et al., 1998).

In contrast to OPCs derived from p27 null mice, the deletion of a related cdk, p21, has no effect on cell cycle arrest during OPC differentiation *in vitro* (Zezula et al., 2001). However, the loss of p21 inhibits oligodendrocyte differentiation *in vitro*, and delays the expression of MBP and PLP *in vivo* compared to wild type mice (Zezula et al., 2001). Together, these results suggest that the cdkis p27 and p21 perform non-redundant functions during OPC differentiation. The expression of p27 is required for cell cycle

arrest, while p21 appears to affect OPC differentiation through a manner independent of cell cycle withdrawal.

The p53 family of proteins, which include p53, p63, and p73, are transcription factors that can activate or repress numerous genes involved in cell cycle arrest in both the G1 or G2/M phase of the cell cycle, apoptosis, and differentiation depending on the cellular context and level at which they are expressed (Levrero et al., 2000). Unlike the p53 gene, which encodes one protein, the p63 and p73 genes express multiple isoforms which either contain a N-terminal transcriptional transactivation domain (TA-p63/73), or a truncated N-terminus that lacks transcriptional activity (Δ Np63/73) (Yang et al., 2002). Previous reports have demonstrated that OPCs express p53 as well as TA and Δ N isoforms of p63 and p73 (Billon et al., 2004; Tokumoto et al., 2001). In addition, p53 and p73, but not p63, affect the timing of oligodendrocyte differentiation *in vitro* (Billon et al., 2004; Eizenberg et al., 1996; Tokumoto et al., 2001)

During OPC proliferation p53 is expressed at low levels and exhibits a cytoplasmic localization (Eizenberg et al., 1996). However, during OPC differentiation, p53 transiently translocates to the nucleus (Eizenberg et al., 1996). The expression of a dominant negative form of p53 significantly inhibits both cell cycle withdrawal and OPC differentiation in the presence of PDGF and T3, suggesting that p53 is a critical component of the T3 controlled effector of the intrinsic timing mechanism (Billon et al., 2004; Tokumoto et al., 2001).

Further studies demonstrate that in addition to p53, the expression of p73 is essential for OPC differentiation (Billon et al., 2004). Prior to differentiation, p73 is localized to the nucleus. However after 5 days of differentiation, p73 expression is up-

regulated and distributed throughout the cell. The antibodies used to detect the localization of p73 react with both the TA and ΔN isoforms, suggesting that the redistribution of a subset of p73 during OPC differentiation may reflect the movement of a specific p73 isoform. Retroviral mediated expression of TAp73 increases the spontaneous differentiation of OPCs cultured in the presence of PDGF, suggesting that this isoform performs a similar function to that of p53 in mediating cell cycle exit during OPC differentiation. In contrast, the expression of $\Delta Np73$ inhibited the generation of oligodendrocytes during OPC differentiation induced by T3 treatment or mitogen withdrawal. In comparison, the expression of p63 isoforms had no effect on OPC differentiation. Furthermore, the expression of $\Delta Np73$ in OPCs derived from p53 null mice also inhibits spontaneous differentiation, suggesting that the function of $\Delta Np73$ does not merely suppress the transcriptional activity of p53.

1.4.3 Transcription factors

The coordinated expression of genes necessary for oligodendrocyte differentiation is regulated in part by the actions of lineage specific transcription factors (Wegner, 2001). Transcription factors involved in the specification and terminal differentiation of oligodendrocytes include the bHLH family members Olig1 and Olig2, SRY-box containing (Sox) family members, Sox8, Sox9, Sox10, and Sox17, the homeobox transcription factor Nkx2.2, and the zinc finger DNA-binding protein Myt1 (Ligon et al., 2006; Nielsen et al., 2004; Sohn et al., 2006; Wegner and Stolt, 2005). While genetic studies in mice have described the role of many of these transcription factors in

oligodendrocyte specification, less is known about their function during oligodendrocyte differentiation and maturation.

The bHLH family members Olig1 and Olig2 are among the earliest markers of OPC specification, and are essential for the generation of oligodendrocytes in the CNS (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Subsequent to OPC specification, Olig1 and Olig2 expression persists, suggesting that they perform additional functions during OPC differentiation and maturation. In support of this hypothesis, myelination is severely impaired in brains of Olig1-null mice despite the generation and proliferation of OPCs (Xin et al., 2005). Decreased myelination in Olig1-null mice is attributed to a failure of OPCs to induce the expression of myelin proteins required for myelinogenesis subsequent to proper morphological differentiation, suggesting that Olig1 is required during a late stage of oligodendrocyte differentiation. In contrast to Olig1, evidence suggests that Olig2 is required during an early stage of OPC differentiation which may be due to its ability to induce the expression of Sox10 (Liu et al., 2007).

A role for Sox10 in oligodendrocyte differentiation is supported by the observation that it can activate the transcription of myelin genes including PLP and MBP (Stolt et al., 2002). Furthermore, neural stem cells derived from Sox10 null mice fail to generate myelinating oligodendrocytes when transplanted into wild-type recipient mice (Stolt et al., 2002). Sox10 is rapidly upregulated at the mRNA level at the onset of differentiation *in vitro* (Dugas et al., 2006). Finally, the siRNA mediated disruption of Sox10 expression during OPC differentiation decreases the expression of MBP but not

myelin oligodendrocyte protein (MOG), suggesting that Sox10 activates a subset of myelin genes during terminal differentiation (Dugas et al., 2006).

Recently, an additional member of the Sox family, Sox17 was identified as a transcription factor that is transiently upregulated during OPC differentiation *in vitro* and *in vivo* (Sohn et al., 2006). During OPC differentiation *in vitro*, Sox17 expression is upregulated and peaks following 3 days in culture during the period when the majority of OPCs transition from a late progenitor to immature oligodendrocyte phenotype. Co-immunostaining for Sox17 and lineage specific cell surface antigens confirmed that Sox17 expression is elevated in O4⁺/GalC⁻ late progenitors relative to early progenitors or immature oligodendrocytes. The disruption of Sox17 expression via siRNA mediated knockdown attenuates OPC differentiation through a mechanism that appears to prevent cell cycle withdrawal. Conversely, overexpression of Sox17 inhibits OPC response to mitogens, increases the numbers of O4⁺ and O1⁺ cells, and upregulates the mRNA levels of several myelin genes.

The inhibitor of DNA binding family of proteins is structurally similar to basic helix-loop-helix (bHLH) transcription factors except that they lack the basic DNA binding domain (Yokota, 2001). The Id proteins act as dominant negative inhibitors of bHLH transcription factors by binding to and sequestering class A bHLH proteins, preventing their ability to dimerize with class B bHLH transcription factors to activate gene transcription (Norton, 2000).

Oligodendrocyte progenitors express the Ids 1-4, and the expression of Id2 and Id4 directly controls the timing of oligodendrocyte differentiation *in vitro* and *in vivo* (Kondo and Raff, 2000; Marin-Husstege et al., 2006; Wang et al., 2001). The expression

of Id4 decreases at the mRNA level as OPCs differentiate in culture, while Id2 appears to be regulated initially by translocation out of the nucleus in response to mitogen withdrawal followed by downregulation at the mRNA level (Kondo and Raff, 2000; Tzeng and de Vellis, 1998; Wang et al., 2001). Overexpression of Id2 or Id4 effectively blocks the generation of GalC⁺ postmitotic oligodendrocytes *in vitro* (Kondo and Raff, 2000; Wang et al., 2001). Furthermore, ectopic expression of Id4 globally decreases the mRNA levels of myelin genes including MBP, PLP, and myelin associated glycoprotein (MAG) (Marin-Husstege et al., 2006). Conversely, genetic deletion of Id4 results in the precocious differentiation of oligodendrocyte progenitors *in vivo* (Marin-Husstege et al., 2006). Finally, bone morphogenetic protein-4 (BMP-4) inhibits oligodendrocyte generation from adult neural progenitors in culture via the upregulation of Id proteins, which directly interact with and suppress the functions of the Olig1 and Olig2 transcription factors (Samanta and Kessler, 2004).

1.4.4 Histone deacetylation

Changes in chromatin conformation through the covalent modification of core histones can result in global changes in gene transcription (Struhl, 1998). The acetylation of lysine residues on the amino-terminal tails of histones by histone acetyltransferases (HATs) generally results in a loosening of chromatin compaction, while the deacetylation by histone deacetylases (HDACs) correlates with chromatin compaction and repression (Turner, 2000; Yoshida et al., 2003).

A previous study showed that an increase in HDAC activity during OPC differentiation is essential for oligodendrocyte lineage progression (Marin-Husstege et al., 2002). In this study, the acetylation of histones H3 and H4 decreased rapidly within the first 24 hours of OPC differentiation initiated through mitogen withdrawal. The inhibition of HDAC activity by Trichostatin A (TSA) decreases branching and blocks OPC differentiation at the O4+/GalC- late progenitor stage. Furthermore, TSA decreases the mRNA levels of key myelin genes, suggesting that global changes in chromatin modification are required for the expression of oligodendrocyte differentiation markers. However, HDAC inhibition had no effect on cell cycle exit during OPC differentiation.

Further studies *in vivo* add support to the role of HDAC activity in the regulation of OPC differentiation (Shen et al., 2005). Specifically, the administration of valproic acid (VPA), an inhibitor of HDAC activity, to rat pups during the first 10 days of postnatal development delays myelin gene expression and results in extensive hypomyelination. Taken together, these studies suggest that intrinsic changes to chromatin structure through the deacetylation of histones are required in addition to cell cycle exit for the terminal differentiation of oligodendrocytes. Furthermore, these studies highlight a critical differentiation checkpoint that occurs during the late progenitor to immature oligodendrocyte transition.

1.5 Extrinsic factors and signaling components

The previous section detailed the current understanding of the role intrinsic factors play in the regulation of oligodendrocyte differentiation. The prevailing model of

oligodendrocyte differentiation, proposed by Martin Raff and his colleagues, suggests that OPC differentiation is controlled by an intrinsic timing mechanism. Specifically, the model states that as OPCs proliferate in response to PDGF, intrinsic components accumulate and/or dissipate within the cell in preparation for differentiation. Thyroid hormone (T3) then activates an effector component of the timing mechanism, which instructs the cells to exit the cell cycle and differentiate. Furthermore, in the absence of PDGF and T3, OPCs rapidly withdraw from the cell cycle and differentiate, suggesting that T3 is not essential for OPC differentiation, but rather coordinates the timing of differentiation in the presence of mitogens.

There are several lines of evidence that argue against an intrinsic timing mechanism for regulating oligodendrocyte differentiation. First, a key prediction of the intrinsic timing model is that cell cycle exit alone is sufficient to initiate the oligodendrocyte differentiation program. However, this appears not to be the case. For example, the overexpression of p27 or a dominant negative form of cdk2 is sufficient to induce cell cycle arrest in OPCs, but not differentiation.

Furthermore, the model suggests that proliferation in response to PDGF is essential for the timing mechanism to operate. If this is the case, one would predict that the intrinsic program would operate appropriately when OPCs are stimulated to divide in the presence of alternative mitogens. In contrast to this hypothesis, OPCs cultured in the presence of FGF-2, a potent mitogen for the lineage, are unable to differentiate past the O4⁺/GalC⁻ late progenitor stage (Gard and Pfeiffer, 1993; McKinnon et al., 1990). In addition, OPCs cultured in presence of PDGF and FGF-2 maintain an undifferentiated state (Tang et al., 2001).

Taken together, these observations suggest that additional factors are required to promote OPCs differentiation subsequent to cell cycle withdrawal. In fact, a number of growth factors, cytokines, and extracellular matrix components have been identified that influence oligodendrocyte differentiation. In this section, we review the literature on a few of these factors that are reported to stimulate oligodendrocyte differentiation.

1.5.1 Thyroid hormone

The pro-hormone thyroxine (T4) and to a lesser degree its active form, triiodothyronine (T3), are synthesized and released into circulation by the thyroid gland. Within cells, T4 is converted to T3 and binds to thyroid hormone receptors which recognize thyroid hormone response elements (TRE) located in the promoters of T3-target genes to activate or repress transcription (Bassett et al., 2003).

A role for thyroid hormone in the development of the oligodendrocyte lineage was initially postulated based on the observation that hypothyroid animals exhibit delayed myelination, while a hyperthyroid state increases myelin protein expression and accelerates myelinogenesis (Walters and Morell, 1981). As discussed previously, it has been proposed that T3 activates an effector component of an intrinsic timing mechanism to enhance the rate of OPC differentiation in the presence of PDGF (Barres et al., 1994; Gao et al., 1997). However, T3 is not essential for OPC differentiation *in vitro*, as oligodendrocyte differentiation proceeds normally in its absence (Barres et al., 1994). Furthermore, the observation that retinoic acid and glucocorticoids can substitute for T3

in promoting OPC differentiation in the presence of PDGF raises the question of the relevance of T3 to this process (Barres et al., 1994).

T3 activates gene expression by binding to thyroid hormone receptors, which are ligand-regulated transcription factors. The thyroid hormone receptors (TR) are encoded by two genes, TR α and TR β (Bassett et al., 2003). The TR α gene produces a functional T3 receptor termed TR α 1, two C-terminal variants designated TR α 2 and TR α 3 which do not bind T3 and act as dominant negative inhibitors, and two N-terminal variants, TR Δ α 1 and TR Δ α 2 which also function as dominant negative antagonists of T3 signaling (Bassett et al., 2003). The TR β gene generates three isoforms TR β 1-3 that bind T3 and regulate gene transcription.

OPCs express TR α 1 and TR α 2 receptors, while the expression of TR β 1 is restricted to mature oligodendrocytes (Billon et al., 2001; Carre et al., 1998). Therefore, the actions of T3 on OPC differentiation are likely mediated by the TR α isoforms. In support of this hypothesis, the generation of oligodendrocytes in the optic nerves of TR α 1 $-/-$ mice is delayed relative to wild type mice (Billon et al., 2002). In addition, OPCs derived from TR α 1 $-/-$ mice are refractory to T3 treatment in culture, and therefore fail to differentiate in the presence of T3 and PDGF. However, TR1 α $-/-$ OPCs are not incapable of differentiating, as treatment with retinoic acid instead of T3 restores their ability to generate the same number of oligodendrocytes as wild type OPCs (Billon et al., 2002). Finally, the over-expression of TR1 α causes a greater than 2-fold increase in the number of oligodendrocytes generated in the presence of PDGF and T3 following 3 days in culture (Billon et al., 2002).

Interestingly, deletion of TR α and TR β in mice does not recapitulate the hypomyelinated phenotype associated with hypothyroidism (Billon et al., 2002). Although a slight delay in myelination in the optic nerve is observed, myelination occurs in the absence of thyroid hormone receptors. The authors suggest that the discrepancy is due to the fact that free thyroid hormone receptors in hypothyroid animals are able to repress T3 target gene expression, which may be responsible for the myelination defects caused by hypothyroidism.

1.5.2 Insulin-like growth factor-I (IGF-I)

Insulin-like growth factor-I (IGF-I) is a 70 amino acid polypeptide growth factor that exhibits 50% homology to proinsulin and promotes the survival, proliferation, and differentiation of multiple cell types (Stewart and Rotwein, 1996). IGF-I signals primarily through the IGF type I receptor (IGF-IR), a type II receptor tyrosine kinase composed of two α and two β subunits linked by disulfide bridges (De Meyts and Whittaker, 2002). IGF-I mediated activation of the IGF-IR is capable of initiating signaling primarily through the MAP kinase and phosphatidylinositol 3-kinase (PI3K) pathways. Cells of the oligodendrocyte lineage express the IGF-IR, and activation of the IGF-IR affects the survival, proliferation, and differentiation of cells of the oligodendrocyte lineage *in vitro* and *in vivo* (McMorris et al., 1993).

A role for IGF-I in oligodendrocyte differentiation was first proposed based on experiments which show that IGF-I significantly enhances the generation of GalC⁺ immature oligodendrocytes *in vitro* (McMorris and Dubois-Dalcq, 1988). IGF-I

increases the expression of myelin proteins, including MBP and CNP (Mozell and McMorris, 1991; Saneto et al., 1988), while inhibiting IGF-I signaling through the exogenous addition of IGFBP-6 decreases the expression of CNP and MAG (Kuhl et al., 2003).

Studies of IGF-I transgenic and knockout mice support the conclusion that IGF-I is an important regulator of OPC differentiation and myelination. First, transgenic mice that overexpress IGF-I exhibit a hypermyelinated phenotype caused by increased myelin production per oligodendrocyte (Carson et al., 1993). The transgenic expression of IGF-I upregulates the mRNA levels of myelin genes, including MBP and PLP (Ye et al., 1995). Conversely, IGF-I $-/-$ mice exhibit hypomyelination characterized by a significant decrease in myelin protein expression (Ye et al., 2002). In addition, the transgenic overexpression of insulin-like growth factor binding protein-1 (IGFBP-1), which negatively regulates the bioavailability of IGF-I, decreases myelin protein expression at the mRNA level, reduces the number of myelinated axons in the anterior commissure, and decreases the thickness of myelin sheaths (Ye et al., 1995).

The mechanisms of IGF-I action on OPC differentiation are unknown. Furthermore, the study of IGF-I signaling during oligodendrocyte differentiation is hampered by the fact that IGF-I is a potent survival factor for OPCs (Cui et al., 2005; Kuhl et al., 2002; Ness and Wood, 2002; Vemuri and McMorris, 1996). Therefore, attempts to study the role of IGF-I on OPC differentiation by inhibiting signaling at the level of the IGF-IR leads to confounding effects due to decreased viability.

1.5.3 Integrins

The integrins are a family of cell surface receptors that transmit signals from the environment to the cell via their ability to bind extracellular matrix (ECM) ligands (Hynes, 2002). Integrins exist as heterodimers formed by the association of one α and one β chain. In total, 18 α and 8 β chains have been identified that generate a diverse array of ECM receptors through the combinatorial interactions of α and β chains (Hynes, 2002). The cells of the oligodendrocyte lineage express at least five integrin receptors, including $\alpha\nu\beta1$, $\alpha\nu\beta3$, $\alpha\nu\beta5$, $\alpha\nu\beta8$, and $\alpha6\beta1$ (Milner and Ffrench-Constant, 1994; Milner et al., 1997), and the activation of specific integrins has been linked to OPC survival, proliferation, and differentiation (Baron et al., 2002; Blaschuk et al., 2000; Colognato et al., 2002).

A role for integrin signaling in OPC differentiation was initially identified for $\alpha\nu\beta5$ (Blaschuk et al., 2000). First, $\alpha\nu\beta5$ is upregulated during OPC differentiation (Milner and Ffrench-Constant, 1994). Furthermore, an $\alpha\nu\beta5$ specific function blocking antibody decreases the numbers of MBP positive oligodendrocytes during differentiation *in vitro* (Blaschuk et al., 2000). In addition, the inhibition of $\alpha\nu$ containing integrins by arginine-glycine-aspartic acid (RGD) containing peptides inhibits OPC myelin membrane synthesis and MBP expression (Cardwell and Rome, 1988).

Further studies provided evidence that $\alpha6\beta1$, which binds laminin and is expressed by OPCs and oligodendrocytes (Milner and Ffrench-Constant, 1994), promotes myelin membrane formation (Chun et al., 2003; Relvas et al., 2001). OPCs expressing a dominant negative $\beta1$ subunit and transplanted into focal demyelinated lesions exhibit a

decreased capacity to remyelinate denuded axons (Chun et al., 2003). *In vitro*, a function blocking anti- $\beta 1$ antibody inhibits the morphological differentiation of OPCs (Chun et al., 2003).

In turn, the analysis of myelination in laminin-2-deficient mice (Lama2^{dy}) support the *in vitro* evidence that $\alpha 6 \beta 1$ contributes to signaling required for proper myelination (Chun et al., 2003). Lama2^{dy} mice exhibit decreased MBP expression in the forebrain, reduced numbers of mature oligodendrocytes, and myelination defects characterized by improper myelin membrane compaction. *In vitro*, the morphological differentiation of OPCs is enhanced by laminin-2 through a signaling mechanism that requires PI3K activation (Chun et al., 2003). Finally, additional studies have shown that integrin signaling activates Fyn, a member of the non-receptor tyrosine kinase family, and that the activation of Fyn is required for the morphological differentiation of OPCs (Liang et al., 2004).

1.5.4 The activation of Fyn

Fyn is a member of the Src family of non-receptor tyrosine kinases (Thomas and Brugge, 1997). In the cells of the oligodendrocyte lineage, Fyn mediates the effects of integrin activation and IGF-I signaling (Colognato et al., 2004; Liang et al., 2004; Sperber and McMorris, 2001).

Several lines of evidence suggest that the activation of Fyn contributes to oligodendrocyte differentiation. First, Fyn is up-regulated during oligodendrocyte differentiation *in vitro*, and its kinase activity increases dramatically preceding the

morphological changes associated with OPC differentiation (Osterhout et al., 1999). Expression of a kinase dead Fyn mutant or siRNA mediated silencing of Fyn expression decreases the morphological complexity of differentiating OPCs (Colognato et al., 2004; Osterhout et al., 1999; Wolf et al., 2001). Fyn regulates process extension and elaboration at least in part through the phosphorylation and activation of members of the Rho family of GTPases, including p190RhoGAP, which regulate the polymerization of the actin cytoskeleton (Liang et al., 2004; Wolf et al., 2001).

Consistent with a proposed role for Fyn in oligodendrocyte differentiation, Fyn ^{-/-} mice exhibit a hypomyelinated phenotype (Sperber et al., 2001; Umemori et al., 1999). Specifically, the myelin sheaths in Fyn ^{-/-} mice are thinner, exhibit irregular compaction, and contain 50% less MBP content compared to wild type mice. Furthermore, transactivation of the MBP promoter is enhanced by Fyn kinase activity (Umemori et al., 1999). Finally, Fyn appears unique among members of the Src family of non-receptor tyrosine kinases in its ability to regulate the myelination and differentiation of OPCs, as mice lacking Src, Yes, or Lyn do not exhibit defects in myelination (Sperber et al., 2001).

1.6 The mammalian target of rapamycin

The mammalian target of rapamycin (mTOR) is a Ser-Thr kinase that integrates signals from growth factors and nutrient availability to regulate a growing number of biological processes, including cell growth, proliferation, differentiation, and autophagy (Dann and Thomas, 2006; Sarbassov et al., 2005a; Wullschleger et al., 2006). mTOR, also known as FRAP, RAFT1, or RAPT1 was identified and cloned following the

discovery of two mutations termed TOR1 and TOR2 which enabled the growth of *Saccharomyces cerevisiae* in the presence of rapamycin (Brown et al., 1994; Chiu et al., 1994; Helliwell et al., 1994; Kunz et al., 1993; Sabatini et al., 1994). Rapamycin, a macrolide antibiotic, binds to FKBP12 (FKBP12 binding protein, $M_r = 12000$) and inhibits mTOR kinase activity through the formation of a ternary complex (Chen et al., 1995; Choi et al., 1996).

In contrast to *Saccharomyces cerevisiae*, higher eukaryotic genomes contain a single TOR gene which encodes a 2549 amino acid protein, that consists of several conserved structural domains, exhibiting ~42% amino acid sequence homology to yeast TOR proteins (Sabatini et al., 1994). The C-terminal region of mTOR contains the kinase domain, which is structurally similar to other members of the phosphatidylinositol 3-kinase related kinase (PIKK) family, which includes ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad-3 related) (Abraham, 2004). Flanking the kinase domain are the FRB domain and a putative negative regulatory domain (NRD). The FRB domain encodes the interaction site between mTOR and the FKBP12-rapamycin binding complex which results in the inhibition of mTOR kinase activity (Chen et al., 1995; Choi et al., 1996). The NRD contains residues phosphorylated in response to activation of the PI3K/Akt pathway by insulin/IGF and other growth factors (Nave et al., 1999; Scott et al., 1998; Sekulic et al., 2000). In addition, mTOR contains two FAT domains (FRAP, ATM, TRAPP2), common to members of the PIKK family, that are thought to influence the proper folding and organization of the kinase domain (Bosotti et al., 2000). The C-terminal FAT domain (FAT-C) is essential for mTOR function, as the deletion of a single amino acid in this domain inhibits mTOR kinase activity (Takahashi et al., 2000).

Finally, the amino-terminus of mTOR contains 20 tandem HEAT (Huntington, EF3, A subunit of PP2A, TOR1) repeats which are believed to function as a scaffolding domain that mediates protein interactions.

mTOR exists in two distinct molecular complexes termed mTORC1 and mTORC2 which regulate the activity of discrete subsets of downstream signaling effectors (Bhaskar and Hay, 2007; Sarbassov et al., 2005a). The TOR complexes are defined by the interaction between mTOR and key adaptor proteins that are thought to regulate mTOR kinase specificity. Specifically, the mTORC1 complex consists of mTOR, the regulatory associated protein of mTOR (raptor), and mLST8 (Kim et al., 2002; Kim et al., 2003). mTORC2 is composed of mTOR, the rapamycin insensitive companion of mTOR (riCTOR), mLST8, and the SAPK-interacting protein (Sin1) (Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004).

The TOR complexes display differential sensitivity to rapamycin inhibition. The mTORC1 complex is acutely sensitive to rapamycin, which inhibits the autophosphorylation of mTOR and disrupts the interaction between mTOR and raptor (Kim et al., 2002). In contrast, chronic exposure to rapamycin is required to disrupt mTORC2 function, through a mechanism that involves the binding and sequestration of mTOR by the rapamycin-FKBP-12 complex (Sarbassov et al., 2006). The known biological functions of the mTORC complexes will be discussed below. Figure 1.2 illustrates the mTORC signaling components and downstream targets.

1.6.1 The mTORC1 complex and downstream signaling effectors

The mTOR-raptor mTORC1 complex influences a number of biological processes including cell growth, ribosome biogenesis, proliferation, and mRNA translation (Sarbasov et al., 2005a). The best characterized functions for mTORC1 include its ability to regulate cell growth and mRNA translation through the regulation of two key downstream substrates, the 4E-BPs and the S6 kinases (Hay and Sonenberg, 2004).

The eIF4E-binding proteins (4E-BP 1-3), in a hypophosphorylated state, act to repress cap dependent translation by binding to and sequestering eukaryotic initiation factor-4E (eIF-4E) (Beretta et al., 1996; Poulin et al., 1998; Rousseau et al., 1996). When activated, the mTORC1 complex phosphorylates multiple Ser-Thr residues on 4E-BPs which disrupts its ability to associate with eIF-4E, enabling cap dependent translation (Burnett et al., 1998; Kim et al., 2002).

The S6 kinases (S6K1 and S6K2) are Ser-Thr kinases named for their ability to phosphorylate the ribosomal protein S6. The S6K1 gene produces two isoforms, p70S6K1 and p85S6K1, the former which contains a truncated N-terminus (Reinhard et al., 1992). Likewise, alternative splicing of the S6K2 mRNA generates two isoforms, p56S6K2 and p54S6K2 (Koh et al., 1999; Lee-Fruman et al., 1999). The p70S6K1 isoform is unique due to its cytoplasmic localization, while p85S6K1 and the S6K2 isoforms primarily reside in the nucleus (Koh et al., 1999; Lee-Fruman et al., 1999; Reinhard et al., 1994; Reinhard et al., 1992). The majority of studies on TOR signaling have focused on the activation of the p70 isoform of S6K1. Finally, Active mTORC1

complex phosphorylates S6K1 on Thr389 and Ser371 which contribute to the activation of S6K1 through multisite phosphorylation (Burnett et al., 1998; Saitoh et al., 2002).

The identification of S6K1 and 4E-BPs as downstream effectors of mTOR signaling was initially based on their sensitivity to inhibition by rapamycin treatment (Burnett et al., 1998). Following the discovery of raptor and the subsequent characterization of the mTORC1 complex, results from siRNA mediated knockdown of raptor and mTOR support the conclusion that S6K1 and 4E-BPs are regulated by the mTORC1 complex (Kim et al., 2002). S6K1 and 4E-BPs contain a TOR signaling (TOS) motif which is believed to facilitate their recognition by the mTORC1 complex through the direct interaction with raptor (Nojima et al., 2003).

Several lines of evidence demonstrate that the mTORC1 complex, via its ability to activate S6K1, functions as a key regulator of cell size. First, siRNA mediated silencing of mTOR, raptor, or S6K1 decreases the mean diameter and volume of mammalian cells (Kim et al., 2002). Furthermore, the deletion of dTOR in *Drosophila* decreases cell size, as does the deletion dS6K but to a lesser degree, suggesting that additional factors downstream of dTOR function in the regulation of cell size (Montagne et al., 1999; Zhang et al., 2000). In addition, myoblasts isolated from S6K1 *-/-*, but not S6K2 *-/-* mice, are smaller in size and are refractory to a further reduction in cell size by rapamycin treatment, suggesting that in muscle, S6K1 is the dominant effector of mTOR dependent regulation of cell size in mammals (Ohanna et al., 2005). S6K1 was believed to control cell size through its ability to regulate the translation of mRNAs containing a 5' oligopyrimidine tract (5'TOP) motif. However, 5'TOP mRNA translation is unaltered in S6K1/S6K2 *-/-* mice, and is sensitive to rapamycin treatment (Pende et al., 2004).

Therefore, the mTORC1/S6K1 dependent mechanism that regulates cell growth is at present unknown.

Activation of the mTORC1 complex occurs primarily in response to growth factor stimulation and nutrient availability. First, growth factor signaling through the PI3K/Akt pathway indirectly activates mTORC1 through the Akt mediated phosphorylation of tuberous sclerosis complex 2 (TSC2), which inhibits the tuberous sclerosis complex (TSC) (Hay and Sonenberg, 2004). The TSC, a complex formed by the association of TSC1 and TSC2, exhibits GTPase-activating protein (GAP) activity that negatively regulates the activity of Rheb (Ras homolog enriched in brain). Rheb is a member of the Ras superfamily of GTPases, which activates mTOR in a GTP dependent manner (Long et al., 2005). To summarize, the activation of Akt suppresses the GTPase activity of the TSC, which enables Rheb to activate mTOR.

The mechanism that activates mTORC1 in response to nutrients is less clear. Two general models have been proposed. The first suggests that amino acids inactivate the TSC complex and is based on the observation that the deletion of TSC1 and TSC2 in *Drosophila* renders cells resistant to amino acid deprivation (Gao et al., 2002). A second model of nutrient sensing by the mTORC1 complex suggests that nutrient availability stabilizes the interaction between mTOR and raptor (Kim et al., 2002). Therefore, when nutrient availability is low, raptor dissociates from mTOR, thus inhibiting the activity of mTORC1.

1.6.2 The mTORC2 complex and downstream signaling effectors

In comparison to the mTORC1 complex, less is known regarding the biological function of the mTOR-riCTOR mTORC2 complex. mTORC2 appears to regulate the organization of the actin cytoskeleton through the activation of protein kinase-C alpha (PKC α) (Jacinto et al., 2004; Sarbassov et al., 2004). In addition, the mTORC2 complex was recently identified as the critical kinase mediating the phosphorylation of Akt Ser 473 *in vitro* (Sarbassov et al., 2006; Sarbassov et al., 2005b). Furthermore, the deletion of rictor in mice results in no detectable phosphorylation of Akt Ser 473 in protein lysates prepared from mouse embryo fibroblasts (Guertin et al., 2006). Interestingly, disruption of mTORC2 activity does not result in a decrease in S6K1 or 4E-BP phosphorylation, suggesting that mTORC2 targets a pool of Akt that is distinct from the pool of Akt upstream of the mTORC1 complex (Jacinto et al., 2004; Sarbassov et al., 2004).

Evidence from knockout mice supports the conclusion that mTORC2 exhibits non-overlapping functions with mTORC1. First, mTOR $-/-$ embryos exhibit embryonic lethality around embryonic day 5.5 shortly after implantation, exhibiting defects in inner cell mass and trophoblast giant cell proliferation (Gangloff et al., 2004; Murakami et al., 2004). Likewise, the genetic deletion of the mTORC1 component, raptor, phenocopies the postimplantation embryonic lethality observed in mTOR $-/-$ embryos (Guertin et al., 2006). In contrast, disruption of mTORC2 function via the genetic deletion of rictor results in embryonic lethality at midgestation around embryonic day 10.5 subsequent to embryogenesis (Guertin et al., 2006; Shiota et al., 2006). The phenotype of rictor knockout embryos is strikingly similar to Akt1/Akt3 double knockout embryos, thus

supporting the idea that the regulation of Akt is an essential function of the mTORC2 complex (Dummler et al., 2006).

In comparison to the mTORC1 complex, little is known regarding the signaling pathways that lead to mTORC2 activation. Recently, a study showed that insulin signaling activates the mTORC2 complexes that contain Sin1.1 or Sin1.2, but not Sin1.5 (Frias et al., 2006). However, the mechanism remains to be defined. In contrast to mTORC1, Rheb does not activate mTORC2, suggesting that the signaling mechanism that links growth factor signaling to mTORC2 is distinct from mTORC1 (Yang et al., 2006).

1.7 mTOR and differentiation

In addition to functioning as a key regulator in the processes of cell growth and translation, several studies have described a role for mTOR signaling in the process of cellular differentiation. In *Drosophila*, TOR signaling is activated downstream of the insulin receptor and controls the timing of neuronal differentiation in the eye (Bateman and McNeill, 2004). In mammalian systems, an mTOR dependent mechanism has been reported to regulate the differentiation of adipocytes and myoblasts.

Specifically, mTOR is activated downstream of IGF-I mediated signaling during myoblast differentiation and regulates the mRNA level of myogenin, a basic helix loop helix transcription factor of the MyoD family required for terminal differentiation and myotube formation (Coolican et al., 1997; Sumitani et al., 2002). Furthermore, the

activation of p70S6K1 correlates with myoblast differentiation, implicating the mTORC1 complex in this process (Sarker and Lee, 2004).

Several studies show that mTOR integrates signaling from insulin stimulation and nutrient availability to regulate adipocyte differentiation (Cho et al., 2004; El-Chaar et al., 2004; Kim and Chen, 2004). mTOR activity is required during several distinct phases of adipocyte differentiation, as the inhibition of mTOR on successive days in culture affects both an early clonal expansion phase, as well as the terminal differentiation of adipocytes (Gagnon et al., 2001; Kim and Chen, 2004; Yeh et al., 1995). During adipocyte differentiation, mTOR regulates the mRNA levels and activity of the peroxisome proliferator-activated receptor- γ (PPAR- γ) and the CCAAT/enhancer-binding protein- α (C/EBP- α), two key transcription factors required for adipogenic gene expression (Kim and Chen, 2004).

Finally, mTOR regulates a subset of cell fate decision in CNS stem cells *in vitro* (Rajan et al., 2003). Specifically, bone morphogenetic protein 4 (BMP4) induces high density cultures of CNS stem cells to undergo glial differentiation through a mechanism that requires the mTOR dependent activation of the signal transducer and activator of transcription 3 (Stat3).

1.8 Statement of thesis

The results of the described studies provide evidence that OPC differentiation is regulated by mechanisms that coordinate cell cycle withdrawal and the expression of genes required for myelination. While the prevailing model of OPC differentiation

suggests that an intrinsic timing mechanism directs the generation of mature oligodendrocytes, several extrinsic factors have been identified that share a responsibility in this process. However, the molecular mechanisms which integrate signals derived from extrinsic and intrinsic factors to promote the generation of mature oligodendrocytes remain to be elucidated.

Of particular interest to our laboratory, IGF-I promotes the generation of GalC⁺ oligodendrocytes *in vitro*, and enhances myelination *in vivo* (Carson et al., 1993; McMorris and Dubois-Dalcq, 1988). The mechanisms responsible for the actions of IGF-I during OPC differentiation are unknown. Furthermore, IGF-I is a potent survival factor for the cells of the oligodendrocyte lineage, which impedes the study of IGF-I function during OPC differentiation at the level of IGF-I receptor activation due to confounding effects on cell viability.

Previous studies from our lab demonstrated that IGF-I signaling through the PI3K pathway activates Akt in a sustained fashion (Ness et al., 2002; Ness and Wood, 2002). The sustained activation of Akt correlates with IGF-I mediated protection from glutamate excitotoxicity, as well as progression to the immature oligodendrocyte stage of oligodendrocyte differentiation. Therefore, we reasoned that the study of signaling effectors downstream of Akt may reveal distinct pathways that mediate IGF-I effects on differentiation, but not survival.

IGF-I mediated activation of the PI3K/Akt pathway leads to the activation of mTOR, a Ser-Thr kinase that regulates cell growth, proliferation, mRNA translation, and differentiation. Relevant to this dissertation, mTOR regulates the differentiation of myoblasts and adipocytes in response to IGF-I/insulin stimulation in part through the

control of the expression and activity of key transcription factors. Taken together, we questioned whether mTOR performs a similar function during oligodendrocyte differentiation.

These observations led us to test the *central hypothesis* of this dissertation: *The activation of mTOR is required for oligodendrocyte differentiation*. To this end, the central hypothesis is tested in the following chapters:

- In *chapter two*, we examine the effects of mTOR inhibition on OPC lineage progression.
- In *chapter three*, we characterize mTOR complex formation and the activation of downstream signaling effectors during oligodendrocyte differentiation
- In *chapter four*, we test the role of S6K1 in mediating the effects of mTORC1 complex activity during oligodendrocyte differentiation.
- In *chapter five*, we detail our attempts to generate an inducible transgenic system to study gene function in the cells of the oligodendrocyte lineage.

Figure 1.1 Stages of oligodendrocyte lineage progression

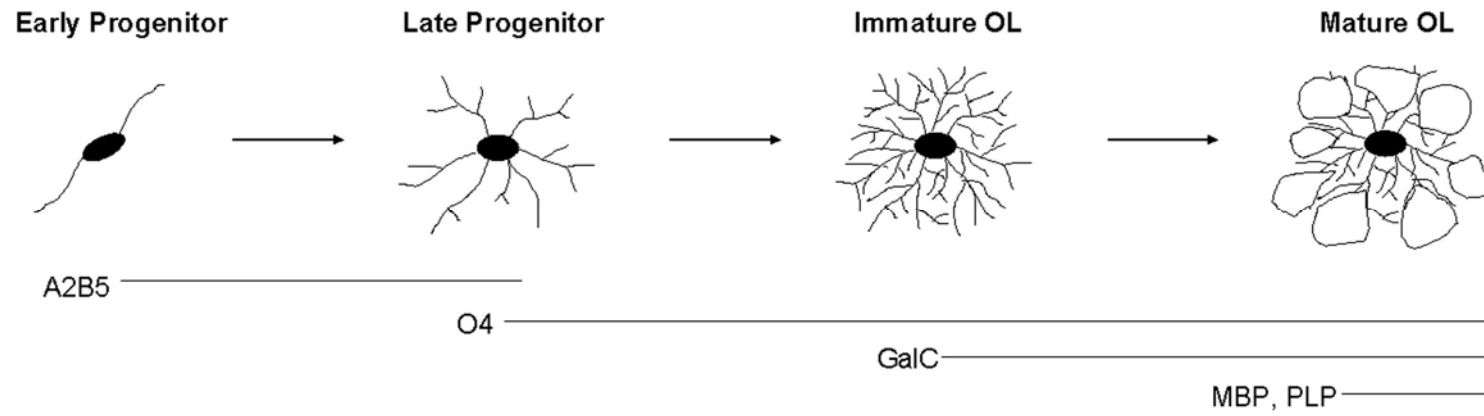


Figure 1.1: Stages of oligodendrocyte lineage progression

Figure 1.2 TOR signaling complexes and downstream targets

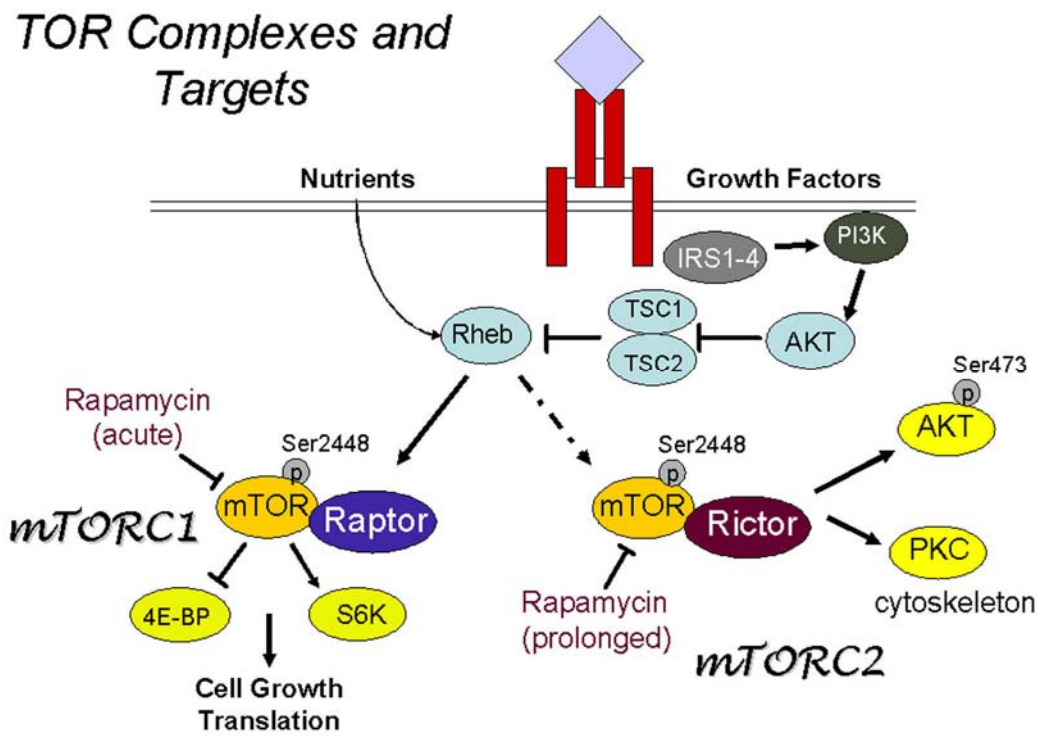


Figure 1.2: TOR signaling complexes and downstream targets

Chapter 2

The activation of the mammalian target of rapamycin (mTOR) is essential for oligodendrocyte differentiation

2.1 Introduction

How do stem and progenitor cells integrate the myriad of extrinsic and intrinsic signals that drive their decisions to proliferate or exit the cell cycle and differentiate? The prevailing thought, in the case of oligodendrocyte progenitors, is that the decision to differentiate into mature oligodendrocytes is controlled by an intrinsic, constitutive pathway initiated solely by the withdrawal of mitogens (Barres et al., 1994; Raff, 2006; Raff et al., 1983; Tokumoto et al., 1999). While several factors, including thyroid hormone (T3) (Ahlgren et al., 1997; Barres et al., 1994), have been identified that enhance the rate of oligodendrocyte generation, a requisite role for these factors in this process is a matter for debate, based on the observation that oligodendrocyte progenitors are able to fully complete a differentiation program in their absence (Barres et al., 1994; Raff et al., 1983).

One caveat to the constitutive model of oligodendrocyte differentiation lies in the fact that a micromolar concentration of insulin, known to activate the IGF type I receptor (LeRoith et al., 1995), is a primary component of the medium used to study oligodendrocyte differentiation *in vitro*. This is an important consideration because several studies have linked the actions of IGF-I to oligodendrocyte differentiation and myelin protein expression *in vitro* and *in vivo* (Carson et al., 1993; McMorris and

Dubois-Dalcq, 1988; Ye et al., 2002). However, the ability to declare IGF-I a true stimulus for differentiation is complicated by the fact that it is also a potent trophic factor for the lineage acting via the PI3K pathway (Cui et al., 2005; Kuhl et al., 2002; Ness and Wood, 2002; Vemuri and McMorris, 1996).

We have approached the problem of whether IGF signaling regulates oligodendrocyte differentiation by focusing on the intracellular signaling pathways downstream of the IGF-IR in an attempt to identify signaling components that distinctly mediate IGF-I effects on oligodendrocyte differentiation, but not survival. Previous studies from our lab demonstrated that IGF-I activates Akt via the PI3K pathway in a sustained fashion (Ness et al., 2002; Ness and Wood, 2002). The sustained activation of Akt correlates with the ability of IGF-I to promote survival of oligodendrocyte progenitors, but also coincides with the transition of late oligodendrocyte progenitors to a post-mitotic, mature stage of differentiation (Ness and Wood, 2002).

Signal transduction downstream of Akt diverges to an array of effector pathways, including glycogen synthase kinase-3 (GSK-3), the Forkhead-box Class O (FoxO) transcription factors, and the mammalian target of rapamycin (mTOR) (Kandel and Hay, 1999). Activation of these pathways influences a number of biological processes including proliferation, survival, protein translation, and differentiation (Coffer et al., 1998; Dummler and Hemmings, 2007). Therefore, we reasoned that the examination of signaling downstream of Akt might identify pathways that specifically regulate the differentiation of oligodendrocyte progenitors.

In this chapter, we examine the role played by the mammalian target of rapamycin (mTOR) in oligodendrocyte differentiation. mTOR is a Ser-Thr kinase that functions as a

key regulator of cell growth and translation in response to PI3K/Akt stimulation and nutrient sensing (Harris and Lawrence, 2003). The activation of mTOR influences a diverse array of biological processes (Sarbasov et al., 2005a). Relevant to this dissertation, mTOR has been implicated in the control of adipocyte and myoblast differentiation (Gagnon et al., 2001; Shu et al., 2002), as well as neuronal differentiation in *Drosophila* (Bateman and McNeill, 2004). Our studies demonstrate that mTOR plays an essential role in oligodendrocyte differentiation.

2.2 Materials and Methods

2.2.1 Materials

Cell culture media (MEM, DMEM/F12), FBS, trypsin, and insulin-selenium-transferrin (ITS) were purchased from GIBCO-BRL (Long Island, NY). Additional N2 supplements, triiodothyronine, and poly-d-lysine were purchased from Sigma (St. Louis, MO). Poly-d-lysine/laminin coated coverslips were acquired from BD Biosciences (San Jose, CA). Recombinant human FGF-2 was purchased from R&D Systems (Minneapolis, MN). For *in vitro* experiments, rapamycin was purchased from Calbiochem (San Diego, CA). Additional quantities of rapamycin, used for *in vivo* injections, were acquired from the NIH (Bethesda, MD). Control and mTOR validated siRNA smart pools were purchased from Dharmacon (Lafayette, CO). Antibodies to MBP, PLP, and CNP were purchased from Chemicon (Temecula, CA). Antibodies to mTOR and active caspase-3 were purchased from Cell Signaling, Inc. (Danvers, MA). Antibodies to p21 and p27

were purchased from BD Biosciences (San, Jose, CA). Trizol reagent and Superscript III reverse transcriptase were purchased from Invitrogen (Carlsbad, CA). Validated QuantiTect primer sets specific for MBP, PLP, Olig2, and Sox10; and the QuantiTect SYBR green PCR detection kit were purchased from Qiagen (Valencia, CA).

2.2.2 Primary cortical oligodendrocyte progenitor cultures

Oligodendrocyte progenitor cells (OPCs) were purified from cortical mixed glial cultures by established methods (Levison and McCarthy 1991). Briefly, brains were removed from postnatal day 0-2 Sprague Dawley rat pups and the cortices were dissected. Cortical pieces were enzymatically digested in 2.5% trypsin and Dnase I followed by mechanical dissociation. Cells were resuspended in MEM-C, which consisted of minimal essential media (MEM) supplemented with 10% FBS, L-glutamine, and 1% Pen-strep, and plated in T75 flasks. The resulting mixed glial cultures were maintained for 10 days.

Purified OPC cultures were prepared by a differential shake described previously (McCarthy and de Vellis, 1980). First, microglia were separated from the cultures by shaking the flasks on a rotary shaker for 1.5h at 260 rpm. OPCs were then isolated following an additional 18h shake. OPCs were subjected to a final purification step to remove residual microglia by plating the cells on a Petri dish for 10 min at 37°C. Purified OPCs were seeded onto poly-d-lysine coated T75 flasks at a density of 2×10^4 cells/cm² in a chemically defined medium termed N2S. N2S consisted of 66% N2B2 (defined below) supplemented with 34% B104 conditioned media, 5ng/ml FGF, and

0.5% FBS. Purified OPCs were amplified for 4-10 days in N2S, passaged once with papain, and plated for experiments. N2B2 media was composed of DMEM/F12 supplemented with 0.66 mg/ml BSA, 10 ng/ml d-biotin, 5 µg/ml insulin, 20 nM progesterone, 100 µM putrescine, 5 ng/ml selenium, 50 µg/ml apo-transferrin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5% FBS.

2.2.3 Differentiation paradigm and rapamycin treatment

To initiate OPC differentiation, an established mitogen withdrawal protocol was followed (Tokumoto et al., 1999). Briefly, OPCs were seeded on to poly-d-lysine coated dishes or poly-d-lysine/laminin coated coverglass at a density of 2×10^4 cells/cm² in N2S media and allowed to recover overnight. The following day, N2S media containing FGF and B104 conditioned media, a source of PDGF, was replaced with mitogen free N2B2 media supplemented with 30 ng/ml triiodothyronine (T3). For rapamycin treatments, a stock solution of rapamycin resuspended in ethanol was diluted into N2B2+T3 to a final concentration of 15nM. Control cultures received vehicle alone (0.002% ethanol). N2B2+T3 differentiation media with or without rapamycin was replenished every 48 hrs during the course of experiments.

2.2.4 Immunocytochemistry and *in vitro* quantification

Purified OPCs were plated onto poly-d-lysine/laminin coated coverglass in N2S media at a density of 2×10^4 cells/cm² and allowed to recover overnight. The following

day, differentiation in the presence or absence of rapamycin at the indicated doses was initiated by mitogen withdrawal in N2B2+T3 differentiation media. Following 3.5 or 5 days of differentiation, the coverslips were rinsed 3 times in PBS. Cells were then fixed with 2% paraformaldehyde for 8 min followed by 3 washes in PBS. A blocking step was performed by incubating the coverslips in diluent (PBS containing 10% fetal calf serum and 10% lamb serum) for 15 minutes. Detection of O4 and GalC cell surface antigens was performed by diluting supernatant from the O4 hybridoma and Ranscht hybridoma 1:3 and 1:2 respectively in diluent and added to the coverslips for 45 minutes at room temperature. Coverslips were washed again in PBS and incubated in diluent containing DAPI (1:1000) and goat anti-mouse-IgM FITC (1:500) and goat anti-mouse-IgG3 TRITC (1:250) secondary antibodies for 45 minutes to detect O4 and GalC respectively. Finally, coverslips were washed 3 times in PBS and mounted on superfrost slides with Aquamount containing 2.5% DABCO. To detect intracellular antigens, including PLP, cells were permeabilized with 0.1% Triton X-100 for 4 minutes following fixation in 2% paraformaldehyde. The detection of PLP was performed by diluting the anti-PLP antibody (Chemicon) 1:100 in diluent and incubated as described above, followed by an incubation step with goat anti-mouse-IgG FITC secondary antibodies.

To quantify the number of O4⁺/GalC⁺ immature oligodendrocytes present in our cultures following 3.5 and 5 days of differentiation, cell counts were performed on a minimum of 6 independent fields (2 fields/3 coverslips/treatment) of photomicrographs captured with a 20X objective. Total counts of O4 and GalC immunoreactive cells were performed, and the number of immature oligodendrocytes per culture was expressed as the percentage of GalC⁺ cells to total O4⁺ cells.

2.2.5 Transfection of siRNA smart pools

For siRNA smart pool transfections, 5×10^6 OPCs were collected and resuspended in 100 μ l of rat oligodendrocyte nucleofector solution (Amaxa). Control or mTOR targeting siRNA smart pools (200 pmols) were added to the cell suspension and transferred to an Amaxa certified cuvette. Cells were electroporated with the Amaxa nucleofection device using a protocol, O-17, optimized for the transfection of primary rat oligodendrocytes. Following electroporation, cells were resuspended in N2S media and plated on poly-d-lysine coated 35 mm dishes. A single transfection yielded enough cells to seed 6-9 35 mm dishes. Cells were allowed to attach and recover overnight in N2S media. The following day, differentiation was initiated in N2B2+T3 media, and cells were cultured for 4 or 5 days prior to protein isolation.

2.2.6 Protein isolation and western immunoblotting

Following treatments, cells were washed twice with ice cold PBS and total cell lysates were harvested in 2X SDS lysis buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 100 mM DTT, 1/50 protease inhibitor (PI) cocktail (Sigma, St. Louis, MO)). The lysates were briefly sonicated and stored at -80°C prior to western analysis. A RC-DC protein assay (BioRad) was performed to determine protein concentration. Approximately 15 μ g of total protein per sample was aliquoted, boiled for 5 minutes and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Bis-Tris mini-gels (Invitrogen). Separated proteins were then transferred to nitrocellulose membranes and blocked in 5% milk/ TBS-0.1% Tween for one hour at room temperature.

Membranes were then incubated in the presence of primary antibodies for MBP (Chemicon, 1:100), mTOR (Cell Signaling (1:1000), p27 (BD Biosciences 1:1000), P21 (BD biosciences 1:1000), active caspase-3 (Cell Signaling, 1:1000), or β -actin (Sigma, 1:5000) diluted in 5% milk/ TBS-0.1% Tween overnight at 4°C. The following day, membranes were washed 3 times for 5 minutes with TBS-0.1% Tween and incubated for 1 hour at room temperature in 5% milk/ TBS-0.1% Tween containing goat anti-rabbit or goat anti-mouse secondary antibodies at a dilution of 1:5000. The detection of HRP conjugated secondary antibodies was performed by enhanced chemiluminescence using the Ultra-LUM imaging device. Protein expression levels were quantified using NIH image 1.62.

2.2.7 RNA isolation and qPCR analysis

For RNA isolation, primary rat OPCs were seeded on poly-d-lysine coated 150mm dishes in N2S media at a density of 2×10^4 cells/cm². The following day, differentiation was initiated in the presence or absence of 15 nM rapamycin in N2B2+T3 differentiation media. On day 3 of differentiation, cells were washed twice in PBS, harvested in 500ul of PBS, and pelleted by centrifugation at 10000xg for 10 minutes. The supernatant was aspirated and cell pellets were stored at -80°C prior to RNA isolation.

Frozen cell pellets were briefly thawed on ice and resuspended in 1 ml of Trizol reagent (Invitrogen). Total RNA was extracted following the TRIZOL isolation protocol. Precipitated total RNA was resuspended in 50 ul of nuclease free water and stored at -

80°C. The mRNA concentration for each sample was measured using a NanoDrop spectrophotometer (NanoDrop Technologies). Three sets of mRNA were isolated from independent experiments for cDNA generation and qPCR analysis. One microgram of total RNA was treated with Dnase I (Invitrogen) and used to generate cDNA. First strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). Concentrations of cDNA were determined by spectrophotometry and stored at -20°C prior to qPCR analysis.

For qPCR reactions, 150 ng of cDNA was used as template in each reaction containing 1X SYBR green detection master mix and 1X QuantiTect primer mix to detect the mRNA expression levels of MBP, PLP, Ugt-8, Sox10, Olig2, Nkx2.2, Id2, or Id4 (Qiagen). Amplification levels were normalized to expression levels of β -actin for each sample. The qPCR reactions were performed on the Applied Biosystems 7900HT Fast Real-time PCR system using associated Sequence Detection Systems Software Version 2.2.2 (SDS2.2.2, Foster City, CA). The thermal reaction profiles for the PCR reactions were performed as follows: 50°C for 2 minutes, 9°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 58°C for 1 minute.

The relative quantity of each mRNA transcript was determined using Sequence Detection Systems Software Version 2.2.2 as follows: First, cycle threshold (Ct) values were measured for each amplification reaction; Next, Δ Ct values were defined as ($Ct_{\text{target}} - Ct_{\text{actin}}$); $\Delta\Delta$ Ct values were computed as ($\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$); finally, the relative quantity (RQ) was defined as $2^{-\Delta\Delta Ct}$. Samples from each experiment were amplified in triplicate.

2.2.8 *In vivo* administration of rapamycin and protein isolation

To investigate the effects of mTOR inhibition on myelination *in vivo*, a series of intraperitoneal (IP) injections of rapamycin was performed. A stock solution of rapamycin dissolved in ethanol was diluted to a final dose of 2.5 mg/kg in Hank's Balanced Saline Solution (HBSS) and delivered via IP injection to postnatal day 14 rat pups for 5 consecutive days. Control animals received vehicle alone (20% ethanol) in HBSS over the same time course. Following injections, pups were returned to the dams. Three experimental (+rapamycin) and three control animals received injections. On postnatal day 20, the rat pups were sacrificed by a lethal injection of a 3:1 ketamine/xylazine cocktail. Brains were removed, dissected in the sagittal plane at the midline and one brain half was embedded for sectioning, while the second half was used for protein extraction. Brain samples for protein isolation were frozen on dry ice and stored at -80°C. Frozen brains were wrapped in aluminum foil, snap frozen in liquid nitrogen, and pulverized. Pulverized tissue was lysed in 1X SDS/ PBS containing protease inhibitor cocktail. Tissue lysates were briefly sonicated, boiled for 5 minutes, and centrifuged at 10000xg for 10 minutes. The supernatants were collected and stored at -80°C. Protein concentration was determined using a RC-DC protein assay. Approximately 20 ug of total protein was aliquoted and separated by SDS-PAGE on 12% Bis-Tris mini-gels. Immunoblotting for MBP, CNP (Chemicon, 1:1000), and β -actin was performed as described previously.

2.2.9 Statistical analysis

Statistical analyses were performed using StatView statistical analysis software. For experiments comparing two treatment groups, an unpaired t-test was performed to assess the statistical significance between treatment groups. For experiments in which multiple treatment groups or time points were examined, one way analysis of variance (ANOVA) was performed to assess the statistical significance between treatment groups. All experiments were repeated at least 3 times.

2.3 Results

2.3.1 The inhibition of mTOR decreases morphological complexity during OPC differentiation

Oligodendrocyte differentiation is characterized in part morphologically by the dramatic transition from simple bipolar early progenitors, to complex multipolar oligodendrocytes. To determine if mTOR regulates the morphological progression of OPCs during differentiation, we examined cultures of differentiating OPCs treated with 15 nM rapamycin by phase contrast microscopy. Following 2 days of differentiation, control and rapamycin treated cells displayed increased branching compared to undifferentiated early progenitors (Figure 2.1 A-C). Control cultures contained a mixed population of simple multipolar cells, characteristic of late progenitors, as well as complex multipolar oligodendrocytes which extended an elaborate network of lacy processes. However, we did not observe any complex multipolar oligodendrocytes in

rapamycin treated cultures. Instead, rapamycin treated cells exhibited a simple multipolar morphology suggesting that they were blocked at the late progenitor stage of differentiation.

2.3.2 Inhibition of mTOR blocks OPC differentiation at the O4⁺/GalC⁻ late progenitor stage

To determine directly whether inhibition of mTOR prevents oligodendrocyte differentiation past the late progenitor stage, we performed immunocytochemistry for the stage specific cell surface antigens O4 and GalC. Differentiating cortical OPC cultures were treated with or without 15 nM rapamycin for 3.5 days, and the total numbers of O4⁺/GalC⁻ late progenitors and O4⁺/GalC⁺ immature oligodendrocytes were quantified.

Representative images of OPCs following 3.5 days of differentiation are shown in (Figure 2.2A-D). Cells in both treatment groups gained the expression of the O4 cell surface antigen, a marker of progression to the late progenitor stage of differentiation. In addition, approximately 30% of cells in control cultures were identified as GalC⁺ immature oligodendrocytes (Figure 2.2E). However, inhibition of mTOR by rapamycin caused a significant reduction in the number of GalC⁺ cells (<1%, P<0.0001; Figure 2.2E), effectively blocking the progression of OPC differentiation at the late progenitor stage.

To determine whether rapamycin delayed rather than blocked differentiation, we repeated the immunocytochemistry for O4 and GalC following 5 days of differentiation. After 5 days of differentiation, ~50% of the OPCs in control cultures reached the GalC⁺ immature OL stage (Figure 2.2F). However, fewer than 10% of the OPCs treated with 15

nM rapamycin progressed past the late progenitor stage (Fig 2.2F, $P < 0.0001$). In addition, we performed a dose response experiment to determine the sensitivity of the transition to the O4+/GalC+ immature OL stage to mTOR inhibition. Doses of rapamycin as low as 0.15 nM caused a 28% reduction ($P < 0.0001$) in the number of OPCs that progressed past the O4+/GalC- late progenitor stage (Figure 2.2F).

2.3.3 mTOR regulates the expression of myelin proteins MBP and PLP

To provide further evidence that mTOR regulates the progression of OPC differentiation past the late progenitor stage, we examined the expression of two myelin proteins, myelin basic protein (MBP) and proteolipid protein (PLP). First, we performed immunocytochemistry for PLP on cells differentiated in the presence or absence of 15 nM rapamycin following 5 days of differentiation. By 5 days of differentiation, we identified a significant number of PLP positive oligodendrocytes in control cultures (Figure 2.3A, B). However, mTOR inhibition completely blocked the expression of PLP (Figure 2.3C, D). In addition, we performed western blot analysis of MBP expression during a differentiation time course. We observed a robust increase in MBP expression in control cultures following 4 days of differentiation that was completely abrogated by mTOR inhibition (Figure 2.3E).

In order to confirm that the effects of rapamycin on OPC differentiation were a specific result of inhibiting mTOR activity, we performed siRNA mediated knockdown of mTOR expression. OPCs were transiently transfected with either a control or mTOR specific siRNA smart pool. One day following transfection, the cells were switched to

differentiation media and kept in culture for 4 days. Western blot analysis of MBP expression was quantified as an index of differentiation. We observed a marked increase in MBP expression in cells transfected with the control siRNA smart pool by 5 days of differentiation (Figure 2.3F). In contrast, transfection with the mTOR siRNA smart pool significantly reduced MBP expression (Figure 2.3F). Accordingly, mTOR expression was also significantly reduced by the transfection of a siRNA smart pool targeting mTOR, while the control siRNA smart pool had no effect on mTOR protein levels (Figure 2.3F).

2.3.4 mTOR inhibition has no effect on OPC viability during differentiation

In addition to the analyses of differentiation parameters, we also determined that rapamycin treatment had no effect on OPC viability. First, we quantified the total numbers of cells following 3.5 and 5 days of differentiation in the presence or absence rapamycin by performing immunocytochemistry for the O4 cell surface antigen. We used O4 as an index of cell number since all of the cells in our cultures acquired O4 expression by these time points. After 3.5 and 5 days of differentiation, we observed no significant change in the total number of cells in cultures treated with rapamycin versus control cultures (Figure 2.4A, B).

To provide further evidence of cell viability, we measured changes in the level of active caspase-3 during a time course of differentiation. Inhibition of mTOR did not increase levels of active caspase-3 at any time point (Figure 2.4C, D). In fact, rapamycin

treatment caused a significant reduction in the observed levels of active caspase-3 following 2 days of differentiation (Figure 2.4D, $P=0.0375$).

Finally, we show that the differentiation block caused by mTOR inhibition is partially reversible. We transiently exposed cells to rapamycin for 1-3 days, after which we replenished the cultures with rapamycin free media for the remainder of the experiment. After six days of differentiation, we quantified the number of GalC⁺ immature oligodendrocytes by immunocytochemistry. Cultures transiently exposed to rapamycin for 1, 2, or 3 days showed a significant increase in the numbers of GalC⁺ cells when compared to cells treated with rapamycin for the duration of the experiment (Figure 2.4E, $P<0.0001$).

2.3.5 mTOR inhibition has no effect on the expression of p27 or p21

Commitment to oligodendrocyte differentiation requires permanent withdrawal from the cell cycle (Casaccia-Bonnet and Liu, 2003). Two cyclin dependent kinase inhibitors (cdkis), p27 and p21, play a critical role in cell cycle exit (Nakayama, 1998). Furthermore, p27 and p21 have defined roles in regulating cell cycle exit during OPC differentiation (Casaccia-Bonnet et al., 1997; Zetzl et al., 2001). In addition, previous reports have demonstrated that mTOR regulates the expression of p27 and p21 in a number of cell types (Ilyin et al., 2003; Kawamata et al., 1998; Leung-Pineda et al., 2004). Therefore, we determined whether mTOR inhibition altered cdki expression levels in cultures of differentiating OPCs. Consistent with previous reports (Durand et al., 1997; Friessen et al., 1997), p27 was expressed at high levels throughout OPC

differentiation (Figure 2.5A, B). In contrast, p21 expression peaked within the first 24 hours of OPC differentiation and then declined (Figure 2.6A, B). Inhibition of mTOR activity by rapamycin had no significant effect on the levels of p27 or p21 at any time point examined.

2.3.6 mTOR regulates the mRNA levels of myelin genes

Our previous data demonstrate that mTOR inhibition significantly reduces the protein levels of the myelin genes MBP and PLP. To gain insight into the mechanism by which mTOR regulates myelin protein expression, we measured MBP and PLP mRNA levels by quantitative PCR. Total RNA isolated from control and rapamycin treated cultures following 3 days of differentiation was used to generate cDNA that was subjected to a validated QuantiTect primer assay (Qiagen) for MBP and PLP. Inhibition of mTOR by rapamycin resulted in an 84% and 64% reduction in MBP and PLP mRNA levels, respectively (Figure 2.7 (A) MBP: $P=0.0032$; (B) PLP: $P=0.0032$).

In addition, we measured the mRNA levels of UDP glycosyltransferase 8 (Ugt-8) following 3 days of differentiation in the presence or absence of rapamycin. Ugt-8 catalyzes the final step in the biosynthesis of cerebroside (Koul et al., 1980), a major component of myelin membranes, and is upregulated during oligodendrocyte differentiation (Dugas et al., 2006). Similar to MBP and PLP, mTOR inhibition caused a 58% reduction in Ugt-8 mRNA levels following 3 days of differentiation. (Figure 2.7C, $P=0.0019$). We focused on 3 days of differentiation because a previous report showed

that the mRNA levels for MBP, PLP, and Ugt-8 are at or near peak levels during this time point (Dugas et al., 2006).

2.3.7 mTOR inhibition has no effect on Olig2, Sox10, or Nkx2.2 mRNA levels

Previous data support the conclusion that mTOR activity is critical for the transition past the late progenitor stage of OPC differentiation. mTOR regulates the expression of several myelin genes at the mRNA level, suggesting that mTOR regulates the decision to commit to oligodendrocyte differentiation rather than just affecting myelin protein levels. One possible explanation is that mTOR modulates the expression of key transcription factors required for differentiation.

Transcription factors involved in oligodendrocyte specification and differentiation include but are not limited to, the basic helix-loop-helix (bHLH) family members Olig1 and Olig2, SRY box containing (Sox) family members Sox8, Sox9, Sox10, and Sox17, and the homeobox containing (Hox) transcription factor Nkx2.2. We have begun to examine the effects of mTOR inhibition on transcription factor expression by measuring the mRNA levels of Olig2, Sox10, and Nkx2.2 during oligodendrocyte differentiation *in vitro*.

We selected Olig2, Sox10, and Nkx2.2 for initial studies based on the following observations: 1) Olig2 is required for oligodendrocyte specification in the vertebrate CNS (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002), but its expression is maintained in mature oligodendrocytes suggesting an additional role for Olig2 in oligodendrocyte maturation (Lu et al., 2000; Takebayashi et al., 2000; Zhou et

al., 2000). The actions of Olig2 in this process may involve the direct control of Sox10 expression (Liu et al., 2007). 2) Sox10 is upregulated in oligodendrocyte progenitors subsequent to their specification and is required for terminal differentiation (Stolt et al., 2004; Stolt et al., 2002). 3) Deletion of Nkx2.2 in mice results in a dramatic decrease in the numbers of MBP and PLP positive oligodendrocytes generated in the CNS despite the presence of appropriate numbers of Olig2⁺ oligodendrocyte progenitors, suggesting Nkx2.2 is required for oligodendrocyte differentiation subsequent to specification (Qi et al., 2001). 4) Olig2, Sox10, and Nkx2.2 have synergistic effects on oligodendrocyte differentiation which requires the appropriate expression and interactions of these transcription factors ((Liu et al., 2007).

In order to examine the mRNA levels of these transcription factors, total RNA isolated from control and rapamycin treated cultures following 3 days of differentiation was used to generate cDNA that was subjected to a validated QuantiTect qPCR primer assay (Qiagen) for Olig2, Sox10, and Nkx2.2 . Interestingly, mTOR inhibition did not significantly alter the mRNA levels of Olig2, Sox10, or Nkx2.2 (Figure 2.8 A-C). We are currently examining the protein levels of Olig2 and Sox10 to determine if mTOR regulates the translation of these mRNAs.

2.3.8 mTOR regulates the mRNA levels of the inhibitor of DNA binding family members, Id2 and Id4

The inhibitor of DNA binding family of proteins is structurally similar to basic helix-loop-helix (bHLH) transcription factors except they lack the basic DNA binding domain (Yokota, 2001). The Id proteins act as dominant negative inhibitors of bHLH

transcription factors by binding to and sequestering class A bHLH proteins, preventing their ability to dimerize with class B bHLH to activate gene transcription (Norton, 2000).

OPCs express the Id(s) 1-4, and the expression of Id2 and Id4 directly controls the timing of oligodendrocyte differentiation *in vitro* and *in vivo* (Kondo and Raff, 2000; Marin-Husstege et al., 2006; Wang et al., 2001). In order to determine whether mTOR regulates the expression of Id2 or Id4 during oligodendrocyte differentiation, we measured their mRNA levels through a validated QuantiTect qPCR primer assay following 3 days of differentiation. Inhibition of mTOR caused a 2.7-fold and 3.8 fold increase in the mRNA levels of Id2 and Id4 respectively relative to control cultures (Figure 2.9A: Id2, P=0.027; B: Id4, P=0.0259).

2.3.9 Systemic administration of rapamycin decreases brain and body weight, but not myelin gene expression

To determine whether mTOR regulates OPC differentiation *in vivo*, we administered rapamycin via intraperitoneal injection to 14 day old rat pups for 5 consecutive days. The experimental group received rapamycin at a dose of 2.5 mg/kg, while the control group received vehicle alone. On postnatal day 20, total body and brain weights were measured. Rapamycin caused a 17% reduction in mean body weight (Figure 2.10A; n=3, P= 0.006). In addition, the mean brain weight from rapamycin treated pups decreased by 18% (Figure 2.10B; n=3, P= 0.0078).

To determine if myelination was altered by the administration of rapamycin, brains were dissected in the sagittal plane at the midline and one brain half was embedded for sectioning, while the second half was used for protein extraction. The

expression levels of MBP and CNP were measured by western immunoblot analysis (Figure 2.10C). Rapamycin had no significant effect on MBP or CNP expression despite reducing mean brain weight.

Because systemic administration of rapamycin failed to alter myelin protein expression, we delivered rapamycin directly into the lateral ventricle of 7 day old pups via surgically implanted osmotic pumps. Four pups received rapamycin at a dose of 0.5mg/kg/day, while four control animals received vehicle alone. All of the pups survived following surgery. However, the pups died within 24 hr of return to the dam possibly due to toxicity of the carrier solution used to solubilize rapamycin.

2.4 Discussion

Our studies reveal an essential role for mTOR in regulating oligodendrocyte differentiation. Specifically, mTOR activation is required for progression past the $O4^+$ /GalC⁻ late progenitor stage. Disruption of mTOR activity by pharmacological inhibition or siRNA mediated knockdown significantly decreased the number of mature oligodendrocytes generated in our cultures. GalC expression, an early indicator of the commitment to oligodendrocyte differentiation, as well as myelin gene expression, was significantly reduced by mTOR inhibition.

Interestingly, we show that the initial phase of OL differentiation, namely the transition to the simple, multipolar $O4^+$ late progenitor stage, is insensitive to mTOR inhibition. In fact, we could not distinguish any discernable differences between control and rapamycin treated cultures during the first 24 hours of differentiation. Furthermore,

the expression of the cdkis p27 and p21 was unchanged by mTOR inhibition, suggesting that the differentiation block observed was not a function of improper withdrawal from the cell cycle. Taken together, these observations imply that distinct extrinsic signals, which specifically activate mTOR, are required to direct late oligodendrocyte progenitors to commit to terminal oligodendrocyte differentiation.

Our analysis demonstrates that mTOR is required for the timely expression of multiple myelin genes including MBP, PLP, and Ugt-8. Rather than solely exerting a translational control of myelin protein expression, mTOR regulates myelin gene expression at the mRNA level. We interpret these data to suggest that mTOR governs factors required for the commitment of oligodendrocyte progenitors to differentiate. To this end, we analyzed the effects of mTOR inhibition on the expression of key transcription factors during oligodendrocyte differentiation. To date, we examined the mRNA levels of Olig2, Sox10, and Nkx2.2, three transcription factors required for the generation of mature oligodendrocytes.

Our results show that the mRNA levels of Olig2, Sox10, and Nkx2.2 were not altered by mTOR inhibition. However, an intriguing possibility is that mTOR exerts a translational control of the expression level of one or more of these transcription factors. In support of this hypothesis, mTOR regulates the translation of specific isoforms of C/EBP α and C/EBP β , members of the CCAAT/enhancer binding (C/EBP) protein family of transcription factors (Calkhoven et al., 2000). Furthermore, the translational control of C/EBP transcription factor isoforms correlates with the cell cycle arrest and differentiation of adipocytes *in vitro* (Calkhoven et al., 2000). Further studies are

required to examine the effect of mTOR inhibition on the protein levels of Olig2, Sox10, and Nkx2.2.

In contrast to the aforementioned transcription factors, our studies demonstrate that mTOR regulates the mRNA levels of members of the inhibitor of DNA binding protein family, Id2 and Id4. The Id proteins act as dominant negative inhibitors of bHLH transcription factors by binding to and sequestering class A bHLH proteins, preventing their ability to dimerize with class B bHLH transcription factors to activate gene transcription (Norton, 2000).

Oligodendrocyte progenitors express the Ids 1-4, and the expression of Id2 and Id4 directly controls the timing of oligodendrocyte differentiation *in vitro* and *in vivo* (Kondo and Raff, 2000; Marin-Husstege et al., 2006; Wang et al., 2001). During OPC differentiation *in vitro*, Id4 expression is rapidly downregulated at the mRNA level (Kondo and Raff, 2000), while Id2 translocates from the nucleus to the cytoplasm (Wang et al., 2001). However *in vivo*, Id2 is ultimately downregulated at the mRNA level during OPC differentiation (Tzeng and de Vellis, 1998). The overexpression of Id2 or Id4 effectively blocks the generation of GalC⁺ postmitotic oligodendrocytes *in vitro* (Kondo and Raff, 2000; Wang et al., 2001). Furthermore, ectopic expression of Id4 globally decreases the mRNA levels of myelin genes including Ugt-8, MBP, PLP, myelin associated glycoprotein (MAG) (Marin-Husstege et al., 2006). Conversely, genetic deletion of Id4 results in the precocious differentiation of oligodendrocyte progenitors *in vivo* (Marin-Husstege et al., 2006). Finally, bone morphogenetic protein-4 (BMP-4) inhibits oligodendrocyte generation from adult neural progenitors in culture via the

upregulation of Id proteins, which directly interact with and suppress the functions of the Olig1 and Olig2 transcription factors (Samanta and Kessler, 2004).

The results of the Id protein overexpression studies are strikingly similar to the differentiation block caused by mTOR inhibition. Furthermore, blocking mTOR activity caused a significant increase in the levels of Id2 and Id4 mRNA transcripts. Our results suggest that mTOR is required for the normal suppression of Id protein function during oligodendrocyte differentiation, as the elevated levels of Id2 and Id4 in rapamycin treated cultures provides at least one mechanistic explanation for the differentiation block caused by disrupting mTOR activity. Further studies are required to confirm that mTOR inhibition results in increased protein levels of Id2 and Id4. In addition, experiments that couple the siRNA mediated suppression of Id2/4 expression with mTOR inhibition will directly test the relationship of these factors in regulating oligodendrocyte differentiation.

While several studies have presented evidence that mTOR modulates the expression of the Id family in other cellular systems (Jankiewicz et al., 2006; Navarro et al., 2001), little is known about the mechanism through which mTOR controls Id protein expression. Oligodendrocyte progenitor differentiation *in vitro* provides a tractable model system to examine the mTOR dependent mechanism that regulates the expression of the Id protein family.

In an attempt to determine the role of mTOR in oligodendrocyte differentiation *in vivo*, we administered rapamycin systemically via intraperitoneal injection beginning on postnatal day 14, for 5 consecutive days. Our results show that delivery of rapamycin during this time significantly decreased mean body and brain weight. However, we were unable to detect any difference in myelin protein content in the brains of rapamycin

treated pups, suggesting that differentiation and myelination were unaltered by the systemic administration of rapamycin.

Why did the systemic administration of rapamycin fail to alter myelination *in vivo*? One possibility is that the dose of rapamycin delivered was not high enough to diffuse into the brain to alter oligodendrocyte lineage progression. Furthermore, the reduction in mean brain weight may have been caused by a systemic effect of rapamycin, rather than acting locally in the CNS. In addition, the possibility exists that the majority of oligodendrocyte progenitors have progressed past a rapamycin sensitive stage of development by postnatal day 14. In fact, a significant degree of myelination has occurred in the forebrain at the level of the corpus callosum by postnatal day 14 in rats (Hamano et al., 1996; Hamano et al., 1998).

To address these concerns, we delivered rapamycin directly into the lateral ventricle of 7 day old pups via surgically implanted osmotic pumps. However, the pups died within 24 hr of return to the dam possibly due to toxicity of the carrier solution used to solubilize rapamycin. Currently, we are performing experiments that couple retroviral lineage tracing with siRNA-mediated silencing of mTOR to study its role in oligodendrocyte differentiation *in vivo*.

Figure 2.1 Rapamycin reduces morphological complexity during OPC differentiation. Phase contrast images of undifferentiated (**A**), control cultures following 2 days of differentiation (**B**), and cultures treated with 15nM rapamycin for 2 days (**C**).

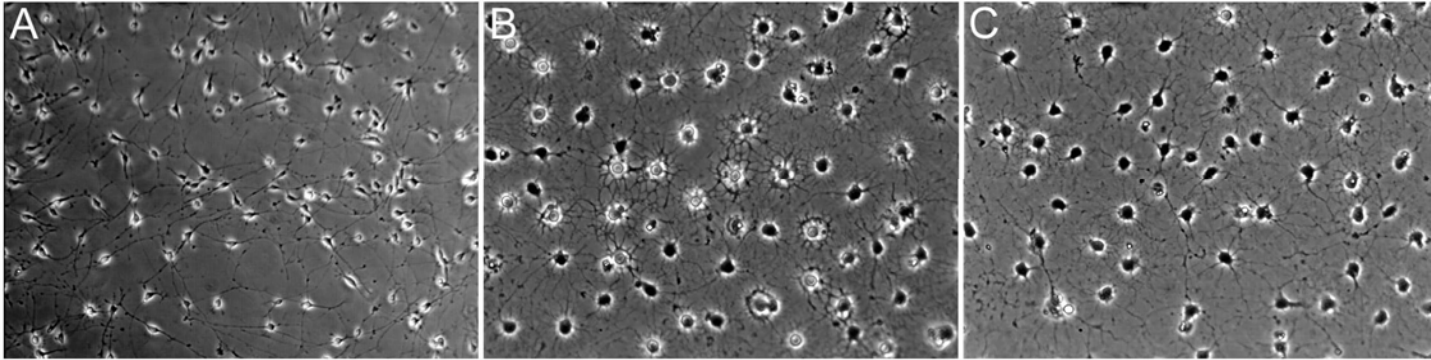


Figure 2.1: Rapamycin reduces morphological complexity during OPC differentiation.

Figure 2.2 Inhibition of mTOR by rapamycin blocks OPC differentiation at the O4⁺/GalC⁻ late progenitor stage. Representative images of O4/GalC IHC performed on OPC cultures differentiated in the absence (**A, B**) or presence (**C, D**) of 15 nM rapamycin for 3.5 days. **E-F:** Quantification of the percentage of GalC⁺ immature oligodendrocytes in control vs. rapamycin treated cultures following 3.5 d of differentiation (E; P<0.0001) or following 5 d of differentiation in control vs. rapamycin treatments of 15 nM, 1.5 nM, and 0.15 nM (F; P<0.0001 vs Ctl for all doses of rapamycin).

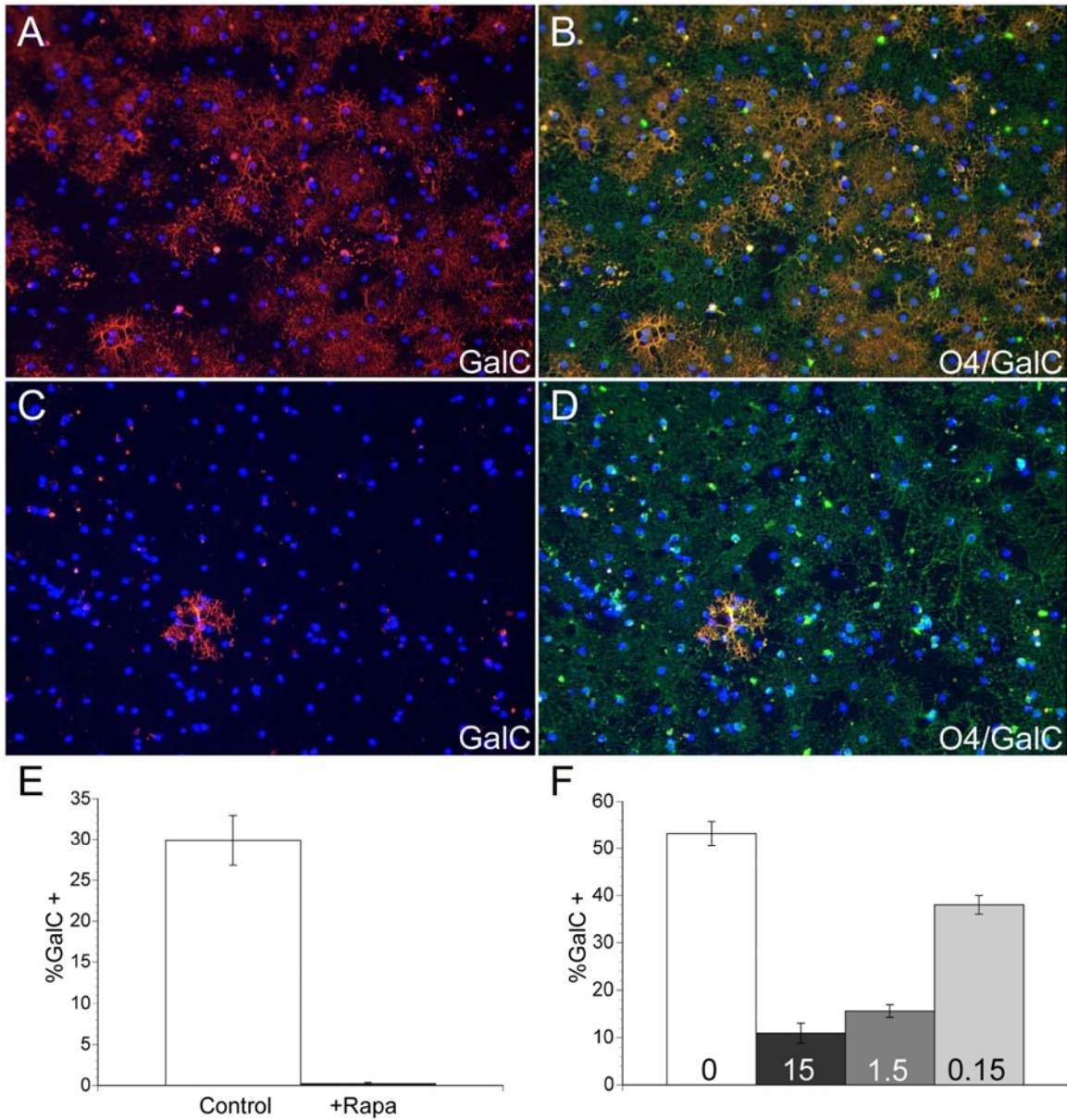


Figure 2.2: Inhibition of mTOR by rapamycin blocks OPC differentiation at the $O4^+/GalC^-$ late progenitor stage.

Figure 2.3 mTOR regulates the expression of key myelin genes, MBP and PLP. A-D: Representative images of PLP IHC are shown for OPCs differentiated in the absence (**A**, **B**) and presence of 15 nM rapamycin (**C**, **D**) for 5 days. **E:** Western immunoblot analysis of MBP expression during 4 consecutive days of OPC differentiation +/- 15nM rapamycin. **F:** Western immunoblot analysis of MBP expression in OPCs transfected with control or mTOR smart pool siRNAs and cultured under differentiation conditions for 4 days.

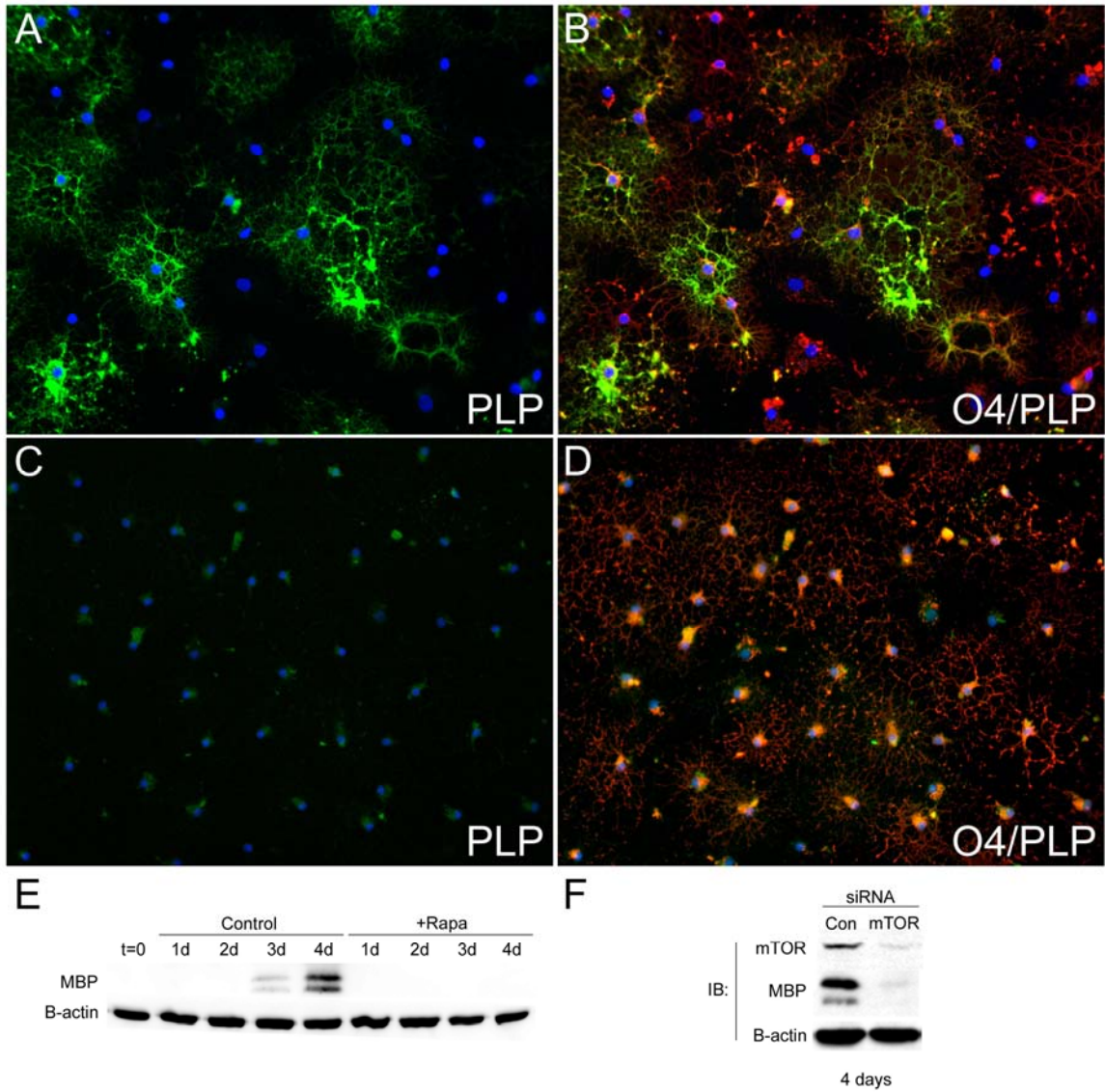


Figure 2.3: mTOR regulates the expression of key myelin genes, MBP and PLP.

Figure 2.4 Rapamycin has no effect on OPC viability. Quantification of O4⁺ total cell number in cultures treated for 3.5 d +/- 15 nM rapamycin (**A**) or for 5 d with rapamycin at the indicated doses (**B**). No significant changes in cell number were observed. **C**: Western immunoblot analysis of active caspase-3 levels +/- 15 nM rapamycin through 4 days of differentiation. **D**: Quantification of active caspase-3 levels, normalized to β -actin, following 2 and 3 d of differentiation +/- 15nM rapamycin. Rapamycin caused a 43% reduction in active caspase-3 levels at 2 d of differentiation, $P=0.0375$. No significant differences were seen after 3 d of differentiation. **E**: Quantification of the percentage of GalC⁺ oligodendrocytes in cultures treated +/- 15nM rapamycin for 6 d, or transiently exposed to rapamycin for 1, 2, or 3 d and then cultured in the absence of rapamycin for the remainder of the experiment. A significant increase in the number of GalC⁺ immature oligodendrocytes was observed in cultures exposed to rapamycin for 1, 2, or 3 days relative to cultures exposed to rapamycin for 6 days ($P<0.0001$) suggesting that the differentiation block is partially reversible.

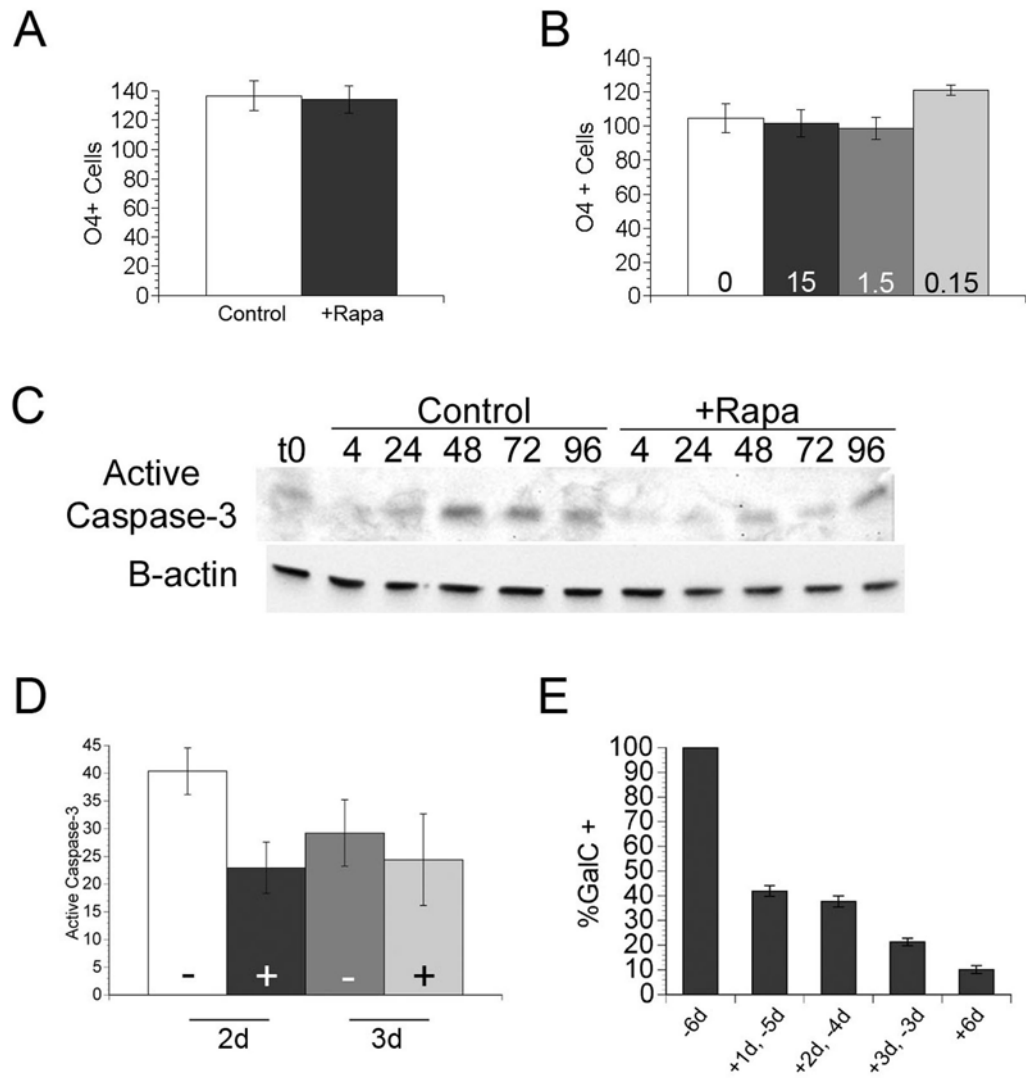


Figure 2.4: Rapamycin has no effect on OPC viability.

Figure **2.5 Rapamycin does not alter the expression of p27 during OPC**

differentiation. A: Representative western blots of p27 expression levels during OPC differentiation over a 4 day time course +/- 15nM rapamycin . **B:** Quantification of p27 expression levels normalized to β -actin. We observed robust expression of p27 during OPC differentiation. Rapamycin did not significantly alter p27 levels at any time point examined.

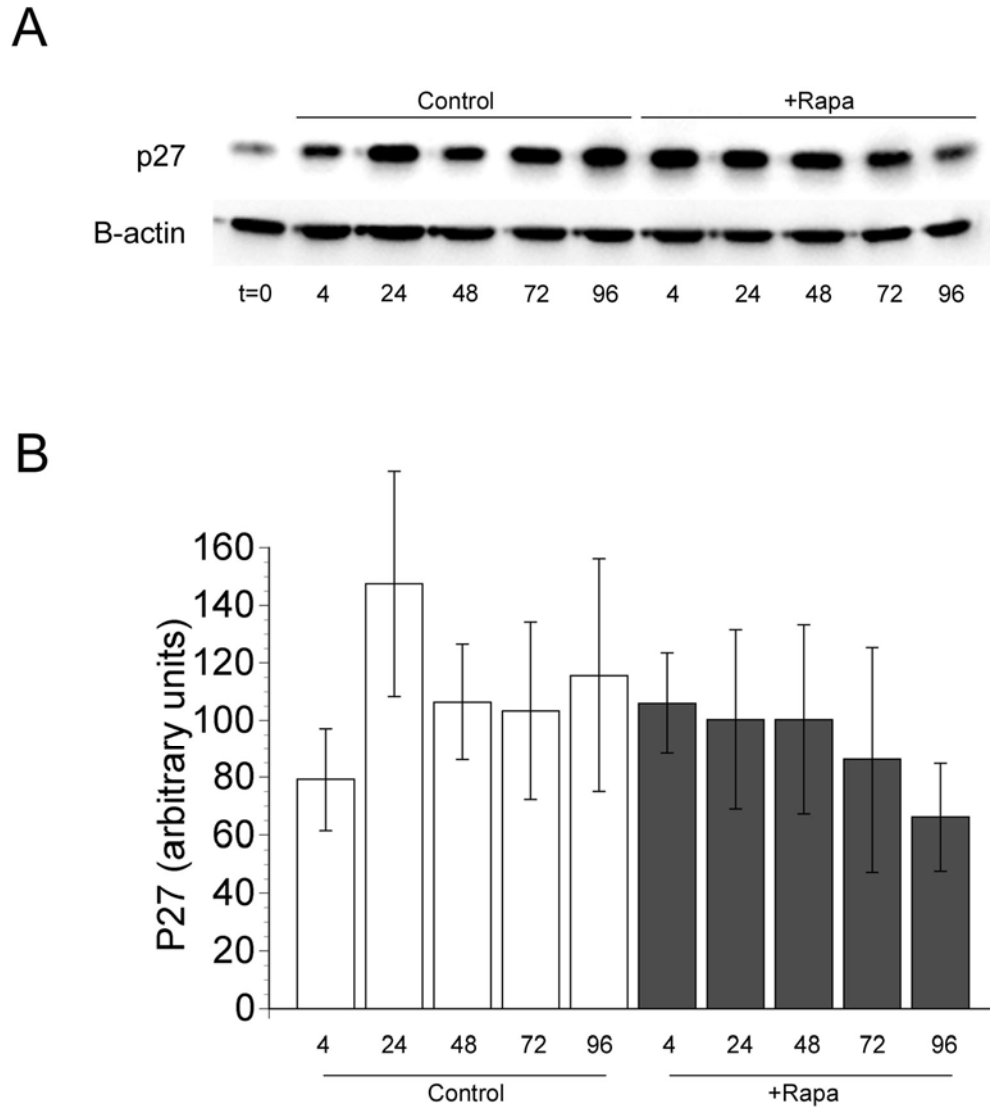
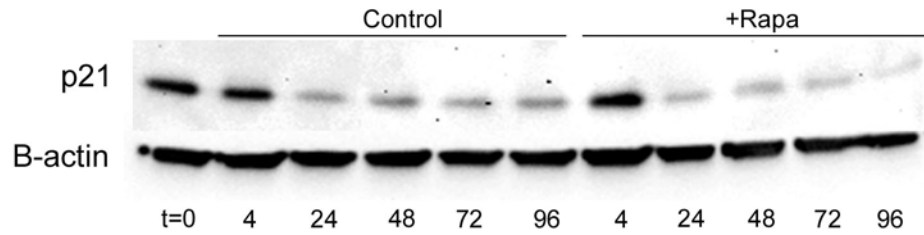


Figure 2.5: Rapamycin does not alter the expression of p27 during OPC differentiation.

Figure 2.6 Rapamycin does not alter the expression of p21 during OPC

differentiation. **A:** Representative western blots of p21 expression levels during OPC differentiation over a 4 day time course +/- 15nM rapamycin. **B:** Quantification of p21 expression levels normalized to β -actin. Expression levels of p21 peaked during the onset of OPC differentiation, then declined through 24 hours. Rapamycin treatment did not significantly alter p21 expression levels.

A



B

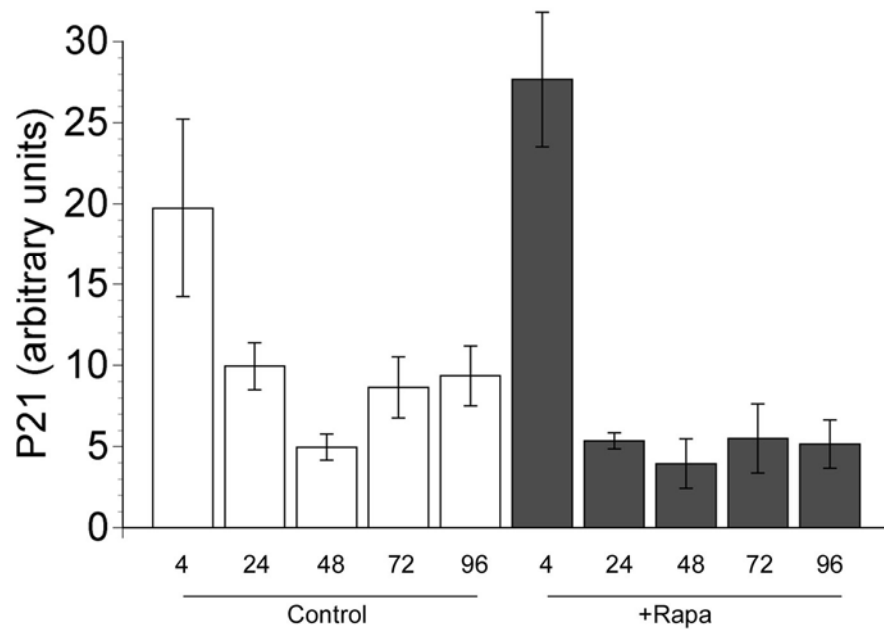


Figure 2.6: Rapamycin does not alter the expression of p21 during OPC differentiation

Figure 2.7 Inhibition of mTOR decreases MBP, PLP, and Ugt-8 mRNA levels. Total RNA was extracted from OPCs after 3 days of differentiation +/- 15 nM rapamycin. MBP (**A**), PLP (**B**), and Ugt-8 (**C**) mRNA levels were quantified using validated QuantiTect primer assays (Qiagen). Rapamycin treatment caused a significant reduction in the mRNA levels of MBP and PLP (MBP, $P=0.0032$; PLP $P= 0.0032$, Ugt-8, $P=0.0014$).

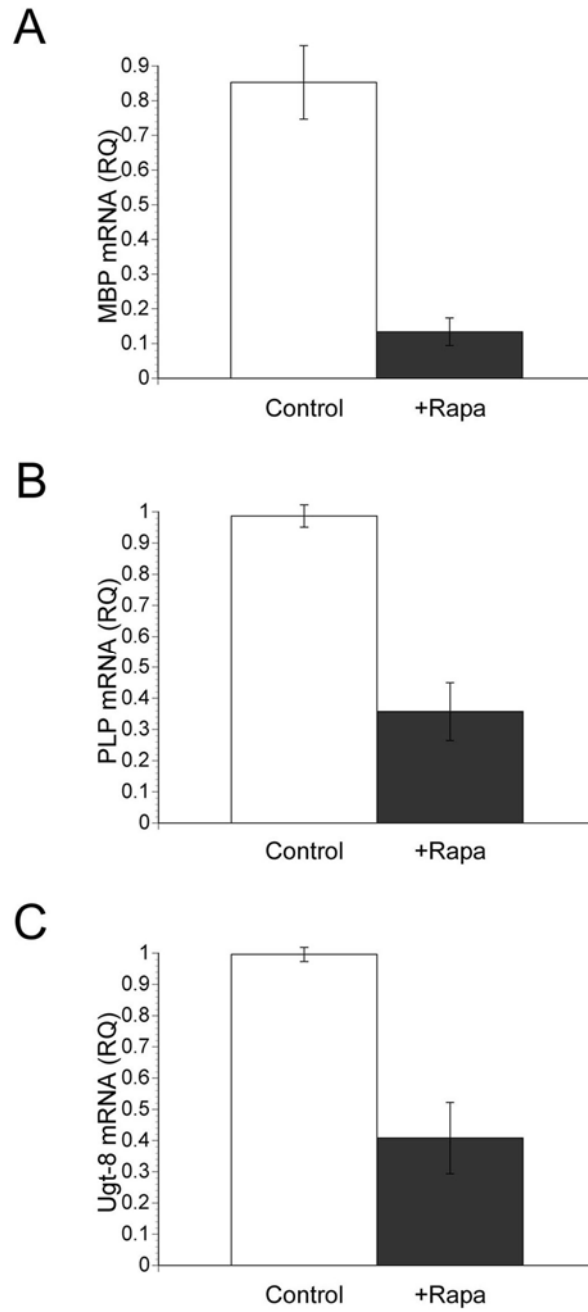


Figure 2.7: Inhibition of mTOR decreases MBP, PLP, and Ugt-8 mRNA levels.

Figure 2.8 Inhibition of mTOR has no effect on the mRNA levels of the transcription factors Olig2, Sox10, or Nkx2.2. Total RNA was extracted from OPCs after 3 days of differentiation \pm 15 nM rapamycin. Olig2 (**A**), Sox10 (**B**), or Nkx2.2 (**C**) mRNA levels were quantified using validated QuantiTect primer assays (Qiagen). Rapamycin treatment did not significantly affect alter the mRNA levels of Olig2, Sox10, or Nkx 2.2.

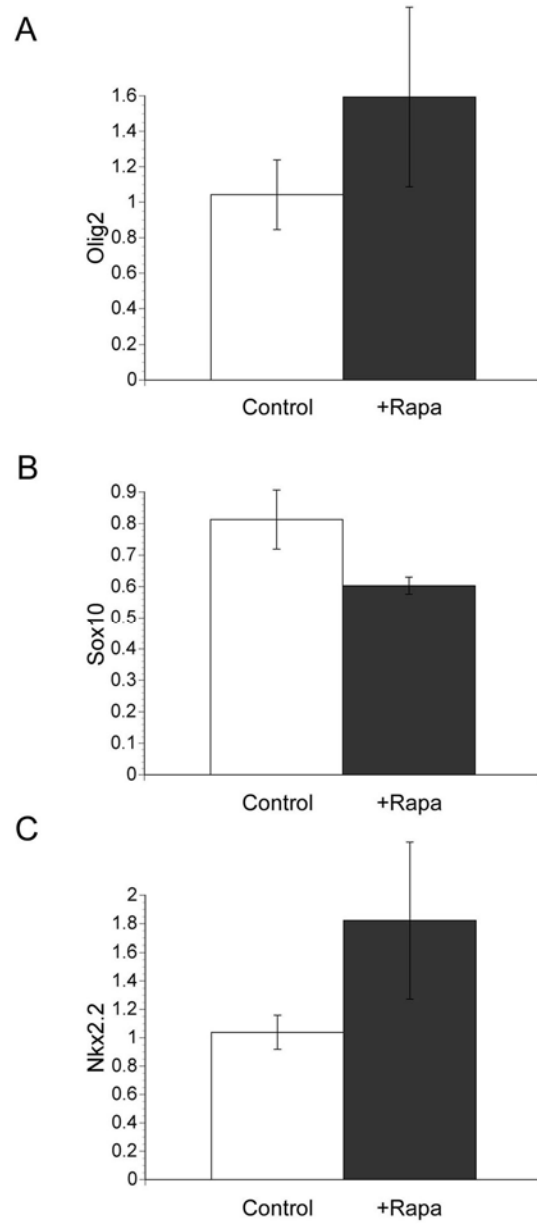


Figure 2.8: Inhibition of mTOR has no effect on the mRNA levels of the transcription factors Olig2, Sox10, or Nkx2.2.

Figure 2.9 mTOR regulates the mRNA levels of the inhibitor of DNA (Id) binding protein family members, Id2 and Id4. **A:** Total RNA was extracted from oligodendrocyte progenitor cultures after 3 days of differentiation +/- 15 nM rapamycin. Id2 (**A**) or Id4 (**B**) mRNA levels were quantified using validated QuantiTect qPCR primer assays (Qiagen). Inhibition of mTOR caused a 2.7 fold and 3.8 fold increase in the mRNA levels of Id2 and Id4 respectively (Id2, P=0.027; Id4, P=0.0259).

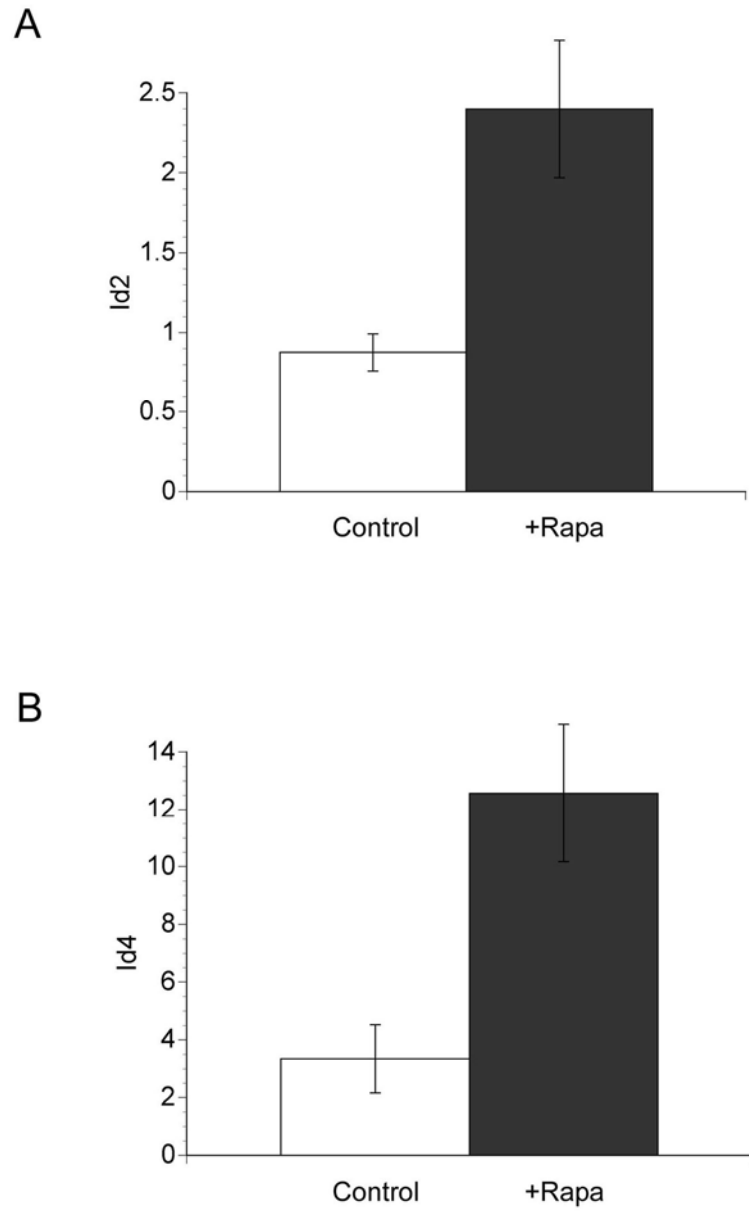


Figure 2.9: mTOR regulates the mRNA levels of the inhibitor of DNA (Id) binding protein family members, Id2 and Id4.

Figure 2.10 Systemic injections of rapamycin reduce body and brain weight, but do not alter MBP and CNPase expression. Rat pups were injected IP with 2.5 mg/kg rapamycin or vehicle for 5 days beginning on postnatal day 14. **A:** Rapamycin caused a 17% reduction in body weight on postnatal day 20 ($P=0.006$). **B:** Brain weights decreased by 18% following administration of rapamycin ($P=0.0078$). **C:** CNPase and MBP expression levels were not significantly reduced by rapamycin injection.

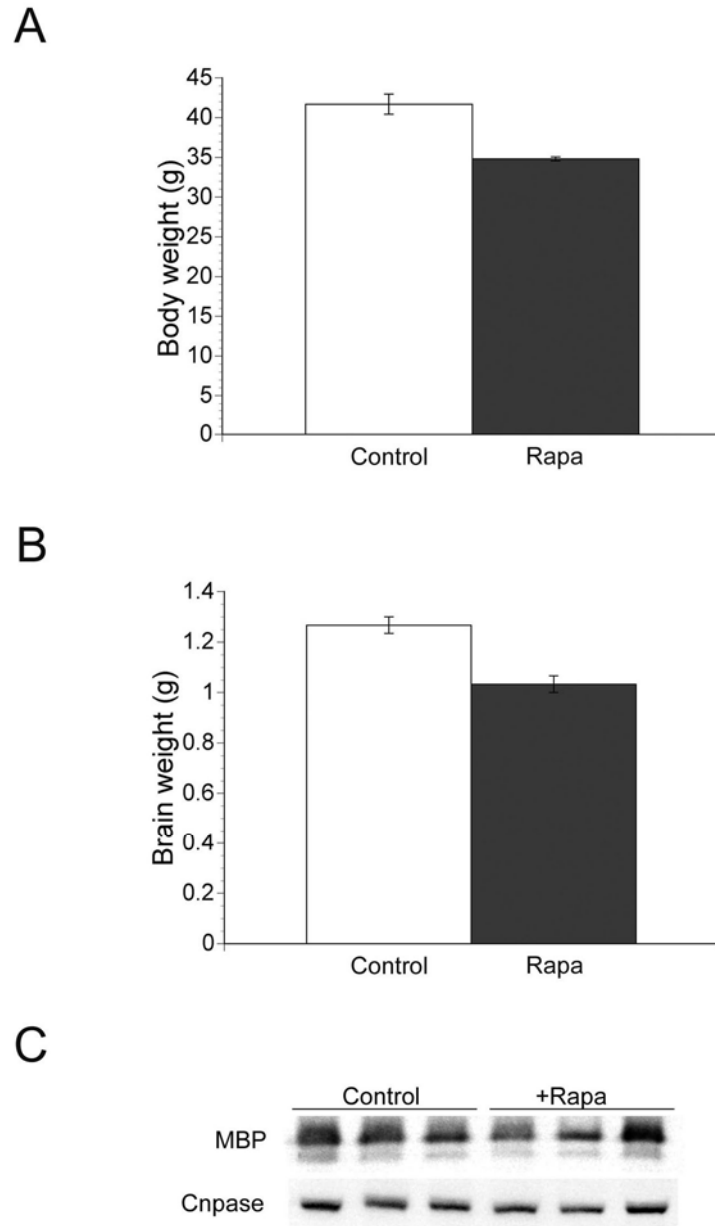


Figure 2.10: Systemic injections of rapamycin reduce body and brain weight, but do not alter MBP and CNPase expression.

Chapter 3

Both mTORC1 and mTORC2 complex activity are required during oligodendrocyte differentiation

3.1 Introduction

Our previous data demonstrate that mTOR is essential for oligodendrocyte differentiation past the late progenitor stage. Here, we explore the mechanism through which mTOR exerts its effects, by examining mTOR complex formation and the activation of downstream signaling effectors.

mTOR forms two unique signaling complexes, termed mTORC1 and mTORC2, defined by the presence of the adaptor proteins raptor and rictor, respectively (Martin and Hall, 2005). Each complex regulates the activity of distinct subsets of downstream signaling effectors. Specifically, the mTORC1 complex is best characterized for its ability to regulate cell growth and protein translation via the phosphorylation of p70S6K1 and 4E-BPs (Hay and Sonenberg, 2004; Kim et al., 2002). p70S6K1, a Ser/Thr kinase, is activated by mTORC1 mediated phosphorylation (Kim et al., 2002). In turn, p70S6K1 acts on an array of downstream substrates, including ribosomal protein S6, to affect protein translation, glucose homeostasis, gene transcription, and cell cycle progression (Ruvinsky and Meyuhas, 2006). In contrast, the mTORC1 regulated phosphorylation of the eIF4E-binding proteins (4E-BPs) represses their ability to bind to and sequester eIF4E, enabling cap dependent translation (Beretta et al., 1996; Hara et al., 2002; Kim et al., 2002).

Less is known about the function of the rictor containing mTORC2 complex. Recently however, mTORC2 was identified as the critical kinase for Akt Ser 473 phosphorylation (Sarbasov et al., 2006; Sarbasov et al., 2005b). mTORC2 also regulates the organization of the actin cytoskeleton in part by modulating the activity of PKC- α (Sarbasov et al., 2004).

TOR complex activity has not previously been studied in cells of the oligodendrocyte lineage. However, the mTORC2 component rictor was recently identified as a mRNA transcript upregulated during oligodendrocyte differentiation (Dugas et al., 2006). In addition, the rictor gene lies within a 2MB region identified as a susceptibility locus for multiple sclerosis (Dugas et al., 2006).

In this chapter, we define the repertoire of mTOR complexes present during oligodendrocyte differentiation. Furthermore, we investigate the role of mTORC1 and mTORC2 complexes in oligodendrocyte differentiation through the disruption of complex formation and the examination of the phosphorylation states of downstream signaling effectors. Finally, we examine the phosphorylation of mTOR *in vivo*, and provide evidence that the activation of an equivalent pathway correlates with myelination during postnatal development *in vivo*.

3.2 Materials and Methods

3.2.1 Materials

Antibodies to raptor, rictor, p-mTOR Ser2448, total mTOR, p-p70S6K1 Thr389, total p70S6K1, p-Akt Ser473, and total Akt were purchased from Cell Signaling (Danvers, MA). Antibodies to 4E-BP were purchased from Bethyl Laboratories (Montgomery, TX). Control, mTOR, rictor, and raptor targeting siRNA smart pools were purchased from Dharmacon (Lafayette, CO). Oligodendrocyte nucleofection kits were obtained from Amaxa biosystems (Gaithersburg, MD). Vector NovaRED substrate kit for peroxidase was purchased from Vector Labs (Burlingame, CA).

3.2.2 Co-immunoprecipitations of mTORC1 and mTORC2 complexes

Primary rat oligodendrocyte progenitor cells were plated on poly-d-lysine coated 100 mm dishes in N2S media. The following day, differentiation was initiated by mitogen withdrawal in N2B2+T3 differentiation media in the presence or absence of 15 nM rapamycin. On days 2 and 3 of differentiation, cells were washed twice with ice cold PBS, harvested in 500 ul of PBS, and pelleted by centrifugation at 10000xg for 5 minutes at 4°C. Cells from 6, 100mm dishes were pooled for each treatment group. Cell pellets were resuspended and lysed in 1 ml of immunoprecipitation (IP) buffer for 30 minutes on ice. IP buffer was composed of 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 50 mM NaF, 0.5 mM orthovanadate, protease inhibitors, and 1% Triton X-100. A portion of the lysate was used to determine protein

concentration by the DC protein assay (BioRad). A total of 600 ug of protein was used for each immunoprecipitation per treatment group. The lysates were pre-cleared by incubation with 30ul of protein A/G agarose at 4°C followed by centrifugation at 10,000xg for 5 minutes. Primary antibodies to mTOR (2 ug) were added to the supernatants and incubated with gentle rotation overnight at 4°C. The following day 30 ul of protein A/G agarose beads was added to the lysates and incubated for 4 hours. Immune complexes were precipitated by centrifugation at 8000xg for 5 minutes and washed three times with ice cold IP buffer. Immune complexes were resuspended in 30 ul of 1X SDS lysis buffer and boiled for 5 minutes. Samples were separated on 3-8% Tris-acetate gels by SDS-PAGE, transferred to nitrocellulose membranes that were subsequently probed with antibodies to detect mTOR, raptor, and rictor.

3.2.3 Transfection of siRNA smart pools targeting mTOR, raptor, and rictor

For siRNA smart pool transfections, 5×10^6 OPCs were collected and resuspended in 100 ul of rat oligodendrocyte nucleofector solution (Amaxa). Control, mTOR, rictor, or raptor targeting siRNA smart pools (200 pmols) were added to the cell suspension and transferred to an Amaxa certified cuvette. Cells were electroporated with the Amaxa nucleofection device using a protocol, O-17, optimized for the transfection of primary rat oligodendrocytes. Following electroporation, cells were resuspended in N2S media and plated on poly-d-lysine coated 35 mm dishes. One transfection yielded enough cells to seed 6-9 35 mm dishes. Cells were allowed to attach and recover overnight in N2S

media. The following day, differentiation was initiated in N2B2+T3 media, and cells were cultured for 4 or 5 days prior to protein isolation.

3.2.4 Protein isolation and western immunoblotting

To analyze the phosphorylation states of mTOR and signaling effectors downstream of the mTORC1 and mTORC2 complexes, primary rat OPCs were plated on poly-d-lysine coated 60 mm dishes in N2S media at a density of 2×10^4 cells/cm². The following day, cells were synchronized by a 12 hour treatment in N1A media. N1A was identical to the N2B2 media described above, minus the addition of FBS and the insulin concentration was lowered to 5 ng/ml, a concentration adequate to stimulate the insulin receptor but not the IGF-IR (LeRoith et al., 1995). Synchronization was performed to minimize variability in the analysis of signaling targets by establishing a baseline for pathway activation. Synchronized cell maintained their undifferentiated state during the incubation in N1A (Frederick TJ and Wood TL, unpublished observation). Next, differentiation was initiated by the addition of N2B2+T3 differentiation media with or without the addition of 15 nM rapamycin. Control cultures received vehicle alone (0.002% ethanol).

Following treatments, cells were washed twice with ice cold PBS and total cell lysates were harvested in 2X SDS lysis buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 100 mM DTT, 1/50 protease inhibitor (PI) cocktail (Sigma, St. Louis, MO). The lysates were briefly sonicated and stored at -80°C prior to western analysis. A RC-DC protein assay (BioRad) was performed to determine protein concentration.

Approximately 15 ug of total protein per sample was aliquoted, boiled for 5 minutes and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on mini-gels (Invitrogen). Separated proteins were then transferred to nitrocellulose membranes and blocked in 5% milk/ TBS-0.1% Tween for one hour at room temperature. Membranes were then incubated in the presence of primary antibodies for p-mTOR Ser2448 (Cell signaling, 1:1000), p-Akt Ser473 (cell signaling, 1:1000), p-p70S6K1 Thr389 (Cell Signaling, 1:1000), 4E-BP (Bethyl Laboratories, 1:1000), or β -actin (Sigma, 1:5000) diluted in 5% milk/ TBS-0.1% Tween overnight at 4°C. The following day, membranes were washed 3 times for 5 minutes with TBS-0.1% Tween and incubated for 1 hour at room temperature in 5% milk/ TBS-0.1% Tween containing goat anti-rabbit or goat anti-mouse secondary antibodies diluted 1:5000. The detection of HRP conjugated secondary antibodies was performed by enhanced chemiluminescence using the Ultra-Lum imaging device (Ultra-Lum, Inc, Claremont, CA). In order to detect total levels of mTOR, Akt, and p70S6K1, separate protein aliquots prepared from the same samples used to examine phosphorylation states were run on mini-gels and probed with antibodies to mTOR (Cell signaling, 1:1000), Akt (Cell signaling 1:1000), or p70S6K1 (Cell signaling, 1:1000). This avoided artifacts caused by stripping and reprobing previous blots. Protein expression levels were quantified using NIH image 1.62.

3.2.5 Delayed addition of rapamycin

Primary rat OPCs were plated on poly-d-lysine/laminin coated coverslips in 24 well dishes in N2S media at a density of 2×10^4 cells/cm². The following day,

differentiation was initiated by the addition of N2B2+T3 differentiation media with or without 15 nM rapamycin. A total volume of 600ul of media was added to each well. For delayed treatments, a 10X stock of rapamycin or vehicle alone was prepared in DMEM/F12 that was diluted directly into the media on 1, 2, or 3 days of differentiation to achieve a final concentration of 15 nM rapamycin. The media for each treatment group was replenished every 48 hours for the duration of the experiment. On day 5 of differentiation, coverslips were washed 3 times in PBS and prepared for the immunocytochemical detection of O4 and GalC cell surface antigens as described. Total numbers of GalC positive immature oligodendrocytes were quantified as described in previous experiments.

3.2.6 IHC detection of p-mTOR Ser 2448 *in vivo*

For developmental analyses of mTOR Ser 2448 phosphorylation *in vivo*, postnatal day 14, 18, and 21 rat pups from the same litter were anesthetized via IP injection of a 3:1 cocktail of ketamine and xylazine. Brains were fixed by transcardiac perfusion with 3% paraformaldehyde and extracted. Extracted brains were further immersed in 3% paraformaldehyde for 4 hours at 4°C. Brains were blocked in a coronal plane, placed in tissue cassettes, and stored in 70% ethanol prior to paraffin embedding. A total of twenty 5 µm sections, two per slide, were prepared from each paraffin embedded tissue sample. Three brain samples were prepared per developmental timepoint per litter. Analyses were performed on a total of 9 brains per timepoint, isolated from 3 independent litters.

For IHC detection, sections were deparaffinized and rehydrated through ethanols. Sections were then incubated in 3% hydrogen peroxide in methanol for 10 minutes at room temperature and rinsed several times with Tris buffered saline (TBS). Antigen retrieval was performed by incubation in proteinase K (5 µg/ml) in 10 mM Tris (pH 8.0) for exactly 10 min @ 37° C. Next, the sections were rinsed in TBS and permeabilized in 0.3 % Triton-X-100 in TBS for 30 minutes at room temperature. Sections were then blocked in TGB superbloc (0.5M Tris PH 7.6, 10% BSA, 10% Goat serum, 0.05% NaN₃) for 1 hour at room temperature. Primary antibodies to p-mTOR Ser 2448 (Cell Signaling, 1:250) were diluted in TGB diluent (0.5M TBS, 20% TGB superbloc, and 0.2% Triton X-100) and incubated overnight in a humid chamber at 4°C. The following day, sections were washed and incubated in TGB diluent containing goat anti-rabbit biotinylated secondary antibodies for 2 hours at 37°C. Next, the sections were washed and incubated in streptavidin-HRP in TBS plus 10% bovine serum 2 hours at 37°C. HRP detection was visualized with NovaRed substrate (Vector Labs). Sections were briefly counterstained with hematoxylin, dehydrated, and coverslipped with cyto seal mounting medium. Photomicrographs of each section were captured using a 20X objective.

3.2.7 Statistical analyses

Statistical analyses were performed using StatView statistical analysis software. For experiments comparing two treatment groups, an unpaired t-test was performed to assess the statistical significance between treatment groups. For experiments in which multiple treatment groups or time points were examined, one way analysis of variance

(ANOVA) was performed to assess the statistical significance between treatment groups.

All experiments were repeated at least 3 times.

3.3 Results

3.3.1 Two distinct mTOR signaling complexes, mTORC1 and mTORC2, are present in OPCs

mTOR interacts directly with the adaptor proteins raptor and rictor to form two distinct signaling complexes, mTORC1 and mTORC2 respectively, which specifically govern the activation of discrete downstream signaling effectors (Sarbasov et al., 2005a). To define the repertoire of TOR complexes present in oligodendrocytes during differentiation, we performed co-immunoprecipitations with antibodies directed to mTOR following 1 and 2 days of differentiation in the presence or absence of 15 nM rapamycin. Immune complexes were separated by SDS-PAGE followed by western blot analysis for mTOR, raptor, and rictor. We observed the formation of both mTORC1 and mTORC2 complexes during oligodendrocyte differentiation (Figure 3.1). In addition, we found that both mTORC1 and mTORC2 dissociate in response to rapamycin treatment (Figure 3.1).

3.3.2 mTORC1 and mTORC2 complexes exert distinct effects on OPC differentiation

In order to delineate the function of mTORC1 and mTORC2 complexes in the regulation of oligodendrocyte differentiation, we performed siRNA mediated knockdown

of rictor and raptor. Specifically, OPCs were transiently transfected with siRNA smart pools specific for mTOR, raptor, or rictor, or with a control siRNA smart pool. One day following transfection, differentiation was initiated in N2B2+T3 media for 5 days. MBP expression was quantified as an index of differentiation by Western immunoblot analysis. We observed a marked increase in MBP expression in cells transfected with a control smart pool of siRNAs by 5 days of differentiation. In contrast, siRNA mediated knockdown of rictor or raptor decreased MBP expression by 44% and 31% respectively. (rictor $P=0.002$, raptor $P=0.0152$; Figure 3.2B).

In addition, we analyzed the efficiency of siRNA mediated knockdown of target genes by Western immunoblotting (Figure 3.3A-C). Each siRNA smart pool significantly reduced the protein levels of the targeted gene compared to control levels (mTOR 81% reduction, $P=0.0201$; B: rictor 34% reduction, $P=0.0094$; C: raptor 51% reduction, $P=0.0348$; Figure 3.3 A). The effects of siRNA smart pools were specific to each gene, as we did not observe a significant decrease in non-targeted TOR signaling components compared to control levels.

3.3.3 mTORC1 and mTORC2 regulate distinct downstream signaling targets during OPC differentiation

In order to gain insight on the functions of mTORC1 and mTORC2 during OPC differentiation, we analyzed the phosphorylation states of key mTOR signaling components. First, we observed the sustained phosphorylation of mTOR Ser 2448 during OPC differentiation (Figure 3.4A). Ser 2448 has been described as an insulin/IGF sensitive site, which is phosphorylated in response to the activation of the PI3K/Akt

pathway by growth factor stimulation (Nave et al., 1999; Sekulic et al., 2000).

Interestingly, rapamycin treatment inhibited phosphorylation of mTOR Ser 2448 (Figure 3.4A).

Because phosphorylation of Ser 2448 is regulated in part by PI3K/Akt activity, we performed western blot analysis of Akt Ser 473. We observed the sustained phosphorylation of Akt Ser 473 during OPC differentiation (Figure 3.4B). However, rapamycin significantly reduced the phosphorylation of Akt Ser 473. We quantified the levels of Akt Ser473 phosphorylation in the presence or absence of rapamycin treatment on days 2 and 3 of differentiation (Figure 3.4C). We focused on days 2 and 3 of differentiation since: 1) Akt Ser 473 phosphorylation peaked during these time points, and 2) progression to the immature oligodendrocyte stage of differentiation coincides with 2 and 3 days of differentiation *in vitro*. We observed a 58% and 61% reduction in the level of Akt Ser 473 phosphorylation on days 2 and 3 respectively in response to rapamycin treatment (day 2, $P=0.0003$; day 3, $P=0.0019$; Figure 3.4 C). Our data are consistent with a previous report showing that the mTORC2 complex regulates phosphorylation of Akt Ser473 (Sarbasov et al., 2006; Sarbasov et al., 2005b).

In the next set of experiments, we examined signaling components regulated by the mTORC1 complex. Specifically, we performed western immunoblot analysis of the phosphorylation states of p70S6K1 and 4E-BPs. We observed an acute increase in the phosphorylation of p70S6K1 Thr389 by 3 hours of differentiation which was maintained through 24 hours (Figure 3.4D) and then declined by 48 hours (data not shown). In addition, we examined the phosphorylation states of 4E-BPs by using an antibody which recognizes both hypo- and hyper-phosphorylated forms of 4E-BP1 and 4E-BP2, the latter

is the most abundant form in the CNS (Ruvinsky and Meyuhas, 2006). Similar to the activation of p70S6K1, hyperphosphorylation of 4E-BP peaked within the first 24 hours of OPC differentiation, and then declined through 48 and 72 hours (Figure 3.4E). Furthermore, rapamycin inhibited the phosphorylation of p70S6K1 and 4E-BP at all time points (Figure 3.4D, E).

3.3.4 mTOR activation is required during the transition to the immature oligodendrocyte stage

In previous experiments, we showed that mTOR activity is required for OPC differentiation past the late progenitor stage. In addition, mTORC1 and mTORC2 complexes activate downstream signaling components during distinct phases of oligodendrocyte differentiation. Specifically, the mTORC1 targets, p70S6K1 and 4E-BP, are activated early during oligodendrocyte differentiation then decline within 48 to 72 hours, while mTORC2 mediated phosphorylation of Akt is sustained throughout differentiation.

Because OPC differentiation *in vitro* takes place gradually over the course of several days, we designed an experiment to determine whether the activation mTOR is required acutely at the onset of differentiation, or whether the sustained activation of mTOR is required specifically during the transition to the immature oligodendrocyte stage. To define the time frame in which mTOR activation promotes OPC differentiation, we delayed the addition of rapamycin to cultures of differentiating OPCs. Specifically, rapamycin was added to OPC cultures following 1, 2, 3, and 4 days in differentiation media. After 5 days of differentiation, the percentage of GalC⁺ positive

immature oligodendrocytes was quantified by immunocytochemistry and compared to control cultures that did not receive rapamycin, or cultures that were treated with rapamycin for the entire 5 day period.

Consistent with previous experiments, we observed ~55% GalC⁺ immature oligodendrocytes in control cultures, while <1% of cells treated with rapamycin for 5 days progressed to the GalC⁺ stage of differentiation. The addition of rapamycin to cultures following 1 and 2 days of differentiation blocked GalC expression as efficiently as the addition of rapamycin at the onset of differentiation (Figure 3.5). In contrast, when rapamycin was added to cultures following 3 and 4 days in differentiation media, we observed a 13-fold and 60-fold increase respectively in the number of GalC⁺ oligodendrocytes compared to cells treated with rapamycin for 5 days (P<0.0001; Figure 3.5).

3.3.5 Phosphorylation of mTOR Ser 2448 correlates with myelination *in vivo*.

To begin to determine whether the PI3K/Akt/mTOR pathway is activated during OPC differentiation *in vivo*, we detected the phosphorylation of mTOR Ser 2448 by IHC in subcortical white matter (WM) during postnatal rat CNS development (Figure 3.6). p-mTOR Ser 2448 was apparent in WM by postnatal day 14 (Figure 3.6A). However, we did not observe p-mTOR staining on d 10 (data not shown). Phosphorylation of mTOR increased in subcortical and striatal WM at postnatal d 18 (Figure 3.6B) and further intensified through d 21 (Figure 3.6C). In contrast, control sections from d 21 rats stained with secondary antibodies alone were devoid of staining (Figure 3.6D).

3.4 Discussion

Our studies demonstrate that mTORC1 and mTORC2 complexes form and are active during oligodendrocyte differentiation. Consistent with previous reports, rapamycin disrupts the formation of mTORC1 and mTORC2 complexes (Kim et al., 2002; Sarbassov et al., 2006). To delineate the roles of mTORC1 and mTORC2 complexes in oligodendrocyte differentiation, we performed siRNA mediated knockdown of raptor and rictor. Surprisingly, we show that both mTORC1 and mTORC2 activity are required for myelin gene expression.

To further define the role of mTORC1 and mTORC2 in oligodendrocyte differentiation, we analyzed the phosphorylation states of key downstream signaling effectors. We show that the phosphorylation of the mTORC1 targets, p70S6K1 and 4E-BP, occurs rapidly during the first 48 hours of oligodendrocyte differentiation but then declines. In contrast, mTORC2 mediated phosphorylation of Akt Ser473 is sustained throughout oligodendrocyte differentiation. In addition, pharmacological inhibition of mTORC complexes by rapamycin abrogates the phosphorylation of these targets.

Because mTORC1 and mTORC2 complexes phosphorylated downstream targets in a temporally distinct fashion, we performed an experiment in which we delayed the addition of rapamycin to cultures of differentiating OPCs to define the critical window of mTOR activation during OPC differentiation. Surprisingly, the addition of rapamycin after 2 days of differentiation was as effective at blocking the generation of GalC⁺ postmitotic oligodendrocytes as the addition of rapamycin at the onset of differentiation.

By 2 days of differentiation, the phosphorylation of the mTORC1 targets, p70S6K1 and 4E-BP, has declined from peak levels, suggesting that their function alone is not sufficient to promote progression through the late progenitor stage. Rather, the activation of targets downstream of mTOR during the transition to the immature oligodendrocyte stage is required. However, these data do not exclude the possibility that the early activation of mTORC1 targets is necessary for differentiation to occur. In fact, disruption of mTORC1 function through the knockdown of raptor suggests that the activation of mTORC1 targets is essential for myelin protein expression. An alternative explanation for these results lies in the possibility that signaling effectors other than p70S6K1 or 4E-BPs mediate the effects of the mTORC1 complex on oligodendrocyte differentiation.

Taken together, these data support a model of mTOR signaling during oligodendrocyte differentiation in which mTORC1 and mTORC2, acting on distinct downstream effectors in a temporal fashion, is essential for the execution of the oligodendrocyte differentiation program. Furthermore, our data suggests that the activation of either mTORC1 or mTORC2 complexes alone is not sufficient to promote mTOR dependent differentiation.

What are the factors that exert the effects of mTORC1 and mTORC2 during differentiation? Our previous data show that Id2 and Id4 expression is regulated at the mRNA level by mTOR, and enhanced levels of Id2 and Id4 correlate with the differentiation block caused by mTOR inhibition. Critical questions moving forward with these studies are: Does mTORC1 or mTORC2 activity regulate Id2/Id4 expression? Do the mTORC1 and mTORC2 signaling pathways converge on the Id family members?

Are there additional effectors of the mTOR complexes in addition to the Ids that regulate oligodendrocyte differentiation? Based on the temporal activation of signaling effectors downstream of the mTORC1 and mTORC2 complexes, it is intriguing to speculate on the existence of additional factors that coordinate to control oligodendrocyte differentiation.

Finally, we show that the phosphorylation of mTOR Ser 2448 correlates with oligodendrocyte differentiation *in vitro* and myelination *in vivo*. We observed the sustained phosphorylation of mTOR Ser 2448 during oligodendrocyte differentiation *in vitro*. In addition, we detected p-mTOR Ser 2448 in the subcortical white matter beginning on postnatal day 14. The presence of p-mTOR increased by postnatal day 18 and further intensified by postnatal day 21. Thus, the phosphorylation of mTOR correlates with the peak of myelination *in vivo* (Hamano et al., 1996; Hamano et al., 1998). These findings suggest that a similar signaling mechanism upstream of mTOR functions during oligodendrocyte differentiation *in vitro* and myelination *in vivo*.

Ser 2448 is defined as an insulin sensitive site, which is phosphorylated by the activation of the PI3K/Akt pathway (Nave et al., 1999; Sekulic et al., 2000). Ser 2448 is located in a putative regulatory domain which contains six serine and threonine residues in the C-terminus of mTOR between the kinase domain and the FATC domain (Harris and Lawrence, 2003). Previous results established a positive correlation between Ser 2448 phosphorylation and mTOR kinase activity and protein synthesis (Reynolds et al., 2002; Sekulic et al., 2000). However, the substitution of alanine in place of Ser 2448, rendering mTOR refractory to phosphorylation at this site, has no effect on mTOR kinase activity (Sekulic et al., 2000). In contrast, the deletion of amino acids 2430-2450 results in a hyper-activated mTOR mutant, suggesting that Ser 2448 lies in a regulatory repressor

domain which integrates growth factor and nutrient signaling to activate mTOR through multi-site phosphorylation (Sekulic et al., 2000). In addition, these studies suggest that Ser 2448 phosphorylation alone is not sufficient to activate mTOR kinase activity by blocking the inhibitory effects of the repressor domain. Therefore, the biological significance of Ser 2448 phosphorylation in response to PI3K/Akt activation is at present unresolved.

Our data provide additional evidence of a correlation between the phosphorylation of mTOR Ser 2448 and the phosphorylation of downstream targets during oligodendrocyte differentiation *in vitro*. Furthermore, p-mTOR Ser 2448 correlates with myelination *in vivo*. Therefore, the study of oligodendrocyte differentiation provides a biologically relevant system to address the significance of mTOR Ser 2448 phosphorylation through future studies.

Figure 3.1 mTORC1 and mTORC2 signaling complexes in OPCs. Western blot analysis of mTORC1 and mTORC2 components immunoprecipitated with an antibody directed to mTOR. Samples were isolated from OPC cultures following 1 and 2 days of differentiation +/- 15 nM rapamycin.

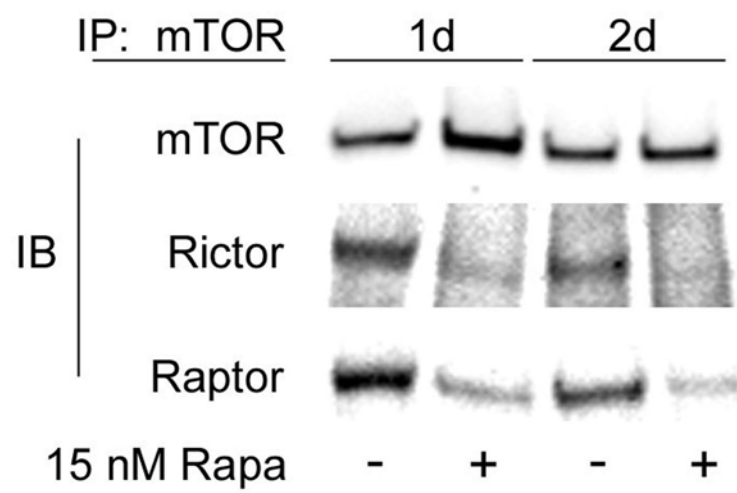


Figure 3.1: mTORC1 and mTORC2 signaling complexes in OPCs.

Figure 3.2 mTORC1 and mTORC2 complexes regulate MBP expression. A: Western immunoblot analysis of MBP expression and mTORC 1/2 complex components at 5 d of differentiation following transient transfection of a control siRNA smart pool or siRNA smart pools targeting mTOR, rictor, or raptor. B: Quantification of MBP expression normalized to β -actin. siRNA mediated knockdown of rictor or raptor decreased MBP expression by 44% and 31% respectively at 5 days of differentiation (rictor siRNA, $P=0.002$; raptor siRNA, $P=0.0152$).

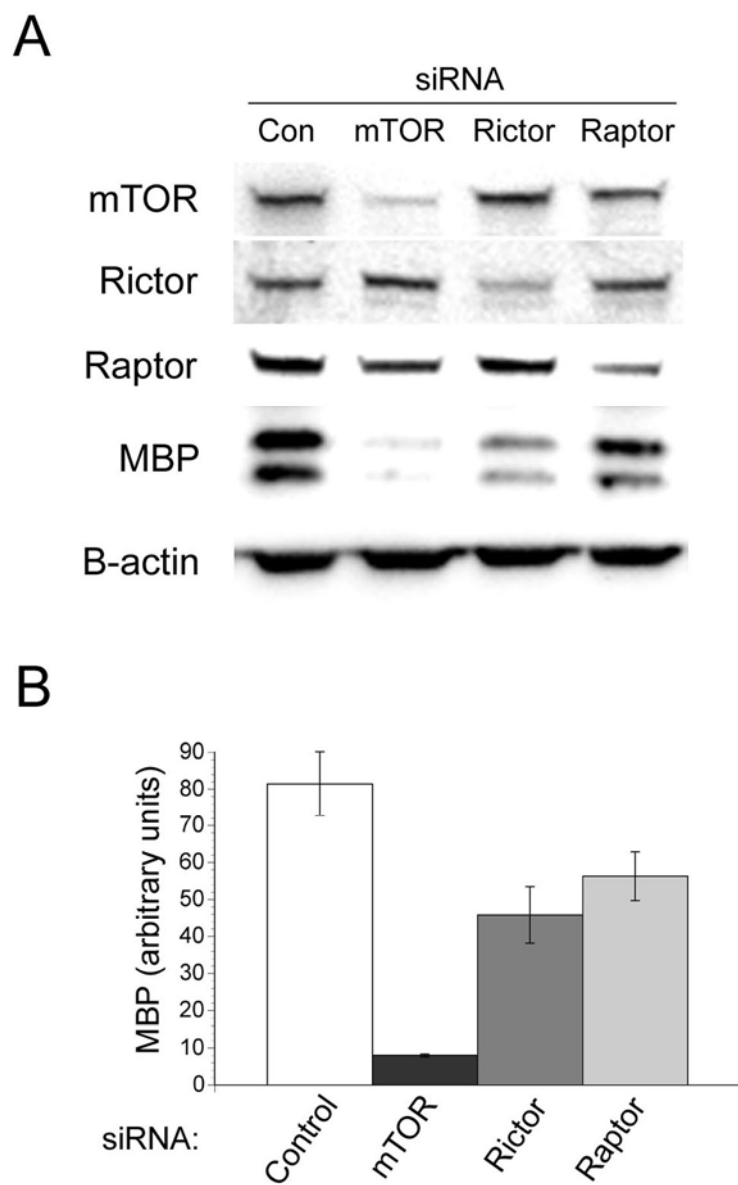


Figure 3.2: mTORC1 and mTORC2 complexes regulate MBP expression.

Figure 3.3 mTOR, rictor, and raptor siRNA smart pools specifically silence target gene expression. Quantification of mTOR (A), rictor (B), and raptor (C) protein levels following 5 days of differentiation in cells transiently transfected with control or target specific siRNA smart pools. A: A siRNA smart pool targeting mTOR caused an 81% reduction in mTOR protein levels versus control (P=0.0201). Smart pools targeting raptor and rictor did not significantly reduce mTOR levels compared to control. B: Transfection with a rictor specific siRNA smart pool resulted in a 34% reduction in rictor protein levels versus control (P=0.0094). Smart pools targeting mTOR and raptor did not significantly effect rictor expression compared to control. C: A raptor targeting siRNA smart pool caused a 51% reduction in raptor protein levels versus control (P=0.0348). Knockdown of mTOR or rictor did not alter raptor expression compared to control.

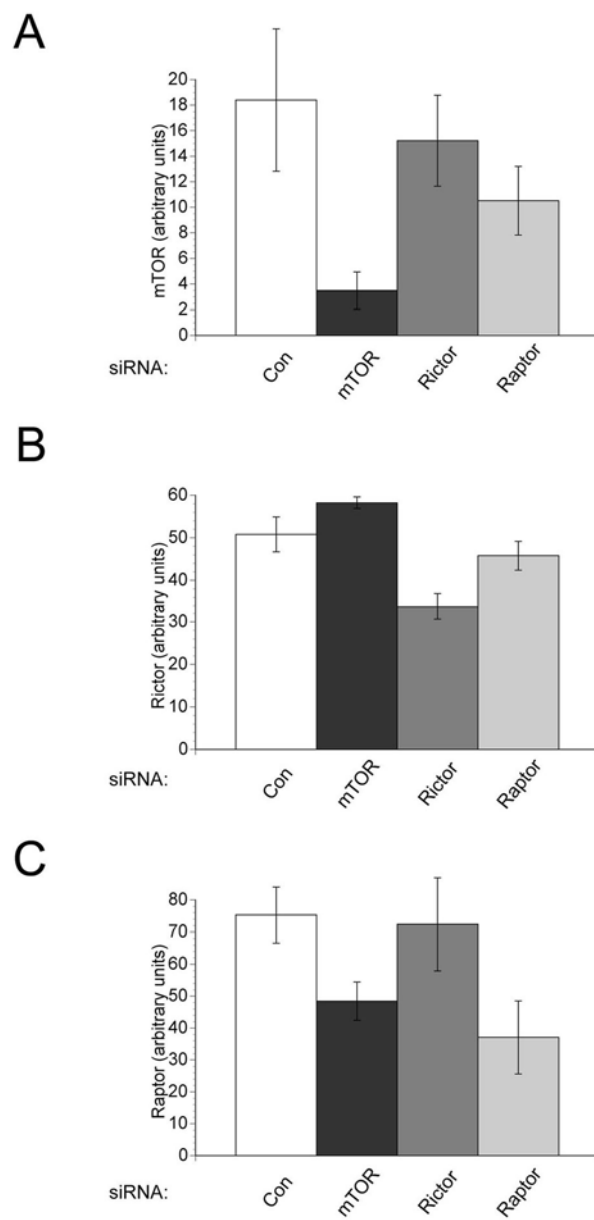


Figure 3.3: mTOR, rictor, and raptor siRNA smart pools specifically silence target gene expression.

Figure 3.4 Activation of mTORC1 and mTORC2 signaling targets during OPC

differentiation. A-C. Western immunoblot analysis of p-mTOR Ser2448 (A) and p-Akt Ser473 (B) during OPC differentiation +/- 15 nM rapamycin. C: Quantification of p-Akt Ser 473/total Akt at 2d and 3d +/- rapamycin. Inhibition of mTOR reduced p-Akt Ser 473 by 58% and 61% on day 2 and day 3 respectively (day 2, P=0.0003: day 3, P=0.0019). D-E: Western immunoblot analysis of p-S6K Thr 389 (D) or 4E-BP (E) during OPC differentiation +/- rapamycin.

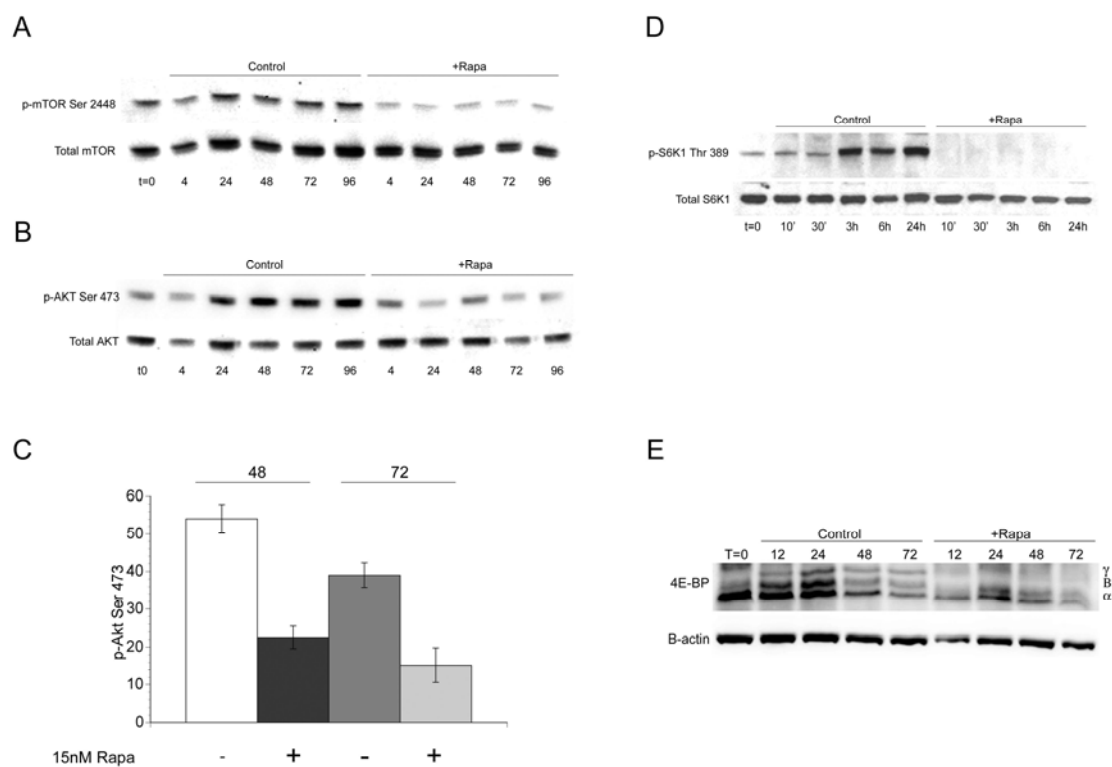


Figure 3.4: Activation of mTORC1 and mTORC2 signaling targets during OPC differentiation.

Figure 3.5 mTOR activation is required during the transition to the immature oligodendrocyte stage. OPCs were cultured +/- 15nM rapamycin in differentiation media for 5d, or rapamycin was added to cultures on days 1, 2, 3, and 4 after the onset of differentiation. A significant increase in GalC⁺ immature oligodendrocytes was observed in rapamycin treated cultures following 3 and 4 d of differentiation compared to cultures exposed to rapamycin for the entire 5 days (P<0.0001).

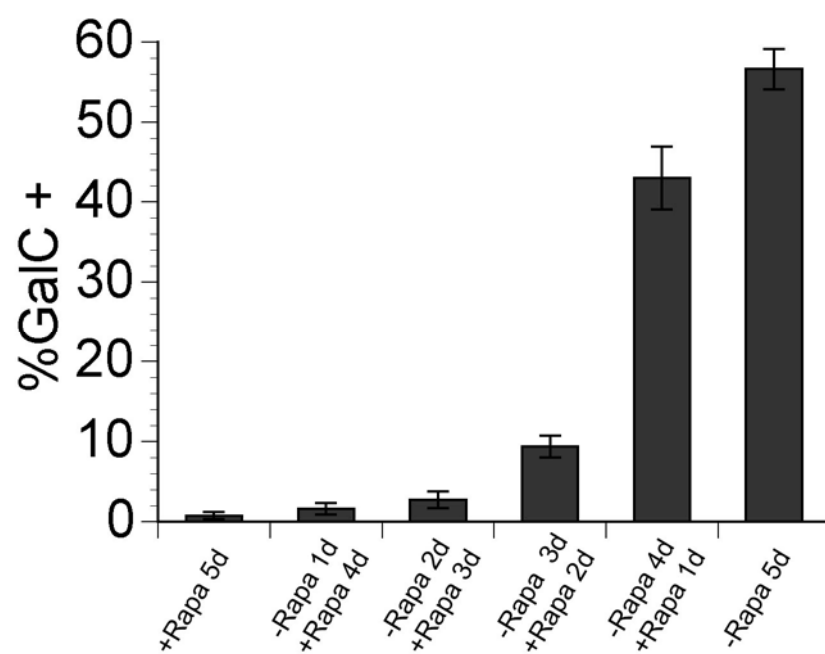


Figure 3.5: mTOR activation is required during the transition to the immature oligodendrocyte stage.

Figure 3.6 IHC detection of p-mTOR Ser 2448 in subcortical white matter during rat postnatal CNS development. A-C: Fixed, paraffin-embedded sections from postnatal d 14 (A), d 18 (B), or d 21 (C) were used for IHC staining of p-mTOR Ser2448 (detected with vector-red). D: A control d 21 section stained with secondary antibody alone. Hematoxylin was used for counter-staining

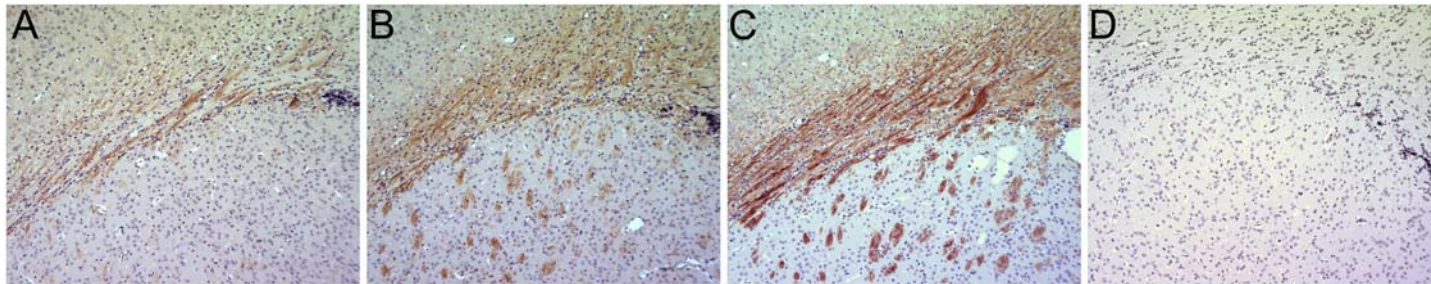


Figure 3.6: IHC detection of p-mTOR Ser 2448 in subcortical white matter during rat postnatal CNS development.

Chapter 4

p70S6K1 mediates the effects of mTORC1 activation during OPC differentiation

4.1 Introduction

The previous studies demonstrate that mTOR, acting through both mTORC1 and mTORC2 complexes on distinct downstream targets, regulates oligodendrocyte differentiation during the O4⁺/GalC⁻ late progenitor to O4⁺/GalC⁺ immature oligodendrocyte transition. The disruption of mTORC1 complex activity, through siRNA mediated knockdown of raptor, resulted in a significant decrease in myelin protein expression. The phosphorylation of the mTORC1 effector substrates, p70S6K1 and 4E-BP, correlated with the acute phase of OPC differentiation, and was inhibited by rapamycin treatment. However, the delayed addition of rapamycin to differentiating cultures of OPCs subsequent to the peak phosphorylation of p70S6K1 and 4E-BP effectively blocked OPC differentiation. These results suggest that the mTORC1 mediated control of p70S6K1 and 4E-BP activity alone is not sufficient to promote OPC differentiation. However, these results do not exclude the possibility that p70S6K1 and 4E-BP are necessary for OPC differentiation to occur.

p70S6K1 is a Ser-Thr kinase that is activated in response to nutrient and growth factor signaling via multi-step phosphorylation by mTOR, PDK1, and PKC ζ (Dufner and Thomas, 1999; Martin and Blenis, 2002). p70S6K1 functions as a key regulator of cell growth and translation via the direct phosphorylation of ribosomal protein S6 (rpS6)

(Fingar et al., 2002; Jenou et al., 1988; Pende et al., 2004). In recent years, several additional p70S6K1 substrates have been identified which extend the biological functions of the kinase to include the regulation of cell survival, proliferation, glucose homeostasis, and gene transcription (Ruvinsky and Meyuhas, 2006).

In this chapter, we characterize the role of p70S6K1 in oligodendrocyte differentiation *in vitro* and *in vivo*. Our results demonstrate that, p70S6K1 is at least one of the critical downstream effectors of the mTORC1 complex that regulates oligodendrocyte differentiation.

4.2 Materials and Methods

4.2.1 Materials

Antibodies used to detect mTOR and p70S6K1 were purchased from Cell signaling Inc. (Danvers, MA). Antibodies to detect MBP, CNP, and Olig2 were purchased from Chemicon (Temecula, CA). Anti- β -actin antibodies were purchased from Sigma (St. Louis, MO). Control, mTOR, and p70S6K1 targeting smart pool siRNAs were purchased from Dharmacon (Lafayette, CO). Luxol Fast Blue was purchased from Sigma (St. Louis, MO). Frozen and paraffin embedded tissue samples from S6K1/2 $-/-$ and wild type mice were a generous gift from Dr. Mario Pende.

4.2.2 Transfections of siRNA smart pools targeting mTOR and p70S6K1

For siRNA smart pool transfections, 5×10^6 OPCs were collected and resuspended in 100 μ l of rat oligodendrocyte nucleofector solution (Amaxa). Control, mTOR, or p70S6K1 targeting siRNA smart pools (10 μ l of a 20 μ M stock) were added to the cell suspension and transferred to an Amaxa certified cuvette. Cells were electroporated with the Amaxa nucleofection device using a protocol, O-17, optimized for the transfection of primary rat oligodendrocytes. Following electroporation, cells were resuspended in N2S media and plated on poly-d-lysine coated 35 mm dishes. One transfection yielded enough cells to seed 6-9 35 mm dishes. Cells were allowed to attach and recover overnight in N2S media. The following day, differentiation was initiated in N2B2+T3 media, and cells were cultured for 4 or 5 days prior to protein isolation.

4.2.3 Protein isolation and western immunoblotting

Following treatments, cells were washed twice with ice cold PBS and total cell lysates were harvested in 2X SDS lysis buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 100 mM DTT, 1/50 protease inhibitor (PI) cocktail (Sigma, St. Louis, MO)). The lysates were briefly sonicated and stored at -80°C prior to western analysis. A RC-DC protein assay (BioRad) was performed to determine protein concentration. Approximately 15 μ g of total protein per sample was aliquoted, boiled for 5 minutes and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Bis-Tris mini-gels (Invitrogen). Separated proteins were then transferred to nitrocellulose membranes and blocked in 5% milk/ TBS-0.1% Tween for one hour at room temperature.

Membranes were then incubated in the presence of primary antibodies for MBP (Chemicon, 1:100), mTOR (Cell Signaling, 1:1000), p70S6K1 (Cell Signaling, 1:1000), or β -actin (Sigma, 1:5000) diluted in 5% milk/TBS-0.1% Tween overnight at 4°C. The following day, membranes were washed 3 times for 5 minutes with TBS-0.1% Tween and incubated for 1 hour at room temperature in 5% milk/TBS-0.1% Tween containing goat anti-rabbit or goat anti-mouse secondary antibodies diluted 1:5000. The detection of HRP conjugated secondary antibodies was performed by enhanced chemiluminescence using the Ultra-LUM imaging device. Protein expression levels were quantified using NIH image 1.62.

4.2.4 Protein isolation from frozen brain specimens

Brains isolated from n=2 S6K1/2 -/- and n=2 wild type mice were obtained from Dr. Mario Pende (Pende et al., 2004). Frozen brains were wrapped in aluminum foil, snap frozen in liquid nitrogen, and pulverized. Pulverized tissue was lysed in 1X SDS/PBS containing a protease inhibitor cocktail. Tissue lysates were briefly sonicated, boiled for 5 minutes, and centrifuged at 10000Xg for 10 minutes. The supernatants were collected and stored at -80°C. Protein concentration was determined using a RC-DC protein assay. Approximately 20 ug of total protein was aliquoted and separated by SDS-PAGE on 12% Bis-Tris mini-gels. Immunoblotting for MBP, CNP (Chemicon, 1:1000), and β -actin was performed as described previously.

4.2.5 Luxol Fast Blue staining

Sections (5 μ m) were prepared from paraffin embedded S6K1/2 $-/-$ and age matched wild type mice. For Luxol Fast Blue staining, the sections were deparaffinized and rehydrated through ethanols and incubated in Luxol Fast Blue staining solution (95% ethanol, 1% Luxol Fast Blue, 10% glacial acetic acid) overnight at 55°C. The following day, sections were rinsed briefly in 95% ethanol, followed by a differentiation step to remove excess stain in lithium carbonate solution (0.05% lithium carbonate in distilled water). Differentiation was continued by several brief rinses in 70% ethanol. Sections were briefly counterstained with hematoxylin, dehydrated through ethanols to xylene, and mounted with cytooseal medium.

4.2.6 IHC detection of Olig2

For IHC detection of Olig2, sections prepared from postnatal day 21 S6K1/2 $-/-$ (n=2) and wild type (n=2) brains were deparaffinized and rehydrated through ethanols. Antigen retrieval was performed by incubation in proteinase K (5 μ g/ml) in 10 mM Tris (pH 8.0) for exactly 10 min @ 37° C. Next, the sections were rinsed in TBS and permeablized in 0.3 % Triton-X-100 in TBS for 30 minutes at room temperature. Sections were then blocked in TGB superbloc (0.5M Tris PH 7.6, 10% BSA, 10% Goat serum, 0.05% NaN₃) for 1 hour at room temperature. Primary antibodies to Olig2 (Chemicon, 1:250) were diluted in TGB diluent (0.5M TBS, 20% TGB superbloc, and 0.2% Triton X-100) and incubated overnight in a humid chamber at 4°C. The following day, sections were washed and incubated in TGB diluent containing goat anti-rabbit

biotinylated secondary antibodies for 2 hours at 37°C, followed by a levamisol block (1:200) in detection buffer (100 mM Tris, pH 8-8.5), for 15 minutes at room temperature. Tissue sections were then rinsed and incubated in Vector Red AP substrate for 30-45 minutes at room temperature in the dark. Incubation in DAPI (1:2500 in TBS) was performed as a counterstain to visualize cell nuclei. Finally, the sections were dehydrated, and coverslipped with cyto seal mounting medium. Photomicrographs of each section were captured using a 20X objective.

4.2.7 Statistical analyses

Statistical analyses were performed using StatView statistical analysis software. One way analysis of variance (ANOVA) was performed to assess the statistical significance between treatment groups. All experiments were repeated at least 3 times.

4.3 Results

4.3.1 The mTORC1 target, p70S6K, is essential for oligodendrocyte differentiation *in vitro*

Data in the previous chapters support the hypothesis that the mTOR-raptor mTORC1 complex is a critical regulator of oligodendrocyte differentiation. Two key targets downstream of mTORC1, p70S6K1 and 4E-BP, are phosphorylated at the onset of oligodendrocyte differentiation *in vitro*. Moreover, the phosphorylation of these targets is inhibited by rapamycin. To determine whether p70S6K1 plays an essential role in

oligodendrocyte differentiation downstream of the mTORC1 complex, we utilized siRNA mediated knockdown of S6K1 during oligodendrocyte differentiation to assess its function. Similar to the previous siRNA experiments, OPCs were transiently transfected with control, S6K1 targeting, or mTOR directed smart pool siRNAs by electroporation with the Amaxa nucleofector device. The following day, the media was replaced to initiate differentiation for 5 days in culture, followed by protein isolation.

We performed western immunoblot analysis of MBP expression as an index of the oligodendrocyte differentiation in transfected cultures. Representative western blots of MBP and siRNA targeted signaling components are shown in Figure 4.1 A.

Consistent with previous data, we observed high expression of MBP in control siRNA transfected cultures following 5 days of differentiation. Interestingly, siRNA mediated knockdown of S6K resulted in a 65% decrease in MBP expression at 5 days ($P=0.0001$; Figure 4.1B). In addition, we observed an 81% decrease in S6K1 expression in S6K1 siRNA smart pool transfected cells compared to control siRNA transfected cells ($P=0.0042$, Figure 4.1C). In contrast, siRNA mediated knockdown of mTOR had no effect on S6K1 levels compared to control (Figure 4.1C).

4.3.2 The S6 kinases are essential for myelin protein expression *in vivo*

Based on data from our *in vitro* studies, we predicted that S6K1 is required for proper myelination *in vivo*. To examine the role of S6K1 during myelination *in vivo*, we performed preliminary analysis of myelin protein content in mice lacking S6K1 and S6K2 (tissues samples were a generous gift from Dr. Mario Pende). We examined S6K

1/2 -/- mice rather than mice lacking S6K1 alone to avoid compensatory effects due to the upregulation of S6K2 (Shima et al., 1998). Protein extracted from the frozen brains of 21 and 14 day old wild type and S6K1/2 -/- mice was used to perform western immunoblot analysis of MBP and CNPase expression (Figure 4.2). S6K1/2 -/- brains showed significantly reduced levels of both MBP and the earlier OL lineage marker 2'3'-cyclic-nucleotide 3' phosphodiesterase (CNPase) at postnatal day 21. CNPase expression was unaltered between wild type and S6K 1/2 -/- brains at postnatal day 14. However, MBP levels were undetectable in samples from S6K 1/2 -/- mice at postnatal day 14, while a low level of MBP expression was observed in wild type brains.

To provide further evidence for a decrease in myelin content in S6K 1/2 -/- brains, we performed histological analysis of paraffin embedded sections from 21 and 14 day of wild type and S6K 1/2 -/- brains. Luxol fast blue (LFB) staining appeared unaltered between wild type and S6K 1/2 -/- brains at postnatal day 21 (Figure 4.3). In contrast, we observed decreased LFB staining in S6K 1/2 -/- brains at postnatal day 14. However, due to a small sample size (n=2 wild type, n=3 S6K 1/2 -/-), we cannot exclude the possibility that the differences observed were merely due to normal variability in the onset of myelination in these animals.

4.3.3 Loss of S6K1/2 does not alter the number or distribution of Olig2 positive oligodendrocytes *in vivo*

Preliminary data suggests that S6K1/2 -/- mice exhibit a decrease in myelin content compared to age matched wild type animals. Because S6K1 has a defined role in cell proliferation (Fingar et al., 2004), we performed analyses to determine whether the

decrease in myelin content was due to a decrease in the total number of oligodendrocytes present in S6K1/2^{-/-} brains rather than to a direct defect in myelination. To this end, immunohistochemical staining for the transcription factor Olig2 was performed. We selected Olig2 as a marker for oligodendrocyte cell number for the following reason: 1. Olig2 expression was not altered by mTOR inhibition *in vitro*, 2. Olig2 expression is maintained throughout oligodendrocyte lineage progression (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000), and 3. Olig2 exhibits a nuclear localization, permitting the direct counts of cell numbers in stained sections. Myelin protein content decreased significantly in S6K1/2^{-/-} brains at postnatal day 21. Therefore, IHC analysis of Olig2 expression was performed on sections from 21 day old wild type and S6K1/2^{-/-} brains. Intense staining for Olig2 was observed throughout the subcortical and striatal white matter in coronal forebrain sections prepared from wild type brains (Figure 4.4). The loss of S6K1/2 did not alter the number or distribution of Olig2 positive cells (Figure 4.4), suggesting that the total number of oligodendrocytes present was unchanged.

4.4 Discussion

The previous studies demonstrate that S6K1 regulates myelin protein expression *in vitro* and *in vivo*. Specifically, siRNA mediated knockdown of S6K1 resulted in a 65% decrease in MBP expression following 5 days of differentiation. In addition, preliminary analysis of S6K1/2^{-/-} brains revealed decreased myelin protein expression compared to wild type controls. The decrease in myelin content observed in S6K1/2^{-/-} brains was not likely due to a defect in the generation of OPCs as the number and

distribution of Olig2 positive progenitors was unaltered relative to wild type mice. Accordingly, S6K1 appears to be at least one signaling effector downstream of the mTORC1 complex required for the proper execution of the oligodendrocyte differentiation program.

Key questions remain concerning the mechanism through which p70S6K1 regulates oligodendrocyte differentiation. Few studies have identified a role for p70S6K1 in the regulation of differentiation in other cell types. One possible explanation may lie in the ability of p70S6K1 to regulate gene transcription through the direct phosphorylation of transcription factors, including a member of the cAMP response element binding (CREB)/ATF family, CREM τ (de Groot et al., 1994).

Alternatively, p70S6K1 was recently identified as a kinase responsible for the phosphorylation of mTOR Ser2448, suggesting the existence of a feedback loop which regulates mTOR activity (Holz and Blenis, 2005). We observed a correlation between the rapamycin sensitive phosphorylation of mTOR Ser 2448 and oligodendrocyte differentiation *in vitro* as well as myelination *in vivo*. Therefore, the observation that the disruption of p70S6K1 expression and activity inhibits oligodendrocyte differentiation may in fact be due to its ability to modulate the activity of mTOR through the phosphorylation of Ser 2448. However, additional experiments are required to determine the biological significance of mTOR Ser 2448 phosphorylation.

Western blot analysis revealed a decrease in myelin protein expression in S6K1/2^{-/-} brains compared to wild type that was pronounced on postnatal d 21. In contrast, we observed a detectable difference in myelin content through Luxol Fast Blue staining primarily on postnatal d 14. This discrepancy may be resolved by considering the

differences in the sensitivity of these analyses. In addition, the inability to directly quantify the intensity of Luxol Fast Blue staining limits its potential to discern subtle differences that may exist in the amount of myelin present in S6K1/2 *-/-* brains on postnatal d 21. We cannot exclude the possibility that the differences in myelin content observed between S6K1/2 *-/-* and wild type brains was merely due to the small number of brains used in our preliminary analysis. Furthermore, the deletion of S6K1/2 did not completely block the generation of myelinating oligodendrocytes *in vivo*. This suggests that mechanisms which may involve additional signaling effectors downstream of mTOR are able to compensate for the loss of S6K1/2 *in vivo*.

However, the siRNA mediated suppression of p70S6K1 *in vitro* clearly reduced the amount of MBP expression during differentiation. Taken together, these data suggest that p70S6K1 is at least one key signaling effector of mTORC1 activity during oligodendrocyte differentiation. Further studies are required to delineate the functions of additional signaling effectors of both the mTORC1 and mTORC2 complexes during oligodendrocyte differentiation.

Figure 4.1 siRNA mediated knockdown of S6K1 inhibits MBP expression. OPCs were transfected with siRNA smart pools targeting mTOR, S6K1, or a control smart pool. A: Representative western blots for MBP, mTOR, and p70S6K1 following 5 days of differentiation. B: Quantification of MBP levels. siRNA mediated knockdown of p70S6K1 resulted in a 65% decrease in MBP expression vs. control (P=0.0001). C: Quantification of p70S6K1 expression levels in S6K1 siRNA transfected samples vs. control at 5 days of differentiation (81% decrease in S6K1 levels for S6K1 siRNA transfected samples vs. control, P=0.0003). The siRNA smart pool targeting mTOR did not significantly affect p70S6K1 levels.

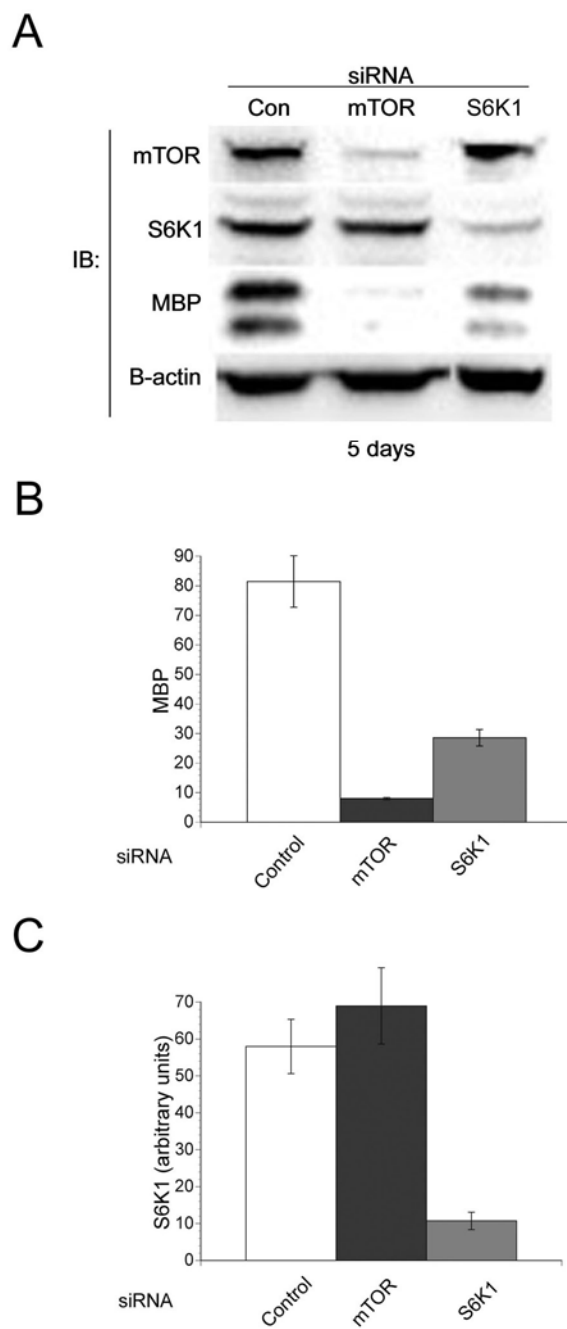


Figure 4.1: siRNA mediated knockdown of S6K1 inhibits MBP expression.

Figure 4.2 S6K 1/2 -/- mice exhibit decreased expression of MBP and CNP. A:

Western immunoblot analysis of MBP and CNP expression in S6K 1/2 knockout mice (n=2) at postnatal days 21 and 14. Protein extracts were prepared from the frozen whole brains of wild type and knockout mice. S6K 1/2 -/- showed decreased expression of the oligodendrocyte specific proteins MBP and CNP at postnatal day 21.

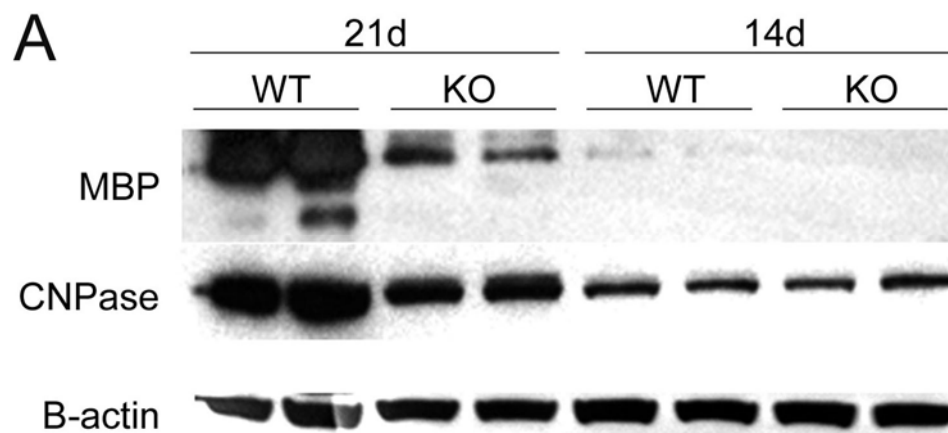


Figure 4.2: *S6K 1/2* $-/-$ mice exhibit decreased expression of MBP and CNP.

Figure 4.3 Histological analysis of S6K 1/2 -/- brains reveals evidence for hypomyelination in forebrain subcortical and striatal white matter. Representative images of luxol fast blue (LFB) stained sections of wild type and S6K 1/2 -/- mice at postnatal days 21 and 14. Wild type and S6K 1/2 -/- mice displayed intense LFB staining at postnatal day 21. At postnatal day 14, sections from S6K 1/2 -/- brains exhibited reduced LFB staining relative to wild type sections.

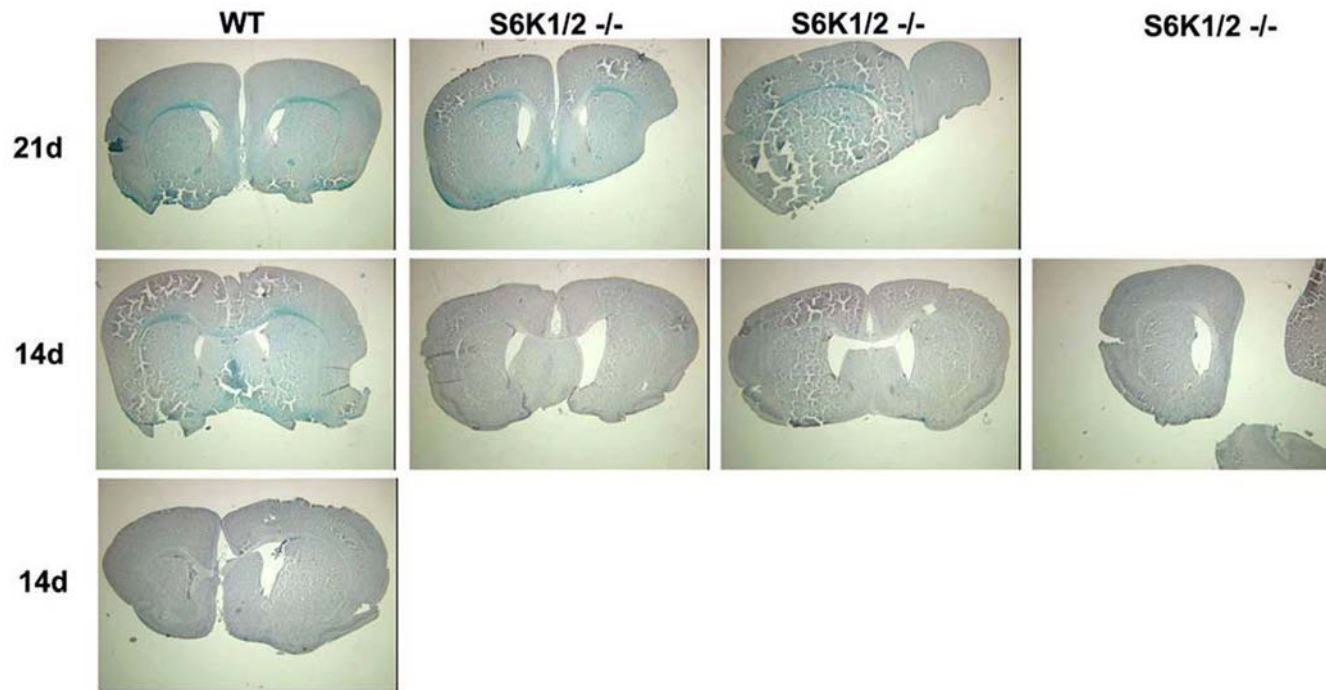


Figure 4.3: Histological analysis of S6K 1/2 -/- brains reveals evidence for hypomyelination in forebrain subcortical and striatal white matter.

Figure 4.4 The number and distribution of Olig2 positive oligodendrocytes is unchanged in S6K 1/2 -/- brains at postnatal day 21. Paraffin embedded sections from postnatal d 21 wild type (A) or S6K 1/2 -/- (B) brains were used for IHC staining of Olig2.

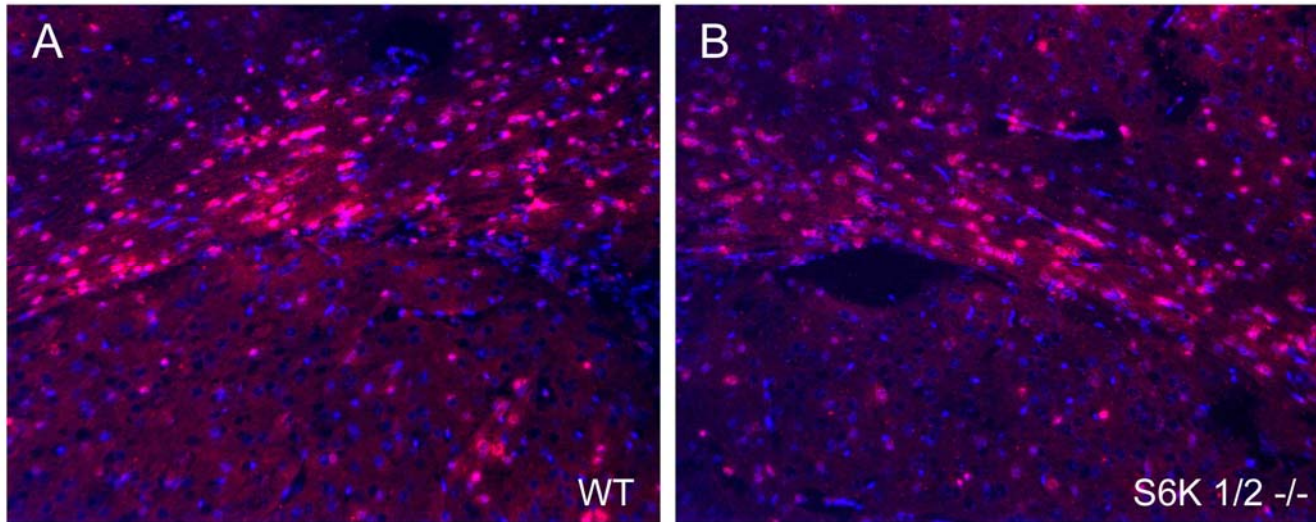


Figure 4.4: The number and distribution of Olig2 positive oligodendrocytes is unchanged in S6K 1/2 -/- brains at postnatal day 21.

Chapter 5

An inducible system for transgenic analysis of gene function in the cells of the oligodendrocyte lineage

5.1 Introduction

In the central nervous system, oligodendrocytes ensheath axon segments with a highly specialized myelin membrane, permitting the rapid conduction of nerve impulses by saltatory conduction (Baumann and Pham-Dinh, 2001). Oligodendrocytes are derived from oligodendrocyte progenitor cells, which undergo an extensive proliferative expansion developmentally to ensure that adequate numbers of oligodendrocytes are generated for proper myelination. In turn, oligodendrocyte progenitors migrate away from germinal ventricular zones into the parenchyma of the brain and spinal cord where they establish contact with axon segments. Having arrived at their final destination, oligodendrocyte differentiation ensues, generating complex multipolar oligodendrocytes capable of myelinating up to 60 independent axon segments (Butt and Ransom, 1989).

In rodents, oligodendrocytes are generated postnatally, with the peak of myelination in the forebrain occurring on postnatal day 21 (Hamano et al., 1996; Hamano et al., 1998). Therefore, the study of gene function in the development of cells of the oligodendrocyte lineage through traditional knockout approaches has been hampered by embryonic and perinatal lethality. Strategies to direct transgenic expression to the cells of the oligodendrocyte lineage through the use of lineage specific promoters have begun to shed light on the functions of certain genes during oligodendrocyte development.

However, direct transgenic expression limits the analysis of gene function to the developmental window in which promoter activity peaks, thus excluding the ability to study gene function during multiple aspects of oligodendrocyte development. Furthermore, the analysis of gene function in adult models of demyelination/remyelination is often impaired by defects that arise during the developmental phase of transgenic expression.

To overcome these obstacles, we sought to generate an inducible system to direct transgenic expression to the cells of the oligodendrocyte lineage. Ideally, this system would permit tightly regulated transgenic expression during any phase of oligodendrocyte development, as well as during stages of remyelination in adult mice. To this end, we employed an inducible strategy based on the Tet-On system (Zhu et al., 2002). The Tet-On system uses a bitransgenic mechanism to regulate inducible gene expression (Figure 5.1). First, a tissue specific promoter drives the expression of the reverse tet-activator transcription factor (rtTA). In the presence of doxycycline, rtTA binds to the tet-responsive element (TRE) in the promoter of the target transgene, activating transcription of the desired mRNA sequence. In the absence of doxycycline, transcription of the target gene is not observed. Furthermore, transgenic expression via the Tet-On system is reversible following induction by the withdrawal of doxycycline.

To direct expression of rtTA to cells of the oligodendrocyte lineage, we employed a 3.7 kb fragment of the 2'3'-cyclic-nucleotide 3' phosphodiesterase (CNP) promoter. The CNP promoter has previously been used to drive the expression of LacZ and EGFP reporters in transgenic mice (Chandross et al., 1999; Gravel et al., 1998; Yuan et al., 2002). CNP expression is primarily restricted to cells of the oligodendrocyte lineage in

the CNS as well as Schwann cells in the PNS. Analysis of CNP-EGFP mice revealed that transgene expression from the CNP promoter is coincident with the expression of the Olig2 transcription factor, an established marker of early oligodendrocyte progenitors (Yuan et al., 2002). In addition, transgene expression driven by the CNP promoter is maintained throughout oligodendrocyte and Schwann cell lineage development as well as in mature oligodendrocytes (Chandross et al., 1999; Yuan et al., 2002).

5.2 Materials and Methods

5.2.1 Materials

The CNP-bsK plasmid, containing a 3.7 kb regulatory sequence of the CNP gene, was a generous gift of Dr. Vittorio Gallo. The coding sequence for the rtTA-M2 variant was a gift from Dr. Wolfgang Hillen. XL polymerase was purchased from Applied Biosystems (Foster city, CA). Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, WI). TOP 10F' chemically competent cells were purchased from Invitrogen (Carlsbad, CA). The Qiaex II gel extraction and DNA purification kits were purchased from Qiagen (Valencia, CA). Doxycycline was purchased from Sigma (St. Louis, MO). Reagents used to perform X-gal staining were provided by Dr. Steve Levison's laboratory. Reagents used to perform *in vivo* bioluminescence imaging were provided by Dr. Ed Gunther's laboratory.

5.2.2 Generation of the CNP-rtTA transgene

To generate the CNP-rtTA transgene, a 1.2 kb fragment of the rtTA-M2 variant was amplified by PCR with high fidelity XL polymerase using PCR primers to add XhoI and HindIII restriction sites to the 5' and 3' ends of the sequence respectively. The amplified product was digested with XhoI and HindIII restriction enzymes and gel purified using a Qiaex II gel extraction kit. In parallel, the CNP-bsK plasmid was digested with XhoI and HindIII and gel purified. The digested rtTA-M2 fragment was ligated in frame with CNP exon0 in the CNP-bsK vector using T4 DNA ligase. Top10 F' chemically competent cells were transformed with 4ul of the ligation reaction and grown on LB-agar/ampicillin plates for 18 hours at 37°C. Colonies were used to seed 3ml mini cultures. Plasmid DNA was purified using a Qiagen mini-prep kit (Qiagen). To confirm that the rtTA sequence was properly ligated in frame with the CNP promoter, mini-prep purified DNA was analyzed by XhoI/HindIII double digest followed by separation on 1% agarose gels visualized with ethidium bromide staining. In addition, positively identified clones were further verified by DNA sequencing performed in the Penn State College of medicine molecular core facility. Sequences were generated using primers flanking the CNP promoter and rtTA sequence as well as a primer across the CNP/rtTA junction. The 4.9 kb CNP-rtTA DNA fragment was digested from positively identified clones, gel purified using a Qiaex II gel extraction kit, resuspended in nuclease free water, and sent to the Penn State College of Medicine transgenic core facility for pronuclear injection.

5.2.3 Generation and identification of CNP-rtTA founders

Pronuclear injections to generate CNP-rtTA founders were performed by Ms. Alane Seidel in the Penn State College of Medicine Transgenic Core Facility. All mice generated for this project were housed in a barrier facility at the Penn State College of Medicine animal facility. In total, 40 potential founders were generated and screened for the presence of the CNP-rtTA transgene. Tail clips from 3 week old pups were used to isolate genomic DNA used for genotyping. Genotyping was performed by PCR analysis. Primer sequences used for PCR identified the CNP-rtTA transgene. Specifically, a sense primer generated to recognize the CNP promoter was used in tandem with an antisense primer specific for the rtTA coding sequence to amplify a 200 bp DNA fragment. By PCR analysis, we identified 3 mice carrying the CNP-rtTA transgene which were used in subsequent studies.

5.2.4 Detection of inducible LacZ expression

Hemizygous CNP-rtTA founders were bred with homozygous Tet-OP LacZ reporter mice (a gift from Dr. Ed Gunther). Dams received doxycycline (2mg/ml, in 5% sucrose) beginning 14 days after giving birth to litters for one week. On postnatal day 21, pups were anesthetized with a 3:1 cocktail of ketamine and xylazine, and tissues were fixed by transcardiac perfusion with 3% paraformaldehyde/0.5% glutaraldehyde and extracted. Tail clips from each pup were collected to isolate genomic DNA used for genotyping. Forebrain vibratome sections (70 μ m) from each pup were prepared in a coronal plane to visualize white matter tracts at the level of the corpus callosum.

For LacZ detection, free floating sections were washed with PBS containing 4mM MgCl₂. Next the sections were incubated in X-Gal staining solution (consisting of 30 mM K₃Fe(CN)₆, 30 mM K₄Fe(CN)₆, 2mM MgCl₂, and 1 mg/ml X-Gal) overnight at 30°C. The following day, sections were rinsed in PBS, 3%DMSO in PBS, rinsed in PBS, and mounted on superfrost slide with Aquamount (Lerner Labs). Photomicrographs of LacZ positive oligodendrocyte progenitors and mature oligodendrocytes were captured with a 100X objective.

5.2.5 Bioluminescence imaging of inducible luciferase expression

Cnp-rtTA/Tet-OP Twnt-Luc mice were transiently anesthetized with isoflurane prior to administering 100 mg/kg ketamine and 10 mg/kg xylazine via intraperitoneal injection. Anesthetized mice then received a 135-mg/kg intraperitoneal injection of D-luciferin (Prolume, CA). Imaging was acquired and processed using an IVIS 50 imaging system and Living Image software (Xenogen, CA). The bioluminescence imaging (BLI) signals were quantified by measuring photon flux within software-generated regions of interest (ROIs). BLI data were acquired between 10 and 15 min after luciferin injection.

5.3 Results

5.3.1 Generation of CNP-rtTA transgenic mice

To generate CNP-rtTA transgenic mice, we ligated the coding sequence for the rtTA-M2 variant (a generous gift of Dr. Wolfgang Hillen) downstream of a 3.7kb fragment of the CNP promoter (a generous gift of Dr. Vittorio Gallo) (Figure 5.2). The rtTA coding sequence was positioned in frame with CNP exon 0 to ensure expression from both the CNP1 and CNP2 transcription initiation sites. Purified DNA was used for several rounds of pronuclear injection performed in the transgenic core facility at the Penn State College of Medicine. In total, 40 potential founders were screened for the presence of the CNP-rtTA transgene by a PCR based genotyping strategy, yielding 3 positively identified founders used in subsequent experiments.

5.3.2 Inducible expression of β -galactosidase in cells of the oligodendrocyte lineage

In order to test the function of the CNP-rtTA transgene, hemizygous CNP-rtTA founders were bred with a homozygous Tet-OP LacZ reporter strain. A schematic of the inducible strategy employed is shown in Figure 5.3. Dams received drinking water containing 2 mg/ml doxycycline in 5% sucrose for one week, beginning at postnatal day 14 for each litter. On postnatal day 21, brains were extracted and coronal forebrain sections were prepared for X-Gal staining. In addition, tail DNA was collected from each pup and used to determine the presence or absence of the CNP-rtTA transgene. We identified several β -Gal positive cells in the cortical and striatal white matter in sections

prepared from bitransgenic mice that received doxycycline. In contrast, we observed no β -Gal positive cells in brain sections of bitransgenic mice that did not received doxycycline. High power magnification revealed that the β -Gal positive cells exhibited the morphological characteristics of early OPCs as well as mature myelinating oligodendrocytes (Figure 5.4). Importantly, we did not identify any β -Gal positive cells that exhibited the morphological characteristics of either neurons or astrocytes.

While we were able to observe the inducible expression of β -Gal in cells of the oligodendrocyte lineage, expression occurred in a mosaic fashion. Fewer than 10% of OL lineage cells were β -Gal positive. In addition, we repeated our analysis of inducible β -Gal expression in adult bitransgenic mice. Again, we observed a mosaic expression pattern of β -Gal in cells of the oligodendrocyte lineage. Several independent founder lines were analyzed with identical results, minimizing the possibility that the mosaic expression pattern we observed was caused by an integration effect of the CNP-rtTA transgene.

5.3.3 Inducible expression of luciferase in CNP-rtTA/ Tet-OP Twnt-Luc mice

Our analysis of inducible expression in CNP-rtTA/ Tet-OP LacZ mice produced mixed results. While we successfully observed doxycycline controlled expression of LacZ in cells of the oligodendrocyte lineage, fewer than 10% of the oligodendrocytes present in the forebrain cortical and striatal white matter of these mice positively induced LacZ expression. One possible explanation for the mosaic expression observed is that the Tet-OP LacZ transgene was silenced in the brains of these mice. To exclude this

possibility and further examine the activity of the CNP-rtTA transgene, we bred CNP-rtTA founders to the Tet-OP Twnt-Luc strain.

Tet-OP Twnt-Luc mice exhibit doxycycline inducible bicistronic expression of Wnt1 and firefly luciferase (Gunther et al., 2003). Adult bitransgenic CNP-rtTA/ Tet-OP Twnt-Luc mice received a 2mg/ml doxycycline, 5% sucrose solution in their drinking water for two weeks prior to bioluminescence imaging of luciferase expression. The inducible expression of luciferase in bitransgenic mice that received doxycycline was compared to control animals which received a 5% sucrose solution alone.

Representative images of bioluminescence imaging signals captured following doxycycline administration are shown in Figure 5.5 A,B. CNP-rtTA/ Tet-OP Twnt-Luc mice which received doxycycline emitted strong bioluminescence signals relative to control mice. However, the signal intensity was primarily localized to the abdomen. Luciferase expression was not apparent in the brain. To further examine luciferase expression, the brains and sciatic nerves were dissected for *ex vivo* imaging (Figure 5.5C). Again, we were unable to detect luciferase expression in the nervous tissues.

5.4 Discussion

The data presented in this chapter detail our attempts to generate an inducible model of transgenic expression targeting the cells of the oligodendrocyte lineage. While we were successful in our efforts to direct transgene expression to oligodendrocytes, we observed a mosaic pattern of expression restricted to fewer than 10% of oligodendrocyte lineage cells in the CNS limiting its utility for subsequent studies. The mosaic expression

observed was not likely due to integration effects of the CNP-rtTA transgene as multiple independent founder lines were analyzed. Furthermore, we observed the mis-expression of transgene expression, primarily in the gut of our bitransgenic mice. To our knowledge, CNP expression in the gut has not been described and is most likely an artifact in our system.

It is difficult to determine why we failed to obtain robust expression from the CNP promoter in our system. One possible flaw may lie in the construct of the CNP-rtTA transgene itself. In order to ensure expression of rtTA from both CNP1 and CNP2 transcription initiation site, we ligated the coding sequence in frame with exon 0 of the CNP promoter. This resulted in the addition of 9 amino acids to the N-terminus of the rtTA protein when transcribed from the CNP2 initiation site. This addition may have altered the DNA binding specificity of rtTA causing the mosaic expression pattern observed.

Figure 5.1 Overview of the Tet-On system. Schematic representation of the Tet-On system (Clontech, 2007). The activator gene regulates the expression of the rtTA transcription factor. In the presence of doxycycline, rtTA binds to the promoter of a response gene containing a TRE recognition sequence to initiate transcription of the desired mRNA sequence.

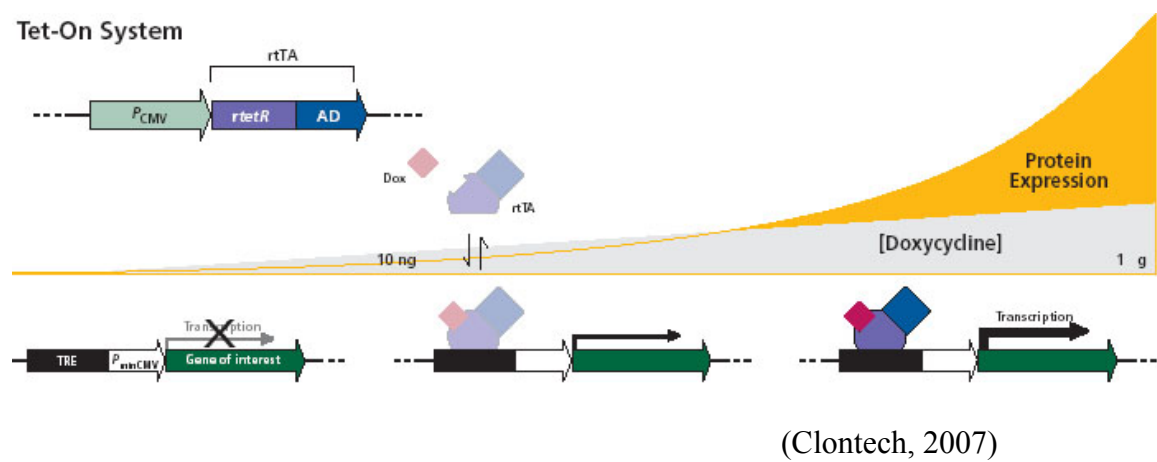


Figure 5.1: Overview of the Tet-On system

Figure 5.2 Schematic of the CNP-rtTA transgene. A 1.2 kb fragment containing the coding sequence for the rtTA transcription factor was ligated downstream of a 3.7 kb sequence of the CNP promoter. The rtTA coding sequence was inserted in frame with CNP exon 0 to ensure expression from both the CNP 1 and CNP 2 transcript initiation sites.

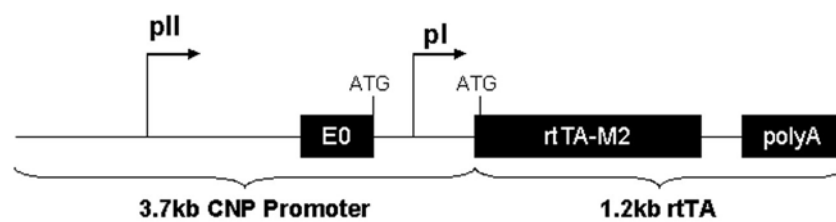


Figure 5.2: Schematic of the CNP-rtTA transgene.

Figure 5.3 Schematic of the bitransgenic strategy used to induce LacZ expression in cells of the oligodendrocyte lineage. A. Diagram of the CNP-rtTA and Tet-OP LacZ transgenes. No expression of LacZ is induced in the absence of doxycycline. B. In the presence of doxycycline, rtTA binds to the tet response element (TRE) in the Tet-OP promoter to induce LacZ expression.

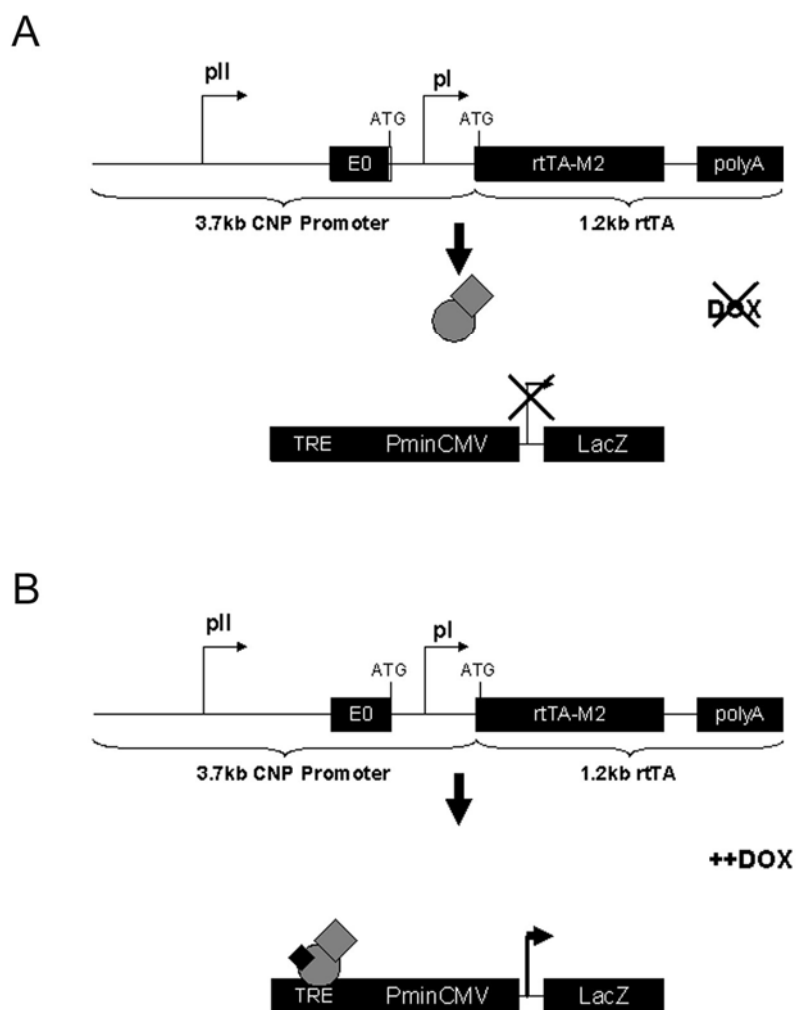


Figure 5.3: Schematic of the bitransgenic strategy used to induce LacZ expression in cells of the oligodendrocyte lineage.

Figure 5.4 Doxycycline induced expression of β -galactosidase in cells of the oligodendrocyte lineage. Representative images of X-Gal staining on brain sections prepared from postnatal day 21 old CNP-rtTA/Tet-OP LacZ mice. A-B: Identification of β -Gal positive cells exhibiting the morphological characteristics of early oligodendrocyte progenitors in subcortical white matter. C-D: β -Gal positive myelinating oligodendrocytes.

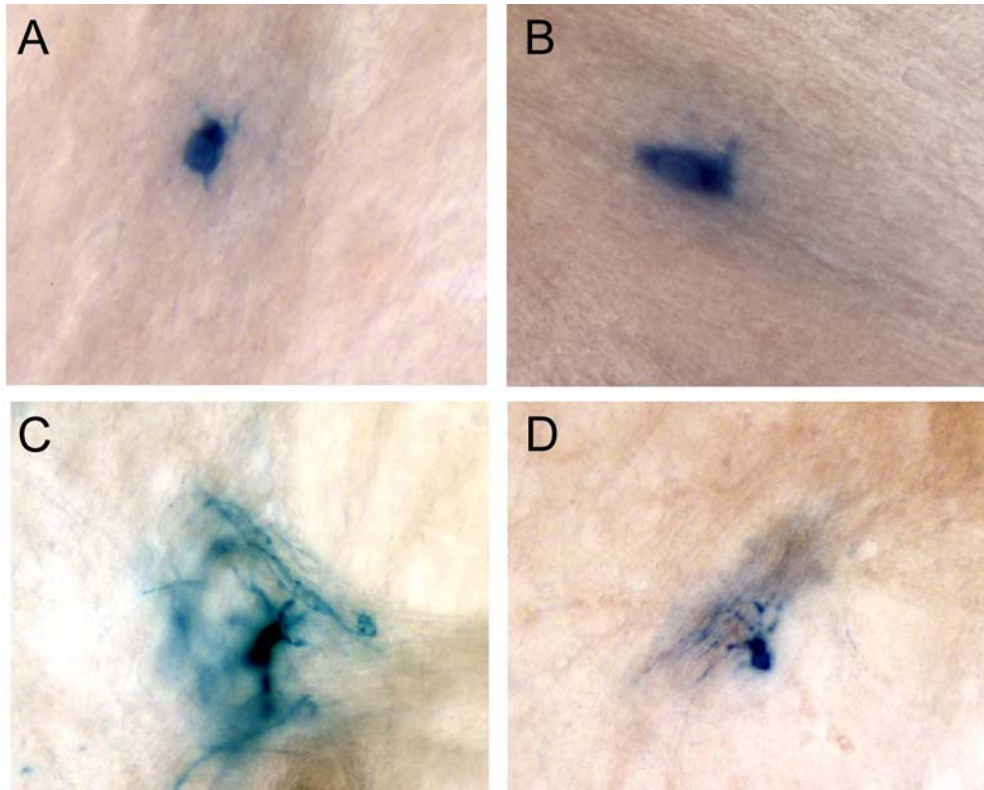


Figure 5.4: Doxycycline induced expression of β -galactosidase in cells of the oligodendrocyte lineage.

Figure 5.5 The distribution of doxycycline-inducible luciferase expression in CNP-rtTA/ Tet-OP Twnt-Luc mice. Representative images of *in vivo* luciferase imaging performed on adult CNP-rtTA/ Tet-OP Twnt-Luc mice following one week of doxycycline administration (2 mg/ml in 5% sucrose). A-B: Dorsal (A) and ventral (B) views of bioluminescent images of bitransgenic CNP-rtTA/ Tet-OP Twnt-Luc following doxycycline administration or control mice which received a 5% sucrose solution alone. C: *Ex vivo* imaging of doxycycline treated and control brains and sciatic nerve (Arrow).

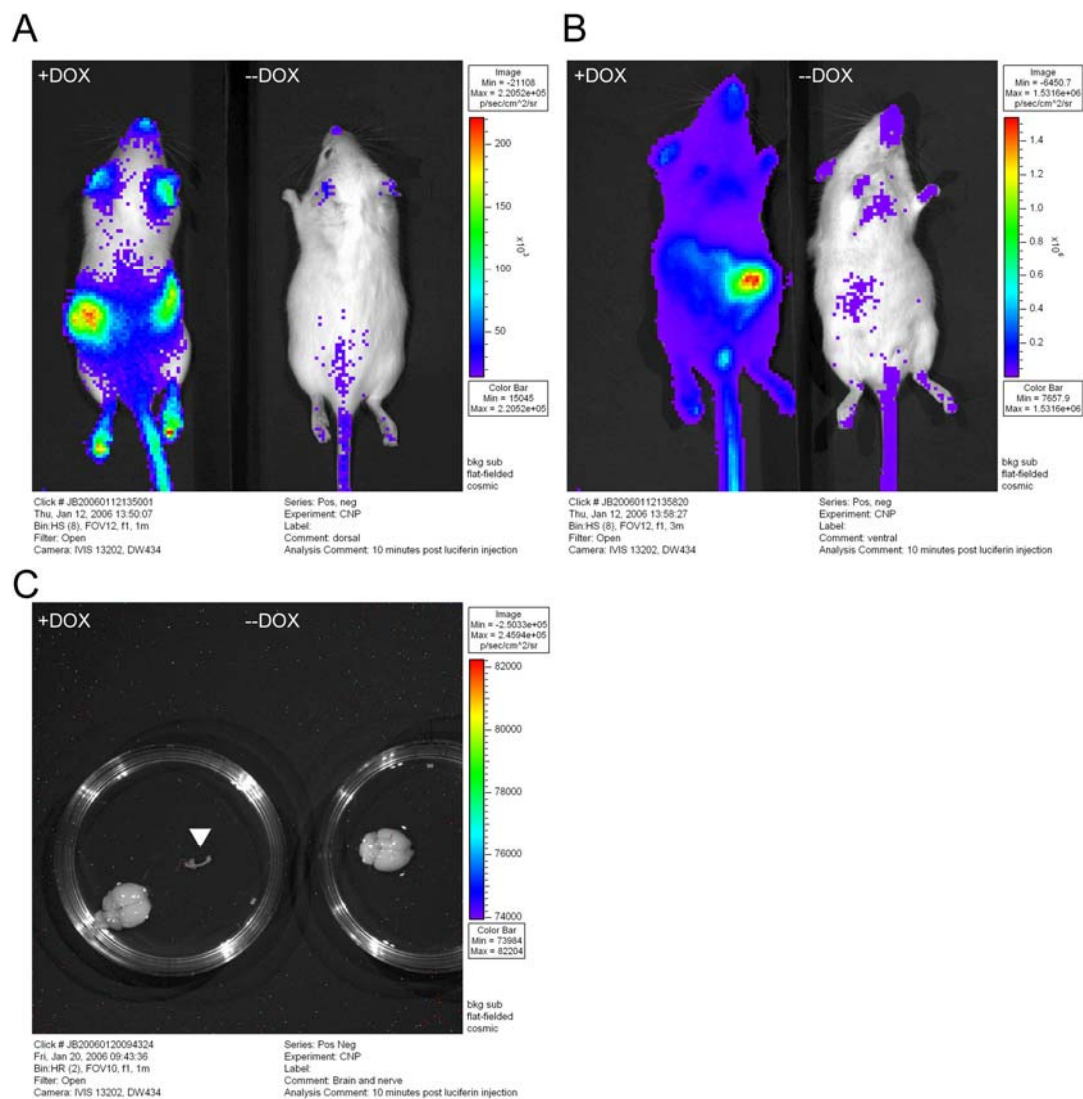


Figure 5.5: The distribution of doxycycline-inducible luciferase expression in CNP-rtTA/ Tet-OP Twnt-Luc mice.

Chapter 6

Final Discussion: Major contributions and future implications

6.1 Summary of results

While several extrinsic and intrinsic factors have been identified that have defined roles in the regulation of oligodendrocyte differentiation, the molecular mechanisms that integrate these signals to coordinate the generation of mature myelinating oligodendrocytes remain poorly defined. The data presented throughout this thesis support the hypothesis that the activation of the mammalian target of rapamycin (mTOR) is essential for oligodendrocyte differentiation.

We have shown evidence to suggest that mTOR, acting through mTORC1 and mTORC2 signaling complexes, activates an array of specific downstream signaling effectors in a temporal fashion beginning at the onset of oligodendrocyte differentiation. Furthermore, the inhibition of mTOR via rapamycin treatment or siRNA mediated knockdown arrests oligodendrocyte lineage progression at the $O4^+$ / $GalC^-$ late progenitor stage.

mTOR regulates the mRNA levels of myelin genes including myelin basic protein (MBP), proteolipid protein (PLP), and UDP glycosyltransferase 8 (Ugt-8), rather than solely affecting the translation of myelin proteins. We interpret these data to suggest that mTOR regulates key factors that control the decision to commit to terminal differentiation rather than simply modulating the expression of a subset of genes during

oligodendrocyte differentiation. In support of this hypothesis, we have identified two members of the inhibitor of DNA binding protein family, Id2 and Id4, that block the differentiation of OPCs and whose expression at the mRNA level is controlled in part by mTOR activity.

Further studies have demonstrated that mTOR exerts its effects on oligodendrocyte differentiation through two unique signaling complexes, mTORC1 and mTORC2. We show that mTORC1 and mTORC2 complexes, defined by the presence of the adaptor proteins raptor and rictor respectively, form and are active during oligodendrocyte differentiation. Furthermore, disruption of mTORC1 or mTORC2 activity via siRNA mediated knockdown of rictor or raptor revealed an essential role for each complex in the control of myelin protein expression.

The examination of signaling effectors downstream of mTORC1 and mTORC2 complexes showed a temporally distinct pattern of target phosphorylation. Specifically, the phosphorylation of p70S6K1 and 4E-BP, targets of the mTORC1 complex, occurred early during oligodendrocyte differentiation but then declined through 48-72 hours of differentiation. In contrast, the phosphorylation of Akt Ser473, a specific substrate for mTORC2, increased within the first 24 hours and was sustained throughout differentiation.

In order to dissect the temporal sequence of mTORC1 and mTORC2 target activation, delayed addition of rapamycin studies were performed to better define the time frame in which mTOR activity is required for differentiation. These studies show that inhibition of mTOR following 2 days of differentiation is as effective at blocking oligodendrocyte differentiation as the inhibition of mTOR from the onset of

differentiation, suggesting that mTOR activity is critical during the timeframe when late progenitors transition to the immature oligodendrocyte stage. The further implications of these studies will be discussed below.

Finally, our studies have determined that mTOR regulates oligodendrocyte differentiation at least in part through its ability to signal to the mTORC1 target p70S6K1. First, we observed the rapid phosphorylation of p70S6K1 Thr389, an essential event regulating p70S6K1 kinase activity (Pearson et al., 1995), at the onset of oligodendrocyte differentiation that was maintained through 24 hours before declining. The phosphorylation of p70S6K1 Thr 389 was completely abrogated by rapamycin. Furthermore, siRNA mediated knockdown of p70S6K1 significantly reduced MBP expression following 5 days of differentiation. In addition, preliminary analysis of S6K1.2 $-/-$ mice showed a decrease in myelin protein content that was not likely due to a decrease in the numbers of oligodendrocyte progenitors present. In total, these data suggest p70S6K1 is a key signaling effector of mTOR during oligodendrocyte differentiation.

Together, these studies provide solid evidence that mTOR is a central player in a signaling pathway that is essential for oligodendrocyte differentiation. Figure 6.1 illustrates a model of the effects of mTOR signaling during oligodendrocyte differentiation. The following sections will discuss the major contributions and implications of these studies. Finally, the relevance of these findings to the development of treatments for demyelinating disorders will be discussed.

6.2 Evidence that extrinsic signals are required to promote the terminal differentiation of oligodendrocytes

Previous studies suggest that oligodendrocyte differentiation is regulated by an intrinsic timing mechanism, stimulated by mitogen withdrawal, that initiates differentiation after a certain number of cell divisions (Barres et al., 1994; Temple and Raff, 1986). These studies propose a model of oligodendrocyte differentiation that occurs via a constitutive pathway intimately coupled to cell cycle exit, independent of extracellular factors required to promote differentiation.

In recent years, the intrinsic model of oligodendrocyte differentiation has been challenged by experiments designed to uncouple cell cycle withdrawal and the onset of differentiation. Specifically, the expression of dominant negative forms of cyclin dependent kinase 2 (cdk2) induced cell cycle arrest in proliferating cultures of oligodendrocyte progenitors, but did not promote differentiation (Belachew et al., 2002). Further studies demonstrate that overexpression of the cyclin dependent kinase inhibitor (cdki) p27 prematurely arrests oligodendrocyte proliferation (Tang et al., 1999). However, cell cycle arrest alone was not sufficient to promote oligodendrocyte differentiation in these cultures, suggesting that additional signals are required.

Growth factors have been identified that suppress oligodendrocyte differentiation. For example, the combination of PDGF and bFGF maintains oligodendrocyte progenitors in an early undifferentiated state (Tokumoto et al., 2001). Furthermore, bFGF inhibits oligodendrocyte differentiation at the O4+/GalC- late progenitor stage (Gard and Pfeiffer, 1993). However, few extrinsic factors that play a bona fide role in the induction of oligodendrocyte differentiation have been identified. Furthermore, there is a paucity of

information regarding the intracellular signaling cascades that coordinate the decision of oligodendrocyte progenitor differentiation.

Our data suggest that activation of mTOR is essential for oligodendrocyte differentiation. Specifically, mTOR activation is required for progression past the late progenitor stage of differentiation as assessed by morphological complexity and the expression of myelin proteins. Inhibition of mTOR reversibly blocks oligodendrocyte differentiation in manner independent of cell cycle arrest, as mTOR did not alter the expression profiles of the cdki(s) p27 or p21, or alter the number of cells present in our cultures. This suggests that extrinsic factors are required to stimulate mTOR signaling to promote the differentiation of oligodendrocyte progenitors. Furthermore, data from the delayed addition of rapamycin experiments suggest that these factors must activate mTOR specifically during the transition to the immature stage of differentiation, as delaying the addition of rapamycin 2 days after the onset of differentiation blocked the progression of late progenitors to the immature oligodendrocyte stage.

mTOR is a serine/threonine kinase that integrates signals derived from growth factor stimulation and nutrient sensing mechanisms (Harris and Lawrence, 2003). At present, the identity of factors that stimulate mTOR activation during oligodendrocyte differentiation is unknown. However, a considerable amount of evidence suggests that insulin-like growth factor 1 (IGF-I) may fulfill this role.

First, we observed the sustained phosphorylation of mTOR on Ser 2448, characterized as an insulin/IGF sensitive site (Nave et al., 1999; Sekulic et al., 2000; Yanocho and Eckhart, 2006), during oligodendrocyte differentiation *in vitro*.

Furthermore, phosphorylation of mTOR at Ser 2448 correlates with myelination in the subcortical white matter of the developing brain.

IGF-I affects the survival, proliferation, and differentiation of cells of the oligodendrocyte lineage *in vitro* and *in vivo*. Relevant to this dissertation, the over-expression of IGF-I in transgenic mice results in a hypermyelinated phenotype caused by increased myelin production per oligodendrocyte (Carson et al., 1993). Conversely, IGF-I $-/-$ mice exhibit hypomyelination characterized by a significant decrease in myelin protein expression (Ye et al., 2002). *In vitro* studies show IGF-I has a profound effect on the generation of GalC⁺ oligodendrocytes by stimulating the differentiation of O4⁺/GalC⁻ late progenitors (McMorris and Dubois-Dalcq, 1988). Furthermore, the media used in our experiments to initiate differentiation contains micromolar levels of insulin, known to stimulate the IGF-IR (LeRoith et al., 1995), and as such should be considered as IGF-I supplemented.

Future studies are required to determine whether IGF-I or additional growth factors stimulate the activity of mTOR during OPC differentiation. Ultimately however, our data show that the activation of mTOR signaling is required for the proper differentiation of oligodendrocytes *in vitro*. These data suggest that OPC differentiation requires signaling derived from extrinsic factors during a specific step of development, namely the transition to the GalC⁺ immature oligodendrocyte stage. However, the transition to the O4⁺ late progenitor stage is not regulated by mTOR signaling, suggesting that the early events that occur during differentiation may in fact occur via an intrinsic program. However our data underscore the importance of the integration of extrinsic and intrinsic signals is required for the generation of mature oligodendrocytes.

6.3 Insight into the temporal activation of mTORC1 and mTORC2 complexes during oligodendrocyte differentiation

mTORC1 and mTORC2 complexes phosphorylated distinct downstream signaling effectors during oligodendrocyte differentiation in a temporal fashion. Specifically, p70S6K1 Thr 389 and 4E-BP, mTORC1 targets, were phosphorylated acutely during the onset of differentiation. However, the phosphorylation of these targets declined by 48 hours. In contrast, the phosphorylation of Akt Ser 473, a substrate of the mTORC2 complex, increased significantly by 24 hours of differentiation and was sustained through 96 hours. Inhibition of mTOR completely abrogated the phosphorylation of mTORC1 targets, and partially yet significantly decreased the phosphorylation of Akt Ser 473.

Interestingly, the disruption of mTORC1 and mTORC2 complex activity via siRNA mediated knockdown of raptor and rictor showed that each complex is essential for myelin protein expression. Taken together, these data suggest that mTOR activity is required during distinct phases of oligodendrocyte differentiation. Specifically, the acute activation of mTORC1 targets constitutes an early effector of mTOR's control of differentiation, while activation of the mTORC2 target, Akt, may be required at later stages of differentiation.

In an attempt to separate these events, we delayed the addition of rapamycin to cultures of differentiating oligodendrocytes to define the critical window of time in which mTOR activity is essential for differentiation. Surprisingly, the addition of rapamycin after 2 days of differentiation was as effective at blocking the generation of GalC⁺

postmitotic oligodendrocytes as was the addition of rapamycin at the onset of differentiation.

By 48 hours of differentiation, phosphorylation of the mTORC1 targets, p70S6K1 and 4E-BP, has declined from peak levels, suggesting that their function alone is not sufficient to promote progression through the late progenitor stage. However, these data do not exclude the possibility that the early activation of these mTORC1 targets is necessary for differentiation to occur. In fact, disruption of mTORC1 function through the knockdown of raptor suggests that the activation of mTORC1 targets is essential for myelin protein expression. Furthermore, we have identified p70S6K1, a mTORC1 target phosphorylated early during differentiation, as an essential effector of mTOR in the regulation of myelin protein expression *in vitro* and *in vivo*.

Taken together, these data support a model of mTOR activation during oligodendrocyte differentiation in which mTORC1 and mTORC2, acting on distinct downstream effectors in a temporal fashion, is essential for the execution of the oligodendrocyte differentiation program. Furthermore, our data suggests that the activation of either mTORC1 or mTORC2 complexes alone is not sufficient to promote mTOR dependent differentiation.

In order to directly test these conclusions, we have initiated a series of “rescue” experiments to identify key signaling effectors downstream of the mTORC1 and mTORC2 complexes that regulate oligodendrocyte differentiation. Specifically, rapamycin resistant p70S6K1, 4E-BP, and Akt mutants will be expressed alone and in combination during oligodendrocyte differentiation in the presence of rapamycin to determine which mTORC effector substrates propagate the mTOR dependent

differentiation signal. Based on our observations that mTORC1 and mTORC2 are both required for the proper expression of myelin proteins, we anticipate that none of the rapamycin resistant mutants acting alone will be able to restore proper differentiation. Instead, mTORC1 and mTORC2 effector substrates acting in combination will be required.

6.4 The sustained activation of Akt is rapamycin sensitive

Previous studies from our lab have shown that IGF-I activates Akt in sustained fashion (Ness et al., 2002; Ness and Wood, 2002). The sustained activation of Akt appears to be a unique property of IGF-I mediated signaling through the PI3K pathway, as neurotrophin-3 (NT-3) mediated activation of the PI3K pathway results in a transient activation of Akt (Ness et al., 2002). This property of IGF-I mediated signaling is of considerable interest because the sustained activation of Akt correlates with the ability of IGF-I to protect oligodendrocyte progenitors from apoptosis caused by glutamate excitotoxicity (Ness and Wood, 2002). However, the mechanism responsible for this phenomenon is unknown.

Current studies demonstrate that the phosphorylation of Akt Ser473 during oligodendrocyte differentiation increases by 24 hours and is sustained through 96 hours. The differentiation medium used in these experiments contains a micromolar concentration of insulin, capable of activating the IGF-IR, which is likely the cause of the observed activation of Akt. Furthermore, the phosphorylation of Akt in our system correlates with the generation of postmitotic GalC⁺ oligodendrocytes.

Interestingly, the inhibition of mTOR via rapamycin treatment resulted in a 58% and 61% reduction in Akt Ser 473 phosphorylation on days 2 and 3 of differentiation (day 2, $P=0.0003$; day 3, $P=0.0019$). The mTOR dependent phosphorylation of Akt during oligodendrocyte differentiation is consistent with previous reports that defined the mTORC2 signaling complex as a critical kinase which mediates Akt Ser 473 phosphorylation in a number of cell types (Sarbasov et al., 2006; Sarbasov et al., 2005b). These results suggest that the sustained activation of Akt occurs at least in part via an mTOR dependent mechanism.

Interestingly, the reduction in Akt Ser 473 phosphorylation caused by rapamycin treatment did not correlate with a decrease in cell viability in our cultures. One possible explanation for this observation is that factors in addition to Akt regulate pro-survival signaling through the PI3K pathway. In support of this hypothesis, a previous study showed that the pretreatment of oligodendrocyte progenitors with a specific inhibitor of Akt or over-expression of a dominant negative form of Akt resulted in the decreased phosphorylation of Akt and GSK-3 β , but did not completely abrogate IGF-I mediated survival during growth factor deprivation (Cui et al., 2005). While the activation of the PI3K pathway is clearly an important regulator of OPC survival (Ness and Wood, 2002; Vemuri and McMorris, 1996; Zaka et al., 2005), it appears that Akt-dependent and independent signal transductions cascades mediate the effects of PI3K activation.

Alternatively, our results show that the sustained phosphorylation of Akt is only partially controlled by an mTOR dependent pathway. We observed a 58% and 61% reduction in the level of Akt Ser 473 phosphorylation on days 2 and 3 respectively in response to rapamycin treatment. In contrast, the phosphorylation of p70S6K1 and 4E-

BP was completely blocked by mTOR inhibition. We interpret these data to suggest that the mTORC2 complex regulates the phosphorylation of a distinct intracellular pool of Akt.

Oligodendrocytes and their progenitors express three isoforms of Akt 1-3 (Ribero and Wood, unpublished observation). Currently, it is unknown whether the mTORC2 complex is capable of phosphorylating each of the Akt isoforms. The intracellular compartmentalization of discrete pools of Akt may govern mTORC2 mediated phosphorylation.

The residual activation of Akt observed in our cultures following rapamycin treatment might signify distinct functions for intracellular pools of Akt defined by either compartmentalization or separate Akt isoforms. For example, the rapamycin sensitive pool of Akt may regulate functions associated with oligodendrocyte differentiation, while the rapamycin insensitive pool of Akt regulates survival. Future experiments to delineate the functions of each Akt isoform expressed by oligodendrocyte progenitors will shed light on the ability of Akt to affect diverse cellular processes including proliferation, differentiation, and survival. Furthermore, the analysis of Akt isoforms will contribute to our understanding of the ability of the mTORC2 complex to regulate the activity of Akt.

6.5 Relevance to Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory disease of the CNS which results in the formation of demyelinated lesions through the degeneration of myelin and the specific loss of oligodendrocytes (Kornek and Lassmann, 2003). Current strategies to

treat MS center around suppressing the inflammatory response through immunomodulatory agents (Hemmer et al., 2006). However, it remains unclear whether reducing inflammation alone is sufficient to initiate the full regeneration of lost myelin (McQualter and Bernard, 2007). Instead, combinatorial strategies that target inflammation as well as promote remyelination are more likely to succeed.

In response to demyelination, the CNS mounts a regenerative process and remyelination by resident adult oligodendrocyte progenitors (Franklin and Hinks, 1999). However, remyelination ultimately fails for unknown reasons, even though adult oligodendrocyte progenitors have been identified in tissue surrounding demyelinated lesions (Blakemore and Keirstead, 1999; Keirstead and Blakemore, 1999). The failure of adult progenitors to repair demyelinated lesions may in part be due to a lack of instructive signals combined with inhibitory factors present in MS lesions that prevent the terminal differentiation of oligodendrocyte progenitors (Franklin, 2002; Kotter et al., 2006; Penderis et al., 2003; Sim et al., 2002).

Our studies have identified mTOR as the central player in a signaling pathway essential for oligodendrocyte differentiation. Therefore, the examination of extrinsic factors that activate and inhibit mTOR signaling may lead to future strategies to promote myelin repair in demyelinating disorders like MS.

While the function of mTOR in the process of remyelination has not been directly tested, the genomic analysis of remyelination in a cuprizone induced mouse model of demyelination identified mTOR as a transcript upregulated during remyelination in wild type mice (Arnett et al., 2003). In contrast, mTOR expression was suppressed in $\text{TNF-}\alpha$ -/- mice, which are defective in their ability to remyelinate following demyelination,

suggesting that mTOR may play an essential role during remyelination. Furthermore, rictor, a component of the TORC2 signaling complex, was recently identified as a transcript upregulated during oligodendrocyte differentiation that is linked to an MS susceptibility locus (Dugas et al., 2006).

In addition, our studies add support to the evidence that the Id family proteins, Id2 and Id4, inhibit the differentiation of oligodendrocyte progenitors (Kondo and Raff, 2000; Marin-Husstege et al., 2006; Samanta and Kessler, 2004; Wang et al., 2001). Inhibition of mTOR activity causes an increase in Id2 and Id4 mRNA levels which correlates with a block of oligodendrocyte differentiation at the $O4^+$ /GalC⁻ late progenitor stage. Characterization of the expression profile of Id proteins in adult oligodendrocyte progenitors in chronic MS lesions may provide valuable insight into the failure of these cells to successfully regenerate lost myelin. Furthermore, mouse models of demyelination/remyelination provide a controlled experimental paradigm to study Id protein function and mTOR signaling during the regenerative process.

Figure 6.1 A model of the effects of mTOR signaling on oligodendrocyte differentiation.

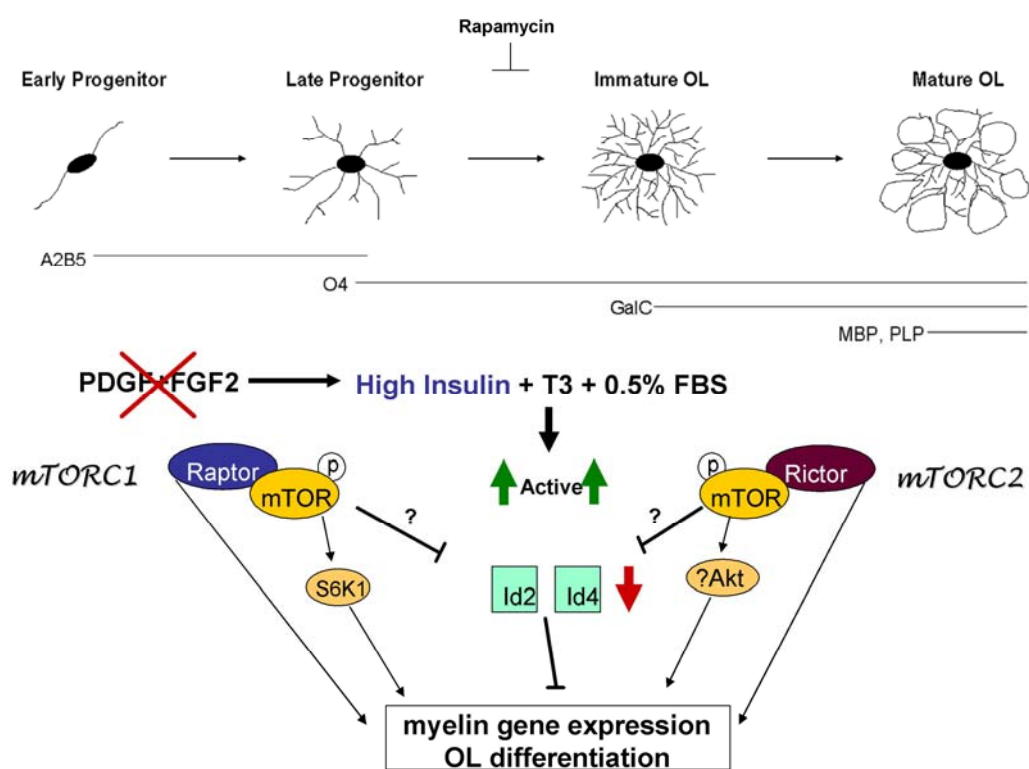


Figure 6.1: A model of the effects of mTOR signaling on oligodendrocyte differentiation.

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