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Erk1/2 MAPK and mTOR Signaling Sequentially Regulates Progression Through Distinct Stages Of Oligodendrocyte Differentiation

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Abstract

Myelination is the culmination of a complex process in which oligodendrocyte (OL) progenitors transition through defined stages in a well-coordinated differentiation program. The signaling mechanisms that regulate this progression are poorly understood. Here we investigate the role of extracellular signal-regulated-kinase-1,-2 (Erk1/2) and the mammalian target of rapamycin (mTOR), downstream effectors of the Ras/Raf/Mek/Erk and PI3K/Akt/mTOR pathways, at specific stages of OL development *in vitro*. Using a panel of developmental stage-specific antigenic markers and pharmacological inhibitors, we provide evidence that Erk1/2 signaling regulates transition of early progenitors to the late progenitor stage and, as a consequence, to the immature OL stage, but not the transition of immature OL to the mature OL stage. In contrast, mTOR signaling is not required for early progenitor transition to late progenitor stage. Surprisingly, it is also not required for the transition of late progenitors to terminally differentiated immature OLs, as has been reported previously, but is required for the next sequential transition of immature OLs to the mature OL stage. Furthermore, mTOR signaling regulates OL cytoskeletal organization and major myelin protein expression. These *in vitro* findings correlate with our *in vivo* data showing that inhibition of mTOR by rapamycin injection attenuated the onset of myelination in the early postnatal brain. Thus, these studies demonstrate that Erk1/2 and mTOR signaling sequentially regulates distinct stages of OL progenitor differentiation and suggest that cells in the OL-lineage require distinct signaling mechanisms to transition through specific stages of their development.

Keywords

oligodendrocyte; myelin; Erk MAPK; mTOR

INTRODUCTION

Failure of efficient remyelination in Multiple Sclerosis and other demyelinating disorders is largely due to the inability of OL progenitors to efficiently differentiate into myelinating OLs (Franklin, 2002; Fancy *et al*., 2011). Therefore, understanding the diverse signaling mechanisms that control the differentiation of OLs through multiple stages of the OL-lineage is essential for developing strategies to promote myelin repair.

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OL progenitors progress through distinct morphological and antigenic stages of maturation prior to myelin formation. These developmental stages have been characterized extensively *in vitro* and are highly correlated *in vivo* (Pfeiffer *et al*., 1993; Warrington *et al*., 1993; Miller, 2002; Emery, 2010). Isolated bipolar *early progenitors* differentiate into multipolar *late progenitors*, which enter terminal differentiation and become postmitotic *immature OLs*. These cells develop complex, branched and intertwined processes before extending networks of membranes, as they become *mature OLs*. This program of differentiation requires multiple integrated extrinsic and intrinsic molecular signals. the intracellular signal transduction pathways that control the transition through distinct stages of the OL-lineage are poorly understood.

Several studies have implicated the Ras/Raf/Mek/Erk and P13K/Akt/mTOR pathways in the proliferation, migration or survival of OL progenitor cells, largely as a result of their activation by various growth factors (*e.g* Bhat, 1995; Baron *et al.*, 2000; Flores *et al.*, 2000; Ebner *et al*., 2000; Yim *et al.*, 2001; Bansal *et al.*, 2003; Cui and Almazan, 2007; Frederick *et al*., 2007; Frost *et al*., 2009; Van't Veer *et al*., 2009). However, there is limited and often contradictory information available about the role of these signaling molecules in the differentiation of OL progenitors to mature myelinating OLs. For example, using pharmacological inhibitors of Mek (PD098059, U0126) in cultures of OL progenitors, Baron *et al.* (2000) and Fyffe-Maricich *et al.* (2011) observed inhibition of differentiation, while Younes-Rapozo *et al.* (2009) found inhibition of process extension by OLs, but not acquisition of myelin proteins. Further, no inhibition or only a transient and partial inhibition of OL progenitor differentiation was observed in mixed primary cortical cultures of Erk1-null or Erk2 conditional single knockout mouse brain, respectively (Fyffe-Maricich *et al.*, 2011). Using rapamycin, an mTOR inhibitor, Tyler *et al.* (2009) reported an inhibition of terminal differentiation of early progenitors to GalC+ immature OLs, while Baron *et al.* (2000), using wortmannin, a PI3K inhibitor, did not observe any inhibition at the same stage of the OL-lineage. Part of the inconsistency between these results may be attributed to differences in culture conditions including inhibitors, source of OL progenitors, culture media and timing of analysis, etc.

To unify potential discrepancies associated with previous studies and provide a more integrated understanding of how these two signaling pathways control the stage specific physiological demands of OL-lineage progression, we investigated the effect of inhibiting these pathways in parallel. We found that inhibition of Erk1/2 with U0126 inhibited the progression of isolated early progenitor to the late progenitor stage with a concomitant delay of maturation to the immature OL stage, but had no effect on the differentiation of immature OLs to the mature OL stage. In contrast, inhibition of mTOR with rapamycin did not block the transition of early or late progenitors or of late progenitors to the immature OL stage, but attenuated their progression to the mature OL stage by inhibiting the expression levels of major myelin proteins and the normal organization of the OL cytoskeleton. The effect of mTOR inhibition on MBP expression was also observed in a more complex environment of a mixed primary culture of mouse brain and correlated with the *in vivo* inhibition of the onset of myelination in the early postnatal brain by rapamycin injection. Together, our data demonstrates that distinct signaling pathways control distinct stages of OL development in a sequential and non-overlapping manner. Importantly, in contrast to previous *in vitro* studies, neither of the pathways was found to directly play an essential role in the commitment of late progenitors to enter terminal differentiation.

MATERIALS AND METHODS

Cell Culture

Enriched cultures of OL progenitors were prepared as described previously (Bansal *et al*., 1996). Mixed primary cultures from neonatal (P1-2) rat telencephalon were shaken overnight in an orbital shaker. Dislodged OL progenitors (and some prepogenitors) were further purified by differential adhesion and complement lysis with anti- GalC. Early progenitors were seeded on poly-L-ornithine (25 ug/ mL, Sigma) coated 4-well plates (30,000 cells/well for immunofluorescence) or 6-well plates (250,000 cells/well for immunoblotting). Following 3 hrs of cell attachment in 5% FCS/DMEM, cells were grown in serum-free, defined medium, N2 [(DMEM supplemented with human transferrin (50 μg/ml), bovine pancreatic insulin (5 μg/ml), 3,3,5-triiodo-L-thyronine (10 ng/ml), sodium selenium (30 nM), D-biotin (10 ng/ml), hydrocortisone (10 nM), sodium pyruvate (0.11 mg/ml), penicillin-streptomycin (10 IU/ml and 100 μg/ml, respectively)], and 0.1% BSA (all from Sigma) in the presence of PDGF for 1-2 days (Preprotech; 10 ng/ml). In some experiments plated cells were not exposed to PDGF at all. The onset of differentiation (day 0) was triggered by PDGF removal with a rinse in N2 and cells were maintained in N2. U0126 and rapamycin are highly selective inhibitors of Mek1/2 (upstream of Erk1/2) and mTOR (Davies *et al*., 2000) used to suppress the MAPK and Akt/mTOR pathways respectively (Fig.1B). Stocks of U0126 (dissolved in DMSO; Calbiochem, LaJolla, CA), rapamycin (dissolved in EtOH; Calbiochem) or dimethysulfoxide (DMSO), as a control, were pre-mixed in N2 and added to the cultures at a final concentration of 10uM and 25uM for U0126 and 22nM for rapamycin. The concentrations of U0126 were based on immunoblotting for phospho-Erk1/2 on isolated OL progenitor cultures (data not shown). Rapamycin was used at similar doses described in previous OL studies for consistency (Baron *et al*., 2000; Tyler *et al*., 2009).

Dissociated primary cultures of neonatal mouse telencephalon were prepared as described previously (Bansal *et al*., 1996) and cells plated at a density of 150,000 cells/cm^2 were grown in N2 defined media following one day in 5% FCS. Rapamycin (22nM final concentration) was added with each medium change during the period indicated in the experiment.

Immunofluorescence Microscopy and quantification of cell numbers

Immunolabelling of cells was performed as described previously (Bansal *et al*., 1996). Primary antibodies used were anti-Olig2 (1:50, IBL), A2B5 (1:25), O4 (1:25, Bansal *et al.*, 1992), O1 (1:25), anti-HPC7 (1:25, Baas and Barnstable, 1998), anti-CNP and anti-MBP (1:100; Sternberger Monoclonals Incorp., Lutherville, MD),. Secondary antibodies include, FITC-conjugated goat anti-mouse IgM, μ-chain specific, for O4, A2B5 and O1; Cy3-conjugated anti-mouse IgG, gama-chain specific for HCP7, CNP, MBP (Jackson Labs, Bar Harbor, Maine) or anti-rabbit IgG for Olig2, and a nuclear label, Hoechst dye (Sigma).

Three to four independent cultures, each with duplicate wells per condition, were analyzed. An average of 125 cells in ~10 fields per well were counted.

Immunoblotting

Cells were lysed in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% NP40 and 1% TX-100, pH 7.4) containing protease and phosphatase inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM orthovanadate, fluoride 50 mM, pyrophosphate 10 mM) by sonication (30 sec; 4°C). After a 10 minute incubation on ice homogenates were centrifuged (15,000 × g, 15 min, 4°C). Proteins were electrophoresed on 12% SDS polyacrylamide gels and transferred onto PVDF membranes.

Membranes were blocked (Tris buffered saline, 0.2% Tween 20, and 5% non fat powdered milk or 5% BSA) prior to incubation in primary antibodies: polyclonal CNP and polyclonal MBP (each at 1:10,000, R. Bansal, Farmington, CT), monoclonal MOG (myelin oligodendrocyte glycoprotein; 1:3,000; C. Linington, Aberdeen, UK) and GAPDH (1:15,000; Biodesign International, Saco, Maine). Following several washes, the membranes were incubated for 30 min in appropriate secondary antibodies conjugated to horseradish peroxidase (1:10,000; Santa Cruz) and developed using the ECL Plus chemiluminescent kit (Amersham, Arlington Heights, IL).

Rapamycin injections and Immunohistochemistry

Starting at postnatal day (P) 6, mice were injected daily with either vehicle or rapamycin intraperitoneally at 10 mg/kg body weight (rapamycin was diluted in the vehicle solution from a stock of 20 mg/ml in 100% ethanol immediately before the injection to a final concentration of 0.8 mg/ml PBS, 5% polyethylene glycol-1000, 5% Tween 80, and 4% ethanol). On P11, brains were removed, drop fixed in 4% paraformaldehyde (overnight at 4°C), cryoprotected in 20% sucrose (overnight at 4°C), embedded in mounting media and sectioned (15 um) coronally. Sections were immersed in 100% ethanol, washed in PBS, blocked for 1hr in 10% normal goat serum, 5% bovine serum albumin, and 0.1% gelatin in PBS and incubated overnight at 4°C in polyclonal anti-rabbit MBP (1:3,000; R. Bansal, Farmington, CT). After three washes in PBS, sections were incubated for 1hr in goat anti-rabbit IgG conjugated to Alexa 488 (1:500; Molecular Probes, Eugene, OR) and Hoechst Blue 33342 (1:1,000), washed in PBS and mounted.

RESULTS

To investigate the role of Erk1/2 and mTOR in the progression of OL-lineage cells through specific stages of differentiation, we evaluated the effects of their pharmacological inhibition by U0126 (Mek inhibitor) or rapamycin (mTOR inhibitor) (Fig. 1B) on the expression of stage-specific antigenic markers and OL morphology. Normal morphology of isolated OL-lineage cells from rat forebrains and key antigenic markers used in this study are shown (Fig. 1A). Early progenitors show a simple morphology and express the transcription factor Olig2 and the lipid antigen A2B5 (Fig. 1Aa). Early progenitors transition into late progenitors that extend additional simple processes and are recognized by the O4 antibody (Fig. 1Ab, Bansal *et al*., 1992). Late progenitors become terminally differentiated immature OLs expressing the lipid galactocerebroside (GalC; recognized by the O1 antibody) and develop more complex branched processes (Fig. 1Ac). Immature OLs also express an unknown antigen HPC7 (Baas and Barnstable, 1998) that co-labels with O1 (data not shown) and can be used interchangeably to mark this stage. Immature OLs soon form a thick ring-like structure and begin to express the myelin protein CNP. We have classified this stage as a more advanced stage of immature OLs (Fig. 1Ac'). Finally, the immature OLs differentiate into mature OLs as they start expressing MBP and later MOG, and the ring-like structure is transformed into an extensive network of thicker processes and membranes (Fig. 1Ad). This multiprocessed morphology is characteristic of neuron-free isolated rat OLs in culture, to be distinguished from the morphology of myelinating OLs *in vivo* or in co-cultures with neurons, where distinct OL processes extend to and wrap the axon. Further, although "mature" OLs in isolation are able to express MBP and PLP and make "myelin-like" membranes, they are not able to make actual myelin or complete their final morphological differentiation as *in vivo*.

Early progenitors purified from mixed primary cultures (McCarthy and DeVellis, 1980) were plated into 4-well or 6-well dishes in defined N2 medium. Following a brief (1-2 day) expansion, PDGF was removed from the media (designated as Day 0) and cells were grown in N2 with or without U0126 or rapamycin, as indicated in each experiment. Note that no

subculture of cells is involved in this culture paradigm. Purity of cultures was 99% as determined by %Olig2+ cells/total Hoescht+ cells and was ~95% as determined by %A2B5+O4 positive cells/total Hoescht+ cells after two days in N2.

Inhibition of Erk1/2 attenuates the progression of early progenitors to late progenitors and concomitantly to immature OLs

In order to examine the requirement of Erk1/2 signaling on the initial stages of the OL developmental pathway, we treated early progenitor cultures with DMSO (control) or with U0126 from 0 to 2 days and immunolabeled cells for O4 and Olig2 (Fig. 2A,B). We found that while >80% of early progenitors (Olig2+/O4-) had progressed to the late progenitor (Olig2+/O4+) stage by day 2, only 20-40% became weakly O4+ late progenitors in U0126-treated cultures (Fig. 2A,B). The effect was more pronounced at 25uM than at 10uM dose. We also investigated the consequence of Erk1/2 inhibition on further progression from the late progenitor stage to the immature OL stage. Cells were treated with U0126 on day 1 (when majority of cells are early progenitors) and analyzed on day 3 for the expression of O1 (Fig. 2C,D). Significantly reduced numbers of O1+ cells were observed in U0126-treated cultures.

We therefore conclude that the transition from the early to the late progenitor stage requires the participation of Erk1/2 activity and that this indirectly affects the appearance of immature OLs.

Inhibition of Erk1/2 does not affect the transition of immature OLs to mature OLs

We next asked whether Erk1/2 signaling is needed for the progression of immature OL (O1+/MBP-) to the mature OL (O1+/ MBP+) stage. We treated cultures with U0126 at day 3 of differentiation (when over 50% of cells were at the immature OL stage) and analyzed cells on day 5 by double labeling with O1 and MBP (Fig. 3). We found that in both DMSO-treated control cultures and U0126-treated cultures, the majority of O1+ immature OLs acquired MBP and elaborated a network of processes characteristic of mature OLs. Therefore, in contrast to its role in early developmental transitions (above), Erk1/2 signaling is not essential for the progression of immature OL to the mature OL stage.

Inhibition of mTOR does not block terminal differentiation of late progenitors to immature OLs

To examine the stage-specific requirements for the PI3K/Akt/mTOR pathway during OL development, we first examined the effect of rapamycin on the transition of early progenitors to late progenitors. Consistent with previous reports (Baron *et al*., 2000; Tyler *et al.*, 2009), we found that rapamycin had no effect on early to late progenitor transition (data not shown). Further, rapamycin-treatment did not have any adverse effects on cell viability over long periods of time. To determine the role of mTOR signaling on the transition of late progenitor to immature OL, we treated cells with rapamycin from day 0 or 1 to day 3 and analyzed by immunolabeling the expression of immature OL markers, O1 (GalC) and HPC7 (Fig. 4). We found no difference in the percent of immature OLs (O1+, HPC7+) that appeared in control and rapamycin-treated cultures. Similarly, no difference was observed even when rapamycin treatment was initiated immediately after plating shaken OL progenitors, without pre-expansion in PDGF (data not shown). Although the numbers of immature OLs was similar, the intensity of O1 and HPC7 immunostaining was somewhat weaker in rapamycin-treated cells than controls, and these OLs did not form a dense ring-like structure like untreated immature OLs (addressed later).

As determined by antigenic marker expression, we conclude that the inhibition of mTOR by rapamycin in our culture conditions does not block terminal differentiation of late progenitors to immature OLs as previously reported (Tyler *et al.*, 2009).

Inhibition of mTOR attenuates the efficient transition of immature OLs to mature OLs

We first investigated the effect of mTOR inhibition by rapamycin on the developmental progression from the O1+ immature OL stage to a slightly later CNP+ immature OL stage as described in Fig. 1A c,c'. We found that CNP was expressed by >80% of O1 positive OLs in both the control and in cultures treated with rapamycin from 0 to 5 days (Fig. 5 A,B). This further demonstrates that mTOR inhibition does not affect differentiation of late progenitors to immature OLs, as they were able to express GalC, and CNP.

We next asked if transition of immature OLs (O1+/MBP-) to mature OLs (O1+/MBP+) was affected by mTOR inhibition (Fig. 5C,D). We found that while in untreated controls approximately 70% of immature OLs expressed MBP by day 5, in the rapamycin treated cultures only about 20% progressed to the MBP+ mature OL stage. Further, the intensity of MBP immuno-reactivity in these OLs was reduced relative to untreated mature OLs.

To complement immunofluorescence microscopy data, we performed immunoblot analysis on parallel cultures to evaluate the effect of rapamycin treatment on myelin protein expression (Fig. 6). Cells grown in the absence or presence of rapamycin for 0-3 and 0-5 days were analyzed for the levels of CNP, MBP and MOG. Consistent with the immunofluorescence results, the expression of CNP was unchanged in cells treated with rapamycin for 3 or 5 days, but the levels of MBP and MOG were significantly reduced. We conclude that mTOR signaling is required for the expression of normal levels of the major myelin proteins by mature OLs.

Inhibition of mTOR adversely affects morphological maturation of immature OLs

As described earlier, cells undergo continuous cytoskeletal alterations prior to acquiring the typical mature OL morphology (Fig. 1A). Notably, the branched processes of immature OL appear to intertwine to form a ring-like structure, which we have classified as a more advanced stage of immature OLs (Fig. 1A c,c'). We found that while in controls this ring-like morphology was evident under both phase contrast microscopy (Fig. 7a) and in O1 immunolabeled cells (Fig 7c), it was rarely seen in rapamycin-treated cultures. Instead, the immature OLs displayed a simpler multiprocess, branched morphology (Fig. 7b,d), characteristic of the early stage of immature OL (Fig. 1A c). Note that these rapamycin-treated immature OLs are antigenically and morphologically distinct from both late progenitors (that do not express O1 and have few unbranched small processes) and mature OLs (that express MBP and have a much more complex network of processes and membranes). We, therefore, conclude that the cytoskeletal organization associated with OL maturation requires the participation of mTOR signaling.

Rapamycin downregulates MBP expression in OLs in dissociated cultures of mouse brain and inhibits the onset of myelination when injected *in vivo*

The studies described so far were conducted on isolated OL progenitor cultures from neonatal rat brains. We next investigated the effects of mTOR inhibition by rapamycin on OLs in the context of a more complex environment of dissociated mouse brain primary cultures where OL development occurs in the presence of signals from other glial cells, mainly astrocytes. With the culture paradigm used, these cultures are virtually devoid of neurons. Cultures were treated with rapamycin from 8-17 days and immunolabeled with anti-MBP (Fig. 8A) and with O4 and HPC7 (data not shown). Consistent with our results in isolated rat OL cultures, we observed a downregulation of MBP immunoreactivity in

rapamycin-treated cultures relative to untreated controls. As for isolated cultures, no inhibition of OL progenitor terminal differentiation to GalC+ stage was observed in dissociated primary cultures (data not shown).

Finally, to further explore the effect of rapamycin inhibition of mTOR on the onset of myelination *in vivo*, we injected mice intraperitoneally daily from P6 to P11 with either vehicle or rapamycin and immunostained brain sections with anti-MBP (Fig. 8B) on P11. Compared to vehicle-injected mice, myelination in the corpus callosum and cingulum of rapamycin-injected mice was reduced. Although this effect of rapamycin on MBP expression and myelination suggests a direct effect on OLs, since it was also seen in isolated OL cultures, the possibility exists that *in vivo* these effects could be secondary as a result of perturbed mTOR signaling in other cells types such as neurons, astrocytes or microglia.

We conclude that inhibition of mTOR attenuates the onset of myelination in the postnatal mouse brain, consistent with its adverse effects on the OL cytoskeleton and the expression of MBP that we observed *in vitro*.

DISCUSSION

Using a panel of stage-specific antigenic markers and pharmacological inhibitors, this study demonstrates that distinct stages in the OL developmental pathway are differentially regulated by Erk1/2 and mTOR signaling *in vitro* (Fig. 9). Specifically, the progression of the early to late progenitor stage requires Erk1/2, but not mTOR signaling; in contrast, the immature OL to mature OL transition requires mTOR, but not Erk1/2. Interestingly, neither of these signaling pathways was found to play a direct role in the terminal differentiation of late progenitors to postmitotic immature OLs. Moreover, activation of mTOR signaling was found to be critical for OL cytoskeletal organization and the expression of normal levels of major myelin protein *in vitro*, and for the onset of myelination in the postnatal mouse brain.

Early progenitors (O4-) and late progenitor (O4+) stages are antigenically, morphologically and functionally distinct maturation stages of OL progenitor that are often grouped as “OPCs”. Transition of early to late progenitors is a critical step in the OL-lineage when cells lose their migratory capacity and become committed to the OL-lineage (Pfeiffer *et al.*, 1993). Our data demonstrates that Erk1/2 regulates this important transition and suggests that, as a consequence, it regulates the appearance of immature OLs. Other studies have also reported a reduction in the appearance of GalC+ immature OLs in their isolated OL progenitor culture systems using pharamacological inhibitors (Baron *et al.*, 2000; Fyffe-Maricich *et al.*, 2011), however the inhibition of O4 expression observed in our study was not reported previously. A possible explanation could be that in one case PD098059 instead of U0126 was used as an Erk inhibitor, and it was added to the cultures when ~50% of the OL-lineage cells were already O4+ (Baron *et al.*, 2000), while in another, only a lower dose of U0126 was used and cells were first analyzed 3 days after treatment (Fyffe-Maricich *et al.*, 2011). Our data showing a requirement of Erk1/2 at early stages of the OL-lineage (perhaps even at the pre-progenitor stage) correlates with *in vivo* findings showing that the genetic disruption of the Erk1/2 in the embryonic brain, using nestin-cre driver, resulted in attenuated OL progenitor generation in the germinal zone and cortex (Imamura *et al*., 2010). Further, our interpretation that Erk1/2 does not play a major role in terminal differentiation of OLs correlates with recent studies on cortical mixed cultures from Erk1-/- or Erk2-single conditional knockout mice (Fyffe-Maricich *et al.*, 2011) and Erk1/2 double knockout mice (unpublished observations), where no reduction or only a partial (25%) transient reduction in the numbers of GalC+ cells was observed in the absence of Erk signaling. Erk1/2 signaling is also dispensable for further lineage progression since immature OLs were able to acquired MBP expression normally in isolated OL-cultures in the presence of U1026 and in the

mixed primary cortical cultures of OL-lineage-specific Erk1/2 conditional knockout-mice (unpublished observations). These results together suggest that while Erk1/2 are important regulators of early progenitor and pre-progenitor stages and mediate regionally specific stimulatory effects of BDNF on myelin protein expression in the basal forebrain OLs (Du *et al*., 2003; Du *et al*., 2006), terminal differentiation and further maturation of cortical OLs are regulated by signals other than Erk1/2. However, it should be noted that these *in vitro* studies do not address if Erk1/2 plays a role in myelin sheath formation and wrapping *per se.*

Transition of late progenitors (O4+/GalC-) to immature OLs (O4+/GalC+) marks a key irreversible event in OL-lineage progression when progenitors become postmitotic and commit to terminal differentiation (Pfeiffer *et al*., 1993; Miller, 2002). Our present results indicate that rapamycin inhibition of mTOR did not affect this critical transition during differentiation, as assessed by the expression of stage-specific antigens. These results are at odds with a recent *in vitro* study that suggests that mTOR is a key regulator that drives the terminal differentiation of late progenitors to GalC+ immature OLs (Tyler *et al*., 2009). The main difference between the two studies is the way OL progenitors were manipulated prior to their exposure to rapamycin. Both studies employed the McCarthy and DeVellis (1980) differential shake-off procedure to obtain early progenitors from the rat brain. While we immediately plated them in defined media following the shake (or after a brief expansion of 1-2 days in PDGF, both with similar results), Tyler and co-workers first expanded the shaken-off OL progenitors in T75 flasks for 4-10 days in N2 media containing 34% B104 media and 5 ng/ml FGF-2, then passaged them before plating for rapamycin-treatment. Prolonged exposure to growth factors and undefined components of B104 and passaging of cells could alter the biology of OL progenitors, as has been observed earlier (Tang *et al.,* 2000; Kondo and Raff, 2000), perhaps reverting them to a more immature progenitor state or slowing down their intrinsic maturation program and response to rapamycin. Thus, it is difficult at this time to definitively conclude whether mTOR regulates OL differentiation at the late progenitor to immature OL transition, as reported earlier (Tyler *et al.,* 2009), or at the immature OL to mature OL stage, as suggested by our present study. If the former were the case (Tyler *et al.,* 2009), one would expect to see decreased numbers of immature OLs (DM20+ premyelinating OLs) upon inhibition of mTOR by rapamycin injection *in vivo*. However, no such decrease in premyelinating OL number was seen in the P7 brains of rapamycin injected mice (Bercury and Macklin, Abstract # PSM01-09 ASN, 2011 and personal communications), suggesting that rapamycin does not block terminal differentiation of late progenitors *in vivo*, supporting the *in vitro* results obtained in our culture system. Despite these differences, both *in vitro* studies are in agreement that rapamycin inhibits the full maturation of MBP+ OLs. To definitively resolve which culture system mimics a true *in vivo* scenario awaits the generation of knockout mice with conditional disruption of mTOR signaling in late progenitors.

Interestingly, we have fortuitously uncovered a role for mTOR signaling in the cytoskeletal maturation of OLs. Specifically, since in our culture system rapamycin did not arrest the progression of differentiation at the early progenitor stage, we were able to see that rapamycin inhibited the characteristic cytoskeletal transformations that occur during the normal progression of the immature OL stages (Fig. 1A c,c'). Consistent with a role of mTOR in cytoskeletal maturation of OLs, recent proteomic analysis found alterations of several cytoskeletal proteins in rapamycin-treated OL cultures (Tyler et al., 2011). In addition, rapamycin treatment also inhibited the expression of major myelin proteins (MBP and MOG) and progression to mature OL stage. Since both the numbers of MBP+ mature OLs and MBP immunofluorescent-intensity per mature OL were reduced, the overall reduction in MBP protein level determined by immunoblotting could be due to a combination of the two factors. The reduced expression of major myelin proteins, accompanied by inhibition of cytoskeletal maturation, could both be mediated through

mTORC1, which is the primary target of rapamycin and is known to regulate protein and lipid synthesis. However, the effect on OL morphology could, in theory, be also mediated through mTORC2 signaling, which is known to regulate cytoskeletal organization and can also be inhibited by prolonged treatment with rapamycin (Zoncu *et al.*, 2011). Thus, mTORC1 and perhaps even mTORC2 signaling enables OLs to acquire the full antigenic and morphological complexity needed for normal myelination.

These *in vitro* observations correlate well with our *in vivo* finding where chronic inhibition of mTOR by daily injections of rapamycin in an early postnatal mouse from P 6-11 resulted in reduced myelination in the brain, demonstrating that mTOR signaling is required at the onset of myelination. The requirement of mTOR continues into the active phase of myelination since reduced myelination was also observed in the brains of mice injected with rapamycin from P21 for three weeks (Narayanan *et al.*, 2009). Moreover, recent studies showing enhanced myelination in transgenic mice with overactive PI3K/Akt/mTOR pathway suggest a role of this pathway in myelin sheath wrapping by OLs, a process requiring major cytoskeletal alterations (Flores *et al*., 2008; Goebbels *et al*., 2010; Harrington *et al*., 2010). Further, rapamycin was shown to reverse this hypermyelination in transgenic mice expressing constitutively active Akt in OLs (Narayanan *et al.*, 2009). Taken together, these findings support our *in vitro* observations suggesting that the PI3K/Akt/mTOR pathway plays a major role in late stages of OL differentiation, especially in the cytoskeletal organization that normally occurs during myelination.

In summary, we demonstrate that, when examined in parallel, there is a sequential and non-overlapping developmental stage-specific requirement of the Ras/Raf/Mek/Erk or the PI3K/Akt/mTOR pathways during OL-lineage progression. Specifically, transition of early progenitors to late progenitors requires Erk1/2 signaling and transition of immature OLs to mature OL requires mTOR signaling. Interestingly, neither pathway directly plays an essential role for the commitment of late progenitors to enter terminal differentiation. This study provides valuable insights about the appropriate timing and nature of the signaling pathways that could serve as useful targets to enhance OL differentiation for remyelination in neurological diseases.

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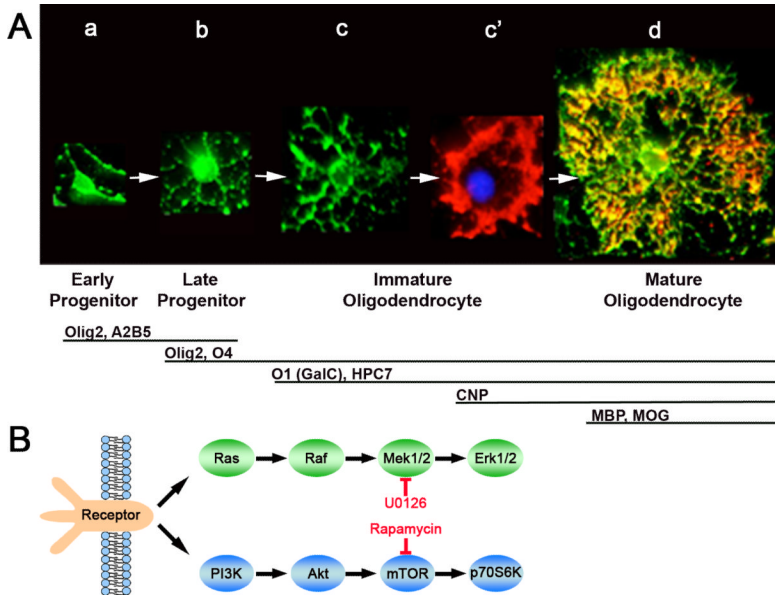

Fig. 1.

A. Immunofluorescence microscopy of enriched OL lineage cells in culture at different stages of the OL developmental pathway. Stage-specific markers and morphology of cells at each stage are shown. **a.** Early progenitor (Olig2+,A2B5+/O4-); **b.** Late progenitor (Olig2+/O4+/GalC-); **c, c'** Two stages of morphological maturation of immature OL (O1(GalC)+/HPC7+/CNP+/MBP-); **d.** Mature OL (O1+/MBP+/MOG+). Note that early progenitors with 1-3 processes (**a**) transition to late progenitor with multiple thin processes (**b**). These late progenitors enter terminal differentiation into immature OLs by elaborating additional more branched processes (**c**), which subsequently intertwine into a transient ring-like structure (**c'**) prior to transforming into an extensive network of membranous processes of mature OLs (**d**). **B.** Simplified diagram of Ras/Raf/Mek/Erk and PI3K/Akt/mTOR signaling pathways showing targets of pharmacological inhibitors, U0126 and rapamycin.

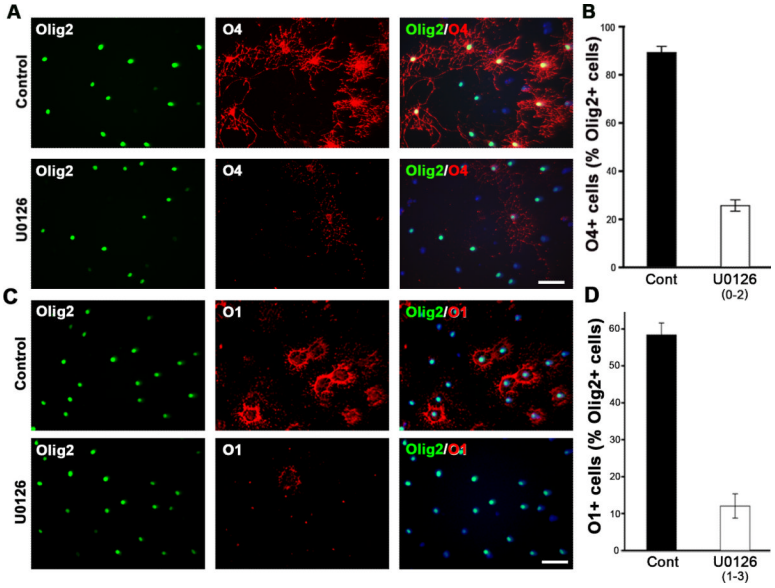

Fig. 2. Inhibition of Erk1/2 attenuates the progression of early progenitors to late progenitors and concomitantly to immature OLs

Early progenitors were grown in the absence (Cont) or presence of U0126 from 0 to 2 days (**A,B**) or 1 to 3 days (**C,D**) and analyzed by double immunolabeling for O4 and Olig2 (**A,B**) or Olig2 and O1 (**C,D**). Quantification of the percentage of late progenitors (O4+/Olig2+) and immature OLs (O1+/Olig2+) shows that inhibition of Erk1/2 signaling significantly attenuates the progression of early progenitors to late progenitors which then results in subsequent reduction in immature OLs. Error bars, SEM, N=3-4 independent cultures in duplicates. Scale bar, 50 um.

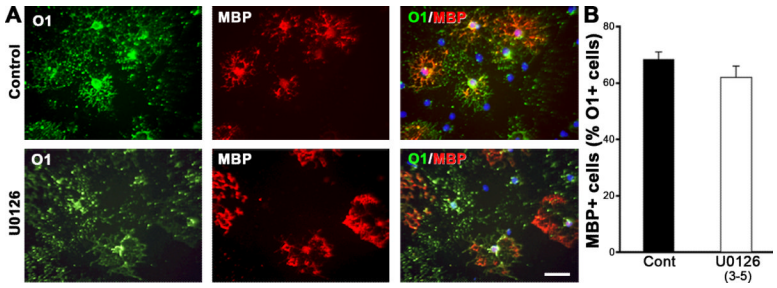

Fig.3. Inhibition of Erk1/2 does not affect the transition of immature OLs to mature OLs

A. Early progenitors were first allowed to differentiate for 3 days in the culture until the majority had differentiated to the immature OL stage. They were then treated with U0126 from day 3 to 5 and analyzed by double immunolabeling for MBP and O1. **B.** Quantification of the percentage of MBP+ mature OLs in DMSO-treated controls (Cont) and in U0126-treated cultures shows that the progression of immature OLs (O1+/MBP-) to mature OLs (O1+/MBP+) remained unaffected by the absence of Erk1/2 signaling. Error bars, SEM, N=3 independent cultures in duplicates. Scale bar, 50 um.

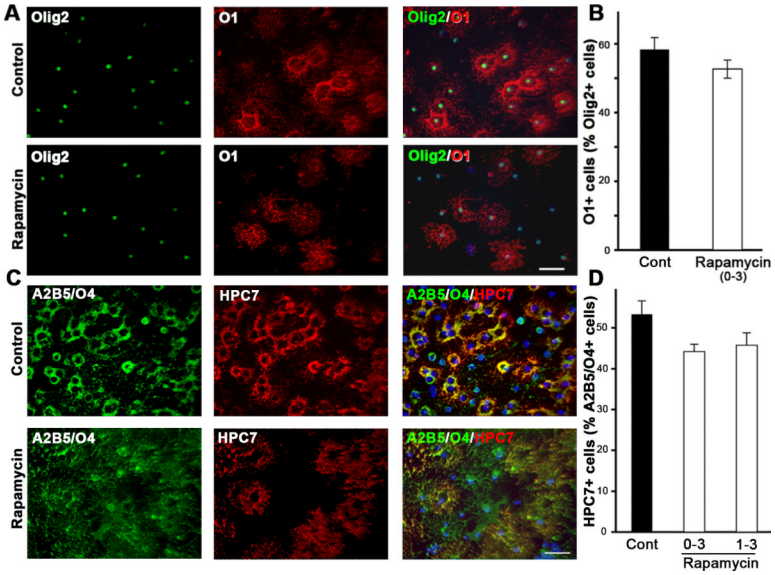

Fig. 4. Inhibition of mTOR does not block terminal differentiation of late progenitors to immature OLs

Early progenitors were grown in the absence (Cont) or presence of rapamycin from 0 to 3 days or 1 to 3 days and analyzed by double immunolabeling for the expression of Olig2 and O1 (**A,B**) or A2B5/O4 and HPC7 (**C,D**). Quantification of the numbers of O1+ or HPC7+ immature OLs, expressed as percentage of total OL-lineage cells (Olig2+ or A2B5+/O4+), shows no difference in rapamycin-treated cultures compared to untreated controls, demonstrating that late progenitors can differentiate normally to immature OLs in the absence of mTOR signaling. Error bars, SEM, N=4 independent cultures in duplicates. Scale bars, 50 um.

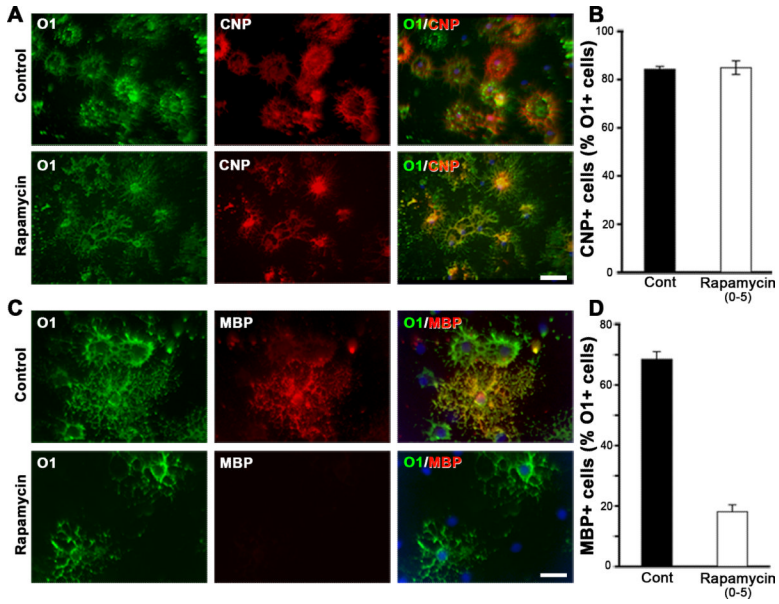

Fig. 5. Inhibition of mTOR attenuates efficient transition of immature OLs to mature OLs

Early progenitors were grown in the absence (Cont) or presence of rapamycin from 0 to 5 days and analyzed by double immunolabeling for O1, and CNP (**A,B**) or O1 and MBP (**C,D**). Quantification of the percentage of O1+ cells that co-express CNP (**B**) or MBP (**D**) shows that while there was no effect of mTOR inhibition on the transition of O1+ immature OLs to CNP+ immature OLs, their progression to MBP+ mature OLs was significantly inhibited. Error bars, SEM, N=4 independent cultures in duplicates. Scale bars, 50 um.

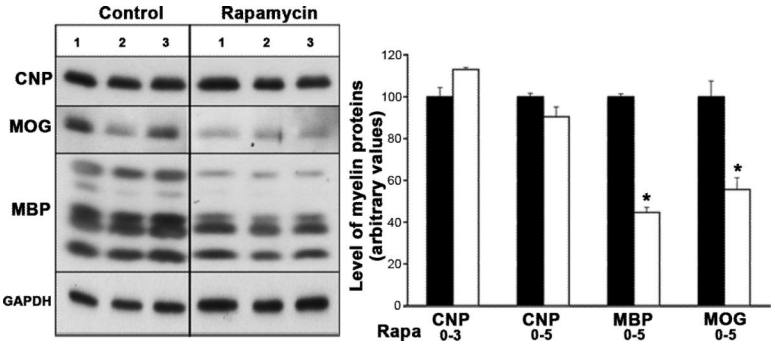

Fig. 6. Inhibition of mTOR does not affect the level of immature OL protein CNP but downregulates mature OL proteins, MBP and MOG

Total protein lysates from cells grown in the absence (solid bars) or presence of rapamycin (open bars) from 0 to 5 days were analyzed by immunoblot analysis for the levels of CNP, MBP and MOG proteins (triplicates are shown). Quantification of the band intensity by NIH image shows that while CNP protein levels (examined also in cells treated from 0 to 3 days) remained unaffected, MBP and MOG levels were significantly reduced in the absence of mTOR signaling. GAPDH was used as a loading control. Error bars, SEM, N=3.

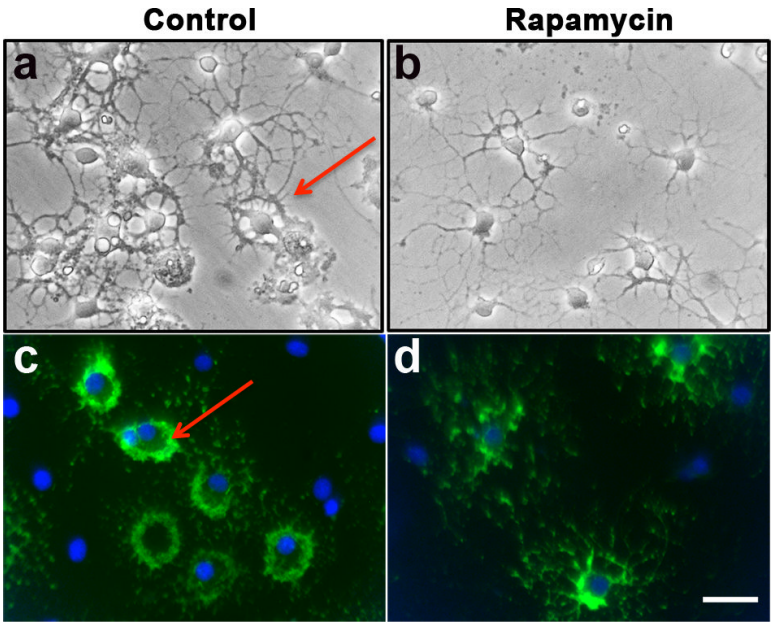

Fig. 7. Inhibition of mTOR adversely affects the morphological maturation of immature OLs

Cells were grown in the absence (Control) or presence of rapamycin from 0 to 3 days and analyzed by phase contrast microscopy (**a,b**) or by immunolabeling with O1 (**c,d**). Note that rapamycin-treated immature OLs failed to acquire the characteristic ring-like morphology with thick intertwined processes like the control immature OLs (arrows). Scale bar, 25 um.

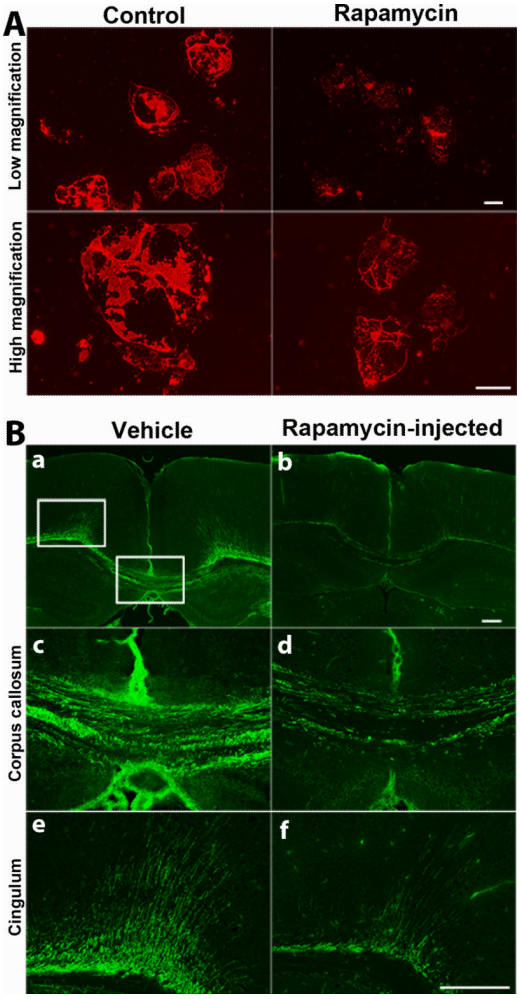

Fig. 8. Rapamycin downregulates MBP expression in mature OLs in dissociated cultures of mouse brain and inhibits the onset of myelination when injected in vivo

A. Dissociated primary cultures of neonatal mouse brains were treated with rapamycin from 8-17 days and immunostained with anti-MBP on day 17. The low magnification images of control and rapamycin treated cultures, taken at the same exposure shows a lower level of MBP immunoreactivity in rapamycin-treated cultures compared to controls. High magnification images of rapamycin-treated cells are shown at twice the exposure as control images to display morphology of OLs more effectively. Representative images from individual cultures of 3 pups are shown. Scale bars, 50 um. **B.** Mice were injected intraperitonially daily from P6 to P11 with either vehicle or rapamycin. Coronal sections from mouse brains were immunostained with anti-MBP on day 11. Representative images out of 3 rapamycin-injected and 3 vehicle-injected mouse brains shows reduced level of myelination in the rapamycin-injected (**b,d,f**) relative to vehicle-injected mice (**a,c,e**). The boxed areas of corpus callosum and cingulum are shown at higher magnification (**c-f**). Scale bars, 200 um.

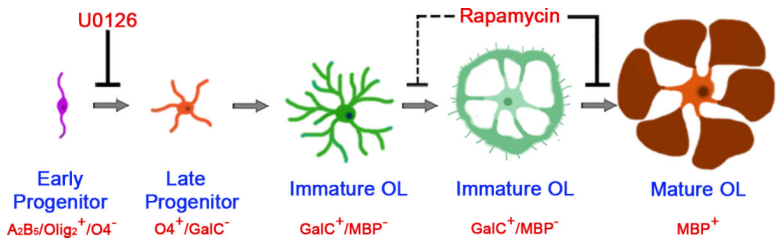

Fig 9. Distinct stages of OL differentiation are sequentially regulated by Erk1/2 and mTOR signaling

Inhibition of Erk1/2 by U0126 interferes with the early progenitor to late progenitor progression (solid line) and subsequently affects the appearance of immature OLs. In contrast, inhibition of mTOR by rapamycin attenuates morphological maturation of immature OLs (dashed line) and their transition to the mature OL stage (solid line). Neither Erk1/2 nor mTOR directly regulates the terminal differentiation of late progenitors to postmitotic immature OLs.