Translational Control of Myelin Basic Protein Expression by ERK2 MAP Kinase Regulates Timely Remyelination in the Adult Brain

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Successful myelin repair in the adult CNS requires the robust and timely production of myelin proteins to generate new myelin sheaths. The underlying regulatory mechanisms and complex molecular basis of myelin regeneration, however, remain poorly understood. Here, we investigate the role of ERK MAP kinase signaling in this process. Conditional deletion of *Erk2* from cells of the oligodendrocyte lineage resulted in delayed remyelination following demyelinating injury to the adult mouse corpus callosum. The delayed repair occurred as a result of a specific deficit in the translation of the major myelin protein, MBP. In the absence of ERK2, activation of the ribosomal protein S6 kinase (p70S6K) and its downstream target, ribosomal protein S6 (S6RP), was impaired at a critical time when premyelinating oligodendrocytes were transitioning to mature cells capable of generating new myelin sheaths. Thus, we have described an important link between the ERK MAP kinase signaling cascade and the translational machinery specifically in remyelinating oligodendrocytes *in vivo*. These results suggest an important role for ERK2 in the translational control of MBP, a myelin protein that appears critical for ensuring the timely generation of new myelin sheaths following demyelinating injury in the adult CNS.

Key words: demyelination; ERK2 MAP kinase; myelin; oligodendrocyte; remyelination

Introduction

Throughout early postnatal development, oligodendrocyte progenitor cells (OPCs) proliferate, migrate, differentiate, and myelinate CNS axons on a predictable schedule (Miller, 2002). The regenerative process of remyelination following demyelinating injury in the adult CNS is thought to proceed through similar mechanisms, although less is understood about the molecular regulation of oligodendrocyte (OL) development in this context (Fancy et al., 2011; Gallo and Deneen, 2014). A more complete appreciation of the relationship between developmental myelination and remyelination will provide a better understanding of human CNS demyelinating disorders such as multiple sclerosis (MS). Remyelination ultimately fails in MS patients, resulting in functional disability as the disease progresses (Prineas and Connell, 1979; Lucchinetti et al., 1996; Patrikios et al., 2006). The reasons for remyelination failure in MS are not completely understood, although a failure in OPC differentiation and/or maturation

has been suggested based on postmortem findings (Wolswijk, 1998; Chang et al., 2000, 2002; Kuhlmann et al., 2008). Efficient and timely remyelination can lead to complete functional recovery (Smith et al., 1979, 1981; Jeffery and Blakemore, 1997; Duncan et al., 2009); therefore, identifying the molecular mechanisms important for successful remyelination is essential for the development of new therapies targeted to restore lost myelin.

Previous studies investigating roles of molecules and signaling pathways important for developmental myelination in the context of remyelination have yielded mixed results. For example, the transcription factor *Olig1* plays important roles during development (Lu et al., 2002; Dai et al., 2015) and is critical during myelin repair (Arnett et al., 2004). Wnt/ β -catenin and Sonic Hedgehog signaling are also important both during development and following demyelination (Orentas et al., 1999; Spassky et al., 2001; Fancy et al., 2009; Ferent et al., 2013). More recent evidence suggests that sustained activation of ERK1 and ERK2 causes significant increases in myelin thickness both during development and following demyelinating injury in the adult mouse spinal cord (Fyffe-Maricich et al., 2013). In contrast, the phosphatase and tensin homolog (PTEN) that regulates PI3K signaling is important for proper myelin thickness and axonal integrity during development but appears dispensable for myelin repair (Harrington et al., 2010).

ERK MAP kinases are critical intracellular molecules that transduce extracellular signals at multiple stages of OL development, and ERK2 plays a specific role in the timing of mouse forebrain myelination during the second postnatal week (Fyffe-Maricich et al., 2011). In the current study, we conditionally de-

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leted *Erk2* from OL-lineage cells to determine whether *Erk2* was also required for timely myelin production following focal demyelination in the adult CNS. We found that ERK2 controls the timing of remyelination not through effects on OPC proliferation, migration, or differentiation, but by directly regulating the efficient translation of the major myelin protein, myelin basic protein (MBP). To explore a potential mechanism for this translational defect, we examined the activation of important components of the translational machinery. We found that *Erk2* conditional knock-out (CKO) OLs were unable to sufficiently activate the ribosomal protein S6 kinase (p70S6K), resulting in decreased phosphorylation and activation of its downstream target, S6 ribosomal protein (S6RP). These results provide important insight into the molecular mechanisms that act downstream of the ERK MAP kinase signaling pathway and point to a critical role for translational control during remyelination.

Materials and Methods

Experimental animals. Heterozygous CNP-Cre mice (Lappe-Siefke et al., 2003) on a mixed 129 C57BL/6 background were interbred with homozygous *Erk2* flox mice (Samuels et al., 2008) to generate CNP-Cre; *Erk2* flox/+ breeders. CNP-Cre; *Erk2* flox/+ mice were then interbred with either *Erk2* flox/flox or *Erk2* flox/+ mice to produce CNP-Cre; *Erk2* flox/flox, referred to as “mutants” or “*Erk2* CKO” mice, and “controls,” which consisted of [WT; *Erk2* flox/flox], [WT; *Erk2* flox/+], and/or [CNP-Cre; +/+] littermates. Age and sex-matched male and female mice were used for all experiments. All mice were kept in microisolation in a pathogen-free environment at the University of Pittsburgh, and all procedures were conducted according to approved Institutional Animal Care and Use Committee guidelines.

Demyelination by lysolecithin injection. All injections were performed on sex-matched pairs of control and mutant adult mice aged 12–16 weeks. Mice were deeply anesthetized and positioned in the stereotaxic frame (Harvard Apparatus). Then 1.5 μ l of 1% L- α -lysophosphatidylcholine (LPC; Sigma) in a 0.9% sodium chloride solution was microinjected at a rate of 8.33 nl/s using a sterile beveled needle and Hamilton syringe. Mice were injected unilaterally into the right corpus callosum using the following stereotaxic coordinates (relative to bregma): 1.1 mm rostral, 1.0 mm lateral, 1.8 mm deep (relative to the surface of the brain). To prevent liquid reflux at the completion of the injection, the needle was left in place for 3 min before removal. The day of injection was considered 0 d post lesion (dpl).

Quantification of lesion size. Lesioned area was determined by staining serial coronal sections through the corpus callosum with luxol fast blue (LFB) to define the borders of the demyelinated area in all three dimensions. 3D-Doctor software (Able Software) was then used to reconstruct each 3D lesion and to quantify the volume. Between three and seven mice were analyzed per genotype at each time point.

Immunolabeling of frozen sections. Mice were perfused in 4% PFA/PBS, postfixed overnight, and cryoprotected in 20% sucrose/PBS. Coronal cryosections (20 μ m) of brain were rinsed in PBS; exposed to antigen retrieval in citrate buffer, pH 6.0, for 10 min; washed with PBS; and then blocked and permeabilized with 0.3% Triton X-100 and 4% goat serum in PBS for 30 min. Sections were incubated with primary antibodies at 4°C overnight, then incubated with secondary antibodies at room temperature for 1 h. Sections were counterstained with DAPI for 3 min. Primary antibodies used were MBP (1:1000; SMI-99; Covance), CC1 (1:100; anti-APC; Calbiochem), Ki67 (1:250; BD Biosciences), OLIG2 (1:250; Millipore), NeuN (1:250; Millipore), GFAP (1:1000; Dako Cytomation), ERK2 (1:250; Santa Cruz Biotechnology), pERK1/2 (rabbit-1:250; Cell Signaling Technology and mouse-1:250; Santa Cruz Biotechnology), IBA1 (1:500; Wako), PLP (1:500; Abcam), MOG (1:250; Santa Cruz Biotechnology), pS6RP Ser240/244 (1:2000; Cell Signaling Technology), anti-pS6RP Ser235/236 (1:1000; Cell Signaling Technology), pmTOR Ser2448 (1:100; Cell Signaling Technology), and pp70S6K Thr421/Ser424 (1:500; Santa Cruz Biotechnology). Alexa Fluor-conjugated IgG (1:500; Invitrogen) was used for secondary antibodies.

Myelin loss was identified using standard LFB histochemical staining (0.1% solution; Electron Microscopy Sciences) and immunostaining for MBP.

Cell counts. For cell number counts in the lesioned tissue, images were acquired from the same area of the lesion ensuring that sections were matched on both a rostrocaudal level as well as mediolateral. LFB and MBP stainings of adjacent sections were used to identify the lesion borders in all dimensions. Cells were counted blindly from at least two sections and from a minimum of three mice per genotype using NIS-Elements software (Nikon Instruments). The same region of the corpus callosum from the contralateral side was analyzed for counts from uninjured tissue. An identical region of interest was created on each image to ensure that an equivalent area of demyelination (or uninjured callosum) was analyzed for each animal. Results are presented as the total number of immunopositive cells per lesion area measured or as the percentage of CC1+ cells that express the protein of interest within the region of interest. NIS-Elements software was also used to determine the percentage of lesioned area with immunopositive cells for Figures 4 and 7. Sections were matched as described above and a binary analysis was performed to calculate the percentage of the area of interest with immunopositive cells.

Fluorescence intensity. NIS-Elements software (Nikon Instruments) was used to determine the sum pERK fluorescence intensity of single CC1+ cells selected from within the lesioned area from *Erk2* CKO and control littermates.

Immunoblotting. Immunoblotting was performed essentially as previously described (Fyffe-Maricich et al., 2011). The lesioned area of corpus callosum was carefully microdissected using a dissecting microscope, homogenized by sonication in RIPA lysis buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Halt protease and phosphatase inhibitor cocktail; Thermo Fisher Scientific) and centrifuged at 10 000 $\times$ g for 10 min at 4°C. An equal amount of total protein lysate was loaded from four mice per genotype onto 4–20% gradient SDS-PAGE gels, transferred to PVDF membranes, and probed overnight at 4°C using the following primary antibodies: pERK1/2 (1:1000; Cell Signaling Technology), GAPDH (1:10,000; Santa Cruz Biotechnology), ERK2 (1:1000; Santa Cruz Biotechnology), pS6RP Ser235/236 (1:1000; Cell Signaling Technology), pS6RP Ser240/244 (1:1000; Cell Signaling Technology), S6RP (1:1000; Cell Signaling Technology), mTOR (1:1500; Cell Signaling Technology), pmTOR Ser2448 (1:1500; Cell Signaling Technology), ERK1 (1:1000; Cell Signaling Technology), p70S6K (1:500; Cell Signaling Technology), pp70S6K Thr389 (1:1000; Cell Signaling Technology), and pp70S6K Thr421/Ser424 (1:1000; Cell Signaling Technology). Quantification was performed using ImageJ software.

In situ hybridization. The *Mbp* probe (GenBank accession number: NM_001025251.2) was generated from adult mouse brain cDNA using primers 5'-ACACACAAGAACTACCCACTACGG-3' and 5'-AGCTAAA TCTGCTGAGGGACAG-3'. The *Mbp* fragment was then subcloned into pGEM-T easy vector (Promega) and linearized by restriction digestion. Digoxigenin-labeled riboprobes were synthesized by *in vitro* transcription with T7 or SP6 polymerase (Roche Applied Science). The *in situ* hybridization procedure was performed as previously described (Domowicz et al., 2008). Briefly, adult mouse brain sections were postfixed with 4% PFA for 15 min, followed by riboprobe incubation overnight at 55°C in hybridization buffer (50% formamide, 5 $\times$ SSC, 1% SDS, 500 μ g/ml tRNA, and 200 μ g/ml heparin). Sense probe hybridization was performed as negative control. Posthybridization washes were performed at 65°C in solution X (50% formamide, 2 $\times$ SSC, and 1% SDS). Samples were then blocked with lamb serum for 1 h and incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Applied Science) for 2 h. Incubation with NBT/BCIP (Roche Applied Science) was used to visualize the RNA duplexes. Select slides were briefly stained with 0.1% cresyl violet acetate for 3 min to visualize cell bodies.

Quantitative RT-PCR. The lesioned area of corpus callosum was carefully microdissected using a dissecting microscope and stored at -80°C until extraction. Total RNA was isolated using TRIzol reagent (Invitrogen) followed by RNeasy clean-up kit (Qiagen). RNA (3 μ g) was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR was performed on an Applied Biosystems Step One Plus Real-Time PCR system. PCR was performed with

TaqMan Gene Expression Probe Mix with the following RNA-specific primers: *Mbp*, Mm01266402_m1; *Plp1*, Mm00456892_m1; *Olig1*, Mm00497537_s1; *Myrf*, Mm01194959_m1; *Gapdh*, Mm99999915_g1 (Life Technologies) and 20 ng of cDNA per reaction. All standard curve reactions and samples were run in triplicate along with no template control reactions for all primer sets. PCR cycling parameters followed manufacturer's recommendations.

Isolation of polysome-bound mRNA. Isolation of polysome-bound mRNA was performed as previously described (del Prete et al., 2007). Briefly, the lesioned area of corpus callosum (~20 μ g) was carefully microdissected at 7 dpl, frozen under liquid nitrogen, and pulverized with mortar and pestle. The sample was lysed with fresh lysis buffer supplemented with RNasin (Promega) and VRC (New England Biolabs) to protect mRNA and the sample was centrifuged briefly to remove nuclei. The supernatant was then supplemented with extraction buffer [0.2 M Tris-HCl, 0.3 M NaCl, 150 μ g/ml cycloheximide (Sigma-Aldrich), 650 μ g/ml heparin (Sigma-Aldrich), 10 mM phenyl-methyl-sulfonyl fluoride (Thermo Fisher Scientific)] to block translation elongation. Mitochondria and membranous debris were then removed via centrifugation. The supernatant was layered onto a 10 ml linear sucrose gradient [15–40% sucrose (w/v), supplemented with cycloheximide (Sigma-Aldrich) and heparin (Sigma-Aldrich)] and centrifuged for 120 min at 38,000 rpm at 4°C with the brake off. Twenty fractions of 550 μ l were collected and digested with proteinase K (Thermo Fisher Scientific). RNAs were recovered by extraction with phenol-chloroform-isoamyl alcohol (Sigma-Aldrich) followed by ethanol precipitation. Absorbance values at 260 nm were determined for each sample. To prevent inhibition of the reverse transcriptase as a result of residual heparin, samples were next precipitated with LiCl (Ambion) before being reverse transcribed. Fractions 1 and 2 were combined for sample 1; fractions 3, 4, and 5 were combined for sample 2; fractions 6–10 combined for sample 3; fractions 11–15 for sample 4; and fractions 16–20 for sample 5. Since the amount of RNA is different in each fraction, potentially resulting in efficiency differences of the reverse transcriptase, the RNA amounts were equalized by adding an appropriate amount of luciferase RNA (Promega) to give a final amount of 500 ng of RNA for each sample for cDNA synthesis. After synthesis of the cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), *Mbp* was amplified from each sample using semiquantitative PCR using the following primers: 5'-ACACACAAGAACTAC CCACTACGG-3' and 5'-AGCTAAATCTGCTGAGGGACAG-3'. Band intensity was quantified using ImageJ software and the amount of *Mbp* in each sample was presented as a percentage of the total *Mbp* for that mouse. $N = 2$ from each genotype.

Statistical analysis. Statistical analysis was performed using the Student's *t* test or by one-way ANOVA for Figure 1 and two-way ANOVA for Figure 7 with Tukey's *post hoc* analysis. Statistical significance was considered for $p < 0.05$.

Results

Increased levels of pERK1/2 are found during active remyelination in the adult mouse corpus callosum

To determine whether ERK MAP kinase signaling plays a role during remyelination, we induced a focal area of demyelination in the corpus callosum of wild-type C57BL/6 adult mice using a

stereotaxic injection of the glial toxin LPC, and then followed the expression of activated ERK1/2 (pERK1/2) throughout the repair process. This particular model of demyelination was chosen because it enables temporal separation of the processes of demyelination and remyelination and the repair process follows a highly predictable time course (Jeffery and Blakemore, 1995; Blakemore and Franklin, 2008). Tissue from the demyelinated area of corpus callosum was microdissected and analyzed by Western blot at 2, 7, 14, and 28 dpl, which are time points corresponding to key steps in the remyelination process: OPC proliferation and migration into the lesioned area, OPC differentiation, and the generation of new myelin sheaths (Nait-Oumesmar et al., 1999). Upregulation of pERK1/2 was first noted at 7 dpl and became statistically significant at 14 and 28 dpl, time points that correspond to when new OLs are generating myelin sheaths in response to the demyelinating injury (Fig. 1*A,B*). Immunolabeling of cells in the lesioned area confirmed significant upregulation of pERK1/2 at both 7 and 14 dpl (Fig. 1*C*). To identify the cell types that responded to the demyelinating injury by activating ERK1/2, double labeling was performed using antibodies directed against NeuN to mark neurons (Fig. 1*D*), GFAP to mark astrocytes (Fig. 1*E*), IBA1 to mark macrophages/microglia (Fig. 1*F*), and CC1 to mark mature OLs (Fig. 1*G,H*). OLs, astrocytes, and macrophages/microglia located within the lesioned area expressed pERK1/2, while neuronal cell bodies in the surrounding cortex did not. These data point to a physiological role for ERK MAP kinase signaling during myelin repair.

Remyelination is delayed when *Erk2* is deleted from OLs

To determine the role of ERK2 during remyelination specifically in cells of the oligodendrocyte lineage, CNP-Cre mice (Lappe-Siefke et al., 2003) were crossed with mice that carry an *Erk2* allele flanked by LoxP sites (Samuels et al., 2008) to create *Erk2* CKO mice that lack *Erk2* in both OPCs and mature OLs. Double immunolabeling of coronal sections from adult corpus callosum with ERK2 and the mature OL marker CC1 confirmed the specific loss of ERK2 in OLs in *Erk2* CKO mice (Fig. 2*A*). We previously showed that CKO of *Erk2* (using either GFAP-Cre or NG2-Cre) results in delayed forebrain myelination that recovers by P21 (Fyffe-Marich et al., 2011). We confirmed normal levels of myelin in uninjured adult CNP-Cre; *Erk2* CKO brain by using the myelin stain LFB and by immunostaining for MBP (Fig. 2*B,C*). To investigate an OL-specific role for ERK MAP kinase signaling in the repair of demyelinated lesions, LPC was injected into the corpus callosum of adult *Erk2* CKO and control littermates to create a focal area of demyelination. Tissue was analyzed at 5, 7, and 21 dpl. Serial sections were stained with LFB and the area of demyelination was identified based on a lack of LFB staining (Fig. 2*D,F,H*, boxed area). At 7 dpl, a striking twofold increase in the relative lesion volume was noted in *Erk2* CKO mice ($n = 7$) compared with control littermates ($n = 5$; $p = 0.004$; Fig. 2*F,G*). Larger lesions at 7 dpl could reflect an increased sensitivity of the mutant mice to LPC resulting in larger initial lesion sizes in response to the toxin. Alternatively, early remyelination may be impaired in *Erk2* CKO mice. To address these questions, 3D-Doctor software was used to reconstruct each lesion and calculate average lesion volumes at each time point during the repair process. The average lesion volume was not significantly different ($p = 0.54$) between control ($n = 4$) and *Erk2* CKO littermates ($n = 3$) at 5 dpl, demonstrating that ERK2 loss does not influence susceptibility to LPC-induced demyelination (Fig. 2*D,E*). Interestingly, by 21 dpl, *Erk2* CKO ($n = 4$) lesion volumes were no longer significantly different ($p = 0.10$) from control littermates

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Figure 1. ERK1/2 is activated in glial cells following LPC-induced demyelinating injury. *A,B*, Western blot analysis and quantification of microdissected tissue from the lesioned area of corpus callosum from WT mice at 2, 7, 14, and 28 dpl. Quantification was performed using ImageJ software ($n = 3$ mice per time point). *C*, Lesioned tissue from corpus callosum was immunostained for pERK1/2 at 2, 7, and 14 dpl. Double labeling at 14 dpl for pERK1/2 with NeuN was used to mark neuronal nuclei (*D*), with GFAP to mark astrocytes (*E*), with IBA1 to mark macrophages and microglia (*F*), and with CC1 to mark mature oligodendrocytes (*G*). *H*, Higher power magnification shows colocalization of pERK1/2 in some, but not all, CC1+ oligodendrocytes at 14 dpl. Dashed lines highlight lesioned area. White arrow points to an oligodendrocyte-expressing pERK1/2 inside the lesion. Values are expressed as $\pm$ SEM, * $p \leq 0.05$, ** $p \leq 0.01$. Scale bars: *C–G*, 100 μ m; *H*, 12.5 μ m.

Figure 2. Remyelination is delayed in the absence of ERK2. **A**, Coronal sections of adult control and *Erk2* CKO brain were colabeled with antibodies against CC1 and ERK2. White arrows highlight ERK2 expression in a string of oligodendrocytes in control mice and a complete lack of ERK2 expression in oligodendrocytes in the *Erk2* CKO mice. DAPI marks cell nuclei in blue. LFB staining (**B**) and MBP staining (**C**) demonstrate normal myelination of the corpus callosum in adult uninjured control and *Erk2* CKO mice. LFB staining and quantification of the volume of demyelinated corpus callosum using serial sections followed by 3D software reconstruction of control and *Erk2* CKO mice at 5 dpl ($n = 4$ controls, $n = 3$ CKO; **D**, **E**), 7 dpl ($n = 5$ controls, $n = 7$ CKO; **F**, **G**), and 21 dpl ($n = 4$ controls, $n = 4$ CKO; **H**, **I**). Black boxes highlight the lesioned area of the callosum. Values are expressed as $\pm$ SEM, $**p = 0.004$. Scale bars: **A**, 50 μ m; **B–D**, **F**, **H**, 1 mm.

Figure 3. The acute response to demyelination is unaffected by loss of ERK2. Immunostaining for Ki67 shows comparable numbers of proliferating cells inside the demyelinated lesion of control (**A**) and *Erk2* CKO mice (**B**) at 5 dpl. Double labeling for Ki67 and OLIG2 demonstrates similar numbers of cycling OPCs in control (**C**) and *Erk2* CKO (**D**) mice. Immunostaining with antibodies against GFAP at 5 (**E**) and 7 dpl (**F**) and IBA1 at 5 (**G**) and 7 dpl (**H**) shows comparable numbers of astrocytes and microglia/macrophages inside the lesioned area at both time points from control and *Erk2* CKO mice. Scale bars: **C, D**, 25 μm; **A, B, E–H**, 100 μm.

($n = 4$), demonstrating that over time OLs in the *Erk2* CKO mice are able to generate new myelin sheaths (Fig. 2*H,I*). Thus, we conclude that in the absence of ERK2, remyelination in the corpus callosum is delayed but not completely inhibited.

The acute response to demyelination is unaffected in *Erk2* CKO mice

Although we observed that the initial lesion sizes were similar, important differences in the acute cellular response of the mutant mice to demyelinating injury could result in the observed delay in remyelination. First, we examined whether OPC proliferation and migration into the lesioned area were affected by the loss of ERK2. Double-labeling of the proliferation marker Ki67 and the OPC marker OLIG2 revealed no significant differences in the percentage of OPCs that were proliferating in *Erk2* CKO and littermate controls (control = $30\% \pm 4$, *Erk2* CKO = $28\% \pm 4$, $p = 0.78$; >100 cells were analyzed per animal, $n = 5$ mice per genotype) at 5 dpl (Fig. 3*A–D*). The number of OLIG2+ OPCs found within the lesioned area was also comparable (control = 170 ± 14 , *Erk2* CKO = 152 ± 24 , $p = 0.53$), suggesting that OPC migration and/or survival were similarly unaffected by the absence of ERK2. Reactive astrocytes can negatively affect remyelination by proliferating to form a glial scar around areas of demyelination (Hammond et al., 2014), therefore, we next examined the number of astrocytes in the lesions. Similar numbers of GFAP+ astrocytes were seen in and around the lesioned area in both mutant and control mice at 5 and 7 dpl (Fig. 3*E,F*). Another major component of the remyelination process that can have both positive and negative effects on myelin regeneration is the innate immune response. Immunostaining of lesioned tissue using anti-IBA1 to mark both macrophages and microglia revealed similar infiltration of immune cells in *Erk2* CKO mice and control littermates at 5 and 7 dpl (Fig. 3*G,H*). These data support the conclusion that the initial or acute response to demyelination is unaffected by the deletion of *Erk2* from OPCs and OLs.

ERK2 CKO mice have a selective deficit in MBP expression early in the myelin repair process

Blocking the expression of pERK1/2 in cell culture hinders the ability of OPCs to transition to mature OLs (Fyffe-Marich et al., 2011; Guardiola-Diaz et al., 2012; Dai et al., 2014). In contrast, *in vivo* exper-

Figure 4. ERK2 is important for the translation of MBP but is dispensable for its transcription during remyelination. Immunostaining with antibodies against PLP (**A**), MBP (**B**), and MOG (**C**) demonstrates a selective deficit in MBP expression in the lesion area of corpus callosum from *Erk2* CKO mice at 7 dpl compared with control littermates. **D**, Colabeling with antibodies against OLIG2 and MBP along the lesion edge shows appropriate localization of MBP to the OL processes in both control and *Erk2* CKO mice. **E**, Quantification confirms a significant decrease in the percentage of the lesion area containing MBP immunopositivity in the *Erk2* CKO mice, while no significant differences between control ($n = 4$) and mutant ($n = 3$) mice were found for PLP or MOG. **F**, Immunostaining demonstrates similar numbers of CC1+ OLs in lesioned corpus callosum from control and *Erk2* CKO mice suggesting that OPC differentiation is unaffected. **G**, *In situ* hybridization shows comparable levels of *Mbp* mRNA from control and *Erk2* CKO mice at 7 dpl. **H**, Quantification demonstrates no significant difference in the percentage of the lesion area containing *Mbp* mRNA between *Erk2* CKO ($n = 3$) and controls ($n = 3$). **I**, High-resolution imaging of *in situ* hybridization for *Mbp* with cresyl violet counterstaining shows proper *Mbp* mRNA localization in *Erk2* CKO mice. **J**, Quantitative RT-PCR confirms comparable levels of *Mbp*, *Plp*, *Mrf*, and *Olig1* mRNA in microdissected lesions from *Erk2* CKO and control littermates. **K**, Representative graph showing (Figure legend continues)

iments have shown that genetic deletion of both *Erk1* and *Erk2* does not affect OPC differentiation during postnatal development (Ishii et al., 2012). To investigate whether a delay in OPC differentiation in *Erk2* CKO mice following demyelinating injury might account for the observed delay in remyelination, we used antibodies against CC1 to mark the cell bodies of mature OLs at the site of demyelination at 7 dpl in *Erk2* CKO and control littermates (Fig. 4F). We saw no significant difference in the number of CC1+ mature OLs (control = 60 ± 7 , *Erk2* CKO = 51 ± 4 , $p = 0.34$) found within the lesioned area, suggesting that ERK2 is dispensable for OPC differentiation during remyelination in adult mice *in vivo*.

Efficient remyelination depends not only on the proliferation, migration, and differentiation of OPCs to mature OLs, but also requires the expression and targeting of myelin proteins to OL processes. To further probe the cause of increased lesion sizes in *Erk2* CKO mice at 7 dpl, we investigated the expression of several myelin proteins. Proteolipid protein (PLP) and MBP are the most abundant myelin proteins in CNS myelin sheaths, while myelin oligodendrocyte glycoprotein (MOG) represents a minor component. Immunostaining of lesioned tissue demonstrated that PLP and MOG were expressed at comparable levels between control and mutant mice (Fig. 4A,C). In contrast, MBP was clearly visible throughout all but the lesion core in control mice, while *Erk2* CKO mice showed significantly decreased MBP expression in the lesion at this time point (Fig. 4B). Double immunolabeling with antibodies against OLIG2 and MBP revealed that MBP was appropriately localized to the OL processes in the small number of OLs found to express MBP along the lesion border in the *Erk2* CKO mice (Fig. 4D). Quantification of the percentage of lesion area with immunopositive staining confirmed a significant reduction in MBP expression in *Erk2* CKO mice (control = $24.5\% \pm 3.3$, *Erk2* CKO = $5.0\% \pm 2.0$, $p = 0.005$; Fig. 4E).

Loss of ERK2 affects the translation but not the transcription of MBP following demyelination

We previously showed that OLs lacking both *Erk1* and *Erk2* have significantly reduced levels of *Mbp* and *Plp* mRNAs, resulting in hypomyelination during postnatal development (Ishii et al., 2012). To determine whether the decreased expression of MBP seen in *Erk2* CKO lesions was the result of a similar defect in transcription, we performed *in situ* hybridization to analyze *Mbp* mRNA expression throughout the lesioned area of corpus callosum (Fig. 4G). Quantification of the percentage of the lesioned area with *Mbp* mRNA expression revealed no significant differences between control and *Erk2* CKO mice at 7 dpl (control = $55.2\% \pm 8.5$, *Erk2* CKO = $61.9\% \pm 1.6$, $p = 0.51$; Fig. 4H). Counterstaining of the *in situ* slides with cresyl violet enabled the visualization of *Mbp* mRNA localization in relation to the cell body (Fig. 4I). The distribution of *Mbp* mRNA appeared similar in control and *Erk2* CKO mice. Quantitative RT-PCR analysis using mRNA isolated from microdissected tissue from the lesioned area of the corpus callosum confirmed that *Mbp* mRNA levels were not significantly different between *Erk2* CKO mice

and control littermates at 7 dpl (Fig. 4J). These data demonstrate that ERK2 is dispensable for the transcription and proper localization of *Mbp* mRNA. Finally, mRNA levels of *Plp* and two transcription factors important for late-stage OL differentiation/myelin initiation (myelin gene regulatory factor; *Myrf*) and for repair of demyelinated lesions (oligodendrocyte transcription factor 1, *Olig1*) were also found to be similar in control and *Erk2* CKO mice at 7 dpl (Fig. 4J). These data show that loss of *Erk2*, when *Erk1* remains functional, does not appear to have a major impact on transcription.

Low levels of MBP protein at 7 dpl in the *Erk2* CKO mice could occur as a result of a defect in protein stability or due to an inhibition in translation. When a given mRNA transcript is to be translated, the large 60S ribosomal subunit joins the small 40S subunit and an 80S translational-competent monosome is formed. Since more than one 80S monosome can translate an mRNA at the same time resulting in the formation of polysomes, the number of polysomes on an mRNA can be used as a measure of the translatability of a particular transcript (Warner et al., 1963; Mašek et al., 2011). To compare the amount of MBP translation in *Erk2* CKO and control littermates at 7 dpl, we used velocity sedimentation to separate *Mbp* mRNA associated with monosomes (relatively low mass) from translationally active polysomes (relatively high mass; del Prete et al., 2007). The lesioned area of the corpus callosum was microdissected from *Erk2* CKO and control littermates and cells were exposed to cycloheximide to block translation elongation by stalling or “freezing” 80S ribosomes on mRNAs. Velocity sedimentation of cellular extracts in sucrose gradients separated monosomes (which segregate in early fractions) from polysomes (found in late fractions) and RNA was purified from each of the 20 fractions that were collected. Absorbance at 260 nm values was plotted for each fraction allowing visualization of peaks corresponding to RNA associated with monosomes compared with polysomes (Fig. 4K). Fractions were then combined to create five samples for each genotype and semiquantitative RT-PCR was used to determine the percentage of the total amount of *Mbp* mRNA that was present in each sample. The largest percentage of *Mbp* transcripts from the control mice were found in the heavy sucrose fractions (samples 4 and 5) suggesting that they were likely bound to a large number of ribosomes. In contrast, a significantly higher percentage of the total *Mbp* mRNA was present in the early fractions (samples 1 and 2) from the *Erk2* CKO mice compared with controls [Sample 1 (control = $5.64\% \pm 1.4$, *Erk2* CKO = $20.34\% \pm 5.4$, $p = 0.008$), Sample 2 (control = $10.52\% \pm 1.58$, *Erk2* CKO = $23.45\% \pm 1.43$, $p = 0.02$)] demonstrating a reduced association with ribosomes indicating inhibited MBP translation in the absence of ERK2 (Fig. 4L). These data suggest that the critical role for ERK2 in remyelination is to allow the efficient and timely translation of MBP.

ERK2 regulates the activation of S6 ribosomal protein

Next, we sought to determine a potential mechanism for the translational inhibition observed in *Erk2* CKO mice during myelin repair. The ribosomal protein S6 (S6RP) is one of 30 distinct ribosomal proteins which, together with 18S rRNA, constitute the smaller 40S ribosomal subunit (Chan and Wool, 1988). S6RP plays a critical role in ribosome function and protein translation and is a known downstream target of both the PI3K/Akt/mTOR and MEK/ERK pathways in several different cell types (Shi et al., 2002; Lehman et al., 2003). To assess whether the activation of ERK2 is required for S6RP phosphorylation specifically in OLs in the context of myelin repair, we quantified the expression of

←
(Figure legend continues.) absorbance at 260 nm from fractions collected following sucrose density fractionation. Boxed areas show the fractions combined to generate the five samples used for RT-PCR. L, Semiquantitative RT-PCR demonstrates that a significantly lower percentage of the total *Mbp* mRNA was present in sample 5 from *Erk2* CKO mice compared with control littermates, while a significantly higher percentage of the total *Mbp* was present in samples 1 and 2. Dashed lines highlight the lesioned area of the corpus callosum. Values are expressed as $\pm$ SEM, * $p \leq 0.05$, ** $p \leq 0.01$. Scale bars: D, 50 μ m, I, 20 μ m, A–C, F, G, 100 μ m.

Figure 5. Oligodendrocyte-specific loss of *Erk2* leads to decreased expression of pS6RP during remyelination. Western blot analysis of microdissected lesioned tissue at 7 dpl (**A**) followed by quantification ($n = 4$ of each genotype; **B**) demonstrates a significant increase in pERK1 relative to total ERK1 in *Erk2* CKO mice compared with littermate controls. **C**, Immunostaining using antibodies against pS6RP at Ser240/244 [pS6RP(240)] and CC1 to mark OLs followed by quantification of the number of double-labeled cells inside the lesioned area (**D**) demonstrated a significant decrease in the number of double-labeled cells in *Erk2* CKO mice ($n = 6$) compared with control littermates ($n = 6$) at 7 dpl. **E**, Colabeling of pmTOR at Ser2448 and CC1 to mark OLs followed by quantification (**F**) showed similar numbers of double-labeled cells in *Erk2* CKO lesions compared with control littermates at 7 dpl. White arrows highlight double-labeled cells. DAPI marks cell nuclei in blue. Values are expressed as $\pm$ SEM, * $p \leq 0.05$, *** $p \leq 0.0001$. Scale bars: **C**, 100 μ m; **E**, 50 μ m.

pS6RP by Western blot using microdissected lesioned tissue at 7 dpl in controls and *Erk2* CKO mice. No significant differences were found in the phosphorylation of S6RP relative to total S6RP at Serine 235/236 [pS6RP(235) (control = 1.21 ± 0.10 , *Erk2* CKO = 1.39 ± 0.23 , $p = 0.49$)]]; however, we did note decreased phosphorylation of S6RP at Serine 240/244 [pS6RP(240) (control = 1.58 ± 0.15 , *Erk2* CKO = 1.24 ± 0.12)] although these results were subtle and did not reach statistical significance ($p =$

0.12; Fig. 5A,B). Since pS6RP is expressed in many cell types within the lesion, it is possible that changes in S6RP phosphorylation occurring specifically in OLs (the only cells that lack ERK2 in this experiment) might go undetected. To address this possibility, we colabeled lesioned tissue with antibodies directed against pS6RP and CC1. We observed a significant decrease in the number of OLs within the lesioned area expressing pS6RP(240) (control = 24 ± 1.02 , *Erk2* CKO = 10 ± 0.79 , $p = 9.63 \times 10^{-7}$;

$n = 6$ mice per genotype; Fig. 5C,D). Only one or two pS6RP(235)+ CC1+ cells were consistently observed within the lesioned area from either control or *Erk2* CKO mice, suggesting that phosphorylation at this particular site may be quite transient or may play a more minor role in oligodendrocytes at this time point post injury. Since S6RP is a well known target of the mechanistic target of rapamycin (mTOR), we next sought to determine whether decreased mTOR activation might occur in the *Erk2* CKO mice due to cross talk between the two signaling pathways. No significant decreases in mTOR expression or activation (pmTOR Ser2448) were found by Western blot in the *Erk2* CKO mice at 7 dpl (Fig. 5A,B). Double immunostaining of tissue sections with antibodies directed against pmTOR(2448) and CC1 confirmed these findings. Similar numbers of pmTOR+ OLs were found inside the lesioned area from control and *Erk2* CKO mice at 7 dpl (control = 13 ± 4.24 , *Erk2* CKO = 13.5 ± 2.12 , $p = 0.899$; Fig. 5E,F). Although these data show that the deletion of *Erk2* does not directly affect the phosphorylation of mTOR itself, it remains possible that the loss of ERK2 negatively impacts mTOR signaling through effects on the activation of other components of the mTORC1 complex. In striking contrast to the decreases found in pS6RP(240), a 3.4-fold increase in activated ERK1 (pERK1) was present at 7 dpl in *Erk2* CKO mice (control = 0.58 ± 0.03 , *Erk2* CKO = 1.98 ± 0.28 , $p = 0.03$; Fig. 5A,B). The defect in S6RP phosphorylation at 7 dpl, despite a large increase in pERK1 in *Erk2* CKO mice, suggests an isoform-specific role for ERK2 in the control of protein translation during this early stage of remyelination.

ERK2 is required for activation of the S6RP upstream kinase p70S6K

We next sought to determine how ERK2 might regulate the activation of S6RP. Two main classes of protein kinases phosphorylate S6RP: the p70 S6 ribosomal protein kinases (p70S6K1/2) and the p90 ribosomal S6 kinase (RSK) family (Hauge and Frödin, 2006; Wood et al., 2013). To enhance protein translation, activated p70S6K (pp70S6K) phosphorylates S6RP at five Serine residues (Ser 235/236/240/244/247), while RSK phosphorylates S6RP exclusively at Ser235/236 (Ferrari et al., 1991; Pende et al., 2004; Roux et al., 2007). The Akt/mTOR pathway is classically thought of as the regulator of p70S6K activation in many cell types including OLs, while the MEK/ERK pathway activates RSK (Wood et al., 2013; Lebrun-Julien et al., 2014). Importantly, the MEK/ERK pathway has also been shown to regulate p70S6K in both cancer and hematopoietic cells (Lehman et al., 2003; Guégan et al., 2014). Because we found very few pS6RP(235)+, CC1+ double-labeled cells in both *Erk2* CKO and control mice, suggesting minimal involvement of RSK at 7 dpl, and we saw significantly decreased pS6RP(240) expression specifically in *Erk2* CKO mice, we hypothesized that ERK2 is required for the activation of p70S6K in OLs at the onset of remyelination. p70S6K requires phosphorylation at multiple sites to achieve full activation. Phosphorylation at Thr421 and Ser424 is thought to activate p70S6K via relief of pseudosubstrate suppression, while phosphorylation at Thr389 within the linker domain most closely correlates with p70S6K activity *in vivo* (Pullen and Thomas, 1997; Weng et al., 1998). The MEK/ERK pathway has been shown to phosphorylate p70S6K at both Thr421/Ser424 and Thr389 in other cell types (Shi et al., 2002). Western blot analysis of microdissected lesioned tissue from the corpus callosum at 7 dpl demonstrated a significant decrease in the phosphorylation of p70S6K at Thr389 and Thr421/Ser424 in *Erk2* CKO mice (Fig. 6A,B). Double labeling for pp70S6K Thr421/Ser424 [pp70S6K(421)] and CC1

followed by cell counts confirmed a significant decrease in pp70S6K(421) in OLs (control = 42 ± 1.5 , *Erk2* CKO = 21 ± 4.7 , $p = 0.037$; Fig. 6C,D). These data demonstrate that loss of signaling through ERK2 in OLs leads to an overall decrease in the activation of p70S6K during remyelination.

Recovery of p70S6K and S6RP phosphorylation correlates with the normalization of MBP expression at 14 dpl in *Erk2* CKO mice

LFB staining and lesion size quantification suggested that remyelination in *Erk2* CKO mice recovered by 21 dpl (Fig. 2H,I), so we next examined whether restoration of MBP expression a week earlier at 14 dpl might account for the eventual recovery of myelin repair. Immunostaining of lesioned tissue followed by quantification revealed that MBP expression was comparable between *Erk2* CKO mice and littermate controls at 14 dpl (Fig. 7A,B). Next, we sought to determine whether the recovery in MBP expression might correlate with an upregulation in phosphorylation of S6RP and p70S6K in the *Erk2* CKO mice, suggesting that over time compensation from another pathway may allow for sufficient activation of the translational machinery to release the block in MBP translation. Sections were colabeled with antibodies directed against pS6RP(240) or pp70S6K(421) and CC1 and double-labeled cells were counted from the area of the lesion. Uninjured tissue from the contralateral side of the corpus callosum was similarly analyzed to determine whether baseline differences in the phosphorylation of these proteins exist as a result of the *Erk2* deletion. No significant difference in the percentage of CC1+ OLs expressing pp70S6K(421) (control = $89.6\% \pm 3.7$, *Erk2* CKO = $75.8\% \pm 2.9$, $p = 0.18$) remained in the lesioned area of *Erk2* CKO mice at 14 dpl (Fig. 7C,D). Similarly, no significant differences were observed between *Erk2* CKO and control littermates in uninjured tissue (control = $25.4\% \pm 2.2$, *Erk2* CKO = $24.8\% \pm 2.8$, $p = 0.99$). Importantly, pp70S6K(421) expression was dramatically increased in the *Erk2* CKO at 14 dpl (*Erk2* CKO = $75.8\% \pm 2.9$) compared with 7 dpl (*Erk2* CKO = $21.9\% \pm 3.0$, $p < 0.0001$) suggesting that this increased level of activation may allow for adequate phosphorylation of downstream targets. In support of this conclusion, no significant differences were found in the percentage of CC1+ OLs expressing pS6RP(240) in lesioned tissue at 14 dpl (control = $20.5\% \pm 1.9$, *Erk2* CKO = $17.2\% \pm 1.0$, $p = 0.72$) and pS6RP(240) expression was significantly increased in *Erk2* CKO mice at 14 dpl (*Erk2* CKO = $17.2\% \pm 1.0$) compared with 7 dpl (*Erk2* CKO = $7.4\% \pm 1.7$, $p = 0.004$; Fig. 7E,F). Similar to pp70S6K(421), analysis of uninjured tissue did not detect any baseline differences in pS6RP(240) between control and *Erk2* CKO mice suggesting that the role for ERK2 in the phosphorylation of these proteins may be specific to either the injured environment or to an environment where the myelination program has been activated.

Since the AKT/mTOR pathway is a known regulator of p70S6K, we next looked to see whether the dramatic recovery in p70S6K phosphorylation in the *Erk2* CKO mice at 14 dpl might occur as a result of compensation through increased levels of pmTOR. Tissue sections were colabeled with antibodies directed against pmTOR and CC1 and double-labeled cells were counted. No significant differences were found in the percentage of CC1+ OLs expressing pmTOR in lesioned tissue from control and *Erk2* CKO mice at 14 dpl (control = $28.8\% \pm 4.1$, *Erk2* CKO = $30.9\% \pm 5.7$, $p = 0.78$).

In contrast to both p70S6K and S6RP, baseline differences in pERK1 expression were detected between control and *Erk2* CKO mice. Since the phospho-specific antibody recognizes the same

Figure 6. ERK2 is necessary for the complete activation of the ribosomal p70S6 kinase in OLs at a critical time point during myelin repair. Western blot analysis of microdissected lesioned tissue from *Erk2* CKO and control littermates at 7 dpl (**A**) followed by quantification (**B**) demonstrates a significant decrease in the phosphorylation of the p70S6K at Thr389 and Thr421/Ser424 relative to total p70S6K in *Erk2* CKO mice. **C**, Immunostaining of lesioned tissue at 7 dpl followed by cell counts (**D**) demonstrates a significant decrease in the number of CC1 and pp70S6K Thr421/Ser424 colabeled cells in the *Erk2* CKO mice. Dashed lines highlight cells along the border of the lesioned area. **E**, A schematic representation of our working model showing convergence of Akt/mTOR and MEK/ERK pathways to regulate the onset of remyelination. Values are expressed as $\pm$ SEM, $*p \leq 0.05$. Scale bar, 100 μ m.

site on both ERK1 and ERK2, we were not able to examine individual expression levels for these two phosphorylated proteins in the control mice. In the *Erk2* CKO mice, however, OLs lack ERK2 so the pERK1/2 antibody will give a readout solely of pERK1 expression. Semiquantitative analysis of the fluorescence intensity in individual CC1+ cells demonstrated significant upregulation of pERK in both control and *Erk2* CKO mice at 7 dpl

compared with uninjured tissue (Fig. 7H). Equivalent total pERK expression was seen in mutant and control mice at all time points (uninjured, $p = 0.98$; 7 dpl, $p = 0.95$; 14 dpl, $p = 0.80$; Fig. 7H). These data show that the level of pERK1 expression alone in *Erk2* CKO mice is comparable to the total combined intensity of pERK1 and pERK2 in OLs from control mice, providing strong support for a specific role for pERK2 in the control of MBP trans-

Figure 7. Increased phosphorylation of p70S6K and S6RP and decreased levels of pERK1 correlate with the recovery of MBP expression at 14 dpl in *Erk2* CKO mice. **A**, Immunostaining for MBP at 14 dpl demonstrates recovery in MBP protein expression in *Erk2* CKO mice. **B**, Quantification confirms no significant difference in the percentage of the lesion area containing MBP immunopositivity in the *Erk2* CKO mice compared with control littermates at 14 dpl. **C**, Immunostaining of lesioned tissue using antibodies against pp70S6K(421) and CC1 at 14 dpl. **D**, Quantification of the percentage of CC1+ OLs expressing pp70S6K(421) in *Erk2* CKO and controls in uninjured tissue at 7 and at 14 dpl. **E**, Colabeling of lesioned tissue at 14 dpl for CC1 and pS6RP(240). **F**, Quantification shows no significant difference in the percentage of CC1+ OLs that express pS6RP(240) in the *Erk2* CKO mice compared with control littermates in uninjured tissue. The percentage of CC1+ OLs expressing pS6RP(240) was significantly decreased in *Erk2* CKO mice at 7 dpl but reached control levels by 14 dpl. **G**, Immunostaining of lesioned tissue from *Erk2* CKO mice using antibodies against pERK1/2 and CC1 demonstrates a robust decrease in pERK1 expression specifically in CC1+ cells (white arrows) between 7 and 14 dpl. **H**, Quantification of pERK fluorescence intensity confirms comparable levels of total pERK in control and *Erk2* CKO mice at all time points analyzed despite deletion of the ERK2 isoform suggesting a massive upregulation of pERK1. Dashed lines highlight lesioned area. DAPI marks cell nuclei in blue. White arrows highlight double-labeled cells. Values are expressed as $\pm$ SEM, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale bars: **A**, **E**, 100 μ m; **C**, **G**, 50 μ m.

lation that cannot be compensated for by pERK1. Furthermore, since the expression of pERK1 drops significantly in *Erk2* CKO OLs between 7 and 14 dpl ($p = 0.009$; Fig. 7*G,H*), correlating with the recovery of S6RP(240) phosphorylation and MBP protein expression, high levels of pERK1 in the absence of ERK2 may in fact be detrimental.

Discussion

Our data demonstrate a critical role for ERK2 in the translational control of MBP, a myelin protein critical for the production of myelin sheaths, during remyelination in the adult brain. Previous studies have implicated the ERK pathway in myelination both *in vitro* and *in vivo* during development (Bhat and Zhang, 1996; Du et al., 2006; Galabova-Kovacs et al., 2008; Younes-Rapozo et al., 2009; Fyffe-Maricich et al., 2011; Furusho et al., 2012; Guardiola-Diaz et al., 2012; Ishii et al., 2012, 2013; J. Xiao et al., 2012; Dai et al., 2014) and following demyelination (Sun et al., 2012; L. Xiao et al., 2012; Fyffe-Maricich et al., 2013); however, the molecular mechanisms and downstream targets have remained poorly defined. Here, we show that loss of *Erk2* in adult OPCs leads to a delay in the expression of MBP following demyelinating injury to the corpus callosum, similar to its role in developmental myelination. We also provide evidence for a downstream mechanism involving p70S6K and S6RP activation that is necessary for the timely translation of MBP, but appears dispensable for the expression of other myelin proteins. It is particularly intriguing that early remyelination was inhibited simply due to lack of MBP protein. This is consistent, however, with previous myelin gene deletion mouse mutants: deletion of MAG, PLP, or CNP has very little impact on the production of myelin itself, although long-term detrimental effects on axonal function clearly occur (Li et al., 1994; Griffiths et al., 1998; Lappe-Siefke et al., 2003). In contrast, *shiverer* mice, which have a deletion in the MBP gene, produce essentially no myelin, illuminating the critical nature of this specific myelin protein (Roach et al., 1985; Molineaux et al., 1986).

Recently, sustained activation of ERK MAP kinase signaling in Schwann cells (the myelinating cells of the peripheral nervous system) was shown to result in minor changes in transcript levels while large-scale changes in protein production led to massive hypermyelination (Sheean et al., 2014). Here, we show for the first time that ERK2 exerts translational control of MBP production following demyelinating injury in the CNS in the absence of changes in mRNA levels. Unlike most myelin protein mRNAs, *Mbp* mRNA is transported within RNA granules that contain all of the necessary molecules for its translation from the nucleus to the distal OL processes, where it is translated locally at the axon-glial contact site (Colman et al., 1982; Barbaresi et al., 1995). Myelin-associated oligodendrocyte basic protein, carbonic anhydrase II, tau, and APP mRNAs are also localized to distal OL processes (Ghandour and Skoff, 1991; LoPresti et al., 1995; Holz et al., 1996; Garcia-Ladona et al., 1997). It is conceivable that ERK2 regulates the translation of multiple locally translated mRNAs in OLs, thus promoting their rapid and efficient translation in the setting of remyelination; future studies will directly address this question. In support of this hypothesis, ERK signaling has been shown to regulate local protein synthesis in response to neuronal activity in hippocampal neurons; a role that is critical for long-lasting forms of synaptic plasticity and memory (Keller et al., 2004).

In the developing CNS, mTOR inhibition causes decreased MBP expression along with decreased pS6RP (Tyler et al., 2009; Bercury et al., 2014; Dai et al., 2014; Lebrun-Julien et al., 2014; Wahl et al., 2014). Interestingly, MBP is the only myelin protein

for which a specific translational defect occurs following conditional deletion of the mTORC1 complex in OLs (Bercury et al., 2014; Lebrun-Julien et al., 2014). Several studies have proposed working models depicting possible signaling mechanisms involving both Akt/mTOR and ERK1/2 signaling pathways to control myelination (Bibollet-Bahena and Almazan, 2009; Bercury et al., 2014; Dai et al., 2014; Wahl et al., 2014). We propose a model where the Akt/mTOR and MEK/ERK pathways converge at the level of p70S6K activation, with each pathway independently sufficient for a basal level of p70S6K activation and subsequent MBP translation (Fig. 6*E*). However, maximal efficiency and thus the appropriate timing of MBP expression during remyelination require a critical threshold that is achieved only through coordinated activation of both pathways. In the absence of ERK2, sufficient activation of p70S6K is eventually reached as a result of phosphorylation by other kinases, enabling the translation of MBP and remyelination to occur after a short delay. Our results illuminate a potential molecular mechanism through which ERK2 MAP kinase signaling regulates the onset of remyelination following demyelinating injury. In support of our model, both ERK and PI3K/Akt/mTOR pathways are required for optimal p70S6K activity in cancer and hematopoietic cells, where phosphorylation of p70S6K at Thr389 appears to be regulated by both ERK and mTOR pathways while phosphorylation at Thr421/Ser424 appears to be modulated by ERK MAP kinase signaling alone (Shi et al., 2002; Lehman et al., 2003; Guégan et al., 2014). It has been proposed that phosphorylation at Thr421/Ser424 by ERK1/2 allows access to Thr389 by other kinases such as mTOR, resulting in optimal p70S6K activity and providing a mechanism where both Akt/mTOR and ERK MAP kinase signaling are needed in cooperation to achieve full activation of p70S6K.

Although MBP was the only protein for which defects in translation were reported in this study, it is possible that ERK2 controls protein translation on a larger scale. p70S6K targets a number of proteins in addition to S6RP that are important for translation. For example, p70S6K impacts the initiation step of translation by phosphorylating the cap-binding complex component eIF4B (Raught et al., 2004), and it controls the elongation step by inactivating the eukaryotic Elongation Factor-2 kinase (Wang et al., 2001). Comprehensive large-scale proteomic studies are underway to determine whether protein translation may be impacted on a more broad level throughout the process of myelin repair in *Erk2* CKO mice. It is also possible that the loss of *Erk2* leads to the delayed translation of MBP as a result of additional and/or alternative mechanisms. The potential involvement of other molecules known to be critical for MBP translation, at least during development, should be considered. Fyn tyrosine kinase plays a central role in the translational regulation of MBP at sites of axon-glial contact (White et al., 2008; Laursen et al., 2009, 2011; Wake et al., 2011; White et al., 2012). Fyn activation results in a breakdown of the RNA granule, releasing *Mbp* mRNA from its inhibitors allowing localized translation to ensue. Interestingly, a recent study demonstrated that Fyn kinase activation promoted a transient increase in the phosphorylation of ERK1/2 (Pérez et al., 2013). It is possible that ERK2 is downstream of Fyn, suggesting that ERK2 may be an important part of the axon-tailored synthesis machinery and that these two important kinases may work together to ensure the timely expression of MBP needed for the rapid expansion of the OL membrane.

Erk1/2 double knock-out (*Erk1/2* dKO) mice initially express MBP protein on schedule, although once they reach the second postnatal week both MBP mRNA and protein levels are dramatically reduced (Ishii et al., 2012). If ERK2 is critical for the onset of

MBP translation, then how is it that *Erk1/2* dKO mice are able to express MBP without delay? First, it is possible that ERK2's role in developmental myelination differs from its role in the context of myelin repair in the adult. This is unlikely, since *Erk2* CKO mice show delayed MBP expression in the forebrain at P10 (Fyffe-Marichich et al., 2011). Alternatively, in the absence of both *Erk1* and *Erk2*, other similar kinases are likely able to compensate and synergize with mTORC1 so that a critical threshold of p70S6K phosphorylation is reached. When only *Erk2* is deleted, ERK1 likely competes for the activation of p70S6K. *In vitro* phosphorylation assays show that pERK1 can phosphorylate p70S6K at Thr421/Ser424, but it does so with poor efficiency compared with pERK2 (Shi et al., 2002). We hypothesize that the 3.5-fold increase in pERK1 that occurs in *Erk2* CKO mice at 7 dpl (Fig. 5A,B) functions in a dominant-negative fashion to inhibit the timely and complete activation of p70S6K, resulting in decreased MBP translation and slower remyelination. Consistent with this hypothesis, we show that the decrease in pERK1 that occurs between 7 and 14 dpl in the *Erk2* CKO mice coincides with the recovery of S6RP phosphorylation and MBP expression (Fig. 7).

The delay in MBP translation seen in the *Erk2* CKO mice, although significant, was only transient (Figs. 2E, 4H). The concept of “timing” is of fundamental importance for the success of remyelination in patients with MS and a window of opportunity appears to exist when OPC differentiation and maturation can successfully occur (Miller and Mi, 2007; Franklin and ffrench-Constant, 2008). This window begins with the expansion of adult OPCs and ends once the lesion environment converts from one of acute inflammation to chronic inflammation (Kotter et al., 2011). Additionally, cell-intrinsic timing limitations exist. Time-lapse microscopy of cocultures (Watkins et al., 2008) and *in vivo* live imaging in zebrafish (Czopka et al., 2013) both demonstrate that individual OLs have only a brief time window, soon after terminal differentiation, in which they are able to form all of their myelin segments. Inhibition of pERK2 in newly differentiated OLs could act as a tipping point to turn an otherwise acute remyelinating lesion into a chronic lesion if the resulting delay in MBP translation causes OLs to miss their “critical window” for new myelin generation. In support of this hypothesis, chronic MS lesions have been found to contain *Mbp* mRNA, but to lack MBP protein (Bauer et al., 2012). It will be important to determine whether defects in ERK MAP kinase signaling may underlie the inability of mature OLs, found in chronic MS lesions, to generate new myelin internodes. Understanding the molecular mechanisms and signaling pathways that drive the process of new myelin generation is a critical first step toward the design of new, effective MS therapies.

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