

# Nucleus-Localized 21.5-kDa Myelin Basic Protein Promotes Oligodendrocyte Proliferation and Enhances Neurite Outgrowth in Coculture, Unlike The Plasma Membrane-Associated 18.5-kDa Isoform

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The classic myelin basic protein (MBP) family of central nervous system (CNS) myelin arises from transcription start site 3 of the Golli (gene of oligodendrocyte lineage) complex and comprises splice isoforms ranging in nominal molecular mass from 14 kDa to (full-length) 21.5 kDa. We have determined here a number of distinct functional differences between the major 18.5-kDa and minor 21.5-kDa isoforms of classic MBP with respect to oligodendrocyte (OLG) proliferation. We have found that, in contrast to 18.5-kDa MBP, 21.5-kDa MBP increases proliferation of early developmental immortalized N19-OLGs by elevating the levels of phosphorylated ERK1/2 and Akt1 kinases and of ribosomal protein S6. Coculture of N2a neuronal cells with N19-OLGs transfected with the 21.5-kDa isoform (or conditioned medium from), but not the 18.5-kDa isoform, caused the N2a cells to have increased neurite outgrowth and process branching complexity. These roles were dependent on subcellular localization of 21.5-kDa MBP to the nucleus and on the exon II-encoded segment, suggesting that the nuclear localization of early minor isoforms of MBP may play a crucial role in regulating and/or initiating myelin and neuronal development in the mammalian CNS. © 2012 Wiley Periodicals, Inc.

**Key words:** myelin basic protein; MBP; oligodendrocytes; myelination; live-cell imaging

In the central nervous system (CNS), myelin arises from oligodendrocytes (OLGs), a highly diverse and plastic lineage (Baumann and Pham-Dinh, 2001; Miller, 2002; Noble et al., 2004; Colognato and French-Constant, 2004; Bradl and Lassmann, 2010; Miron et al., 2011). The OLGs proceed through a regulated differentiation pathway, culminating in myelination of

axons (Pfeiffer et al., 1993; Miller, 1996). The OLG is at the mature stage when myelin basic protein (MBP) and proteolipid protein (PLP) are expressed, and extensive processes and myelin-like sheets form and extend around an axon. The MBP family comprises developmentally regulated members arising from different transcription start sites of the Golli (gene of oligodendrocyte lineage) complex, with further differential splicing and combinatorial posttranslational modifications (Campagnoni et al., 1993; Pribyl et al., 1993; Givogri et al., 2001). The “classic” MBP isoforms arise in more highly differentiated and mature OLGs, from transcription start site 3 of Golli, and comprise splice isoforms ranging in nominal molecular mass from 14 to 21.5 kDa. These MBPs are fundamental structural proteins of CNS myelin, particularly the predominant (in mature myelin) 18.5-kDa isoform, being essential for myelin development and stability (Readhead et al., 1990; Fitzner et al., 2006; Simons and Trotter, 2007; Aggarwal et al., 2011a,b; Simons et al., 2012).

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The 18.5-kDa MBP isoform is an intrinsically disordered protein with numerous conformational transitions and multiple binding partners, including cytoskeletal proteins, calcium-activated calmodulin, and SH3-domain-containing proteins, indicative of multifunctionality (for review see Harauz et al., 2004, 2009; Boggs, 2006, 2008; Harauz and Libich, 2009; Libich et al., 2010). These associations and their functional significance have recently been demonstrated in cellula using primarily the conditionally immortalized N19-OLG cell line, which closely resembles an immature OLG or its immediate precursor, particularly the O2A progenitor (Foster et al., 1993, 1995; Verity et al., 1993; Byravan et al., 1994). Thus, the N19-OLG cell line is valuable for exploring MBP's multifunctionality in glia. We have shown 18.5-kDa MBP to inhibit calcium influx into N19-OLGs (Smith et al., 2011), to interact with Fyn to affect process development in these cells (Smith et al., 2012a), and to associate with cytoskeletal and SH3-domain proteins in membrane ruffles upon phorbol ester stimulation (Smith et al., 2012b). The mRNA for this isoform comprises a minimal 21-nucleotide 3'-untranslated region (3'UTR), that causes it to be trafficked to the peripheral processes of developing OLGs (Ainger et al., 1997). The translocation is believed to aid in the immediate insertion of the highly positively charged MBP directly into the OLG myelin membrane (Hardy et al., 1996; Carson et al., 1998; Barbarese et al., 1999; Maier et al., 2008). It has also been suggested that the association of the basic protein with phosphatidylinositol-4,5-bis-phosphate (PI[4,5]P2) plays a further role in its membrane targeting (Nawaz et al., 2009). The differential targeting of MBP mRNAs is essential for establishing and maintaining OLG polarity (Baron and Hoekstra, 2010; Simons et al., 2012).

The full-length 21.5-kDa transcript includes exon II (exon 6 in Golli numbering), and the 26-residue exon II-encoded segment is conserved in higher vertebrates (Smith et al., 2012c). One of the primary differences between the two classic MBP isoforms is that 21.5-kDa MBP is targeted predominantly to the nucleus and 18.5-kDa MBP to the cytoplasmic plasma membrane leaflet, within OLGs (Colman et al., 1990; Gillespie et al., 1990; Staugaitis et al., 1990; Allinquant et al., 1991; de Vries et al., 1997). The transport of exon II-containing MBP into the nucleus has been determined in HeLa cells to be an active process (meaning that it is time, temperature, and energy dependent; Pedraza et al., 1997). However, threonine pseudophosphorylation of 18.5-kDa MBP can cause it to translocate to the nucleus from the cytoplasm in N19-OLGs (Smith et al., 2012a, cf. Boggs et al., 2006; Nardozzi et al., 2010). At high N19-OLG density in culture, the 21.5-kDa isoform translocated from the nucleus to the cytoplasm (Smith et al., 2012b) and also after phorbol ester treatment of low-confluence cultures (Smith et al., 2012a). We have recently shown that the 21.5-kDa MBP isoform has a nontraditional PY-nuclear localization signal (NLS) in the 26-residue segment encoded by exon II (Smith

et al., 2012c). Classic MBP isoforms are thus another example of proteins that play roles both at the plasma membrane and in the nucleus (Benmerah et al., 2003).

The expression of exon II-containing MBP transcripts is enriched during active early myelination in the human and mouse and in immature OLGs in culture (Barbarese et al., 1978; Kamholz et al., 1988; Jordan et al., 1989; Pedraza, 1997; Pedraza et al., 1997) and has been suggested to be associated specifically with differentiation (de Vries et al., 1997). In geriatric rats, exon II-lacking isoforms are virtually absent (Sugiyama et al., 2002). Curiously, 21.5-kDa MBP is temporospatially expressed in developing rat molars (Kim et al., 2008). The functional implications remain unresolved, but it was suggested that this might play a regulatory role in the terminal histodifferentiation of odontogenic cells, perhaps involving the release of neurotrophins and/or neurotransmitters. It has been proposed that the exon II-containing MBP isoforms, which are expressed early in development, may have regulatory effects on the myelination program, particularly because of their active transport into the nucleus (Staugaitis et al., 1996; Pedraza et al., 1997).

The current study has determined a number of distinct functional differences between the major 18.5-kDa and minor 21.5-kDa isoforms of classic MBP with respect to OLG proliferation and survival. We have found that, in contrast to 18.5-kDa MBP, 21.5-kDa MBP overexpression increases proliferation of early developmental immortalized N19-OLGs by elevating levels of active ERK1/2 and Akt1 kinases and of ribosomal protein S6 (rpS6). Coculture of N2a neuronal cells with N19-OLGs transfected with the 21.5-kDa isoform, but not the 18.5-kDa isoform, caused the N2a cells to have increased neurite outgrowth and process branching complexity. The same phenomenon was observed in N2a cells treated with conditioned medium from these N19-OLGs. These roles of 21.5-kDa MBP are thus dependent on subcellular localization to the nucleus, suggesting that the nuclear localization of early minor isoforms of MBP may play a crucial role in regulating and/or initiating myelin development in the rodent CNS.

## MATERIALS AND METHODS

### Plasmids

Previously described plasmids coding for RFP-tagged versions of 18.5-kDa and 21.5-kDa recombinant murine MBPs possessing a 3' untranslated region (UTR), namely, pERFP-C1-rmMBP-18.5C1-UTR and pERFP-C1-rmMBP-21.5-UTR, were used throughout these investigations (Smith et al., 2011, 2012a,b). For the 18.5-kDa isoform, the second "C1" designation means that the protein emulates the unmodified C1 component of MBP, which is highly post-translationally modified in vivo (Kim et al., 2003; Harauz et al., 2009; Harauz and Libich, 2009; Zhang et al., 2012). The designation "UTR" means that a 21-nt 3'UTR was added to each gene (Smith et al., 2011). Henceforth, the proteins encoded by these plasmids will usually be referred to

more simply as 18.5-kDa MBP or 21.5-kDa MBP, keeping in mind that they are fused to RFP at the N-terminus (RFP-MBP-18.5 and RFP-MBP-21.5).

Two additional plasmids coding for RFP-tagged 18.5-kDa MBP containing a 2xNLS (nuclear-localization signal, amino acids KKKRK), and for RFP-tagged 21.5-kDa MBP containing a 2xNES (nuclear export signal, amino acids LQLPPLERLTL) were used for relocalization experiments in this study (Fig. 1A). To produce the 2xNLS insert, the primers NLS-Fp (5'-CCGGAAAGAAGAAGAGAAAGAAGAAGAAGAGAAAGT-3') and NLS-Rp (3'-TTTCTTCTTCTTCTTCTTCTTCTTCTTTCAGGCC-5') were annealed together. To produce the 2xNES insert, the primers NES-Fp (5'-CCG GACTGCAGCTGCCGCCGCTGGAACGCCTGACCCCT GCTGCAGCTGCCGCCGCTGGAACGCCTGACCCCTGT-3') and NES-Rp (5'-CCGGACAGGGTCAGGCGTTCCAGCGGC GGCAGCTGCAGCAGGGTCAGGCGTTCCAGCGGCGGCA GCTGCAGT3') were annealed together. To anneal the oligonucleotides, a thermal cycler was programmed for a 1°C/min decreasing gradient temperature profile beginning at 94°C and ending at 65°C, with a final hold at 4°C. After this annealing process, a further reaction was used to phosphorylate the 5'-ends of the annealed complementary oligonucleotides. Each 100- $\mu$ l reaction contained 50  $\mu$ l of the annealed oligonucleotide PCR, 10  $\mu$ l of 10  $\times$  T4 polynucleotide kinase buffer (New England Biolabs, Mississauga, Ontario, Canada), 1.3  $\mu$ l of 2.55 mM ATP, 2.5  $\mu$ l of T4 polynucleotide kinase, and 36.2  $\mu$ l MilliQ H<sub>2</sub>O. The resulting reaction was precipitated, purified, and ligated into the previously constructed pERFP-C1-rmMBPC1-UTR and pERFP-C1-rmMBP21.5-UTR plasmids (Smith et al., 2011). The resulting constructs were confirmed by sequencing (Laboratory Services Division, University of Guelph). Henceforth, the proteins encoded by these new plasmids will be referred to simply as RFP-2xNLS-MBP-18.5 and RFP-2xNES-MBP-21.5.

### Culture, Transfection, Fluorescence Microscopy, and Live-Cell Imaging of N19-OLGs

The cell culture of the immortalized N19-OLG line (Foster et al., 1993, 1995; Verity et al., 1993; Byravan et al., 1994), transfection, fluorescence and immunofluorescence microscopy, and live-cell imaging were performed as previously described (Smith et al., 2011, 2012a,b,d).

### Statistical Analyses of Proliferation

For proliferation assays, bromodeoxyuridine (BrdU) incorporation was measured from 15 randomly selected fields from three separate experimental replicates, and the numbers of BrdU-positive cells were counted for each field of view. A paired *t*-test ( $P = 0.05$ ) was used to determine that the duplicate sample sets did not differ significantly. Afterward, the recorded measurements from each duplicate experiment were grouped into a larger sample set of  $n = 60$ . The N19-OLGs from each experiment were then compared with one another using an ANOVA table ( $P = 0.05$ ), and the means of each variant and the SEM for each trait were determined. The difference in means was further analyzed using the Tukey means comparison test ( $P = 0.05$ ) to determine which treatments were different from one another.

### Western Blotting

Western blots of N19-OLG lysates were performed as previously described (Smith et al., 2011). The following antibodies were obtained from commercial sources: rabbit anti-Akt (pan; Cell Signaling, Beverly, MA; catalog No. 4691), used at 1/1,000 dilution; rabbit antiphospho-Akt-Ser473 (Cell Signaling; catalog No. 4060), 1/2,000 dilution; rabbit anti-p44/42 MAPK (ERK1/2; Cell Signaling; catalog No. 4695), 1/500 dilution; rabbit antiphospho-p44/42 MAPK (ERK1/2-Thr202/Tyr204; Cell Signaling; catalog No. 4370), 1/1,000 dilution; rabbit anti-bovine neurofilament (Serotec, Bicester, United Kingdom; catalog No. AHP245); mouse anti-MBP monoclonal (Chemicon, Temecula, CA, catalog No. MAB382; or Covance, Berkeley, CA, catalog No. SMI99); mouse anti-GAPDH (Applied Biological Materials, Richmond, British Columbia, Canada; catalog No. G041), 1/1,000 dilution; goat anti-rabbit (Alexa Fluor 488; Invitrogen, Carlsbad, CA); and rabbit IgG peroxidase conjugate (Sigma, St. Louis, MO; catalog No. A-9169).

Lysates were pooled from six transfections to account for differences between treatments. Western blots were detected using an enhanced chemiluminescence (ECL Advance Western Blotting Detection kit; GE Healthcare Life Sciences, Montréal, Québec, Canada).

### ELISA for Ribosomal Protein S6, p38 MAPK, SAPK/JNK, and Their Phosphorylated Forms

Protein lysates were collected from N19-OLG cell cultures 48 hr after transfection. Lysates from 18 separate transfections were pooled together and were used for ELISA at protein concentrations ranging from 0.1 to 1.0 mg/ml (PathScan Total S6 Ribosomal Protein Sandwich ELISA Kit; catalog No. 7203; PathScan Phospho S6 Ribosomal Protein [Ser 235/236] Sandwich ELISA Kit; catalog No. 7205; PathScan Phospho-SAPK/JNK [Thr183/Tyr185] Sandwich ELISA Antibody Pair; catalog No. 7217; PathScan Phospho-p38 MAPK [Thr180/Tyr182] Sandwich ELISA Kit; catalog No. 7946). The ELISA was performed according to the manufacturer's instructions (Cell Signaling). ELISA substrate detection was made via an automated microplate fluorescence reader (Polarstar Omega; BMG Laboratory Technologies, Ortenburg, Germany). Results shown are the mean and SEM of triplicate measurements made on the same protein lysate from each pooled treatment.

### Coculture of N2a Neuronal Cells With N19-OLGs

For N2a/N19 cocultures, N19-OLGs were transfected with the desired construct and were exposed to the transfection reagent for 48 hr. After this transfection period, the medium was removed, and the cell cultures were washed twice with 2 ml Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), and then the medium was replaced. The N2a cells were trypsinized and plated at a density of  $1.0 \times 10^6$  cells/ml and were cultured for an additional 48 hr at 37°C prior to fixation and microscopy. After transfection, the medium was removed, and the cell cultures were washed twice with 2 ml DMEM containing 10% FBS. The cells were then trypsinized in 75  $\mu$ l of 0.25% trypsin,



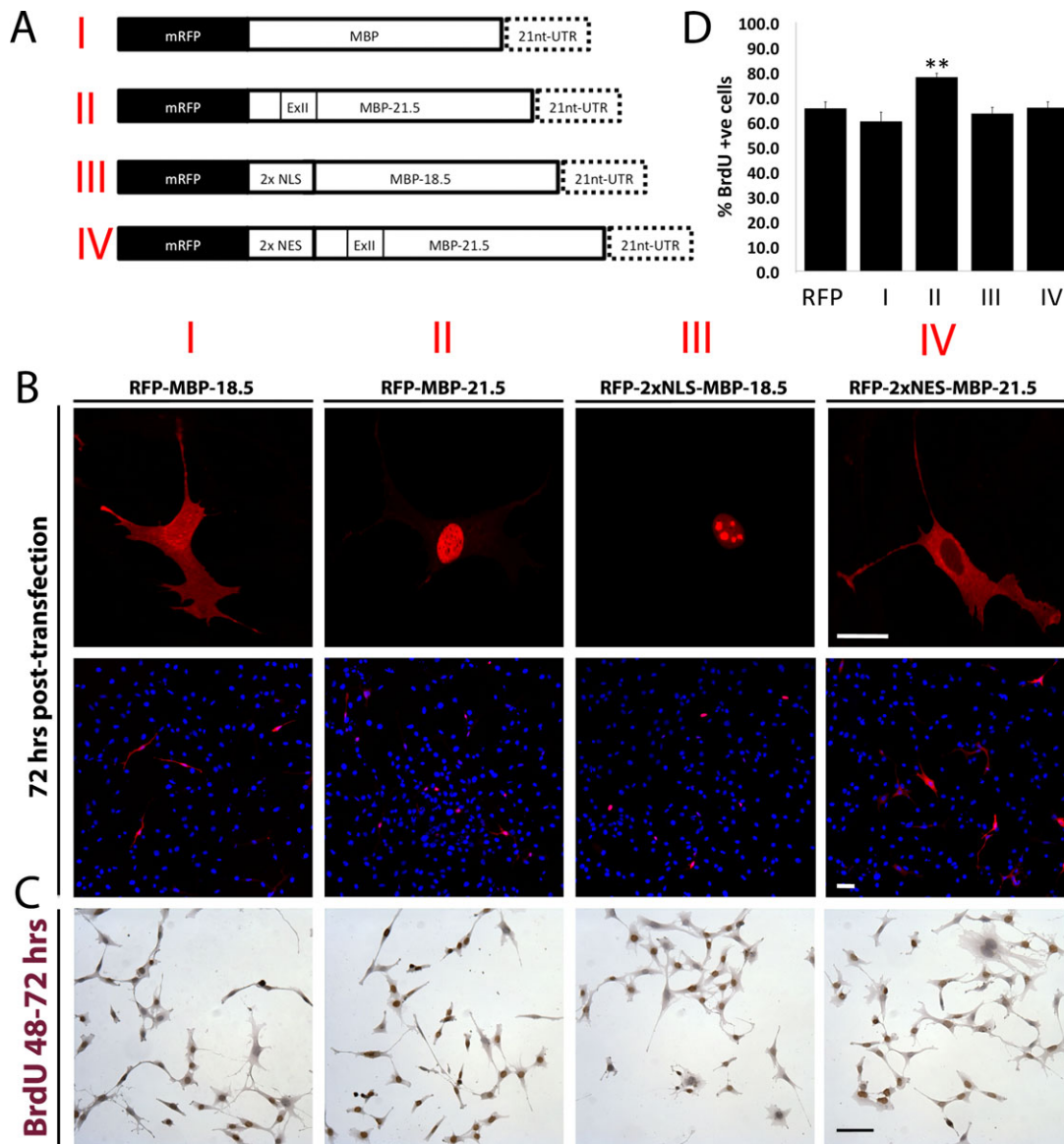


Fig. 1. Classic MBP constructs, subcellular targeting, and proliferation assays. **A**: Schematic diagram of the RFP-tagged 18.5-kDa and 21.5-kDa MBP constructs, and their complementary reverse localization variants, that were used throughout this study to distinguish whether the nuclear localization of MBP, or the isoform specificity, was required for phenotypic gain of function. The inclusion of exon II in the 21.5-kDa isoform and the minimal 21-nt 3'-untranslated region (UTR) are also shown for completeness. **B**: Fluorescence micrographs of cultured N19-OLGs 3 days posttransfection expressing 18.5-kDa or 21.5-kDa MBP isoforms and their complementary reverse localization variants (red). The 2xNLS signal added as a linker between RFP and 18.5-kDa MBP traffics the protein to the nucleus, whereas the 2xNES added to 21.5-kDa MBP exports the protein out of the nucleus to the cytoplasm and plasma membrane. **C**: Cultured N19-OLGs expressing the four MBP constructs were processed for BrdU incorporation following a 24-hr BrdU pulse (BrdU administered between 48 hr and 72 hr posttransfection). The BrdU was

detected using immunohistochemical diaminobenzidine staining. **D**: The percentage of total cells incorporating BrdU was determined by a blinded observer counting 10 random fields in three different experiments. A statistically significant increase in number of cells incorporating BrdU of 13% (\*\* $P = 0.05$ ) was observed for RFP-MBP-21.5, and no increases in cell proliferation were seen in cultures transfected with either RFP-MBP-18.5 or RFP-2xNLS-MBP-18.5. Cell cultures transfected with RFP-2xNES-MBP-21.5 did not show any increases in cell proliferation, supporting the conclusion that the nuclear localization of 21.5-kDa MBP is required for cell proliferation. The 18.5-kDa MBP isoform, when localized to the nucleus, also did not increase the rate of cell proliferation, suggesting that this process requires the presence of the exon II-encoded segment. A color version of this figure can be found in the online version of this article. Scale bars = 20  $\mu\text{m}$  in row 1; 50  $\mu\text{m}$  in rows 2, 3. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

subsequently plated in the neuronal cell culture dishes, and grown for an additional 48 hr prior to fixation and microscopy.

The N2a cell cultures grown in monoculture were also treated with conditioned media removed from MBP-transfected N19-OLG cultures. In this experiment, N2a cells were plated either at low density ( $0.2 \times 10^6$  cells/ml) or at high density ( $1.0 \times 10^6$  cells/ml) and were supplemented with a 1:1 ratio of conditioned medium from N19-OLG cultures after 48 hr of transfection. These conditioned N2a cultures were cultured for an additional 48 hr at 37°C prior to fixation and microscopy.

## RESULTS

### Nuclear Localization and the Exon II-Encoded Segment of 21.5-kDa MBP Increase the Rate of N19-OLG Proliferation in Contrast to the Classic 18.5-kDa MBP Isoform

In N19-OLGs in culture, the 21.5-kDa MBP splice isoform is targeted predominantly to the nucleus and unmodified 18.5-kDa MBP to the cytoplasmic plasma membrane leaflet (Smith et al., 2011, 2012a–c). These two constructs have been previously described (Smith et al., 2011) and are shown schematically in Figure 1A. In this study, two additional constructs were generated utilizing the SV40 large T-antigen monopartite nuclear localization signal (NLS; i.e., KRKKK) or the leucine-rich nuclear export signal (NES; i.e., LQLPPLERPTL; Kalderon et al., 1984; Wen et al., 1995). Signal linkers were inserted between the RFP and MBP genes in the fusion proteins, either a 2xNLS sequence within RFP-MBP-18.5 to override its inherent plasma membrane targeting signals or a 2xNES sequence within RFP-MBP-21.5 to override its inherent nuclear targeting (Fig. 1A). The proteins encoded by these new plasmids were named RFP-2xNLS-MBP-18.5 and RFP-2xNES-MBP-21.5, respectively. Figure 1B demonstrates that the localization patterns of these four constructs were as expected.

We found next that expression of 21.5-kDa MBP in the immature N19-OLG line increases N19-OLG proliferation, in contrast to 18.5-kDa MBP (Fig. 1C). To assess the rate of cell proliferation, we exposed the N19-OLG culture transfected with either 21.5-kDa or 18.5-kDa MBP to bromodeoxyuridine (BrdU) for 48 hr posttransfection and cultured the cells for an additional 24 hr. There was a statistically significant 13% increase in BrdU incorporated into the nuclei of N19-OLG cultures expressing 21.5-kDa MBP compared with the RFP control (Fig. 1D).

We next assessed whether the nuclear localization of MBP was responsible for the differences seen in N19-OLG proliferation and whether 18.5-kDa MBP was capable of increasing cell proliferation if localized to the nucleus. When RFP-2xNES-MBP-21.5 was expressed in N19-OLGs, there was no increase in BrdU-positive cells, in contrast to RFP-MBP-21.5, demonstrating that the nuclear localization of 21.5-kDa MBP, and not just

its expression within the cell, was necessary for the observed increase in cell proliferation (Fig. 1C,D). Furthermore, RFP-2xNLS-MBP-18.5 did not cause increases in cell proliferation based on BrdU nucleotide incorporation.

Taken together, these results indicate that 21.5-kDa MBP localization within the nucleus is responsible directly or indirectly for stimulating cell proliferation and also that these proliferative effects are splice isoform specific and require the segment encoded by exon II, insofar as 18.5-kDa MBP was unable to induce increases in cell proliferation when localized to the nucleus.

### Overexpression of 21.5-kDa MBP Increases the Phosphorylation of ERK1/2 and Akt1

With an observed increase in cell proliferation in N19-OLG cultures expressing 21.5-kDa MBP, we next probed the activity of several convergent and regulatory proteins that can control cell proliferation. The mitogen-activated protein kinases (MAPKs) are activated by phosphorylation cascades, and they, in turn, phosphorylate seryl and threonyl residues in their downstream substrates (Raman et al., 2007; Plattner and Bibb, 2012). The MAPKs participate in intrinsic messenger cascades, integrating a number of signal transducers that regulate cellular processes, including cell differentiation, proliferation, and death (Crews et al., 1992; Matsuda et al., 1992; Errede et al., 1995; Lewis et al., 1998; Plotnikov et al., 2011). The MAPKs comprise extracellular signal-regulated protein kinases (ERKs), p38 MAPKs, and c-Jun NH2-terminal kinases, all of which are functional in oligodendrocytes (for review see Stariha and Kim, 2001a,b; Bhat et al., 2007; Fragoso et al., 2007; Haines et al., 2008; Chew et al., 2010). The ERKs in particular have been demonstrated to participate in oligodendrocyte differentiation (Stariha et al., 1997; Younes-Rapoza et al., 2009; Fyffe-Maricich et al., 2011; Guardiola-Diaz et al., 2012; Ishii et al., 2012; Sun et al., 2012; Xiao et al., 2012).

Fluorescence immunostaining for phosphorylated ERK1/2-Thr202/Tyr204 of nontransfected cultures, or of cultures transfected with RFP or RFP-MBP-18.5, showed that they had low levels (blue on the colorimetric grading scale) of phospho-ERK1/2 (Fig. 2, right). The phospho-ERK1/2 was present in the cell body and processes, with less in the nuclei, of nondividing cells, indicated by speckled DAPI staining in the nucleus (Fig. 2, left), but was mainly in the nucleus of dividing cells, indicated by homogeneous and condensed DAPI staining of the nuclei (Fig. 2, left, dividing cells indicated by arrowheads). However, cultures transfected with RFP-MBP-21.5 had 100–200 times (green to red on the colorimetric grading scale) more phospho-ERK1/2 in the nuclei of dividing cells than in nondividing cells in the culture or in the nontransfected cultures or cultures transfected with other variants. They also had low levels of phospho-ERK1/2 in the cell body and cytoplasm in contrast to dividing cells in the other cultures.

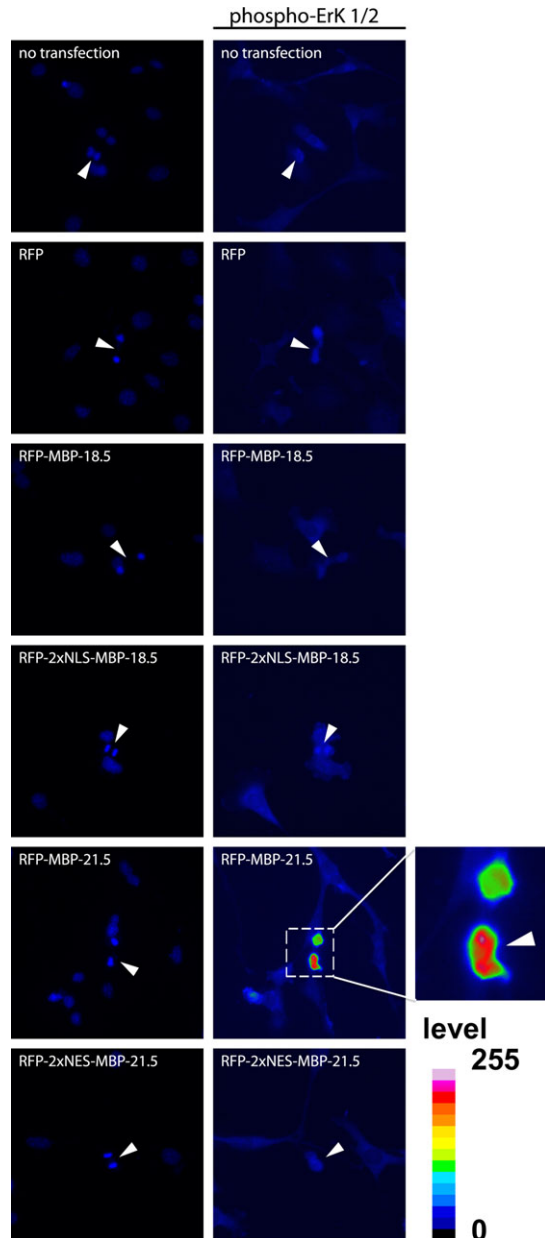


Fig. 2. Immunofluorescence micrographs of N19-OLG cultures transfected with RFP-MBP constructs. All images were acquired using strict image acquisition settings that were the same for each micrograph. Staining of DNA in nuclei with DAPI (blue) is shown in the left panels. Nondividing cells have nuclear speckles, whereas dividing cells have more homogeneous, concentrated staining because of condensed DNA (arrowheads). Antiphospho-ERK1/2-Thr202/Tyr204 antibody staining is shown in the right panels in which the images were pseudocolored, with higher intensity pixels represented as warmer colors and lower intensity pixels as cooler colors, as indicated by the colorimetric gradient scale. Fluorescence immunostaining of cultures transfected with RFP-MBP-21.5 for pERK1/2-Thr202/Tyr204 revealed that both transfected and nontransfected N19-OLG cells in culture had 100–200 times elevated levels of phospho-ERK1/2 in the nuclei during mitosis compared with nontransfected cells or with cells transfected with RFP or other MBP variants. A color version of this figure can be found in the online version of this article. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

Interestingly, imaging for RFP-MBP (not shown) in cultures examined in other experiments revealed that both transfected and nontransfected N19-OLG cells present in the RFP-MBP-21.5-transfected culture had elevated levels of phospho-ERK1/2 in the nuclei during mitosis. This observation suggested that the transfected cells secreted a factor that affected the nontransfected cells. The dramatic increase in phospho-ERK1/2 in transfected N19-OLG cultures was dependent on the nuclear localization of 21.5-kDa MBP, insofar as cultures expressing RFP-2xNES-MBP-21.5 did not have increased levels of phosphorylation compared with the RFP control cultures. Conversely, transfection with the MBP-18.5 variant containing an NLS (RFP-2xNLS-MBP-18.5) did not cause increased phospho-ERK1/2 in the nuclei of dividing cells (Fig. 2, right).

To confirm these changes, the N19-OLG cell cultures expressing RFP-MBP-18.5, RFP-2xNLS-MBP-18.5, RFP-MBP-21.5, or RFP-2xNES-MBP-21.5 (Fig. 2) were harvested, and whole-cell lysates were examined using Western blotting to assess the phosphorylation status of several cell proliferation cascades, including the p38 $\alpha$  MAPK, SAPK/JNK, p44/42 MAPK (ERK1/2), and Akt1 (also known as *protein kinase B*; Hers et al., 2011). Transfection of RFP alone, which served as a vehicle control, caused noticeable increases in the phosphorylation status of several proteins as a result of the transfection itself (Fig. 3). However, we detected a considerable increase in phosphorylated ERK1/2-Thr202/Tyr204 in cell cultures transfected with RFP-MBP-21.5 compared with RFP and RFP-MBP-18.5. We also observed a smaller increase in the levels of pAkt-Ser473 in cell cultures transfected with RFP-MBP-21.5 compared with RFP-MBP-18.5, RFP-2xNLS-MBP-18.5, and RFP-2xNES-MBP-21.5. The NLS did not cause 18.5-kDa MBP to induce Akt or ERK phosphorylation, but the NES prevented 21.5-kDa MBP from having this effect and even decreased ERK phosphorylation relative to RFP alone (Fig. 3). Compared with the RFP control, no significant differences were seen in phosphorylated p38 MAPK-Thr180/Tyr182 or SAPK/JNK-Thr183/Tyr185 in cultures of N19-OLGs expressing MBP variants (results not shown).

### The 21.5-kDa MBP Isoform Increases the Endogenous Levels of rpS6 and Its Phosphorylation at Ser235/236

The small ribosomal subunit protein S6 (rpS6), S6 kinase, and the S6 signaling pathway play a key role in the regulation of cell growth, proliferation, and size (for review see Dufner and Thomas, 1999; Volarevic and Thomas, 2001). Mitogens and extracellular growth factors can induce the activation of p70 S6 kinase, resulting in downstream phosphorylation of the rpS6 ribosomal protein. Phosphorylation of rpS6 correlates with an increase in translation of mRNA transcripts that contain an oligopyrimidine tract in their 5'UTR (Peterson and Schreiber, 1998). Overall, these events lead to a general

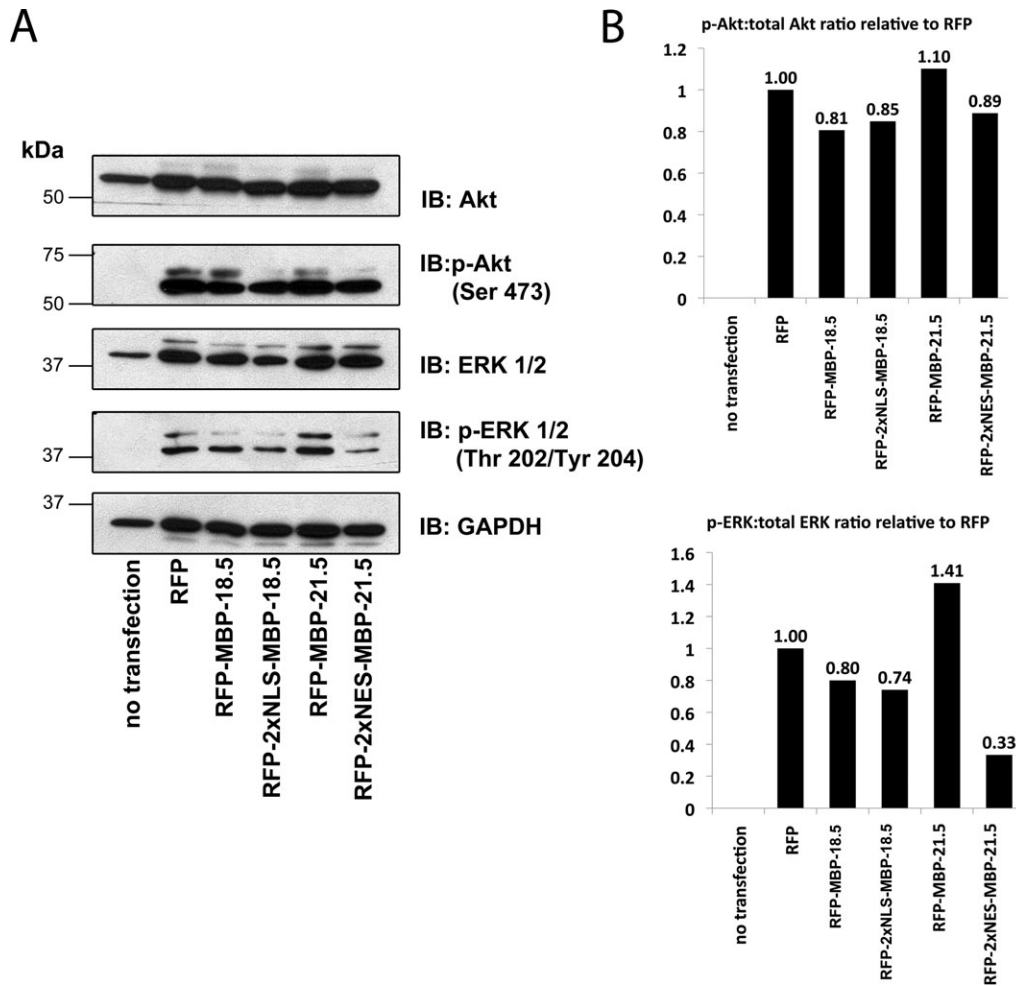


Fig. 3. **A**: Western blots from N19-OLG whole cell lysates following transfection with 18.5-kDa and 21.5-kDa MBP constructs and their complementary reverse localization variants (lysates from six separate transfections were pooled). Cells were transfected, and lysates were harvested 72 hr posttransfection, pooled, and probed for endogenous Akt, ERK1/2, pAkt-Ser473, and pERK1/2-Thr202/Tyr204. **B**: Densitometry measurements from the Western analyses in A were used to determine the ratio of the phosphorylated proteins

to the total levels of Akt and ERK. Two equally loaded gels from the same lysates were used for these experiments to avoid complications arising from membrane stripping. A small increase in the ratio of pAkt-Ser473 in N19-OLG cell cultures transfected with RFP-MBP-21.5 compared with RFP-MBP-18.5, RFP-2xNLS-MBP-18.5, and RFP-2xNES-MBP-21.5 was observed, whereas a considerable increase in the ratio of pERK1/2-Thr202/Tyr204 was detected in cell cultures transfected with RFP-MBP-21.5.

upregulation of the ribosomal biosynthetic machinery, which can increase the capacity required for cell growth and proliferation.

To examine whether the expression of 18.5-kDa and/or 21.5-kDa MBP, and their complementary reverse localization variants, affected total rpS6 ribosomal protein and/or phospho-Ser235/236 rpS6, we next analyzed the cell lysates of transfected N19-OLG cultures via ELISA (Fig. 4). We found that transfection of RFP alone caused a large increase in total endogenous rpS6 (Fig. 4A) and also that it was highly phosphorylated at residues Ser235/236 (Fig. 4B). However, transfection of RFP-MBP isoforms caused similar increases in rpS6. The expression of 21.5-kDa MBP caused a small but significant increase in phosphorylation of S6 at residues

Ser235/236 compared with the RFP control and 18.5-kDa MBP, and this effect was prevented by the NES (Fig. 4B,C). Localization of MBP-18.5 (RFP-2xNLS-MBP-18.5) in the nucleus, however, decreased phosphorylation of rpS6. Also of interest was RFP-BG21, which is a Golgi protein that arises earlier in development prior to classic MBP expression but has subcellular localization patterns within the nucleus similar to the pattern observed for 21.5-kDa MBP (Reyes and Campagnoni, 2002; Fulton et al., 2010). Transfection of RFP-BG21 also decreased phosphorylation of rpS6 relative to RFP alone and to 18.5-kDa MBP. Taken as a whole, these observations show further that 21.5-kDa MBP promotes cell growth and proliferation via MAPK pathways, unlike the 18.5-kDa MBP isoform, and that this process



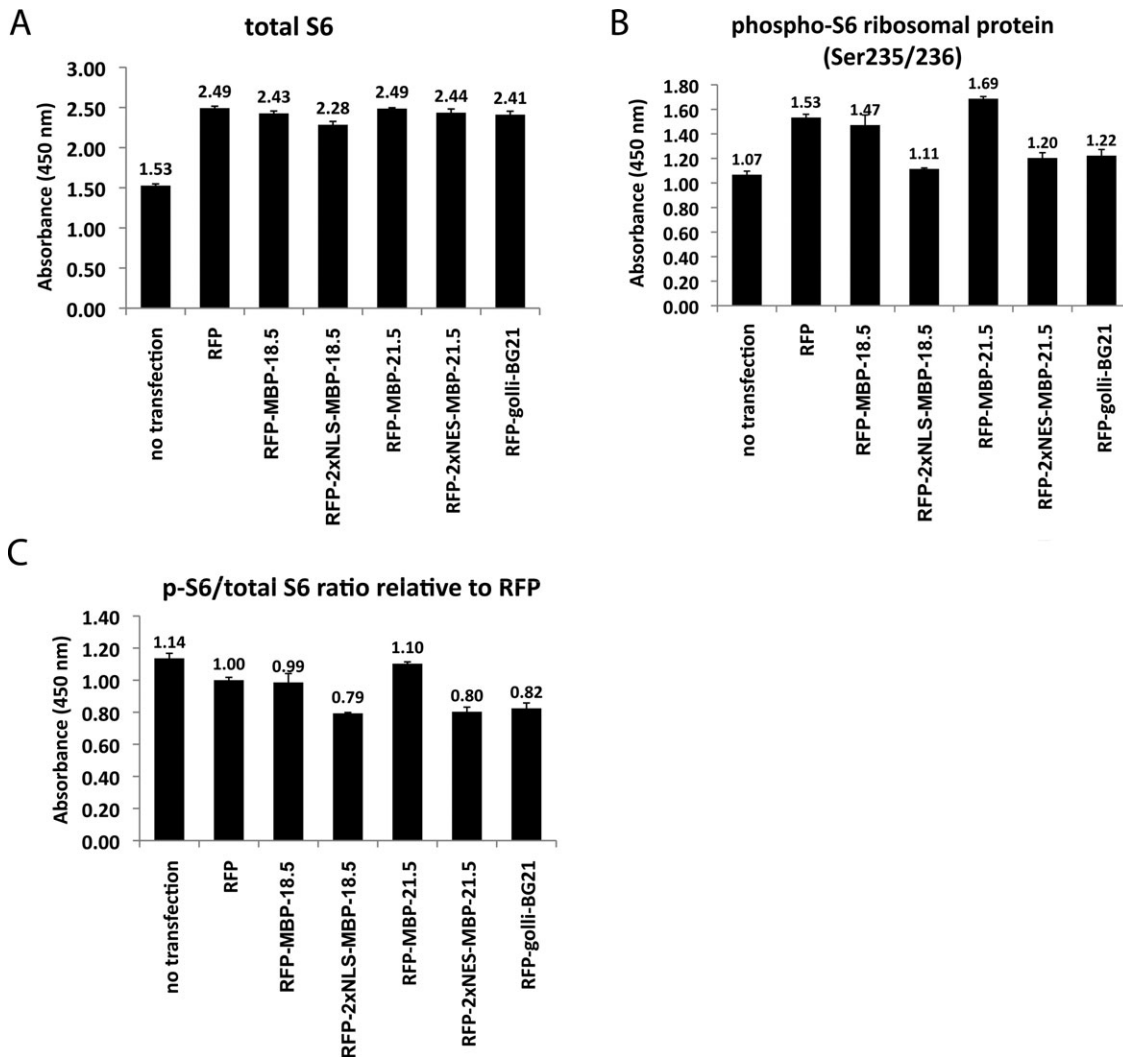


Fig. 4. The N19-OLG cell cultures were probed for levels of total endogenous rpS6 ribosomal protein (**A**) and of rpS6 phosphorylated at Ser235/236 (**B**) as detected by the PathScan Phospho-S6 Ribosomal Protein (Ser235/236) Sandwich ELISA Antibody Pair (Cell Signaling; catalog No. 7205), following 72 hr posttransfection as suggested by the manufacturer. The ratio of phospho-rpS6 (denoted p-S6) to total rpS6 (denoted total S6) is shown in **C**. Lysates were

assayed at a protein concentration of 0.45 mg/ml and were pooled from a total of 12 separate transfection experiments for each construct. The absorbance readings were measured at 450 nm. Compared with the RFP control and other variants, overexpression and nuclear localization of RFP-MBP-21.5 increases phosphorylation of the rpS6 ribosomal protein (p-S6).

is dependent on its subcellular localization within the nucleus.

### N2a Cells Demonstrate Increased Branching Complexity and Neurite Outgrowth When Cocultured With N19-OLGs Expressing 21.5-kDa MBP

The Neuro-2a neuroblastoma (N2a) cell line, previously used to examine cell proliferation, differentiation, and neurite outgrowth (Brown et al., 1998; Sun et al., 2010), was used in these studies to examine neuroglial communication between N2a neurons and N19-OLGs expressing different isoforms of MBP. Briefly, OLGs

were transfected with each construct coding for RFP-MBP-18.5, RFP-MBP-21.5, or RFP. After 48 hr of expression, the N19-OLGs were cocultured with the N2a cells for an additional 48 hr. We observed that N2a cells had increased branching complexity in cocultures when 21.5-kDa MBP was expressed in N19-OLGs, which was not evident in cocultures that were expressing 18.5-kDa MBP (Fig. 5A, arrowheads). We next wanted to ascertain whether these changes in N2a phenotype were mediated by cell-cell contact or through a soluble trophic factor. We found that the observed increases in N2a branching complexity, and neurite outgrowth, occurred in regions of the culture chamber where N2a cells were not in contact with N19 OLGs. They also



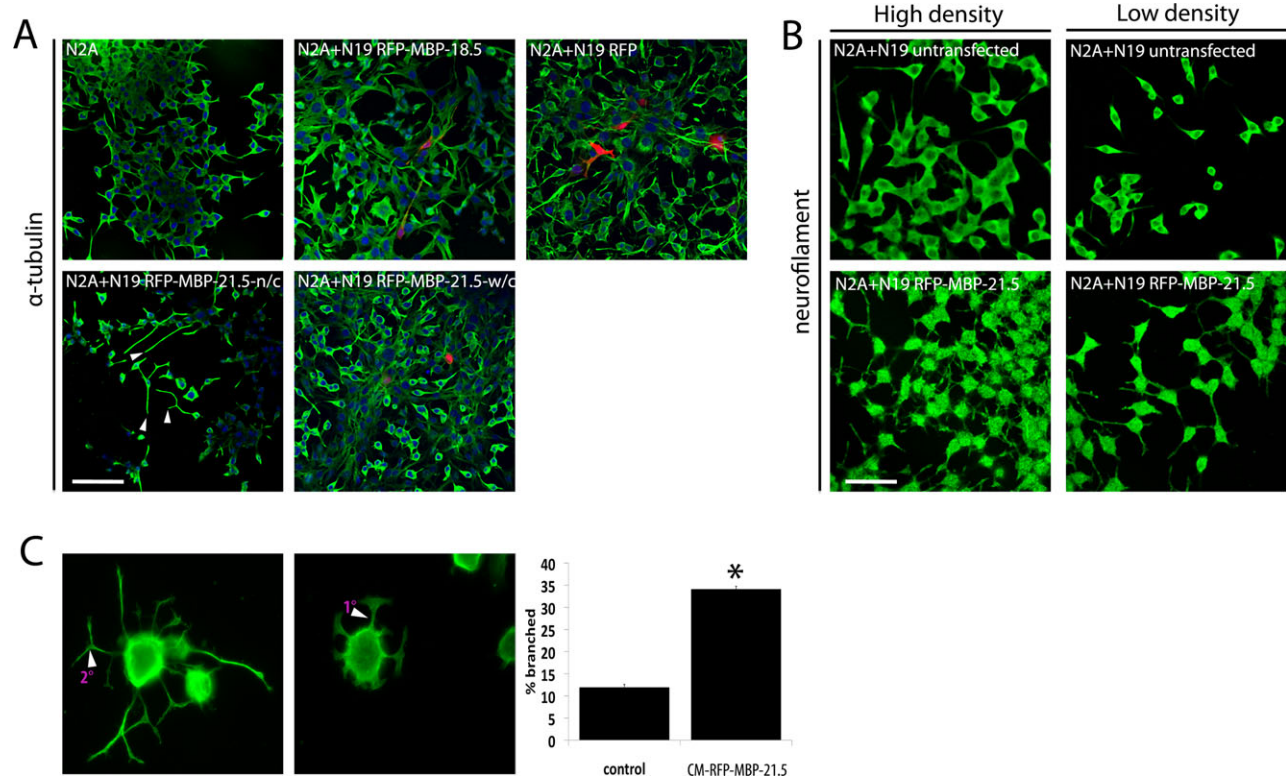


Fig. 5. **A:** The N19-OLG cells transfected with different MBP isoforms grown in coculture with N2a neuronal cells. Cell cultures were stained for endogenous tubulin (green), allowing the morphological distinction of tubulin-rich N2a neurons from N19-OLGs. Overexpression of RFP and RFP-MBPs (red) can be observed in N19-OLGs, and cell nuclei are labeled with DAPI (blue). The N19-OLGs were exposed to transfection for 48 hr prior to trypsinization. They were then transferred and plated at a density of  $1.0 \times 10^6$  cells/ml, together with N2a cells. The cocultures were grown for an additional 48 hr at  $37^\circ\text{C}$  prior to fixation, staining, and microscopy. The N2a neurons cocultured with N19-OLGs overexpressing the 21.5-kDa MBP isoform showed increases in neurite outgrowth and process branching complexity compared with those cocultured with N19-OLGs overexpressing 18.5-kDa MBP, RFP alone, or no transfection control. This result, for cells with possible contact between N2a cells and N19-OLGs (w/c), was also seen for N2a cells cocultured with N19-OLGs overexpressing 21.5-kDa MBP that were cultured in the same cell chamber, but in regions with no contact (n/c) between N2a cells and N19-OLGs (OLG-N2a). **B:** The N2a cells grown in monoculture and stained for neurofilament heavy chain (green), imaged 48 hr after a 50% supplementation of conditioned media from N19-OLG cultures overexpressing 21.5-kDa MBP,

demonstrated increased branching complexity, but not a consistent change in process length, compared with the control. This result shows that this phenotype is attributable to a secreted factor from N19-OLG cultures overexpressing 21.5-kDa MBP and that N19-OLG-N2a cell-cell contact is not required. The N2a cells, at both high and low density, were affected similarly by conditioned media from N19-OLG cultures overexpressing 21.5-kDa MBP, indicating that N2a-N2a cell-cell contact is also not required. **C:** Quantification of branching complexity from cell cultures shown in B. The N2a cultures supplemented with conditioned media from N19-OLG cultures overexpressing 21.5-kDa MBP demonstrated a statistically significant threefold increase in secondary process branching (examples shown in micrographs, with branching indicated by arrowheads) compared with the control. Values represent mean  $\pm$  standard deviation for four experiments with 80 cells counted per experiment. \* $P = 0.05$  by Student's *t*-test. Micrograph at left shows a cell that was categorized as having secondary ( $2^\circ$ ) branching, and that at right is an example of a cell that was characterized as having only primary ( $1^\circ$ ) branching. A color version of this figure can be found in the online version of this article. Scale bars =  $100 \mu\text{m}$ . [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

occurred in the presence of conditioned medium from the N19 cells and thus were independent of N19-N2a cell-cell contact (Fig. 5B,C). A change in process length was not consistently observed. The N2a cells grown at either high or low density in the presence of conditioned medium also showed these changes, indicating further that N2a cell-cell contact was also not required. These results, in total, suggested that mitogens and/or trophic factors secreted from the 21.5-kDa MBP-expressing N19-OLGs were responsible for these changes in pheno-

type by some extracellular signaling mechanism. It has been reported previously that nontransfected N19 cells cocultured with PC12 cells caused neurite outgrowth as a result of release of growth factors, both nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF; Byravan et al., 1994). However, we did not observe this effect with N2a cells. It occurred only when the N2a cells were cocultured with N19 cells transfected with 21.5-kDa MBP or with conditioned medium from 21.5-kDa MBP-transfected N19 cells. Nevertheless,

NGF and BDNF are likely candidates to be confirmed in future studies (Du et al., 2006; Van't Veer et al., 2009; Xiao et al., 2010, 2012).

## DISCUSSION

Here we have studied further the role of the 21.5-kDa MBP isoform, which has an additional 26 amino acids encoded by exon II of the classic MBP gene that cause it to translocate to the nucleus (Smith et al., 2012c). Although both 18.5-kDa and 21.5-kDa isoforms are intrinsically disordered, one notable difference may be that the 21.5-kDa isoform has greater potential to dimerize *in vitro* because of a single cysteinyl residue encoded by exon II (Nye et al., 1995); its *in vivo* quaternary structure is unknown. The exon II-encoded segment also has a higher positive charge density (proportion of basic residues) than segments encoded by other exons (Hill et al., 2005; Hill and Harauz, 2005), which may affect its interactions with nuclear proteins. The *in vitro* interactions of 21.5-kDa MBP with cytoskeletal proteins and with phospholipid membranes are not appreciably different from those of 18.5-kDa MBP (Boggs et al., 2000, 2005; Hill et al., 2005; Hill and Harauz, 2005), but the former isoform may have different targets *in vivo*, including in the nucleus.

It has been reported that transfection of 21.5-kDa MBP into human hepatoma carcinoma (Hep-G2) cells resulted in increased proliferation and blocking of apoptotic pathways (Pan et al., 2009). Here, using BrdU incorporation, we have shown that 21.5-kDa MBP promotes cell proliferation in N19-OLGs in contrast to the 18.5-kDa MBP isoform and that loss of its subcellular localization from the nucleus abolishes this phenotype in tissue culture (Fig. 1). Furthermore, when the major 18.5-kDa isoform of MBP was directed to the nucleus, we did not observe proliferation in culture, which suggests that there are some features specific to the exon II-encoded sequence or other developmental cues that may be required for increases in cell proliferation.

The onset of expression of 21.5-kDa MBP occurs at the developmental stage at which OPC/O2A cells transition into immature OLGs, in which positive regulation of cell proliferation of potential myelinating OLGs would be important. The OPC/O2A cells respond to a number of growth factors such as platelet-derived growth factor (PDGF- $\alpha$ ), neurotrophin (NT-3), and fibroblast growth factor (FGF) that promote self-renewal and proliferation. Further exposure of OPC/O2A cells to cytokines, such as transforming growth factor- $\beta$  (TGF- $\beta$ ) or brain-derived neurotrophic factor (BDNF) can regulate the balance between self-renewal and OLG differentiation (Noble et al., 1988; McKinnon et al., 1990, 1993; Louis et al., 1993; McTigue et al., 1998; Baron et al., 2000).

We have also found here that 21.5-kDa MBP expression in N19-OLGs increases the levels of phosphorylated ERK1/2, Akt1, and rpS6 (Figs. 2–4). Activated (i.e., phosphorylated at Thr202/Tyr204)

ERK1/2 MAP kinases have been shown to respond to a number of extracellular stimuli or cues, including mitogens, growth factors, and cytokines (Roux and Blenis, 2004; Baccarini, 2005; Meloche and Pouyssegur, 2007), and are implicated in oligodendroglial differentiation (Younes-Rapozo et al., 2009; Fyffe-Maricich et al., 2011; Ishii et al., 2012; Xiao et al., 2012). It has also been shown that ERK1/2 activation must be sustained until late G1 phase for successful S-phase entry (Yamamoto et al., 2006). In the current study, we have observed significant increases in ERK1/2 activation in cells that were undergoing mitosis in N19-OLG cell cultures that were transfected with 21.5-kDa MBP. There was an increase in ERK1/2 activation during mitosis in all dividing N19-OLGs, even those not expressing 21.5-kDa-MBP. These data suggest that this activation response may be due to a secreted factor, which might have also increased BrdU incorporation in nontransfected cells in the MBP-21.5-transfected culture.

The p90 ribosomal S6 kinases (RSKs) are downstream of the Ras/ERK1/2 signaling cascade and have been shown to phosphorylate rpS6 exclusively at Ser235/236 *in vitro* and *in vivo* using a mammalian target of rapamycin (mTOR)-independent mechanism (Roux et al., 2007). Our data are consistent with these previous reports in that we observe increases in the phosphorylation of rpS6 at Ser235/236 in N19-OLG cultures expressing 21.5-kDa MBP. It has been established that activation of the ERK1/2 pathway by growth factors, or by conditionally active Raf-1, induces an immediate increase in ribosomal RNA transcription, whereas inactivation of the pathway by MEK1/2 inhibitors suppresses basal and stimulated ribosomal transcription (Stefanovsky et al., 2001). It is reasonable to hypothesize that the 21.5-kDa MBP isoform may be modulating cell proliferation and cell cycle progression through the ERK1/2 regulated signaling pathway and an mTOR-independent mechanism (cf. Tyler et al., 2009; Guardiola-Diaz et al., 2012).

## CONCLUSIONS AND OUTLOOK

Previously, the exon II-containing MBP isoforms (21.5-kDa and 17.22-kDa in the mouse) have been shown to have high expression levels only in developing OLGs (Barbarese et al., 1978). The 21.5-kDa isoform has been localized to the nucleus in various cell lines (Colman et al., 1990) but also to “detergent-insoluble” myelin fractions (Pfeiffer et al., 1993) and more specifically to the radial component of mature myelin, where it has been found to be enriched along with myelin 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), actin, and tubulin (Karthigasan et al., 1994, 1996). We have also shown earlier that, although 21.5-kDa MBP was localized primarily to the nucleus of N19-OLGs, it partially redistributed to the cytosol and to membrane ruffles on stimulation with phorbol ester (Smith et al., 2012b) and also partially redistributed to the cytosol after culture at high cell density

(Smith et al., 2012a). The exon II-containing 21.5- and 17.22-kDa MBP isoforms may have specific functional, and not solely structural, roles at different stages of myelination, in the radial component of mature myelin (see discussion in Boggs, 2006; DeBruin and Harauz, 2007) and in the OLG nucleus or cytosol and membrane under different conditions.

Here we show further that nuclear localization of 21.5-kDa MBP stimulates proliferation of N19-OLGs through the ERK1/2, Akt1, and ribosomal protein S6 pathways and promotes neurite outgrowth and branching by N2a cells through secretion of a soluble trophic factor, such as BDNF or NGF. This study thus suggests that the nuclear localization of early minor isoforms of MBP may play a crucial role in regulating and/or initiating myelin and neuronal development in the mammalian CNS.

Mice with a double knockout of FcR $\gamma$  (the  $\gamma$ -chain of immunoglobulin Fc receptors) and Fyn (a nonreceptor tyrosine kinase) were found to be resistant to cuprizone-induced demyelination (Seiwa et al., 2007). The levels of phosphorylated 21.5-kDa MBP in particular were found to be decreased in such mice as well as in mice exhibiting demyelination from age or cuprizone treatment. Of interest also is a recent, independent study in which it was shown the Golli isoforms stimulate oligodendrocyte progenitor cell proliferation in the remyelinating adult mouse brain after cuprizone withdrawal (Paez et al., 2012). The roles of the classic 21.5-kDa MBP isoform and its phosphorylated forms, in myelination and remyelination after cuprizone or other causes of demyelination, require further study. In summary, the 21.5-kDa MBP isoform increases proliferation of early developmental N19-OLGs; levels of phosphorylated ribosomal protein S6 and of active ERK1/2 and Akt1 kinases are increased; these phenomena are abolished when nuclear import is inhibited; and N2a neurite outgrowth is increased in coculture with N19-OLGs.

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