Classical 18.5-and 21.5-kDa Isoforms of Myelin Basic Protein Inhibit Calcium Influx Into Oligodendroglial Cells, in Contrast to Golli Isoforms

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Abstract

The myelin basic protein (MBP) family arises from different transcription start sites of the golli (gene of oligodendrocyte lineage) complex, with further variety generated by differential splicing. The “classical” MBP isoforms are peripheral membrane proteins that facilitate compaction of the mature myelin sheath but also have multiple protein interactions. The early developmental golli isoforms have previously been shown to promote process extension and enhance Ca^{2+} influx into primary and immortalized oligodendrocyte cell lines. Here, we have performed similar studies with the classical 18.5- and 21.5-kDa isoforms of MBP. In contrast to golli proteins, overexpression of classical MBP isoforms significantly reduces Ca^{2+} influx in the oligodendrocyte cell line N19 as well as in primary cultures of oligodendroglial progenitor cells. Pharmacological experiments demonstrate that this effect is mediated by voltage-operated Ca^{2+} channels (VOCCs) and not by ligand-gated Ca^{2+} channels or Ca^{2+} release from intracellular stores. The pseudo-deiminated 18.5-kDa and the full-length 21.5-kDa isoforms do not reduce Ca^{2+} influx as much as the unmodified 18.5-kDa isoform. However, more efficient membrane localization (of overexpressed, pseudo-deiminated 18.5-kDa and 21.5-kDa isoforms of classical MBP containing the 21-nt 3'-untranslated region transit signal) further reduces the Ca^{2+} response after plasma membrane depolarization, suggesting that binding of classical MBP isoforms to the plasma membrane is important for modulation of Ca^{2+} homeostasis. Furthermore, we have found that the mature 18.5-kDa isoform expressed in oligodendrocytes colocalizes with VOCCs, particularly at the leading edge of extending membrane processes. In summary, our findings suggest a key role for classical MBP proteins in regulating voltage-gated Ca^{2+} channels at the plasma membrane of oligodendroglial cells and thus also in regulation of multiple developmental stages in this cell lineage.

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Keywords

myelin basic protein (MBP); oligodendrocytes; glia; RNA transport signal (RTS); voltage-gated calcium channels; calcium imaging

The myelin basic protein (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). Golli proteins are expressed from transcription start site 1 in myelin-producing cells (Landry et al., 1996, 1997, 1998) and also in macrophages and T cells of the immune system (Feng et al., 2002, 2006). Golli isoforms promote process extension and enhance potassium-induced calcium influx into primary and immortalized (N19) oligodendrocytes (OLGs), a phenomenon dependent on plasma membrane targeting (Paez et al., 2007).

Classical MBP isoforms arising from transcription start site 3 (which range in size from 14 kDa to the full-length 21.5-kDa transcript and henceforth are referred to simply as MBP for simplicity) primarily facilitate compaction of the adult central nervous system (CNS) myelin sheath and also interact with actin, tubulin, calmodulin, and SH3-domain proteins (Boggs, 2006, 2008; Harauz et al., 2009). Classical MBP isoforms have intricate mRNA translocation and regulation properties. It has been demonstrated by microinjection in primary OLGs that fluorescently labeled mRNA coding for mouse 14-kDa MBP forms granules in the cell body, which are subsequently transported through OLG extensions to areas of compact myelin (Ainger et al., 1993, 1997). This transportation and localization of MBP mRNA depends on a minimal 21-nucleotide 3'-untranslated region (3'UTR). This minimal sufficient mRNA transport signal (RTS) packages the mRNA into "granules" that are trafficked in a microtubule-dependent manner to the peripheral processes of developing OLGs (Ainger et al., 1997). The translocation is suggested to aid in the immediate insertion of newly translated MBP directly into the OLG plasma membrane (Hardy et al., 1996), thus targeting the highly charged protein to the plasma membrane (Maier et al., 2008). In contrast, although the early 21.5-kDa isoform of MBP is present in compact myelin, it has predominantly nuclear localization within OLGs, suggesting additional roles in the nucleus (Pedraza et al., 1997; Pedraza and Colman, 2000).

Here, we have constructed several fluorescently tagged recombinant versions of classical murine MBP isoforms: 1) MBP-C1, which emulates the predominant, minimally modified 18.5-kDa C1 component of healthy myelin (Bates et al., 2000); 2) MBP-C8 with six R/K-to-Q substitutions to mimic deimination in the highly modified C8 component (Bates et al., 2002), which is found in increased proportion in multiple sclerosis (MS; Moscarello et al., 2007); and 3) an exon II-expressing 21.5-kDa isoform (MBP-21.5), which is expressed earlier in development compared with the 18.5-kDa C1 and C8 isoforms. We show here that, in contrast to golli proteins, these classical MBP isoforms significantly decrease calcium influx into OLGs and, furthermore, that the 21-nucleotide 3'UTR further enhances the calcium response in comparison with MBPs without this signal. These results demonstrate that efficient transport of MBP to the OLG plasma membrane is dependent on its 3'UTR,

and that one of the functions of classical MBP may be to maintain constant levels of Ca^{2+} within the OLG cytoplasm.

MATERIALS AND METHODS

Plasmid Construction

Recombinant DNA molecules or plasmids were produced using restriction endonucleases and other enzymes provided by New England Biolabs Ltd. (Mississauga, Ontario, Canada). Polymerase chain reaction (PCR) amplifications were performed with a Bio-Rad (Hercules, CA) thermal cycler PCR system using ProofStart DNA Polymerase (Qiagen Inc., Mississauga, Ontario, Canada) with the following cycling parameters: initial denaturing temperature of 95°C for 5 min; followed by 45 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec; followed by a final 4°C hold. For murine MBP-RFP constructs, full-length cDNAs coding for 18.5-kDa recombinant murine MBPC1, MBPC8, and 21.5-kDa MBP21.5 were obtained from previously described pET22b protein overexpression vectors (Bates et al., 2002). The MBPC1, MBPC8, and MBP21.5 variants were cloned into the pERFP-C1 vector using BspEI and XbaI restriction sites. (n.b., The “C1” of the RFP vector designation is not to be confused with the “C1” charge component of MBP.) The BspEI restriction site was introduced to all three corresponding cDNAs using the common forward primer RMBPFp1, and the XbaI site was introduced to MBPC1 and MBP21.5 using primer RMBPRp1. For MBPC8, the primer RMBPRp2 was used. Amplified products were digested with BspEI and XbaI and were ligated into the pERFP-C1 vector. Next, two oligonucleotides, namely, UTRFp1 and UTRRp1, were annealed together to produce a synthetic 21-nucleotide 3'UTR insert. 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-μl reaction contained: 50 μl of the annealed oligonucleotide PCR, 10 μl of 10 × T4 polynucleotide kinase buffer (New England Biolabs), 1.3 μl of 2.55 mM ATP, 2.5 μl of T4 polynucleotide kinase, and 36.2 μl ddH₂O. The resulting reaction was precipitated, purified, and ligated into pERFP-C1-MBPC1, pERFP-C1-MBPC8, and pERFP-C1-MBP21.5 constructs that had previously been digested with XhoI. (Again, the “C1” of the RFP vector is different from the MBP “C1” charge component.) The resulting constructs were confirmed by sequencing (Laboratory Services Division, University of Guelph). The GFP-tagged versions of these MBP isoforms were constructed by the same procedure using the pEGFP-C1 backbone vector (Clontech, Mountain View, CA). A complete list of all primers used for recombinant DNA construction can be found in Table I.

Primary Cultures of Cortical Oligodendrocyte Precursor Cells

Enriched oligodendrocytes were prepared as described (Amur-Umarjee et al., 1993). First, cerebral hemispheres from 1-day-old mice were mechanically dissociated and were plated on poly-D-lysine-coated flasks in Dulbecco's modified Eagle's and Ham's F12 media (1:1 vol/vol; Invitrogen Life Technologies, Burlington, Ontario, Canada), containing 100 μg/ml gentamycin and supplemented with 4 mg/ml dextrose anhydrous, 3.75 mg/ml HEPES buffer, 2.4 mg/ml sodium bicarbonate, and 10% fetal bovine serum (FBS; Omega Scientific). After

24 hr, the medium was changed and the cells were grown in DMEM/F12 supplemented with insulin (5 µg/ml), transferrin (50 µg/ml), sodium selenite (30 nM), T₃ (15 nM), d-biotin (10 mM), hydrocortisone (10 nM), 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO), 1% horse serum, and 1% FBS (Omega Scientific). After 9 days, oligodendrocytes were purified from the mixed glial culture by the differential shaking and adhesion procedure as described elsewhere (Suzumura et al., 1984) and allowed to grow on polylysine-coated coverslips in defined culture media (Agresti et al., 1996) with added PDGF (10 ng/ml) and bFGF (10 ng/ml; Peprotech). Twenty-four hours after plating, the cells were transiently transfected with pERFP-C1-MBPC1 and pERFP-C1-MBPC8. Briefly, 1.0 µg of plasmid DNA was mixed with Lipofectamine 2000 (Invitrogen), and the mixture was placed on 35-mm Petri dishes containing 80% confluent oligodendrocytes. Oligodendrocytes were then further cultured for 48 hr in defined culture media (Agresti et al., 1996) with added PDGF (10 ng/ml) and bFGF (10 ng/ml) before being used for Ca²⁺ imaging experiments.

Cell Line Culture and Transfection

Tissue culture reagents were purchased from Gibco/Invitrogen (Invitrogen Life Technologies). The FuGene HD transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). The N19 immortalized oligodendroglial cell line was grown in DMEM high-glucose media supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin and cultured in 10-cm plates at 34°C/5% CO₂. At 70–80% confluence (4–7 days), cells were detached using 0.25% trypsin for 5 min, and were seeded onto 2-cm plates containing a glass coverslip. Cells were grown overnight to a confluence of 15% prior to transfection using 100 µl serum-free media, 0.75–3.0 µg of plasmid DNA, and 4 µl of FuGene HD. The DNA was allowed to complex for 5 min at room temperature and was directly added to cells following incubation. Cells were cultured for an additional 48 hr at 34°C prior to treatment, fixation, or immunoprocessing.

Immunofluorescence, Microscopy, and Image Analyses

After protein expression, untreated cells were fixed directly using 4% formaldehyde in phosphate-buffered saline (PBS) solution for 15 min with gentle rocking. Samples requiring immunoprocessing were permeabilized using 0.1% vol/vol Triton X-100 for 20 min and were subsequently washed once with 1 ml PBS. Slides were blocked for 1 hr using 10% normal goat serum (NGS), and, after this incubation, the primary antibody (1:100) was added and incubated for an additional 1 hr. The slides were then washed three times with 1 ml PBS, and the secondary goat anti-mouse A488 antibody (1:400 dilution) was applied for 20 min. Once again, the slides were washed four times with 1 ml PBS and were mounted using ProLong Gold AntiFade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Slides were viewed using a Leica epifluorescent microscope (DMRA2), an Olympus spinning disc confocal microscope (IX81), or a multiphoton scanning confocal microscope (Leica DM6000). Images were processed in ImageJ software (National Institutes of Health) and were compiled in Adobe Photoshop CS3.

Immunoblotting

The N19 cell cultures grown in 10-cm plates were transfected with 8 µg of plasmid DNA encoding the various MBP isoforms. After 48 hr of expression, cells were harvested in 400

μl PLC (passive lytic components) lysis buffer supplemented with fresh protease inhibitors (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). From each transfection crude lysate, a 20-μl aliquot from each sample was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was transferred using a semidry system (Bio-Rad) to a polyvinylidene fluoride (PVDF) membrane. The following antibodies were obtained from commercial sources: antibody for β-actin (1:2,500 dilution; Sigma; A-5316 clone AC-74), rabbit polyclonal anti-Ca_v1.2 (1:100 dilution; Millipore, Bedford, MA; catalog No. AB5156 against the α-subunit), rabbit IgG peroxidase conjugate (1:20,000 dilution; Sigma; A-9169). Immunoblots were detected using an enhanced chemiluminescence (ECL) advanced Western blotting detection kit (GE Healthcare Life Sciences, Piscataway, NJ).

Calcium Imaging

Methods were similar to those described previously (Colwell, 2000; Michel et al., 2002; Paz Soldan et al., 2003). Briefly, a cooled CCD camera (ORCA-ER; Hamamatsu, Hamamatsu City, Japan) was added to the Olympus (Melville, NY) spinning disc confocal microscope to measure fluorescence. To load the dye into cells, the coverslips were washed in serum and phenol red-free DMEM, and the cells were incubated for 45 min at 37°C, 5% CO₂ in the same medium containing a final concentration of 4 μM Fura-2 AM (TefLabs, Austin, TX) plus 0.08% pluronic F-127 (Invitrogen), washed four times in DMEM, and stored in DMEM for 0–1 hr before being imaged (Paz Soldan et al., 2003). Resting calcium levels were measured in serum-free HBSS containing 2 mM Ca²⁺ but no Mg²⁺. Other measurements were made in HBSS. Calcium influx and resting Ca²⁺ levels were measured on individual cells, and the results were pooled from five separate coverslips representing five separate cell preparations for each condition. The fluorescence of Fura-2 was excited alternatively at wavelengths of 340 and 380 nm by means of a high-speed wavelength-switching device (Lambda DG-4; Sutter Instruments, Novato, CA). Image analysis software (SlideBook 4.1; Intelligent Imaging Innovations, San Diego, CA) allowed the selection of several “regions of interest” within the field from which measurements were taken. To minimize bleaching, the intensity of excitation light and sampling frequency was kept as low as possible. In these experiments, measurements were normally made once every 2 sec.

Calibration of Ca²⁺ Signals

Free [Ca²⁺] was estimated from the ratio (R) of fluorescence at 340 and 380 nm, using the following equation: $[Ca^{2+}] = K_d \times \text{slope factor} \times (R - R_{\min}) / (R_{\max} - R)$ (Grynkiewicz et al., 1985). The K_d was assumed to be 140 nM, whereas values for R_{min} and R_{max} were all determined via calibration methods. An in vitro method (Fura-2 Ca²⁺ imaging calibration kit; Invitrogen) was used to estimate values. With this method, glass coverslips were filled with a high-[Ca²⁺] solution (Fura-2 plus 10 mM Ca²⁺), a low-[Ca²⁺] solution (Fura-2 plus 10 mM EGTA), and a control solution without Fura-2. Each solution also contained a dilute suspension of 15-μm-diameter polystyrene microspheres to ensure uniform coverslip/slide separation and facilitate microscope focusing. The fluorescence (F) at 380 nm excitation of the low-[Ca²⁺] solution was imaged, and the exposure of the camera was adjusted to

maximize the signal. These camera settings were then fixed, and measurements were made with 380 and 340 nm excitation of the three solutions. Here, $R_{\min} = F_{340 \text{ nm in low-[Ca}^{2+}\text{]}}/F_{380 \text{ in low-[Ca}^{2+}\text{]}}$; $R_{\max} = F_{340 \text{ in high-[Ca}^{2+}\text{]}}/F_{380 \text{ in high-[Ca}^{2+}\text{]}}$; $S_f = F_{380 \text{ in low-[Ca}^{2+}\text{]}}/F_{380 \text{ in high-[Ca}^{2+}\text{]}}$.

Statistical Analysis

Because ectopic protein expression experiments inherently result in differential protein levels within individual cells throughout an entire cell population, in this current study, cell cultures for calcium investigations were transfected with the same quantity (1 μg) of each plasmid for each experiment. Furthermore, changes in calcium levels were recorded for over 500 cells per treatment to gather an accurate overall effect of each MBP isoform on VOCC activity within a cell population. Data are presented as mean $\pm$ SEM unless noted otherwise. For the Fura-2 experiments, statistical comparison between different experimental groups was performed by analysis of covariance.

RESULTS

Oligodendroglial Cells Overexpressing Classical MBP Isoforms Exhibit Decreased Ca^{2+} Influx After Membrane Depolarization

The conditionally immortalized N19 oligodendroglial line was chosen as a relevant stage-specific cell line to examine expression of different MBP isoforms in tissue culture (Verity et al., 1993). This cell line stains positively for both NG2 and A2B5 antibodies, which are markers for oligodendroglial progenitor cells (OPCs), lack expression of classical MBP and proteolipid mRNAs (Foster et al., 1993), and have been successfully employed in other OLG calcium homeostasis studies, including specifically examining the effect of golli overexpression (Paez et al., 2007; Jacobs et al., 2009; Fulton et al., 2010).

To determine whether classical MBPs have a role in Ca^{2+} homeostasis similar to that of golli, we first overexpressed different GFP-MBP constructs into N19 cells and stimulated the cells using high $[\text{K}^+]$ at a concentration of 20 mM. Intracellular concentrations of Ca^{2+} were measured in individual Fura-2-loaded cells using an Olympus spinning disc confocal microscope equipped with calcium-imaging software. High $[\text{K}^+]$ was applied to the cells by a fast and local perfusion system, and the Fura-2 ratios in selected cells were plotted with respect to the time of stimulation (Fig. 1A; n.b., each line represents an analysis of a single cell). Using this treatment, we detected a change in intracellular $[\text{Ca}^{2+}]$ following 6 min of K^+ treatment and found that the N19 cells overexpressing MBP-21.5 and MBP-C1 had significantly lower intracellular Ca^{2+} concentrations than the transfected cells with unmodified GFP vector (control; Fig. 1A–D). In contrast to golli proteins, classical MBP isoforms appeared to decrease Ca^{2+} uptake significantly after plasma membrane depolarization in N19 cells.

The Effect of MBP on N19 Cell Ca^{2+} Homeostasis Is Mediated Through Voltage-Operated Ca^{2+} Channels

Previously, golli proteins were shown to regulate $[\text{Ca}^{2+}]$ in OLGs specifically through voltage-gated calcium channels (VOCCs; Paez et al., 2009a; Fulton et al., 2010). To

determine whether the decreased calcium levels were due to an effect of MBP on VOCCs, we used several pharmacological agents that have been recognized to modulate VOCCs in previous studies (Kostyuk and Krishtal, 1977; Lansman et al., 1986). Verapamil and nifedipine are specific L-type VOCC blockers, whereas Bay K 8644 is an L-type Ca^{2+} agonist that prolongs single channel open time without affecting the close time. Here, Bay K 8644 enhanced the amplitude of Ca^{2+} uptake in control cells by ~30%, but this VOCC agonist was less effective in N19 cells overexpressing MBP-21.5 and MBP-C1, producing only a ~10% increase in the amplitude of Ca^{2+} uptake. These data clearly show an inhibitory effect of classical MBP isoforms on N19 voltage-operated Ca^{2+} channels. In support of this conclusion, we observed that the decreases in Fura-2 signal in N19 cells overexpressing classical MBP isoforms were abolished in the presence of zero $[\text{Ca}^{2+}]$ and were blocked by Cd^{2+} , verapamil, and nifedipine, confirming that these changes in intracellular Ca^{2+} result from MBP-mediated inhibition of Ca^{2+} influx via VOCCs (Fig. 2). Altogether, these results indicate that overexpression of MBP modulates Ca^{2+} levels in N19 cells via VOCCs.

To examine how MBP regulates calcium homeostasis in its entirety, we investigated the effect of several agonists that activate or block different routes that generate Ca^{2+} signaling in OLGs. We performed a number of additional stimulation experiments using glutamate, ATP, thapsigargin, and caffeine in N19 OLGs transfected with MBP-C1 and MBP-C8.

To analyze the effect of classical MBP isoforms on ligand-gated Ca^{2+} -channels, Ca^{2+} uptake was stimulated in N19 cells using ATP and glutamate. We performed these experiments in either the presence or the absence of extracellular Ca^{2+} , which allowed us to assess the participation of metabotropic and ionotropic ATP and glutamate receptors. Oligodendrocytes can exhibit Ca^{2+} responses to ATP, either through P2X receptors, which are ligand-gated, nonselective Ca^{2+} channels, or through P2Y receptors, which are metabotropic receptors linked to G-protein activation and to IP₃-dependent Ca^{2+} release. Treatment of N19 cells overexpressing MBP-C1 or MBP-C8 with ATP, with or without extracellular Ca^{2+} , displayed no difference vs. control (Fig. 3A). Glutamate also activates ionotropic receptors, which gate membrane ion channels permeable to Ca^{2+} and meta-botropic receptors, which are coupled to G-proteins. Ionotropic glutamate receptors (i.e., AMPA/kainate) are expressed by OLGs and OPCs. Similarly to ATP, no significant differences in intracellular Ca^{2+} responses between groups were found after glutamate stimulation (Fig. 3A). Taken together, these data demonstrate that classical MBPs do not affect ionotropic or metabotropic glutamate and ATP receptors, further supporting the notion that classical MBP modulates calcium homeostasis via VOCCs in OLGs.

To assess Ca^{2+} efflux from intracellular stores, we analyzed Ca^{2+} responses in N19 cells in the presence of caffeine and thapsigargin. Release of Ca^{2+} from intracellular stores occurs via inositol 1,4,5-triphosphate (IP₃) or ryanodine receptors, and Ca^{2+} is subsequently resequenced into stores via sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPases (SERCAs) (Berridge, 2004). In many cell types, SERCA inhibition leads to elevation of cytosolic $[\text{Ca}^{2+}]$, secondary to leakage of Ca^{2+} from stores. Thapsigargin is used to inhibit SERCA pumps in cultured cells, and it has been demonstrated that SERCA-mediated store depletion activates store-operated Ca^{2+} entry in glial cells (Simpson et al., 1997). The results presented here show that inhibition of SERCA pumps by thapsigargin, or ryanodine receptor activation

by caffeine, evoked significant Ca^{2+} release from intracellular stores in N19 cells in the presence of normal extracellular $[\text{Ca}^{2+}]$ (Fig. 3B). Our data clearly indicate that, with these pharmacological treatments, the MBP-transfected N19 cells did not demonstrate a decrease in intracellular Ca^{2+} concentration compared with controls (Fig. 3B). Additionally, the effect of extracellular Ca^{2+} on caffeine and thapsigargin responses was examined in parallel cultures by perfusion with caffeine or thapsigargin in Ca^{2+} -free medium. This procedure evoked a slow-onset transient elevation of intracellular $[\text{Ca}^{2+}]$ in all groups. This response was smaller in amplitude and, under this experimental condition, we did not find any appreciable difference between MBP-transfected and nontransfected N19 cells (Fig. 3B), suggesting that Ca^{2+} release from intracellular stores is not affected by the expression of classical MBP isoforms. In summary, we found a significant decrease in intracellular Ca^{2+} concentration only in cells overexpressing classical MBP isoforms depolarized with high $[\text{K}^+]$, indicating that the calcium changes induced by MBP expression are mediated exclusively by the inhibition of voltage-gated Ca^{2+} uptake.

OPCs Transfected With Classical MBP Isoforms Exhibit Decreased Ca^{2+} Uptake

To confirm the notion that MBP proteins play a role in Ca^{2+} homeostasis in OLGs, we transfected primary cultures of cortical OPCs with different MBP constructs and examined the effect of depolarization with high $[\text{K}^+]$ to induce Ca^{2+} uptake in the cells. As expected, the experiment showed a significant decrease in Ca^{2+} influx induced by high $[\text{K}^+]$ in cells overexpressing MBP-C1 and MBP-C8 vs. control OPCs (Fig. 4). During potassium stimulation, Bay K 8644 enhanced the amplitude of Ca^{2+} uptake in OPCs, but, in cells overexpressing MBP-C1 and MBP-C8, this VOCC agonist was significantly less effective than in control OPCs (Fig. 4C). In addition, we observed that the decreases in Fura-2 signal in OPCs overexpressing classical MBP isoforms were abolished in the presence of zero $[\text{Ca}^{2+}]$ and were blocked by verapamil, confirming that these changes in intracellular Ca^{2+} concentration result from Ca^{2+} influx via VOCCs (Fig. 4C).

The 3' mRNA UTR is Necessary for Efficient Membrane Localization of Overexpressed MBP

Unlike golli proteins, classical MBP isoforms arising from the third transcription start site do not possess an N-terminal myristoylation site, although they are N-terminal acylated (for review see Harauz et al., 2009; Harauz and Libich, 2009). Deletion of the first 45 or 110 amino acids from the N-terminus of the J37 golli has been shown to abolish its role in calcium influx in N19 OLGs, and, furthermore, a single point mutation from Gly2→Ala2 of both J37 or BG21 golli also reversed the effects of calcium influx normally observed with golli overexpression (Feng et al., 2006; Paez et al., 2007). These studies demonstrate that close association of the protein with the plasma membrane is required for the enhancement of Ca^{2+} entry into OLGs.

Efficient transportation and localization of classical MBP mRNA into the plasma membrane of OLGs in CNS myelin is dependent on a minimal 21-nucleotide 3'UTR present in the mRNA transcript (Ainger et al., 1993, 1997). To evaluate the importance of this 3'UTR, we next produced a series of RFP-MBPs containing the minimal 3' mRNA transit signal, for comparison with the series described above that lack this signal. This signal was inserted

into each plasmid directly following the termination codon of each fusion protein. When expressed in N19 OLGs, the UTR region obviously increased the efficiency of membrane targeting and localization of MBP-C1 and MBP-C8, although it was too low to detect when evaluating the localization of MBP-21.5 (Fig. 5A). To illustrate further the differences in protein localization of MBP-C1 (without 3' UTR) vs. MBP-C1-UTR, a graph plotting fluorescent pixel intensity over the length of the cell was generated; it shows an increase in the amount of localized protein present in the membrane extensions of the OLGs and exclusion from the nucleus when compared with DAPI counter-stain (Fig. 5B).

MBPs Possessing the 3' UTR Further Enhance the Ca²⁺ Response in N19 Cells

Considering the increases in membrane localization observed with MBP variants containing a 3' UTR, along with previous studies demonstrating that calcium response in OLGs overexpressing golli is dependent on membrane targeting (Paez et al., 2007), we next compared whether there were any differences in calcium response in OLGs overexpressing classical MBPs possessing the 3' UTR compared with the constructs devoid of this signal. Both the MBP-C8 and the MBP-21.5 isoforms containing 3' UTRs further decreased calcium entry into OLGs, although no difference was observed for MBP-C1 (Fig. 6). Possibly this isoform is already adequately expressed in the plasma membrane to inhibit Ca²⁺ uptake maximally.

VOCCs Are Concentrated in the Extending Membrane Processes of N19 Cells and Colocalize With MBPs

Our results described above support the suggestion that the close membrane association of MBP may modulate VOCCs by direct or indirect protein-protein interactions. To establish the subcellular relationship between MBPs and L-type VOCCs in OLGs, we employed a series of immunofluorescence studies, specifically investigating L-type Ca²⁺ channels. Previously, by using time-lapse confocal microscopy to monitor protein concentration, golli proteins were shown to modulate calcium influx in OLGs at golli-enriched "patches" of the plasma membrane where process retraction and extension was occurring (Paez et al., 2007). Our immunostaining of cultured N19 OLGs showed that, during cell spreading, VOCCs were concentrated at the leading edge of extending membrane OLG processes, and these results were consistent with previous notions of golli and VOCC interactions (Fig. 7A). Cells expressing MBP-C1-UTR or MBP-C8-UTR showed areas of colocalization of 18.5-kDa MBP with L-type VOCCs, particularly at the leading edge of extending membrane processes, whereas cells expressing MBP-21.5-UTR showed no obvious areas of colocalization of 21.5-kDa MBP with L-type VOCCs (Fig. 7B).

Overexpression of MBP-21.5 Changes the Intracellular Distribution of VOCCs in N19 Cells

One notable observation when viewing OLG cultures stained for VOCCs was that overexpression of MBP-21.5-UTR changed the cellular distribution of L-type VOCCs in N19 OLGs compared with MBP-C1-UTR, MBP-C8-UTR, or control experiments (Fig. 7C). This staining pattern was less intense in the OLG extensions, and L-type VOCC localization was observed predominantly in the cell body of MBP-21.5-UTR transfected cells, similarly to other surrounding cells in culture that were not transfected. The global changes observed in all N19 cells in culture exposed to MBP-21.5-UTR-transfected cells suggest that the

distribution of L-type VOCCs is potentially mediated by a soluble factor and/or cell–cell interactions, as opposed to direct effects caused exclusively in OLGs overexpressing the protein. Alternatively, 18.5-kDa MBP might have a positive effect in directing VOCCs to membrane processes. With a decreased staining pattern observed in OLG processes, we performed a Western blot to determine whether MBP-21.5-UTR was altering protein expression levels of VOCCs. Total protein was harvested from cultures of OLGs expressing MBP isoforms, and no significant differences in VOCC expression were observed between MBP isoforms (Fig. 7D).

DISCUSSION

MBP is required for tight compaction of the multi-lamellar structure of CNS myelin, holding together the apposing cytosolic leaflets of the oligodendrocyte membrane. Recently it has been appreciated that MBP is more than a simple, structural scaffolding protein; it can participate in a number of biological interactions ranging from PIP₂/PIP₃ sequestration, cytoskeletal actin and tubulin polymerization and stability, Ca²⁺-calmodulin binding, to binding with SH3-domain proteins (for review see Boggs, 2006; Harauz et al., 2009). The current study analyzed Ca²⁺ response in N19 and primary OPC cultures overexpressing different charge components and splice isoforms of classical MBP. The conditionally immortalized N19 OLG cell line expresses many well-established markers of OLGs, including Olig2, NG2, Sox2, and Sox9 (Foster et al., 1993; Verity et al., 1993; Byravan et al., 1994; Bongarzone et al., 1996). When microinjected into the subventricular zone (SVZ) of the developing brain of *shiverer* mice, N19 cells migrate and form myelin-like sheaths around the axons (Foster et al., 1995). Using this cell line primarily, we have shown a new functional role for classical MBP, demonstrating that it participates in calcium homeostasis in OLGs. Furthermore, we address the physiological importance and subtleties of inherent protein trafficking signals contained within transcribed nascent MBP mRNA.

MBP Decreases Calcium Influx Following Plasma Membrane Depolarization in Oligodendroglial Cells

Electrophysiological experiments have shown at least six pharmacologically distinct types of VOCCs (P/Q-, L-, N-, R-, and T-type) that are heterologously distributed in the rat CNS (Snutch et al., 1990; Ishibashi et al., 1995). The regional distribution and functional roles of VOCCs in excitable cells such as neurons have been shown to be important for neurotransmitter release and muscle contraction (Olivera et al., 1994; Akaike, 1997; Santafe et al., 2001). More recently, it has been shown that VOCCs, specifically L-, N-, and R-type, are also expressed in supporting glial cells, including OLGs, and play an important role in OPC/OLG migration and maturation (Takeda et al., 1995; Wang et al., 1996; Simpson and Armstrong, 1999; Bergles et al., 2000; Paez et al., 2007, 2009a, b).

In contrast to the earlier developmental golgi proteins (Paez et al., 2007), a significant decrease was found in intracellular Ca²⁺ concentrations in N19 and OPCs overexpressing classical MBP after plasma membrane depolarization, demonstrating a negative regulation of these proteins on OLG voltage-gated Ca²⁺ channels. Metal ions can inhibit and block VOCCs, preventing influx of calcium into cells (Kostyuk and Krishtal, 1977; Lansman et al.,

1986). Our results have shown that Cd^{2+} completely inhibited the MBP-induced Ca^{2+} effect after high- $[\text{K}^+]$ stimulation. Moreover, pretreatment with verapamil or nifedipine, specific VOCC blockers, abolished the effect of calcium reduction observed in OLGs that are overexpressing MBPs, further supporting the conclusion that classical MBP isoforms modulate calcium influx through VOCCs.

Golli and classical MBP proteins arise from the same gene complex and are differentially expressed throughout OLG maturation, and it is reasonable to propose that one of the roles of classical MBP may be to down-regulate intracellular Ca^{2+} concentrations following golli expression, eventually leading to cellular events that may promote decreased migration and augment maturation of OLGs.

Intracellular Ca^{2+} concentrations can be altered from a number of different routes, so we examined calcium response in the presence of several agonists to activate different mechanisms that generate Ca^{2+} signaling in OLGs. Oligodendrocytes exhibit Ca^{2+} responses to glutamate, which activate ionotropic receptors, that gate Ca^{2+} through membrane ion channels and/or metabotropic receptors that are coupled to G-proteins (Dingledine et al., 1999). Although it has been shown that OLGs express ionotropic glutamate receptors such as α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate (McDonald et al., 1998), we found no significant change in intracellular Ca^{2+} concentrations in response to glutamate in N19 OLGs over-expressing classical MBPs.

Oligodendrocytes have also been shown to respond to ATP by activating P2X or P2Y receptors, which are ligand-gated nonselective Ca^{2+} channels or metabotropic receptors that respond to G-protein activation and IP3-dependent Ca^{2+} release, respectively (Kirischuk et al., 1995; Takeda et al., 1995; von Kukelgen and Wetter, 2000; Khakh, 2001). Through these mechanisms, ATP can initially produce an increase in $[\text{Na}^+]$, triggering a secondary increase in intracellular $[\text{Ca}^{2+}]$ through VOCCs. The N19 OLGs overexpressing classical MBP isoforms following ATP treatment displayed no difference in Ca^{2+} concentrations vs. control, suggesting that these types of Ca^{2+} -channels are not affected by expression of classical MBPs. [A recent, independent study has shown binding of golli proteins to STIM1, the master regulator of store-operated Ca^{2+} channels (Walsh et al., 2010); Walsh and colleagues propose that Golli-BG21 functions to regulate store-operated Ca^{2+} influx via a direct interaction with STIM1, but the exact molecular mechanism underlying this interaction has yet to be defined.]

Efficient MBP Membrane Localization is Dependent on a Minimal 21-nt 3' UTR Sequence

Several studies to date have used ectopic expression of fluorescently tagged MBP to investigate functional properties of the protein, including subcellular localization, roles in myelination, and interactions with PIP_2 in the plasma membrane (Barbarese et al., 1988; Pedraza and Colman, 2000; Nawaz et al., 2009). Efficient transport and local translocation of MBP mRNA to the cell plasma membrane has been shown to depend on a minimal RTS (Ainger et al., 1997). The MBP mRNA has been shown to contain a *cis*-acting element termed the *A2 response factor* found within this 3'UTR region, which binds to the heterogeneous nuclear ribonucleoprotein (hnRNP), together forming "granules" that are eventually exported from the nucleus to the cytoplasm, where they are trafficked to

oligodendrocyte processes in a microtubule-dependent manner (Ainger et al., 1997; Munro et al., 1999; Carson and Barbarese, 2005).

Previous investigations examining MBP expression using constructs with and without the presence of a 3'UTR RTS have observed indistinguishable MBP localization (Nawaz et al., 2009). Here, we have shown both quantitatively through arbitrary fluorescence intensity and with enhanced calcium response in N19 OLGs that this RTS makes a significant contribution to protein localization and function at the plasma membrane. However, this UTR seems to play a less significant role in VOCC calcium regulation for the MBP-C1 component compared with MBP-C8 and MBP-21.5. As the least-modified form of classical MBPs, MBP-C1 has a net charge of +19, which has been shown to associate via stronger electrostatic interactions with the lipid membrane compared with MBP-C8 (Bates et al., 2002, 2003; Musse et al., 2006). It is possible that highly positively charged MBP-C1 naturally associates with the membrane on the cytosolic leaflet and that the 3'UTR does not further enhance its interactions with VOCCs.

Overexpression of MBP-21.5 Changes the Heterologous Distribution of L-Type VOCCs

We demonstrate in this study that Ca^{2+} channels are not evenly distributed in the membrane of glial precursor N19 cells overexpressing MBP-21.5. Such an uneven distribution of ion channels or receptors has been described before, e.g., for γ -aminobutyric (GABA) receptors in Bergmann glial cells (Muller et al., 1993; Muller and Kettenmann, 1995). It has been shown that the low-voltage-activated Ca^{2+} channels are concentrated at the processes; the high-voltage-activated (L-type) are also present in the somatic region of OPCs (Kirischuk et al., 1995). The present study showed that MBP-21.5 overexpression in N19 cells resulted in redistribution of L-type VOCCs compared with the later developmental 18.5-kDa MBP isoform, suggesting that it may play a different role. It is also possible that 18.5-kDa MBP causes redistribution and enrichment of VOCCs at the leading edge of extending processes of OLGs compared with 21.5-kDa MBP. The L-type VOCC immunostaining was less intense in process extensions and appeared to be more concentrated at the somatic region of transfected N19 cells with MBP-21.5. We assume that the glial Ca^{2+} channels are linked to the cytoskeleton via linker proteins such as ankyrin or spectrin, similar to other ion channels in neurons (Srinivasan et al., 1988). The interaction of classical MBP isoforms with cytoskeletal proteins (actin and tubulin) is well known (Boggs et al., 2005; Hill et al., 2005; Hill and Harauz, 2005), and changes in cytoskeletal dynamics resulting from overexpression of MBP-21.5 are one possible explanation for this phenomenon. Alternatively, it is possible that the overexpression of MBP-21.5 may change the quantity of VOCCs within the plasma membrane, promoting internalization and degradation of $\text{Ca}_v1.2$ channels, resulting in decreased Ca^{2+} entry. A further explanation for these observed differences in subcellular distribution of VOCCs is that the typical ramified morphology of N19s OLGs could be altered by MBP-21.5 overexpression, resulting in reduced process length and a polygonal cell shape.

MBPs Play a Key Developmental Role in Calcium Homeostasis

The results described here suggest that classical MBPs play a key role in down-regulating Ca^{2+} homeostasis, in contrast to golgi proteins. Recent studies have shown that activation of

PKC, which can regulate VOCC activity, may affect both process extension and migration of OPCs (Paez et al., 2010). Along with golgi proteins, classical MBP isoforms contain numerous potential mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) phosphorylation sites (for review see Harauz et al., 2009; Harauz and Libich, 2009), which may participate directly or indirectly in complex developmental and spatial temporal regulation of OPC migration and OLG differentiation.

Our current results suggest that overexpression of classical MBP isoforms significantly reduces potassium-induced Ca^{2+} influx in OLGs, and further extensive pharmacological and immunofluorescence experiments demonstrate that this effect is mediated specifically by voltage-gated calcium uptake and does not involve Ca^{2+} release from intracellular stores or ligand-gated Ca^{2+} channels (i.e., glutamate/ATP). Efficient membrane localization of overexpressed classical MBP containing the 21-nucleotide 3'UTR transit signal further reduces the Ca^{2+} response after plasma membrane depolarization. These data suggest that binding of classical MBPs to the plasma membrane is important for modulating Ca^{2+} homeostasis in oligodendroglial cells, by regulating voltage-gated Ca^{2+} channels. In MS, activation of peptidylarginine deiminases by increased intracellular calcium levels results in increased deimination of MBP, contributing to destabilization of myelin (Musse et al., 2006, 2008). Thus, variations observed in calcium influx induced by different MBP isoforms may have implications in MS.

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Fig. 1.

Ca^{2+} uptake was stimulated in N19 cells overexpressing different GFP-MBP constructs using high $[\text{K}^+]$ (20 mM). High $[\text{K}^+]$ was applied to N19 cells by a fast and local perfusion system. The time of addition of K^+ -containing external solution is indicated by the horizontal bar. Note that each trace corresponds to a single cell. Fura-2 imaging of Ca^{2+} response in N19 cells transfected with unmodified GFP vector (control; **A**), MBP-21.5 (**B**), and MBP-C1 (**C**). **D**: The bar plot shows the average amplitude calculated from the responding cells ($n > 500$ cells for each condition), expressed as percentage of change of the emission intensities and the percentage of responding cells in each experimental group. Values are expressed as mean $\pm$ SEM of at least four independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. cells transfected with unmodified GFP vector (control). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com).]

Fig. 2.

Fura-2 imaging of Ca^{2+} response to 20 mM K^+ in N19 cells overexpressing different GFP-MBP constructs. Decreases in Fura-2 signal in N19 cells overexpressing classical MBPs (MBP-21.5 and MBP-C1) were abolished in the absence of Ca^{2+} , and were blocked by Cd^{2+} (10 μM), verapamil (25 μM), and nifedipine (25 μM), confirming that these changes in intracellular $[\text{Ca}^{2+}]$ result from Ca^{2+} influx via VOCCs. In support of this conclusion, Bay K 8644 (5 μM), an L-type Ca^{2+} channel agonist, enhanced the amplitude of Ca^{2+} uptake. Values are expressed as mean $\pm$ SEM of at least six independent experiments ($n > 500$ cells for each condition). * $P < 0.05$, ** $P < 0.01$ vs. cells transfected with unmodified GFP vector (control). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 3.

Fura-2 imaging was employed to evaluate the ability of a number of agonists to elicit a Ca^{2+} response in N19 cells overexpressing different RFP-MBP constructs. The Ca^{2+} uptake was stimulated in control N19 cells (transfected with unmodified RFP vector) and in N19 cells overexpressing MBP-C1 and MBP-C8, using glutamate (100 μM) and ATP (100 μM ; **A**) and using thapsigargin (100 μM) and caffeine (2 mM; **B**). The graphs show the average amplitude calculated from the responding cells, expressed as percentage of change of the emission intensities. Each agonist was applied for 120 sec by a fast and local perfusion system. Values are expressed as mean $\pm$ SEM of at least two independent experiments ($n > 250$ cells for each experimental condition). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 4.

The Fura-2-based video-imaging approach was employed to evaluate the ability of high $[\text{K}^+]$ to elicit a Ca^{2+} response in primary OPCs overexpressing different RFP-MBP constructs. **A:** Two OPCs overexpressing RFP-MBP-C1 (yellow arrowheads) respond with a increase in Ca^{2+} uptake after 7 min of 20 mM K^+ stimulation. In the untransfected control cells (yellow asterisks), the Ca^{2+} transients induced by high $[\text{K}^+]$ were significantly higher. An increased Fura-2 fluorescence ratio is indicated by warmer colors (going from blue to red). **B:** Fura-2 imaging of Ca^{2+} response in OPCs transfected with unmodified RFP vector (control) and RFP-MBP-C1. The time of addition of K^+ -containing external solution is indicated by the horizontal bar. Note that each trace corresponds to a single cell. **C:** Decreases in Fura-2 signal in OPCs overexpressing MBP-C1/C8 were abolished in the absence of Ca^{2+} , and were blocked by verapamil (25 μM). On the other hand, Bay K 8644 (5 μM) enhanced the amplitude of Ca^{2+} uptake, but not if MBP was present. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 500$ cells for each condition). $**P < 0.01$ vs. cells transfected with unmodified RFP vector (control). Scale bar = 25 μm . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 5.

Fluorescent (epifluorescent) micrographs of N19 cells 2 days posttransfection, expressing different isoforms of RFP-MBP. **A:** All expression constructs shown containing the 3'UTR sequence localize efficiently to the plasma membrane processes and are distributed farther from the nucleus compared with the constructs devoid of this RTS, although the MBP-21.5 isoform localized primarily to the nucleus compared with its MBP-C1 and MBP-C8 isoforms. **B:** Fluorescence micrographs of N19 cells, 2 days posttransfection, coding for two different versions of MBP-C1. Fluorescence intensity trace for each image compares MBP-C1 and MBP-C1-UTR, showing that MBP mRNA is efficiently transported to the plasma membrane with the addition of the 3'UTR transit signal. For intensity comparison, an OLG from cell cultures expressing either MBP-C1 or MBP-C1-UTR was chosen for analysis. For each selected micrograph, the blue line represents DAPI staining, and the red line represents MBP. Each cell is representative of the predominant protein localization phenotype observed in greater than 95% of transfected cells. Scale bar = 20 μm . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 6.

Fura-2 imaging of Ca²⁺ responses to 20 mM K⁺ in N19 cells, comparing calcium response in RFP-MBP constructs with and without the 3'UTR. The graph shows the average amplitude calculated from the responding cells, expressed as percentage of change of the emission intensities. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 300$ cells for each experimental condition). ** $P < 0.01$, *** $P < 0.001$ vs. cells transfected with MBP-21.5 and MBP-C8 without the 3'UTR. Control: cells transfected with unmodified RFP vector. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 7.

Fluorescent micrographs of immunostained VOCCs in cultured N19 OLGs during different stages of cell spreading (**A**). The VOCCs in OLGs were concentrated at leading edges of extending membrane processes in initially spreading cells, compared with OLGs at more advanced stages of spreading. Fluorescence micrographs of N19 cells 2 days posttransfection expressing different isoforms of RFP-MBP (red) with nuclei counterstained with DAPI (blue; **B**). Cells were immuno-processed for L-type VOCCs that were detected using a conjugated Alexa 488 secondary antibody (green). Merged images of both MBP-C1 and MBP-C8 isoforms show areas of obvious colocalization and/or juxtaposition at the leading edge of extending membrane processes as shown in high-magnification insets (arrows). The MBP-21.5 isoform does not show obvious areas of colocalization with stained L-type VOCCs compared with MBP-C1 and MBP-C8, but it may be too low to detect in the processes. **C**: Fluorescence micrographs of N19 cell cultures overexpressing different isoforms of RFP-MBP. Cells were immunoprocessed for L-type VOCCs that were detected using a conjugated Alexa 488 secondary antibody (green). Cultures with N19 cells overexpressing MBP-C1, MBP-C8, or unmodified vector show heterologous distributions of VOCCs throughout the cell body as well as at the distal membrane process extensions. The N19 cells overexpressing MBP-21.5 have a reduced distribution of VOCCs in cell processes as a result of RFP-MBP-21.5 (RFP not shown). **D**: Immunoblot of total protein lysate from N19 cultures transfected with various RFP-MBP isoforms detecting the α -subunit of L-type VOCCs. Compared with equally loaded β -actin, N19 cells overexpressing MBPs have the same expression level of L-type VOCCs compared with cells transfected with unmodified RFP vector (indicated by “vector”). Scale bar = 20 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I

Primers Used From the Construction of Recombinant GFP- and RFP-MBP Expression Vectors Used Throughout This Study^{*}

Primer	Sequence (from 5' to 3')
RMBPRp1	AGAGAGAGAGATCCGGAATGGCATCACAGAAGAGACCCTCACA
RMBPRp1	GAATTCTAGACTCGAGGCGTCTCGCCATGGGAGATCC
RMBPRp2	GAATTCTAGACTCGAGTTGTCTCGCCATGGGAGATCC
UTRFp1	TCGAGTAAGCCAAGGAGCCAGAGAGCATGC
UTRRp1	TCGAGCATGCTCTCTGGCTCCTTGGCTTAC

^{*} All primers were ordered from University of Guelph Laboratory Services Division.