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Histone Deacetylase 11 Regulates Oligodendrocyte-Specific Gene Expression and Cell Development in OL-1 Oligodendroglia Cells

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Abstract

Both *in vivo* and *in vitro* studies indicate a correlation between reduced acetylation of histone core proteins and oligodendrocyte development. The nature of these histone modifications and the mechanisms mediating them remain undefined. To address these issues we utilized OL-1 cells, a rat non-transformed oligodendrocyte cell line, and primary oligodendrocyte cultures. We found that the acetylated histone H3 at lysine 9 and lysine 14 (H3K9/K14ac) is reduced in both the myelin basic protein (MBP) and proteolipid protein (PLP) genes of maturing oligodendroglial OL-1 cells, and furthermore, this temporally correlates with increases in MBP, PLP, and histone deacetylase (HDAC) 11 expression. Disruption of developmentally-regulated histone H3 deacetylation within the MBP and PLP genes by the HDAC inhibitor trichostatin A blunts MBP and PLP expression. With its increased expression, interaction of HDAC 11 with acetylated histone H3 and recruitment of HDAC 11 to the MBP and PLP genes markedly increases in maturing OL-1 cells. Moreover, suppressing HDAC 11 expression with small interfering RNA significantly: 1) increases H3K9/K14ac globally and within the MBP and PLP genes, 2) decreases MBP and PLP mRNA expression, and 3) blunts the morphological changes associated with oligodendrocyte development. Our data strongly support a specific role for HDAC 11 in histone deacetylation and in turn the regulation of oligodendrocyte-specific protein gene expression and oligodendrocyte development.

Keywords

epigenetic; acetylation; myelination; MBP; PLP

INTRODUCTION

Oligodendrocytes, the myelin-producing cells in the central nervous system (CNS), develop from neural stem cells (NSC) and oligodendrocyte progenitors (OPC) in the ventricular and subventricular zones in the brain and the spinal cord (Cameron-Curry and Le Douarin, 1995; Hirano and Goldman, 1988; Levison and Goldman, 1993). The development of NSC and OPC appears to be directed and controlled by epigenetic regulation of the expression of the genes involved in the cellular specification and differentiation. The mechanisms that control these processes remain to be entirely defined.

Acetylation of histone core proteins, a dynamic process that is regulated by two classes of enzymes, i.e., histone acetyltransferases (HATs) and histone deacetylases (HDACs), has

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been implicated in NSC and OPC development. Development of cultured adult rat NSC into mature oligodendrocytes correlates with a reduction in the acetylation of histones H3 and H4 (Hsieh et al., 2004). Similarly, histone H3 acetylation in rat corpus callosum is significantly reduced with increasing age, although the activity of both HAT and HDAC appears to be reduced during the same period (Shen et al., 2005). Furthermore, suppressing HDAC activity with non-specific inhibitors significantly blunts oligodendrocyte development in cultured NSC (Hsieh et al., 2004) and OPC (Liu et al., 2003), and reduces myelination in developing rats (Shen et al., 2005). The above studies, however, do not address whether the histone modification occurs on oligodendrocyte-specific genes and/or on genes important for oligodendrocyte development, and whether such modifications have a function in gene expression. Moreover, it is not known whether the HDAC inhibitors used directly affect the expression of oligodendrocyte-specific genes and the development of oligodendrocytes by modifying histones on these genes, or indirectly by other mechanisms.

Four classes of HDACs have been identified (Gregoretti et al., 2004). Class I HDACs include HDAC 1, 2, 3, and 8, and class II HDACs are comprised of HDAC 4, 5, 6, 7, 9 and 10. Class III HDACs include the Sir2 (silent information regulation)-like family of NAD-dependent deacetylases, which are insensitive to HDAC inhibitors, such as vaproic acid (VPA) and trichostatin A (TSA). HDAC 11 is the sole known member of mammalian class IV HDAC. In both humans (Gao et al., 2002) and mice (Liu et al., 2008) high levels of HDAC 11 are expressed only in a few of organs, including brain, heart and kidney, suggesting a specialized function for HDAC 11 in these organs.

In this report, utilizing OL-1 cells, a non-transformed oligodendrocyte precursor line, and cultured primary oligodendrocytes, we evaluated the role of histone modification during oligodendrocyte development by directly evaluating the associations of such modifications with oligodendrocyte-specific gene expression, cell growth and HDAC function. Our results indicate that histone H3K9/K14 deacetylation, mediated at least in part by HDAC 11, occurs within the genes encoding proteolipid protein (PLP) and myelin basic protein (MBP), two major oligodendrocyte-specific proteins, and that this deacetylation and HDAC 11 activity are required for proper oligodendrocyte-specific protein gene expression and oligodendrocyte development.

MATERIALS AND METHODS

OL-1 Cell Culture

Generation and maintenance of OL-1 cells was described in detail elsewhere (Lagarde et al., 2007). Briefly, OL-1 cells were grown in a serum-free defined medium (SFM) supplemented with 30% B104 conditioned medium (B104 CM). SFM contains insulin (10 μg/ml, Sigma, St. Louis, MO) and transferrin (25μg/ml, Sigma). B104 CM was obtained by culturing sub-confluent B104 neuroblastoma cells with SFM for 2 days. B104 cells were maintained by culturing with DMEM supplemented with 10% fetal calf serum (FCS).

For RNA experiments, cells (4–5 $\times 10^5$ cells/dish) were plated into 60 mm dishes and cultured with B104 CM-containing SFM overnight to recover from passage shock. Experiments were initiated by replacing B104 CM-containing medium with B104 CM-free SFM supplemented with 30 nM triiodothyronine (T3) to promote maturation. For Western immnuoblot, protein immunoprecipitation, and chromosome immunoprecipitation (ChIP) studies, Cells (4–5 $\times 10^5$ cells/dish) were plated into 100 mm dishes and expanded by growing in B104 CM-containing SFM. After 3–4 days, when cell density reached 3 – 4 $\times 10^6$ per dish, B104 CM-containing SFM was replaced with B104 CM-free SFM supplemented with 30 nM T3. Cells collected immediately before removal of B104 CM-containing SFM were designated as 0 hr controls.

Primary Neural Cell Culture

Enriched cultures of OPC, astrocytes and microglia were obtained from brains of Wistar rats at age of postnatal day (P) 1 – 2, using the method described by McCarthy and De Vellis (1980). OPC were expended in DMEM/F12 SFM supplemented with 30% B104 CM, 0.5% FCS and 10 ng/ml basic fibroblast growth factor (bFGF, Invitrogen, Carlsbad, CA). OPC were then disassociated from dishes using papain, and seeded into 24-well-plates ($2 – 3 \times 10^4$ cells/well). To promote maturation, cells were cultured in SFM supplemented with T3 and 20 ng/ml human insulin-like growth factor-I (Genentech, South San Francisco, CA) for 2–3 days. Astrocytes and microglia were maintained using DMEM supplemented with 10% FCS. Enrichments of OPC were >90%, based on immunostaining of parallel independent cultures with NG2, NeuN and GFAP antibodies.

RNA Isolation and Quantative Real-time PCR (qRT-PCR)

Total RNA was extracted using a Trizole (Invitrogen) or a Picopure RNA kit (Arcturus, Sunnyvale, CA). Reverse transcription was performed using random decamers as primers and Superscript II reverse transcriptase (Invitrogen). The abundance of resultant mRNA-derived cDNA was determined by qRT-PCR analysis, using Sybr Green PCR Master Mix (Qiagen, Valencia, CA), primers specific for genes of interest, and a LightCycler (Roche, Indianapolis, IN) or Realplex cycler (Eppendorf, Westbury, NY). Each set of primers spans at least two introns so that contaminating genomic DNA either can not be amplified due to large product size or can be easily identified based on its size on agarose gel. The sequences of each primer set are listed in the supplemental Tables S1 and S2. PCR using these primer sets produced cDNA amplicons of the expected size(s). The specificity of each real-time PCR target was confirmed by melt temperature analysis using a program supplied with the Lightcycler or Realplex and agarose gel fragmentation of amplicons. The identities of HDAC 10 and HDAC 11 PCR amplicons, as well as those of HAT p300, CBP and GCN5, were confirmed by DNA sequencing.

To quantify mRNA abundance, a standard curve for each target mRNA, as well as for 18S rRNA or β-actin, was generated from serial dilutions of cDNAs derived from independent OL-1 cell cultures. The relative abundance of mRNA of interest in each sample was determined based on its corresponding standard curve, and normalized against the abundance of 18S rRNA or β-actin mRNA.

Transfection of Small Interfering RNA (siRNA)

For RNA expression, cells (5×10^4 OL-1 cells or 3×10^4 OPC) were plated into each well of 24-well-plates. For Western immunoblot and ChIP experiments, 2.3×10^6 OL-1 cells were plated into 100 mm dishes. After 6 – 8 hr in culture to recover from passage shock, cells were transfected with siRNA duplexes (pre-designed and synthesized by Qiagen) specific for rat HDAC 11 or with a “scrambled” negative control siRNA, using HiPerFect transfection reagent (Qiagen) according to the manufacturer’s protocol. The target sequences of the siRNAs are as follows: 5′ end HDAC 11 siRNA: 5′-aagcgtgtatatatcatggat-3′; and 3′ end HDAC 11 siRNA: 5′-acggctactcacagaacatta-3′.

Protein Western Immunoblot and Immunoprecipitation Analysis

Acid-soluble nuclear proteins were isolated as previously reported (Sassone-Corsi et al., 1999). Protein concentration was determined using a BCA protein kit (Pierce, Rockford, IL). Aliquots of nuclear protein (5–10 μg) were separated on polyacrylamide gels and transferred onto PVDF nylon membranes (Amersham, Arlington Heights, IL). Membranes were incubated with antibody specific for di-acetylated histone H3 at lysine 9 and lysine 14 (H3K9/K14ac, 1:3000), acetylated histone H4 at lysine 8 (H4K8ac, 1:2,000), mono-, di- and

tri-methylated H3 at lysine 4 (H3K4me, 1:3,000), or total histone H3 (1:8,000, Millipore). Antibodies against H3K9/K14ac, H4K8ac, H3K4me and histone H3 were purchased from Millipore (Billerica, MA). Specific immunoreactivity was visualized using an ECL kit. Images of specific protein bands on x-ray films were digitally scanned and quantitatively analyzed using a computer-assisted image analysis system (Image-Pro, Media Cybermetics, Silver Spring, MD).

For immunoprecipitation assays, cells were lyzed with a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, 1 mM PMSF, 0.5% triton-X 100) containing 20 ng/ml TSA. After adjusting the NaCl concentration in the cell lysates to 100 mM by adding additional NaCl, cell debris was removed by centrifugation. Aliquots of 250 μg total protein were subjected to immunoprecipitation with an HDAC 11 antibody (Abcam, Cambridge, MA or Lifespan Bioscience, Seattle, WA). Antibody-antigen complexes were precipitated by agarose-conjugated protein G. After washes with a washing buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM PMSF, 0.5% triton-X 100) in presence of 20 ng/ml TSA, immunoprecipitants were subjected to Western immunoblot analysis using an antibody against total H3, H3K9ac or H4K8ac. Rabbit IgG or no antibody addition was used as a negative control.

ChIP

OL-1 cells were collected and pooled (~1×10^7 cells per sample), and genomic DNA-nuclear proteins were cross-linked by incubating cells with 1% formaldehyde. ChIP was performed using the method of Aparicio et al. (Apariciao et al., 2005), except that sonication was used to fragment DNA. Using a Sonic Dismembrator (Model 500, Fisher Scientific), genomic DNA was fragmented, with the majority of the fragments being 500–1,100 bp. For HDAC 11 ChIP assay, cells were incubated with dimethyl 3,3′-dithiobispropionimidate (5 mM) for 30 minutes, followed by 1% formaldehyde. Genomic DNA was fragmented by MNase digestion. The majority of fragments again were 400–800 bp in size. The addition of cross-link enhances detection of weakly binding nuclear proteins to genomic DNA (Fujita and Wade, 2004). DNA-protein complexes were immunoprecipitated using a rabbit antibody against H3K9/K14ac, total histone H3, RNA polymerase II (Millipore), or HDAC 11, respectively. Rabbit IgG or no antibody addition was used as negative control. Rabbit IgG does not effectively precipitate DNA-protein complexes. Immunoprecipitated DNA fragments were purified using the QiaQuick PCR Purification Kit (Qiagen). The relative abundance of DNA products was quantified by qRT-PCR using specific primers (see supplemental Table S3 for primer sequences).

Statistics

One way ANOVA was used to test statistical significance among multiple groups, and followed by comparison of each group mean using Newman-Keuls Student test. To test statistical significance between two groups *Student-t* test was used. All tests were performed assisted with the software SigmaStat for Windows (SPSS, Inc., Chicago, IL).

RESULTS

During maturation promoted by B104 CM removal, as we previously reported (Lagarde et al., 2007), OL-1 oligodendrocyte cells underwent significant morphology changes and exhibited a marked increase in the expression of mRNA for oligodendrocyte-specific proteins, MBP, PLP, myelin-associated glycoprotein (MAG), and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (see supplemental Figure S1). In contrast, the abundance of mRNA for precursor-specific proteins and other proteins was reduced by 2 – 13 fold during the same period (supplemental Figure S1). These findings are consistent with changes

observed in developing oligodendrocytes *in vivo* and *in vitro* (Avellana-Adalid et al., 1996; Young and Levison, 1997).

With cell maturation, i.e., with increasing time in culture and increased expression of oligodendrocyte-specific protein genes, OL-1 cells exhibited a gradual reduction in the abundance of H3K9/K14ac (Figure 1), being ~70%, ~25% and ~10% of that in immature control cells at 3, 24 and 48 hr after withdrawal of B104 CM, respectively. These results are consistent with previous findings of others showing reduced histone acetylation in mature oligodendrocytes in corpus callosum (Shen et al., 2005), cultured adult hippocampal NSC (Hsieh et al., 2004), and cultured OPC (Marin-Husstege et al., 2002). In contrast, overall cellular H3K4me showed no significant changes (Figure 1).

To directly determine whether such reductions in histone H3 acetylation also occurs within oligodendrocyte-specific protein genes, we used ChIP and qRT-PCR assays and examined H3K9/K14 acetylation in the promoter, coding and intron regions of the MBP and PLP genes. For promoter regions, we examined DNA sequences extending from the transcription start site to −1,037 bp for MBP and −915 bp for PLP of their 5′ regions (Figure 2A). It has been shown that DNA within the 1 kb MBP and PLP 5′ regulatory region is capable of directing transgene expression (Berndt et al., 1992; Berndt et al., 2001; Clark et al, 1998; Nave and Lemke, 1991). In addition, we also examined a region containing an anti-silence element (ASE) in intron 1 of the PLP gene, which has been shown to be essential for specific PLP expression in oligodendrocytes (Li et al., 2002; Meng et al., 2005; Wight and Dobretsova, 2004), and a region containing 188 bp of intron and the first 37 bp of exon 2.

Consistent with the changes in overall H3K9/K14ac, the abundance of H3K9/K14ac in both the MBP and PLP genes was significantly reduced in mature OL-1 cells 48 hr after withdrawal of B104 CM (Figure 2A). Compared to immature OL-1 cells that were cultured in presence of B104 CM, mature OL-1 cells exhibited a 40 – 60 % reduction in H3K9/K14ac from the upstream promoter region through to the coding region of the MBP gene. Similarly, H3K9/K14ac associated with PLP gene was reduced by 30 – 85 % in mature OL-1 cells. While no changes were observed for the β-actin gene (data not shown), H3K9/K14ac in the cyclin D1 and nestin genes also was significantly decreased, being 11 – 35% of that in immature OL-1 cells (supplemental Figure S2).

In contrast, in maturing OL-1 cells methylation of histone H3 at lysine 4 (H3K4me) and recruitment of RNA polymerase II, an enzyme that is required for mRNA synthesis, were significantly increased within the promoter regions of the MBP and PLP genes (Figure 2A), a finding consistent with an active expression of these genes.

To further determine whether acetylation of histone H3 within the MBP and PLP genes has a similar pattern of changes during early development, developing OL-1 cells were collected 3, 6 and 24 hr after B104 CM withdrawal, and subjected to ChIP analysis. Maturing OL-1 cells exhibited no significant changes in H3K9/K14ac abundance in the MBP gene during the first 6 hr of development. This, however, was followed by a marked reduction 24 hr after B104 CM withdrawal (Figure 2B). When compared to immature OL-1 cells, the abundance of H3K9/K14ac in all regions in the MBP gene examined was decreased by 70% to 75% 24 hr after withdrawal of B104 CM. The acetylation of H3K9/K14 in the PLP gene exhibited a similar pattern of change. Although H3K9/K14ac in the promoter (−602 bp to −174 bp, fragment 2) and promoter/coding (−322 bp to +108 bp, fragment 3) regions of the PLP gene appeared to be increased (by ~47% and ~30%, respectively) 3 hr after initiation of cell maturation, these increases did not meet tests of statistical significance (Figure 2B). These ChIP analysis results are consistent with the global loss of H3K9/K14ac observed in our Western immunoblot analysis.

We next investigated whether aberrant acetylation of H3K9/K14 in the MBP and PLP gene alters the expression of these two genes. Treatment of OL-1 cells with the HDAC inhibitors TSA or VPA for 24 hr markedly increased both global H3K9/K14ac (Figure 3A) and the H3K9/K14ac in both the MBP and PLP genes (Figures 3B). In contrast, TSA had no apparent effect on H3K4me within the PLP gene (Figure 4). TSA or VPA also significantly blunted developmentally-upregulated expression of MBP and PLP mRNAs (Figure 3C), and that of MAG, CNP, Olig1 and Olig2 mRNAs (supplemental Figure S3), as well as the growth of cell processes (Figure 3D). Each of these findings that are in line with the report by Casaccia-Bonnefil and associates (Liu et al., 2003; Shen et al., 2005). In addition, TSA further decreased the developmentally-reduced cyclin D1 mRNA expression, while it increased the developmentally-decreased nestin mRNA expression (supplemental Figure S3).

To determine whether increased HDAC abundance and/or decreased HAT abundance account for the reduction in H3K9/K14ac, the expression of HDAC 1 through 11 and HATs (p300, CBP and GCN5) was quantified in maturing cells after B104 CM withdrawal (Figure 5). Compared to immature OL-1 cells, mature OL-1 cells exhibited moderate increases (50 to 100%) in p300, CBP and GCN5 mRNA 24 hr and 48 hr after B104 withdrawal (Figures 5A). After 24 hr and 48 hr mRNAs for HDAC 2 through HDAC 9 exhibited either no change or were modestly reduced in maturing cells (Figures 5B). The abundance of HDAC 10 and HDAC 11 mRNA, however, was markedly increased in OL-1 cells with maturation, being ~300% and ~500% of their immature controls, respectively. The abundance of HDAC 1 mRNA also appeared to be increased in mature OL-1 cells, but the increase was not significant.

Further examination showed that the abundance of HDAC 11 mRNA began to increase as early as 3 hr after B104 CM withdrawal, and the increase became significant 6 hr after B104 CM withdrawal, while HDAC 10 mRNA was not increased significantly until 24 hr after B104 CM withdrawal (Figure 5C). Temporally the increase in HDAC 11 mRNA correlated well with the increase in MBP and PLP mRNA. The latter mRNAs also began to significantly increase 6 hr after B104 CM withdrawal (Figure 5D). These relatively early and dramatic rises in the magnitude of HDAC 11 mRNA abundance during OL-1 cell maturation, as well as their strong temporal correlation with the expression of MBP and PLP mRNA, prompted us to further investigate HDAC 11 actions, rather than those of HDAC 10.

To evaluate its interaction with acetylated H3, HDAC 11 protein co-immunoprecipitation was performed. While acetylated H3K9 (H3K9ac) could be readily detected in protein complexes immunoprecipitated from immature and mature OL-1 cell lysates with an antibody specific for HDAC 11, significantly more histone H3 and H3K9ac was observed in the immunoprecipitated protein complexes derived from maturing OL-1 oligodendrocytes, as compared to that from immature OL-1 cells (Figure 6A). Taken together with the fact that HDAC 11 immunoreactivity was predominantly located in cell nuclei (data not shown), these data are consistent with its role in histone deacetylation.

Recruitment of HDAC 11 to the promoter region of the MBP and PLP genes was also increased during OL-1 cell development. As shown in the Figure 6B, maturing OL-1 cells exhibited a 40 – 120 % increase in HDAC 11 recruitment to the promoter region of the MBP and PLP genes, as compared to that in immature OL-1 cells that were cultured in presence of B104 CM. These data suggest that the increased HDAC 11 within the MBP and PLP genes accounts for the reduced H3K9/K14ac in the loci during development.

Next to more directly investigate HDAC 11 influence on MBP and PLP mRNA expression, HDAC 11 expression was suppressed using a specific siRNA during OL-1 cell maturation.

Based on transfection of OL-1 cultures with a Fluro Alexa 488-labled HDAC 11 siRNA, about 60% of immature OL-1 cells were transfected 24 hours after incubation with HDAC 11 siRNA, and HDAC 11 siRNA remains stable in OL-1 cells 48 hr after transfection (data not shown). Compared to the cells treated with a scrambled negative control siRNA, immature OL-1 cells transfected with siRNAs for the 5′ or 3′ end of the HDAC 11 mRNA, or combination of both, exhibited ~40% to ~48% reduction in HDAC 11 mRNA. No change in the abundance of mRNA for HDAC 1, HDAC 6 or HDAC 10 was observed in the HDAC 11 siRNA treated cultures, indicating a specific blunting of HDAC 11 mRNA expression (see supplemental Figure S4).

To determine the effects of the siRNA HDAC 11 mRNA knockdown on the expression of oligodendrocyte-specific protein mRNA during maturation, OL-1 cells were transfected with a combination of the two HDAC 11 siRNAs. After 24 hr, B104 CM containing medium was removed. OL-1 cells were cultured with B104 CM-free SFM for another 24 hr to promote cell maturation, and then subjected to analysis. A schematic diagram of the treatment schedule is shown as Figure 7A. Compared to that in control siRNA-treated OL-1 cells, the abundance of HDAC 11 mRNA was decreased by 30 – 40% in HDAC 11 siRNA treated OL-1 cells, while there were no significant changes in mRNA for HDAC 1 and 10 (Figure 7B), consistent with the data from immature OL-1 cells (see above). In the cells treated with the HDAC 11 siRNAs the abundance of H3K9/K14ac associated with the promoter of the MBP or PLP genes was significantly increased, being ~200 to ~300% of that in controls (Figure 7D). The abundance of global H3K9/K14ac also was increased by ~2 fold, while no significant change in H4K8ac was observed (Figure 7C).

Suppressing HDAC 11 expression by siRNA significantly blunted the developmentally-increased expression of mRNA for MBP, PLP and MAG (by 30 – 35%) during OL-1 cell maturation (Figure 7E). HDAC 11 siRNA treated OL-1 cells also showed a 20% decrease in CNP mRNA. This change, however, did not meet tests of statistical significance. Similar to TSA treatment, HDAC 11 siRNA also blocked the growth of OL-1 cell processes (Figure 8). Compared to controls, OL-1 cells treated with HDAC 11 siRNA exhibited ~38% decreases in the number of primary processes (9.97 ± 0.31/cell in controls vs. 6.20 ± 0.28/cell in HDAC 11 siRNA treated cells, $P<0.001$), and ~44% decreases in secondary processes (16.80 ± 0.49/cell in controls vs 9.50 ± 0.45/cell in HDAC 11 siRNA treated cells, $P<0.001$).

In line with our previous findings that oligodendrocyte lineage cells are a major site of neural HDAC 11 expression in the developing mouse brain, cultured oligodendrocytes derived from neonatal rat brains expressed high level of HDAC 11 mRNA, while astrocytes and microglia only showed a modest expression of HDAC 11 mRNA, being ~6% and ~4% of that in oligodendrocytes, respectively (Figure 9A). With increasing maturation, the abundance of HDAC11 mRNA gradually increased, being about 300% of that in OPC 24 hr and 72 hr after initiation of maturation process (Figure 9B). Suppression of HDAC 11 mRNA expression in developing OPC by HDAC 11 siRNA significantly blunted the expression of MBP and PLP expression (Figure 9C).

DISCUSSION

The evidence reported here strongly supports a pivotal role for deacetylation of histone H3K9/K14 in the regulation of oligodendrocyte-specific protein gene expression and cell process growth, and thus in oligodendrocyte development. Our data also indicate that the reduction in histone H3K9/K14 acetylation is likely mediated, at least in part, by HDAC 11 activity. Specifically, we show that: 1) the acetylation of histone H3K9/K14 in both the PLP and MBP genes, as well as overall cellular H3K9/K14 acetylation, is significantly reduced in

maturing OL-1 oligodendroglial cells, and this negatively correlates with an increase in MBP and PLP mRNA expression (OL-1 oligodendroglial cells share the characteristics of primarily cultured OPC as judged by morphologic development, and regulation of histone modification and oligodendrocyte-specific protein gene expression); 2) inhibition of HDAC activity by the HDAC inhibitor TSA increases H3K9/K14ac within the MBP and PLP genes and blunts their expression; 3) HDAC 11 expression increases and its recruitment to the MBP and PLP genes and the protein complexes containing H3K9ac significantly increases in maturing OL-1 cells, and 4) suppressing HDAC 11 expression with specific siRNAs significantly increases H3K9/K14ac and blunts the developmentally promoted MBP and PLP mRNA expression and morphological maturation in OL-1 cells and primary oligodendrocyte cultures.

While histone acetylation is often linked to activation of specific genes (Jenuwein and Allis, 2001; Roh et al., 2007), accumulating evidence indicates that deacetylation and HDAC activity also are important for gene expression and cell development. For example, histone hypoacetylation and HDAC activity have been correlated with the expression of oligodendrocyte-specific protein genes and the development of oligodendrocyte lineage cells in rodents (Hsieh et al., 2004; Shen et al., 2005) and in fish (Cunliffe and Casaccia-Bonnefil, 2006). Similarly, interferon-β gene expression appears to require histone deacetylation, as do some interferon target genes (Nusinzon and Horvath, 2003; 2005; 2006). In the former studies, however, a direct association of deacetylation and specific HDAC activity with the expression of oligodendrocyte-specific protein genes and/or the differentiation of oligodendrocyte lineage cells can not be made, because the HDAC inhibitors used are not specific for a HDAC and they often influence the expression of multiple genes, including those associated with cell proliferation and death (Hall et al., 2002; Stadler et al., 2005; Wang et al., 2002; Wetzel et al., 2005), as well as other cellular events (Hao et al., 2004). In the current study, we utilize ChIP assays and directly demonstrate that histone H3K9/K14 deacetylation occurs in both the promoter and other regions of the MBP and PLP genes during OL-1 cell maturation. Furthermore, HDAC inhibitors and HDAC 11 siRNA significantly increases H3K9/K14 acetylation in these regions and blunts the developmentally-induced expression of MBP and PLP mRNA, as well as cellular process growth. Because treatment of cells with HDAC inhibitor TSA has little effect on H3K4 methylation within the PLP gene, our results suggest that modification of H3 acetylation alone can affect the expression of oligodendrocyte-specific protein genes, and thus strongly support a crucial role for H3K9/K14 deacetylation in the expression of oligodendrocyte-specific protein genes and the development of oligodendrocyte lineage cells.

The mechanisms that lead to an active expression of the MBP and PLP genes following histone deacetylation remain to be elucidated. It has been reported that both HAT and HDAC activity are required for the expression of the interferon-β gene and some of its target genes. Histone H3 acetylation of interferon-β promoter regions transiently increases before mRNA expression increases (Agalioti et al., 2002; Parekh and Maniatis, 1999). This transient increase in H3 acetylation followed by deacetylation appears to be required for recruitment of RNA polymerase II and other factors necessary for the transcription of these genes (Agalioti et al., 2002; Nusinzon and Horvath, 2003; 2005; 2006; Parekh and Maniatis, 1999; Sakamoto et al., 2004). Whether the same occurs in maturing oligodendrocytes remains to be determined. Shen et al. (2005) also reported that the abundance of HAT p300 and CBP in the corpus callosum of developing rats is transiently increased in the first week of postnatal life, a time when MBP and PLP begin to be expressed; they then gradually decrease in the next 2 weeks. We also observed that GCN5, p300 and CBP mRNA are moderately increased in maturing OL-1 cells, and that the acetylation of H3K9/K14 in several regions of the PLP gene exhibits a 30% to 47% increase 3 hr after initiation of cell maturation (although these increases do not meet statistical significance). These data suggest

that in oligodendrocytes acetylation of H3K9/K14, as well as acetylation at other sites, could increase at earlier developmental times, followed by a reduction in H3K9/K14 acetylation.

Deacetylation of histone H3 in the MBP and PLP genes in developing oligodendrocytes appears to be mediated, at least in part, by HDAC 11 activity. We show that in maturing OPC and OL-1 cells HDAC 11 mRNA gradually increases in a fashion that correlates negatively with decreased histone H3K9/K14 acetylation and positively with an increase in mRNAs for oligodendrocyte-specific proteins. Moreover, HDAC 11 protein is preferentially localized in the nuclei of OL-1 cells as shown by immunostaining. Consistently, our previous data (Liu et al., 2008) also show that in developing mouse brain HDAC 11 is predominately located in the nuclei of oligodendrocytes and some neurons, but not astrocytes, and that HDAC 11 expression gradually increases during a period when the expression of oligodendrocyte-specific proteins is significantly increased. In line with its role in deacetylation of H3K9/K14 within the MBP and PLP genes, HDAC 11 recruitment to acetylated H3K9 and to the promoter region of the MBP and PLP genes significantly increases in maturing oligodendrocytes. Our strongest evidence supporting a role for HDAC 11 in deacetylation of histone H3 within the MBP and PLP genes, as well as in oligodendrocyte development, however, comes from specific suppression of HDAC 11 expression. Blocking HDAC 11 mRNA expression with a specific siRNA significantly increases global H3K9/K14 acetylation and that within the MBP and PLP genes, while it blunts MBP and PLP mRNA expression and cell process growth. Taken together, these data strongly suggest that HDAC 11 recruitment to H3K9/K14 within the MBP and PLP genes increases during oligodendrocyte development, and the increased HDAC 11 in turn deacetylates acetylated H3K9/K14 at these loci. Our conclusion that HDAC 11 in part mediates deacetylation of histone H3, however, does not exclude a role for HDAC 10, as well as for other HDACs, in regulation of histone H3 deacetylation. Because HDAC 10 mRNA also moderately increases during OL-1 cell development, it is possible that it also contributes to the reduction of histone acetylation in developing OL-1 cells.

Our data demonstrate that blunting HDAC 11 mRNA expression in maturing OL-1 cells specifically increases the global acetylation of H3K9/K14, as well as H3K9/K14 acetylation within the MBP and PLP genes, but has no effect on histone H4K8 acetylation. Consistent with the former observation, HDAC 11 recruitment to protein complexes that contain H3K9ac, but not those that contain H4K8ac (data not shown), significantly increases in maturing OL-1 oligodendrocytes. These findings suggest that H4K8ac likely is not a target for HDAC 11 activity in developing oligodendroglial cells. These observations may not be fully consistent with the data of Gao et al. (2002). They showed that purified human HDAC 11, derived from an overexpressed human cDNA, significantly increased HDAC activity and reduced histone H4 acetylation in a cell-free system. In their study, however, the site(s) of histone H4 acetylation was not addressed, nor were the effects of human HDAC 11 on histone H3 studied (Gao et al., 2002). Explanations for this apparent discrepancy include the possibility that HDAC 11 activity is species or tissue specific and/or acts in an amino acid residue-dependent manner.

In summary, we have provided evidence that strongly supports a role for histone H3 deacetylation in the expression of oligodendrocyte-specific protein genes, and that HDAC 11 plays a pivotal role in the regulation of histone H3 acetylation in oligodendrocytes. In man HDAC 11 does not appear to bind mSin3A, SMRT or N-CoR (Gao et al., 2002), three transcriptional co-repressor complexes that often participate in HDAC inhibition of gene expression. These findings are consistent with our studies and with the possibility that HDAC 11 acts as a transcriptional co-activator to maintain transcriptional architecture in the oligodendrocyte-specific protein genes. The exact mechanisms by which histone deacetylation and HDAC 11 influence oligodendrocyte-specific protein gene expression,

however, remain to be defined, and it is possible that HDAC 11 effects on the expression of these genes is indirect.

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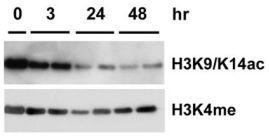

Figure 1.

Representative Western immunoblot analysis of diacetylated H3K9/K14 (H3K9/K14ac) and methylated H3K4 (H3K4me). After probing with H3K9/K14ac, the membrane was stripped and re-probed with H3K4me. The duration after B104 CM withdrawal is indicated at the top of the panel.

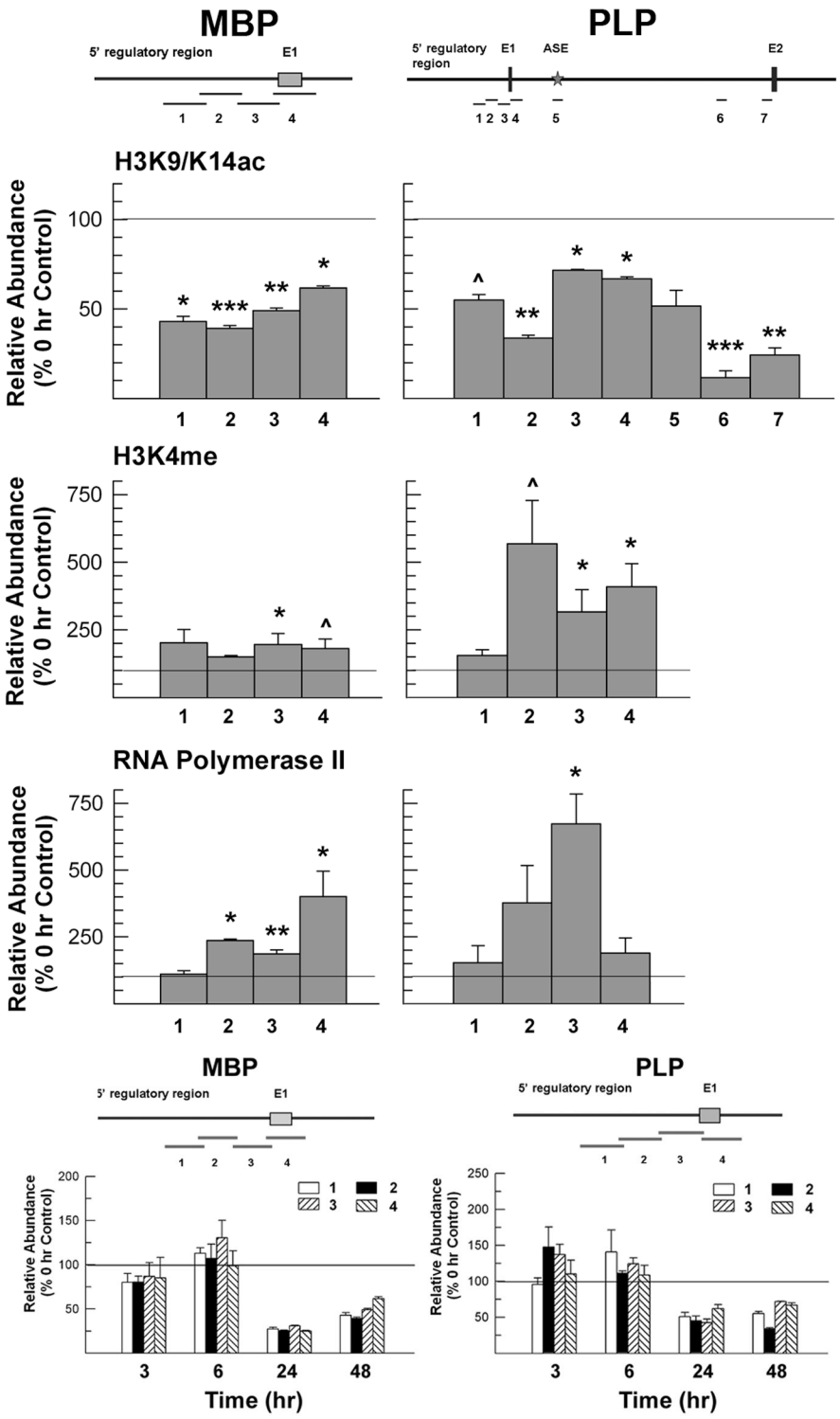

Figure 2.

Abundance of H3K9/K14ac, H3K4me, and RNA polymerase II associated with the MBP and PLP genes during OL-1 cell development. **Panel A**. Abundance of H3K9/K14ac, H3K4me, and RNA polymerase II associated with the MBP and PLP genes in OL-1 cells after 48 hr maturation. The abundance of modified histone H3 and RNA polymerase II

associated with the MBP and PLP genes were determined using ChIP analysis and qRT-PCR. **Panel B**. Acetylation of histone H3K9/K14 in the MBP and PLP genes during OL-1 cell development. Cells were collected for ChIP analysis 3, 6 and 24 hr after B104 CM withdrawal. Data for 48 hr were derived from the experiments shown in Panel A. The positions of PCR amplified DNA fragments relative to each target gene are indicated and numbered in diagrams above each figure. The diagram of the gene structure is not precisely to scale. Relative abundance is expressed as percentage of that from samples immediately before withdrawal of B104 CM (designated as 0 hr controls). Values represent mean ±SE from 3 – 4 samples. ^, P=0.06; *, P<0.05; **, P<0.01; ***, P<0.001, versus 0 hr control.

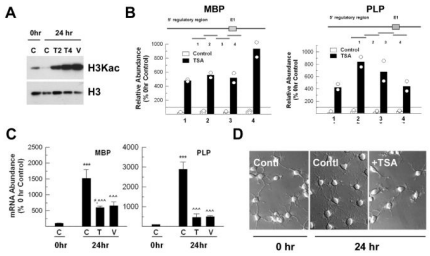

Figure 3.

Influence of HDAC inhibitors on H3K9/K14 acetylation, MBP and PLP mRNA expression, and cell morphology in OL-1 cells. **Panel A**. Representative Western immunoblot analysis of acetylated H3K9/K14 (H3Kac) in cells treated for 24 hr without (C) or with the HDAC inhibitors TSA (T2, 20 ng/ml; T4, 40 ng/ml TSA), or VPA (V, 40mM). After stripping, the membrane was re-probed with an antibody against histone H3 (H3, bottom panel). **Panel B**. ChIP assays of H3K9/K14ac in the MBP and PLP genes. OL-1 cells were treated without (control) or with 20 ng/ml TSA for 24 hr. The abundance of H3K9/K14ac is expressed as percentage of that in 0 hr controls. Each dot represents the value of each sample, and the bars represent the average of two samples. **Panel C.** HDAC inhibitor suppression of oligodendrocyte-specific protein mRNA expression. Cells were treated for 24 hr without (control; C) or with TSA (T, 20 ng/ml) or VPA (V, 40mM). The abundance of mRNA is expressed as percentage of that in 0 hr controls. Values represent mean ±SE from 3 – 4 samples. #, P=0.06; *, P<0.05; **. P<0.01; ***, P<0.001, compared to 0 hr controls. ^, P<0.05; ^^. P<0.01; ^^^, P<0.001, compared to 24 hr untreated controls. **Panel D**. TSA alteration of development-induced cell process growth. Cells treated without (Contl) or with 20 ng/ml TSA (+TSA) were photomicrographed using a Nikon inverted TE300 microscope with a Hoffman modulator. Note that the number of cell processes in cultures treated with TSA for 24 hr are far fewer than those of controls 24 hr after B104 CM withdrawal and similar to those of 0 hr controls.

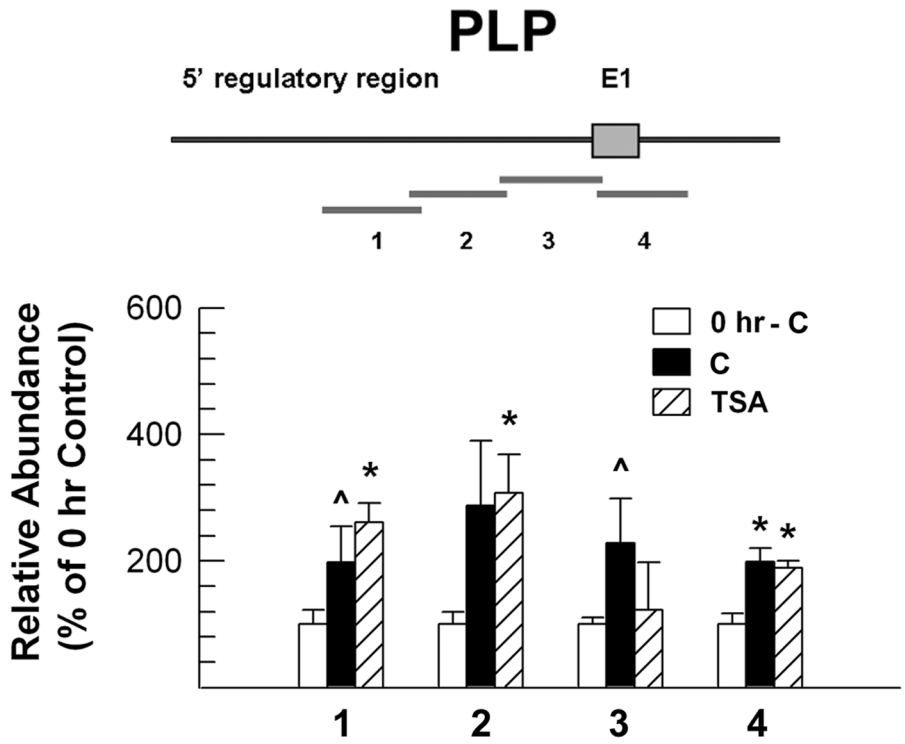

Figure 4.

Influence of the HDAC inhibitor TSA on H3K4 methylation in the promoter region of the PLP gene in OL-1 cells. Cells were treated for 24 hr without (C) or with 20 ng/ml TSA. The abundance of H3K4me is expressed as percentage of that in 0 hr controls (0 hr-C). Values represent mean ±SE from 3 – 4 samples. ^, P=0.06; *, P<0.05, compared to 0 hr controls.

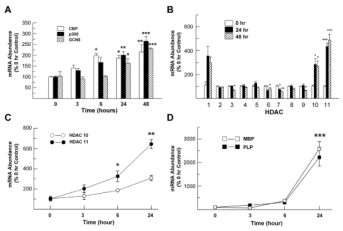

Figure 5.

Expression of mRNAs for HATs (Panel A), HDACs (Panels B and C), and oligodendrocyte-specific proteins (Panel D) in developing OL-1 cells. B104 CM was withdrawn for times indicated to promote cell maturation. The abundance of mRNA in maturing OL-1 cells is expressed as percentage of that in 0 hr control. Values represent mean ±SE from 3 – 4 samples. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, compared to mRNA abundance in 0 hr controls.

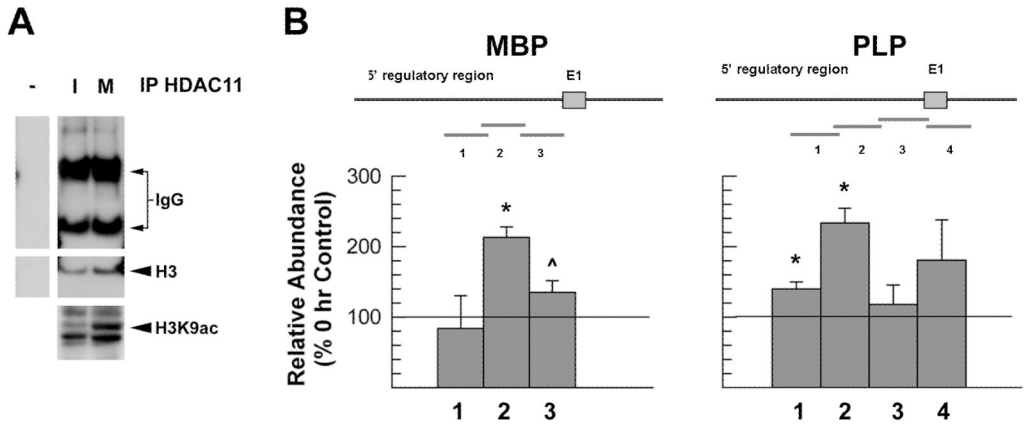

Figure 6.

Interaction of HDAC 11 with acetylated H3 within the MBP and PLP promoter in OL-1 cells during development. **Panel A**. HDAC 11 immunoprecipitation (IP) with H3K9ac. Total protein was extracted from mature (M) or immature (I) OL-1 cells, and subjected to IP with an HDAC 11 antibody. Co-immunoprecipited histone H3 and H3K9ac then were analyzed using Western immunoblot with an antibody against histone H3 and H3K9ac, respectively. Note that the abundance of co-immunoprecipitated H3 and H3K9ac (indicated by an arrowhead at the right side of the panel) is increased in the mature OL-1 cells. **Panel B**. HDAC 11 recruitment to the MBP and PLP genes. The positions of fragments relative to each target gene are indicated and numbered in diagrams above each panel. Relative abundance is expressed as percentage of that in immature Ol-1 cells (designated as 0 hr controls). Values represent mean ±SE from 3 – 4 samples. ^, $P<0.1$; *, $P<0.05$, versus control.

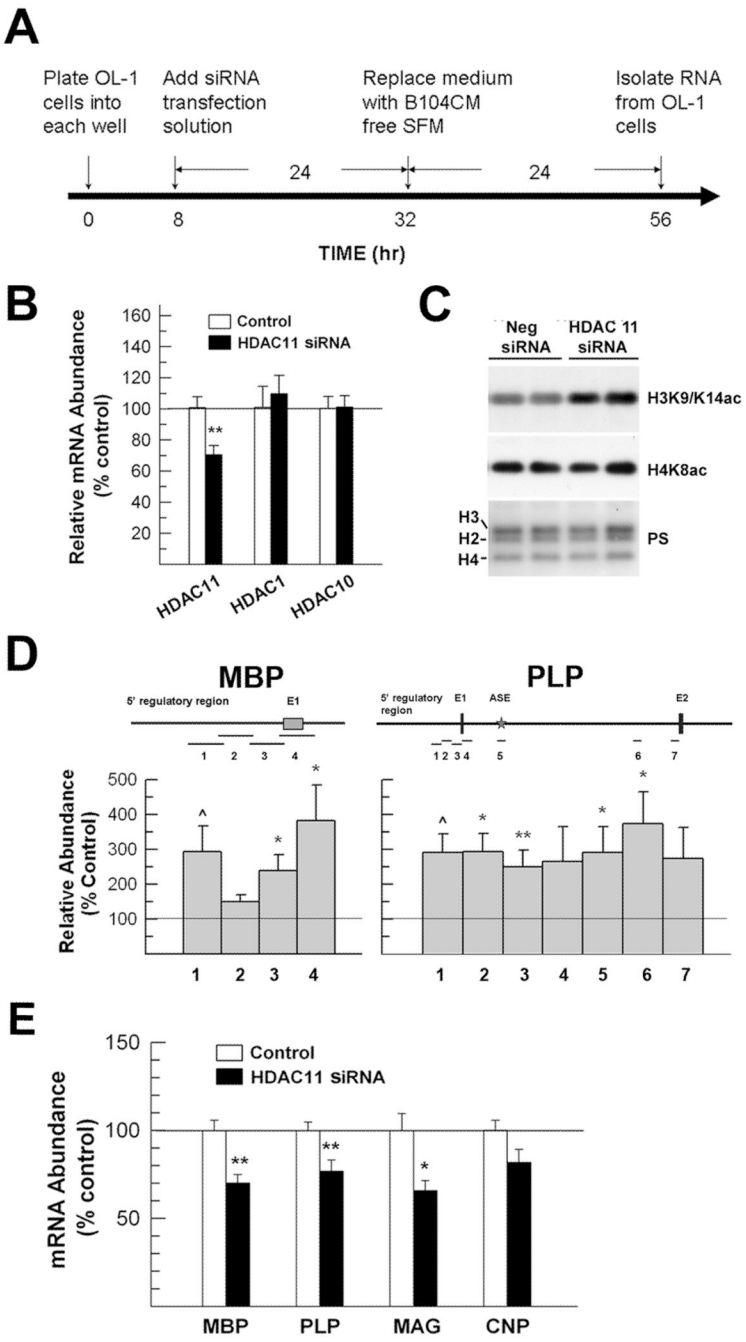

Figure 7.

HDAC 11 siRNA suppression of HDAC 11 mRNA expression, and increase of H3K9/K14ac in maturing OL-1 cells. **Panel A**. Schematic diagram of HDAC 11 siRNA treatment. OL-1 cells were treated with HDAC 11 siRNA (7.5 nM both 3′ and 5′ end HDAC 11 siRNA) or a scrambled negative control siRNA (Neg siRNA, 15 nM) for 24 hr, followed by culturing in B104 CM-free SFM for 24 hr. **Panel B**. Expression of mRNAs for HDAC 11, 1 and 10 in OL-1 cells treated with HDAC 11 siRNA or control siRNA. **Panel C**. Representative Western immunoblot analysis of acetylated H3K9/K14 (H3K9/K14ac) and H4K8 (H4K8ac). Bottom panel shows an image of Poncea S staining (PS) of the blot. Bands of histone H2, H3 and H4 are indicated at left of the panel. **Panel D**. Acetylation of H3K9/

K14 in the MBP and PLP genes in maturing OL-1 cells treated with HDAC 11 siRNA. The abundance of H3K9/K14ac is expressed as percentage of that in cells treated with control siRNA. The positions of PCR amplified DNA fragments relative to each target gene are indicated and numbered in diagrams above each figure. **Panel E**. HDAC 11 siRNA suppression of oligodendrocyte-specific protein mRNA expression in maturing OL-1 cells. Values in panels B, D and E represent mean ±SE from 3 – 5 samples. ^, P=0.06; *, P<0.05; **, P<0.01, compared to cells treated with control siRNA.

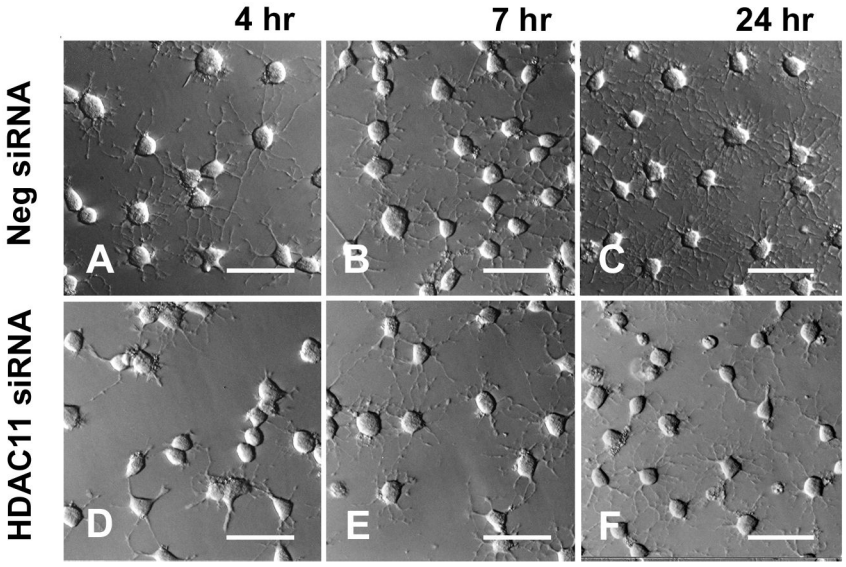

Figure 8.

HDAC 11 siRNA blockage of cell process growth during OL-1 cell development. OL-1 cells treated with a combination of both 3′ and 5′ end HDAC 11 (HDAC11 siRNA, 7.5 nM each, Panels D, E, and F) or with 15 nM scrambled negative control siRNA (Neg siRNA, Panel A, B, and C) for 24 hr. B104 CM was removed for 4 hr (panels A and D), 7 hr (Panels B and E) and 24 hr (Panels C and F) hr to promote maturation. Scale bars = 20 μm.

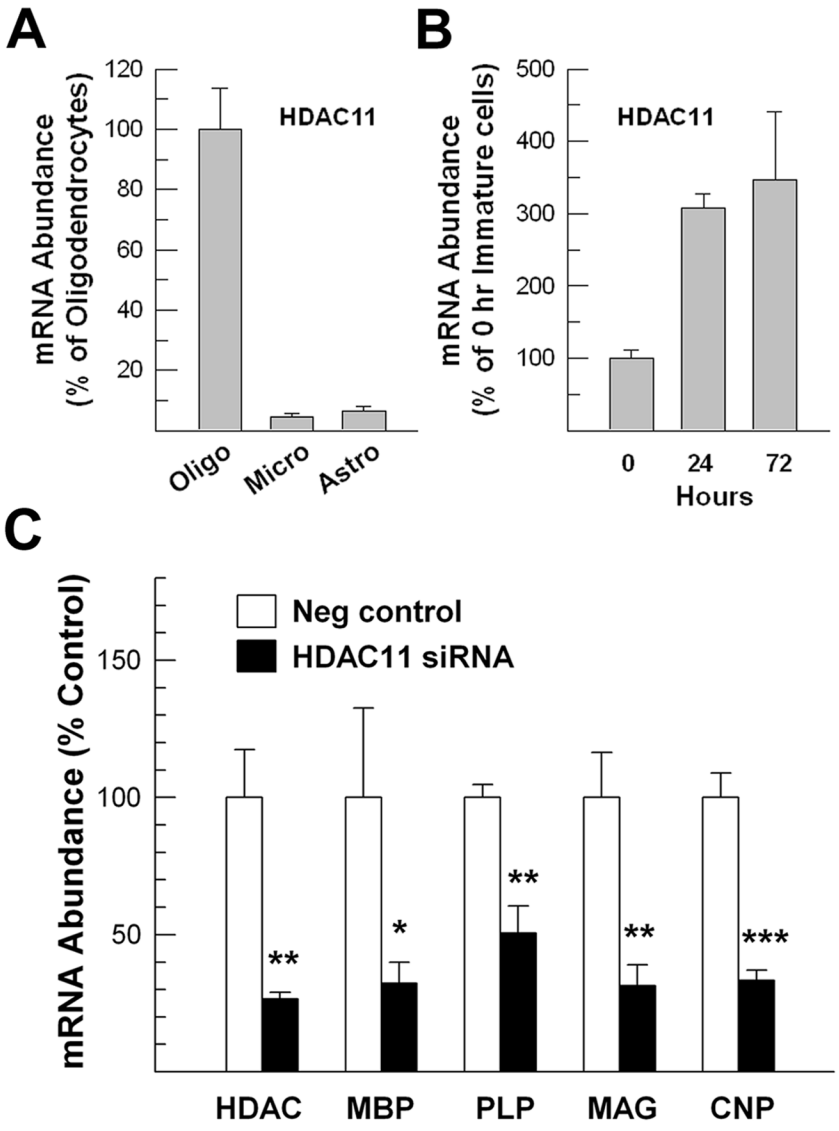

Figure 9.

HDAC 11 expression in cultured neural cells and HDAC 11 siRNA suppression of oligodendrocyte-specific protein mRNA expression in enriched rat oligodendrocytes. **Panel A**. HDAC 11 mRNA abundance in enriched oligodendrocytes (Oligo), microglia (Micro) and astrocytes (Astro). **Panel B**. Developmental expression of HDAC 11 mRNA in oligodendrocytes 0 hr, 24hr and 72 hr after removal of B104 CM with bFGF to promote maturation. **Panel C**. Suppression of HDAC 11 mRNA and oligodendrocyte-specific protein mRNA expression by HDAC 11 siRNA in maturing oligodendrocytes. OPC were enriched from cerebral cortexes of P1-2 rats, and treated with HDAC 11 siRNA as described previously in Figure 8. Values represent mean ±SE from 3 – 6 samples. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, compared to cells treated with negative control siRNA.