DNA Methylation–Related Chromatin Remodeling in Activity-Dependent *Bdnf* Gene Regulation

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In conjunction with histone modifications, DNA methylation plays critical roles in gene silencing through chromatin remodeling. Changes in DNA methylation perturb neuronal function, and mutations in a methyl-CpG–binding protein, MeCP2, are associated with Rett syndrome. We report that increased synthesis of brain-derived neurotrophic factor (BDNF) in neurons after depolarization correlates with a decrease in CpG methylation within the regulatory region of the *Bdnf* gene. Moreover, increased *Bdnf* transcription involves dissociation of the MeCP2–histone deacetylase–mSin3A repression complex from its promoter. Our findings suggest that DNA methylation–related chromatin remodeling is important for activity-dependent gene regulation that may be critical for neural plasticity.

Neuronal adaptive responses involve long-lasting functional and structural synaptic changes, which require alterations in neuronal gene expression (*1*). Epigenetic modifications, including DNA cytosine methylation, have been implicated in gene regulation during embryonic development, genomic imprinting, and X-chromosome inactivation (*2*). However, whether DNA methylation also influences the long-lasting changes in gene expression that occur during neural plasticity or neuronal maturation has yet to be reported.

The gene silencing effect of DNA methylation is mediated by a family of methylcytosine-binding proteins including MeCP2, which is abundantly expressed in the central nervous system (CNS) (*3*, *4*). Mutations in the *Mecp2* gene have been linked to a neurodevelopmental disorder, Rett syndrome (*5*). MeCP2 deficiency in mice results in Rett syndrome–like abnormalities, supporting a potential role for MeCP2 in regulating neuronal function (*5*–*8*). The enzymes that establish and maintain specific DNA-methylation patterns, including the de novo DNA methyltransferases 3a and 3b and the maintenance DNA methyltransferase 1 (Dnmt1) (*9*, *10*), are present in neurons and appear to be critical for their function. Indeed, mice that lack Dnmt1 in the CNS show perturbations in neuronal function (*11*). However, how alterations in DNA methylation or MeCP2 deficiency affect the gene expression that is critical for neuronal function is still unknown.

BDNF has been implicated in adult neural plasticity, including learning and memory (*1*). The genomic structure of the *Bdnf* gene is unusual in that it contains multiple promoters that are used to generate mRNAs containing different noncoding exons spliced upstream of a common coding exon (*12*) (fig. S1). These distinct sets of *Bdnf* promoters are used to achieve precise temporal and spatial control of BDNF production (*12*). The best-characterized *Bdnf* promoter is immediately upstream of the rat exon III, which is similar in sequence to the mouse *Bdnf* exon IV promoter. The rat promoter is activated upon membrane depolarization in cultured cortical and hippocampal neurons by means of KCl (50 mM) treatment, which leads to calcium influx, activating signaling cascades and inducing the expression of an array of genes involved in neural plasticity (*13*–*16*). We observed that when mouse E14 (embryonic day 14) cortical cultures are treated with 50 mM of KCl, transcription of *Bdnf* from the exon IV mouse promoter was enhanced (Fig. 1A). To investigate a role for DNA methylation in neural plasticity, we compared the amount of *Bdnf* exon IV–specific expression in E18 control and hypomethylated *Dnmt1*$^{-/-}$ mouse brains (*11*, *17*). The amounts of *Bdnf* mRNA in *Dnmt1* mutant brains were three to four times as high as those in littermate controls (Fig. 1B). This finding suggests that both the neuronal activity evoked by KCl and the hypomethylation caused by *Dnmt1* deletion lead to enhanced expression of *Bdnf*.

To directly examine whether DNA methylation affects activity-dependent *Bdnf* expression, we analyzed a 404–base pair (bp) region of the mouse *Bdnf* exon IV promoter; this region was located immediately upstream of the transcription initiation site (base pair +1). The region from base pair +1 to base pair –170 of the rat exon III promoter contains three critical cis-responsive elements that are 100% conserved in the mouse exon IV promoter (*13*–*16*). A schematic of the mouse *Bdnf* gene is shown in Fig. 1C, which displays the previously described Ca^{2+}-responsive elements: (i) the calcium-responsive element 1 (CaRE 1: 5′-CTATTT**CG**AG-3′, located between base pairs –72 and –63) (*15*); (ii) an upstream stimulatory factor–binding site (E-box: 5′-ATCATATGAC-3′, located between base pairs –51 and –42) (*16*); and (iii) a cyclic adenosine monophosphate (cAMP) response element (CRE: 5′-TCA**CG**TCA-3′, located between base pairs –38 and –31) (*13*, *14*). Encompassing these three critical elements are 8 CpG sites within the *Bdnf* exon IV promoter (Fig. 1C). We determined whether methylation of these sites could inhibit calcium activation of the promoter. We introduced site-specific methylation into the 404-bp *Bdnf* exon IV promoter–luciferase reporter construct through polymerase chain reaction (PCR) amplification (fig. S1) (*17*). Cells transfected with these methylated constructs were treated and maintained in KCl to induce depolarization. Luciferase activity was then monitored as a measure of exon IV promoter activity (*17*). Although methylation of the –148 CpG site had no impact on promoter activities, methylation of the CpGs at the –109, –66 (CaRE), –35 (CRE), and –24 sites all significantly decreased *Bdnf* IV promoter activity induced by KCl (Fig. 1D). These data suggest that CpG methylation at a particular site or sites can suppress activity-dependent transcription of *Bdnf*.

To assess whether CpG methylation patterns within the *Bdnf* promoter change upon depolarization, we examined the methylation status within the region from base pair –163 to base pair +60 (*17*). Analyses were performed for both untreated and depolarized mouse E14 cortical cells after 3 days in vitro (DIV). Immunostaining with neuronal, glial, and progenitor markers showed that approximately 90% of the cells in these cultures were postmitotic neurons. We first used a methylation-sensitive single-nucleotide primer extension (MS-SNuPE) assay (*17*) to compare methylation at six individual CpG sites between control and KCl-treated cultures (fig. S2A). Results from at least four independent experiments indicated that three sites (–111, –109, and –24) were significantly less methylated in depolarized cortical neurons (Fig. 2A). We also measured the level of DNA methylation by sequencing individual clones or the whole population of PCR products from bisulfite-converted DNA (*17*). Figure 2B shows that 9 out of 11 CpG sites (between base pairs –163 and +60) exhibited a trend of demethylation upon depolarization (fig. S2, B and C). Correlating with elevated *Bdnf* expression in *Dnmt1*$^{-/-}$ brains, we found that several sites in the exon IV promoter were almost completely demethylated in cultured E14 mutant cortical

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cells (≤7.5% methylation) (fig. S2D). Collectively, these findings indicate that DNA-methylation patterns in postmitotic neurons can undergo dynamic changes in response to depolarization, and a lower level of DNA methylation correlates with a higher level of *Bdnf* gene transcription in neurons.

We hypothesized that the association of methylcytosine-binding proteins with methylated DNA within the exon IV promoter represses *Bdnf* gene transcription. Chromatin immunoprecipitation (ChIP) analysis of MeCP2 and the CRE-binding protein (CREB) indicated that MeCP2 was more tightly associated with the exon IV promoter

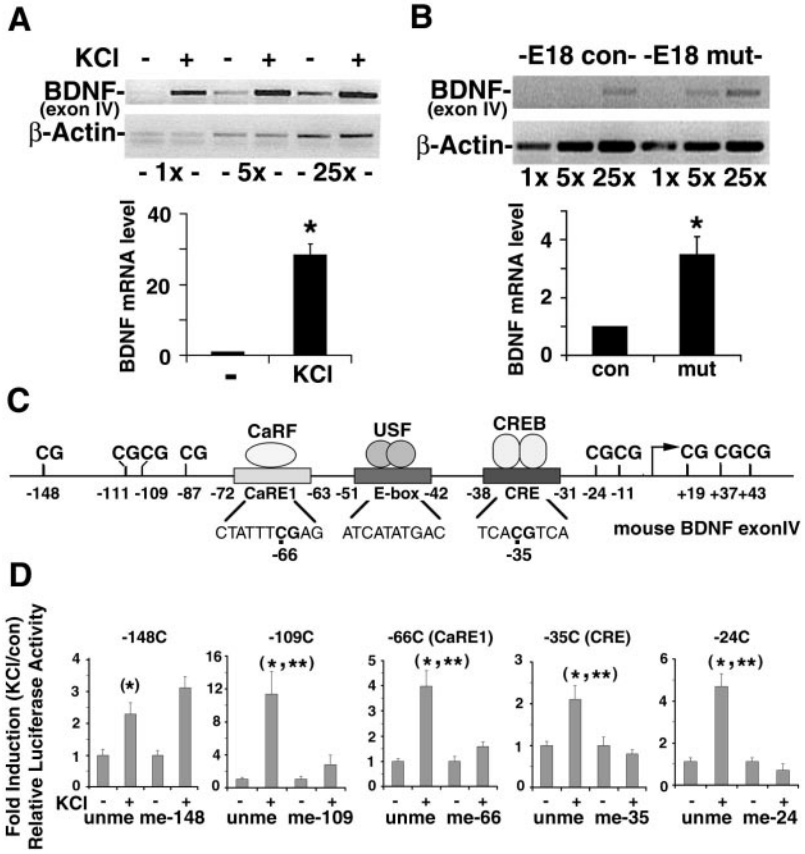

Fig. 1. Transcription of mouse *Bdnf* exon IV upon depolarization in wild-type versus DNA methylation–deficient mouse brain. (**A**) Reverse transcription (RT)–PCR showing increased exon IV mRNA in response to depolarization in E14 wild-type mouse cortical neuronal cultures after 2 DIV. Serial dilutions with 1×, 5×, and 25× cDNA input are shown. β-actin expression serves as a control. (Bottom panel) Relative amounts of *Bdnf* mRNA (*Bdnf* exon IV/β-actin) were measured with quantitative real-time PCR (*, $P < 0.05$, $n = 3$, t test). (**B**) RT-PCR for mouse *Bdnf* exon IV from E18 CNS-specific *Dnmt1* conditional knockout (mut) and control littermate (con). (Bottom panel) Quantitative real-time PCR measuring the relative ratio of *Bdnf* exon IV to β-actin (*, $P < 0.05$, $n = 3$, t test). (**C**) A schematic depicting the regulatory elements within the mouse exon IV promoter that are important for activity-dependent transcription. (**D**) Exon IV promoter luciferase constructs with site-specific methylation at base pairs –148C (cytosine), –109C, –66C, –35C, and –24C, as well as their unmethylated counterparts, were introduced into 2-DIV E14 cultures. Luciferase activity was then measured with and without KCl treatment after 24 hours. (For each site: *, $P < 0.05$, compared with the unmethylated non-KCl treated control; **, $P < 0.05$, compared with the KCl-treated methylated samples, $n \geq 12$, analysis of variance with posthoc Fisher's protected least significant difference test).

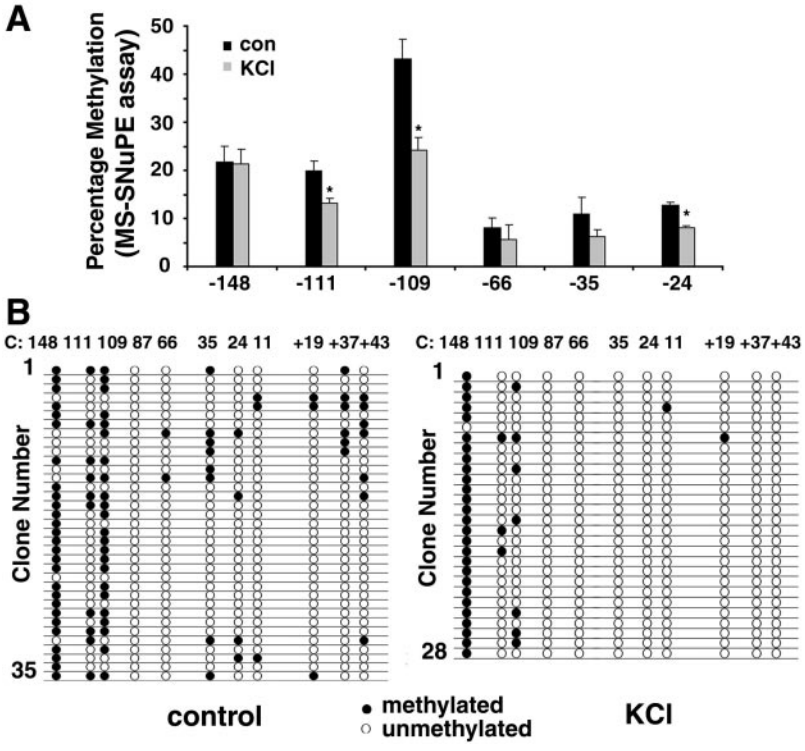

Fig. 2. Activity-dependent methylation changes within the *Bdnf* exon IV promoter. (**A**) Phosphoimager quantification of the MS-SNuPE assays of methylation at CpG sites of *Bdnf* exon IV promoter from the region of base pairs –24 to –148 (*, $P < 0.05$, KCl-treated samples compared with control, $n \geq 4$). (**B**) Bisulfite sequencing analysis performed on 11 CpG sites near the transcription initiation site of exon IV. Analysis performed on clones derived from DNA from untreated and depolarized E14 3-DIV cultures. The discrepancy between the two assays at the site of base pair –148 is addressed in fig. S2. C, cytosine.

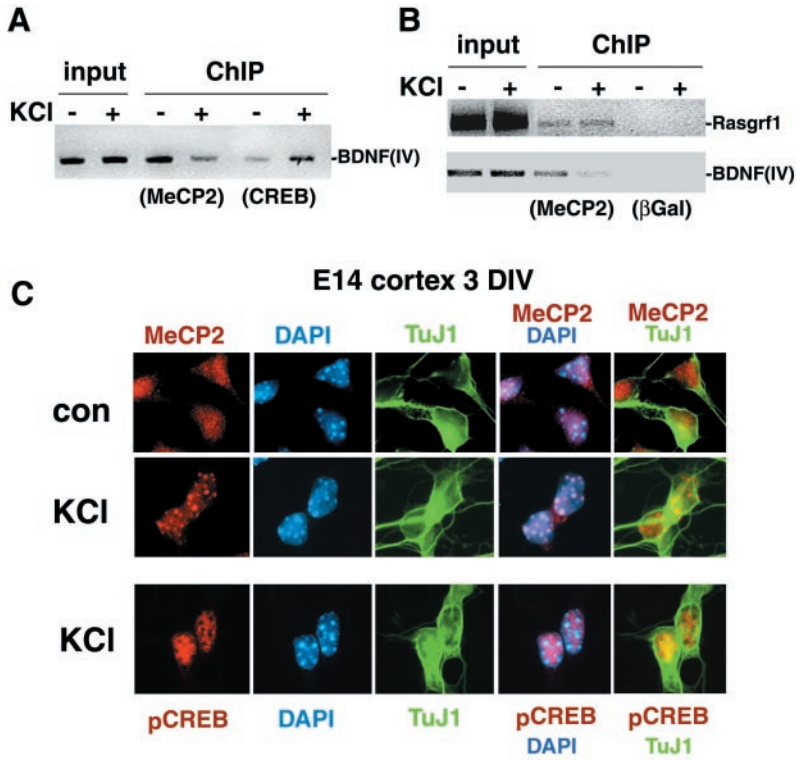

Fig. 3. Reciprocal association of MeCP2 and CREB with the *Bdnf* exon IV promoter. (**A**) ChIP assays of MeCP2 and CREB binding within the *Bdnf* (IV) promoter region of base pairs +1 to –200 before and after depolarization. (**B**) Association of MeCP2 with the differentially methylated domain of the imprinted gene *Rasgrf1* in response to depolarization. (**C**) Staining of 3-DIV E14 cortical neurons cultured in the presence or absence of KCl with the neuronal marker TuJ1 and MeCP2. KCl-treated cortical neurons were also double stained with pCREB and TuJ1. The levels of MeCP2 are similar in cells with and without KCl treatment (fig. S4A).

when cells were not depolarized. However, after depolarization, MeCP2 partially dissociated from the promoter, whereas CREB became more tightly associated (Fig. 3A). We also found that MeCP2 association with the differentially methylated domain of an imprinted gene, *Rasgrf1* (*18*), did not change after depolarization (Fig. 3B). These ChIP assays suggest that MeCP2 dissociates from specific gene loci upon depolarization. Consistent with the notion that depolarization may induce a redistribution of MeCP2 within the nucleus, we found that the more diffused nuclear staining pattern of MeCP2 in nondepolarized E14 mouse cortical cells became more punctate upon depolarization (Fig. 3C) (*17*). MeCP2 staining overlaps with 4′,6′-diamidino-2-phenylindole (DAPI)–positive puncta, indicative of heavily methylated pericentromeric heterochromatin structures (*3*, *19*). The MeCP2/DAPI overlapping pattern was also observed in adult cortical neurons in vivo. It is possible that depolarization accelerates neuronal maturation, leading to a global redistribution of MeCP2. In contrast, Ser133 phospho-CREB (pCREB) appears to be excluded from the DAPI puncta (Fig. 3C). Together, these data are consistent with a role for MeCP2 as a transcriptional repressor and for pCREB as a transcriptional activator, as well as consistent with the reciprocal association of MeCP2 and pCREB with the BDNF promoter in control and depolarized neurons.

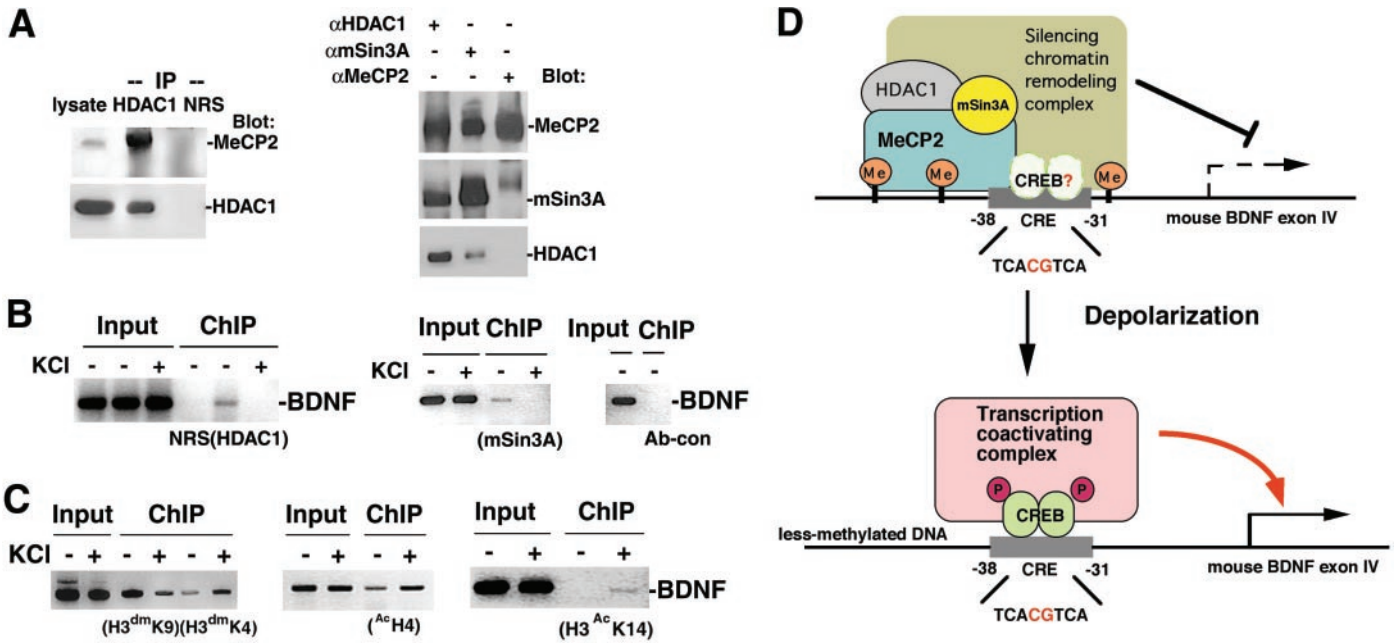

Fig. 4. Involvement of chromatin remodeling in DNA methylation–related and MeCP2-mediated regulation of *Bdnf* exon IV. (**A**) Coimmunoprecipitation assays with 3-DIV E14 neuronal culture to determine coassociation of HDAC1, mSin3A, and MeCP2. Immunoprecipitates were analyzed by Western blot. Normal rabbit serum (NRS) was used as a negative control. The association of the proteins can be detected using the HDAC1 and mSin3A antibodies. Our MeCP2 antibody (Upstate) was raised against a C-terminal peptide, which failed to pull down the complex. This could be due to the possible antibody-triggered disruption of the protein complex and/or because only a subset of MeCP2 associates with the mSin3A-HDAC1 complex. ChIP assays show the association of HDAC1 and mSin3A (**B**), $H3^{dm}K9$ and $H3^{dm}K4$, and $H3^{Ac}K14$ and $^{Ac}H4$ (**C**) with *Bdnf* exon IV promoter with primers specific for a region of ~200 bp directly upstream of transcription initiation. NRS and no antibody immunoprecipitates (Ab-con) were used as controls for ChIP assay specificity. (**D**) A proposed mechanism by which DNA methylation–related chromatin remodeling regulates neuronal activity-dependent *Bdnf* gene expression.

MeCP2 may participate in gene silencing by serving as a bridge linking DNA methylation with chromatin remodeling by recruiting histone deacetylases (HDACs) through the corepressor mSin3A (*4*, *20*, *21*) or histone methyltransferases such as SUV39H1 (*22*, *23*). Coimmunoprecipitation assays (*17*) showed that MeCP2, HDAC1, and mSin3A associated with one another in 3-DIV E14 cortical cells (Fig. 4A). ChIP assays indicated that HDAC1 and mSin3A were also associated with the *Bdnf* IV promoter when the cells were not depolarized (Fig. 4B). However, both proteins become dissociated from the promoter upon membrane depolarization, consistent with the notion that activity-dependent *Bdnf* gene expression involves dissociation of the MeCP2-HDAC1-mSin3A repression complex (Fig. 4B). Additionally, histone H3 and H4 acetylation becomes more evident within the *Bdnf* exon IV promoter in depolarized cortical neurons as indicated by ChIP assays (Fig. 4C).

MeCP2 can interact not only with HDACs but also with the histone H3 lysine (K) 9 methyltransferase, SUV39H1, and trigger dimethylation of histone H3 on K9 ($H3^{dm}K9$), providing a histone code, indicative of inactive chromatin structure (*22*). In contrast, dimethylation of histone H3 at the K4 position ($H3^{dm}K4$) provides a histone code for the active, euchromatin structure (*24*–*27*). We performed ChIP analyses with antibodies against both $H3^{dm}K9$ and $H3^{dm}K4$ (*17*) and found more $H3^{dm}K9$ association with the *Bdnf* exon IV promoter before depolarization and stronger $H3^{dm}K4$ association after depolarization (Fig. 4C). These data suggest that, upon depolarization, the *Bdnf* exon IV promoter switches from an inactive to an active chromatin structure.

We propose a model in which DNA methylation and its related chromatin remodeling play critical roles in regulating gene transcription in response to neuronal activity (Fig. 4D). CpG methylation at any critical site may increase the likelihood of MeCP2 binding, which can recruit HDACs and the H3-K9 methyltransferase to mediate inactive chromatin remodeling, or may directly induce chromatin compaction to repress gene expression (*28*). Dissociation of MeCP2 and its associated corepressors from the *Bdnf* promoter after depolarization allows for active chromatin remodeling and subsequent gene activation by transcription coactivators. Recent evidence suggests that MeCP2 phosphorylation can also induce its dissociation from methylated DNA (*29*); however, this modification appears to be transient (fig. S4B). Therefore, additional mechanisms such as changes in DNA methylation may be required for sustained dissociation of MeCP2 from the active promoter. Collectively, our findings suggest that dynamic changes in DNA methylation can occur in postmitotic neurons, and that methylation-mediated chromatin remodeling may play critical roles in long-lasting neuronal adaptive responses.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S4
References

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