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REST and Its Corepressors Mediate Plasticity of Neuronal Gene Chromatin throughout Neurogenesis

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Summary

Regulation of neuronal gene expression is critical to central nervous system development. Here, we show that REST regulates the transitions from pluripotent to neural stem/progenitor cell and from progenitor to mature neuron. In the transition to progenitor cell, REST is degraded to levels just sufficient to maintain neuronal gene chromatin in an inactive state that is nonetheless poised for expression. As progenitors differentiate into neurons, REST and its corepressors dissociate from the RE1 site, triggering activation of neuronal genes. In some genes, the level of expression is adjusted further in neurons by CoREST/MeCP2 repressor complexes that remain bound to a site of methylated DNA distinct from the RE1 site. Expression profiling based on this mechanism indicates that REST defines a gene set subject to plasticity in mature neurons. Thus, a multistage repressor mechanism controls the orderly expression of genes during development while still permitting fine tuning in response to specific stimuli.

Introduction

Nervous system development relies on a network of transcriptional repressors and activators that control the orderly acquisition and maintenance of neuronal traits (reviewed in Anderson and Jan [1997], Edlund and Jessell [1999], Munoz-Sanjuan and Brivanlou [2002], and Sasai [1998]). Although many of these factors have been studied individually, a unifying framework, if it exists, that underlies the sequential stages of neurogenesis, from stem cell to terminal differentiation, has not been identified. Moreover, whether genetic plasticity in terminally differentiated neurons exploits the same mechanisms utilized during development is also not known.

The transcriptional repressor REST (RE1 silencing transcription factor, also called NRSF), as a key regulator of many neuronal genes (Chong et al., 1995; Schoenherr and Anderson, 1995), is a good candidate for providing a unifying framework. REST is a zinc finger protein that binds to a conserved 23 bp motif known as RE1 (repressor element 1, also called NRSE) (Chong et al., 1995; Schoenherr and Anderson, 1995) in a large number of genes encoding fundamental neuronal traits (Schoenherr et al., 1996) such as ion channels, synaptic vesicle proteins, and neurotransmitter receptors. REST mediates active repression via recruitment of histone deacetylases by its corepressors mSin3 and CoREST (Ballas et al., 2001). Silencing of neuronal genes by REST in differentiated nonneuronal cells occurs in conjunction with CoREST (Andres et al., 1999), which recruits additional silencing machinery including the methyl DNA binding protein MeCP2 and the histone H3 K9 methyltransferases G9a and SuVar39H1 (Lunyak et al., 2002; Roopra et al., 2004; Shi et al., 2003). Thus, while mechanisms of REST silencing outside the nervous system have been illuminated, roles for the REST repressor complex during nervous system development have been largely unexplored.

Mice lacking the REST gene die very early in development (Chen et al., 1998). This phenotype precludes in-depth study, but the mice display a highly crenulated ventricular zone accompanied by massive cell death (J. Leheste and G.M., unpublished data). Similarly, overexpression of REST in the developing spinal cord of chicken embryos causes neuronal pathfinding errors (Paquette et al., 2000) and, in neuronal cell lines, blocks the developmental acquisition of sodium channel excitability in response to growth factor (Ballas et al., 2001). Finally, hundreds of genes, many of them neuronal, contain REST binding sites as identified through computational analyses (Bruce et al., 2004). These data, taken together, suggest a link between acquisition of a neuronal phenotype during development and REST function.

Here, we show that the REST complex regulates its target genes differentially at different developmental stages. REST repression in embryonic stem (ES) cells utilizes a mechanism independent of both histone H3 K9 methylation and DNA methylation at the REST binding site, creating a chromatin status poised for subsequent activation. Unlike the case in ES cells, REST protein is downregulated posttranslationally in neural progenitors. The low REST concentration likely permits selective upregulation of REST target genes. Differentiation of progenitors to mature neurons occurs with loss of REST repressor complex from the RE1 site of neuronal genes as the *REST* gene itself is transcriptionally repressed. While some of these genes are expressed to high levels by default, other genes, such as *Calbindin* and brain-derived neurotrophic factor (*BDNF*), are expressed at lower levels due to the continued presence of CoREST and MeCP2 on an adjacent site of methylated DNA in neurons. Membrane depolarization increases the level of expression of the *BDNF* gene through selective release of MeCP2 but not CoREST from its methylated site. The persistence of CoREST after REST departure may provide a platform for the dynamic assembly and disassembly of repressor complexes required for plasticity in mature neurons.

Results

The Transition from ES to Neural Stem/Progenitor Cell Involves Posttranslational Degradation of REST

A starting point for our studies was mouse ES cells. Embryonic stem cells can form embryoid bodies (EBs)

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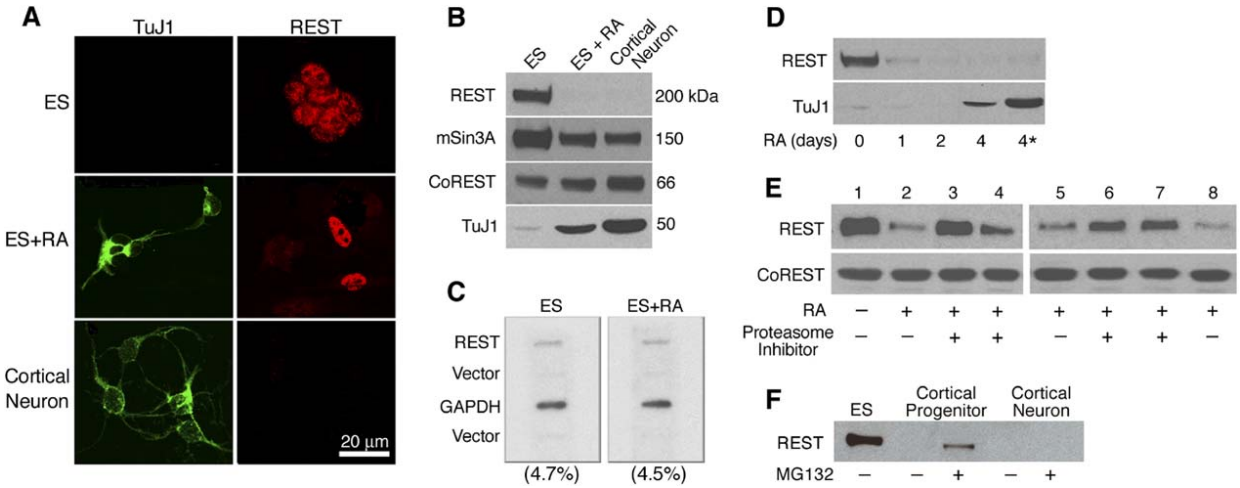

Figure 1. REST Protein Is Degraded Selectively at an Early Stage of Neuronal Differentiation

(A) Immunostaining for REST (red, right) and neuronal β-tubulin (TuJ1, green, left) in embryonic stem (ES) cells and mature neurons. Embryoid bodies (EBs) treated with retinoic acid (RA) for 4 days followed by 2 days in culture without RA (ES+RA).

(B) Western blot analysis of REST repressor components and TuJ1 in ES cells and mature neurons (ES+RA as in (A).

(C) Nuclear run-on analysis comparing transcriptional rates (%) for the *REST* gene relative to *GAPDH* in nuclei from ES cells and from EBs treated for 3 days with RA (ES+RA). Radiolabeled RNA from the cells was hybridized to immobilized cDNA in vector (slots 1 and 3) and vector alone (slots 2 and 4).

(D) Western blot analysis of cell extracts after short-term (1–2 day) or long-term (4 day) treatments of EBs with RA. Asterisk indicates 4 days +RA followed by 2 days in culture without RA.

(E) Western blot analysis of extracts from EBs treated for 14 hr simultaneously with RA and the proteasome inhibitors MG132 (lane 3) or lactacystin (lane 4), or with RA for 14 hr followed by addition of MG132 (lane 6) or lactacystin (lane 7) for another 5 hr. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (lane 8) served as a control.

(F) Western blot analysis of REST in cell extracts from E12.5 cortices (cortical progenitors) or cortical neurons treated with (+) or without (–) MG132.

in culture that, upon exposure to retinoic acid (RA), differentiate into neurons. The differentiation occurs by pathways that recapitulate neuron generation in vivo (Wichterle et al., 2002). We exploited this relatively simple system to analyze REST function as early as the pluripotent stem cell stage and throughout differentiation. REST protein was present in the nuclei of dividing undifferentiated ES cells, none of which express the neuronal-specific β-tubulin gene TuJ1 (Figure 1A, top panel). It is likely that REST functions as a repressor at this stage; it was bound to the RE1 site in the neuronal *Calbindin D28K* (*Calbindin*) gene, both REST and CoREST functioned as repressors in functional assays of ES cells, and CoREST was in complexes with HDAC (see Figure S1 in the Supplemental Data available with this article online).

With exposure of EBs to RA, approximately 80% of the cells differentiated into neurons (data not shown). Neurons that stained positively for the neuronal marker TuJ1 were negative for REST (Figure 1A, middle panel). The two REST-positive nuclei in Figure 1A represent nonneuronal cells. The reciprocity of REST and TuJ1 was not an in vitro phenomenon because postmitotic cortical neurons also lacked REST (Figure 1A, bottom panel). Moreover, the disappearance of REST was selective because its corepressors, CoREST and mSin3A, persisted in the mature neurons (Figure 1B). CoREST was still in complexes with HDAC (Figure S1C), suggesting that CoREST mediates repression in neurons in the absence of REST.

Although ES and neural stem cells each have the capacity to self renew, the differentiation capacity of the latter and other neural progenitors is restricted to a neural lineage (Temple, 2001). As a repressor of neuronal genes, we reasoned that modulation of REST expression or function or both might be responsible for the transition from ES to neural stem/progenitor cell. To test this idea, REST properties were examined in early stages of ES cell differentiation and in neural progenitors isolated from cortices of E12.5 mouse embryos. Because changes in transcription factor levels during development are often controlled at the mRNA level, we examined the transcriptional rate of *REST* before and after 3 days of RA treatment of EBs. Surprisingly, the transcriptional rates remained constant (Figure 1C), and REST mRNA levels remained high (40%–50% relative to ES cells) (data not shown). In contrast, the amount of REST protein was reduced dramatically within the first 24 hr of RA treatment and was undetectable thereafter (Figure 1D), suggesting that REST was downregulated posttranslationally at this stage of differentiation. Indeed, treatment of EBs with the proteasome inhibitors MG132 or lactacystin resulted in stabilization (Figure 1E, lanes 1–4) or restoration (Figure 1E, lanes 5–7) of REST protein. The RA-induced proteasomal degradation was specific for REST because neither RA nor proteasome inhibitors had any effect on CoREST protein (Figure 1E).

Interestingly, there was an interval of time, 1–2 days after RA treatment, during which REST was downregu-

lated but neuronal genes, such as TuJ1, were not yet expressed (Figure 1D). Because these cells were dividing and nestin positive (Figure S2), we suspected that they represented the lineage-restricted neural stem/progenitor cells and that the transition from ES cells to this stage was triggered by the proteasomal degradation of REST protein. To test whether this same stage existed in vivo, cortical progenitors were analyzed for REST expression. Immunostaining of acutely isolated cells or cells allowed to proliferate for 3 days in culture in the presence of FGF indicated that, as in the RA-treated EBs, cortical progenitors were mostly nestin positive and TuJ1 negative (Figure S2). Importantly, REST protein was undetectable by Western blot analysis of cortical progenitors, but its presence was restored upon treatment with proteasome inhibitor (Figure 1F). Regulation by proteasomal degradation was specific for this stage, because proteasome inhibitor was unable to restore REST protein in postmitotic cortical neurons (Figure 1F).

Transition from Neural Progenitor, where REST Is Bound Selectively to Its Targets, to Mature Neuron Requires Dismissal of REST from Neuronal Gene Chromatin and Its Transcriptional Repression

The apparent absence of REST protein at early stages of ES cell differentiation and in cortical progenitors (Figures 1D and 1F) at a time when neuronal genes were not yet expressed (Figures 1D and 2E) prompted us to analyze the chromatin in these cells. Surprisingly, REST was still bound to the RE1 region of neuronal genes in cortical progenitors (Figure 2A, left panel), and EBs treated for 1–2 days with RA (data not shown). Importantly, however, REST was absent from the chromatin of the same genes in postmitotic neurons (Figure 2A, right panel). Furthermore, quantitative ChIP analysis showed that the amount of REST associated with a specific RE1 was similar in undifferentiated ES cells and acutely isolated or dividing cortical progenitors in culture (Figure 2B, E12.5 and E12.5+3 DIV), declining to negligible levels with differentiation to mature cortical neurons (Figure 2B).

To confirm that REST was a repressor in cortical progenitors, the cells were purified by sorting for a surface carbohydrate marker (LeX) enriched in neural stem/progenitor cells (Capela and Temple, 2002) and then infected with adenovirus expressing a dominant-negative (D/N) form of REST (Ballas et al., 2001). Real-time RT-PCR analysis demonstrated that overexpression of D/N REST resulted in derepression of the *BDNF*, *Calbindin*, and *Synaptotagmin 4* genes (Figure 2C). Although REST occupied the *Nav1.2* gene in cortical progenitors (Figure 2A), D/N REST had no effect on its expression, perhaps reflecting regulation by additional mechanisms at this stage.

The global degradation of REST in progenitors (Figure 1F) at a time when REST was still bound to neuronal gene chromatin (Figures 2A and 2B) was not predicted. However, if REST target genes exhibited different affinities for REST, the decreased REST concentration could result in differential gene expression. We searched the genome for new REST targets and identified a consensus RE1 in the proneural gene *MASH1*, which encodes a transcription factor important in specifying neuronal identity as early as the progenitor cell stage (Casarosa et al., 1999). REST was present on the *MASH1* gene in ES cells, but its presence was reduced dramatically (5-fold) in cortical progenitors (Figure 2D, left panel) in conjunction with increases (150-fold) in *MASH1* mRNA levels (Figure 2D, right panel). In contrast, the mRNA levels for neuronal genes did not increase until around the time of terminal differentiation (Figure 2E). Although all the REST-regulated genes were upregulated with the same time course, the levels of expression of the *Calbindin* and *BDNF* genes were an order of magnitude lower than that of the *Synaptotagmin 4* and *Nav1.2* genes, likely due to distinct regulatory mechanisms (see below).

In neurons, the proteasomal pathway no longer regulated REST (Figure 1F), and *REST* mRNA levels were significantly lower than in the progenitors (Figure 2F). To test whether this was due to HDAC-mediated transcriptional repression of the *REST* gene, ES cells, cortical progenitors, and neurons were treated with the HDAC inhibitor trichostatin A (TSA). TSA had no effect on *REST* expression in ES or cortical progenitors (Figure 2F), consistent with active transcription of the *REST* gene at this stage (Figure 1C). In contrast, treatment of cortical neurons with TSA resulted in a 5-fold increase in *REST* expression (Figure 2F), suggesting that the *REST* gene was itself transcriptionally repressed and that HDAC was involved in the mechanism. Chromatin immunoprecipitation (ChIP) analysis of the *REST* promoter ultimately revealed the presence of an HDAC complex that contained mSin3A, CoREST, and MeCP2 (Figure 2G). Intriguingly, the RA receptor (RAR) and its corepressor, N-CoR, were part of this repressor complex, bound to a region containing a RA receptor element (RARE), located 400 bp upstream of the transcriptional start site (Figure 2G).

Neuronal Gene Chromatin in ES Cells Is Maintained in a Poised State via a Specialized REST Repressor Complex that Permits Low Basal Expression of Neuronal Genes

Because only ES cells and neural progenitors and not terminally differentiated nonneuronal cells have the capacity to acquire a neural phenotype, we reasoned that neuronal genes in ES cells and progenitors might contain a distinct pattern of epigenetic modifications.

The REST repressor complex on the RE1 site was analyzed in ES cells, cortical progenitors, and cortical neurons and compared to fibroblasts in which the genes are silent (Lunyak et al., 2002; Roopra et al., 2004). The core complex was present on genes in all of the nonneuronal cells but was absent in neuronal cells lacking REST (Figure 3A). The histone H3 K9 methyltransferase G9a, however, was present at much higher levels on neuronal genes in fibroblasts than in the pluripotent ES cells (Figure 3B). Additionally, dimethylated histone H3 K9 (diMeK9H3), a modification associated with silencing, was detected only in the fibroblasts (Figure 3C). Because this modification is associated with DNA methylation (Bird, 2002) and silencing (reviewed in Bannister et al. [2002] and Fischle et al. [2003]), we examined the methylation status of the RE1 site. Bisul-

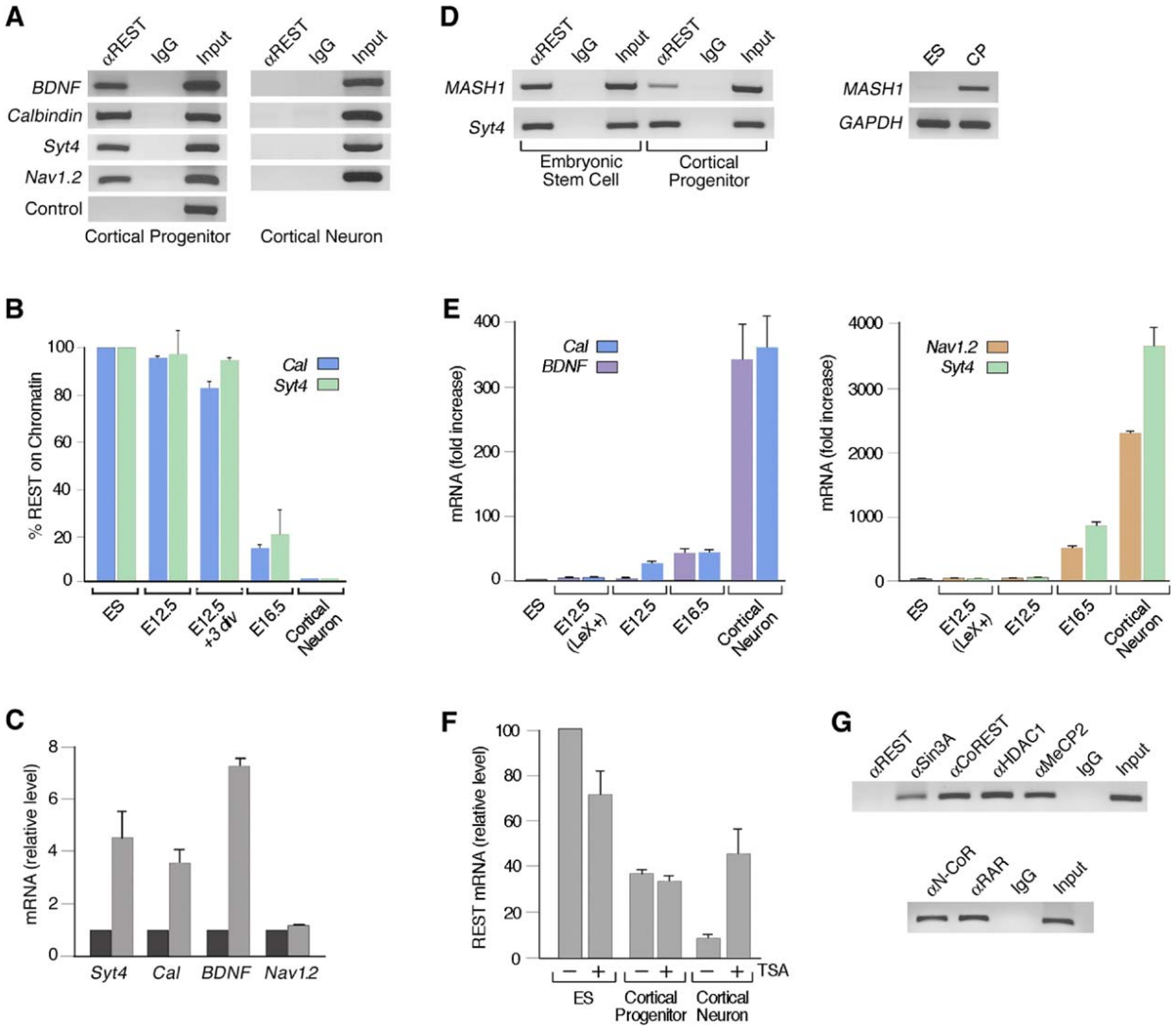

Figure 2. Transition to a Mature Neuron Requires Dismissal of REST from the Chromatin of Neuronal Genes and Its Transcriptional Repression

(A) ChIP analysis indicating REST on the RE1 site of four neuronal genes in cortical progenitors but not neurons. Control DNA is the 3′ UTR of *Synaptotagmin 4* (*Syt4*) gene.

(B) Disappearance of REST from the RE1 sites of *Calbindin* (*Cal*) and *Synaptotagmin 4* (*Syt4*) genes during neurogenesis measured by quantitative ChIP analysis. DIV, days in vitro. E12.5, E16.5, cortices from E12.5 or E16.5 embryos. Error bars represent SD based on three independent experiments.

(C) Real-time RT-PCR measurements of mRNA from LeX-enriched cortical progenitors infected with adenovirus bearing dominant-negative REST and GFP (gray bars) or GFP alone (black bars). *BDNF*, *BDNF* exon IV. Error bars as in (B).

(D) (Left) Semiquantitative ChIP analysis of REST bound to the RE1 site of the proneural gene *MASH1*. (Right) Semiquantitative RT-PCR analysis of *MASH1* mRNA levels in ES cells and LeX sorted cortical progenitors.

(E) Real-time RT-PCR analysis of mRNA levels of neuronal genes at indicated developmental stages. *BDNF*, *BDNF* exon IV. Error bars as in (B).

(F) Real-time RT-PCR analysis of *REST* mRNA levels in cells not treated (–) or treated (+) with the HDAC inhibitor TSA. Error bars as in (B).

(G) ChIP analysis of the *REST* gene in cortical neurons using primers surrounding a RARE site in the promoter region. Antibodies are as indicated (αRAR, anti-RARα+anti-RARβ).

fite genomic sequencing analysis indicated that the RE1 motif and flanking region in the *Calbindin* (Figure 3D and Figure S3) and *Synaptotagmin 4* (data not shown) genes was densely methylated in fibroblasts but not ES cells.

Di- and triMeK4H3, usually associated with actively transcribed genes, was absent from fibroblasts (Figure 3C and Lunyak et al. [2002]). Unexpectedly, although ES cells are not known to express neuronal genes, these modifications were present on the RE1 sites of *Calbindin* and *Synaptotagmin 4* genes (Figure 3C). This finding prompted a more critical evaluation of gene expression. Indeed, RNA polymerase II occupied the *Calbindin* and *Synaptotagmin 4* genes in ES cells but not fibroblasts (Figure 3B). Further, their mRNA was detected by RT-PCR in ES cells (Figure 3E) and ES cells

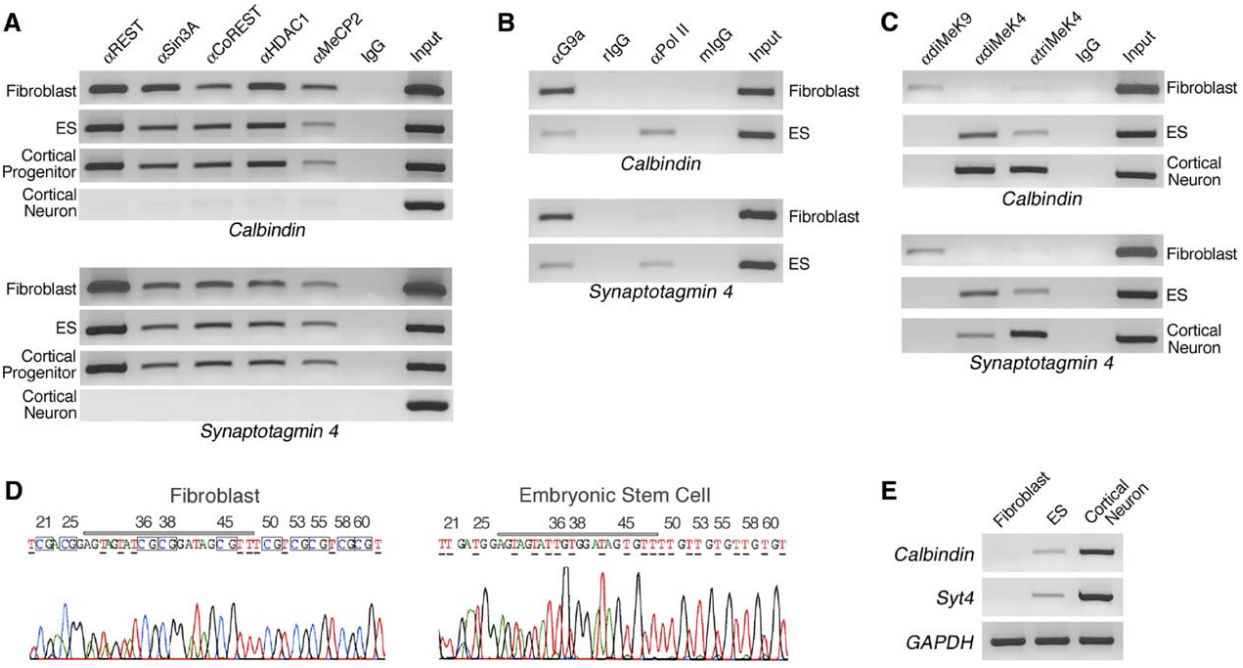

Figure 3. A REST Complex on the RE1 Permits Low Basal Expression Levels of Neuronal Genes in ES Cells due to Distinct Epigenetic Modifications

(A) ChIP analysis of a core REST repressor complex in indicated cell types.

(B) ChIP analysis for two REST target genes using indicated antibodies. RNA polymerase II (Pol II). Control antibodies are rat (r) and mouse (m) IgG.

(C) ChIP analysis of neuronal genes using indicated antibodies to methylated histone H3. For di- and triMeK4 in ES cells and cortical neurons, ChIP DNAs were amplified under semiquantitative conditions.

(D) Bisulfite genomic sequencing analysis of the RE1 region (bar) of the *Calbindin* gene. Methylated CpGs are enclosed in boxes, and nonmethylated cytosines are indicated by Ts.

(E) RT-PCR analysis of neuronal gene mRNAs and *GAPDH* as a control. Cycles of amplification were increased for fibroblasts to show complete absence of neuronal mRNAs.

enriched for LeX (data not shown), although the levels of expression were much greater in cortical neurons (Figures 2E and 3E).

A pattern emerged from examination of the relative amounts of di- and triMeK4H3 associated with six neuronal genes (Figure 3C and Figure S4), all of which contained an RE1 site within 1 kb of their transcriptional start sites. Because they are different antibodies, comparisons of absolute levels of di- and triMeK4H3 for specific genes are invalid. However, for all the genes, the levels of triMeK4H3 were higher in mature neurons than in ES cells, consistent with their higher expression levels in neurons (Figure S4). In contrast, the relative amounts of diMeK4H3 in ES cells and neurons were gene dependent (Figure 3C and Figure S4); however, the ratio of tri- to diMeK4H3 was always higher in neurons (Figure 3C and Figure S4).

To determine how these epigenetic differences in ES cells and fibroblasts might impact on differences in the plasticity of neuronal chromatin, the ability of TSA and the demethylating agent 5′-aza-2-deoxycytidine (5-azadC) to derepress the *Calbindin* and other neuronal genes was examined. Only in ES cells did these treatments exhibit an effect, resulting in a dramatic and synergistic derepression of the *Calbindin* gene (Figure 4A) and additive derepression of the *Synaptotagmin 4* and *Nav1.2* genes (Figure S5). Because the RE1 region of these genes is not methylated in ES cells, however, the derepression by 5-azadC suggested regulation by a site of DNA methylation distinct from the RE1 site.

Methylated DNA Binds a REST Corepressor Complex in ES Cells and Persists as a Platform for Dynamic Regulation of Neuronal Genes in Mature Neurons

Bisulfite genomic sequencing analysis identified eight consecutive methylated CpGs (mCpGs) in the *Calbindin* promoter region in ES cells (Figure 4B, left panel, and Figure S6) that were demethylated by 5-azadC treatment (Figure 4B, right panel). A site of methylated DNA has been also identified in *BDNF* promoter IV in neurons (Martinowich et al., 2003). ChIP analysis revealed the presence of the same REST repressor complex on the RE1 and mCpG sites of the *Calbindin* and *BDNF* promoters in ES cells, irrespective of the distances of the RE1 site from the mCpG site (Figure 4C). The unexpected presence of REST on the mCpG regions may be explained by a gratuitous association with its corepressors, present on the mCpG sites, or by a looping of the DNA between the RE1 and mCpG sites (see Discussion).

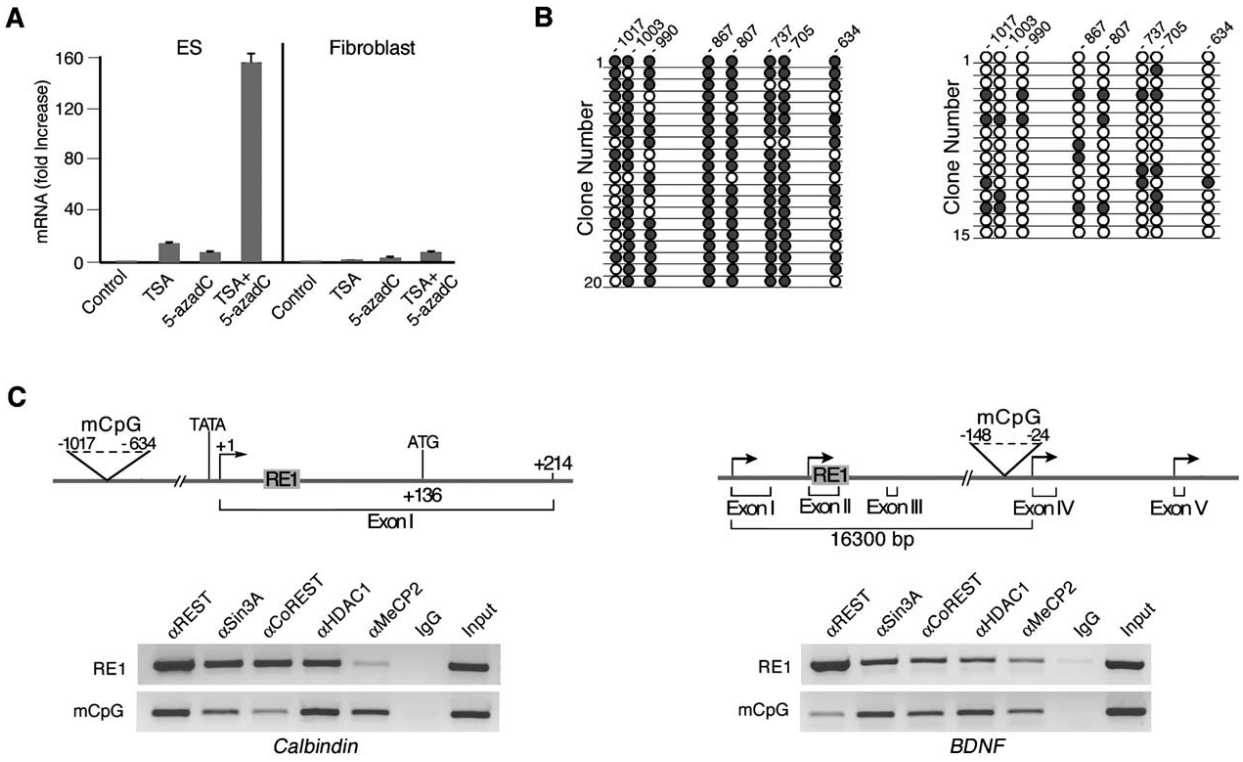

Figure 4. Methylated CpGs in *Calbindin* and *BDNF* Promoters Bind an Additional Corepressor Complex in ES Cells

(A) Real-time RT-PCR analysis of *Calbindin* mRNA levels in cells with no treatment (control) or after exposure to HDAC inhibitor (TSA) and/or demethylating agent (5-azadC). Error bars represent SD based on three independent experiments.

(B) Clonal analysis of bisulfite genomic sequencing of a region of mCpGs in the *Calbindin* gene in ES cells (left) that is demethylated by 5-azadC (right). Closed circles, mCpG; open circles, CpG.

(C) (Top) Schematic diagrams for the *Calbindin* (left) and *BDNF* (right) genes. Arrows indicate transcriptional start sites. (Bottom) ChIP analyses of the RE1 and mCpG sites of *Calbindin* and *BDNF* genes.

The eight mCpGs in *Calbindin* in ES cells remained methylated throughout differentiation, including the stage of postmitotic cortical neurons. In fact, the *Calbindin* promoter was more methylated in the neurons relative to ES cells, containing an additional three consecutive mCpGs (Figure 5A). Moreover, MeCP2 and the other corepressors, including CoREST, remained on the mCpG site after loss of REST from the RE1 site (Figure 5B), suggesting an additional regulatory mechanism by REST corepressors in neurons in the absence of REST.

Membrane Depolarization Activates Neuronal Genes Selectively through a Mechanism Involving MeCP2 and CoREST

It was shown recently that membrane depolarization of cortical neurons resulted in derepression of mouse and rat *BDNF* exons (IV and III, respectively) due to phosphorylation-mediated release of MeCP2 from a mCpG site in their promoters that binds a MeCP2/mSin3A/HDAC repressor complex (Chen et al., 2003; Martinowich et al., 2003). Our data indicated that, like the mCpG site of the *Calbindin* promoter, a CoREST/HDAC corepressor complex was also recruited to the mCpG site of *BDNF* promoter IV in cortical neurons (Figure 5B), suggesting similar regulatory mechanisms for these genes. To test this, a real-time RT-PCR analysis was performed. Although the *Calbindin* gene and *BDNF* exon IV were expressed to similarly low levels in cortical neurons, upon membrane depolarization, *BDNF* exon IV expression increased by almost two orders of magnitude, while *Calbindin* gene expression did not change (Figure 5C). Because depolarization promoted *BDNF* but not *Calbindin* gene expression, we asked whether depolarization, which induced phosphorylation of MeCP2 (Figure 5D and Chen et al. [2003]), induced global or gene-specific release of MeCP2 from mCpG promoters. ChIP analysis before and after 3 hr of depolarization (+KCl) revealed that, while MeCP2 was released from the mCpG promoter of *BDNF* exon IV, it remained bound to the mCpG promoter of *Calbindin* (Figure 5E), suggesting that phosphorylation-mediated release of MeCP2 was specific for the induced gene. Unlike the case for MeCP2, CoREST gel mobility was unchanged (Figure 5D) and CoREST remained bound to the methylated promoters of both *BDNF* and *Calbindin* genes (Figure 5E).

One of the events occurring with depolarization is an accumulation of MeCP2 into heterochromatic puncta. This accumulation was reported as occurring after long-term (3–4 days) treatment with KCl (Martinowich et al., 2003). To determine if MeCP2 and CoREST differed in their cellular localization, immunostaining was performed after short-term treatment with KCl. MeCP2

maintained its nuclear localization (Figure 5D) and formed puncta at 5 hr after treatment with KCl (Figure 5F, +KCl) but as early as 90 min (data not shown). The puncta persisted for the duration of the treatment (Figure 5F, 5 hr and 24 hr +KCl) and then dissipated within 19 hr (Figure 5F) and as early as 4 hr after removal of KCl (data not shown). In contrast to MeCP2, CoREST became partially cytoplasmic (Figure 5D) and did not form puncta (Figure 5F, compare merged images).

The Corepressor Complex Occupying the *Calbindin* and *BDNF* Genes Likely Regulates a Larger Set of Genes in Cortical Neurons

The finding that two REST-regulated genes involved in neuronal plasticity, *Calbindin* and *BDNF*, were each occupied by a mCpG corepressor complex prompted us to search for other neuronal genes that might be regulated similarly. We identified *BDNF* exon I as well as four additional RE1-containing genes that were upregulated in cortical neurons relative to ES cells (Figure 6). Like *Calbindin* and *BDNF* exon IV, all these genes were further upregulated in cortical neurons by interference with HDAC activity (+TSA) and, in some cases, also by depolarization (+KCl) (Figure 6). A cDNA microarray analysis performed on mRNA purified from ES cells, cortical neurons, and TSA-treated cortical neurons identified an additional 14 RE1-containing genes that appear to be in this gene class (Figure S7), suggesting that they may also be involved in plasticity (Figure 7B, class II). Another class of REST-regulated genes, represented by *Nav1.2* and *Synaptotagmin 4*, was identified. Genes in this class were also upregulated in cortical neurons, but interference with HDAC activity did not result in further upregulation of these genes (data not shown), suggesting that this gene class is not regulated by corepressors in cortical neurons (Figure 7B, class I).

Discussion

Our findings point to REST as a key regulator of neuronal genes during neural differentiation, both by its presence in progenitors and its absence upon terminal differentiation. A summary of the regulation of REST during neurogenesis is shown in Figure 7A. REST protein is present at its highest level in the nuclei of pluripotent ES cells. As ES cells differentiate to progenitors, REST mRNA levels stay relatively constant, but REST protein is downregulated posttranslationally to minimal levels. The downregulation of REST by the proteasomal pathway (Figure 1) likely occurs in an ubiquitin-independent manner, similar to several other proteins such as c-Jun and IκBα (reviewed in Hoyt and Coffino [2004]), as we were unable to detect polyubiquitylated REST (data not shown). We propose that the decrease in REST concentration plays two roles: it facilitates a rapid transition to terminal differentiation as REST leaves the chromatin of neuronal genes; and it may permit differential expression of a subset of genes in progenitors that have lower affinity REST binding sites. As such, this mechanism may play a fundamental role in the progression of ES cells to lineage-restricted neural progenitors. Supporting the differential binding of REST to RE1 sites is the recent finding that, in U373 glioma cells, where REST is present at low levels, REST occupies some genes, whereas other genes require elevated levels of REST for occupancy (Bruce et al., 2004).

The release of REST from neuronal gene chromatin as progenitors differentiate into neurons is concomitant with its own repression. This likely occurs through recruitment of an unliganded RAR repressor complex to a RARE site located upstream from the transcriptional start site (Figure 7A). RARs play major roles throughout development (reviewed in Sucov and Evans [1995]), and repression through RAR signaling, for example, is required for head formation in *Xenopus* embryos (Koide et al., 2001; Weston et al., 2003). REST repression by the RAR repressor complex may act as a catalyst for exit from the cell cycle and the onset of terminal differentiation. Recently, small noncoding double-stranded RNAs containing a RE1/NRSE sequence have been reported to trigger the activation of neuronal genes during adult hippocampal stem cell differentiation by converting REST from a repressor to an activator (Kuwabara et al., 2004). Our data differ from this mechanism, as we do not detect REST on the chromatin of neuronal genes in postmitotic neurons, and interference with REST binding in progenitors leads to upregulation of the target genes. Future studies will clarify whether a specific signal triggers the dissociation of REST from the RE1 of neuronal genes during cortical neuron differentiation or whether transcriptional downregulation of REST is sufficient to elicit this developmental effect.

In ES cells and neural progenitors, REST represses neuronal genes by recruiting a core corepressor complex to the RE1 site that consists of CoREST, mSin3, HDAC, and MeCP2 (Figure 7B). Despite a similar core complex in fibroblasts, there are striking differences between neuronal gene chromatin in these cells and pluripotent ES cells. In fibroblasts, the REST complex is associated with much higher levels of the histone methyltransferase G9a (Figure 3). Moreover, interfering with HDAC activity does not result in derepression of neuronal genes in fibroblasts. In contrast, in ES cells and presumably in neural progenitors as well, there is diminished G9a associated with neuronal chromatin, and recruitment of HDAC by mSin3, CoREST, and MeCP2 to the RE1 site plays a significant role in maintaining repression. This role for HDAC may help explain the recent finding that inhibition of HDAC activity in adult hippocampal neural progenitors is sufficient to induce neuronal differentiation (Hsieh et al., 2004).

The predominant epigenetic modifications in the RE1 region of silenced neuronal genes in fibroblasts are DNA hypermethylation and diMeK9H3. In ES cells, the RE1 site is hypomethylated, and diMeK9H3 is absent and replaced by di- and triMeK4H3. The significance of di- and triMeK4H3 modifications on both actively transcribed and inactive genes is poorly understood. However, all neuronal genes analyzed in this study exhibited higher levels of triMeK4H3 in neurons relative to ES cells, whereas the relative amount of diMeK4H3 was either unchanged or higher in neurons. Interestingly, the ratio of tri- to diMeK4H3 also correlated with the level of expression of the neuronal genes (Figure S4). Higher ratios of tri- to diMeK4H3 have also been reported for active genes in the chicken *β-globin* locus during development (Schneider et al., 2004). Thus, increases in the

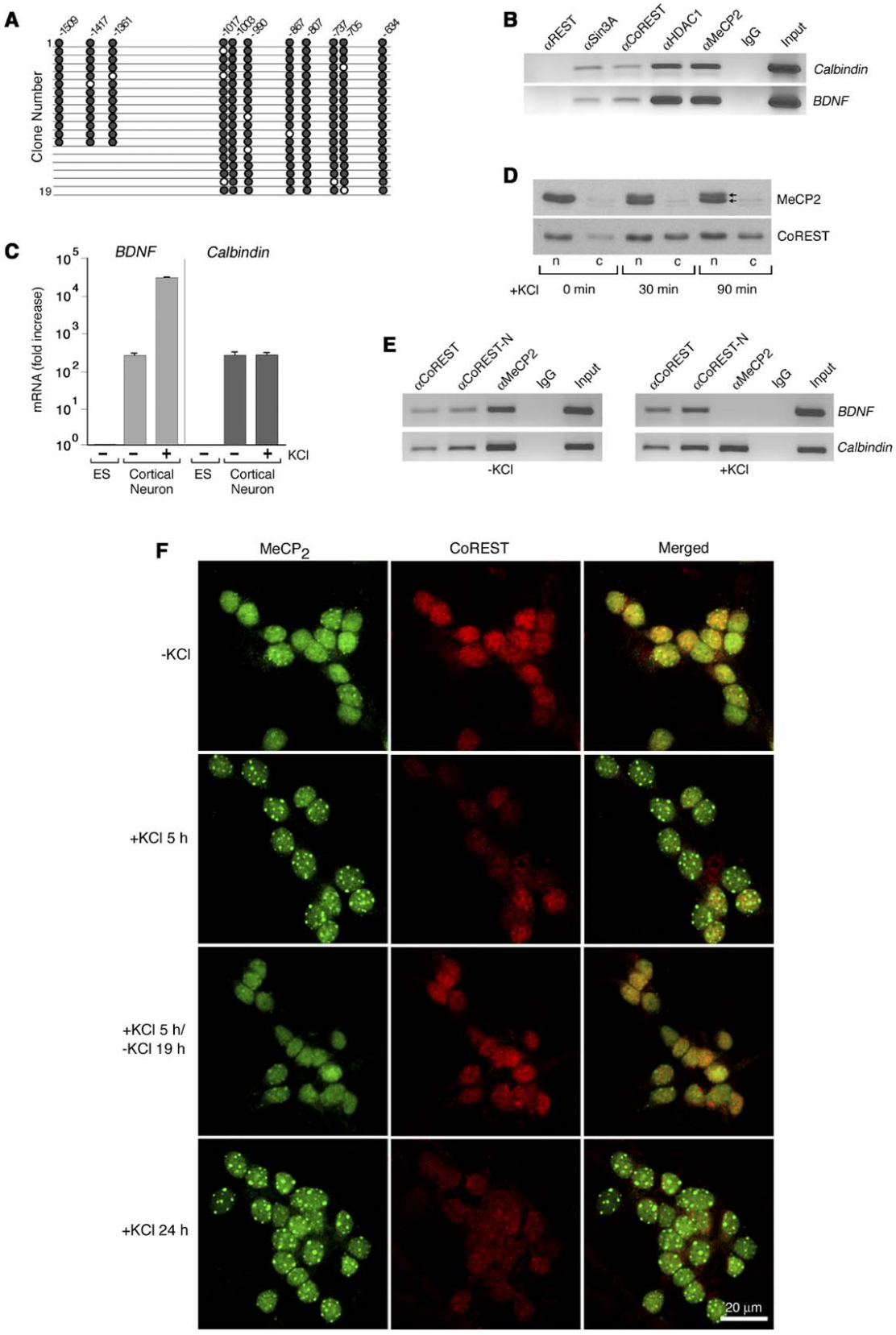

Figure 5. CoREST/MeCP2 Corepressor Complexes Are Associated with Neuronal Gene Chromatin in Neurons in the Absence of REST

(A) Bisulfite genomic sequencing analysis of a region of methylated DNA in the *Calbindin* promoter in cortical neurons. Closed circles, mCpG; open circles, CpG.

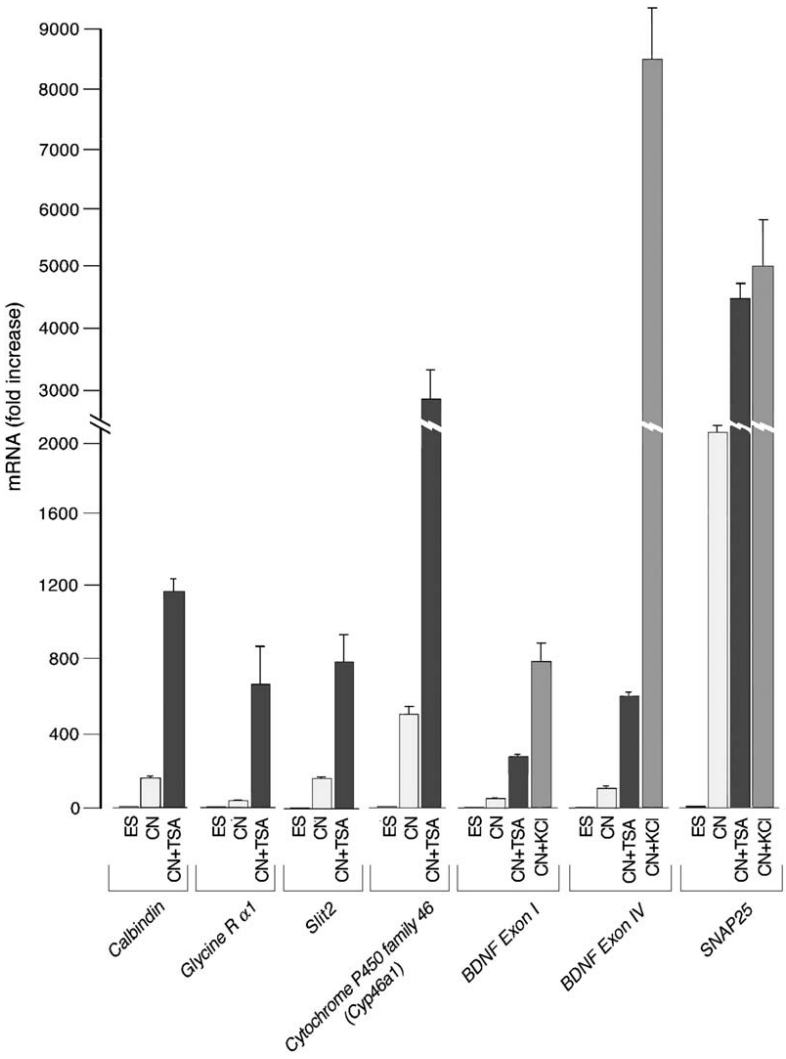

Figure 6. A Class of RE1-Containing Genes Is Upregulated by the HDAC Inhibitor TSA in Cortical Neurons

Real-time RT-PCR analysis of mRNAs transcribed from RE1-containing genes. Cortical neurons were not treated (CN) or treated with HDAC inhibitor (CN+TSA) or depolarized (+KCl) for 10 hr. The fold increases are relative to mRNA levels in ES cells. Error bars represent SD based on three independent experiments.

amount of tri- rather than diMeK4H3 may better reflect a state of gene activation.

In summary, in conjunction with the epigenetic modifications, the REST complex in ES/progenitor cells mediates an active repression mechanism that allows a very low basal level of neuronal gene expression. We propose that this repressor complex helps maintain genes in a state that is permissive for subsequent induced levels of expression. The dual functionality of the REST complex at the RE1 sites of neuronal genes in ES and terminally differentiated nonneuronal cells likely contributes importantly to their differential abilities to further differentiate. A recent paper by Shi et al. (2004) suggests that gene repression is mediated by activity of an H3K4 demethylase (KIAA0601) that is recruited by CoREST to the RE1 site of neuronal genes in differentiated nonneuronal cells (Hakimi et al., 2002; Shi et al., 2004). How this activity, which is specific for diMeK4H3, relates to the changes in neuronal gene activation during development has yet to be determined.

Generally, the activation of neuronal genes in postmitotic neurons depends on the release of the REST repressor complex from the RE1 site. The extent to which different neuronal genes are regulated by the loss of REST, however, differs. Specifically, our data suggests two classes of REST-regulated genes (Figure 7B). Class

(B) ChIP analysis of corepressors occupying the mCpG regions in *Calbindin* promoter and *BDNF* promoter IV in cortical neurons.
(C) Real-time RT-PCR analysis of mRNAs of two REST-regulated genes with no treatment (–) or following treatment (+) with KCl for 10 hr. Error bars represent SD based on three independent experiments.
(D) Western blot analysis of nuclear (n) or cytoplasmic (c) extracts from cortical neurons with no treatment (0 min) or treatment with KCl for indicated time periods.
(E) ChIP analysis of the mCpG regions of the *Calbindin* and *BDNF* genes before (–KCl) and after (+KCl) membrane depolarization for 3 hr. Equal amounts of input chromatin were immunoprecipitated with the indicated antibodies.
(F) Immunostaining for MeCP2 (green) and CoREST (red) in cortical neurons treated with KCl for 5 or 24 hr, or treated for 5 hr with KCl and cultured for additional 19 hr in the absence of KCl (–KCl). Images are representative of three independent experiments.

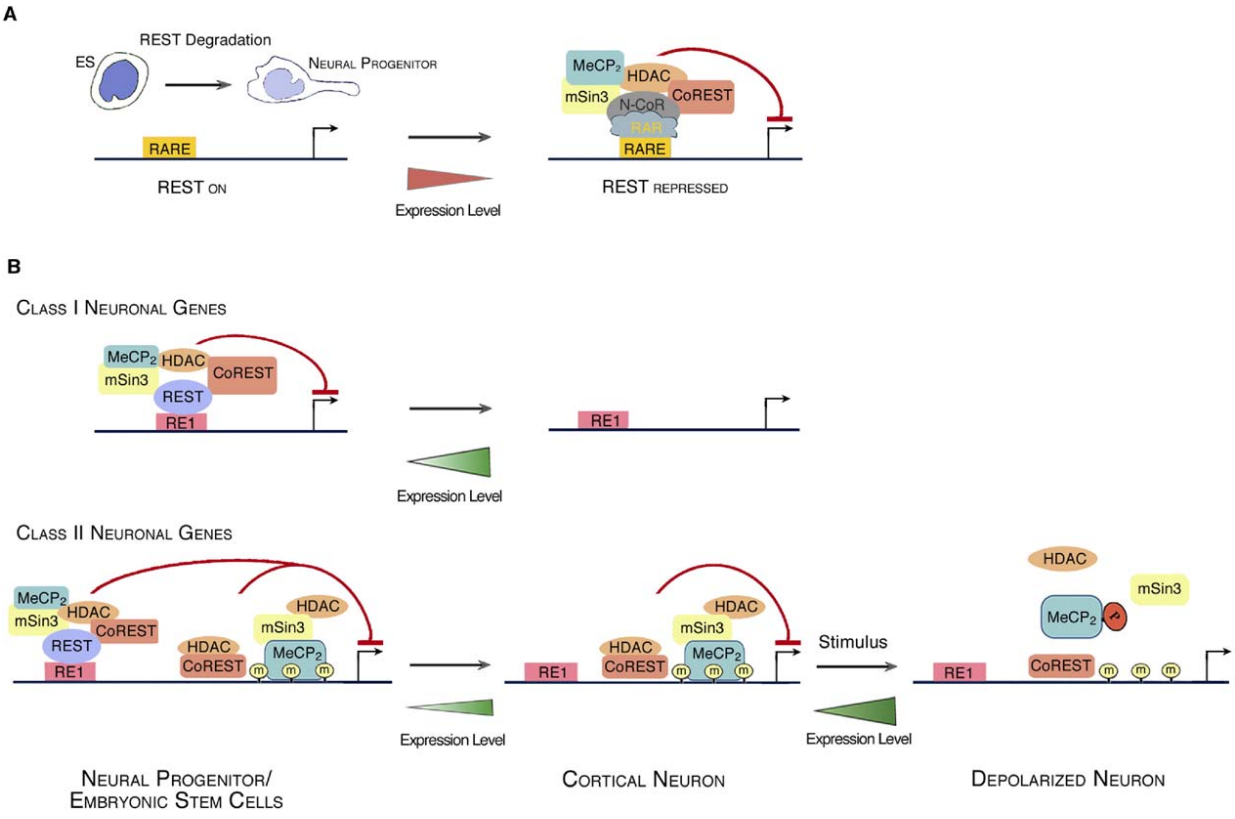

Figure 7. Schematic Models for the Differential Regulation of REST and Its Target Genes during Development and in Mature Cortical Neurons

(A) REST is downregulated by two different mechanisms during neural development. Top cartoon shows REST protein expressed to highest levels in ES cells (dark blue nuclei). In transit to neural progenitor stage, REST protein levels are reduced dramatically by posttranslational degradation (light blue nuclei). In ES cells and progenitors, the REST gene is actively transcribed. As progenitors exit the cell cycle and differentiate into mature neurons, the REST gene is repressed via binding of the unliganded RA receptor (RAR) repressor complex to the retinoic acid receptor element (RARE).

(B) Two classes of RE1-containing genes are regulated differentially in postmitotic cortical neurons. For class I genes, shown in the top diagram, the REST complex occupies the RE1 site and represses gene expression in neural progenitor/ES cells. Levels of expression are increased maximally by default as REST, and thus its corepressors, dissociates from the RE1 site in the transition to cortical neurons. For class II genes, in neural progenitor/ES cells, a REST repressor complex on the RE1 is accompanied by CoREST and MeCP2 corepressor complexes residing on a site of mCpGs (yellow filled circles) in the promoter region. These genes are expressed to lower levels than class I genes (smaller green arrow) in cortical neurons. The transition to mature neurons is accompanied by loss of REST and corepressors from the RE1 site but persistence of the CoREST and MeCP2 repressor complexes at the mCpG site. Upon a specific stimulus, such as membrane depolarization, CoREST remains bound to chromatin, while MeCP2, together with mSin3 and HDAC, leaves the mCpG site of some genes, allowing higher levels of expression.

I genes are expressed by default, relying solely on dissociation of the REST repressor complex from the RE1 site for maximal expression. In contrast, class II genes, typified by *BDNF* and *Calbindin*, contain, in addition to the REST complex at the RE1 site, CoREST and MeCP2 complexes on a distinct site of mCpGs in their promoters that remained bound throughout differentiation. We have represented the complexes at the two sites in a linear fashion (Figure 7B, class II), but other scenarios are possible. Recently, MeCP2 was shown to silence genes via a looped structure involving distinct sites (Horike et al., 2005). Our ChIP analyses (Figure 4C) suggest that a similar event may be occurring between the RE1 and mCpG sites in the *Calbindin* and *BDNF* genes. Indeed, crosstalk by corepressors on a site of mCpGs and a distinct DNA element has been proposed for regulation of the Hairy2a gene during *Xenopus* development (Stancheva et al., 2003).

Upon differentiation into cortical neurons, class II genes are expressed initially at relatively low levels due to the persistence of the CoREST and MeCP2 repressor complexes after REST leaves their chromatin. The two complexes, however, are regulated differentially. Upon membrane depolarization, MeCP2 becomes phosphorylated and, along with HDAC and mSin3, dissociates from the chromatin of the induced genes, typified by *BDNF*, resulting in maximal levels of expression (Figure 7B and Chen et al. [2003], Martinowich et al. [2003]). In contrast to MeCP2, CoREST remains bound, directly or indirectly, to the mCpG region, suggesting that CoREST may serve as an anchor for the dynamic recruitment of its corepressor partners or, alternatively, become a coactivator in response to specific stimuli. The latter idea is supported by our previous findings that CoREST interacts with components of the SWI-SNF chromatin-remodeling complex that mediates both repression and

gene activation (Battaglioli et al., 2002). MeCP2 and CoREST are also redistributed differentially. While MeCP2 forms nuclear puncta, CoREST becomes partially cytoplasmic. Whether the dynamic redistribution of MeCP2 and CoREST is related to their gene regulatory functions during membrane depolarization has yet to be determined.

The *Calbindin* and *BDNF* genes are representative of a larger class of genes whose expression, due to the presence of the CoREST/MeCP2 corepressors, is upregulated in neurons by interfering with HDAC activity and, in some cases, also by depolarization (Figure 6 and Figure S7). Many of these genes have been implicated in plasticity in vivo, but the stimuli are likely to be specific for the individual genes. For example, although the *Calbindin* gene is not regulated by depolarization in cortical neurons, a calcium-sensitive promoter element in *Calbindin* gene has been identified in Purkinje cells (Arnold and Heintz, 1997), suggesting that it is regulated dynamically in a cell type-specific manner in vivo.

The regulation of class II genes by two repressor complexes, and the selective repression by REST only in nonneuronal cells, may help explain a paradox of Rett Syndrome (RTT), a disorder due to a global defect in MeCP2 (reviewed in Nan and Bird [2001], Van den Veyver and Zoghbi [2001]). Although MeCP2 represses genes outside and within the nervous system, inexplicably, the effects of MeCP2 mutations in RTT patients and mouse models are observed primarily in the nervous system (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002). Our model predicts that, in nonneuronal cells, the presence of the REST complex on the chromatin is sufficient to repress neuronal genes even in the absence of MeCP2. In neurons, where REST is absent, CoREST and MeCP2 remain bound to methylated DNA and are the predominant mechanisms for regulation of class II genes (Figure 7B). Indeed, the lack of MeCP2 in mouse models causes a 2-fold increase in steady-state levels of *BDNF* exon III expression in rat cortical neurons (Chen et al., 2003) and subtle transcriptional changes in the brain (Tudor et al., 2002), which could upset the balance of neuronal function, thus resulting in neurological disorder. The subtle changes in gene expression, however, may be due to the continuous presence of CoREST/HDAC complex on the methylated DNA in the absence of MeCP2 and resultant repression, at least in part, of the class II genes. Future studies of class II gene regulation, under stimulated and nonstimulated conditions, may provide further insight to the nervous system phenotype in Rett models.

Experimental Procedures

ES Cell Culture and Differentiation

Mouse ES cells (129/SvJ) were propagated on irradiated mouse embryonic fibroblasts. Prior to experiments, the ES cells were cultured on 0.1% gelatin-coated dishes using ES medium containing 15% fetal bovine serum (HyClone), nucleosides (×100, Specialty Media), DMEM, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 10 mM HEPES, penicillin-streptomycin, and 55 μM βME, supplemented with 1000 U/ml ESGRO (all from GIBCO-BRL). ES cells were differentiated to neurons by forming embryoid bodies (EBs) for 3 days followed by 4 days' treatment with 1 μM retinoic acid (RA) as described (Bain et al., 1995). EBs were dissociated and cultured on poly-D-lysine without RA for an additional 2 days (Bain et al., 1995).

Primary Cultures of Cortical Progenitors and Neurons

Cortical progenitors were isolated from E12.5 mouse embryos and cultured in neurobasal medium supplemented with bFGF (BD Biosciences) as described (Slack et al., 1998). When required, acutely isolated cortical progenitors or cultured progenitors were labeled with MMA (CD15 FITC, BD Biosciences) (Capela and Temple, 2002) and sorted by green fluorescence (488 nm) for LeX-positive cells using a Becton Dickinson FACS Vantage. Cortical neurons were prepared from E15.5–E16.5 embryos. Cortices were dissociated with papain (Worthington) and cultured on poly-D-lysine/laminin-coated dishes in neurobasal medium without bFGF. After 3 days, cells were treated with 4 μM cytosine arabinoside (Sigma) for another 2 days. All experiments were performed on 6 day-in-vitro (6 DIV) cultures except for membrane depolarization studies where 5 DIV cultures were used.

Drug Treatments

EBs were either treated simultaneously for 14 hr with 1 μM RA (Sigma), and 8 μM MG132 or lactacystin (Calbiochem), or for 14 hr with RA followed by 5 hr with 12 μM MG132 or lactacystin or 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cortices from E12.5 embryos were treated for 5 hr with 10 μM MG132 and cortical neurons for 7 hr with 20 μM MG132. Treatment with TSA (Calbiochem) was for 14 hr for all cell types. ES cells and cortical progenitors were treated with 100 nM and 200 nM, respectively, and cortical neurons and fibroblasts (NIH3T3) with 300 nM TSA. ES or NIH3T3 cells were treated with 5-azadC (Sigma) (0.2 μM or 5 μM, respectively) for 40 hr. When cells were treated with TSA and 5-azadC, TSA was added after 26 hr of culture with 5-azadC for another 14 hr.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and probed with rabbit anti-REST-C (generated against the last 300 amino acids of the C terminal), a mouse anti-CoREST (generated by Dr. J. Trimmer, University of California, Davis), rabbit anti-MeCP2 (a generous gift from Dr. M. Greenberg, Harvard Medical School, Boston), mouse anti-β-tubulin (TuJ1; Covance, Inc.), or mouse anti-nestin (Chemicon) primary antibodies, followed by incubation with the appropriate secondary antibody conjugated to Alexa Fluor (Molecular Probes) as described previously (Ballas et al., 2001). For REST immunostaining, 4% paraformaldehyde was added to the cells followed by high-power microwave heating for 20 s. Images were collected on a Zeiss confocal laser scanning LSM 510 microscope.

Viral Vectors and Infection of Cortical Progenitors

REST cDNA lacking sequences coding for the amino- and carboxy-terminal repressor domains (*REST* ΔNΔC) (Ballas et al., 2001) was cloned into the pAdTrackCMV vector containing GFP. Adenovirus was assembled in HEK293 cells and purified following the manufacturer's instructions (Ambion). Cortical progenitors were cultured for 4 days, then dissociated with papain, labeled with CD15 FITC, and sorted for LeX by green fluorescence as described above. Sorted progenitors were cultured for 2 days and then infected with Adeno virus (24 MOI) for 30 hr.

Chromatin Immunoprecipitation

ChIP assays were performed as described previously (Ballas et al., 2001). Crosslinked chromatin was sonicated to generate fragments with an average length of 300–400 bp. The chromatin was subjected to immunoprecipitation using the following polyclonal antibodies: anti-REST p73 (Chong et al., 1995) or anti-REST-C (see above); anti-CoREST (Andres et al., 1999); anti-CoREST-N (was generated against the first 100 amino acids of the N terminal); anti-mSin3A, anti RARα, anti RARβ (all from Santa Cruz); anti-H3-dimethyl K4 and anti-H3-dimethyl K9 (Upstate Biotechnology); anti-HDAC1, anti-H3-trimethyl K4, anti-N-CoR, mouse anti-Pol II (all from Abcam); anti-MeCP2 (see above); anti-G9a (a generous gift from Dr. Y. Nakatani, Harvard Medical School, Boston). Unless otherwise stated, DNA was subjected to 50 cycles of PCR. When quantitative ChIP was performed, the abundance of the immuno-

precipitated DNA in a sample was normalized to the amount of signal in the input DNA, using an ABI PRISM 7700 Sequence Detector and SYBR-green PCR master mix (PE Applied Biosystem). All quantitative ChIP was verified by PCR amplification of the same ChIP DNA and analysis on agarose gel. For the specific set of primers used for ChIP and quantitative ChIP, see Supplemental Data.

RNA Isolation, RT-PCR, and Quantitative Real-Time RT-PCR Analysis

Total RNA was prepared using RNeasy (Qiagen) and treated with DNase, RNase-free, (Ambion, DNA-free kit). For reverse transcription, First Strand Superscript II (Invitrogen) was used and quantitative real-time PCR was performed in an ABI PRISM 7700 Sequence Detector using SYBR-green PCR master mix (PE Applied Biosystem). Relative abundance of the specific mRNAs was normalized to *GAPDH* mRNA. For the primer sets used for RT-PCR and for quantitative real-time RT-PCR, see Supplemental Data.

Western Blot Analysis

Nuclear and cytoplasmic cell extracts were prepared by the modified Dignam method as described (Grimes et al., 2000). For whole-cell extracts, cells were lysed directly in nuclear lysis buffer. The following antibodies were used for Western blot analysis: anti-REST p73 or anti-REST-C, anti-CoREST, anti-MeCP2, anti-mSin3A, anti-β-tubulin (TUJ1), anti-HDAC1 (all described above).

Nuclear Run-On Transcription

Nuclear run-on transcription was performed essentially as described (Ashe et al., 1997) with the following modifications: nuclei (2×10^7) were incubated for 30 min at 30°C in with an equal volume of 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.3 M KCl containing 1 mM of each ribonucleotide (ATP, CTP, GTP), 5 mM dithiothreitol, 100 U RNase inhibitor (Roche), and [α-^{32}P] UTP (100 μCi of 3000 Ci/mmol). Labeled RNA was isolated and hybridized (1×10^7 cpm) overnight at 42°C to nitrocellulose membrane filters with immobilized plasmids carrying the indicated cDNA clones. The plasmid pUBS-SK-*mREST*, containing *REST* cDNA corresponding to the eight zinc-finger motif, was a generous gift from Dr. D. Anderson (Caltech, Pasadena); *GAPDH* cDNA, corresponding to *GAPDH* mRNA positions 213–1117, was subcloned as EcoRI/ BamHI fragment into pSP73 vector (Promega). Hybridization signals were quantified using a Molecular Dynamics PhosphorImager.

Bisulfite Genomic Sequencing

Bisulfite genomic sequencing was performed essentially as described (Clark et al., 1994). For experimental details and primer sets used, see Supplemental Data.

Microarray Hybridization and Analysis

Microarray analysis was performed at the Genomic Informatics Center (University of Rochester Medical School, Rochester, NY) under the supervision of Dr. Andrew Brooks. For experimental details, see Supplemental Data. The RE1 site was identified in the selected genes using an algorithm based on scoring the occurrence of each nucleotide in the 23 bp consensus sequence for 20 experimentally confirmed REST binding sites in mammals.

Supplemental Data

Supplemental Data include seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/121/4/645/DC1/.

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Supplemental Data

REST and Its Corepressors Mediate Plasticity of Neuronal Gene Chromatin throughout Neurogenesis

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Supplemental Experimental Procedures

Expression Plasmids and Transient Transfection Analysis

Gal4 expression constructs and the UAS *Nav1.2-CAT* reporter plasmids were described previously (Ballas et al., 2001). Transfection of ES cells and repression assays were performed as in (Ballas et al., 2001). Reporter plasmid (5 μg) was mixed in a molar ratio of 1:0.6 with the different Gal4 constructs and co-transfected using the FuGene 6 transfecting reagent (Roche). CAT assays were performed 40 hours post transfection.

Communoprecipitation

Coimmunoprecipitation was performed as described (Ballas et al., 2001) using anti-CoREST antibodies.

Bisulfite Genomic Sequencing

Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega), digested (2 μg) with specific restriction enzymes with recognition sites flanking the test DNA region, and purified (Wizard genomic DNA purification kit; Promega). The sulfonation reaction was carried out as follows: DNA was denatured with 0.3 M NaOH, in 22 μl reaction volume, for 15 min at 75^0C, after which fresh solutions of 10 mM hydroquinone (12 μl) and 6.24 M Urea/4 M sodium bisulfite (208 μl) were added. Samples were subjected to 25 PCR cycles of 55^0C for 15 min, and 95^0C for 45 sec. DNA was purified (Quiquick; QIAGEN), desalted with 0.3 M NaOH, ethanol precipitated, and resuspended in 25 μl of 1 mM Tris-HCl pH 8.0. The purified DNA (4 μl) was subjected to PCR amplification using Platinum Taq DNA polymerase (Invitrogen) and primer sets designed for the converted DNA. The PCR products were gel-purified, subcloned, and transformed, using TOPO TA cloning kit (Invitrogen). Individual clones were sequenced using the BigDye terminator cycle sequencing kit (PE Applied Biosystems) and an ABI PRISM 310 DNA sequencer (Perkin Elmer). Clones were chosen from three independent experiments.

Microarray Hybridization and Analysis

Total RNA was generated from three independent experiments and reverse transcribed to cDNA followed by addition of an initiation site for T7 RNA polymerase at the 3' end. cRNA was generated from 1 μg of the modified cDNA using biotinylated UTP and CTP and fragmented (20 μg from each sample) for 35 min at 94^0C in 200 mM Tris-actetate (pH 8.1), 500 mM KOAc, and 150 mM MgOAc. Samples were subjected to gene expression analysis via the Affymetrix Murine 430Plus high-density oligonucleotide array that currently queries 42,000 mouse probe sets. Iobion's GeneTraffic MULTI was used to perform Robust Multi-Chip Analysis (RMA) that is a median polishing algorithm used in conjunction with both background subtraction and quantile normalization approaches. Data was analyzed by Statsitical Analysis of Microarrays (SAM). For more details see http://fgc.urmc.rochester.edu.

Primer Sets Used for Different Applications

ChIP Primers

Calbindin RE1, forward 5'-GAG AAC TCC GGA GGA CGC CCG AAC-3', reverse 5'-GAT GGT TGG GAT GAA CAA GCA CAG TC-3'; *Calbindin* mCpG promoter, forward 5'-GGA GGA AAC ACA AAT GTG GTG AAT TCC A-3', reverse 5'- GCT GCG CTG CTT CTA TCT CTA GGT GA-3'; *BDNF* RE1, forward 5'-GAG GAA TAA GTC AGA AGT GTC TAT CTA-3', reverse 5'-GTG TGA GCC GAA CCT CGG AAA AGA C-3'; *BDNF* mCpG promoter/exon IV, forward 5'-GTT GTT GCT GCC TAG ATA ATG ACA GGC-3', reverse 5'-GAG AAG CTC CAT TTG ATC TAG GCA GAG-3'; *Synaptotagmin 4* RE1, forward 5'-GCG TCA ATT CGG GTT TCA GCC ACG TC-3', reverse 5'- CAA GTT CAG CAA CTT GCT CAC CGA ATT C-3'; *Synaptotagmin 4* (3' UTR control), forward 5'-GGT GTT GGC CAA GTT TTC ATA AGA TAT TC-3', reverse 5'-GCT ACC CTT CTT

ATG ATG AGA CTG TAT C-3'; *Nav1.2* RE1, forward 5'- GAT CTT AAC CTC TGA GCA ATC TTT C-3', reverse 5'-GCT GAG TAT ATG ACC TGT TCA ATA C-3'; *REST* promoter –1932 to –1602, forward 5'-GAG TGT AGT TTC GCA CAC AGG ACT CA-3', reverse 5'-GTG TCT GAA GAT AGC TAC AGC GTA CAC A; *REST* promoter –1276 to –952, forward 5'-GAT TCA ATG CAC GGA GCT TGC ACT GAA-3', reverse 5'-GGA AGC AGA GCT TCA GGT TTC TCT GA-3'; *REST* promoter –555 to –341, forward 5'-GAA AGG TCT TCA CTT TGT AGC TAT GGC-3', reverse 5'- GGC TGC ACA AGT CTG TAA TCC CTA CTT-3'; *MASH1* RE1, forward 5'-GTC CGA TAA ATC ATC TTT CCT TGT GTT TG-3', reverse 5'-GTC TCG GTG CTG AGT AAT TAA AAT TGC AG-3'; *SNAP25* RE1, forward 5'-GCT ACA GGC GAG CAT GTG CTG CAA AG-3', reverse 5'-GTG AGG AAG AGA GCA GGC AAT TGT CTG-3'; *Glycine Receptor alpha 1* RE1, forward 5'-GGA TTT CTT CCT TTC ATG GAG CTG GCC-3', reverse 5'-GTC GTG CAG GTT CAG CAC CAC GGA-3'; *NMDA1* RE1, forward 5'-GGA TGA CTG TCC CTG GTT CTC TTG TAT-3', reverse 5'-GTG CTG TCC GAG GTG CTG AAG CGT A-3'; *GAD1* RE1, forward 5'-TCC AAA TGC CAA GGA CAG TTG TCG-3', reverse 5'-AGG TAG ACT GAG GGA CTC ATG ACT-3'.

Quantitative ChIP Primers

Calbindin RE1, forward 5'- GCT CCG CGC ACT CTC AAA-3', reverse 5'-GAG ATG ACT GCA GGT GGG ATT C-3'; and *Synaptotagmin 4* RE1, forward 5'-AGA CAA GCT TTT CAG AAG AGC CA-3', reverse 5'-CCG CGC TCT CTA TCA GCA AT-3'; *MASH1* RE1, forward 5'-TCC ATA TTG TAG TCA CCA TCT TTT CAG-3', reverse 5'-TTA CTC TTG ACC ATG CAA TTA AAT ACA TT-3'; *SNAP25* RE1, forward 5'-GGA CGG CGA TCC GGC TGC AGC-3', reverse 5'-GAT CTC AGA GAG AAA ATT TGG CCT C-3'; *Glycine Receptor alpha 1* RE1, forward 5'-GCT CTG ACA CCT CGT CCC TCT A-3', reverse 5'-GAA GCC AGA GAG CGC CAC T-3'; *NMDA1* RE1, forward 5'-GCA GCT GTC TTT TTC GCC TT-3', reverse 5'-CTG AAG CGT ATT GGG CGC-3'; *GAD1* RE1, forward CGC ACC TGC AGT GAA CAC C-3', reverse 5'-AAG ACT TCA GCA CCG AGG ACA-3'.

RT-PCR Primers

MASH1, forward 5'-GGA ACT GAT GCG CTG CAA ACG CCG-3', reverse 5'-GTT GGT AAA GTC CAG CAG CTC TTG TT-3'; *Calbindin*, 5'-GTT TCG TGT ATC CTT TAG CTA GTG TGT-3', reverse 5'-TCT AAA GTC ACT GCT TCC AAA TAC GTG C-3'; *Synaptotagmin 4*, forward 5'-GGT GTT GGC CAA GTT TTC ATA AGA TAT TC-3', reverse 5'-GCT ACC CTT CTT ATG ATG AGA CTG TAT C-3'; *GAPDH*, forward 5'-GAC TCC ACT CAC GGC AAA TTC AAC GG-3', reverse 5'-CAT TGC TGA CAA TCT TGA GTG AGT TGT C-3'.

Quantitative RT-PCR Primers

Synaptotagmin 4, forward 5'-AAT GAG GTG ATT GGA CGG TTG-3', reverse 5'-AGT GCC CCC CAC CGC-3'; *Nav 1.2*, forward 5'-GGC ACA ATC AGT GCT GGT ACC-3', reverse 5'-CAG CAA GGG ATT CCC TGG T-3'; *Calbindin*, forward 5'-GCT GCA GAA CTT GAT CCA GGA-3', reverse 5'-TCC GGT GAT AGC TCC AAT CC-3'; *BDNF* exon I, forward 5'-GCC GGC TGG TGC AGA A-3', reverse 5'-GCC TTG TCA AGC TAG GGC G-3'; *BDNF* exon IV, forward 5'-CCC AGC CTC TGC CTA GAT-3', reverse 5'-TGC ATG GCG GAG GTA ATA CTC-3'; *REST*, forward 5'-ACC GGG TCA GGA TCT TCT CA-3', reverse 5'-GCC CTG TTA GGG AAA CCT CC-3'; *GAPDH*, forward 5'-AGA AGG TGG TGA AGC AGG CA-3', reverse 5'-CGA AGG TGG AAG AGT GGG AG-3'; *MASH1*, forward 5'-TCG TCC TCT CCG GAA CTG AT-3', reverse 5'-TAG CCG AAG CCG CTG AAG-3'; *SNAP25*, forward 5'-ACG GGA GCA GAT GGC CA-3', reverse 5'-CCC GGG CAT CGT TTG TT-3'; *Glycine Receptor alpha1*, forward 5'-TGC CTG CTC TTC GTG TTC TCT-3', reverse 5'-TGT TGC CGA GAC ACA AAG TTG-3'; *NMDA1*, forward 5'-GGT GGC CGT GAT GCT GTA C-3', reverse 5'-TCG CTG TTC ACC TTA AAT CGG-3'; *GAD1*, forward 5'-GGG TGG TGG ACT GCT CAT GT-3', reverse 5'-AAT TGG CCC TTT CTA TGC CG-3'; *Slit2*, forward 5'-CAT TTG TGG ACG AGG TTG AGA A-3', reverse 5'-CTA GAG ACG CGC TTA GGA GGC-3'; *Cyp46a1*, forward 5'-CCC GTG TCC ATG CAG GAC-3', reverse 5'-AGC TGC CTT GGC CAG GA-3'.

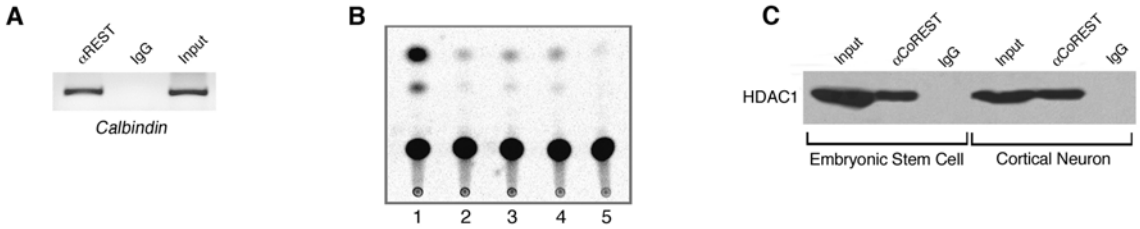
Bisulfite Genomic Sequencing Primers

For *Calbindin* promoter and RE1-containing 5' UTR regions: -1540 to-1322, forward 5'-GTA AAT ATG GGT TAA TGG AGG AAG TGT-3', reverse 5'-CAA ATA CTT TAC TAA ATC CTA CAA ATA ATA CA-3'; -1059 to –683, forward 5'-GGA GGA AAT ATA AAT GTG GTG AAT TTT AT-3', reverse 5'-CAC CAA ACT AAC CAA TAA CTC C-3'; -558 to -242, forward 5'-GTT TAA AAA GTA ATA ATA GTA AGA GTA GTA GA-3', reverse 5'-CAC TCC TAA ATA TTA AAA TAA AAC AAC ATA CC-3'; -151 to +165, forward 5'-GTT TTA GTT ATT TTT TGG GAA GAG AG-3', reverse 5'-CAA AAA TAA CTA CAA ATA AAA TTC TAC C-3'.

Supplemental References

Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., Burger, C., Moniwa, M., Davie, J. R., Bowers, W. J.*, et al.* (2001). Regulation of neuronal traits by a novel transcriptional complex. Neuron *31*, 353-365.

Figure S1. REST Is a Repressor in Embryonic Stem Cells

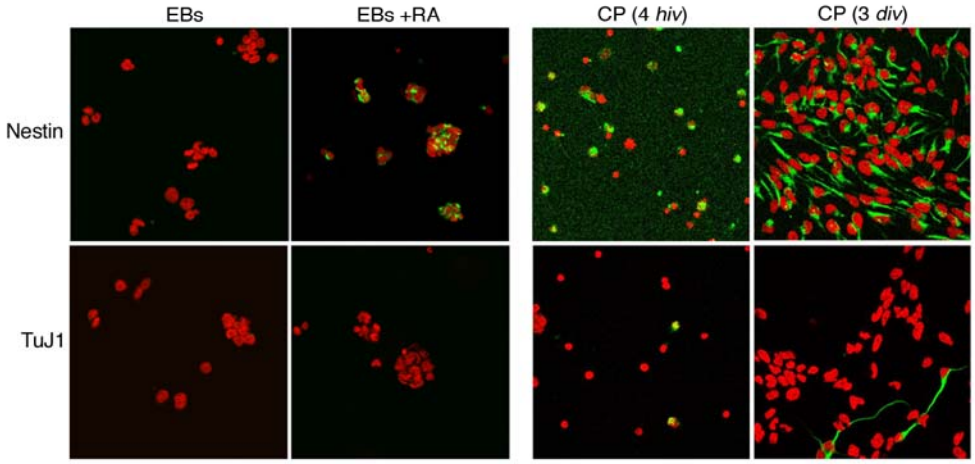


(A) Chromatin immunoprecipitation (ChIP) assay shows occupancy of REST (α REST) on the RE1 site of the *Calbindin* gene in embryonic stem (ES) cells.

(B)Transient CAT assay in ES cells shows repression of UAS-*Nav1.2* -*CAT* reporter gene by Gal4 fusion proteins: Lane 1, Gal4 alone; lane 2, full-length REST; lane 3, REST C-terminal; lane 4, REST N-terminal; lane 5, full-length CoREST.

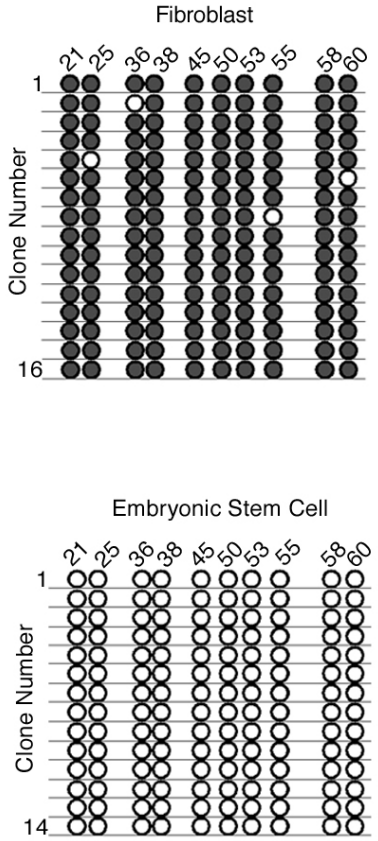
(C) Western blot shows HDAC1 in CoREST (α CoREST) immunocomplexes in Embryonic Stem cells and Cortical Neurons.

Figure S2. Immunostaining Shows RA-Treated Embryoid Bodies and Cortical Progenitors both Express Nestin but Not Neuronal β-Tubulin



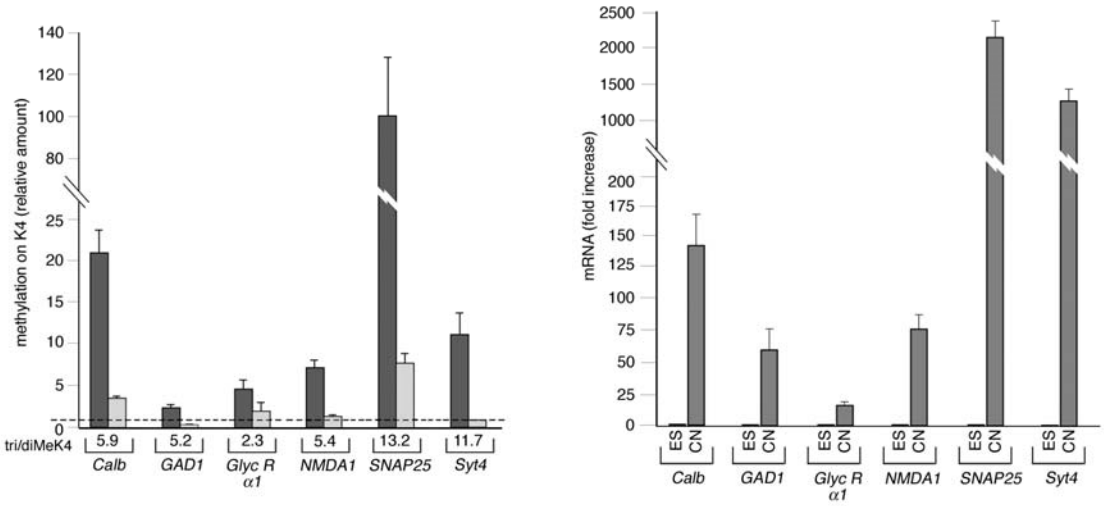
Left Panel: Embryonic bodies (EBs) and EBs treated with RA for two days (EBs+RA). EBs were partially dissociated before staining. Nuclei stained with propidium iodide (red). Note the presence of nestin–positive cells (green) in RA-treated cells (top, right square) but not in the untreated cells (top, left square), and the absence of neuronal β-tubulin (TuJ1) positive cells (bottom two squares) at both of these stages. Right Panel: Cortical Progenitors (CP) after culture for 4 hours (4 *hiv*) or 3 days (3 *div*). Note the prevalence of nestin-positive cells (green) in the dividing progenitors (top two squares) and relative paucity of neuronal β-tubulin-positive cells (TuJ1; green; bottom two squares).

Figure S3. Bisulfite Genomic Requencing Analysis of the RE1 Region of the *Calbindin* Gene Showing Dense Methylation in Fibroblasts but Not ES Cells



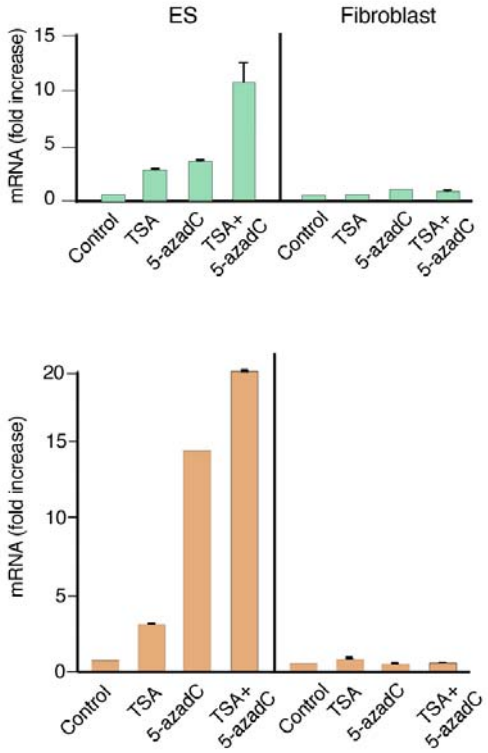
Closed circles, mCpGs; open circles, CpGs.

Figure S4. Methylation on Histone H3 K4 (MeK4H3) and Expression Levels of REST Target Genes



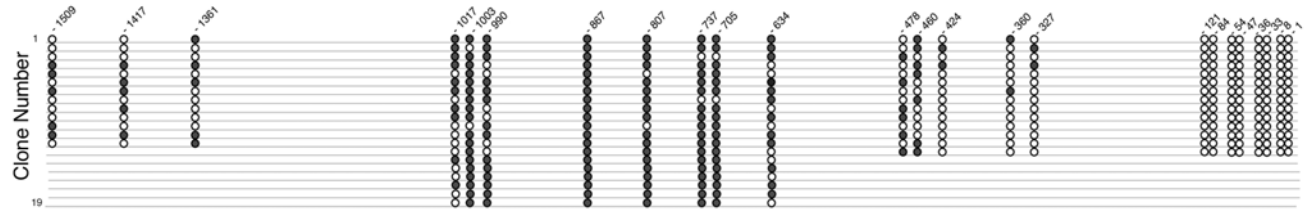
Left Panel: Quantitative ChIP analysis of the RE1 regions of the different neuronal genes in ES cells and cortical neurons using antibodies to di and triMeK4H3. Absolute values of diMeK4H3 (gray bars) and triMeK4H3 (black bars) in neurons are relative to the amounts of di or triMeK4H3 in ES cells (dotted line). Ratios of tri to diMeK4H3 in cortical neurons are shown for each gene in brackets. Right Panel: Real time RT-PCR measurements of mRNAs levels in Cortical Neurons (CN) compared to ES cells. Error bars represent SD based on four independent experiments.

Figure S5. Real-Time RT-PCR Measurements of *Synaptotagmin 4* (Upper Graph) and *Nav1.2* (Lower Graph) mRNAs after Treatment of ES Cells or Fibroblasts with TSA and/or 5-azadC



Error bars represent SD based on three independent experiments.

Figure S6. Bisulfite Genomic Sequencing Analysis Showing the Distribution of mCpGs in the *Calbindin* Promoter Region in ES Cells



Closed circles, mCpGs; open circles, CpGs.

Figure S7. Genes, identified by microarray analysis, whose expression in cortical neurons is up regulated after interference with HDAC activity

	Accession Number	Gene Name	Description
1.	NM _007387	Acp2	Lysosomal acid phosphatase
2.	UNKNOWN	Cntnap2/Caspr2	Neurexin
3.	NM _030719	Gats	GABA transporter
4.	NM _010298	Glrb	Glycine Receptor β subunit
5.	NM _025898	Napa	NSF attachment protein
6.	NM _008845	Pip5k2a	PI4-Phosphate 5-kinase
7.	NM _009538	Plag11/Zac1	Zinc finger protein
8.	NM _009062	Rgs4	Regulator of G protein signaling
9.	NM _139228	Rhbd14	Rhomboid receptor
10.	NM _028052	Synpr	Synaptoporin
11.	NM _009382	Thy1	Cell adhesion molecule
12.	NM _009508	Viaat	Vesicular Inhibitory Amino Acid Transporter
13.	NM _009513	VMP	Vesicular Membrane Protein
14.	NM _009525	Wnt5b	Wnt signaling protein

All of these genes contain a consensus REST binding site (RE1).