Neuron
Article

# A Calcium-Dependent Switch in a CREST-BRG1 Complex Regulates Activity-Dependent Gene Expression

Zilong Qiu[1] and Anirvan Ghosh[1,*]
[1]Neurobiology Section, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093-0366, USA
*Correspondence: aghosh@ucsd.edu
DOI 10.1016/j.neuron.2008.09.040

## SUMMARY

**CREST plays a critical role in activity-dependent development, but its mechanism of action is not well understood. Here, we show that a CREST-BRG1 complex regulates promoter activation by orchestrating a calcium-dependent release of a repressor complex and a recruitment of an activator complex. In resting neurons, transcription of the c-fos promoter is inhibited by BRG1-dependent recruitment of a phospho-Rb-HDAC repressor complex. Upon calcium influx, Rb becomes dephosphorylated at serine 795 by calcineurin, which leads to release of the repressor complex. At the same time, there is increased recruitment of CBP to the promoter by a CREST-dependent mechanism, which leads to transcriptional activation. The CREST-BRG1 also binds to the NR2B promoter, and activity-dependent induction of NR2B expression involves a release of HDAC1 and recruitment of CBP, suggesting that this mechanism may be generally involved in regulating calcium-dependent transcription of neuronal genes.**

## INTRODUCTION

One of the most remarkable features of the nervous system is that its structure and function can be modified by sensory input. For example, the pioneering work of Hubel and Wiesel showed that changes in postnatal visual experience can lead to lasting changes in cortical connectivity in cats (reviewed in Hubel and Wiesel, 1998). It is now well established that neuronal activity plays a critical role in controlling many aspects of neural development and function, including neuronal viability, migration, morphogenesis, and plasticity (reviewed in Katz and Shatz, 1996).

The effects of activity in the nervous system are primarily mediated by calcium signaling. Calcium influx leads to posttranslational modification of synaptic proteins as well as induction of new gene expression. The lasting effects of neuronal activity, such as activity-dependent dendritic growth, long-term plasticity in sensory systems, and memory consolidation, require calcium-dependent transcription (Ghosh and Greenberg, 1995; West et al., 2001; Redmond et al., 2002). Much of our understanding of calcium-dependent transcription has come from studies on the regulation of immediate early genes (IEGs), such as c-*fos*, which are rapidly induced upon calcium influx (reviewed in Ghosh and Greenberg, 1995). Among the major insights from these studies has been the recognition that a CREB-CBP complex plays a central role in regulating calcium-dependent transcription (reviewed in Mayr and Montminy, 2001; Chrivia et al., 1993; Chawla et al., 1998; Hu et al., 1999). Recent progress in chromatin biology and epigenetics suggests that covalent modification of histones, such as acetylation and methylation, also serve a critical role in regulating gene expression (Rosenfeld et al., 2006), but the role of these modifications in calcium-dependent transcription has not been extensively explored.

To gain additional insight into the mechanisms that mediate activity-dependent transcription, we previously carried out a screen for calcium-dependent transactivators and cloned a factor called Calcium RESponsive Transactivator (CREST; Aizawa et al., 2004). Here, we report on our investigation of the mechanisms by which calcium signaling regulates transcription via the CREST complex. We show that CREST binds to CBP and BRG1 via distinct domains. Whereas the association with CBP facilitates transcription, the association with BRG1 suppresses CREST-mediated transcription in resting neurons. Transcriptional repression by BRG1 is mediated by the retinoblastoma protein (Rb), which recruits a histone deacetylase (HDAC) complex to the promoter. Calcium-influx leads to release of the HDAC complex from Rb via calcineurin-dependent dephosphorylation of Rb. These findings reveal a regulatory mechanism for calcium-dependent transcription that is likely to play a critical role in mediating adaptive responses in the nervous system.

## RESULTS

### Characterization of the CREST-BRG1 Complex and Bidirectional Regulation of Calcium-Dependent c-*fos* Transcription by CREST and BRG1

To identify components of the CREST complex, we carried out a yeast two-hybrid screen and identified a homolog of BAF250b as a CREST-interacting protein (Shu-Ching Hu and A.G., unpublished data). BAF250b is a component of the BRG1 chromatin-remodeling complex (Nie et al., 2000; Olave et al., 2002), which suggested that CREST and BRG1 might be part of the same complex. Coimmunoprecipitation experiments

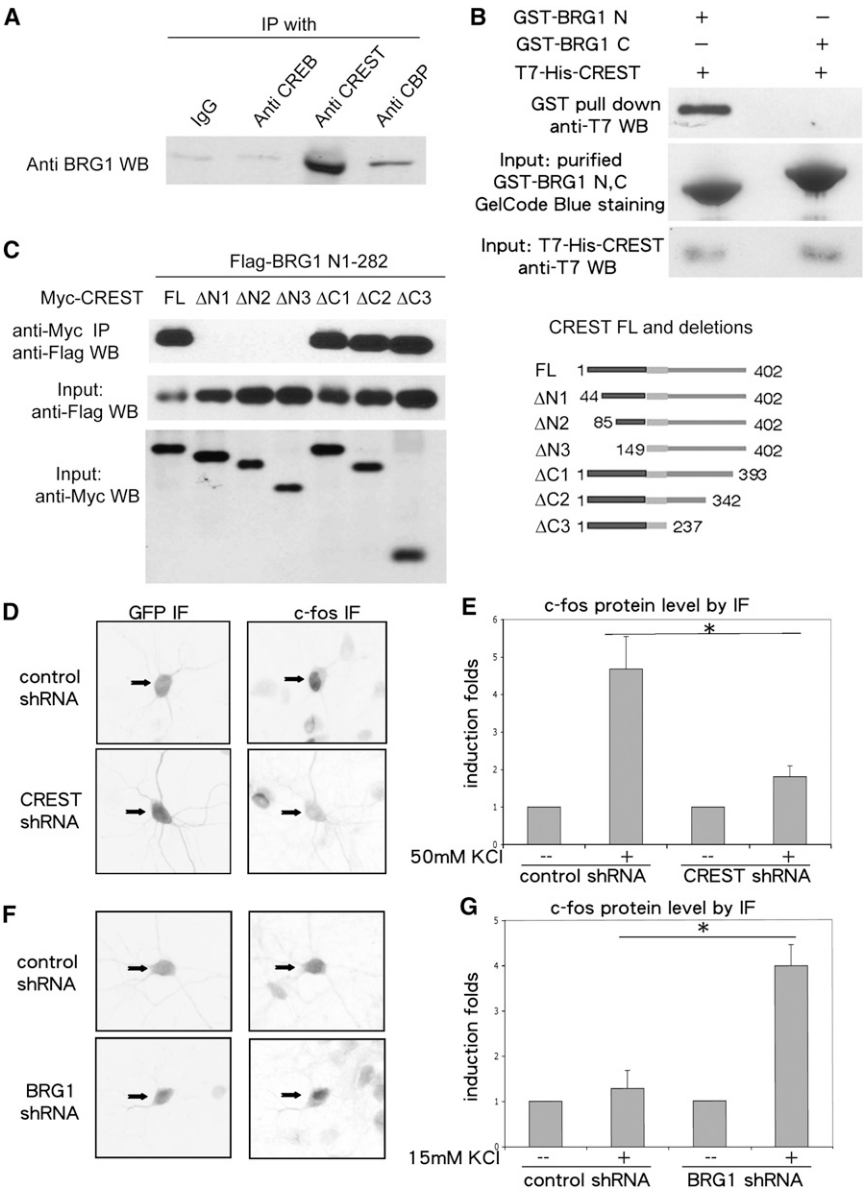


**Figure 1. CREST and BRG1 Are Present in a Complex and Exert Opposite Effects on Calcium-Dependent c-*fos* Expression**

(A) Immunoprecipitation of CREST with BRG1. Cortices from postnatal 4 days rat were dissected, homogenized, and immunoprecipitated with antibodies as indicated. The immunoprecipitates were resolved by SDS-PAGE, and the western blot was probed with an anti-BRG1 antibody.

(B) Interaction between the N terminus of BRG1 and CREST. His-T7-CREST, GST-BRG1 N, and GST-BRG1 C were expressed in *E. coli* and purified. His-T7-CREST was combined with GST-BRG1 N and GST-BRG1 C, respectively. The mixture was incubated for 30 min at room temperature. After immobilizing GST-BRG1 N and GST-BRG1 C with GST affinity beads, mixtures were washed extensively, resolved by SDS-PAGE, and the western blot was probed with anti-T7 antibody.

(C) Interaction between the N terminus of CREST and BRG1. HEK293 cells were transfected with Flag-tagged BRG1 N terminal aa 1–282 along with myc-tagged CREST deletion constructs (shown on the right) and immunoprecipitated with an anti-myc antibody. The myc immunoprecipitates were separated by SDS-PAGE and probed with an anti-Flag antibody (upper blot). Similar amounts of input Flag-tagged proteins in each sample were confirmed by probing with an anti-Flag antibody (middle blot). Similar amounts of input myc-tagged proteins in each sample were confirmed by probing with an anti-myc antibody (lower blot).

(D) Examples of cortical neurons transfected with pSuper-control shRNA or pSuper-CREST shRNA and immunostained with the antibodies indicated, after 50 mM KCl stimulation for 4 hr.

(E) Quantifications of c-*fos* protein levels measured by immunofluorescence intensity. n = 12 cells.

(F) Examples of cortical neurons transfected with pSuper-control shRNA and pSuper-BRG1 shRNA and immunostained with the antibodies indicated, after 15 mM KCl stimulation for 4 hr.

(G) Quantifications of c-*fos* protein levels measured by immunofluorescence intensity. n = 12.

Asterisks indicate significance at $p < 0.05$. Error bars represent +SD.

indicated that endogenous CREST associated with BRG1 in cortical neurons (Figure 1A). To determine whether this was due to a direct interaction between BRG1 and CREST, we purified GST fusion proteins of the N and C terminus of BRG1 and tested the ability of BRG1 to bind to purified His-CREST using a GST pull-down assay. As shown in Figure 1B, the N terminus of BRG1 (amino acid 1–282) directly interacts with CREST. To identify the domain of CREST that interacts with BRG1, we cotransfected CREST deletion constructs and the N terminus fragment of BRG1 in 293 cells and examined association by coimmunoprecipitation. These experiments revealed that the N terminus of CREST is required for its interaction with BRG1 (Figure 1C). We have previously shown that the C-terminal domain of CREST interacts with CBP (Aizawa et al., 2004). Thus, CREST can directly interact with both CBP and BRG1, raising the possibility that the BRG1-CREST-CBP protein complex regulates calcium-dependent gene expression.

To determine whether CREST and BRG1 contribute to calcium-dependent gene expression, we examined the consequence of shRNA-mediated knockdown of CREST and BRG1 on depolarization-induced c-*fos* expression (Figures 2A and S1). c-*fos* is the prototypical calcium-regulated gene and has been widely used to study mechanisms of calcium-dependent transcription (Sheng and Greenberg, 1990). The pSuper shRNA vector that was used in these experiments contains the PGK-GFP cassette, which allows transfected neurons to be identified based on GFP expression. After transfecting pSuper vector-based short hairpin RNA against CREST and BRG1 into primary cortical neurons, respectively, we measured depolarization-induced c-*fos* protein level by immunofluorescence. Reduction

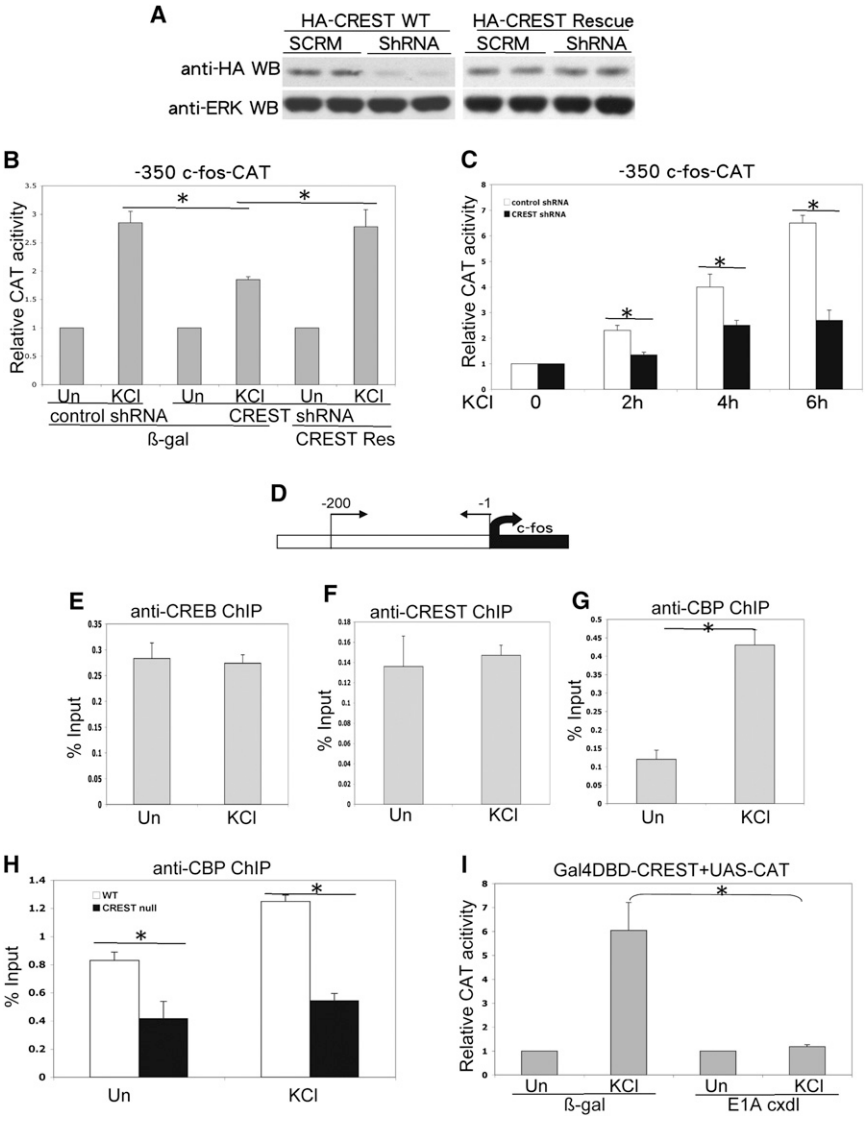


**Figure 2. CREST Regulates Calcium-Dependent c-*fos* Expression via CBP Recruitment**

(A) Inhibition of CREST expression by CREST short hairpin RNA. HEK293 cells were transfected with HA-CREST and HA-CREST rescue constructs along with pSuper vector harboring scrambled shRNA and shRNA against rat CREST. The cell lysates were separated by SDS-PAGE and probed with an anti-HA antibody (upper blot). Similar amounts of input proteins in each sample were confirmed by probing with an anti-ERK1 antibody (lower blot).

(B) Relative CAT activity in E18 cortical neurons transfected with mouse −350 c-*fos*-CAT reporter along with pSuper vector harboring control shRNA and CREST-specific shRNA constructs at 3 DIV. The same amounts of β-galactosidase and HA-CREST rescue constructs were cotransfected to perform rescue experiments. Transfected cells were stimulated for 4 hr with 50 mM KCl as indicated at 5 DIV.

(C) Relative CAT activity in E18 cortical neurons transfected with mouse −350 c-*fos*-CAT reporter along with pSuper vector harboring control shRNA and CREST-specific shRNA constructs at 3 DIV. Transfected cells were stimulated for the number of hours indicated, at 5 DIV (50 mM KCl).

(D) Diagram for c-*fos* promoter analyzed by chromatin immunoprecipitation assay.

(E–G) Chromatin immunoprecipitation on c-*fos* promoter. Rat E18 cortical neurons were stimulated with KCl (50 mM) for 10 min at 5 DIV and immunoprecipitated with antibodies as indicated. PCR reactions with endogenous c-*fos* primers (upper panel) were used to amplify endogenous c-*fos* promoter-specific DNA segments from −200 to −1. Real-time PCR was performed, and signals were normalized as percentage of input. ChIP with IgG was always less than 0.05% of input (Figure 7).

(H) Chromatin immunoprecipitation in CREST null and wild-type neurons. E15 cortical neurons from CREST KO and wild-type mice were cultured and stimulated for 10 min with KCl (50 mM) at 5 DIV. After immunoprecipitation with anti-CBP antibody, PCR reactions with endogenous c-*fos* promoter primers were used to amplify c-*fos* promoter-specific segments. Real-time PCR was performed, and signals were normalized as percentage of input.

(I) Relative CAT activity in E18 cortical neurons transfected with Gal4DBD-CREST UAS-CAT reporter along with β-galactosidase and E1A cxdl constructs at 3 DIV stimulated as indicated at 5 DIV (KCl 50 mM).

Asterisks indicate significance at $p < 0.05$. Error bars represent SD.

of CREST expression strongly inhibited depolarization-induced c-*fos* expression (Figures 1D and 1E). In contrast, c-*fos* immunofluorescence was significantly increased following knockdown of BRG1 (Figures 1F and 1G). These observations suggest that CREST and BRG1 exert opposite regulatory influences on calcium-activated c-*fos* transcription—whereas CREST appears to facilitate c-*fos* transcription, BRG1 appears to suppress calcium activation of c-*fos*.

To determine whether BRG1 negatively regulates CREST-mediated transcription, we examined the consequences of suppressing BRG1 expression in cortical neurons using the BRG1 shRNA construct. Expression of BRG1 shRNA led to a marked increase in KCl-induced activation of Gal4-CREST, indicating that endogenous BRG1 negatively regulates CREST-mediated transcription (Figure S2A). The effects of BRG1 shRNA were reversed by overexpression of human BRG1 that is resistant to the shRNA against rat BRG1 (Figure S2A). Expression of BRG1 shRNA did not affect calcium activation of Gal4-CREB, indicating that suppression of BRG1 expression does not lead to a general increase in calcium-dependent transcription (Figure S2B). Consistent with a repressive role for BRG1 on CREST-mediated transcription, we found that expression of WT hBRG1 strongly suppressed calcium activation of Gal4-CREST (Figure S2C). Mutation of the N-terminal domain of CREST, which disrupts the association of

CREST with BRG1, abrogated the ability of BRG1 to inhibit activation of Gal4-CREST (Figure S2D). Expression of WT BRG1 did not suppress calcium activation of Gal4-NeuroD1, indicating that BRG1 overexpression does not lead to a general suppression of calcium-dependent transcription (Figure S2E). Thus, BRG1 directly binds to the N terminus of CREST and exhibits a negative effect to CREST-mediated transcription.

### Regulation of the c-*fos* Promoter by the CREST-CBP Complex

To gain further insight into the role of CREST in calcium activation of the c-*fos* promoter, we examined the effects of expressing a CREST shRNA construct on depolarization-induced activation of a c-*fos* reporter. As shown in Figure 2A, the CREST shRNA sharply reduced expression of HA-CREST in transfected 293 cells. This suppression could be reversed by expression of a HA-CREST rescue construct that contains the same sense mutations in shRNA targeting sequences (Figure 2A). Consistent with the effects on endogenous c-*fos* expression, the expression of CREST shRNA significantly decreased KCl induced activation of −350 c-*fos*-CAT, a construct that includes all major transcriptional regulatory sites (Figure 2B). More importantly, this effect was reversed by coexpressing a shRNA resistant HA-CREST construct, indicating that this effect is specifically due to the loss of CREST. We found that knocking down CREST significantly reduces calcium-mediated c-*fos* reporter expression at various time points up to 6 hr (Figure 2C).

To examine the effect of depolarization on the recruitment of CREB, CREST, and CBP to the c-*fos* promoter, we performed chromatin immunoprecipitation (ChIP) experiments on extracts of control and KCl-treated neurons using CREB, CREST, and CBP antibodies (Figures 2D–2G). In this and all subsequent experiments, the ChIP experiments were quantified using real-time quantitative PCR. All three proteins were found to be bound to the endogenous c-*fos* promoter both before and after KCl stimulation. Whereas the binding of CREB and CREST to the promoter was largely unaffected by KCl stimulation, there was a significant increase in the association of CBP with the promoter (Figure 2G). Since CREST binds to CBP, we next asked whether CREST contributes to the recruitment of CBP to the c-*fos* promoter by comparing CBP association with the promoter in cortical cultures from wild-type and CREST knockout mice (Aizawa et al., 2004). As shown in Figure 2H, recruitment of CBP to the c-*fos* promoter was significantly decreased in CREST null neurons in both unstimulated and KCl-stimulated cultures. This observation, together with the fact that CBP can mediate calcium-dependent transcription, suggested that the transactivation activity of CREST may rely on CBP (Chawla et al., 1998; Hu et al., 1999; Impey et al., 2002). Indeed, Gal4-CREST-mediated reporter gene expression was totally abolished when CBP function was inhibited by the viral protein E1A (cxdl) (Hu et al., 1999) (Figure 2I). These data indicate that CREST mediates calcium activation of the c-*fos* promoter via recruitment of CBP.

### Regulation of the c-*fos* Promoter by the BRG1-Rb-HDAC Complex

We next investigated the mechanism by which BRG1 regulates the c-*fos* promoter. BRG1 has extensively been studied as a component of a chromatin-remodeling complex, but its role in calcium-dependent transcription has not been explored. Consistent with the observation that BRG1 shRNA leads to an increase in calcium activation of the c-*fos* expression (Figure 1G), expression of BRG1 shRNA increased expression of the c-*fos*-CAT reporter, whereas overexpression of wild-type BRG1 led to a suppression of KCl activation of c-*fos* CAT (Figures 3A and 3B). To determine whether transcriptional repression of the c-*fos* promoter by BRG1 might involve recruitment of an HDAC complex (Fass et al., 2003; Kumar et al., 2005), we examined the effect of BRG1 in the presence of trichostatin A (TSA), an HDAC inhibitor. Not only did BRG1 expression not inhibit KCl-induced transactivation in the presence of TSA, it actually enhanced transactivation, indicating that the effect of BRG1 on c-*fos* activation requires HDAC activity (Figure 3C). The fact that BRG1 expression increases transcription in the presence of TSA suggests that BRG1 might also play a positive role in calcium-dependent transcription that is occluded by HDAC recruitment. In contrast to its effect on BRG1-transfected neurons, TSA did not reverse the inhibitory effect of acidic CREB on calcium activation of c-*fos*-CAT (Figures S3A and S3B), suggesting that HDACs might specifically inhibit transcription of the c-*fos* promoter via the BRG1 complex. Consistent with this possibility, coimmunoprecipitation experiments from cortical lysates showed that HDAC1, HDAC2, and to some extent HDAC3 and mSin3A associate with BRG1 (Figure 3D).

We next investigated the mechanism by which BRG1 recruits the repressor complex to the promoter. Specifically, we decided to consider the possibility that the retinoblastoma protein might mediate recruitment of a repressor complex to BRG1. Rb and BRG1 have been independently reported to suppress c-*fos* expression, but whether their actions are mechanistically linked is not known (Robbins et al., 1990; Dunaief et al., 1994; Murphy et al., 1999). We found that Rb could be coimmunoprecipitated with both BRG1 and HDAC1 from cortical lysates (Figures 3E and 3F), suggesting that the three proteins may be part of one repressor complex. To determine whether association of BRG1 and Rb was involved in BRG1-mediated repression of c-*fos* activation, we examined the effects of a BRG1 mutant (LXCXE-RXRXE) that prevents association with Rb (Zhang et al., 2000). As shown in Figure 3G, this BRG1 mutant was unable to inhibit KCl activation of c-*fos*-CAT, indicating that the repressive effect of BRG1 requires interaction with Rb. We also found that a mutant of Rb that does not bind HDAC (RbΔexon22; Zhang et al., 2000) potentiated KCl activation of c-*fos*-CAT, further strengthening the evidence that a BRG1-Rb-HDAC complex negatively regulates c-*fos* expression (Figure 3H).

To exert a repressive effect, the BRG1-Rb-HDAC complex needs to be recruited to the promoter. The interaction of BRG1 with CREST is unlikely to help with promoter recruitment, since CREST does not contain a DNA-binding domain. One possibility is that the BRG1 complex is recruited to the promoter via an Sp1 binding site since BRG1 associates with Rb, and the suppression of c-*fos* expression by Rb depends on Sp1 binding sites (Robbins et al., 1990; Udvadia et al., 1993; Sohm et al., 1999). To determine whether BRG1 associates with the c-*fos*-CAT promoter in a region that includes the Sp1 site, we carried out chromatin immunoprecipitation (ChIP) experiments in neurons transfected with c-*fos*-CAT constructs including different

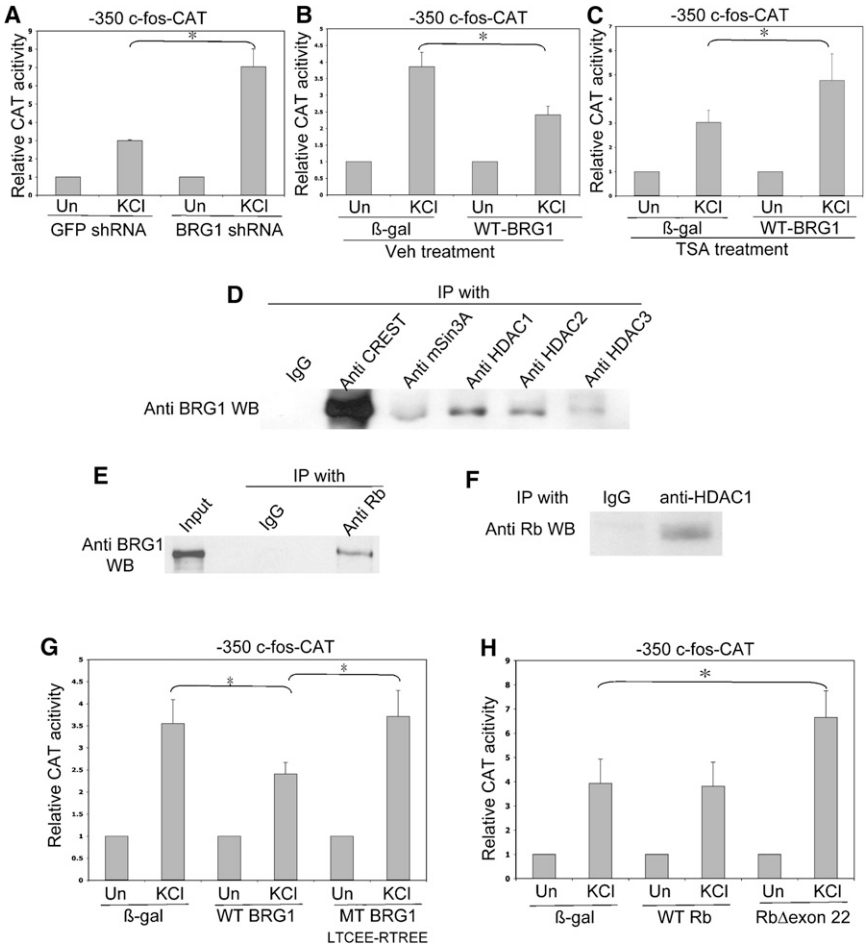


**Figure 3. BRG1 Recruits HDAC and Rb to Inhibit c-*fos* Activation**

(A) Relative CAT activity in E18 cortical neurons transfected with mouse −350 c-*fos*-CAT reporter along with shRNA against GFP and rat BRG1 at 3 DIV and stimulated as indicated at 5 DIV (KCl 50 mM).

(B) Relative CAT activity in rat E18 cortical neurons transfected with mouse −350 c-*fos*-CAT reporter along with β-galactosidase and wild-type BRG1 constructs at 3 DIV and stimulated as indicated at 5 DIV (KCl 50 mM) with the presence of DMSO.

(C) Relative CAT activity in rat E18 cortical neurons transfected with mouse −350 c-*fos*-CAT reporter along with β-galactosidase and wild-type BRG1 construct at 3 DIV and stimulated as indicated at 5 DIV (KCl 50 mM) with TSA pretreatment (TSA 250 ng/ml).

(D) Immunoprecipitation of BRG1 with class I HDAC complex. Cortices from postnatal 4 day rats were dissected, homogenized, and immunoprecipitated with antibodies as indicated. The immunoprecipitates were resolved by SDS-PAGE, and the western blot was probed with an anti-BRG1 antibody.

(E) Immunoprecipitation of BRG1 with Rb. Nuclear fractions of rat E18 cortical neurons were collected at 5 DIV and immunoprecipitated with IgG and anti-Rb antibody. The immunoprecipitates were resolved by SDS-PAGE, and western blot was probed with an anti-BRG1 antibody.

(F) Immunoprecipitation of Rb with HDAC1. Nuclear fractions of rat E18 cortical neurons were collected at 5 DIV and immunoprecipitated with IgG and anti-HDAC1 antibody. The immunoprecipitates were resolved by SDS-PAGE, and western blot was probed with an anti-Rb antibody.

(G) Relative CAT activity in rat E18 cortical neurons transfected with mouse −350 c-*fos*-CAT reporter along with β-galactosidase, wild-type BRG1, and mutated BRG1 (LTCEE-RTREE) constructs at 3 DIV and stimulated as indicated at 5 DIV (KCl 50 mM).

(H) Relative CAT activity in rat E18 cortical neurons transfected with mouse −350 c-*fos*-CAT reporter along with β-galactosidase, wild-type retinoblastoma protein, and mutated Rb protein (Δexon 22) constructs at 3 DIV and stimulated as indicated at 5 DIV (KCl 50 mM).

Asterisks indicate significance at $p < 0.05$. Error bars represent +SD.

promoter elements using a forward primer in the c-*fos* promoter and a reverse primer in the CAT sequence (Figure S4A). As shown in Figure S4B, BRG1 could be efficiently precipitated with −290 c-*fos*-CAT but not with −67 c-*fos*-CAT, suggesting that it binds to the c-*fos* promoter between −67 and −290. This region includes the Sp1 site, but not the CRE or SRE sites. Consistent with the possibility that the BRG1 complex is recruited to the Sp1 site, we were able to coprecipitate BRG1 with Sp1, but not with CREB (Figure 4A; Kadam and Emerson, 2003). Furthermore, we found that BRG1 failed to bind with c-*fos*-CAT reporter that carried a mutation in the SP1 binding site (−73 CCGCCC to AAATTT) (Figure 4B). These observations indicate that the BRG1 complex is recruited to the c-*fos* promoter via its interaction with Sp1.

## Mechanism of Release of the HDAC Complex after Calcium Stimulation

We next investigated whether calcium stimulation leads to a release of the BRG1 repressor complex from the c-*fos* promoter by examining the association and dissociation of various factors with the promoter before and after depolarization. Chromatin immunoprecipitation experiments showed that Sp1, BRG1, and Rb were associated with the promoter both before and after KCl stimulation (Figures 4C–4H). There was even an increased recruitment of BRG1 after stimulation. In contrast, HDAC1 and mSin3A were associated with the promoter before stimulation but were released from the promoter upon KCl stimulation (Figures 4G and 4H). Thus, calcium stimulation leads to a rapid release of HDAC1 and mSin3A from the c-*fos* promoter.

To determine whether Rb is required for HDAC1 recruitment onto the c-*fos* promoter, we expressed myc-HDAC1 together with a scrambled shRNA and shRNA against rat Rb in rat cortical neurons (Liu et al., 2005). Chromatin immunoprecipitation of myc-HDAC1 indicated that binding of HDAC1 to the c-*fos* promoter was significantly reduced in Rb shRNA-expressing cells (Figures 4I and 4J). This, together with the coimmunoprecipitation data for Rb and HDAC1 (Figure 3F), indicates that Rb is required for recruitment of HDAC1 onto the c-*fos* promoter and implies that calcium influx must lead to a release of the HDAC1 from the c-*fos* promoter.

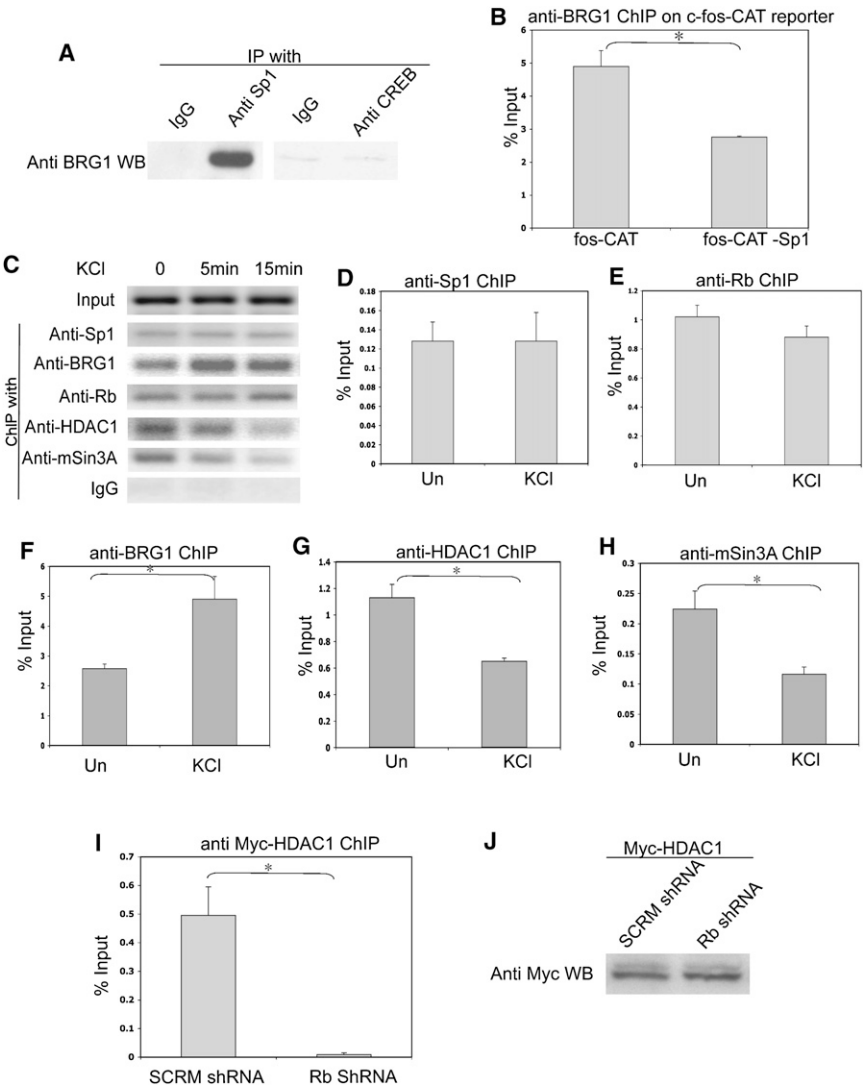


**Figure 4. Role of Sp1 in Recruitment of BRG1 on the c-*fos* Promoter, and Release of Repressor Complex from c-*fos* Promoter upon Stimulation**

(A) Immunoprecipitation of BRG1 with Sp1. Cortices from postnatal 4 day rat were dissected, homogenized, and immunoprecipitated with antibodies as indicated. The immunoprecipitates were resolved by SDS-PAGE, and the western blot was probed with an anti-BRG1 antibody.

(B) Chromatin immunoprecipitation of BRG1 with c-*fos*-CAT reporter. Rat E18 cortical neurons were transfected with mouse −350 c-*fos*-CAT or −350 c-*fos*-CAT (-Sp1), and immunoprecipitated with antibodies as indicated. PCR reactions with ChIP F and ChIP R primers were used to amplify reporter-specific DNA segments. Real-time PCR was performed, and signals were normalized as percentage of input. ChIP with IgG was less than 0.05% of input. Asterisks indicate significance at $p < 0.05$. Error bars represent +SD.

(C) Chromatin immunoprecipitation on c-*fos* promoter. Rat E18 cortical neurons were stimulated with KCl (50 mM) for 5 min and 15 min at 5 DIV and immunoprecipitated with antibodies as indicated. PCR reactions with endogenous c-*fos* primers (upper panel) were used to amplify endogenous c-*fos* promoter-specific DNA segments from −200 to −1.

(D–H) Real-time PCR results of ChIP experiments. Data are collected from two sets of independent experiments, each with triplicate samples. Asterisks indicate significance at $p < 0.05$. Error bars represent SD.

(I) Chromatin immunoprecipitation of myc-HDAC1 with endogenous c-*fos* promoter. Myc-HDAC1 along with pSilencer 1.0 vector harboring scrambled shRNA or shRNA against rat Rb1 gene were transfected into rat E18 cortical culture at 3 DIV. Cells were collected and ChIPed with anti-Myc antibody at 6 DIV. Endogenous c-*fos* promoter primers were used to amplify enriched promoter segments that associated with mycHDAC1. Real-time PCR was performed, and signals were normalized as percentage of input. Asterisks indicate significance at $p < 0.05$. Error bars represent +SD.

(J) Input control shows that the same amount of myc-HDAC1 was expressed in transfected neurons in the two conditions.

In the next set of experiments, we investigated the mechanism by which the HDAC1 complex was released from the c-*fos* promoter upon calcium stimulation. To determine whether this might be mediated by a posttranslational modification of Rb, we examined whether Rb phosphorylation was regulated by calcium signaling. We decided to focus on serine 795, as that site has been reported to be phosphorylated by MAP kinase, and MAP kinase has been implicated in calcium-dependent activation of c-*fos* (Garnovskaya et al., 2004). As shown in Figure 5A, Rb was phosphorylated at serine 795 in unstimulated neurons. Surprisingly, KCl stimulation did not lead to an increase in Rb phosphorylation at this site and instead led to a rapid dephosphorylation of S795 (Figure 5A). The dephosphorylation of Rb at S795 suggested that this was mediated by a calcium-dependent phosphatase, and we decided to examine the possibility that Rb dephosphorylation might be mediated by calcineurin, a major calcium-regulated phosphatase in neurons that has been implicated in transcriptional regulation (Hogan et al., 2003; Kingsbury et al., 2007). To determine whether calcineurin activity was required for calcium-induced dephosphorylation of Rb, we examined the effects of cyclosporin A, a calcineurin inhibitor. As shown in Figure 5A, KCl-induced dephosphorylation of Rb was completely blocked by cyclosporin A, indicating that calcineurin activity was required for Rb dephosphorylation. In complementary experiments, we found that purified calcineurin was effective in dephosphorylating Rb immunoprecipitated from cortical lysates (Figure 5B). Calcium-induced dephosphorylation of Rb was not affected by the CaM kinase inhibitor KN93,

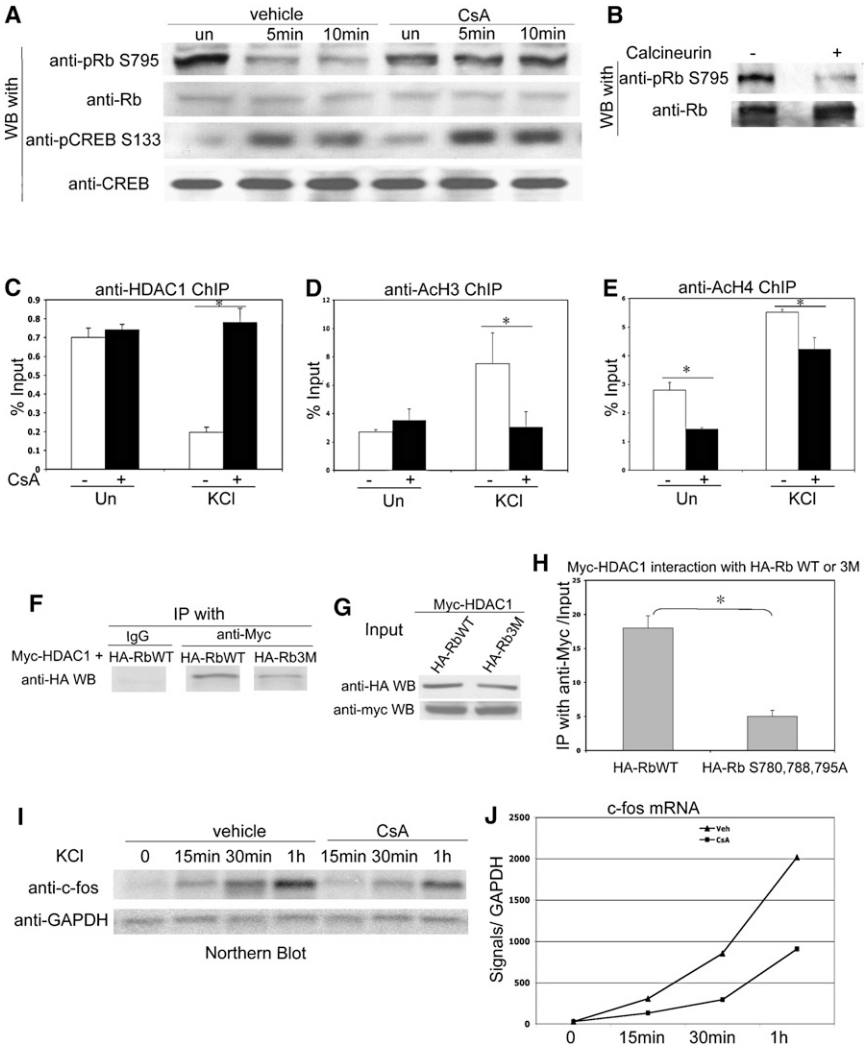


**Figure 5. Calcineurin-Dependent Dephosphorylation of Rb and Release of HDAC1 from c-*fos* Promoter**

(A) Assessment of Rb phosphorylation at S795 in unstimulated and stimulated neurons using a phospho-specific antibody and effect of cyclosporin A on depolarization-induced Rb dephosphorylation. Whole-cell lysates from E18 cortical cultures stimulated as indicated with 50 mM KCl were resolved by SDS-PAGE and probed with an anti-phospho-Rb S795, anti-pCREB S133 antibody, and anti-Rb, anti-CREB antibody sequentially.

(B) Effect of calcineurin on Rb phosphorylation at S795. Rb was immunoprecipitated from rat E18 cortical cultures and treated with or without activated CaM and human recombinant active calcineurin at 30°C. Reactions were resolved by SDS-PAGE and probed with the antibodies indicated.

(C) Effect of cyclosporin A on association of HDAC1 with the c-*fos* promoter. Rat E18 cortical cultures were pretreated with vehicle or cyclosporin A for 1 hr and then left unstimulated or stimulated with 50 mM KCl for 10 min. Cells were lysed for chromatin immunoprecipitation, immunoprecipitated with antibodies as indicated, and PCR reactions with endogenous c-*fos* promoter primers were used to amplify c-*fos* promoter-specific segments associated with the indicated proteins. Real-time PCR was performed, and signals were normalized as percentage of input.

(D and E) Effect of cyclosporin A on histone acetylation of the c-*fos* promoter. Rat E18 cortical cultures were pretreated with vehicle or cyclosporin A for 1 hr and then left unstimulated or stimulated with 50 mM KCl for 10 min. Cells were lysed for chromatin immunoprecipitation, immunoprecipitated with antibodies as indicated, and PCR reactions with endogenous c-*fos* promoter primers were used to amplify c-*fos* promoter-specific segments associated with the indicated proteins. Real-time PCR was performed, and signals were normalized as percentage of input. Asterisks indicate significance at $p < 0.05$. Error bars represent +SD.

(F and G) Immunoprecipitation of HDAC1 with Rb WT and phosphorylation mutant. Rat E18 cortical cultures were transfected with myc-HDAC1 with HA-Rb WT and HA-Rb 3M, respectively. Cells are harvested 3 days after transfection, immunoprecipitated with anti-myc antibody, and resolved on SDS-PAGE and blotted with anti-HA antibody.

(H) Quantification of coimmunoprecipitation experiments. Data are collected from two sets of independent experiments. Asterisks indicate significance at $p < 0.05$. Error bars represent +SD.

(I) Effects of calcineurin on endogenous c-*fos* expression upon KCl stimulation. Rat E18 cortical neurons were pretreated with vehicle or cyclosporine A, following which they were stimulated with 50 mM KCl, as indicated. Total RNA was collected with Trizol reagent (Invitrogen), resolved by RNA agarose gel, and probed by an anti-c-fos cDNA probe labeled with $P^{32}$ followed by radioautography with X-ray films.

(J) Quantification of northern blot. Intensity of blots was measured using Image J.

indicating that calcium regulation of Rb dephosphorylation is independent of CaM kinase activity (Figure S5).

To determine whether calcineurin activity was required for the release of HDAC1 from the c-*fos* promoter, we examined the effects of cyclosporin A on association of HDAC1 with the c-*fos* promoter as measured by chromatin immunoprecipitation. As shown in Figure 5C, treatment with cyclosporin A completely blocked the release of HDAC1 from the c-*fos* promoter. Consistent with a role for HDAC1 release in c-*fos* activation, we found that treatment with cyclosporin A led to a significant decrease in the association of acetylated histone H3 and H4 with the c-*fos* promoter (Figures 5D and 5E).

We next examined whether the association of HDAC1 with Rb was dependent on the specific phosphorylation sites on Rb. Serine 780, serine 788, and serine 795 comprise a set of key phosphorylation sites on the Rb protein (Knudsen and Wang, 1997). Whereas we could effectively coimmunoprecipitate HA-WT-Rb and Myc-HDAC1 from transfected cells, Rb constructs that carried triple mutations in S780, S788, and S795 did not coprecipitate with Myc-HDAC1 with the same efficiency, suggesting that these phosphorylation sites are important for the association of Rb and HDAC1 (Figures 5F–5H). Consistent with a role for calcineurin in calcium activation of the c-*fos* promoter, we found that treatment with cyclosporin A significantly attenuated KCl-induced

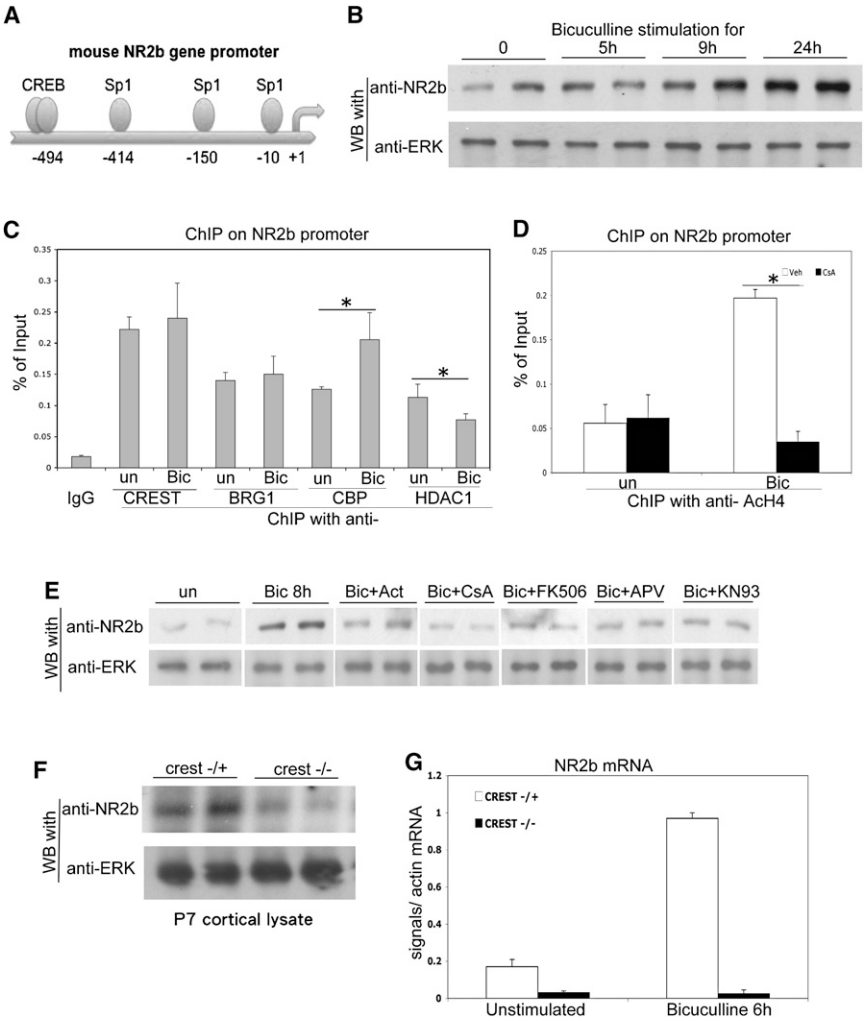


**Figure 6. CREST Mediates Activity-Dependent Regulation of NR2B Expression**

(A) Schematic representation of the NR2B promoter. The promoter includes several Sp1 sites and a distal CREB site.

(B) Activity-dependent regulation of NR2B expression. Rat E18 cortical neurons were cultured for 16 DIV and stimulated with bicuculline for the period indicated. Proteins were separated by SDS-PAGE and blotted with NR2B and ERK antibodies as indicated.

(C) Association of the CREST complex with the NR2B promoter. Lysates from unstimulated and bicuculline-stimulated (1 hr) E18 cortical cultures were processed for chromatin immunoprecipitation using the antibodies indicated. PCR reactions with NR2B primers were used to amplify endogenous NR2B promoter-specific DNA segments from −200 to −1.

(D) Activity-dependent changes in histone acetylation at the NR2B promoter. Lysates from unstimulated and bicuculline-stimulated (1 hr) E18 cortical cultures were treated with vehicle or cyclosporine A (CsA) and processed for chromatin immunoprecipitation using anti-acetylated histone H4 antibodies and NR2B primers.

(E) Signaling pathways involved in activity-dependent regulation of NR2B expression. Rat E18 cortical neurons were cultured for 16 DIV, pretreated by the indicated blockers for 1 hr, and stimulated with bicuculline for 8 hr. Proteins were separated by SDS-PAGE and blotted with NR2B and ERK antibodies as indicated.

(F) Levels of NR2B proteins are reduced in CREST null brains. Cortical lysates from P7 *crest*$^{-/-}$ and *crest*$^{+/-}$ mice were collected, separated by SDS-PAGE, and blotted with NR2B and ERK antibodies as indicated.

(G) CREST is required for activity-dependent regulation of NR2B expression. Embryonic 15 days cortical neurons from *crest*$^{-/-}$ and *crest*$^{+/-}$ were cultured for 12 DIV and stimulated with bicuculline for 6 hr. Total RNA was collected, reverse-transcribed, and analyzed with NR2B-specific primers using real-time PCR. NR2B-specific signals, normalized with actin mRNA levels, are shown.

expression of endogenous c-*fos* (Figures 5I and 5J), as well as KCl activation of the c-*fos*-CAT reporter (data not shown).

These observations indicate that calcium influx leads to a calcineurin-dependent dephosphorylation of Rb, which leads to a release of HDAC1 and activation of gene transcription. In the context of these experiments, it is noteworthy that calcineurin also contributes to calcium-dependent activation of MEF2-mediated transcription by regulating dephosphorylation (Flavell et al., 2006; Shalizi et al., 2006).

## Role of CREST in Activity-Dependent Regulation of NR2B Expression

Finally, we were interested in determining whether the CREST complex was involved in regulating activity-dependent expression of other genes that have a direct effect on neuronal physiology. To identify activity-dependent genes that may be regulated by CREST, we carried out a microarray analysis of genes induced by treating cortical cultures with bicuculline for 6 hr. Bicuculline blocks GABA-A receptors and leads to increased network activity by suppressing inhibition (Hardingham et al., 2002). Analysis of this dataset showed that expression of the NMDA receptor subunit NR2B was significantly increased by bicuculline treatment (Figure 6).

The NR2B promoter contains multiple Sp1 sites, suggesting that, as in the case of c-*fos*, NR2B expression might also be regulated by a BRG1-CREST complex (Sasner et al., 1996; Klein et al., 1998; Figure 6A). To determine whether proteins of the CREST-BRG1 complex associate with the NR2B promoter, we carried out chromatin immunoprecipitation experiments and found that CREST, BRG1, CBP, and HDAC1 bind to the NR2B promoter (Figure 6C). As with the c-*fos* promoter, stimulation led to an increase in CBP recruitment and a decrease in HDAC1 recruitment at the NR2B promoter (Figure 6C). Consistent with transcriptional activation, chromatin immunoprecipitation

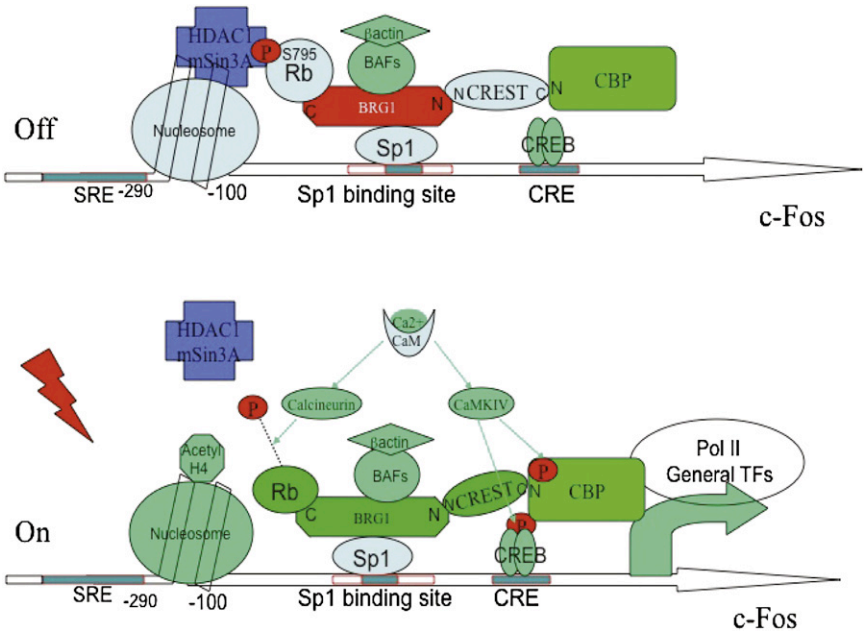


**Figure 7. Proposed Mechanism for Calcium-Dependent Activation of the Proximal *c-fos* Promoter**

In unstimulated neurons, transcription is repressed due to recruitment of an HDAC complex to the Sp1 site via BRG1 and Rb. Calcium stimulation leads to dephosphorylation of Rb via a calcineurin-dependent mechanism and a release of the HDAC complex. At the same time, the transcriptional coactivator CBP (which is recruited to the promoter by CREST and CREB) is phosphorylated by CaM kinase IV and mediates transcriptional activation.

experiments revealed a marked increase in the levels of acetylated histone A4 antibody associated with the NR2B promoter following bicuculline stimulation. This increase was blocked by the calcineurin inhibitor, cyclosporin A. Western blot analysis showed that an increase in NR2B protein levels was detectable by 5 hr after the onset of bicuculline stimulation and continued to increase over a period of 24 hr (Figure 6B). The bicuculline-induced increase in NR2B expression was blocked by actinomycin D (suggesting a requirement for transcription) and by cyclosporin A and FK506 (suggesting a requirement for calcineurin) (Figure 6E). The increase in NR2B expression also required NMDA receptor activation and CaM Kinase II activity (Figure 6E).

To determine whether the CREST complex was involved in regulating NR2B expression in vivo, we examined NR2B expression in crest heterozygous (control) and crest null neurons. Analysis of NR2B expression in cortical lysates showed that NR2B levels were significantly reduced in crest null mice at P7 (Figure 6F). To determine whether activity-dependent upregulation of NR2B expression required CREST function, we examined NR2B mRNA levels in unstimulated and bicuculline-stimulated cultures in control and crest null cultures and found that the bicuculline-induced increase in NR2B expression was completely absent in crest null neurons (Figure 6G). These findings strongly suggest that the activity-dependent increase in NR2B expression involves a switch from a repressor to activator complex and requires CREST function.

## DISCUSSION

The observations described here reveal a regulatory mechanism involved in calcium-dependent transcription. Previous studies on this problem have tended to focus on recruitment of transcriptional activators, with a particular focus on the CREB-CBP complex. Our investigation of CREST-mediated transcription reveals a much more intricate mechanism of transcriptional regulation. In resting neurons, the CREST-mediated transcription complex is negatively regulated by the BRG1-Rb-HDAC1 complex. Upon calcium stimulation, HDAC1 is released from the complex and CBP is recruited, which facilitates transcriptional activation. The contribution of CREST to calcium-dependent transcription is independent of the CRE and represents a major mechanism for calcium-dependent transcriptional activation.

An important contribution of this work is the identification of a role of a BRG1 complex in regulating activity-dependent transcription. The BRG1 complex has been extensively studied in the past few years as a core component of a chromatin remodeling complex. Here, we find that the BRG1 complex plays a major role in negatively regulating transcriptional activation of specific promoters, which is subject to regulation by calcium signaling. The BRG1 complex associates with the c-*fos* promoter via its interaction with Sp1. In unstimulated neurons, BRG1 recruits Rb, which in turn recruits HDAC1 and mSin3A to the promoter to suppress gene expression. Depolarization-dependent calcium influx leads to a calcineurin-dependent dephosphorylation of Rb at S795 and a release of HDAC1 from the promoter. This derepression mechanism works in concert with calcium-dependent activation of a CREST-CBP complex to stimulate transcription. Based on these observations, we suggest that a calcium-dependent switch from a BRG1-Rb-HDAC1 repressor complex to a BRG1-CREST-CBP activator complex plays a critical role in transcriptional activation (Figure 7).

### Interaction of CREST with BRG1 and Characterization of the BRG1-Associated Repressor Complex

CREST is 62% identical in protein sequence with a proto-oncoprotein SYT, and the two proteins share several molecular interactions. We had previously shown that CREST interacts with CBP (Aizawa et al., 2004), and here we show that CREST directly binds BRG1. Recent results from the Crabtree group also indicate that CREST is tightly associated with the BRG1 complex (Wu et al., 2007). Similar to CREST, SYT can directly interact with N terminus of BRG1 (Perani et al., 2003). Moreover, SYT is found to interact with p300, a transcription activator similar

with CBP (Eid et al., 2000). The similarities between the CREST and SYT complexes suggest that SYT-mediated transcription might also be bidirectionally regulated by BRG1 and CBP/p300. Whether SYT can also mediate calcium-dependent transcription remains to be determined.

BRG1 is the core component of an ATPase-dependent chromatin remodeling complex. ATPase-dependent chromatin remodeling complexes use the energy from ATP hydrolysis to move nucleosomes, unfold DNA, and facilitate transcription. They are conserved from yeast to mammals. BRG1 has ATPase activity and is the human homolog of SNF2b, which is a component of the SWI/SNF complex in yeast. Besides the SWI/SNF family, there are two other ATPase-dependent chromatin remodeling complexes, the chromodomain and helicase-like domain (CHD) family and the ISWI family (de la Serna et al., 2006). These three families use different ATPases as core enzymes. Although the BRG1 chromatin remodeling complex has been studied mainly with regard to its role in transcriptional activation, previous observations hint at a negative role in regulating gene expression. For example, after inactivating the SWI/SNF chromatin remodeling complex in yeast, it was found that most of the genes whose expression is altered are upregulated, suggesting that they are negatively regulated by the SWI/SNF complex (Holstege et al., 1998). Similarly, a previous study reported that overexpression of BRG1 could inhibit the basal level c-*fos* expression in various in vitro cell lines via the existence of Rb protein, although the mechanism was not identified (Murphy et al., 1999). The observations presented here clearly show that the BRG1 complex can directly repress transcriptional activation by recruitment of a repressor complex to the promoter.

Our experiments strongly suggest a repressive role of BRG1 at the c-*fos* promoter. Inhibition of BRG1 expression by a BRG1 shRNA increases calcium-dependent activation of the c-*fos* promoter, and overexpressing BRG1 inhibits calcium-dependent activation of the promoter. Furthermore, BRG1 associates with the c-*fos* promoter and can associate with retinoblastoma protein and an HDAC complex to regulate promoter activation. The association of BRG1with an HDAC complex was first reported by Sif and colleagues (Sif et al., 2001; Battaglioli et al., 2002), but the mechanism of recruitment was not known. Our observations indicate that recruitment of HDAC1 by BRG1 is mediated by the Rb protein. Interestingly, the inhibitory effect of BRG1 requires ATPase activity, which is consistent with a previous study (Z.Q. and A.G., unpublished data; Murphy et al., 1999). It will be of interest to determine whether recruitment of Rb and HDAC1 by BRG1 requires chromatin remodeling.

Since BRG1, Rb, and HDAC1 do not have sequence-specific DNA-binding domains, they must be recruited to specific promoter sites via an indirect mechanism. Our results indicate that the transcription factor Sp1 associates with BRG1, providing a potential mechanism for recruitment of the BRG1 complex to the c-*fos* promoter. This interpretation is consistent with previous observations that Rb can negatively regulate c-*fos* expression via the so-called Rb control element (RCE), which corresponds to the Sp1 binding site CCG/ACCC (Udvadia et al., 1993). The Sp1 binding site is directly upstream of the cAMP responsible element (CRE), TGACGTAG. Although the Sp1 site was identified a long time ago, its role in calcium-dependent transcription had not been explored. We have found that both the CRE and the Sp1 site contribute to calcium-dependent activation of the c-*fos* promoter (Figure S6). ChIP experiments indicate that both CREB and Sp1 are bound to the c-*fos* promoter before and after KCl stimulation. Following calcium influx, there is a rapid release of HDAC1 from the promoter and an increase in CBP recruitment. This switch is likely to be critical for the transcription activation program.

## Release of the Repressor Complex by Calcium/Calcineurin Signaling

What is the mechanism by which calcium influx leads to recruitment of CBP and the release of HDAC1? CBP is recruited to the promoter by association with phosphorylated CREB, as well as by interaction with CREST. The significance of the CREST-CBP interaction for transcription activation is highlighted by the fact that calcium activation of Gal4-CREST is completely blocked by expression of E1A cxdl domain, and association of CBP with the c-*fos* promoter is significantly reduced in CREST null neurons. As previously described, the association of CREST and CBP is mediated by the C-terminal domain of CBP (Aizawa et al., 2004).

The recruitment and release of HDAC1 to the promoter is mediated by BRG1. BRG1 interacts with Rb, which in turn interacts with HDAC1. Calcium-dependent release of HDAC1 appears to be mediated by calcium-induced dephosphorylation of Rb. We find that Rb is phosphorylated at serine 795 in resting neurons. This site becomes rapidly dephosphorylated upon calcium stimulation. Inhibiting calcineurin activity prevents dephosphorylation of Rb, as well as release of HDAC1 from the promoter. These observations reveal a mechanism by which calcineurin can regulate association of a histone deacetylase with a promoter and show that the recruitment and release of histone modifiers can be regulated by calcium signaling.

## Implication for Mechanisms of Plasticity

Much of our understanding of molecular mechanism that mediate adaptive responses in the nervous system has come from investigations of activity-dependent gene expression (Ghosh and Greenberg, 1995). For example, CREB was identified as a mediator of cAMP- and calcium-dependent transcription before it was implicated as a mediator of learning and memory processes (Silva et al., 1998). It is likely that the transcription regulatory mechanisms identified here will also play key regulatory roles in mediating activity-dependent development and plasticity. Consistent with such a possibility, we find that CREST is associated with the NR2B promoter, and activity-dependent increases in NR2B levels require CREST function. We also find that CREST and BRG1 are associated with promoters of several plasticity-related genes, including Arc and zif268, suggesting that activity-dependent transcription of these genes might also depend on the BRG1-CREST complex (Figures S7A and S7B). The NR2B, Arc, and zif268 promoters all contain Sp1 sites (Wang et al., 2005; Waltereit et al., 2001), and it will be interesting to determine whether BRG1 is recruited to these promoters via Sp1 and if activity-dependent transcription of these promoters

involves release of HDAC1, as we find to be the case for c-*fos* expression.

The potential importance of the BRG1-CREST complex in adaptive responses is also supported by the fact that this complex recruits CBP, which has been implicated in the regulation of cognitive functions such as learning and memory (Alarcon et al., 2004; Korzus et al., 2004). Our observation that calcium signaling regulates release of HDAC1 from the BRG1-Rb suggests that this is an important regulatory step in activity-dependent gene expression and may be important for learning and memory. Consistent with such a possibility, it was recently reported that intracerebroventricular injections of HDAC inhibitors sodium butyrate and TSA significantly facilitated associative learning, such as fear conditioning and spatial learning (Fischer et al., 2007). Our findings suggest that the calcium-dependent switch in BRG1-CREST-associated complexes could be generally involved in mediating adaptive changes in neurons that underlie neural development and long-term plasticity.

## EXPERIMENTAL PROCEDURES

### Plasmids

The following plasmids used in this study have been previously described: UAS-CAT, Gal4-CREST and deletion mutants, HA-CREST, Myc-CREST, and E1A cxdl (Aizawa et al., 2004; Hu et al., 1999). pcDNA3-WT BRG1-flag construct was made by enzymatic digestion-based subcloning from original human BRG1 constructs from Dr. Anthony Imbalzano at University of Massachusetts. pcDNA3-BRG1N(1-282)-flag, GSTBRG1N(1-283), and GST-BRG1C(1371-1573) were obtained from Dr. Beverly Emerson at the Salk institute. pcDNA3-MT BRG1 (LTCEE-RTREE)-flag was made from pcDNA3-wtBRG1-flag using the QuikChange II kit from Stratagene. Retinoblastoma protein wild-type and Δexon 22 constructs were from Dr. James DeCaprio at Dana-Farber Cancer Institute. Human HDAC1 cDNA was obtained from Dr. M.G. Rosenfeld at UCSD. The c-*fos*-CAT reporter used included the mouse c-*fos* gene from −350 to +50 fused with the CAT gene. −107 c-*fos*-CAT contains sequences from −107 to +50 fused with CAT gene. Sp1 mutation changes −73 CCGCCC to AAATTT. CRE mutation changes −65 TGACGTAG to TGGGAGTG.

### Molecular Biology Reagents

General molecular biology reagents, competent cells, real-time PCR mix, and reverse transcription kits are from Biopioneer Inc., San Diego, CA, http://www.biopioneerinc.com.

### Antibodies

Antibodies were obtained from the following sources: BRG1 G7, CREB, CBP C22, HDAC1, HDAC2, HDAC3, mSin3A, Rb IF8, Sp1, and control rabbit IgG (Santa Cruz Biotechnology); HA 16B12 (Covance); c-*fos* ab5 clone (EMD PC38), Rb phospho-specific S795 (Assay Bio Tech); myc 9E10 (Roche); and Flag M2 (Sigma). Antibodies against CREST were generated in our lab (Aizawa et al., 2004).

### Cell Culture

E18 rat cortical cells were cultured and transfected in 12-well plates as previously described (Song and Ghosh, 2004). Cultures were used at 3–5 DIV. We used 50 mM KCl depolarization to activate calcium-dependent transcription. For TSA treatment, cells were pretreated with either trichostatin A (250 ng/ml) or ethanol (for the vehicle group) prior to stimulation for 2 hr. For CsA treatment, cells were pretreated with cyclosporin A (Calbiochem) (1μM) or DMSO prior to stimulation for 1 hr. For VT-11R treatment, cells were pretreated with 11R-VIVIT (Calbiochem, 480401) (1 μM) or DMSO prior to simulation for 1 hr.

### Immunoprecipitation

For endogenous IP, cortices from postnatal 4 day rat were dissected in cold HBSS buffer, homogenized in RIPA buffer with protease inhibitor cocktail (Roche), and immunoprecipitated with 2 μg antibodies for each reactions in 4°C overnight and followed by 30 μl of a 50% slurry of mixed protein-A/G agarose (Santa Cruz) for 1 hr for each reaction. The precipitates were then washed four times with the lysis buffer, then eluted by boiling-SDS lysis, and resolved by the 6% SDS-PAGE. The gel was transferred to nitrocellulose membranes, and the membrane was blocked with 5% milk in TBST buffer for 1 hr. It was then incubated overnight at 4°C with the anti-BRG1 G7 antibody (Santa Cruz Biotechnology) at 1:2000 dilution, washed three times in TBST, and the signals were revealed by HRP reaction using the Super signal chemiluminescent substrate (Pierce).

For nuclear fraction IP, nuclear fractions from 5 DIV cultured cortical neurons were collected with Nuclear Complex co-IP kit (Active Motif, Cat#54001) as manual described. Gentle procedures were applied to all steps.

For co-IP in 293T cells, HEK293T cells were plated on 60 mm plates precoated with poly-D-lysine in 3 ml of high-glucose DMEM supplemented with fetal bovine serum (10%), and transfection was performed when the cells reached 50% confluence. Lipofectamine 2000 (Invitrogen) was used for transfection. A total of 5 μg of DNA was used per well in 6-well plates at a molar ratio of 1:1 for myc-tagged and HA-tagged constructs. Cells were harvested 24 hr later. The cells were rinsed with cold PBS, harvested, and lysed for 20 min at 4°C in a modified RIPA buffer. 10% of the supernatant was saved for the input control, and the rest was incubated with 2 μg anti-HA antibody 16B12 (Covance) or anti-myc 9E10 (Roche) overnight at 4°C. The immune complex was isolated by addition of 30 μl of a 50% slurry of mixed protein-A/G agarose (Santa Cruz) for 1 hr, washed three times with the lysis buffer, then eluted by boiling-SDS lysis, and resolved by the 10% SDS-PAGE. The gel was transferred to nitrocellulose membranes, and the membrane was blocked with 5% milk in TBST buffer for 1 hr. It was then incubated overnight at 4°C with the anti-Flag M2 (Sigma) at 1:5000 dilution, washed three times in TBST, and the signals were revealed by HRP reaction using the Supersignal chemiluminescent substrate (Pierce).

### GST Pull-Down Assay

GST pull-down assay was performed as described before (Aizawa et al., 2004). Overnight express TB medium from Novagen (Cat#71491-4) was used to generate recombinant protein from *E. coli*.

### Transactivation Assay

Detailed procedures for CAT assay have been described before (Hu et al., 1999). Cells are harvested 20 hr after stimulation unless other indicated. CAT assay experiments were done with triple duplicates. Paired t tests were performed using GraphPad InStat version 3.0a for Macintosh, GraphPad Software, San Diego, CA, www.graphpad.com.

### Chromatin Immunoprecipitation

ChIP assays were performed as originally described, with minor modifications (Song and Ghosh, 2004). Primers: c-*fos* F: ATCCTACATGCGGAGGGTC CAGGA, c-*fos* R: AGTAGTAGGCGCCTCAGCTGGCCG. For reporter-based chromatin IP, a different primer pairs was used. ChIP F: AGTGACGTAGGA AGTCCATC, ChIPR: CTCCCGGGGATCCTCTAGAG. Egr1 promoter ChIP primers are −149 to +23 of mouse Egr1 gene. Arc promoter primers amplify −200 to −1 of mouse Arc gene.

For real-time PCR experiments, SYBR Green PCR master mix from Applied Biotechnology was used. ABI 7000 was used to perform experiments. Datasets are from at least two independent experiments, each of which has triple duplicates. Paired t tests were performed using GraphPad InStat version 3.0a for Macintosh, GraphPad Software.

### In Vitro Dephosphorylation Assay

Immunoprecipitation purified Rb proteins from 5 DIV E18 rat cortical culture combine reaction assay buffer, CaM, and with or without recombinant active calcineurin (BIOMOL), incubating at 30°C for 2 hr. Reaction products are analyzed by western blot with the antibody indicated.

### Short Hairpin RNA Target Design

pSuper retro neo GFP vector from Oligoengine was used to harbor CREST-specific short hairpin RNA. Workstation software from Oligoengine was used

to design short hairpins. CREST-specific shRNA target sequence is GGTCAG CAGTATGGAAGCT. Scrambled sequence (control) is ATAGACGCGCACG CACACT. Rescue construct was designed to replace original DNA bases by introducing same sense mutations, which are resistant to short hairpin RNA and don't change the amino acid sequences. Replacing sequence is GGA CAACAATACGGTAGTT. Target sequence for rat BRG1 is CCAAAGCAAC CATCGAACT. Target sequence for rat Rb1 is GGAGCACGAGTGTAATGTA. pSilencer 1.0 vector from Ambion is also used for harboring rat Rb1 shRNA.

### Measurements of Dendritic Growth

GFP expressing plasmid was transfected into rat E18 cortical cultures at 3 DIV, pretreatment and KCl stimulation were done at 5 DIV. Immunostaining and imaging experiments were carried out at 24 hr after stimulation. Image J software from NIH was used for measuring dendritic growth. Sholl analysis plug-in could be downloaded from http://wwwbiology.ucsd.edu/labs/ghosh/software/index.html. Paired t tests were performed using GraphPad InStat version 3.0a for Macintosh, GraphPad Software.

## SUPPLEMENTAL DATA

The Supplemental Data can be found with this article online at http://www.neuron.org/supplemental/S0896-6273(08)00843-X.

## ACKNOWLEDGMENTS

We would like to thank Drs. A. Imbalzano, Jean Y. Wang, B. Emerson, J. DeCaprio, and Xiang-Ting (Tina) Wang, Ping Zhu, and M.G. Rosenfeld for providing plasmids; Drs. Stefanie Otto, Yang Xu, and Hoseok Song for helping with real-time PCR experiments; Dr. Megan Williams for helping with imaging experiments; Drs. M. Montminy, G.R. Crabtree, Ping Zhu, and members of the Ghosh Lab for valuable discussion and comments on the manuscript; and Mrs. Fang Gao for instructions about Word and Excel software. We would like to give special thanks to Drs. G.R. Crabtree and J. Wu for sharing data prior to publication. This work was supported by NIH grant MH068578 (A.G.).

Accepted: September 29, 2008
Published: December 10, 2008

## REFERENCES

Aizawa, H., Hu, S.C., Bobb, K., Balakrishnan, K., Ince, G., Gurevich, I., Cowan, M., and Ghosh, A. (2004). Dendrite development regulated by CREST, a calcium-regulated transcriptional activator. Science *303*, 197–202.

Alarcon, J.M., Malleret, G., Touzani, K., Vronskaya, S., Ishii, S., Kandel, E.R., and Barco, A. (2004). Chromatin acetylation, memory, and LTP are impaired in CBP+/− mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. Neuron *42*, 947–959.

Battaglioli, E., Andres, M.E., Rose, D.W., Chenoweth, J.G., Rosenfeld, M.G., Anderson, M.E., and Mandel, G. (2002). REST repression of neuronal genes requires components of the hSWI.SNF complex. J. Biol. Chem. *277*, 41038–41045.

Chawla, S., Hardingham, G.E., Quinn, D.R., and Bading, H. (1998). CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. Science *281*, 1505–1509.

Chrivia, J.C., Kwok, R.P., Lamb, N., Hagiwara, M., Montminy, M.R., and Goodman, R.H. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature *365*, 855–859.

de la Serna, I.L., Ohkawa, Y., and Imbalzano, A.N. (2006). Chromatin remodelling in mammalian differentiation: lessons from ATP-dependent remodellers. Nat. Rev. Genet. *7*, 461–473.

Dunaief, J.L., Strober, B.E., Guha, S., Khavari, P.A., Alin, K., Luban, J., Begemann, M., Crabtree, G.R., and Goff, S.P. (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell *79*, 119–130.

Eid, J.E., Kung, A.L., Scully, R., and Livingston, D.M. (2000). p300 interacts with the nuclear proto-oncoprotein SYT as part of the active control of cell adhesion. Cell *102*, 839–848.

Fass, D.M., Butler, J.E., and Goodman, R.H. (2003). Deacetylase activity is required for cAMP activation of a subset of CREB target genes. J. Biol. Chem. *278*, 43014–43019.

Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M., and Tsai, L.H. (2007). Recovery of learning and memory is associated with chromatin remodelling. Nature *447*, 178–182.

Flavell, S.W., Cowan, C.W., Kim, T.K., Greer, P.L., Lin, Y., Paradis, S., Griffith, E.C., Hu, L.S., Chen, C., and Greenberg, M.E. (2006). Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. Science *311*, 1008–1012.

Garnovskaya, M.N., Mukhin, Y.V., Vlasova, T.M., Grewal, J.S., Ullian, M.E., Tholanikunnel, B.G., and Raymond, J.R. (2004). Mitogen-induced rapid phosphorylation of serine 795 of the retinoblastoma gene product in vascular smooth muscle cells involves ERK activation. J. Biol. Chem. *279*, 24899–24905.

Ghosh, A., and Greenberg, M.E. (1995). Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science *268*, 239–247.

Hardingham, G.E., Fukunaga, Y., and Bading, H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat. Neurosci. *5*, 405–414.

Hogan, P.G., Chen, L., Nardone, J., and Rao, A. (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev. *17*, 2205–2232.

Holstege, F.C., Jennings, E.G., Wyrick, J.J., Lee, T.I., Hengartner, C.J., Green, M.R., Golub, T.R., Lander, E.S., and Young, R.A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717–728.

Hu, S.C., Chrivia, J., and Ghosh, A. (1999). Regulation of CBP-mediated transcription by neuronal calcium signaling. Neuron *22*, 799–808.

Hubel, D.H., and Wiesel, T.N. (1998). Early exploration of the visual cortex. Neuron *20*, 401–412.

Impey, S., Fong, A.L., Wang, Y., Cardinaux, J.R., Fass, D.M., Obrietan, K., Wayman, G.A., Storm, D.R., Soderling, T.R., and Goodman, R.H. (2002). Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV. Neuron *34*, 235–244.

Kadam, S., and Emerson, B.M. (2003). Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes. Mol. Cell *11*, 377–389.

Katz, L.C., and Shatz, C.J. (1996). Synaptic activity and the construction of cortical circuits. Science *274*, 1133–1138.

Kingsbury, T.J., Bambrick, L.L., Roby, C.D., and Krueger, B. (2007). Calcineurin activity is required for depolarization-induced, CREB-dependent gene transcription in cortical neurons. J. Neurochem. *103*, 761–770.

Klein, M., Pieri, I., Uhlmann, F., Pfizenmaier, K., and Eisel, U. (1998). Cloning and characterization of promoter and 5′-UTR of the NMDA receptor subunit epsilon 2: evidence for alternative splicing of 5′-non-coding exon. Gene *208*, 259–269.

Knudsen, E.S., and Wang, J.Y. (1997). Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol. Cell. Biol. *17*, 5771–5783.

Korzus, E., Rosenfeld, M.G., and Mayford, M. (2004). CBP histone acetyltransferase activity is a critical component of memory consolidation. Neuron *42*, 961–972.

Kumar, A., Choi, K.H., Renthal, W., Tsankova, N.M., Theobald, D.E., Truong, H.T., Russo, S.J., Laplant, Q., Sasaki, T.S., Whistler, K.N., et al. (2005). Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. Neuron *48*, 303–314.

Liu, D.X., Nath, N., Chellappan, S.P., and Greene, L.A. (2005). Regulation of neuron survival and death by p130 and associated chromatin modifiers. Genes Dev. *19*, 719–732.

Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev. Mol. Cell. Biol. *2*, 599–609.

Murphy, D.J., Hardy, S., and Engel, D.A. (1999). Human SWI-SNF component BRG1 represses transcription of the c-fos gene. Mol. Cell. Biol. *19*, 2724–2733.

Nie, Z., Xue, Y., Yang, D., Zhou, S., Deroo, B.J., Archer, T.K., and Wang, W. (2000). A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. Mol. Cell. Biol. *20*, 8879–8888.

Olave, I.A., Reck-Peterson, S.L., and Crabtree, G.R. (2002). Nuclear actin and actin-related proteins in chromatin remodeling. Annu. Rev. Biochem. *71*, 755–781.

Perani, M., Ingram, C.J., Cooper, C.S., Garrett, M.D., and Goodwin, G.H. (2003). Conserved SNH domain of the proto-oncoprotein SYT interacts with components of the human chromatin remodelling complexes, while the QPGY repeat domain forms homo-oligomers. Oncogene *22*, 8156–8167.

Redmond, L., Kashani, A.H., and Ghosh, A. (2002). Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron *34*, 999–1010.

Robbins, P.D., Horowitz, J.M., and Mulligan, R.C. (1990). Negative regulation of human c-fos expression by the retinoblastoma gene product. Nature *346*, 668–671.

Rosenfeld, M.G., Lunyak, V.V., and Glass, C.K. (2006). Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev. *20*, 1405–1428.

Sasner, M., and Buonanno, A. (1996). Distinct N-methyl-D-aspartate receptor 2B subunit gene sequences confer neural and developmental specific expression. J. Biol. Chem. *271*, 21316–21322.

Shalizi, A., Gaudilliere, B., Yuan, Z., Stegmuller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J.W., and Bonni, A. (2006). A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. Science *311*, 1012–1017.

Sheng, M., and Greenberg, M.E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron *4*, 477–485.

Sif, S., Saurin, A.J., Imbalzano, A.N., and Kingston, R.E. (2001). Purification and characterization of mSin3A-containing Brg1 and hBrm chromatin remodeling complexes. Genes Dev. *15*, 603–618.

Silva, A.J., Kogan, J.H., Frankland, P.W., and Kida, S. (1998). CREB and memory. Annu. Rev. Neurosci. *21*, 127–148.

Sohm, F., Gaiddon, C., Antoine, M., Boutillier, A.L., and Loeffler, J.P. (1999). The retinoblastoma susceptibility gene product/Sp1 signalling pathway is modulated by Ca2+/calmodulin kinases II and IV activity. Oncogene *18*, 2762–2769.

Song, M.R., and Ghosh, A. (2004). FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. Nat. Neurosci. *7*, 229–235.

Udvadia, A.J., Rogers, K.T., Higgins, P.D., Murata, Y., Martin, K.H., Humphrey, P.A., and Horowitz, J.M. (1993). Sp-1 binds promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression. Proc. Natl. Acad. Sci. USA *90*, 3265–3269.

Waltereit, R., Dammermann, B., Wulff, P., Scafidi, J., Staubli, U., Kauselmann, G., Bundman, M., and Kuhl, D. (2001). Arg3.1/Arc mRNA induction by Ca2+ and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. J. Neurosci. *21*, 5484–5493.

Wang, G., Balamotis, M.A., Stevens, J.L., Yamaguchi, Y., Handa, H., and Berk, A.J. (2005). Mediator requirement for both recruitment and postrecruitment steps in transcription initiation. Mol. Cell *17*, 683–694.

West, A.E., Chen, W.G., Dalva, M.B., Dolmetsch, R.E., Kornhauser, J.M., Shaywitz, A.J., Takasu, M.A., Tao, X., and Greenberg, M.E. (2001). Calcium regulation of neuronal gene expression. Proc. Natl. Acad. Sci. USA *98*, 11024–11031.

Wu, J.I., Lessard, J., Olave, I.A., Qiu, Z., Ghosh, A., Graef, I.A., and Crabtree, G.R. (2007). Regulation of dendritic development by neuron-specific chromatin remodeling complexes. Neuron *56*, 94–108.

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W., and Dean, D.C. (2000). Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell *101*, 79–89.