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Crebinostat: A Novel Cognitive Enhancer that Inhibits Histone Deacetylase Activity and Modulates Chromatin-Mediated Neuroplasticity

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Abstract

Long-term memory formation is known to be critically dependent upon *de novo* gene expression in the brain. As a consequence, pharmacological enhancement of the transcriptional processes mediating long-term memory formation provides a potential therapeutic strategy for cognitive disorders involving aberrant neuroplasticity. Here we focus on the identification and characterization of small molecule inhibitors of histone deacetylases (HDACs) as enhancers of CREB (cAMP response element-binding protein)-regulated transcription and modulators of chromatin-mediated neuroplasticity. Using a CREB reporter gene cell line, we screened a library of small molecules structurally related to known HDAC inhibitors leading to the identification of a probe we termed crebinostat that produced robust activation of CREB-mediated transcription. Further characterization of crebinostat revealed its potent inhibition of the deacetylase activity of recombinant class I HDACs 1, 2, 3, and class IIb HDAC6, with weaker inhibition of the class I HDAC8 and no significant inhibition of the class IIa HDACs 4, 5, 7, and 9. In cultured mouse primary neurons, crebinostat potently induced acetylation of both histone H3 and histone H4 as well as enhanced the expression of the CREB target gene *Egr1* (early growth response 1). Using a hippocampus-dependent, contextual fear conditioning paradigm, mice systemically administered

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crebinostat for a ten day time period exhibited enhanced memory. To gain insight into the molecular mechanisms of memory enhancement by HDAC inhibitors, whole genome transcriptome profiling of cultured mouse primary neurons treated with crebinostat, combined with bioinformatic analyses of CREB-target genes, was performed revealing a highly connected protein-protein interaction network reflecting modules of genes important to synaptic structure and plasticity. Consistent with these findings, crebinostat treatment increased the density of synapsin-1 punctae along dendrites in cultured neurons. Finally, crebinostat treatment of cultured mouse primary neurons was found to upregulate *Bdnf* (brain-derived neurotrophic factor) and *Grn* (granulin) and downregulate *Mapt* (tau) gene expression—genes implicated in aging-related cognitive decline and cognitive disorders. Taken together, these results demonstrate that crebinostat provides a novel probe to modulate chromatin-mediated neuroplasticity and further suggests that pharmacological optimization of selective of HDAC inhibitors may provide an effective therapeutic approach for human cognitive disorders.

Keywords

Cognitive enhancer; histone deacetylases; epigenetic; chromatin; acetylation; CREB

1.1 Introduction

Numerous studies have described a key role for the transcription factor CREB (cAMP response element-binding protein) in neuroplasticity underlying learning and memory (Silva et al., 1998; Benito & Barco, 2010; Sakamoto et al., 2011). Early work in *Drosophila melanogaster* showed that CREB is required for olfactory memory (Yin et al., 1994). Knockout of CREB in mice impairs fear conditioning memory (Bourtchouladze et al., 1994). Finally, enhancement of hippocampal-dependent memory by histone deacetylase (HDAC) inhibitors depends on CREB and its interaction with the coactivator CBP (CREB-binding protein) (Vecsey et al., 2007). CBP has lysine acetyltransferase activity, and can acetylate lysines in histone N-terminal tails. Pointing to the importance of these mechanisms to human cognition, loss of function of CBP is known cause the human genetic disorder Rubinstein-Taybi syndrome (Rubinstein & Taybi, 1963; Petrij et al., 1995), a congenital neurodevelopmental disorder defined by characteristic postnatal growth deficiencies, dysmorphology and intellectual disability. The identification of dysregulated histone acetylation due to loss of CBP in Rubinstein-Taybi syndrome patients (Murata et al., 2001), and in the corresponding mouse models (Alarcón et al., 2004; Korzus et al., 2004; Wood et al., 2005), serves as one of the first examples of a now growing list of human diseases with cognitive deficits that can be considered as ‘chromatinopathies’ due to causally involved mutations in regulators of the structure or function of chromatin and gene expression (reviewed in Levenson & Sweatt 2005; van Bokhoven 2011; Haggarty & Tsai 2011). In addition to these primary chromatinopathies, it is also increasingly recognized that certain neurodegenerative disorders with cognitive deficits, such as Alzheimer’s disease (Gräff et al., 2012) and Huntington’s disease (Giralt et al., 2012), involve a significant component of epigenetic dysregulation as a downstream consequence of disease pathophysiology. Taken together, these findings point to CREB-mediated transcription as being of paramount importance to the study of human cognitive disorders and efforts to develop novel cognitive enhancers.

The CREB transcriptional pathway is activated by intracellular signaling triggered by increases in intracellular cAMP concentration, or a variety of other signaling pathways (Silva et al. 1998; Johannessen et al., 2004; Benito & Barco, 2010). Typically, the final effector of these signaling pathways is a kinase that phosphorylates CREB at serine 133. The coactivator histone acetyltransferase CBP is then recruited to phospho-(S133)-CREB, which

is bound to cyclic-AMP response elements (CREs) in gene promoters. The complex of CREB-CBP then interacts with the general transcriptional machinery to induce activation of transcription of CREB target genes (Goldman et al., 1997). Termination of this transcriptional pathway is mediated by phosphatases that dephosphorylate CREB (Mauna et al., 2011). Finally, the pathway can also be downregulated by proteasome-mediated degradation of CREB (Garat et al., 2006).

Inhibitors of HDACs regulate CREB-dependent transcription (e.g. Fass et al., 2003) and enhance cognition (reviewed in Haggarty & Tsai, 2011). In the case of contextual fear conditioning, enhancement of cognition by HDAC inhibitors is dependent on the functioning of CREB (Vecsey et al., 2007). HDACs are a family of 18 isoforms that catalyze the deacetylation of the ε-amino group of lysine side chains in histone N-terminal tails, and also in numerous other intracellular proteins (Grozinger & Schreiber, 2002; Choudhary et al., 2009). The catalytic mechanism of 11 of these HDACs is critically dependent on a divalent zinc cation coordinated by conserved histidine and aspartate residues within the catalytic site (Bressi et al., 2010). Zinc-dependent HDAC isoforms have been categorized into classes based on structural analyses of the active sites and other domains in these proteins and inhibitor sensitivity (Bradner et al., 2010): class I (HDACs 1, 2, 3, and 8); class IIa (HDACs 4, 5, 7, and 9); class IIb (HDACs 6 and 10); and class IV (HDAC11).

The HDAC isoforms that regulate CREB-dependent transcription and cognition have not been fully identified (reviewed in Fischer et al., 2010). HDACs could potentially regulate CREB-dependent transcription via several mechanisms. First, HDACs could reverse CBP-catalyzed histone acetylation events that mediate transcriptional activation (e.g Valor et al., 2011). Indeed, an HDAC inhibitor failed to enhance cognition in mice with a focal depletion of CBP in the hippocampus (Barrett et al., 2011). Second, HDACs could counteract CBP and the related lysine acetyltransfrease p300 activation by auto-acetylation (Thompson et al., 2004). Also, HDAC1- and HDAC8-protein phosphatase 1 complexes have been shown to mediate CREB dephosphorylation (Canettieri et al., 2003; Gao et al., 2009). Further work will be required to fully identify the HDAC isoforms that regulate CREB-dependent transcription and cognition, and their mechanisms of action.

Here, we describe a chemical-genetic approach to identify novel HDAC inhibitors that regulate CREB-dependent transcription, neuronal histone acetylation, and enhance learning and memory *in vivo*.

2. Results

2.1 CREB reporter gene screen

To identify novel enhancers of CREB-dependent transcription, we used a PC12 cell line stably transfected with a reporter gene driven by six tandem CREs (Figure 1A; Meinkoth et al., 1990). CREB reporter gene induction by forskolin, an adenylate cyclase activator, is triggered by an elevation of cAMP levels, which stimulates a well described pathway involving protein kinase A (PKA)-mediated phosphorylation of CREB and recruitment of CBP (Johannessen et al., 2004). Activation of this pathway is further enhanced by inhibition of phosphodiesterase activity by rolipram (Xia et al., 2009). These facts underscore the effectiveness of this reporter gene assay in identifying novel small molecules with memory enhancing activity (Xia et al., 2009), such as rolipram (e.g. Hosseini-Sharifabad et al., 2012).

Treatment with the forskolin (10 μM; 4 hours) produced ~4-fold induction of the CREB reporter gene (Figure 1B). This inducible reporter gene signal was detectable when the cells

were grown in 384-well microtitre plates in a format compatible with high-throughput screening. Forskolin induction of this reporter gene is further enhanced by the HDAC inhibitor trichostatin A (Fass et al., 2003; Figure 1B). We then used this assay to screen a library of ~1,200 small molecules designed to inhibit HDACs (Tang et al., 2011; Figure 1C and Supplementary Figure 1). These compounds contained one of three biasing elements—metal chelating hydroxamic acid, which may bind to the zinc in the HDAC catalytic site; ortho-hydroxy anilide; or carboxylic acid—that are present in known HDAC inhibitors. The biasing elements were coupled to 4, 5, or 6 carbon linkers ending in a hydrazone moiety. Reaction with aldehyde-containing "caps" resulted in a library of novel potential HDAC inhibitors modeled after the classic "cap-linker-biasing element" structure of known HDAC inhibitors (Figure 1C) (Grozinger & Schreiber, 2002; Cole, 2008; Tang et al., 2011).

The range of cellular activities of the compounds in the CREB reporter gene screen both in the absence and presence of forskolin (4 hour treatments) are shown in Figure 1D. We found compounds that activated the reporter gene in both conditions. Thus, we chose to focus on compounds that enhanced reporter gene activation only in the presence of forskolin as a means of selecting compounds that could target CREB-mediated transcription when CBP is in an activated state due to PKA activation by cAMP. The structure of the most active forskolin-dependent compound, which we termed "crebinostat", is shown in Figure 1D.

To confirm that crebinostat was indeed an HDAC inhibitor, we resynthesized it and confirmed its structure and purity (Supplementary Figures 1, 2), and then we tested its ability to inhibit the enzymatic activity of a panel of recombinant HDACs 1-9. We used a modified version of an *in vitro* assay with HDAC class-specific substrates (Bradner et al., 2010) in which the HDACs deacetylate a tripeptide substrate derived from histone H4 acetylated lysine 12 (AcH4K12) residue, which is then cleaved by trypsin, releasing a fluorophore (Fass et al., 2010). This substrate was chosen on the basis of its robust deacetylation by class I HDACs, including HDAC2, as well as its relevance to cognition--decreased acetylated histone H4 lysine 12 is found in the brain in mice showing ageing-related cognitive decline (Guan et al., 2009; Peleg et al., 2010). Table 1 shows the IC_{50}s for inhibition of HDACs 1-9 by crebinostat, and for comparison, the related hydroxamic acid SAHA (suberoylanilide hydroxamic acid). Crebinostat is approximately 10-fold more potent than SAHA for inhibition of class I HDACs 1-3.

2.2 Regulation of neuronal histone acetylation by crebinostat

To test the cellular HDAC inhibitory activity of crebinostat, we measured its ability to induce histone acetylation in mouse primary cultured forebrain neurons. We chose to focus on AcH4K12 (the physiological correlate of our *in vitro* biochemical assay substrate), as well as AcH3K9, because these acetylation states have been implicated in cognition enhancement in normal and aged mice (Fischer et al., 2007; Guan et al., 2009; Peleg et al., 2010). To quantify these acetylation marks, we developed a high throughput, immunofluorescence-based assay using laser-scanning cytometry (Figure 2A; Supplementary Figure 3). Treatment with crebinostat for 24 hr induced robust acetylation of AcH4K12 and AcH3K9 in a dose dependent manner (Figure 2B). EC_{50} values were 0.29 μM and 1.9 μM (AcH4K12) and 0.18 μM and 1.0 μM (AcH3K9) for crebinostat and SAHA, respectively. As predicted by its improved potency in the *in vitro* enzymatic assays, crebinostat was ~6-fold more potent than SAHA at inducing histone acetylation in neurons.

To test for effects of crebinostat beyond these two particular acetylated residues on histone H4 and histone H3, we also performed western blotting of chromatin isolated from crebinostat-treated neurons with a general anti-acetylated lysine antibody (Figure 2C), which revealed strong increases in acetylation of histone H3, histone H4 as well as histone H2A and H2B, which are histone known to be hypo-acetylated upon loss of CBP in Rubinstein-

Taybi syndrome patient lymphoblastoid cell lines (Lopez-Atalaya et al., 2012). Taken together, these data indicate that crebinostat is a highly potent cell-active HDAC inhibitor capable of altering histone acetylation states related to cognition enhancement in neurons.

2.3 Testing of crebinostat in contextual fear conditioning paradigm in mice

Our ultimate goal is to discover novel HDAC inhibitors that affect neuroplasticity not only *in vitro* with cultured neurons but also *in vivo* to enhance learning and memory. In order to first determine if crebinostat was brain penetrant upon systemic administration, a pharmacokinetic profile was determined with a single IP dose (25 mg/kg) chosen to match that used in the fear conditioning experiments. Crebinostat had a maximum concentration (C_{max}) in brain of 60 nM at 30 minutes post-injection, and a half-life ($T_{1/2}$) of 0.72 hrs. (Figure 3A; see Supplementary Figure 4 for more details). Given crebinostat's enzymatic activity IC_{50} values in the single digit nM range (Table 1), this level of brain exposure is consistent with the concentrations needed to inhibit HDACs, particularly for HDAC1/2/3/6. Furthermore, this exposure profile further defines the pharmacokinetic properties sufficient for hydroxamate-based HDAC inhibitors to enhance memory—pulsatile, rather than continuous, exposure, is sufficient.

Having determined that crebinostat was brain penetrant, we tested the effect of chronic (10 day) treatment with crebinostat (25 mg/kg), administered systemically via daily intraperitoneal injections, on performance in a mouse contextual fear conditioning model (Figure 3B). In this fear conditioning model, mice cease to locomote ("freeze") in a context-dependent manner once they have learned the association between the context and a footshock given only in this context. Figure 3C shows that crebinostat produced robust enhancement of context-dependent freezing behavior. Importantly, no significant differences were detected between vehicle and crebinostat treated animal groups in exploratory activity immediately prior to foot shock, indicating that chronic crebinostat treatment did not cause a state-dependent effect that confounded the measurement of percent freezing post-shock (Figure 3D). We also measured the effect of crebinostat injections on histone acetylation in the hippocampus by giving one additional dose of crebinostat or vehicle 24 hours after the fear conditioning test, and harvesting brains one hour after these injections. By western blot analysis, we found that crebinostat induced a trend towards an increase in total hippocampal acetylation of both H4K12 and H3K9, measured one hour after IP injection (Supplementary Figure 5); however, this effect was ultimately not statistically significant. This lack of correlation between changes in histone acetylation and the cognition enhancing effects of crebinostat suggests that either the effects of crebinostat on the particular histone acetylation marks we investigated are subtle, or transient in nature, or alternatively, that appropriate pharmacodynamic markers for HDAC inhibitor-mediated cognition enhancement at various time points and in different brain regions, still need to be discovered.

2.4 Regulation of *Egr1* gene expression by crebinostat

In light of its robust cognition-enhancing effects, and with the desire of identifying other pharmacodynamic markers of HDAC inhibitor-mediated cognitive enhancement, we next sought to determine whether crebinostat would enhance the expression of CREB-target genes thought to play a role in learning and memory. We chose to focus initially on *Egr1*, an immediate early gene that is activated in the brain in animals undergoing memory training tasks, and may be required for memory formation (Malkani et al., 2004). Furthermore, *Egr1* was recently reported to be an HDAC2 target gene (Guan et al., 2009), as well as a member of a gene set comprising a molecular signature for intellectual disability disorders (Hashimoto et al., 2011). *Egr1* is a CREB target gene, with two CREs in its proximal promoter, along with binding elements for SRF/ELK and SP1 (Sakamoto et al., 1994; Tur et al., 2010; see Figure 4A). Chromatin immunoprecipitation assays have shown that HDAC2

also binds to the *Egr1* promoter, and overexpression of HDAC2 suppresses neuronal *Egr1* expression, making it a useful marker of chromatin-mediated neuroplasticity that may be relevant to memory formation (Guan et al., 2009). Figure 4A shows that both crebinostat and SAHA (24 hour treatments) induced Egr1-EGFP reporter gene activity in primary cultured cortical neurons. Representative images of EGFP fluorescence from DMSO and crebinostat-treated cultures are shown in Figure 4A. Crebinostat was again found to be more potent than SAHA, consistent with its greater potency in inducing neuronal histone acetylation. Finally, crebinostat also activated expression of endogenous *Egr1* in primary cultured neurons (Figure 4B). Taken together, these results demonstrate that crebinostat treatment not only increases histone acetylation in neurons, but also leads to the regulation of gene expression that is known to be involved in memory formation and other forms of neuroplasticity.

One intriguing aspect of crebinostat regulation of *Egr1* expression was that crebinostat treatment alone induced *Egr1* in mouse cortical neurons (Figure 4), whereas activation of the CREB reporter gene in PC12 cells by crebinostat required the presence of forskolin (Figure 1). These data suggest that crebinostat regulation of CREB target genes can be dependent on factors specific to individual cell types, gene promoters, and/or the concurrence of ongoing signal transduction events in cells. To further explore the effects of crebinostat on gene expression in cortical neurons in the context of neuronal stimulation, we treated cultures for 6 hours with the neuronal signaling inducers forskolin, KCl, or BDNF in the absence and presence of crebinostat. We added crebinostat 18 hours prior to stimulation with the signaling inducers, and maintained crebinostat in the cultures during the 6 hour stimulations. Using a multiplexed, NanoString nCounter gene expression assay, which is based on direct digital detection of mRNA molecules of interest, we measured the effects of these treatments on the expression levels of 53 genes selected on the basis of previous observations of their responsiveness to HDAC inhibitor treatments, or data suggesting them as candidate genes involved in neuroplasticity induced by HDAC inhibition. For the vast majority of the genes, crebinostat regulation was additive with signal inducer regulation (Supplementary Table 5). However, Figure 4C shows one exception: crebinostat regulation of *Homer1* expression in cortical neurons was synergistic with BDNF stimulation. These data, together with the *Egr1* data (Figure 4B), underscore the notion that crebinostat regulation of gene expression is dependent on gene promoter- and signaling context-specific factors.

2.5 Analysis of the transcriptome of neurons treated with crebinostat

To further dissect the mechanism of cognition enhancement by crebinostat, and to identify additional potential pharmacodynamic markers for HDAC inhibitor-mediated cognition enhancement beyond those tested in our candidate gene approach described above, we hypothesized that besides the CREB target gene *Egr1*, crebinostat would regulate the expression of additional CREB target genes. To test this hypothesis, we generated whole genome transcriptome profiles with Illumina bead arrays from samples of mouse primary cultured neurons treated with crebinostat (1 μM) for 24 hrs. We analyzed triplicate samples obtained in two replicate experiments involving DMSO and crebinostat treatment. After normalizing the microarray data, the distributions of the data were remarkably consistent across replicates and experiments. Upon applying a statistical significance threshold ($p < 0.05$), we found that crebinostat upregulated 624 genes and downregulated 706 genes by at least 1.5 fold (Figure 5; Supplementary Figure 6; Supplementary Table 1).

To identify CREB target genes within the list of crebinostat regulated genes, we cross-referenced published CREB ChIP-seq data sets (Kim et al., 2010; Martianov et al., 2010; MacGillavry et al., 2011). We determined that 153 of the upregulated genes and 152 of the downregulated genes are experimentally validated CREB target genes (Supplementary Table

2). On the basis of their reported effects on cognition, synaptic function, or neuronal development, a subset of the CREB target genes upregulated by crebinostat were selected for further validation by RT-PCR (Table 2). Using independent neuronal cultures from those used for the whole genome transcriptome analysis, crebinostat treatment was verified to upregulate all of the genes in Table 2, with many showing greater fold-changes than observed on the microarray format, likely due to signal compression inherent in microarray data. These results provide strong validation of the original CREB reporter gene assay used to identify crebinostat, and further underscore the importance of HDAC activity as a key regulator of CREB-mediated transcription in cultured neurons.

To test the possibility that additional transcription factors were regulated by crebinostat, we searched for enrichment for genes with promoters containing known binding sites for specific transcription factors within the set of crebinostat regulated genes. To perform this analysis, we used the gene set enrichment analysis tool (Subramanian et al., 2005) on the Broad Institute's MSigDB website (http://www.broadinstitute.org/gsea/msigdb/index.jsp). This analysis revealed strong statistical enrichment for genes with promoters having binding sites for several transcription factors, including SP1, MYC, and NFAT (Supplementary Table 4). Further work will be required to fully analyze crebinostat regulation of transcription mediated by these factors; however, for the remainder of the present study, we focused on crebinostat regulation of CREB target genes.

2.6 Analysis of crebinostat responsive synaptic CREB target gene networks

We next examined the possibility that crebinostat regulation of CREB target gene expression would modulate the activity of networks of proteins involved in synaptic function in neurons. To perform this analysis, we cross-referenced published databases and literature identifying synaptic proteins (Kennedy et al., 2005; Collins et al., 2006; Pocklington et al., 2006; Waite & Eickholt, 2010; Bayes et al., 2011), and identified 38 synaptic CREB target genes regulated by crebinostat (10 upregulated and 28 downregulated). To visualize these networks, we input the lists of up- and down-regulated synaptic CREB target genes, mapped to their human homologs, into the VisANT integrative visual analysis tool for biological pathways and networks (www.visant.bu.edu) and queried for protein-protein interactions between these and other proteins in the VisANT database. The resulting networks, consisting of the up- or down-regulated synaptic CREB target gene "nodes", and protein-protein interactions illustrated as connecting lines ("edges"), are illustrated in Figure 5 (C, D). The full set of interactions indicated in Figure 5 is contained in Supplementary Table 3.

Several functional sub-networks (or "modules") were evident within the crebinostat-responsive CREB target gene synaptic interactome. First, amongst genes upregulated by crebinostat (Fig. 5A), one module consisted of five components of the protein translational machinery. One protein in particular, Eef2, has been reported to respond to synaptic activity by mediating local dendritic synthesis of BDNF (Verpelli et al., 2010). Mice with deficient activity of the kinase that phosphorylates and activates Eef2 (Eef2k) show impaired associative learning (Gildish et al., 2012). In addition, crebinostat upregulation of CTNNA1 (α-catenin) is expected to impact a module, including CTNNB1 (β-catenin) that plays an important role in synaptic plasticity (Magushak & Ressler, 2012). A module of 13 proteins regulating neurite outgrowth or the cytoskeleton was apparent within the set of synaptic CREB target genes downregulated by crebinostat (Fig. 5B). Three of these downregulated proteins (DPYSL4, DPYSL5, and OMG) are known to negatively regulate neurite outgrowth (Kottis et al., 2002; Aylsworth et al, 2009; Brot et al., 2010). In addition, one downregulated protein (CTNND2) was reported to be a negative regulator of β-catenin signaling (Bareiss et al., 2010). Lastly, two of the downregulated proteins (CDK5R1 and MAPT) are dysregulated under pathological conditions of neurodegeneration (Iqbal et al.,

2010; Su & Tsai, 2011). These computational analyses suggest that HDAC inhibitors such as crebinostat can enhance memory by regulating modules of genes that function together to coordinate key changes in synaptic physiology.

2.7 Characterization of the effect of crebinostat on synapse formation

The observation that several of the CREB target genes upregulated by crebinostat promote synapse formation, organization, and stability led us to hypothesize that crebinostat would regulate some aspect of synapse morphology in our primary mouse forebrain cultures. To test this hypothesis, we used immunofluoresence staining to detect synapsin I punctae along dendrites identified as Map2-positive. While our previous experiments investigating crebinostat's effect on histone acetylation were performed in primary neuronal cultures grown in 96-well plates, these synaptic morphology experiments were performed on cultures grown on glass coverslips, featuring a lower density of neurons to allow more precise determination of neuronal process morphology using higher resolution imaging. Figure 6 shows that, in these cultures, crebinostat treatment (1 μM) for 24 hrs produced a robust increase in histone acetylation, as we observed in our 96-well plate cultures. This treatment with crebinostat produced no detectable toxicity (Supplementary Figure 7). Although the effect of crebinostat on histone acetylation was apparent and statistically significant when mean nuclear intensity was quantified (Figure 6I); this effect was even larger when imaged nuclei were parsed into signal intensity bins, and the data were analyzed to determine the percentage of nuclei crossing a "high" signal intensity threshold (Figure 6J).

In addition to its effect on neuronal histone acetylation, crebinostat produced a visually apparent increase in the number of synapsin I punctae along Map2-positive dendrites (Figure 6C versus 6D; Figure 7A). To quantify crebinostat's effects on dendritic synapsin I punctae, we analyzed images using ImageJ image processing and analysis software. First, we determined that crebinostat did not alter total dendrite length per image (Figure 7C), indicating that crebinostat did not produce a gross change in neuronal morphology. Second, we found that crebinostat did not alter synapsin I punctae size or staining intensity of those punctae that could be measured (Figure 7D, E). Thus, there was no difference in the detectability of synapsin I punctae staining signals between control and crebinostat-treated cultures. Finally, we found that crebinostat treatment produced a nearly 2-fold increase in dendritic synapsin I punctae density (Figure 7B). We propose that crebinostat may increase synaptic density by upregulating the expression of genes involved in synaptic formation or maintenance, which we speculate may be involved in its enhancement of memory formation. The effect of crebinostat on synaptic density seen here is consistent with evidence for a key role of HDAC2 in regulating synaptic density *in vivo* (Guan et al., 2009).

2.8 Regulation of genes implicated in human cognitive disorders by crebinostat

Given the ability of crebinostat to regulate chromatin-mediated neuroplasticity at the level of CREB target genes involved in synaptic structure and plasticity along with its ability to enhance memory in mice raises the question of whether crebinostat could reverse molecular pathology underlying human diseases of cognition. Frontotemporal dementia is a hereditable form of dementia, primarily involving behavioral and language deficits that can be caused by haploinsufficiency at the *Grn* gene (granulin) locus (Gass et al., 2006). A recent report indicated that the HDAC inhibitor, SAHA, can upregulate the expression of *Grn* (granulin) in cell lines (Cenik et al., 2011). Indeed, here we extend these results by demonstrating that crebinostat can also upregulate *Grn* mRNA expression in mouse forebrain primary neuronal cultures (Figure 8A). Mutations in the *Mapt* gene encoding the microtubule-associated protein tau can also cause FTD (Lee at al., 2001). These gain-of-function mutations lead to pathological aggregation of tau in neurons. We found that crebinostat downregulated *Mapt* mRNA expression in primary cultured neurons (Figure 8B). A reduction in the expression of

aggregating forms of tau might help reverse cognitive deficits (Sydow et al., 2011). In addition, overexpression of the heat shock protein HSP70 can induce clearance of neurotoxic amyloid-β peptide in a mouse model of Alzheimer's disease (Hoshino et al., 2011). Indeed, crebinostat also upregulated *Hspa1b* (*Hsp70)* mRNA expression in primary cultured neurons (Figure 8C). Lastly, crebinostat also upregulated *Bdnf* mRNA expression (Figure 8D). In support of the potential therapeutic relevance of these findings, several lines of evidence suggest that elevating the level of BDNF in the brain might enhance neuroplasticity and produce therapeutic benefit in disorders of cognition (Nagahara & Tuszynski, 2011). BDNF has also been implicated recently as a key factor in the consolidation of fear memory and in reversing ageing-related cognitive decline (Lubin et al., 2008; Zeng et al., 2011). Taken together, these data demonstrate the ability of crebinostat to regulate the expression of key genes involved in neuroplasticity.

3. Discussion

Recent molecular, cellular, and behavioral studies indicate that pharmacological inhibition of HDACs can ameliorate cognitive deficits produced by ageing or neurodegeneration (Guan et al., 2009; Peleg et al., 2010; Fischer et al., 2010; Gräff et al., 2012). Harnessing the therapeutic potential of HDAC inhibitors linked to cognitive enhancement requires knowledge of the specific HDAC family member(s) involved, and the downstream transcriptional pathways regulated by these HDACs that may be involved in memory. With the long-term goal of determining if these HDAC-regulated pathways can be targeted for therapeutic benefit in the context of disorders of cognition, we have sought to identify improved small-molecule probes of HDACs relevant to neuroplasticity. Figure 9 shows potential mechanisms by which one such probe molecule, crebinostat, could have these effects by enhancing CREB-dependent transcription (see Introduction and Results for details).

Crebinostat was selected from amongst a library of over 1,200 small molecules designed to target the HDAC family (Tang et al., 2011) on the basis of its strong activity in the primary CREB reporter gene assay. Consistent with its strong cellular activity, crebinostant was found to potently inhibit the class I HDACs 1, 2, 3, and class IIb HDAC6, and to a lesser extent the class I HDAC8 (Table 1), suggesting that one or more of these isoforms repress CREB-dependent transcription. Indeed, HDAC1 has been reported to promote CREB dephosphorylation through the recruitment of PP1 (Canettieri et al., 2003). In addition, HDAC2 has recently been demonstrated to repress transcription of the CREB target genes *Egr1* and *cFos* in the brain (Guan et al., 2009). Finally, epistasis experiments in *C. elegans* indicate that HDAC3 can oppose CREB-mediated neuroprotection (Bates et al., 2006). While our data with crebinostat do not support a role for class IIa HDACs, it has been reported that HDAC4 can repress CREB-dependent transcription (Bolger & Yao, 2005). Moreover, we were not able to study the effects of crebinostat on the enzymatic activity of HDACs 10 and 11 *in vitro* due to current limitations in our ability to develop robust biochemical assays for these isoforms, and thus the role of these two HDACs is uncertain. Further work will be required to fully elucidate which HDAC isoforms repress CREB-dependent transcription, and whether different isoforms play distinct roles in a cell-type specific or signaling-dependent manner. It will also be important to determine whether class I HDAC isoform-selective inhibitors can enhance CREB-dependent transcription and cognition.

Our results demonstrating that crebinostat treatment increases the number of synapsin I positive punctae, reflective of the assembly and clustering of pre-synaptic vesicles along Map2 staining dendrites, are consistent with those described by our group recently for a structurally related HDAC inhibitor, synapsinostat, which we showed could enhance

synaptogenesis in a microfluidics-based co-culture assay (Shi et al., 2012). However, data reported here indicate that crebinostat is capable of either enhancing the formation of synapses, or preventing their loss, or both, and both the pre-synaptic and post-synaptic contributions to this process occur in primary cultured mouse neurons tested here, in contrast to the artificial conditions of the co-culture assay.

Our transcript profiles generated from primary mouse neurons treated with crebinostat allowed genome-wide assessment of the effects of a memory enhancing probe on the regulation of gene expression. As anticipated from early studies on the effects of HDAC inhibitors on CREB-mediated transcription (Fass et al., 2003), as well as systematic studies of neural-activity dependent changes in gene expression (Benito et al., 2011), we observed both upregulation and downregulation of gene expression. The analyses performed here, particularly the intersection with global datasets of promoter occupancy by CREB through chromatin immunoprecipitation experiments, and synaptome constituent identification proteomic studies, represent one of the first efforts to generate a complete picture of the synaptic regulatory protein interaction network modulated by activation of CREB target genes by an HDAC inhibitor that is known to enhance cognition in mice. We found that crebinostat upregulated the expression of several experimentally validated CREB target genes (Table 2) that have been reported to play a role in cognition, synaptic function, or neuronal development. For example, one of the networks of genes regulated in our expression profiling in response to crebinostat was that of the Ras GTPase pathway. Dysregulation of Ras-MAPK signaling has been implicated in multiple human genetic disorders that include cognitive deficits, including neurofibromatosis, Noonan syndrome, Costello syndrome, LEOPARD syndrome, CFC syndrome, and Legius syndrome (reviewed in van Bokhoven, 2011). Thus, beyond Rubinstein-Taybi syndrome, these findings point to a potential broader application of HDAC inhibitors in treating intellectual disabilities, although the specific consequence of each disease causing mutation on downstream signaling and how these altered neuronal cell states respond to HDAC inhibitor treatment will need to be carefully investigated.

It is possible that there are additional CREB target genes in our list of transcripts upregulated by crebinostat (Supplementary Table 1)—these genes might be identified in future CREB ChIP-seq studies performed on brain tissue. It will be important to identify the full set of CREB target genes that are regulated in the brain by crebinostat, and to determine which of these contribute to the cellular events that mediate its effect on synapse density and enhancement of memory. It will also be important to fully understand how the proteins encoded by these CREB target genes interact to form networks that modulate synaptic physiology in the brain (Figure 7). A full understanding of these networks will allow us to identify critical modules that drive and control network activity—these modules may be ideal targets for effective therapeutic intervention in disorders of cognition and altered neuroplasticity.

While not addressed here, it will be important to consider the regulation of acetylation of non-histone substrates as part of gaining a comprehensive understanding of the role of HDACs in mediating cognition and neuroplasticity. For example, Yeh et al. (2004) implicated acetylation of the transcription factor NF-kappaB in memory formation, although the precise HDACs involved in mediating this effect and its relevance to different types of memory and forms of neuroplasticity remain unclear. More recently, quantitative proteomics studies performed in non-neuronal cell lines have identified a total of 1,750 acetylated proteins and 3,600 individual lysine acetylation sites in these proteins (Choudhary et al., 2009), many of which were sensitive to HDAC inhibitors such as SAHA. Taken together, these findings point to the need in the future better understand the possible contribution of

non-histone substrates to the short-term and long lasting effects of HDAC inhibitors such as crebinostat on neuroplasticity. Indeed, such studies are ongoing in our lab.

While there are attractive features of crebinostat as a probe of chromatin-mediated neuroplasticity and memory, there are limitations to the *in vivo* pharmacological properties of crebinostat as revealed by the analysis of the brain-to-plasma C_{max} ratio of 0.02, exposure (AUC_{last}) ratio of 0.02, and $T_{1/2}$ in brain of ~43 min following IP administration of crebinostat (Supplementary Figure 4). Now that these limitations are known, future optimization of the pharmacological properties of this series of memory enhancers will aim to increase overall brain exposure and the brain:plasma ratio. While ultimately the optimal exposure profile for HDAC inhibitors for memory enhancement remains to be rigorously determined, and may vary for different behavioral paradigms, we speculate that the pulsatile nature of crebinostat exposure in the brain may be advantageous for the following reasons: 1) a pulsatile type of exposure may mimic aspects of salient cognitive experiences that occur in short duration yet induce long-lasting memories; 2) pulsatile exposure has been reported to be optimal in inducing neuroprotection (Langley et al., 2008); and 3) pulsatile exposure may minimize undesired effects of long-term HDAC inhibition. An interesting challenge for future studies will be to understand and delineate, amongst different classes of HDAC inhibitors, with different binding kinetics and residence times on different HDAC targets, which parameters determine the optimal pharmacokinetic profiles for HDAC inhibition leading to memory enhancement and other behavioral effects.

4. Conclusions

In summary, a critical step in the probe development efforts summarized here that lead to the identification of the novel memory enhancer crebinostat was to demonstrate efficacy in neuronal cell-based assays that read out 1) HDAC-regulated acetylation; and 2) transcriptional processes that can be related to mechanisms implicated in learning and memory. In particular, acetylation of histone H4 lysine 12 (AcH4K12), CREB-dependent transcription, and *Egr1* expression all have been linked with synaptic plasticity and memory (Benito & Barco, 2010; Sakamoto et al., 2011; Malkani et al., 2004; Guan et al., 2009; Peleg et al., 2010; Fischer et al., 2010). With our ability to perform these assays in miniaturized forebrain and cortical primary neuronal cultures in 96-well plates, in combination with the use of *in vitro* enzymatic assays to assess potency toward recombinant HDACs that have been validated with other known memory enhancers targeting HDACs (Fass et al., 2010), we are now poised to screen new libraries of small molecules to identify novel HDAC inhibitors that enhance learning and memory. We expect that our future screening efforts will identify novel compounds with diverse patterns of HDAC isoform selectivity, potency, and structural properties that can be optimized further for *in vivo* use. A recent example of our efforts in this direction has been the identification of a novel class of macrocyclic HDAC inhibitors, from a screen of a stereochemically and skeletally diverse library of small molecules (Marcaurelle et al., 2010), that display complex mixed competitive/non-competitive enzyme inhibition kinetics, and that were capable of increasing AcH4K12 levels in our primary mouse neuronal culture. It is anticipated that the systematic testing of such small-molecule probes in our platform of cellular assays and *in vitro* enzymatic assays will lead to the discovery of a novel class of CREB-enhancing HDAC inhibitors, as well as small molecules targeting other epigenetic mechanisms, that will potentially be useful as therapeutics for neurodegenerative and neuropsychiatric diseases that involve dysregulated chromatin-mediated neuroplasticity.

Materials and Methods

CREB reporter gene assay

PC12 cells with a stably incorporated 6X-CRE-β-galactosidase CREB reporter gene (Meinkoth et al., 1990) were seeded into poly-D-lysine coated 384-well plates at a density of 25,000 cells per well. The following day, cells were treated with HDAC inhibitors (20 μM) in the absence or presence of forskolin (10 μM) for 4 hours. Cells were then processed for a β-galactosidase assay (Applied Biosystems Galacto-Star) according to the manufacturer's instructions, and luminescence was read on an Envision plate reader (Perkin-Elmer).

In Vitro HDAC enzymatic assays

Assays were run in 384-well plates with recombinant HDACs 1-9 (BPS Bioscience) under conditions appropriate for Michaelis-Menten kinetic analysis with the use of acetylated tripeptide substrates at K_m that are amide-coupled to 7-amino-4-methylcoumarin that can detect either class I/IIb (substrate MAZ1600) or class IIa/HDAC8 (substrate MAZ1675) HDAC activity exactly as described in Fass et al., 2010.

Neuronal histone acetylation assays

Mouse primary neuronal cultures were generated in factory precoated poly-D-lysine black/clear bottom 96-well plates (BD Biosciences #BD356692) treated overnight with 75 l/well of laminin [0.05 mg/ml] (Sigma #L2020) in PBS buffer. E17 embryonic mouse forebrain was dissociated into a single cell suspension by gentle trituration following trypsin/DNAse digestion (trypsin: Cellgro #25-052-Cl; DNAse: Sigma #D4527). Cells were plated at a density of 12,500 cells per well in 100 l of Neurobasal medium (Gibco #21103-049) containing 2% B27 (Gibco #17504-044), 1% Penicillin/Streptomycin/Glutamine (Cellgro #30-009-CI) and cultured at 37°C with 5% CO_2. After 13 days, cultures were treated with HDAC inhibitors by pin transfer of compound (185 nl per well) using a CyBi-Well vario pinning robot (CyBio Corp., Germany) and subsequently incubated for 24 hrs at 37°C with 5% CO_2. Cells were then fixed with 4% formaldehyde for 10 min. Following two washes with phosphate-buffered saline, cells were permeabilized and blocked with blocking buffer composed of 0.1% Triton X-100, 2% BSA in PBS. For immunofluorescence imaging of histone modifications, cells were stained with anti-acetyl-H3K9 (Millipore, cat# 07-352) or anti-Ac-H4K12 (Millipore, catalog #04-119) antibodies, and Alexa488-conjugated secondary antibody (Molecular Probes). Cellular nuclei were identified by staining with Hoechst 33342 (Invitrogen, H3570). Cell nuclei and histone acetylation signal intensity were detected and measured using a laser-scanning microcytometer (Acumen eX3, TTP Laptech). Acumen Explorer software was used to identify a threshold of histone acetylation signal intensity such that, in the absence of HDAC inhibitor, >99.5 % of cells had intensity levels below the threshold. In the presence of HDAC inhibitors, cells with histone acetylation signal intensities above the threshold were scored as "bright green nuclei". The percentage of nuclei scoring as bright green was quantified for each HDAC inhibitor. This percentage was then normalized to DMSO controls.

Western blotting of neuronal histone acetylation

Protein lysates were collected from mouse forebrain primary cultures grown *in vitro* for 13 days (DIV13) on poly-D-lysine/laminin-coated 6-well plates (2 million cells/well) and treated with DMSO or crebinostat (1 μM) for an additional 24 hours. Western blotting was performed using standard conditions and immunoblotting with a general anti-acetylated lysine antibody (Cell Signaling Technology, catalog #9814). Histone H2A, H2B, H3, and H4 bands were identified by molecular weight and comparison to staining patterns of antibodies for the individual histones.

Egr1 reporter gene assay

Assays were conducted on mouse cortex primary neuronal cultures from transgenic animals expressing an Egr1-EGFP reporter gene exactly as described in Guan et al., 2009.

NanoString nCounter gene expression assay

Mouse forebrain primary cultures, grown in poly-lysine- and laminin-coated 6-well plates, were treated for 24 hours with DMSO or crebinostat (1 μM) starting on DIV13. During the last 6 hours of this treatment, neuronal stimuli (or vehicle) were added at the following concentrations: forskolin (50 μM); KCl (55 mM); or BDNF (20 ng/ml). RNA was isolated with the RNeasy kit (Qiagen) according to the manufacturer's instructions. Triplicate samples were collected and analyzed using Nanostring nCounter Analysis System according to the manufacturer's instructions (http://www.nanostring.com/products/gene_expression.php). A custom CodeSet was designed to probe for 53 genes listed in Supplementary Table 5. See Supplementary Table 6 for this list of probes, as well as negative and positive control probes, contained in the CodeSet. Raw data were background subtracted using negative control probes, and then normalized to the average signal from the positive control probes.

Transcriptome profiling

Mouse forebrain primary cultures, grown in poly-lysine- and laminin-coated 6-well plates, were treated for 24 hours with DMSO or crebinostat (1 μM) starting on DIV13. RNA was isolated with the RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using ArrayScript (Ambion). Triplicate samples from two independent cultures (total n=6) were collected and analyzed using Illumina mouse whole-genome-6 microarrays. Total RNA from the samples was normalized to 20 ng/μl, and the Illumina TotalPrep-96 RNA Amplification Kit (Applied Biosystems, PN #4393543) protocol was used for amplification in a semi automated process. The total RNA underwent reverse transcription to synthesize first-strand cDNA. This cDNA was then converted into a double-stranded DNA template for transcription. *In vitro* transcription synthesized aRNA and incorporated a biotin-conjugated nucleotide. The aRNA was then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate. Labeled cRNA was normalized to 150 ng/μl and hybridized to Illumina's MouseWG-6 v2.0 Expression BeadChip. The labeled RNA strand was hybridized to the bead on the BeadChip containing the complementary gene-specific sequence. After a 16 hr hybridization, the beadchips were washed and stained using a Cy3 streptavidin conjugate. Illumina's BeadArray Reader was used to measure the fluorescence intensity at each addressed bead location. Raw data were annotated with Genome Studio (Illumina), and then quantile normalized and baseline transformed to the median of the DMSO control samples using GeneSpring software (Agilent). Probes failing to score as "present" in 100% of samples from at least one treatment condition (DMSO or crebinostat) were removed. A statistical threshold ($p < 0.05$, paired t-test with Benjamini-Hochberg multiple comparisons correction) and a fold change criterion (1.5) were then applied to generate lists of genes up- and down-regulated by crebinostat. All raw data will be deposited at NIH Gene Expression Omnibus (GEO) Database.

Protein-protein interaction network analysis

Mouse genes scoring in the Illumina microarray data as upregulated by crebinostat were cross-referenced to published lists of CREB target genes identified in ChIP-seq experiments (Kim et al., 2010; Martianov et al., 2010; MacGillavry et al., 2011). Synaptic CREB target genes were identified by cross-referencing published databases and literature identifying synaptic proteins (Kennedy et al., 2005; Collins et al., 2006; Pocklington et al., 2006; Waite

& Eickholt, 2010; Bayes et al., 2011). Gene symbols for all crebinostat-regulated synaptic CREB target genes were converted to human orthologs and loaded as nodes into the VisANT (http://visant.bu.edu/). Searches conducted with nodes consisting of the crebinostat up- and down-regulated synaptic CREB target genes produced the protein-interaction networks shown in Figure 5 C and D, respectively.

Quantitative RT-PCR assays of gene expression

Primary culture treatments, RNA isolation, and cDNA synthesis were performed as described for the Illumina microarray experiments. mRNA levels for crebinostat-regulated genes were measured by quantitative real-time PCR using TaqMan probe sets (Applied Biosystems) and normalized to levels of *Gapdh* mRNA.

High resolution neuronal histone acetylation and synapsin I punctae imaging

Mouse primary forebrain cultures were grown on glass coverslips coated with poly-lysine and laminin. Cultures were treated with DMSO or crebinostat (1 μM) for 24 hrs and then processed for immunofluorescence staining according to standard methods (4% formaldehyde fixation, 1 hour at 25°C), followed by staining with AcH4K12, Map2, and synapsin I antibodies (Millipore, catalog number 04-119; EnCor Biotechnology, catalog number CPCA-MAP2; Synaptic Systems, catalog number 106001) and standard fluorophore-coupled secondary antibodies. Fluorescent images were acquired on a Zeiss Axio Observer.Z1 microscope with a 63X objective. To quantify AcH4K12 fluorescence, signal intensity of 10-25 nuclei was measured from duplicate crebinostat or DMSO treatments in three independent cultures. To quantify synapsin I punctae, z-stacks (step size 0.5 m) were acquired from 15 sites on coverslips from three independent cultures, treated with DMSO or crebinostat, using a Leica confocal microscope with a 40X objective. Z-stacks were exported to ImageJ (http://rsbweb.nih.gov/ij/) and collapsed into single images for further processing. After applying thresholds delimiting specific signals (held constant over all images), Map2 staining was skeletonized, and total dendrite length was quantified as the total length of the skeletonized image. Next, Map2 staining was dilated to create a dendrite mask. Total size and intensity of synapsin I puntae staining contained within the dendrite mask were measured. The density of synapsin I punctae was determined as the ratio of number of synapsin I punctae over total dendrite length.

Animals

All mice used in this study for behavior were male C57BL/6J mice (9-10 weeks old) obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under 12:12 light/dark cycles, with food and water available ad libidum. Transgenic Tg(Egr1-EGFP)GO90Gsat mice containing the Egr1-EGFP bacterial artificial chromosome (BAC) reporter gene (BX1225) were obtained originally on an FVB/NTac background from the Jackson Laboratory (Bar Harbor, ME) and maintained through backcrossing to C57BL/6J mice. Positive mice were detected through EGFP fluorescence. Embryos of heterozygous mice were used for generating cortical neuron cultures. All procedures were performed in accordance with the Committee for Animal Care of the Division of Comparative Medicine at the Massachusetts Institute of Technology and with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals: Eighth Edition. Pregnant female C57BL6/J mice used for generating embryonic cultures of neurons for histone acetlyation and gene expression analysis were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under 12:12 light/dark cycles, with food and water available ad libidum. All procedures were performed in accordance with the Massachusetts General Hospital Subcommittee on Research Animal Care and with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals: Eighth Edition. All possible efforts were

made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

Compound administration

Crebinostat was administered in a vehicle consisting of 5% DMSO, 30% Cremophor, and 65% saline via daily intraperitoneal injections (25 mg/kg) for 10 days alternating daily between left and right sides of the abdomen prior to behavioral testing with an additional dose 1 hr after training on day 11.

Measurement of crebinostat concentration in the brain and plasma

A total 27 C57BL/6 mice were weighed before dose administration. Mice were dosed intraperitoneally with a solution formulation of crebinostat at 25 mg/kg dose. Blood samples (approximately 60 L) and tissues (brain) were collected from a set of three mice at pre determined time points (pre-dose, 0.08, 0.25, 0.5, 1, 2, 4, 8, and 24 hr). Immediately after collection of blood, brain tissues were collected from a set of three mice at the same time point. Plasma was harvested by centrifugation of blood and stored below −70°C prior to analysis. Brain samples were homogenized using ice-cold phosphate buffer saline (pH 7.4) and homogenates were stored below −70°C prior to analysis. Total homogenate volume was three times the tissue weight. Crebinostat was detected in plasma and brain homogenates by LC-MS/MS. The plasma and brain concentration-time data for crebinostat were used for the pharmacokinetic analysis. Brain concentrations were converted to ng/g from ng/mL considering total homogenate volume and brain weight where the density of brain was considered as 1, which is equivalent to plasma density. Pharmacokinetic analysis was performed using NCA module of WinNonlin Enterprise Version 5.2.

Behavioral procedures for contextual fear conditioning and open field locomotion

For contextual fear conditioning training, mice were placed in a TSE Multi-conditioning System training chamber (the context) for a period of 3 minutes followed by two foot shocks (2 seconds; 0.8 mA constant current) applied 30 seconds apart. After the last shock, the mice remained in the chamber for an additional 15 seconds before being returned to their home cage. The memory test was performed 24 h later by re-exposing the mice to the conditioning context for 3 minutes. Freezing, defined as the lack of movement (except for respiration) with an associated crouching posture, was recorded every 10s by a trained observer for 3 minutes (a total of 18 sampling intervals). Freezing was expressed as a percentage of the number of positive freezing observations divided by the total number of sampling intervals (18). Open field locomotion was the amount of horizontal movement (distance traveled in cm) in the training chamber recorded during the first three minutes of the contextual fear-conditioning training procedure, prior to shock administration, using the TSE Fear Conditioning software package.

Chemical synthesis of novel HDAC inhibitors

A library of compounds was made following a published procedure using hydrazone coupling chemistry (Vegas et al., 2007; Patel et al., 2009; Tang et al., 2011). HDAC inhibitor biasing elements coupled to 4, 5 or 6 carbon linkers ending in a hydrazone bond were reacted with aldehyde-containing caps (see scheme in Figure 1C). QC of the synthesized compounds was performed on a Waters 2545 HPLC equipped with a 2998 diode array detector, a Waters 3100 ESI-MS module, using a XTerraMS C18 5μm, 4.6×50 mm column at a flow rate of 5 ml/min with a linear gradient (95% A: 5% B to 100% B 90sec and 30sec hold at 100% B, solvent A = water + 0.1% formic acid, solvent B = acetonitrile + 0.1% formic acid).

Preparation of crebinostat (7-(2-([1,1′-biphenyl]-4-ylmethylene)hydrazinyl)-N-hydroxy-7-oxoheptanamide)

4-phenyl benzaldehyde (1.16 g, 6.34 mmol) was added to a solution of amine hydroxamic acid linker, 7-hydrazinyl-N-hydroxy-7-oxoheptanamide (1.0 g, 5.28 mmol) in methanol (25 ml) in a round bottom flask. A catalytic amount of difluoroacetic acid (20 μl) was added into the reaction mixture and the solution was stirred for 1 hr at room temperature. The solid precipitate was filtered out and purified by recrystallization from methanol to yield the desired product, crebinostat, as a white solid (1.232 g, 66% yield). Purity was checked by LC/MS and ^{1}H NMR spectra from Varian 400 MHz FT NMR (Supplementary Figure 2). ^{1}H NMR (400 MHz, DMSO-d6) δ ppm relative to TMS 8.67 (s, 1H, CH-benzylidenimin), 8.18 (s, 1H, NH-sec. amide), 8.00 (s, 1H, NH-sec. amide), 7.68-7.74 (m, 6H), 7.45-7.49 (t, 2H, *J* = 8 Hz), 7.36-7.39 (t, 1H, J = 6 Hz), 3.14-3.16 (bs, 2H), 2.48-2.49 (bs, 2H), 1.49-1.59 (m, 4H), 1.22-1.31 (bs, 2H).

Statistical analysis

Details on the specific statistical model used in each experiment can be found in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AcH4K12	acetylated histone H4 lysine 12
AcH3K9	acetylated histone lysine 9
Bdnf	(brain-derived neurotrophic factor)
CRE	cAMP response element
CREB	cAMP response element-binding protein
CBP	CREB binding protein
Egr1	early growth response 1
Grn	(granulin)
HDAC	histone deacetylase
Mapt	(tau)
SAHA	suberoylanilide hydroxamic acid

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Highlights

- Identified “crebinostat”, a novel HDAC inhibitor, in a CREB reporter gene screen.
- Crebinostat inhibits HDACs 1-3 and 6; and induces histone acetylation.
- Crebinostat regulates neuronal genes involved in synaptic function and cognition.
- Crebinostat enhances performance in fear conditioning learning in mice.

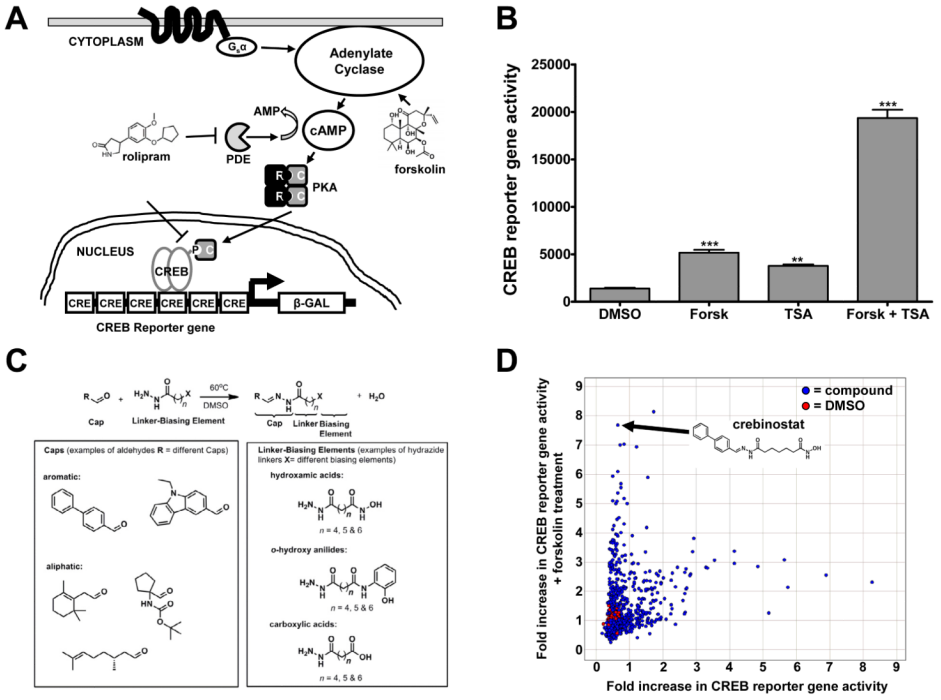

Figure 1. Discovery of crebinostat in a CREB reporter gene assay screen

(A) Prototypical pathway for regulation of the CREB reporter gene by cAMP. cAMP generated by adenylate cyclase induces protein kinase A (PKA) to phosphorylate and activate CREB. Phosphodiesterase (PDE) activity degrades cAMP and terminates activation of the pathway. This reporter gene has exquisite sensitivity to reveal the bioactivity of two well-known small molecule modulators of adenylate cyclase and PDE, respectively: forskolin and rolipram (Hagiwara et al., 1992; Xia et al., 2009). **(B)** Induction of CREB reporter gene activity measured through a β-galactosidase luminescence assay in a stable PC12 cell line by forskolin and trichostatin A (4 hr treatments) in cultures grown in 384-well microtiter plates. *** = $p < 0.0001$, ** = $p < 0.001$ (one-way ANOVA; post hoc tests with Bonferroni correction). **(C)** Synthetic strategy for generation of a library of novel compounds structurally biased towards inhibition of HDACs. Cap and linker-biasing element building blocks were mixed in DMSO and incubated at 60°C for 12 hours to promote coupling to form the final novel compounds as products. The lower boxes show examples of both types of building blocks used in the library. **(D)** Novel HDAC inhibitor compounds were tested (20 μM; 4 hrs treatment; n=2), in the presence of forskolin (10 μM) using CREB reporter PC12 cells. Levels of compound-induced activity were normalized to DMSO in the absence or presence of forskolin. Inset shows location of crebinostat in the primary CREB reporter gene screen with its strong activation of the reporter only in the presence of forskolin.

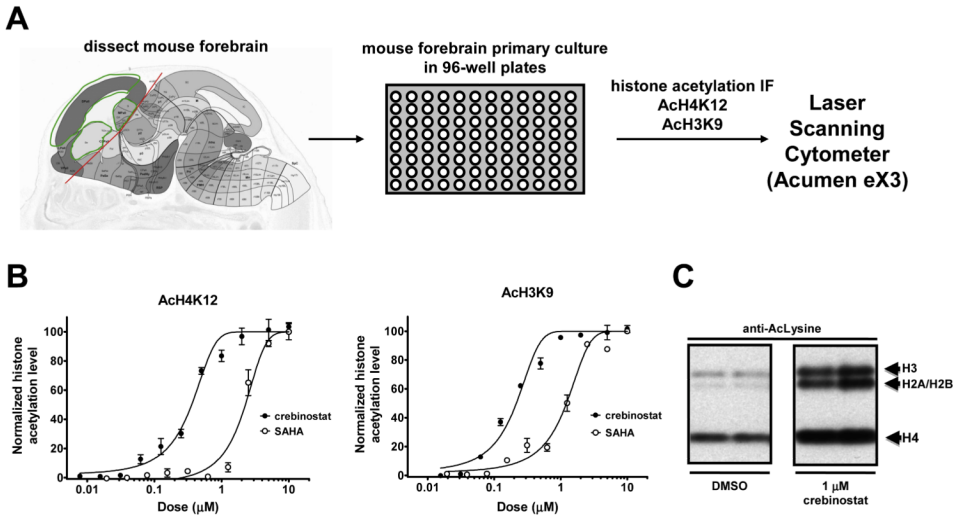

Figure 2. Crebinostat potently increases histone acetylation in neurons

(A) High-throughput neuronal histone acetylation immunofluorescence assay. Mouse forebrain (image from the Allen Brain Atlas—www.brain-map.org) primary cultures were grown in 96-well plates, treated with crebinostat or DMSO for 24 hrs, and then fixed and stained for AcH4K12 or AcH3K9 according to standard immunofluorescence methods. Histone acetylation signals were detected using laser scanning cytometer. **(B)** Dose response plots for induction of histone acetylation in cultured primary mouse neurons by crebinostat and SAHA for 24 hrs. EC_{50} values were 0.29 μM and 1.9 μM (AcH4K12) and 0.18 μM and 1.0 μM (AcH3K9) for crebinostat and SAHA, respectively. **(C)** Western blot analysis of chromatin extracted from cultured primary mouse neurons treated with crebinostat (1 μM) for 24 hrs using a general anti-acetylated lysine antibody that recognizes acetylated histone H3, H4, H2A, and H2B. Crebinostat strongly increases the acetylation of all four major components of nucleosomes in neurons.

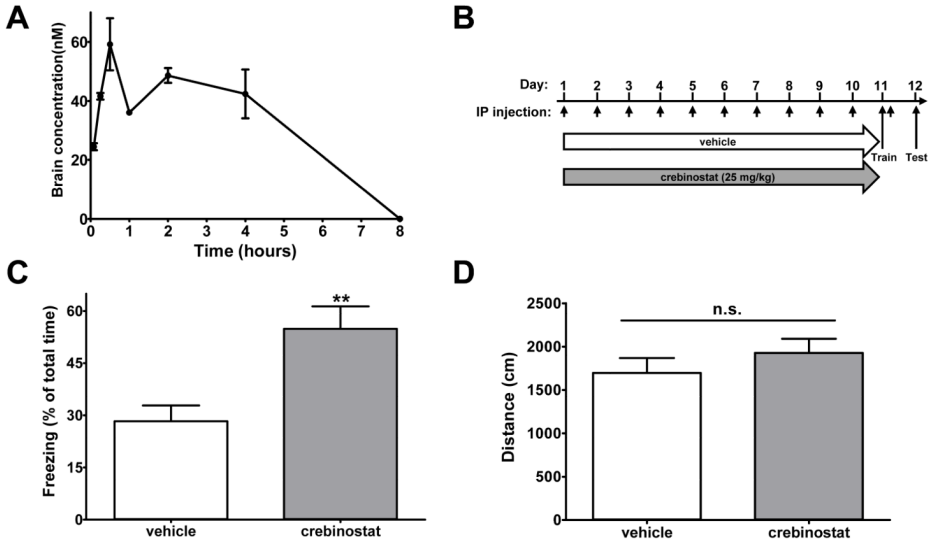

Figure 3. Crebinostat enhances memory of contextual fear conditioning in mice

(A) Concentration of crebinostat in the brain following IP injection. **(B)** Timeline of contextual fear conditioning paradigm. Intraperitoneal injections of vehicle or crebinostat (25 mg/kg) were given once daily for 10 days. On day 11, animals were trained in the contextual fear conditioning paradigm and then given one more injection one hour after the training. Animals were then tested for conditioned "freezing" behavior 24 hrs later on day 12 by re-exposure to the conditioning context. **(C)** Quantification of freezing behavior for vehicle or crebinostat-treated mice. Data are mean percent freezing; n = 10 animals per group. *** = $p < 0.001$ (unpaired t-test). **(D)** Quantification of exploratory open field locomotion (horizontal movement) during the three minutes immediately prior to the footshock. n.s. = not statistically significant (unpaired t-test). Data shown are from one representative experiment; these results were reproduced in three separate experiments analyzed independently by two trained experts.

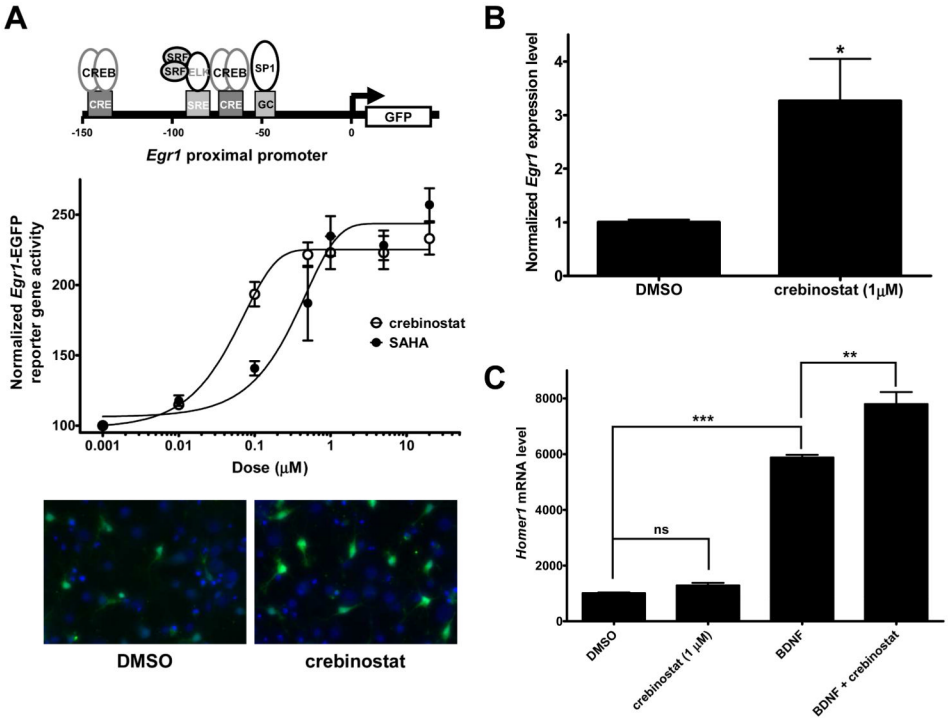

Figure 4. Crebinostat induces the immediate early gene *Egr1*

(A) *Upper panel*: Neuronal Egr1-EGFP reporter gene. *Egr1* responds robustly and rapidly to a variety of neuronal signaling events via the action of kinase cascades on three signal-responsive transcription factors that bind to its proximal promoter: CREB, SRF/ELK1 ternary complex (serum response factor/Ets-like transcription factor 1); and SP1 (specificity protein 1). *Lower panel*: Activation of the Egr1-EGFP reporter gene by crebinostat and SAHA in primary cultured cortical neurons. Cortical neurons were treated for 24 hours at the indicated doses**.** The graph illustrates the dose response for induction of EGFP fluorescence by crebinostat and SAHA (mean +/- standard deviation). **(B)** Representative images of neuronal EGFP fluorescence (20X magnification) from DMSO and crebinostat treated cultures. **(C)** Induction of endogenous *Egr1* mRNA in mouse forebrain primary cultures by 24 hrs treatment with crebinostat. *Egr1* mRNA levels measured by RT-PCR were normalized to *Gapdh* mRNA levels and then plotted as fold change versus the DMSO condition. * = $p < 0.05$ (unpaired t-test).

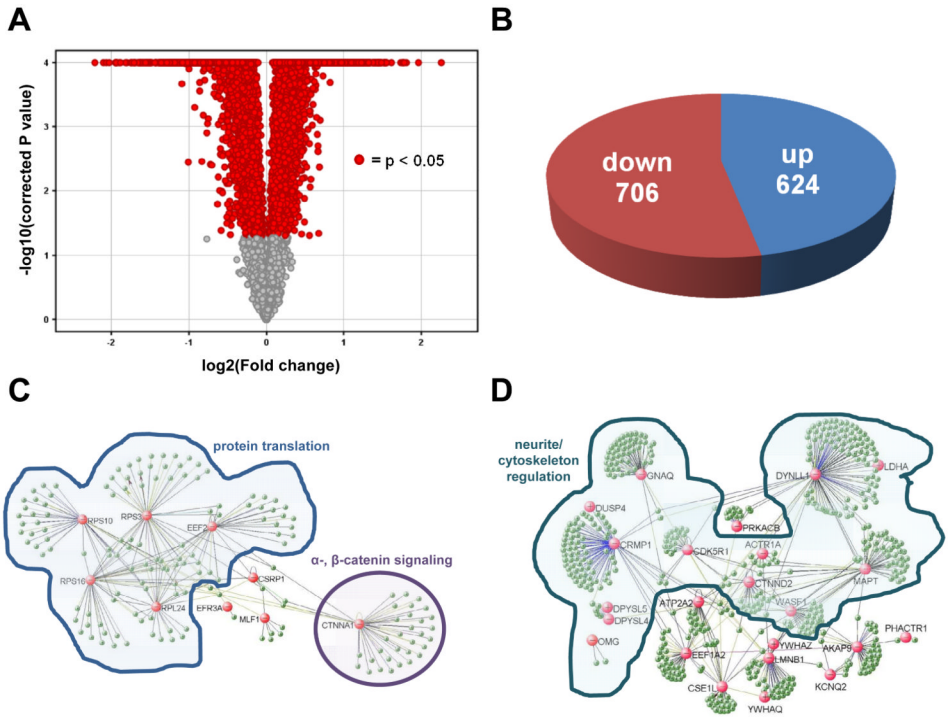

Figure 5. Regulation of the neuronal transcriptome by crebinostat treatment

(A) Volcano plot of normalized Illumina microarray data from RNA samples made from mouse forebrain primary cultured neurons treated for 24 hrs with DMSO vehicle or crebinostat (1 μM). Upregulated or downregulated transcripts meeting a statistical significance threshold ($p < 0.05$) are indicated in red. **(B)** Pie chart reflecting the total number of transcripts up- or down-regulated by crebinostat. **(C and D)** Protein-protein interaction network reveals connectivity of synaptic CREB target genes up- or down-regulated by crebinostat. Nodes (circles) represent proteins encoded by the crebinostat regulated genes and edges (lines) connecting nodes represent known protein-protein interactions. Modules consisting of nodes with related functions are circled.

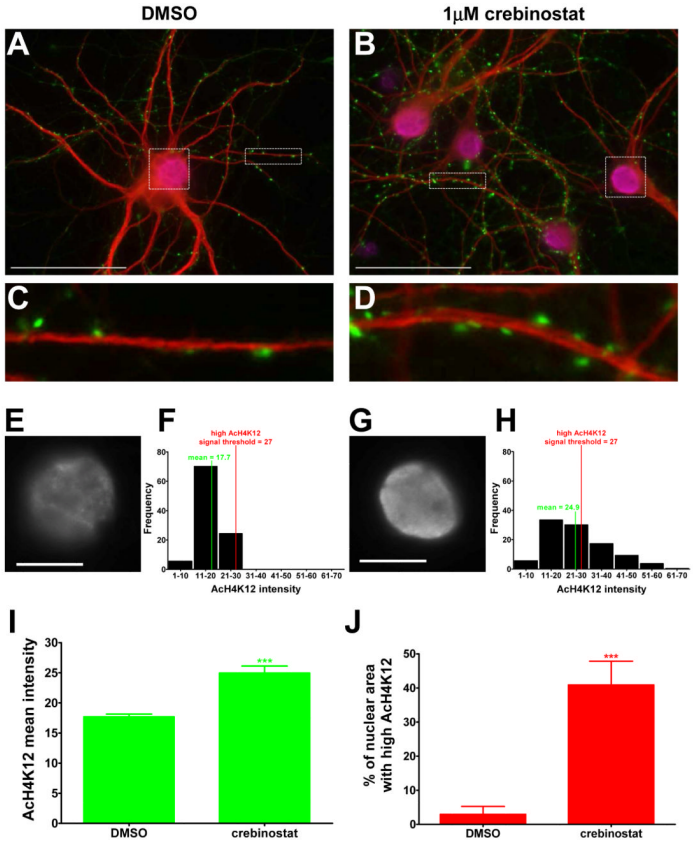

Figure 6. Crebinostat increases histone acetylation and synapsin I punctae along dendrites in primary cultured neurons

Immunofluorescence staining of neurons treated for 24 hrs with DMSO as a vehicle **(A, C, E)** or crebinostat (1 μM) **(B, D, G)** for a dendrite marker (Map2, in red); acetyl-histone H4 lysine 12 (magenta); and synapsin I (green). Scale bar = 50 μm. **(C, D)** Enlarged view from representative images of dendrites with synapsin I punctae. **(E, G)** Enlarged nuclei stained for acetyl-histone H4 lysine 12 (AcH4K12). Scale bar = 10 μm. **(F, H)** Histogram showing the frequency distribution of average acetyl-histone H4 lysine 12 (AcH4K12) fluorescence intensity levels in nuclei. N = 10 (DMSO) or 28 (1 μM crebinostat). Green bar = mean intensity; red bar = threshold chosen to define “high” acetylation levels. **(I)** Quantification of mean nuclear acetyl-histone H4, lysine12 fluorescence intensity levels after 24 hrs DMSO or crebinostat treatment. **(J)** Quantification of percentage of nuclear area with high levels of acetyl-histone H4, lysine12 signal. A nuclear pixel was defined as having a “high” level of acetylation if it had a signal intensity ≥ a threshold of 27 (arbitrary units; indicated by red line in panels **F** and **H**). *** = $p < 0.001$ (unpaired t-test).

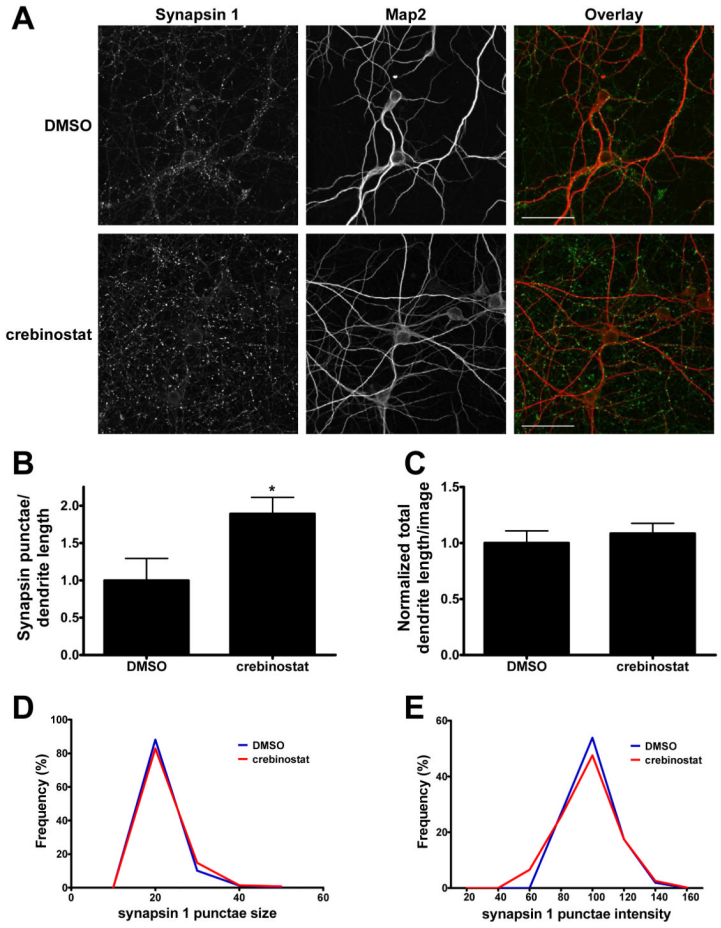

Figure 7. Crebinostat increases synapsin I punctae density along dendrites

(A) Representative immunofluorescence images of neurons stained for the dendrite marker Map2 and synapsin I following 24 hr treatments with DMSO or crebinostat (1 μM). The right panel shows the overlay of the two images (Map2 in red; synapsin I in green). Scale bar = 50 μm. ImageJ analysis was used to quantify the density of synapsin I punctae along Map2-positive dendrites **(B)**, the total amount of Map2-positive dendritic length per field (* = $p < 0.05$; unpaired t-test) **(C)**, the frequency distribution of the size of synapsin I punctae **(D)**, and the frequency distribution of the mean signal intensity for synapsin I staining in punctae **(E)**. 15 fields from three independent cultures were analyzed for each graph.

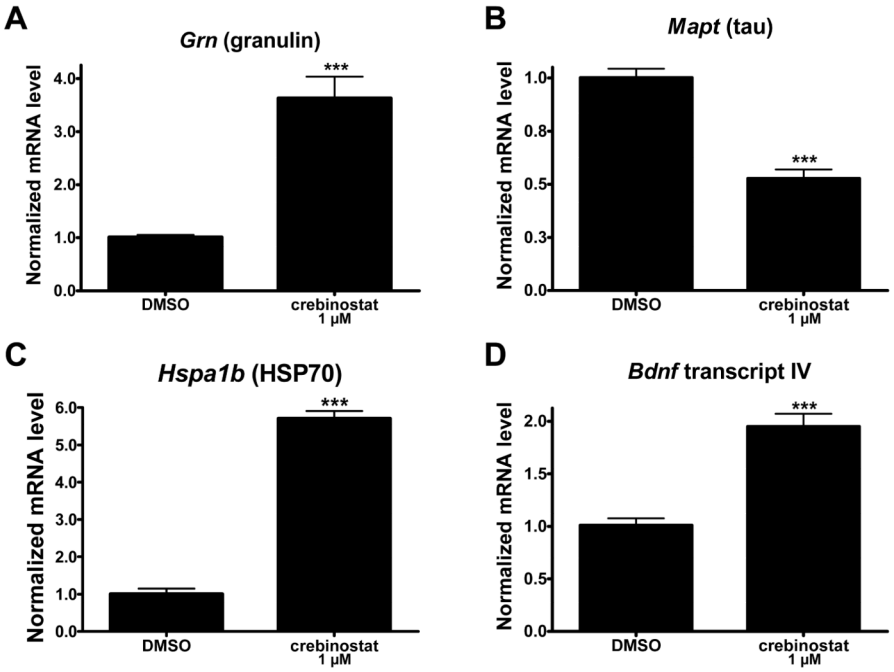

Figure 8. Regulation of granulin, tau, Hsp70, and BDNF gene expression by crebinostat

Primary cultured mouse forebrain neurons were treated for 24 hours with DMSO or crebinostat (1 μM). **A**) *Grn* (granulin), **B**) *Mapt* (tau), **C**) *Hspa1b (Hsp70)*, and **D**) *Bdnf* transcript IV mRNA levels were measured using TaqMan real time PCR assays and normalized to *Gapdh* mRNA levels. *** = $p < 0.001$ (unpaired t-test).

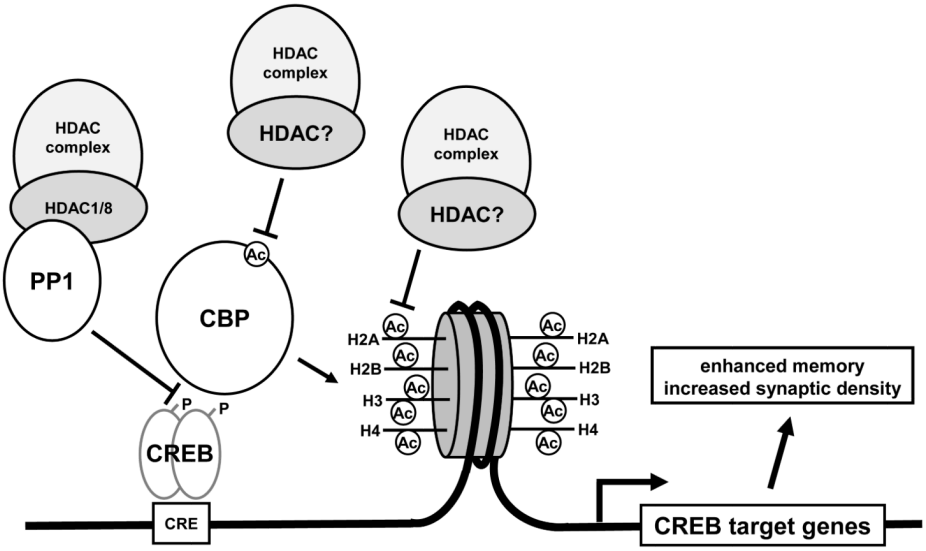

Figure 9. Potential models of regulation of CREB-dependent transcription, synaptic function, and memory by HDACs

"Ac" marks acetylation events reported in the literature that are likely to be key control points for CREB activity. HDACs may regulate CREB-dependent transcription by reversing any of these acetylation events. CBP = CREB-binding protein; PP1 = protein phosphatase 1; H2A, H2B, H3, and H4 = histone protein N-terminal tails.

Table 1

In vitro potency of crebinostat and SAHA[a]

	Class I				Class IIa				Class IIb
Compound	**HDAC1**	**HDAC2**	**HDAC3**	**HDAC8**	**HDAC4**	**HDAC5**	**HDAC7**	**HDAC9**	**HDAC6**
SAHA	7.0 ± 1.1	10.2 ± 1.1	21 ± 2	1155 ±290	>2000	>2000	>2000	>2000	15.3 ± 1.3
crebinostat	0.7 ± 0.04	1.0 ± 0.08	2.0 ± 0.3	> 2000	>2000	>2000	>2000	>2000	9.3 ± 0.6

[a] IC_{50} (nM) values for inhibition of enzymatic activity of HDACs 1-9. Data from two experiments (each performed in duplicate) are shown as mean ± S.D.

Table 2

CREB-target genes upregulated by crebinostat.

Gene	Fold upregulation (Illumina array)	Fold upregulation (RT-PCR)	CREB target gene reference	Function (reference)
Egr1	-	3.9	Kim et al., 2010	inducible transcription factor (Malkani et al., 2004
Sdcbp	2.2	2.3	Martianov et al., 2010	synapse formation in development (Hirbec et al., 2005)
Sema5a	2.1	3.6	Martianov et al., 2010	axon guidance (Kantor et al., 2004)
Aldh2	2.0	2.3	MacGillavry et al., 2011	neuronal mitochondrial detoxification (Ohsawa et al., 2008)
Pik3c3	1.6	2.2	Martianov et al., 2010	dendritic spine stability (Wang et al., 2011)
Fzd5	1.6	5.2	Martianov et al., 2010	Wnt-mediated synaptogenesis (Sahores et al., 2010)