

Hippocampal Focal Knockout of CBP Affects Specific Histone Modifications, Long-Term Potentiation, and Long-Term Memory

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To identify the role of the histone acetyltransferase (HAT) CREB-binding protein (CBP) in neurons of the CA1 region of the hippocampus during memory formation, we examine the effects of a focal homozygous knockout of CBP on histone modifications, gene expression, synaptic plasticity, and long-term memory. We show that CBP is critical for the *in vivo* acetylation of lysines on histones H2B, H3, and H4. CBP's homolog p300 was unable to compensate for the loss of CBP. Neurons lacking CBP maintained phosphorylation of the transcription factor CREB, yet failed to activate CREB:CBP-mediated gene expression. Loss of CBP in dorsal CA1 of the hippocampus resulted in selective impairments to long-term potentiation and long-term memory for contextual fear and object recognition. Together, these results suggest a necessary role for specific chromatin modifications, selectively mediated by CBP in the consolidation of memories. *Neuropsychopharmacology* (2011) **36**, 1545–1556; doi:10.1038/npp.2011.61; published online 20 April 2011

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INTRODUCTION

Chromatin, the complex of DNA and associated proteins, is a physical barrier to transcription mechanisms. The manipulation of chromatin is critically involved in regulating gene expression for a number of neuronal processes, including the formation of long-term memory (Barrett and Wood, 2008). Chromatin-modifying complexes, which contain histone-modifying enzymes, regulate access to the underlying genomic DNA by relaxing chromatin structure and providing docking sites for additional regulatory factors (Kouzarides, 2007). The enzymes that regulate levels of histone acetylation are histone acetyltransferases (HATs) and histone deacetylases (HDACs). One such enzyme, the HAT CREB-binding protein (CBP) is the best studied of the three HATs implicated in the regulation of transcription during memory and synaptic plasticity (reviewed in Barrett and Wood, 2008).

To date, six different types of *Cbp* mutant mice have been generated to examine the role of CBP in memory (Oike *et al*, 1999; Bourtchouladze *et al*, 2003; Alarcon *et al*, 2004; Korzus *et al*, 2004; Wood *et al*, 2005, 2006; Chen *et al*, 2010). However, none of these *Cbp* genetically modified mice were designed to target a single brain region and only one generated a complete knockout of CBP, as a homozygous knockout of *Cbp* results in embryonic lethality (Tanaka *et al*, 1997). These mice, therefore, may have phenotypes reflecting residual CBP or compensatory mechanisms. To overcome current limitations, we have examined *Cbp*^{lox/flox} mice, in which a focal deletion of *Cbp* can be generated by Cre recombinase introduced by adeno-associated virus (AAV-Cre). This approach allows us to generate a homozygous knockout of *Cbp* and limit the deletion to a specific brain region. Further, AAV-Cre can be introduced into adult mice, which limits potential developmental compensation, either pre- or postnatal, that may be observed if the deletion was induced earlier in life, such as in Cre-expressing mouse lines.

This approach allowed us to address key open questions regarding the role of CBP in histone acetylation and long-term memory. For example, *in vitro* and cell culture studies indicate that other HATs, such as p300, can acetylate many of the same residues as CBP. However, whether these HATs can compensate at specific residues in the brain for

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a lack of CBP is unknown. We also examined whether CREB phosphorylation can be uncoupled from CREB:CBP-mediated gene expression *in vivo*, as has been shown in cell culture (Chawla *et al*, 1998; Hardingham *et al*, 1999). Lastly, the effect of a homozygous deletion of *Cbp* on long-term potentiation (LTP) has never been examined. In this study, we addressed these questions and found that homozygous deletion of *Cbp* reveals a role for CBP in the regulation of a specific profile of histone modifications, hippocampal LTP, and hippocampus-dependent long-term memory. These findings provide new insight into CBP's *in vivo* role in the regulation of histone modification and gene expression required for long-term memory processes.

METHODS

Subjects and Surgical Procedures

CBP conditional knockout mice (*Cbp*^{flx/flx}) were generated as described in detail in Kang-Decker *et al* (2004) and are maintained on a C57BL/6 background. Cre recombinase can be introduced to delete the sequence between the loxP sites, producing deletion of CBP (Kang-Decker *et al*, 2004; Kasper *et al*, 2006; Xu *et al*, 2006). At 2 weeks before the experiments, mice were anesthetized using isoflurane and infused with adeno-associated virus expressing Cre-recombinase (AAV-Cre), either serotype AAV2/2 (made in the lab of RWG), or AAV2/1 (purchased from Penn Vector Core; University of Pennsylvania; Kaspar *et al*, 2002). The infusion cannula was positioned over the hippocampus (AP -2.0 mm; ML ± 1.5 mm from bregma) and lowered 0.2 mm/15 s to a depth of -1.5 mm (DV from bregma). It was allowed to sit for 2 min and then 1.0 µl of virus was injected at a rate of 6 µl/h and allowed to diffuse for 2 min. The cannula was removed 0.2 mm initially, followed by a 1 min pause, and then 0.1 mm/15 s. All deletions were confirmed by immunohistochemistry (as described below). Mice were 8–12 weeks old and had free access to food and water in their home cages. Lights were maintained on a 12:12 h light/dark cycle, with all behavioral testing performed during the light portion of the cycle. All experiments were conducted according to National Institutes of Health guidelines for animal care and use and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Immunohistochemistry

Tissue preparation and immunohistochemical analysis was performed as described previously (Vecsey *et al*, 2007; Malvaez *et al*, 2010). Briefly, coronal sections of paraformaldehyde-fixed, cryoprotected brains were cut with a cryostat at a thickness of 20 µm at the level of the hippocampus and collected in PBS.

The following primary antibodies were used: anti-CBP (C-20; 1:1000), and anti-p300 (C-20; 1:1000) antibodies were purchased from Santa Cruz Biotechnology. Anti-H3K14ac (1:1000), anti-H4K12ac (1:500), and anti-phospho-CREB (1:1000) antibodies were purchased from Millipore. Anti-H4K8ac (1:1000) was purchased from Cell Signaling. Anti-H2BK12ac (1:500) was purchased from Abcam.

Floating sections were blocked for 1 h at room temperature in 8% normal goat serum (NGS, Jackson Immuno-Research Laboratories) with 0.3% Triton X-100 in PBS and then incubated overnight at 4°C in 2% NGS, 0.3% Triton X-100 in PBS with primary antibody. The sections were then incubated for 2 h at room temperature with goat anti-rabbit IgG-FITC (1:1000, Chemicon International). All sections were washed three times for 5 min each in PBS before and after each incubation step and mounted on slides using ProLong Gold antifade reagent with DAPI (Invitrogen).

All images were acquired using an Olympus (BX51, Japan) microscope using a ×4 or ×20 objective, CCD camera (QImaging), QCapture Pro 6.0 software (QImaging), combined with ImageJ software (NIH).

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed to examine *c-fos* expression. Tissue was collected by taking 1 mm punches from dorsal hippocampal slices in the area of the focal deletion in *Cbp*^{flx/flx} mice as confirmed by immunohistochemistry for CBP and equivalent regions in *Cbp*^{+/+} mice. RNA was isolated using RNeasy minikit (Qiagen, Carlsbad, CA). cDNA was made from 200 ng total RNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Sciences). Primers were derived from the Roche Universal ProbeLibrary: *c-fos* left primer 5'-ggggcaaa gtagagcagcta-3'; *c-fos* right primer 5'-agctccctcctccgattc-3'; probe, 5'-atggctgc-3'; *Cbp* left primer 5'-caggcaggtgtttcac agg-3'; *Cbp* right primer 5'-gcatgttcagagggttaggg-3'; probe, 5'-cctggagc-3'. The *c-fos* and *Cbp* probes are conjugated to the dye FAM. GAPDH left primer 5'-atggtaaggtcgggtgta-3'; right primer 5'-aatcctcactttgccactgc-3'; probe 5'-tggcggta ttgg-3'; GAPDH probe is conjugated to Lightcycler Yellow 555. The non-overlapping dyes and quencher on the reference gene allow for multiplexing in the Roche Light-Cycle 480 II machine (Roche Applied Sciences). All values were normalized to GAPDH expression levels. Analysis and statistics were performed using the Roche proprietary algorithms and REST 2009 software based on the Pfaffl method (Pfaffl, 2001, Pfaffl *et al*, 2002).

Contextual Fear Conditioning

Training and testing took place in four PhenoTyper conditioning chambers as previously described (Lattal *et al*, 2007). Mice were placed into the conditioning chamber and received a 2 s, 0.75 mA scrambled foot shock at 2.5 min after placement into the chamber. Mice were removed from the chamber after 3 min. During testing, mice received one 5 min exposure to the conditioned context in the absence of shock 1 h or 24 h after conditioning. Conditioning was assayed by measuring freezing behavior, the complete absence of movement (Fanselow, 1980). Freezing was scored during conditioning as well as testing.

Object Recognition

Training and testing for location-dependent object recognition (Rooszendaal *et al*, 2010) and novel object recognition

(Mumby *et al*, 2002, 2005; Winters *et al*, 2004; Balderas *et al*, 2008) was carried out as previously described (Rooszendaal *et al*, 2010; Stefanko *et al*, 2009). Briefly, before training, mice were handled 1–2 min for 5 days and then habituated to the experimental apparatus (white rectangular open field, 30 × 23 × 21.5 cm) 5 min a day for 4 consecutive days in the absence of objects. During training, mice were placed into the experimental apparatus with two identical objects (A1 and A2; either 100 ml beakers, 2.5 cm diameter, 4 cm height; or large blue Lego blocks, 2.5 × 2.5 × 5 cm), and were allowed to explore for 10 min or 3 min (Stefanko *et al*, 2009). During the retention test, (90 min for short-term memory or 24 h for long-term memory), mice explored the experimental apparatus for 5 min. For the object recognition memory test (ORM), one familiar object (A3) and one novel object (B1) were placed in the same location as during training. For the object location memory test (OLM), one familiar object (A3) was placed in the middle of the box (a novel location) and another familiar object (A4) was placed in the same location as during training. All combinations and locations of objects were used in a balanced manner to reduce potential biases due to preference for particular locations or objects.

Slice Preparation and Recording

Hippocampal slices were prepared from *Cbp^{fllox/fllox}* and *Cbp^{+/+}* mice (approximately at 2 months of age). Following isoflurane anesthesia, mice were decapitated, and the brain was quickly removed and submerged in ice-cold, oxygenated dissection medium containing (mM): 124 NaCl, 3 KCl, 1.25 KH₂PO₄, 5 MgSO₄, 0 CaCl₂, 26 NaHCO₃, and 10 glucose. Transverse hippocampal slices (300 μm) through the mid-third of the septotemporal axis of the hippocampus were prepared using a Leica vibrating tissue slicer (Model:VT1000S) before being transferred to an interface recording containing preheated artificial cerebrospinal fluid of the following composition (mM): 124 NaCl, 3 KCl, 1.25 KH₂PO₄, 1.5 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃, and 10 glucose and maintained at 31 ± 1 °C. Slices were continuously perfused with this solution at a rate of 1.75–2 ml/min, whereas the surface of the slices were exposed to warm, humidified 95% O₂/5% CO₂. Recordings began following at least 2 h of incubation.

Field excitatory postsynaptic potentials were recorded from CA1b stratum radiatum using a single glass pipette (2–3 MΩ). Bipolar stainless steel stimulation electrodes (25 μm diameter, FHC) were positioned at two sites (CA1a and CA1c) in the apical Schaffer collateral–commissural projections to provide activation of separate converging pathways of CA1b pyramidal cells. Pulses were administered in an alternating manner to the two electrodes at 0.05 Hz using a current that elicited a 50% maximal response. After establishing a 10–20 min stable baseline, LTP induced by delivering 5 or 10 ‘θ’ bursts, with each burst consisting of four pulses at 100 Hz and the bursts themselves separated by 200 ms (ie, θ-burst stimulation or TBS). The stimulation intensity was not increased during TBS. Data were collected and digitized by NAC 2.0 Neurodata Acquisition System (Theta Burst Corporation, Irvine, CA) and stored on a disk.

RESULTS

Generation of Focal Homozygous *Cbp* Deletion Using *Cbp^{fllox/fllox}* Mice and AAV-Cre

To generate a homozygous gene deletion of *Cbp*, we used mice carrying loxP sites flanking exon 9 of *Cbp* (*Cbp^{fllox/fllox}* mice; Kang-Decker *et al*, 2004), in which Cre recombinase excises exon 9 of *Cbp*. Cre recombinase was delivered via adeno-associated virus (referred to as AAV-Cre). *Cbp^{fllox/fllox}* mice have previously been shown to be indistinguishable from their wild-type littermates and to have a normal lifespan (Kang-Decker *et al*, 2004). We find no difference in CBP protein or mRNA in *Cbp^{fllox/fllox}* mice not exposed to AAV-Cre (Supplementary Figure S1A, B). *Cbp^{fllox/fllox}* and *Cbp^{+/+}* mice were administered intrahippocampal infusions of AAV-Cre (1 μl per side) serotype 2/2 (AAV2/2-Cre) or 2/1 (AAV2/1-Cre) to create a small (Figure 1a and b) and a large (Figure 1c and d) hippocampal CBP deletion, respectively. These serotypes, while having the same viral genome, have different coat proteins that allow them to transduce cells with different efficiencies (Burger *et al*, 2004) and therefore allow us to create deletions of CBP affecting more or less of the hippocampus. Both serotypes transduce neurons at a higher efficiency than other cell types (Burger *et al*, 2004). On the basis of the finding by Scammell *et al* (2003), showing that by 2 weeks post-AAV-Cre injection, a floxed gene can be deleted in the hippocampus, we chose to examine mice at 2 weeks post injection. We observed a complete deletion of CBP at every time point examined (at 2 weeks and at 1 month post-AAV-Cre infusion shown in Supplementary Figure S1C) and all behavior and immunohistochemistry was carried out between 2 and 6 weeks following AAV-Cre infusion. All deletions were verified with immunohistochemistry following behavior.

AAV2/2-Cre created a deletion limited to a portion of dorsal CA1 and did not affect the cortex. AAV2/1-Cre created a deletion that encompassed dorsal CA1, CA3, and the dentate gyrus, as well as more ventral CA1 and affected the region of the cortex closest to the deletion in the hippocampus (Supplementary Figure S1). This deletion does not induce cell death, indicated by the fact that nuclei are intact, as demonstrated by DAPI staining (Figure 1). Further, there is no indication of cell death by Fluoro-Jade B staining (Schmued and Hopkins, 2000a, b; data not shown). AAV does not lead to an immune response or impair basal synaptic transmission (Scammell *et al*, 2003; see also Figure 4).

p300 is considered a functional homolog of CBP because of its high sequence similarity with CBP (Eckner *et al*, 1994) and overlapping interaction partners (Arany *et al*, 1995; Lundblad *et al*, 1995). p300 therefore may be expected to compensate for a lack of CBP. However, we observed no change in p300 in the region of CBP deletion (Figure 1) and therefore, similarly to findings in other CBP mutant mice (Kasper *et al*, 2006; Chen *et al*, 2010), it does not appear that p300 is being upregulated to compensate for a loss of CBP.

CBP is Involved in the Acetylation of Residues on Histones H2B, H3, and H4 in the Dorsal CA1

CBP has been shown *in vitro* to acetylate a number of histone residues involved in transcriptional regulation

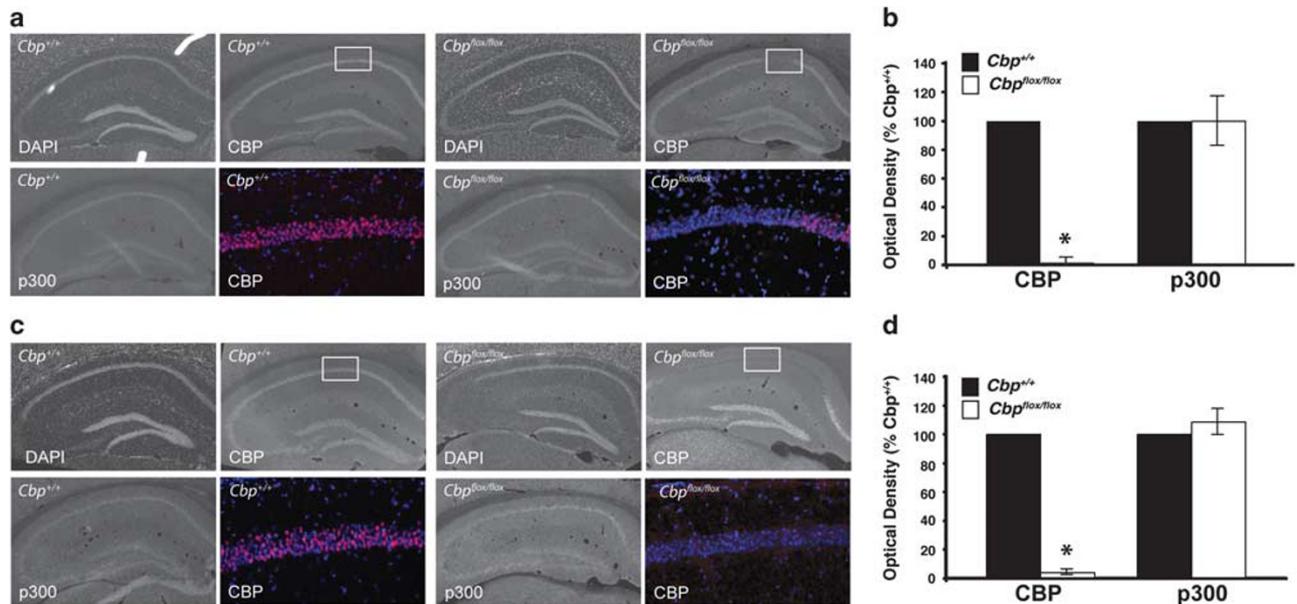


Figure 1 AAV-Cre infusion in $Cbp^{flox/flox}$ mice induces a focal loss of CBP, but no change in expression of CBP homolog p300. Perfusion-fixed immunostained coronal brain slices were prepared from $Cbp^{flox/flox}$ and $Cbp^{+/+}$ mice at 3–6 weeks following intrahippocampal infusions of AAV-Cre, either serotype 2/2 or 2/1 (AAV2/2-Cre or AAV2/1-Cre). Quantification of immunostaining is shown on right. (a) Representative images showing DAPI labeling and CBP and p300 immunoreactivity in hippocampi of AAV2/2-Cre infused $Cbp^{+/+}$ and $Cbp^{flox/flox}$ mice. The color images are $\times 20$ magnifications of the regions boxed in white, labeled with DAPI (blue), and CBP (pink). The $Cbp^{+/+}$ mice displayed expression of CBP throughout CA1, CA3, and the dentate gyrus. However the $Cbp^{flox/flox}$ mice display a focal loss of CBP in area CA1 of the hippocampus, while exhibiting no difference in p300 expression in this region. (b) Quantification of CBP and p300 optical density. (c) Representative images showing DAPI labeling and CBP and p300 immunoreactivity in hippocampi of AAV2/1-Cre infused $Cbp^{+/+}$ and $Cbp^{flox/flox}$ mice. The color images are $\times 20$ magnifications of the regions boxed in white, labeled with DAPI (blue), and CBP (pink). The $Cbp^{+/+}$ mice displayed expression of CBP throughout CA1, CA3, and the dentate gyrus. However the $Cbp^{flox/flox}$ mice display a loss of CBP, while exhibiting no difference in p300 expression in this region. (d) Quantification of CBP and p300 optical density. * $p < 0.05$.

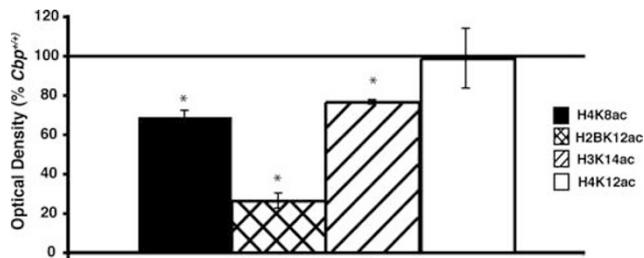


Figure 2 Focal homozygous gene deletion of Cbp results in altered histone acetylation. Hippocampal sections from $Cbp^{flox/flox}$ and $Cbp^{+/+}$ mice infused with AAV2/1-Cre adjacent to sections immunolabeled for CBP were immunolabeled for H4K8ac, H2BK12ac, H3K14ac, and H4K12ac. Optical density of $\times 20$ magnifications of area CA1 of the hippocampus was quantified. * $p < 0.05$.

(Ogryzko *et al*, 1996; Kouzarides, 2007). We therefore examined the role of CBP in histone acetylation *in vivo* by assessing acetylation of specific histone residues in neurons lacking CBP. Acetylation of H3K14, H4K8, and H4K12 has been shown to correlate with learning (Levenson *et al*, 2004; Chwang *et al*, 2006, 2007; Peleg *et al*, 2010). Lysine 12 on H2B, lysine 14 on H3, lysine 8 on H4, known targets of CBP (Kouzarides, 2007), were all significantly reduced in the dorsal CA1 region of the hippocampus of $Cbp^{flox/flox}$ mice compared with $Cbp^{+/+}$ mice (Figure 2). Lysine 12 on H4 is not known to be a target of CBP (Kouzarides, 2007) and was not different between $Cbp^{flox/flox}$ mice and $Cbp^{+/+}$ mice (Figure 2). These results suggest that *in vivo*, in the

hippocampus, CBP is critical for acetylation of specific histone residues, and that p300 is unable to fully compensate for the loss of CBP.

CREB Phosphorylation is Uncoupled From CREB:CBP-Mediated Gene Expression *In Vivo*

To determine whether signaling upstream of CBP and histone acetylation was affected by the deletion of CBP, we examined phosphorylation of CREB in $Cbp^{flox/flox}$ and $Cbp^{+/+}$ mice 10 min following fear conditioning. This time point was chosen based on studies showing maximal phospho-CREB following fear conditioning in the hippocampus (Stanciu *et al*, 2001). $Cbp^{flox/flox}$ and $Cbp^{+/+}$ mice received bilateral intrahippocampal AAV2/2-Cre infusions and 2 weeks later were trained in the contextual fear conditioning paradigm. At 10 min following training, mice were perfused. We observed no change in CREB phosphorylation (Figure 3a and b), indicating that signaling upstream of CBP is intact.

To determine whether CREB:CBP-mediated target gene expression is affected by the CBP deletion, we examined *c-fos* expression in $Cbp^{flox/flox}$ and $Cbp^{+/+}$ mice at 1 h following contextual fear conditioning (Stanciu *et al*, 2001), as transcription of immediate early genes such as *c-fos* initiated by patterned synaptic activity is necessary for synaptic plasticity and long-term memory (reviewed in Alberini, 2009). $Cbp^{flox/flox}$ and $Cbp^{+/+}$ mice received bilateral intrahippocampal AAV2/1-Cre infusions and at 2 weeks later were trained in the contextual fear conditioning

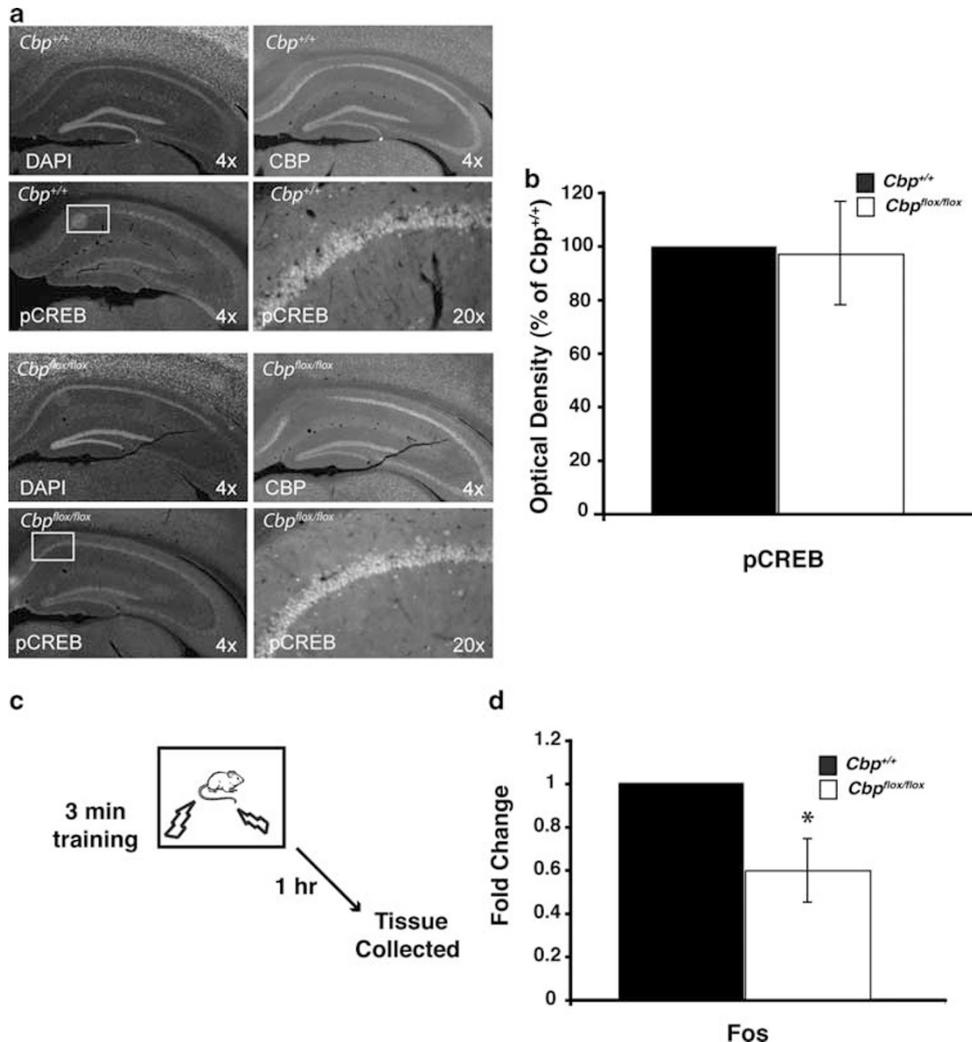


Figure 3 Phosphorylation of CREB is unaltered while expression of *c-fos* is reduced following fear conditioning in the absence of CBP. (a) Representative images showing DAPI labeling and pCREB immunoreactivity in hippocampi of *Cbp*^{+/+} and *Cbp*^{lox/lox} mice. Mice were infused with AAV2/2-Cre and at 2 weeks later were fear conditioned. Perfusion-fixed immunostained coronal brain slices were prepared from mice killed at 10 min following fear conditioning. The *Cbp*^{+/+} mice displayed expression of CBP throughout CA1, CA3, and the dentate gyrus. However, the *Cbp*^{lox/lox} mice display a focal loss of CBP, but exhibit no difference in pCREB expression in this region (bottom right two panels). (b) Quantification of pCREB immunostaining indicates no difference in pCREB in *Cbp*^{+/+} and *Cbp*^{lox/lox} mice in the region of CBP deletion. (c) Mice were infused with AAV2/1-Cre and at 2 weeks later were fear conditioned. At 1 h following training, quantitative RT-PCR shows that *c-fos* expression is significantly decreased in the dorsal hippocampus of *Cbp*^{lox/lox} mice compared with wild-type littermates. * $p < 0.05$.

paradigm. At 1 h following training (Figure 3c), tissue was collected by taking 1 mm punches from dorsal hippocampal slices in the area of the focal deletion in *Cbp*^{lox/lox} mice ($n = 3$), as confirmed by immunohistochemistry. Equivalent regions were analyzed from *Cbp*^{+/+} punches. In this experiment, AAV2/1 was used in order to generate larger deletion sizes, so that 1 mm punches could be taken from some slices and immunohistochemistry could be performed on adjacent slices to confirm the deletion. The expression of *c-fos* was significantly reduced after fear conditioning in *Cbp*^{lox/lox} dorsal hippocampus as compared with *Cbp*^{+/+} controls (Figure 3d). Together, these results suggest that although upstream signaling events appear normal, resulting in CREB phosphorylation, without CBP there is a failure to activate *c-fos* expression. This uncoupling between phospho-CREB and CREB:CBP-mediated gene expression is similar to previous

observations showing this dissociation in cell culture (Chawla *et al*, 1998; Hardingham *et al*, 1999).

Focal Homozygous *Cbp* Deletion Using *Cbp*^{lox/lox} Mice and AAV-Cre Impairs LTP in Hippocampus

Acute hippocampal slices were used to test for changes in synaptic physiology associated with focal deletion of *Cbp* using AAV2/1-Cre, as verified by immunohistochemistry for CBP following recording. Stimulation pulses were delivered to the Schaffer-commissural projections at 3 min and field EPSPs recorded from the proximal apical dendrites of field CA1b. Baseline synaptic transmission, as assayed by input/output curves (Figure 4a) and paired-pulse facilitation (Figure 4b), was not detectably different in the slices prepared from *Cbp*^{+/+} or *Cbp*^{lox/lox} mice. However,

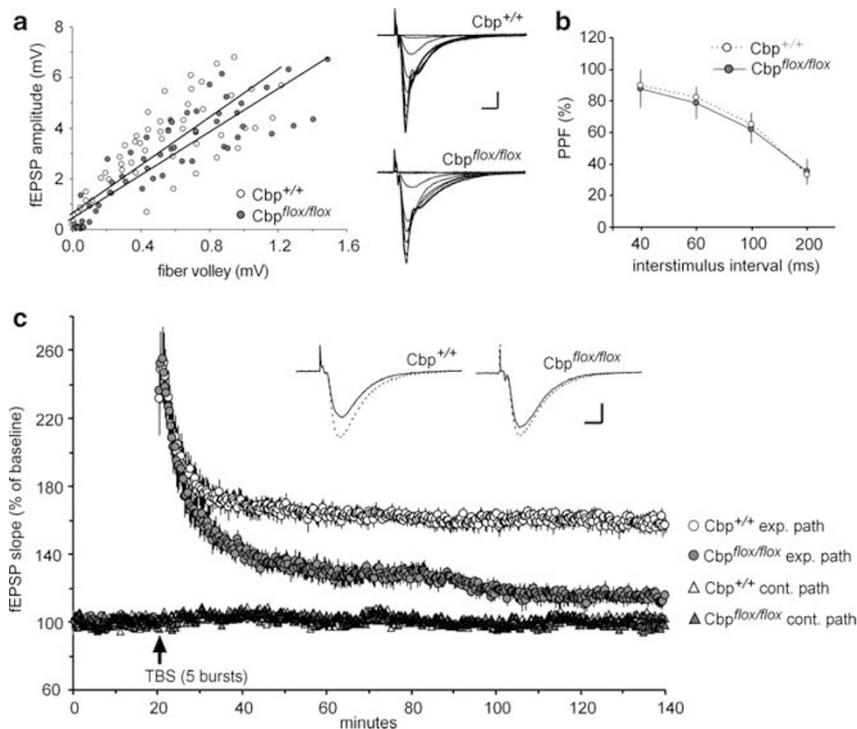


Figure 4 Local deletion of CBP disrupts LTP in hippocampal slices. Field EPSPs evoked by stimulation of either of two electrodes placed in the Schaffer-commissural projections were recorded in CA1b stratum radiatum of slices prepared from *Cbp*^{+/+} and *Cbp*^{flox/flox} mice infused with AAV2/1-Cre ($n = 7$ slices/group). (a) Input/output curves compare amplitudes of the presynaptic fiber volley to the field excitatory postsynaptic potentials (fEPSP) amplitude across a range of stimulation currents (10, 20, 30, 40, and 50 μ A). Slopes for the linear regression lines were not detectably different between the two groups. Representative traces are shown at right (scale bar: 1 mV/5 ms). (b) Paired pulse facilitation of the initial slope of the synaptic response was comparable (40, 60, 100, and 200 ms inter-pulse intervals) in the two groups of slices. (c) A train of five θ -bursts delivered to one of the two stimulation electrodes (exp. path) produced stable potentiation in slices from *Cbp*^{+/+} mice (open circles) but not in *Cbp*^{flox/flox} slices (dark circles) ($p < 0.001$ at the end of the 2 h post-TBS recording session). Synaptic responses recorded in the pathway (cont. path) that did not receive TBS remained stable throughout the experiment for both groups (open triangle, *Cbp*^{+/+}; dark triangle, *Cbp*^{flox/flox}). Insets show field excitatory postsynaptic potentials (fEPSP) traces collected during baseline testing (solid line) and at 2 h after TBS (dotted line). Scale: 1 mV/5 ms.

LTP was clearly impaired in the latter group. A single train of five θ -bursts (TBS) produced a potentiation effect that decayed to levels well below that observed in *Cbp*^{+/+} slices (Figure 4c). The difference between the two groups in mean percent LTP at 2 h post TBS was highly significant (*Cbp*^{+/+}: $+58 \pm 11\%$ relative to baseline; *Cbp*^{flox/flox}: $+14 \pm 10\%$; $n = 7$ /group, $p < 0.001$). Control (no TBS) inputs recorded on the same slices did not change from baseline over the 2-h test period in either group (*Cbp*^{+/+} = $-2 \pm 3\%$; *Cbp*^{flox/flox} = $+3 \pm 6\%$), indicating that the deletion did not affect the synapse specificity of LTP or the stability of evoked EPSPs. Importantly, the pronounced potentiation recorded at 2 min post TBS was equivalent in the two groups ($+144 \pm 47\%$ *Cbp*^{+/+} and $+144 \pm 50\%$ relative to baseline from *Cbp*^{flox/flox}). This strongly suggests that the complex signaling pathways underlying the induction and initial expression of LTP were left intact by CBP deletion. It follows from this that the essential problem produced by the deletion involves a failure of LTP to properly stabilize.

Past studies have overcome failures of LTP stabilization in learning impaired mouse models by using supra-threshold levels of θ -burst stimulation (Lauterborn *et al*, 2007). We tested for this effect in *Cbp*^{flox/flox} mice using trains of 10 θ bursts to induce LTP. We found that the percent potentiation at 2 h post TBS ($n = 3$; $+18 \pm 5\%$) was about the same as that found at this time point in the 5 burst

studies ($p = 0.56$) but significantly reduced compared with LTP induced with 10 burst in slices from *Cbp*^{+/+} mice ($n = 5$; $+67 \pm 22\%$ *Cbp*^{+/+}, $p = 0.005$). It is thus unlikely that the impairment involves a shift in the threshold for engaging the LTP consolidation machinery.

Long-Term Memory for Contextual Fear is Impaired in Mice With a Focal Hippocampal Deletion of CBP

To examine the effect of a homozygous *Cbp* deletion in the dorsal hippocampus on memory formation, we measured the effect of small (using AAV2/2-Cre) and large (using AAV2/1-Cre) hippocampal CBP deletions on long-term memory for contextual fear conditioning. In contextual fear conditioning, mice learn to associate a foot shock with a context in a single training session (Maren and Quirk, 2004). Memory for this association is dependent on the hippocampus (Maren, 2001) and dorsal CA1 has been demonstrated to be involved in retrieval of contextual fear conditioning (Hunsaker and Kesner, 2008). *Cbp*^{flox/flox} and *Cbp*^{+/+} mice were administered intrahippocampal infusions of AAV2/2-Cre or AAV2/1-Cre, fear conditioned at 2 weeks later, and tested either at 1 or 24 h following fear conditioning (different sets of mice used for 1 or 24 h time points). Deletions were verified with immunohistochemistry for CBP following behavior. Both *Cbp*^{flox/flox} mice infused

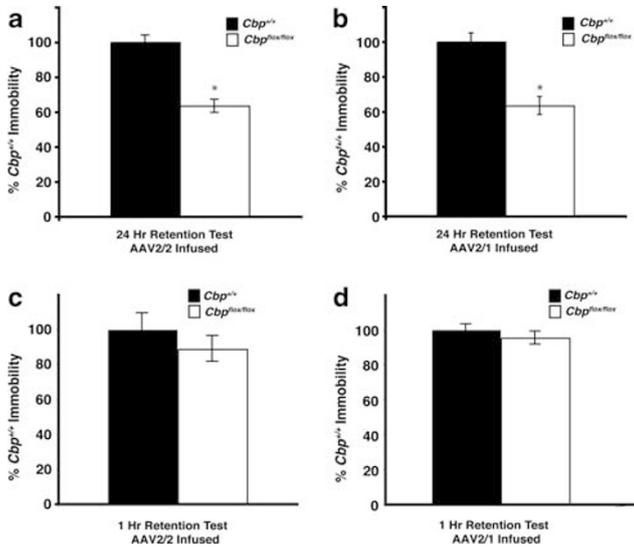


Figure 5 CBP deletion in the hippocampus impairs long-term, but not short-term memory. Memory for contextual fear conditioning was measured as immobility and is displayed as percent of wild type. (a) *Cbp*^{+/+} and *Cbp*^{flox/flox} mice were infused with AAV2/2-Cre and at 2 weeks later were trained and tested for contextual fear conditioning. *Cbp*^{flox/flox} mice ($n=9$) exhibited a significant decrease in levels of freezing in a 24 h retention test compared with *Cbp*^{+/+} mice ($n=8$). * $p<0.05$. (b) *Cbp*^{flox/flox} and *Cbp*^{+/+} mice were administered intrahippocampal infusions of AAV2/1-Cre. At 2 weeks later they were given contextual fear conditioning training. *Cbp*^{flox/flox} mice ($n=9$) exhibited a significant decrease in levels of freezing in a 24 h retention test compared with *Cbp*^{+/+} mice ($n=8$). * $p<0.05$. (c) *Cbp*^{+/+} and *Cbp*^{flox/flox} mice were infused with AAV2/2-Cre and at 2 weeks later were trained and tested at 1 h for contextual fear conditioning. *Cbp*^{flox/flox} mice ($n=9$) and *Cbp*^{+/+} mice ($n=9$) were not significantly different. (d) *Cbp*^{+/+} and *Cbp*^{flox/flox} mice were infused with AAV2/1-Cre and at 2 weeks later were trained and tested at 1 h for contextual fear conditioning. *Cbp*^{flox/flox} mice ($n=9$) and *Cbp*^{+/+} mice ($n=10$) were not significantly different.

with AAV2/2 (Figure 5a) and those infused with AAV2/1 (Figure 5b) exhibited significantly reduced levels of freezing when re-exposed to the conditioned context during the 24 h test ($p<0.05$). There were, however, no differences between *Cbp*^{flox/flox} mice and *Cbp*^{+/+} controls not administered AAV-Cre when tested at 24 h after training (Supplementary Figure S1D). In a different set of mice, there were no differences during a 1 h test ($p>0.05$; Figure 5c and d). This experiment demonstrates that a focal CBP deletion in a subregion of CA1 is sufficient to impair long-term memory for contextual fear, but not short-term memory.

Long-Term Memory for Object Location, but not Memory for the Object Itself, is Impaired in Mice With a Focal Hippocampal Deletion of *Cbp*

To further understand the role of CBP in long-term memory formation, we examined the effect of a hippocampal homozygous *Cbp* deletion using two different object recognition tasks. As no differences were observed between *Cbp*^{flox/flox} mice infused with AAV2/2-Cre and AAV2/1-Cre, *Cbp*^{flox/flox} and *Cbp*^{+/+} mice were administered intrahippocampal infusions of AAV2/1-Cre. They were trained 2 weeks later, and tested at 24 h following training. During training, mice were placed in an arena with two identical objects

for a 10-min training session, which we have previously shown to result in long-term memory (Stefanko et al, 2009; Roozendaal et al, 2010; Haettig et al, 2011), and then tested at 24 h later in the same arena with one familiar object moved to a novel location (OLM; Figure 6a). AAV2/1-Cre infused *Cbp*^{flox/flox} mice exhibited no significant long-term memory for the object location as compared with *Cbp*^{+/+} mice ($p<0.05$; Figure 6b). However, *Cbp*^{flox/flox} mice not infused with AAV-Cre exhibited long-term memory for the object location and were not significantly different than *Cbp*^{+/+} controls ($p>0.05$; Supplementary Figure S1E).

To examine whether short-term memory was affected by a hippocampal *Cbp* deletion, we trained a different group of AAV2/1-Cre infused *Cbp*^{flox/flox} mice with two identical objects and tested at 90 min later for OLM (Figure 6c). AAV2/1-Cre infused *Cbp*^{flox/flox} mice exhibited discrimination for the novel location that was not significantly different than that of *Cbp*^{+/+} mice ($p>0.05$; Figure 6d). This result indicates that *Cbp*^{flox/flox} mice with a focal deletion of *Cbp* in the hippocampus have unimpaired short-term memory for object location.

We next examined whether the focal *Cbp* deletion affected long-term memory in a standard novel object recognition task (ORM; see Figure 6e). In this task, there is no change in context or object location, but one of the familiar objects is replaced with a novel object. The dorsal hippocampus has been shown to encode information regarding context and location (O'Keefe and Burgess, 1999; Fanselow, 2000; Maren and Holt, 2000; Smith and Mizumori, 2006), whereas other brain regions, such as insular cortex, have been shown to be critical for long-term memory for the object itself (Balderas et al, 2008; Roozendaal et al, 2010). This distinct neural circuitry for the ORM and OLM tasks can reveal the regional specificity of our *Cbp* deletions. In a recent study, we used these tasks to demonstrate that focal deletion of HDAC3 in the hippocampus of HDAC3^{flox/flox} mice via AAV2/1-Cre affects long-term OLM, but not long-term memory for the object itself (ORM; McQuown et al, 2011).

Cbp^{flox/flox} and *Cbp*^{+/+} mice were administered intrahippocampal infusions of AAV2/1-Cre, trained at 2 weeks later in ORM (see Figure 6e). Both *Cbp*^{flox/flox} and *Cbp*^{+/+} mice showed a significant preference for the novel object and were not significantly different from each other ($p>0.05$; Figure 6f). These results demonstrate that focal deletions of CBP in the hippocampus impair long-term OLM, but not long-term memory for the object itself.

Long-Term Memory is not Rescued by Systemic Administration of an HDAC Inhibitor in Mice With a Focal Deletion of CBP

Previously our laboratory has shown that HDAC inhibition is able to enhance long-term memory and synaptic plasticity (Vecsey et al, 2007) and rescue long-term memory deficits in CBP mutant mice when the mice are tested on object recognition (Stefanko et al, 2009), but not when they are tested on object-location recognition (Haettig et al, 2011). We therefore examined the ability of the HDAC inhibitor sodium butyrate (NaBut) to ameliorate memory impairments in *Cbp*^{flox/flox} mice. As before, *Cbp*^{flox/flox} and *Cbp*^{+/+} mice were administered intrahippocampal infusions of

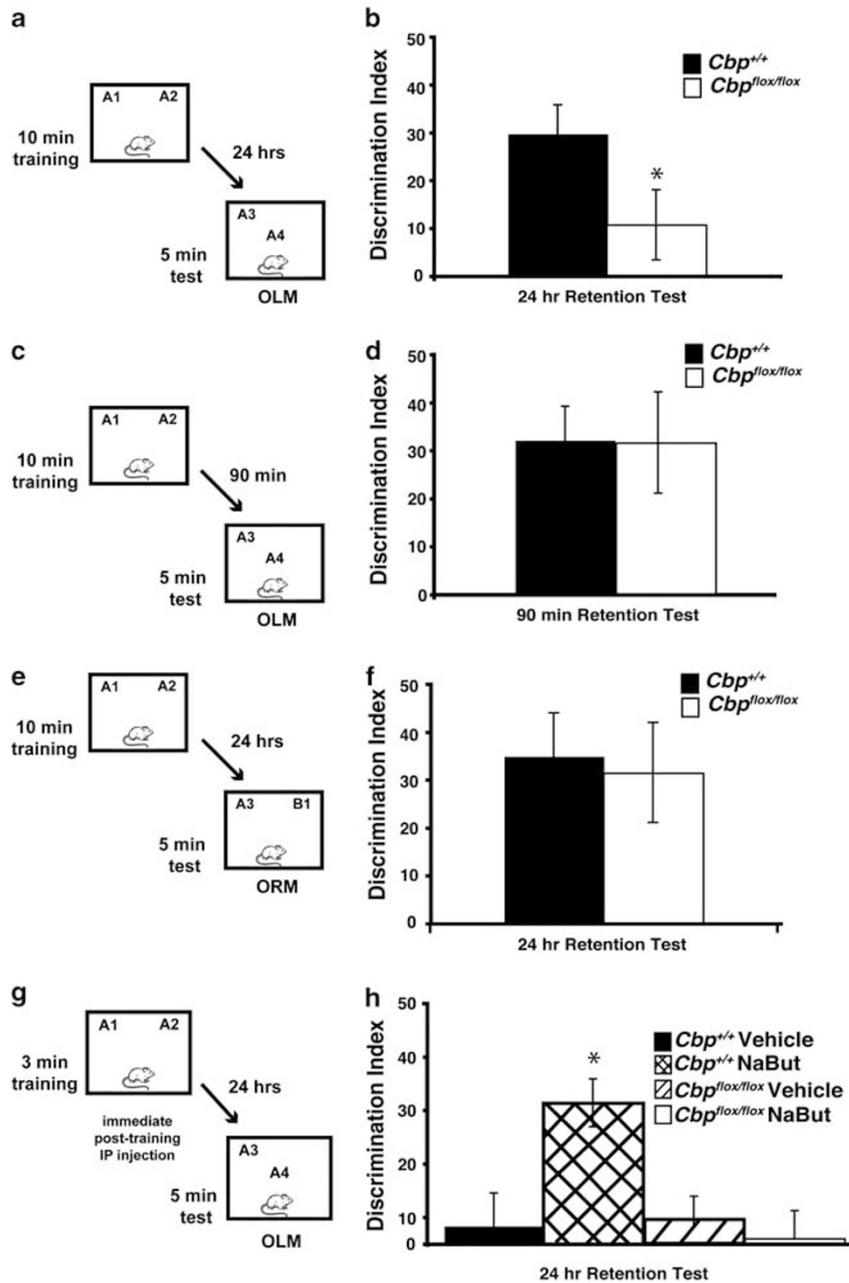


Figure 6 CBP deletion in the hippocampus leads to impairment in hippocampus-dependent location-dependent object recognition that is not rescued by HDAC inhibition. (a) Mice received 10 min training in an environment with two identical objects and received a retention test 24 h later for object location memory (OLM) in which one object is moved to a new location. (b) AAV2/1-Cre infused *Cbp*^{flx/flx} ($n = 12$) mice exhibit a significant 24 h long-term memory deficit ($p < 0.05$) in a hippocampus-dependent object location recognition task as compared with *Cbp*^{+/+} mice ($n = 9$). (c) Mice received 10 min training in an environment with two identical objects and received a 90 min OLM test, in which one object is moved to a new location. (d) AAV2/1-Cre infused *Cbp*^{flx/flx} ($n = 7$) mice exhibit normal 90 min short-term memory for a familiar location as compared with *Cbp*^{+/+} mice ($n = 8$). (e) Mice received 10 min training in an environment with two identical objects and received a retention test at 24 h later for object recognition memory (ORM) in which one object is replaced with a novel one. (f) AAV2/1-Cre infused *Cbp*^{flx/flx} mice ($n = 4$) exhibit normal 24 h long-term memory for a familiar object as compared with *Cbp*^{+/+} mice ($n = 9$) in a hippocampus-independent object recognition task. (g) Mice received subthreshold training (3 min) in an environment with two identical objects immediately followed by i.p. injection of sodium butyrate (NaBut) and received a retention test 24 h later in which one object is moved to a new location. (h) *Cbp*^{+/+} mice treated with NaBut ($n = 7$) exhibited a significant preference for the novel object while *Cbp*^{+/+} mice treated with vehicle ($n = 11$) and *Cbp*^{flx/flx} mice treated with vehicle ($n = 9$) or NaBut ($n = 9$) did not show a significant preference. * $p < 0.05$.

AAV2/1-Cre, trained at 2 weeks later, and tested at 24 h following training. During training, mice were placed in an arena with two identical objects for a 3-min training session (Figure 6g). The 3-min training session was used because this subthreshold training does not lead to long-term

memory formation in wild-type mice unless it is paired with HDAC inhibition (Stefanko *et al*, 2009; McQuown *et al*, 2011; Haettig *et al*, 2011). Immediately following training, mice were administered systemic injections of NaBut (1.2 g/kg). Mice were tested at 24 h later for object

location recognition. NaBut administration led to expression of long-term memory in wild-type mice. However, there was no difference between vehicle and NaBut treated *Cbp^{flox/flox}* mice (Figure 6h). These results further support the conclusion that CBP is critical for long-term memory formation in the hippocampus and even when histone deacetylation is inhibited, p300 cannot compensate for a loss of CBP.

DISCUSSION

Currently, there is little known about the actual histone modifications regulated by CBP *in vivo*, especially in the brain. Furthermore, no study to date has examined the effects of a complete focal deletion of CBP on modifications of specific histone residues in neurons and the subsequent impact on long-term memory. We demonstrate that a homozygous focal *Cbp* knockout in the dorsal hippocampus leads to impaired long-term memory but normal short-term memory and that systemic administration of an HDAC inhibitor does not rescue memory in these mice. In neurons in area CA1 of the hippocampus, this loss of CBP leads to a reduction in acetylation of several histone residues thought to be direct targets of CBP, which correlates with significantly reduced *c-fos* expression in the neurons of the focal deletion. These results indicate that CBP has unique functions in regulating histone modifications associated with long-term memory formation that cannot be compensated for by p300 in neurons of the hippocampus.

Although the HATs p300, PCAF, and CBP have all been implicated in the modification of histones necessary for the regulation of gene expression underlying memory formation, the specific role of each in this process is still unclear (Oliveira *et al*, 2006, 2007, 2011; Barrett and Wood, 2008; Maurice *et al*, 2008). The functions of p300 and CBP have been considered redundant until recently (Stern and Berger, 2000) and although they have been previously shown to have non-overlapping functions (Kawasaki *et al*, 1998; Kung *et al*, 2000; Kasper *et al*, 2002; McManus and Hendzel, 2003), they are still often referred to as CBP/p300. Although both p300 and CBP have been shown to have similar targets *in vitro* (Ogryzko *et al*, 1996; Kouzarides, 2007), our data suggest p300 cannot fully compensate for CBP *in vivo*. p300 appears to be normally expressed in the hippocampus of *Cbp^{flox/flox}* receiving AAV-Cre, similar to a previous study showing that *Cbp^{flox/flox}* mice crossed with CaMKII α -Cre transgenic mice exhibit normal p300 expression in neurons lacking CBP (Kang-Decker *et al*, 2004; Chen *et al*, 2010). Supporting a previous behavioral study showing that CBP and p300 have different roles in motor learning, with CBP function being more critical for motor skill learning than p300 function (Oliveira *et al*, 2006), the findings reported here indicate that CBP and p300 may have unique functions in the brain, specifically in the hippocampus.

In the late 1990s, two studies demonstrated that the phosphorylation of CREB could be uncoupled from CREB-mediated gene expression (Chawla *et al*, 1998; Hardingham *et al*, 1999). Chawla *et al* (1998) proposed a model in which CREB:CBP-mediated gene expression requires two steps: The phosphorylation of CREB (resulting in the recruitment

of CBP) and the phosphorylation of CBP for its activation. This model suggests how different signaling cascades may initiate different transcriptional pathways in spite of converging on a common transcription factor like CREB. Thus, even though upstream signaling may result in phosphorylation of CREB at serine 133 (see Figure 3a and b), such actions may be insufficient to activate CREB:CBP-mediated gene expression in the absence of CBP (Chawla *et al*, 1998; Hardingham *et al*, 1999; see Figure 3d). Loss of CBP resulted in a decrease in histone acetylation on multiple histones as well as decreased expression of *c-fos*, a well-known downstream target of CREB and CBP. These data agree with previous cell culture studies and also indicate that pCREB should not be used as an absolute marker of CREB-mediated gene expression.

Previously, we and others have found long-term memory impairments in CBP mutant mice (Oike *et al*, 1999; Bourchouladze *et al*, 2003; Alarcon *et al*, 2004; Korzus *et al*, 2004; Wood *et al*, 2005, 2006; Chen *et al*, 2010). However, no study to date has examined a homozygous knockout of CBP in a site-specific and temporally defined manner. Our data demonstrate that a CBP focal deletion, whether large or small, leads to long-term, but not short-term, memory deficits. Mice with the large deletion exhibit impaired long-term memory for location-dependent object recognition, but normal long-term memory for object recognition in which the location was not changed. Further, systemic administration of the HDAC inhibitor NaBut was not able to enhance memory in mice with a focal deletion of CBP, similar to findings previously published (Vecsey *et al*, 2007; Haettig *et al*, 2011). Together, these findings further support a role for CBP in hippocampus-dependent long-term memory formation.

Very recently, Chen *et al* (2010) found that CaMKII α -Cre driven deletion of *Cbp* in *Cbp^{flox/flox}* mice resulted in impairments in both short- and long-term memory. The impairments in short-term memory are particularly interesting as no other study examining genetically modified CBP mutant mice has found short-term memory impairments (Oike *et al*, 1999; Bourchouladze *et al*, 2003; Alarcon *et al*, 2004; Korzus *et al*, 2004; Wood *et al*, 2005, 2006). Chen *et al* (2010) suggested this may be because a full CBP knockout had never been examined with regard to learning and memory before their study. However, it is possible that there are developmental confounds because of the use of CaMKII α , which begins driving expression in all forebrain neurons after birth and reaches maximum expression over the next few weeks of life (Kojima *et al*, 1997). This could have consequences on the developing hippocampus considering a homozygous knockout of CBP is embryonic lethal (Tanaka *et al*, 1997). In this study, AAV-Cre was used to generate a complete CBP knockout in adult mice only in the dorsal hippocampus, thus reducing the possibility of confounding developmental or behavioral performance effects. Indeed, we observed no effect on basal synaptic transmission, upstream signaling events leading to phosphorylation of CREB, or short-term memory.

Our data demonstrate that a small focal CBP deletion in the dorsal CA1 area of the hippocampus is sufficient to disrupt long-term memory formation for contextual fear conditioning. Although it is difficult to directly compare the effects of the smaller and larger deletions, our data also

suggest that a more extensive hippocampal CBP deletion does not create a larger disruption in memory. Compared with the smaller CBP deletions, larger CBP deletions described in this study not only affected more of area CA1, but CA3 and the dentate gyrus as well. Other studies have demonstrated different roles for different regions of the hippocampus in specific types of memory (Nakazawa *et al*, 2003; Lee and Kesner, 2004; Matus-Amat *et al*, 2004; Hunsaker and Kesner, 2008), and therefore it is interesting that our results indicate no difference between a deletion of CBP solely in area CA1 and one affecting multiple areas of the hippocampus. However, previous results indicate that while dorsal and ventral CA1 lesions as well as ventral CA3 lesions all impair memory for contextual fear conditioning, dorsal CA3 lesions do not (Hunsaker and Kesner, 2008). The larger deletions cover more of dorsal CA1 and extend into dorsal CA3 and while they also extend somewhat further ventrally, they are mainly restricted to the dorsal hippocampus. As such, our data fit well with lesion data, suggesting that in neurons that are critical for long-term memory, CBP is critical for the formation of this memory.

As predicted from the behavioral results, we found that CBP deletion results in a pronounced impairment to LTP in hippocampal field CA1b. The effect is surprisingly discrete: multiple measures of baseline transmission appeared normal, as did the induction and early expression of potentiation. The selective loss of LTP consolidation is fully consistent with our behavioral results indicating that the CBP:CREB system is not required for the initial phases of learning but instead has an essential role in transferring newly acquired information into long-term memory. The LTP findings also provide clues about the plasticity-related cellular mechanisms affected by the deletion. Past studies indicate that LTP consolidation involves rapid reorganization of the subsynaptic cytoskeleton (Fukazawa *et al*, 2003; Kramar *et al*, 2006) and a somewhat delayed protein synthesis event (Stanton and Sarvey, 1984; Frey and Morris, 1997; Nguyen and Kandel, 1997). The protracted time course over which potentiation decays in the *Cbp* knockouts is as expected for a defect in the synthesis stage of stabilization. Nguyen and Kandel (1997) showed that with a similar LTP induction, a transcription blocker resulted in impaired LTP, with a decay mirroring what we observe here. The LTP and basal synaptic transmission findings support our behavioral findings indicating that CBP is critical for long-term memory formation, but in adult neurons is not involved in basal synaptic function or short-term memory.

In summary, this study represents the first time a focal homozygous knockout of *Cbp* in a single region of the adult brain has been investigated, which is critically important, as it has revealed exciting functions of CBP. Although loss of CBP leaves upstream signaling events intact, indicated by unchanged phosphorylation of CREB, it disrupts downstream events such as the acetylation of histones, *c-fos* expression, and behavior. Together, these findings elucidate the mechanism by which CBP may be regulating coordinate gene regulation during memory formation.

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DISCLOSURE

The authors declare no conflict of interest.

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