Pharmacological Activators of the NR4A Nuclear Receptors Enhance LTP in a CREB/CBP-Dependent Manner

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Nr4a nuclear receptors contribute to long-term memory formation and are required for long-term memory enhancement by a class of broad-acting drugs known as histone deacetylase (HDAC) inhibitors. Understanding the molecular mechanisms that regulate these genes and identifying ways to increase their activity may provide novel therapeutic approaches for ameliorating cognitive dysfunction. In the present study, we find that Nr4a gene expression after learning requires the cAMP-response element binding (CREB) interaction domain of the histone acetyltransferase CREB-binding protein (CBP). These gene expression deficits emerge at a time after learning marked by promoter histone acetylation in wild-type mice. Further, mutation of the CREB–CBP interaction domain reduces Nr4a promoter acetylation after learning. As memory enhancement by HDAC inhibitors requires CREB–CBP interaction and Nr4a gene function, these data support the notion that the balance of histone acetylation at the Nr4a promoters is critical for memory formation. NR4A ligands have recently been described, but the effect of these drugs on synaptic plasticity or memory has not been investigated. We find that the 'C-DIM' NR4A ligands, para-phenyl substituted di-indolylmethane compounds, enhance long-term contextual fear memory and increase the duration of long-term potentiation (LTP), a form of hippocampal synaptic plasticity. LTP enhancement by these drugs is eliminated in mice expressing a dominant negative form of NR4A and attenuated in mice with mutation of the CREB–CBP interaction domain. These data define the molecular connection between histone acetylation and Nr4a gene expression after learning. In addition, they suggest that NR4A-activating C-DIM compounds may serve as a potent and selective means to enhance memory and synaptic plasticity.
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INTRODUCTION

Epigenetic mechanisms, such as the posttranslational modification of histone proteins, are important regulators of long-term memory and long-term potentiation (LTP; Bridi and Abel, 2013a; Peixoto and Abel, 2013). Epigenetic histone marks are associated with learning and memory in brain regions including hippocampus, cortex, and amygdala (Bridi and Abel, 2013a). One of these marks, histone acetylation, is clearly correlated with active transcription (Hebbes and Thorne, 1988), and pharmacological inhibitors of histone deacetylase (HDAC) enzymes have been consistently shown to enhance hippocampal LTP (Guan et al, 2009; Levenson et al, 2004; Vecsey et al, 2007) and various forms of long-term memory (Haettig et al, 2011; Hawk et al, 2011; Lattal et al, 2007; Stefanko et al, 2009). However, the critical genes regulated by histone acetylation to enhance memory and

plasticity are only now becoming clear (Hawk et al, 2012; Bridi and Abel, 2013b).

The Nr4a nuclear receptor transcription factor subfamily comprised three closely related genes (Nr4a1/Nur77/HZF-3, Nr4a2/Nurr1/NGFI-B, and Nr4a3/NOR-1/TEC; Maxwell and Muscat, 2006) encoding 'orphan' nuclear receptors that regulate transcription in a concentration-dependent manner (Johnson et al, 2011). Researchers have shown that NR4A disruption impairs learning and memory formation (Colón-Cesario et al, 2006; McNulty et al, 2012; Rojas et al, 2007). Marcelo Wood's group has shown that NR4A1 and NR4A2 are involved in distinct forms of object location memory (McNulty et al, 2012) and demonstrated roles for NR4A2 in extinction (Malvaez et al, 2013) and the formation of cocaine-context-associated memories (Rogge et al, 2013). We have found that NR4A function is critical for long-term contextual fear memory and hippocampal LTP and that learning-induced Nr4a expression is upregulated by HDAC inhibition and critical for the enhancement of memory and LTP by HDAC inhibition (Vecsey et al, 2007; Hawk et al, 2012; Bridi and Abel, 2013b; Poplawski et al, 2014).

Although the NR4A orphan receptors lack identified endogenous ligands, pharmacological agents that activate them have been reported (Dubois et al, 2006; Hintermann et al, 2007; Ordentlich et al, 2003; Pearen and Muscat, 2010;

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Wansa et al, 2003; Zhan et al, 2008). Recently, a class of putative NR4A activators, the para-phenyl substituted di-indolylmethane analogs—or 'C-DIM' compounds—has been described (Chintharlapalli et al, 2004; Cho et al, 2007; Inamoto et al, 2008; Lei et al, 2008; Li et al, 2012). Importantly, these C-DIM compounds bind nuclear NR4A1 to activate or antagonize gene expression (Lee et al, 2009, 2014), penetrate the blood–brain barrier and are well-tolerated in vivo (Cho et al, 2007; De Miranda et al, 2013, 2015).

The Nr4a genes are targets of the plasticity-regulating cAMP-PKA-CREB (cAMP-response element binding) signaling pathway (Impey et al, 2004; Kovalovsky et al, 2002; Lemberger et al, 2008). CREB-binding protein (CBP) is a transcriptional co-activator with histone acetyltransferase (HAT) activity. CBP mutation impairs long-term memory formation (Alarcón et al, 2004; Korzus et al, 2004; Oike et al, 1999; Wood et al, 2005; Wood et al, 2006). Memory-disrupting CBP mutations include point mutations affecting the HAT domain (Korzus et al, 2004) and the kinase-inducible CREB interaction (KIX) domain of this co-activator (Wood et al, 2006). CBP–CREB interaction mutants are particularly interesting, because they selectively eliminate activity-induced interaction of CBP with phosphorylated CREB (Kasper et al, 2002). This CBP$^{kix/kix}$ mutation allows us to assess the link between CREB activation, a classical marker of experience, and CBP recruitment, which brings HAT activity to specific target genes.

Because of their regulation both by CREB and histone acetylation, and their importance for the enhancement of plasticity by HDAC inhibition, we hypothesized that learning-induced Nr4a gene expression would be CBP regulated. We used mutant mice with disruption in the CREB-interacting KIX domain of CBP, to examine the role of CBP in regulating Nr4a gene expression and promoter acetylation following hippocampus-dependent contextual fear learning. As these experiments supported the hypothesis that the CREB–CBP pathway regulates histone acetylation at Nr4a promoters, we explored the potential of putative NR4A activators and ligands, the 'C-DIM' compounds, to enhance hippocampus-dependent contextual fear memory as well as LTP in hippocampal slices. We found that Nr4a gene expression and promoter acetylation are attenuated by mutation of the KIX domain of CBP. Importantly, we found that the C-DIM putative NR4A activators enhanced the formation of long-term fear memory and also enhanced LTP in a fashion that was impacted by mutations reducing Nr4a expression or function.

MATERIALS AND METHODS

Animals

Mice were maintained under standard conditions consistent with National Institute of Health guidelines and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Food and water were available ad libitum. Adult mice (2–6 months old) were used for all experiments. Mice were kept on a 12 h light/12 h dark cycle, with all experiments performed during the light cycle. Unless otherwise noted, male mice were used in all experiments.

CBP$^{kix/kix}$ mutant mice were produced as described previously (Kasper et al, 2002; Wood et al, 2006). Mice heterozygous for the triple point mutation in CBP (Tyr650Ala, Ala654Gln, and Tyr658Ala) were backcrossed on a C57BL/6J background for at least 10 generations. Heterozygous mutant mouse (CBP$^{kix/wt}$) crosses produced homozygous mutant mice and wild-type (CBP$^{wt/wt}$) littermates for experiments. PCR genotyping for the mutant CBP allele used PureTaq RTG beads (GE-Amersham) with the primers KIX-F and KIX-R (Table 1), and the following thermal cycling parameters: 94 °C for 3 min, (94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s) × 34 cycles, 72 °C for 10 min. Male and female CBP$^{kix/kix}$ and CBP$^{wt/wt}$ mice were used in biochemical and electrophysiological experiments. Sexes were balanced among groups to avoid sex-driven bias in our results.

Nr4aDN transgenic mutant mice were produced as described previously (Hawk et al, 2012; Bridi and Abel, 2013b). Briefly, Nr4aDN transgenic mice express a dominant-negative NR4A construct under the control of the tetracycline operator (tetO). The tetO-Nr4aDN strain was crossed with transgenic CaMKII-tetracycline transactivator (CaMKII-tTA) mice (Mayford et al, 1996) to produce a double-transgenic line with postnatal expression of the Nr4aDN construct in excitatory forebrain neurons. The dominant-negative NR4A construct is a form of NR4A1 that contains the dimerization domain and DNA-binding domain but lacks the transactivation domain, containing instead an HA and a YFP tag (Hawk et al, 2012; Robert et al, 2006). Double-transgenic (CaMKII-tTA+; Nr4aDN+) males were bred to C57BL/6J females. From the resulting litters, experimental Nr4aDN mutant mice were double transgenic, while non-transgenic and single-transgenic littermates served as controls. Mice were raised in the absence of doxycycline. Male Nr4aDN mice were used for electrophysiological experiments.

Table 1 Primer Sequences for Genotyping and Gene-Specific Nr4a Promoter ChIP

Name	Purpose	Sequence
TCRA-F	tTA genotyping	5′-CAA ATG TTG CTT GTC TGG TG-3′
TCRA-R	tTA genotyping	5′- GTC AGT CGA GTG CAC AGT TT-3′
tTA-F	tTA genotyping	5′-CGC TGT GGG GCA TTT TAC TTT AG -3′
tTA-R	tTA genotyping	5′-CAT GTC CAG ATC GAA ATC GTC-3′
EGFP-F	Nr4aDN genotyping	5′-CCT ACG GCG TGC AGT GCT TCA GC-3′
EGFP-R	Nr4aDN genotyping	5′-CGG CGA GCT GCA CGC TGC GTC CTC-3′
Actb-F	Nr4aDN genotyping	5′-GAT GAC GAT ATC GCT GCG CTG GTC G-3′
Actb-R	Nr4aDN genotyping	5′-GCC TGT GGT ACG ACC AGA GGC ATA C-3′
KIX-F	CBP-kix genotyping	5′-TAG TTC CCT TGT GCC ACC TT-3′
KIX-R	CBP-kix genotyping	5′-TCC CAG TGA TAC CAG CAT ACC-3′
Nr4a1-F	ChIP primer	5′-CCC TTG TAT GGC CAA AGC TC-3′
Nr4a1-R	ChIP primer	5′-CTC CGC AGT CCT TCT AGC AC-3′
Nr4a2-F	ChIP primer	5′-CCG TTC CCA CCT TAA AAT CA-3′
Nr4a2-R	ChIP primer	5′-CTG CCA ACA TGC ACC TAA AG-3′
Nr4a3-F	ChIP primer	5′-GAG GGA GGA GGA GGG TGA CGT A-3′
Nr4a2-R	ChIP primer	5′-CAT AGA GTG CCT GGA ATG CGA GA-3′
LINE1-F	ChIP primer	5′-AAA CGA GGA GTT GGT TCT TTG AG-3′
LINE1-R	ChIP primer	5′-TTT GTC CCT GTG CCC TTT AGT GA-3′

Abbreviation: CHIP, chromatin immunoprecipitation.

Drugs

DIM-C-pPhOCH$_3$ was purchased from Sigma-Aldrich (D7946 SIGMA). DIM-C-pPhBr was generously provided by Dr Stephen Safe of Texas A&M University. For electrophysiology experiments C-DIM compounds were dissolved in 100% DMSO to a stock concentration of 5 mM, aliquoted into amber tubes, and stored at − 20° C. C-DIM drugs were administered to hippocampal slices at a final concentration of 2.5 μM in artificial CSF (aCSF). For behavioral experiments using oral gavage DIM-C-pPhOCH$_3$ was dissolved in corn oil to a final dosage of 25 mg/kg.

Behavior

Fear conditioning before gene expression and chromatin immunoprecipitation (ChIP) analysis was performed as previously described (Vecsey et al, 2007), with handling for 3 days before conditioning. Briefly, the conditioning protocol entailed a single 2 s, 1.5 mA footshock terminating 2.5 min after placement of the mouse in the novel chamber. Conditioning was quantified by measuring freezing behavior, the absence of non-respiratory movement (Fanselow, 1980; Maren and Quirk, 2004), using automated scoring software (Clever Systems, Reston, VA).

A modified protocol was used for fear-conditioning experiments with the oral administration of DIM-C-pPhOCH$_3$. Mice were habituated to handling and oral gavage using corn oil for 5 consecutive days before fear conditioning. On the day of conditioning mice were administered either vehicle (corn oil) or DIM-C-pPhOCH$_3$ (at a dosage of 25 mg/kg dissolved in corn oil) 1 h before conditioning with a single 2 s, 0.5 mA footshock. Long-term fear memory was assayed 24 h after conditioning by re-exposing mice to the fear conditioning chamber for 5 min and measuring the percent of time spent in freezing behavior.

RNA Preparation

Hippocampi were dissected on ice after fear conditioning. Conditioning and dissections alternated between control and experimental groups. RNA was prepared using a modified Trizol RNA extraction followed by RNeasy (Qiagen) purification and DNA-free (Ambion) DNase treatment. RNA concentration was determined using a NanoDrop spectrophotometer (ThermoFisher Scientific).

cDNA Synthesis

cDNA was produced using the RETROscript kit (Ambion). For each reaction, 1 μg of total RNA was added to a 20 μl total reaction volume composed of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl$_2$, 5 mM dithiothreitol, 500 μM each dNTP, 5 μM random decamer, 10 units RNase inhibitor, and 100 units MMLV-RT. Control reactions were performed lacking template or reverse transcriptase. Reactions were performed at 44 °C for 1 h, followed by heat inactivation at 100 °C for 10 min. Reactions were diluted to 2 ng/μl in water to 500 μl final volume.
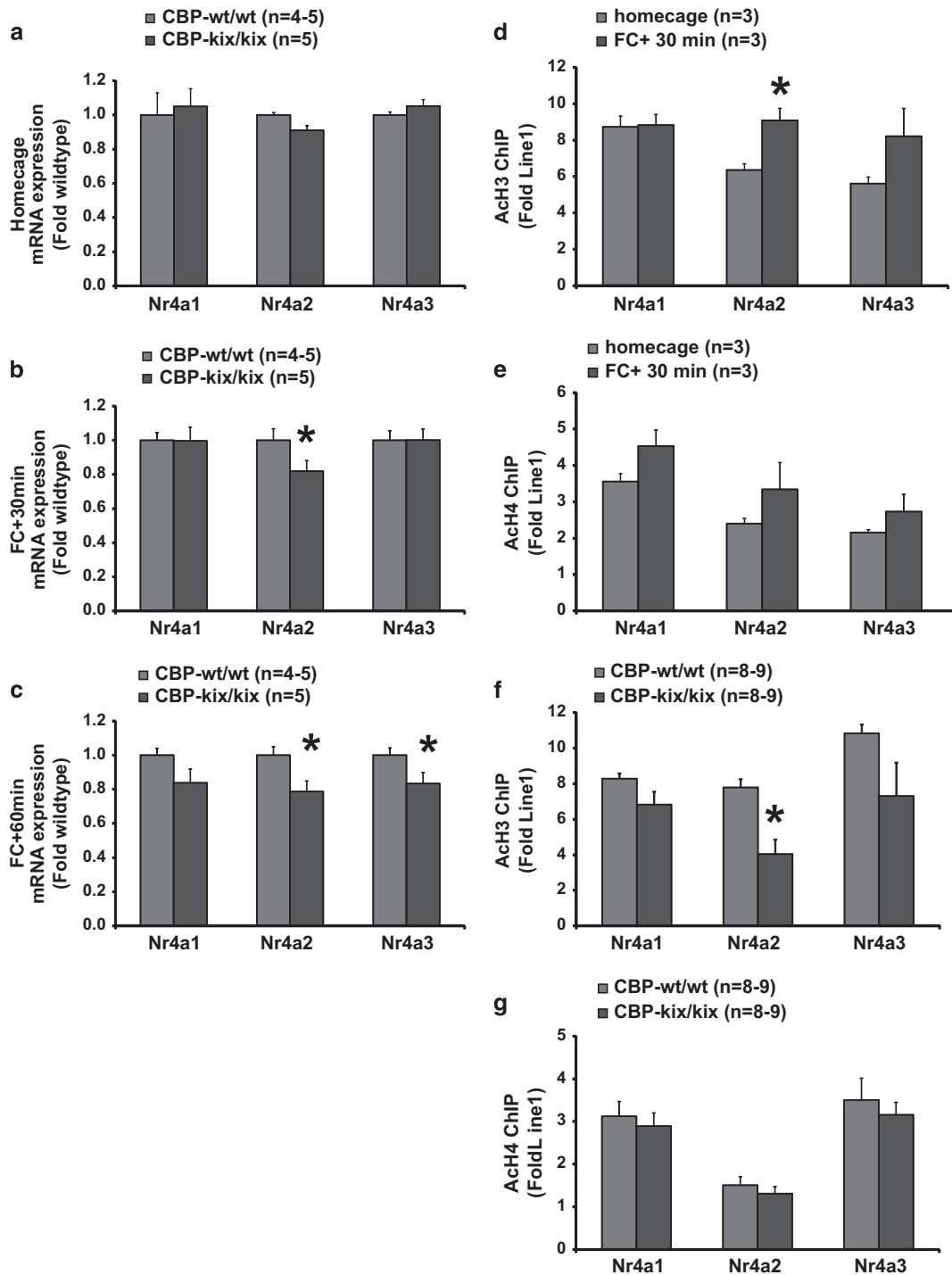
Real-Time PCR

qPCR was performed on the ABI7500 Fast using three separate housekeeper genes for normalization (Gapdh, Tuba4a, and Hprt). Relative quantification of gene expression was based on the ABI users' bulletin using a ΔΔCt method and described previously (Vecsey et al, 2007). Fold difference in mean value for biological replicates is presented and each sample is a distinct biological replicate.

Chromatin Immunoprecipitation

ChIP assays were performed as previously described (Vecsey et al, 2007). Briefly, finely chopped hippocampi were cross-linked in 2% paraformaldehyde in PBS. Nuclei were prepared and cross-linked chromatin was extracted as described (Vecsey et al, 2007). Chromatin isolated from nuclei was sonicated to between 200 and 1000 bp using the Bioruptor (Diagenode) with high-power sonication for 30 min with a 1.5 min rest between 1 min pulses of sonication. Soluble chromatin quantity and fragmentation size was assessed using agarose gel electrophoresis and Nanodrop (Wilmington, DE) spectrophotometry. For each ChIP assay, 2 μg chromatin and 2 μg antibody (anti-acetyl histone H3, 1 : 1000, Millipore 06–599; anti-acetyl H4, 1 : 1000, Millipore #06–866) were incubated overnight at 4 °C. Mock immunoprecipitation with pre-immune IgG was performed in parallel. Immunoprecipitation was performed with 100 μl protein G plus agarose beads (Invitrogen) at 4 °C for 2 h. Beads were washed in low salt buffer, high-salt buffer, LiCl buffer, and TE buffers as described previously (Vecsey et al, 2007). Chromatin–antibody conjugates were released from beads in 1% SDS, 100 mM NaHCO$_3$ elution buffer. Cross-linking was reversed by overnight incubation at 65 °C in the presence of 200 mM NaCl, followed by proteinase K treatment for 1 h at 55 °C. DNA was isolated using MinElute spin columns (Qiagen). Quantitative real-time PCR was performed with gene promoter-specific ChIP primers (Nr4a1-F, Nr4a1-R, Nr4a2-F, Nr4a2-R, Nr4a3-F, Nr4a3-R, LINE1-F, and LINE1-R) as listed in Table 1. Amplification detection was performed using PowerSYBR green mix (ABI) on the ABI7500 Fast real-time PCR system using 7500 standard cycling parameters.

Electrophysiology

Electrophysiological recordings were performed as previously described (Bridi and Abel, 2013b; Park et al, 2014; Vecsey et al, 2007). Mice were killed by cervical dislocation and their hippocampi quickly removed and dissected in ice-cold oxygenated aCSF (124 mM NaCl, 4.4 mM KCl, 1.3 mM MgSO$_4$•7H$_2$O, 1 NaH$_2$PO$_4$•H$_2$O, 26.2 mM NaHCO$_3$, 2.5 mM CaCl$_2$•2H$_2$O, 10 mM glucose). Transverse hippocampal slices 400 μm thick were cut with a Stoelting tissue chopper, transferred to an interface recording chamber, and maintained in oxygenated aCSF at 28.0 °C for at least 2 h before recording. Single-pathway recordings employed a single bipolar stimulating electrode of nichrome wire (A-M Systems) placed in stratum radiatum of CA1 to elicit action potentials in axons from CA3. Field potentials (fEPSPs) were recorded using an aCSF-filled glass micro-electrode (A-M Systems) of resistance 1–6 MΩ placed in

a CBP-wt/wt (n=4-5)
CBP-kix/kix (n=5)

Homecage mRNA expression (Fold wildtype)

Nr4a1   Nr4a2   Nr4a3

b CBP-wt/wt (n=4-5)
CBP-kix/kix (n=5)

FC+30min mRNA expression (Fold wildtype)

Nr4a1   Nr4a2 *   Nr4a3

c CBP-wt/wt (n=4-5)
CBP-kix/kix (n=5)

FC+60min mRNA expression (Fold wildtype)

Nr4a1   Nr4a2 *   Nr4a3 *

d homecage (n=3)
FC+ 30 min (n=3)

AcH3 ChIP (Fold Line1)

Nr4a1   Nr4a2 *   Nr4a3

e homecage (n=3)
FC+ 30 min (n=3)

AcH4 ChIP (Fold Line1)

Nr4a1   Nr4a2   Nr4a3

f CBP-wt/wt (n=8-9)
CBP-kix/kix (n=8-9)

AcH3 ChIP (Fold Line1)

Nr4a1   Nr4a2 *   Nr4a3

g CBP-wt/wt (n=8-9)
CBP-kix/kix (n=8-9)

AcH4 ChIP (FoldL ine1)

Nr4a1   Nr4a2   Nr4a3

Figure I Hippocampal *Nr4a* gene expression and promoter histone acetylation increases after contextual fear learning and depends on cAMP-response element binding (CREB)–CREB-binding protein (CBP) interaction. *Nr4a* family transcript levels were examined in wild-type (CBP$^{wt/wt}$) and CBP-KIX domain mutant mice (CBP$^{kix/kix}$) at two time points after contextual fear training (a–c), and *Nr4a* promoter acetylation levels after training were examined (d–g). (a) Without training (homecage), no significant differences were detected in CBP$^{kix/kix}$ mice in comparison to littermate controls (CBP$^{wt/wt}$, $n = 5$; CBP$^{kix/kix}$, $n = 5$; *Nr4a1*, $p = 0.970$; *Nr4a2*, $p = 0.496$; *Nr4a3*, $p = 0.910$). (b) At 30 min after training, a defect emerges in *Nr4a2* gene expression (CBP$^{wt/wt}$, $n = 5$; CBP$^{kix/kix}$, $n = 5$; *Nr4a1*, $p = 0.999$; *Nr4a2*, $p = 0.028$; *Nr4a3*, $p = 0.583$). (c) At 60 min after training, significant deficits were observed in expression of both *Nr4a2* and *Nr4a3* in comparison to littermate controls (CBP$^{wt/wt}$, $n = 4$; CBP$^{kix/kix}$, $n = 5$; *Nr4a1*, $p = 0.072$; *Nr4a2*, $p = 0.013$; *Nr4a3*, $p = 0.036$). (d) Acetylation of histone H3 is elevated at the *Nr4a2* promoter 30 min after contextual fear training (homecage, $n = 3$; FC+30 min, $n = 3$; *Nr4a1*, $p = 0.827$; *Nr4a2*, $p = 0.0495$; *Nr4a3*, $p = 0.127$). (e) No significant differences were identified in histone H4 acetylation at the *Nr4a* promoters 30 min after contextual fear training (homecage, $n = 3$; FC+30 min, $n = 3$; *Nr4a1*, $p = 0.127$; *Nr4a2*, $p = 0.513$; *Nr4a3*, $p = 0.275$). (f) At 30 min after training, CBP$^{kix/kix}$ mice have significantly reduced levels of histone H3 acetylation at the *Nr4a2* gene promoter (*Nr4a1*: CBP$^{wt/wt}$ $n = 8$, CBP$^{kix/kix}$, $n = 9$, $p = 0.772$; *Nr4a2*: CBP$^{wt/wt}$ $n = 8$, CBP$^{kix/kix}$ $n = 8$, $p = 0.049$; *Nr4a3*: CBP$^{wt/wt}$ $n = 9$, CBP$^{kix/kix}$ $n = 8$, $p = 0.309$). (g) No significant changes were observed in acetylation of histone H4 at the *Nr4a* family promoters (*Nr4a1*: CBP$^{wt/wt}$ $n = 8$, CBP$^{kix/kix}$ $n = 8$, $p = 0.605$; *Nr4a2*: CBP$^{wt/wt}$ $n = 9$, CBP$^{kix/kix}$ $n = 9$, $p = 0.434$; *Nr4a3*: CBP$^{wt/wt}$ $n = 9$, CBP$^{kix/kix}$ $n = 8$, $p = 0.539$). Data are expressed as a ratio of acetylation relative to the LINE-1 retrotransposon. (*$p < 0.05$.)

stratum radiatum of CA1. Data were collected with Clampex software (Molecular Devices, Palo Alto, CA) and analyzed with Clampfit (Molecular Devices). Peak fEPSP amplitude was required to be at least 5 mV. Stimulus intensity during recordings was set to produce 40% of the maximum response. Probe stimulation occurred once per minute. Baseline responses were recorded for 20 min. Following baseline recording, a C-DIM compound or an equivalent volume of DMSO was administered for 40 min. LTP was induced using a single 1 s, 100 Hz train of stimuli, 20 min into the drug treatment. Recordings lasted for 3 h after beginning drug treatment.

Data Analysis

Statistical analysis was performed in SPSS (version 17), STATISTICA (version 11), and JMP (version 5). For behavioral experiments, ANOVAs were performed followed by Student–Newman–Keuls *post hoc* tests. Gene expression and ChIP statistical analysis used non-parametric Kruskall–Wallis ANOVAs. For electrophysiology data, analyses were performed as previously described (Bridi and Abel, 2013b; Park *et al*, 2014; Vecsey *et al*, 2007). Initial fEPSP slope was used to quantify potentiation, normalized to the averaged 20-minute baseline value. Only one slice per animal was included in any treatment condition; whenever possible, recordings were made from multiple slices per mouse to minimize animal use. To examine differences in the effects of vehicle *vs* DIM-C-pPhOCH$_3$ or vehicle *vs* DIM-C-pPhBr in controls and mutants, we performed repeated-measures ANOVAs on the final 20 min of the recordings, followed by Tukey's *post-hoc* test when appropriate (Vecsey *et al*, 2007). Experimenters were blind to genotype and genotypes were confirmed after experimentation. Data are expressed as mean $\pm$ SEM. Significance is indicated by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

RESULTS

The CREB Interaction Domain of CBP is Required for Nr4a Gene Expression after Contextual Fear Learning

As the *Nr4a* genes are regulated by CREB (Impey *et al*, 2004; Lemberger *et al*, 2008), we examined whether recruitment of CBP to phosphorylated CREB by the KIX interaction domain is required for hippocampal expression of the *Nr4a* genes induced by contextual fear conditioning. We used mice carrying homozygous knock-in mutations in the KIX domain of CBP (CBP$^{kix/kix}$ mice; Kasper *et al*, 2002). Previously, we have used CBP$^{kix/kix}$ mice to show that disruption of CREB/CBP interaction blocks the enhancement of Schaffer collateral LTP by administration of the HDAC inhibitor trichostatin A (Vecsey *et al*, 2007) and impairs long-term contextual fear memory (Wood *et al*, 2006).

We first examined *Nr4a1*, *Nr4a2*, and *Nr4a3* gene expression under homecage conditions in CBP$^{kix/kix}$ mutant mice and CBP$^{wt/wt}$ controls. We did not detect any differences in *Nr4a* mRNA levels between genotypes (*Nr4a1*, $p = 0.970$; *Nr4a2*, $p = 0.496$; *Nr4a3*, $p = 0.910$), indicating that the disruption of CREB/CBP interaction does not affect baseline *Nr4a* transcription (Figure 1a). We have

seen previously that hippocampal *Nr4a* expression is upregulated rapidly after learning (Hawk *et al*, 2012), so we assayed *Nr4a* mRNA levels at two time points after single-trial contextual fear conditioning, at 30 min and at 60 min post training.

At 30 min post fear conditioning, *Nr4a2* mRNA levels were significantly reduced in CBP$^{kix/kix}$ mutants compared to CBP$^{wt/wt}$ controls ($p = 0.028$; Figure 1b), whereas no significant differences were found in mRNA levels of either *Nr4a1* ($p = 0.999$) or *Nr4a3* ($p = 0.583$; Figure 1b). When we analysed tissue collected 60 min after fear conditioning, we observed significant deficits in the level of both *Nr4a2* ($p = 0.013$) and *Nr4a3* ($p = 0.036$) mRNA in CBP$^{kix/kix}$ mice compared to controls (Figure 1c). A non-significant trend towards a deficit in expression of *Nr4a1* was also observed ($p = 0.072$) (Figure 1c). These results suggest that disrupting CREB/CBP interaction inhibits activity-dependent upregulation of *Nr4a2* and *Nr4a3* expression.

Histone Acetylation at Nr4a Gene Promoters after Fear Conditioning Depends on CREB–CBP Interaction

All three *Nr4a* nuclear receptor genes are CREB-regulated, and CREB mutation attenuates the activity-induced expression of *Nr4a1* and *Nr4a2* in the hippocampus (Impey *et al*, 2004; Lemberger *et al*, 2008). Further, increased expression of *Nr4a1* and *Nr4a2* after contextual fear conditioning is augmented by administration of the HDAC inhibitor trichostatin A (Vecsey *et al*, 2007). Therefore, it was somewhat unexpected that expression of *Nr4a2* and *Nr4a3*, but not *Nr4a1*, was affected by mutation of the CREB interaction domain of CBP (Figure 1b and c). One scenario that could explain these results is that the promoters of *Nr4a2* and *Nr4a3*, but not of *Nr4a1*, are regulated by CBP-dependent histone acetylation.

To investigate the relationship between chromatin acetylation and learning-induced *Nr4a* gene expression, we examined histone acetylation at baseline and 30 min after contextual fear conditioning using ChIP with antibodies specific to acetylated histone H3 and acetylated histone H4. We found that H3 acetylation was significantly increased at the *Nr4a2* promoter ($p = 0.0495$) 30 min after fear conditioning relative to baseline (Figure 1d). There was no significant change in H3 acetylation at the *Nr4a1* promoter ($p = 0.827$), although there was a non-significant trend towards increased acetylation at the *Nr4a3* promoter ($p = 0.127$; Figure 1d). Acetylation of histone H4 was not significantly altered at any of the *Nr4a* gene promoters (*Nr4a1*, $p = 0.127$; *Nr4a2*, $p = 0.513$; *Nr4a3*, $p = 0.275$) (Figure 1e).

Next, we used CBP$^{kix/kix}$ mutant mice to investigate the importance of CREB–CBP interaction in the regulation and histone acetylation of the *Nr4a* genes. Because of the differences in post-training *Nr4a2* and *Nr4a3* expression and histone H3 acetylation we observed in CBP$^{kix/kix}$ mice, we hypothesized that CBP$^{kix/kix}$ mutants would also exhibit reduced histone acetylation at the *Nr4a2* promoter. Indeed, compared with controls, CBP$^{kix/kix}$ mice showed lower levels of histone H3 acetylation at the *Nr4a2* promoter ($p = 0.049$), but not at the promoters of *Nr4a1* ($p = 0.772$) or *Nr4a3* ($p = 0.309$; Figure 1f). No differences were found in the histone H4 acetylation (*Nr4a1*, $p = 0.605$; *Nr4a2*, $p = 0.434$; *Nr4a3*, $p = 0.539$; Figure 1g). These results indicate that

rapid, learning-induced expression of *Nr4a2* is regulated by activity-dependent, CBP-mediated histone acetylation. This is consistent with our previous research, which has shown that CREB–CBP interaction is required for upregulated *Nr4a* gene expression following fear conditioning and HDAC inhibitor administration (Vecsey *et al*, 2007).

Administration of Putative NR4A-Activator DIM-C-pPhOCH3 Enhances Long-Term Contextual Fear Memory

We have previously demonstrated that perturbation of NR4A function disrupts both normal memory consolidation (Hawk *et al*, 2012) and synaptic plasticity (Bridi and Abel, 2013a,b), but it has not been investigated whether targeted activation of the NR4A transcription factors could enhance hippocampal plasticity or cognition. We hypothesized that administration of an NR4A-activating compound would enhance the formation of hippocampus-dependent long-term contextual fear memory. Two recent studies (De Miranda *et al*, 2013, 2015) have found that the NR4A-activating C-DIM compounds cross the blood-brain barrier after oral administration, and exert neuroprotective effects in a mouse model of Parkinson's disease. Based on these findings, we administered 25 mg/kg DIM-C-pPhOCH$_3$ via oral gavage to C57Bl/6J mice 1 h before contextual fear conditioning with a moderate (0.5 mA) footshock (Figure 2a). A test of long-term contextual fear memory 24 h later revealed that the C-DIM treatment group exhibited significantly higher freezing behavior than did the vehicle treatment group (vehicle, $n = 19$, freezing $= 45.9 \pm 2.8\%$; C-DIM-pPhOCH$_3$, $n = 20$, freezing $= 58.9 \pm 3.1\%$; *t*-test, $p = 0.004$; Figure 2b), indicating that pharmacological activation of NR4A does enhance the formation of hippocampus-dependent long-term memory.

Enhancement of Hippocampal LTP by C-DIM Compounds is an NR4A-Dependent Manner

We hypothesized that administration of NR4A-activating compounds would enhance hippocampal synaptic potentiation induced by stimulation normally below the threshold for induction of long-lasting LTP. Therefore, we investigated the effects of two putative NR4A-activating C-DIM compounds (DIM-C-pPhOCH$_3$ and DIM-C-pPhBr) on LTP in wild-type and mutant mice expressing a dominant-negative NR4A construct (*Nr4a*DN), which blocks NR4A transcription

factor function (Bridi and Abel, 2013b; Hawk *et al*, 2012), as well as in CBP$^{kix/kix}$ and CBP$^{wt/wt}$ mice. In unstimulated slices from control animals, neither 2.5 μM DIM-C-pPhOCH$_3$ nor 2.5 μM DIM-C-pPhBr led a change in synaptic strength relative to vehicle-treated slices (vehicle, $n = 4$; pPhOCH$_3$, $n = 4$; pPhBr, $n = 4$; vehicle *vs* pPhOCH$_3$, $p > 0.05$; vehicle *vs* pPhBr, $p > 0.05$), indicating that the C-DIMs do not elicit LTP in the absence of stimulation (Figure 3e). Both control and *Nr4a*DN transgenic mice exhibited short-lived LTP in the presence of vehicle when a weak stimulus was applied (control+veh, $n = 4$, $104.2 \pm 10.0\%$; *Nr4a*DN+veh, $n = 4$, $100.8 \pm 13.6\%$; Tukey's
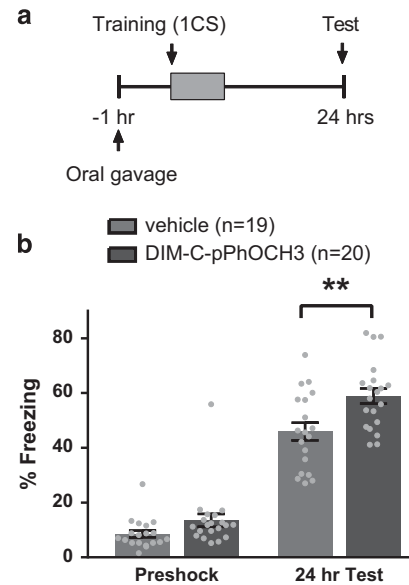
Figure 2 C-DIM administration enhances hippocampus-dependent long-term contextual fear memory. (a) Diagram of the contextual fear-conditioning paradigm. Wild-type C57Bl6/J mice were orally administered a single dose of corn oil or 25 mg/kg DIM-C-pPhOCH3 60 min before fear conditioning in a novel context with a moderate 0.5 mA footshock. Mice were returned to the training context 24 h later and time spent freezing was used to assay long-term memory. (b) Mice in the vehicle ($n = 19$) and C-DIM ($n = 20$) treatment groups did not exhibit significantly different levels of pre-shock freezing (vehicle, $45.9 \pm 2.8\%$; C-DIM, $58.9 \pm 3.1\%$; *t*-test, $p = 0.004$). C-DIM administration resulted in significantly higher levels of freezing compared to vehicle treatment during the long-term memory test 24 h after training (*t*-test, **p < 0.01).

Figure 3 C-DIM administration enhances hippocampal LTP and is attenuated by expression of a dominant-negative NR4A construct. (a) A single 1 s, 100 Hz tetanus induces short-lived LTP in *Nr4a*DN transgenic mice and WT littermate controls. (b) Administration of 2.5 μM DIM-C-pPhOCH3 around the time of LTP induction enhances LTP in control slices relative to vehicle treatment, but not in *Nr4a*DN mutants (repeated measures ANOVA: genotype (*Nr4a*DN *vs* control), F = 16.7, *p* = 0.0098; treatment (veh *vs* DIM-C-pPhOCH3), F = 33.8, *p* = 0.00034; genotype × treatment, F = 14.4, *p* = 0.0017. Tukey's *post-hoc*: control+pPhOCH3 *vs* *Nr4a*DN+pPhOCH3, *p* = 0.000292; control+veh *vs* control+pPhOCH3, *p* = 0.00214; *Nr4a*DN+veh *vs* control+pPhOCH3, *p* = 0.000205; other comparisons, *p* > 0.4). (c) Administration of 2.5 μM DIM-C-pPhBr around the time of LTP induction enhances LTP in control slices relative to vehicle treatment, but not in *Nr4a*DN mutants (repeated measures ANOVA: genotype (*Nr4a*DN *vs* control), F = 8.7, *p* = 0.01; treatment (veh *vs* DIM-C-pPhBr), F = 21.7, *p* = 0.00031; genotype × treatment, F = 7.5, *p* = 0.015. Tukey's *post-hoc*: control+pPhBr *vs* *Nr4a*DN+pPhBr, *p* = 0.00291; control +veh *vs* control+pPhBr, *p* = 0.000788; *Nr4a*DN+veh *vs* control+pPhBr, *p* = 0.000642; other comparisons, *p* > 0.5). (d) fEPSP slope averaged over the last 20 min for each experimental group. (e) In slices from wild-type control mice, administration of either tested C-DIM compound, in the absence of LTP-inducing stimulation, did not lead to significant changes in synaptic strength (vehicle *vs* pPhOCH3, *p* > 0.05; vehicle *vs* pPhBr, *p* > 0.05). (*p < 0.05, **p < 0.01, ***p < 0.001, arrows indicates LTP induction, black bars indicate drug or vehicle treatment, representative traces show synaptic response during baseline and at end of recording).

post-hoc, $p = 0.998$; Figure 3a). Slices from controls exhibited enhanced LTP in the presence of 2.5 μM DIM-C-pPhOCH$_3$ (control+pPhOCH$_3$, $n = 5$, $205.9 \pm 12.4\%$; Tukey's *post-hoc*, control+pPhOCH$_3$ *vs* control+veh, $p = 0.00082$) or of 2.5 μM DIM-C-pPhBr (control+pPhBr, $n = 5$, $209.7 \pm 19.3\%$; Tukey's *post-hoc*, control+pPhBr *vs* control+veh, $p = 0.0008$;

Figure 3b and c). However, expression of the *Nr4a*DN construct blocked LTP enhancement by DIM-C-pPhOCH$_3$ (*Nr4a*DN+pPhOCH$_3$, $n = 6$, $122.4 \pm 6.8\%$; Tukey's *post-hoc*, *Nr4a*DN+pPhOCH$_3$ *vs* *Nr4a*DN+veh, $p = 0.488$; Figure 3b) and by DIM-C-pPhBr (*Nr4a*DN+pPhBr, $n = 6$, $124.6 \pm 8.7\%$; Tukey's *post-hoc*, *Nr4a*DN+pPhBr *vs* *Nr4a*DN+veh,
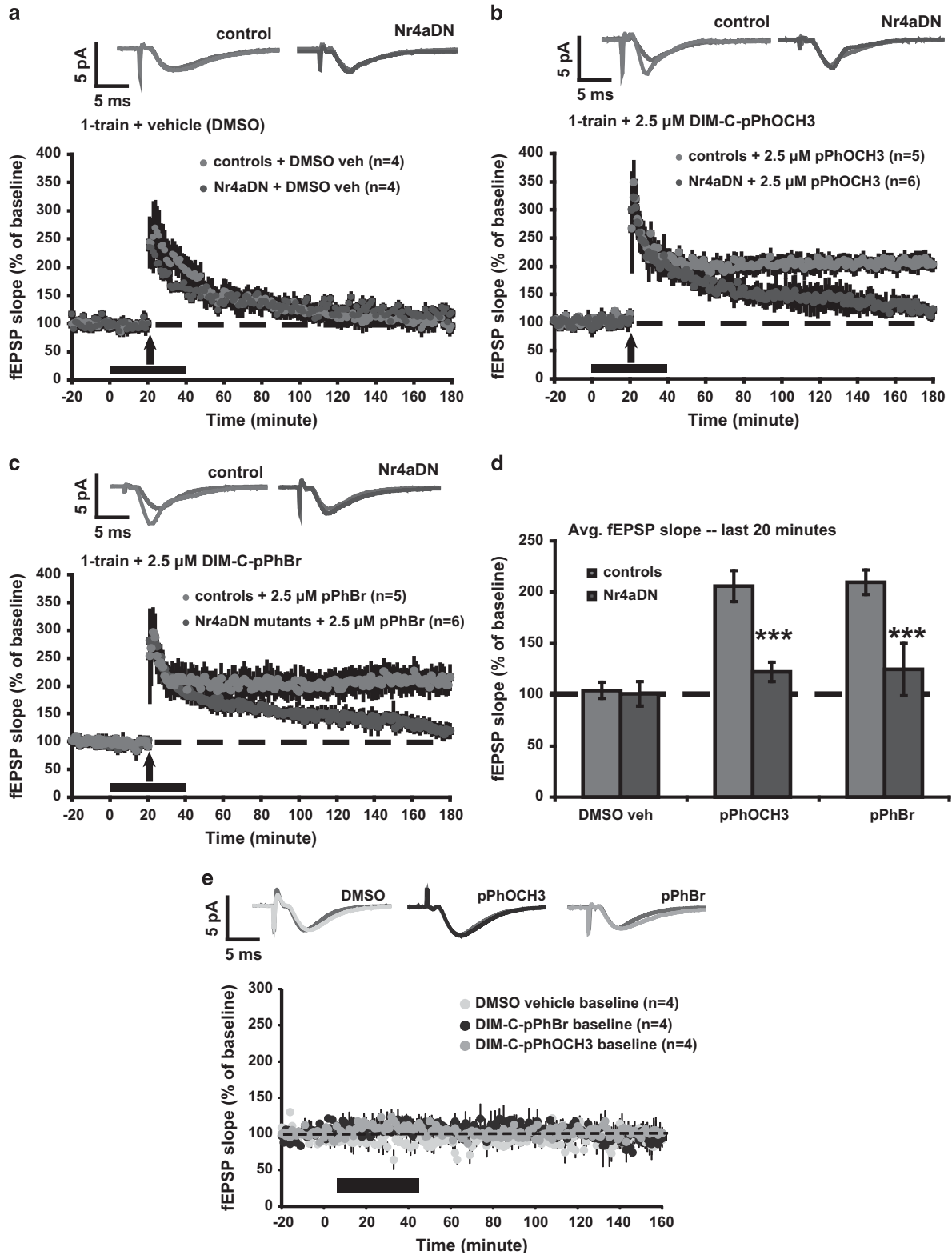
$p = 0.525$; Figure 3c and d). These data show that putative NR4A ligands enhance LTP in a manner requiring NR4A function.

In hippocampal slices from CBP$^{wt/wt}$ control and CBP$^{kix/kix}$ mutant mice, a single tetanus induced short-lived potentiation in the presence of vehicle (CBP$^{wt/wt}$+veh, $n = 7$, $99.8 \pm 7.6\%$; CBP$^{kix/kix}$+veh, $n = 7$, $108.0 \pm 12.0\%$; Tukey's *post-hoc*, $p = 0.968$; Figure 4a). In CBP$^{wt/wt}$ controls, administration of either DIM-C-pPhOCH$_3$ (CBP$^{wt/wt}$+pPhOCH$_3$, $n = 5$, $205.8 \pm 15.3\%$; Tukey's *post-hoc*, CBP$^{wt/wt}$+pPhOCH$_3$ *vs* CBP$^{wt/wt}$+veh, $p = 0.0002$) or DIM-C-pPhBr

(CBP$^{wt/wt}$+pPhBr, $n = 5$, $224.2 \pm 11.7\%$; Tukey's *post-hoc*, CBP$^{wt/wt}$+pPhBr *vs* CBP$^{wt/wt}$+veh, $p = 0.0002$) led to long-lasting potentiation compared to vehicle-treated slices (Figure 4b–d). In CBP$^{kix/kix}$ mutant mice, application of DIM-C-pPhOCH$_3$ enhanced LTP compared with vehicle-treated slices, but potentiation was still significantly lower than in CBP$^{wt/wt}$ controls (CBP$^{kix/kix}$+pPhOCH$_3$, $n = 5$, $154.4 \pm 9.5\%$; Tukey's *post-hoc*, CBP$^{kix/kix}$+pPhOCH$_3$ *vs* CBP$^{kix/kix}$+veh, $p = 0.0197$; CBP$^{kix/kix}$+pPhOCH$_3$ *vs* CBP$^{wt/wt}$+pPhOCH$_3$, $p = 0.047$; Figure 4b and d). In DIM-C-pPhBr-treated CBP$^{kix/kix}$ slices
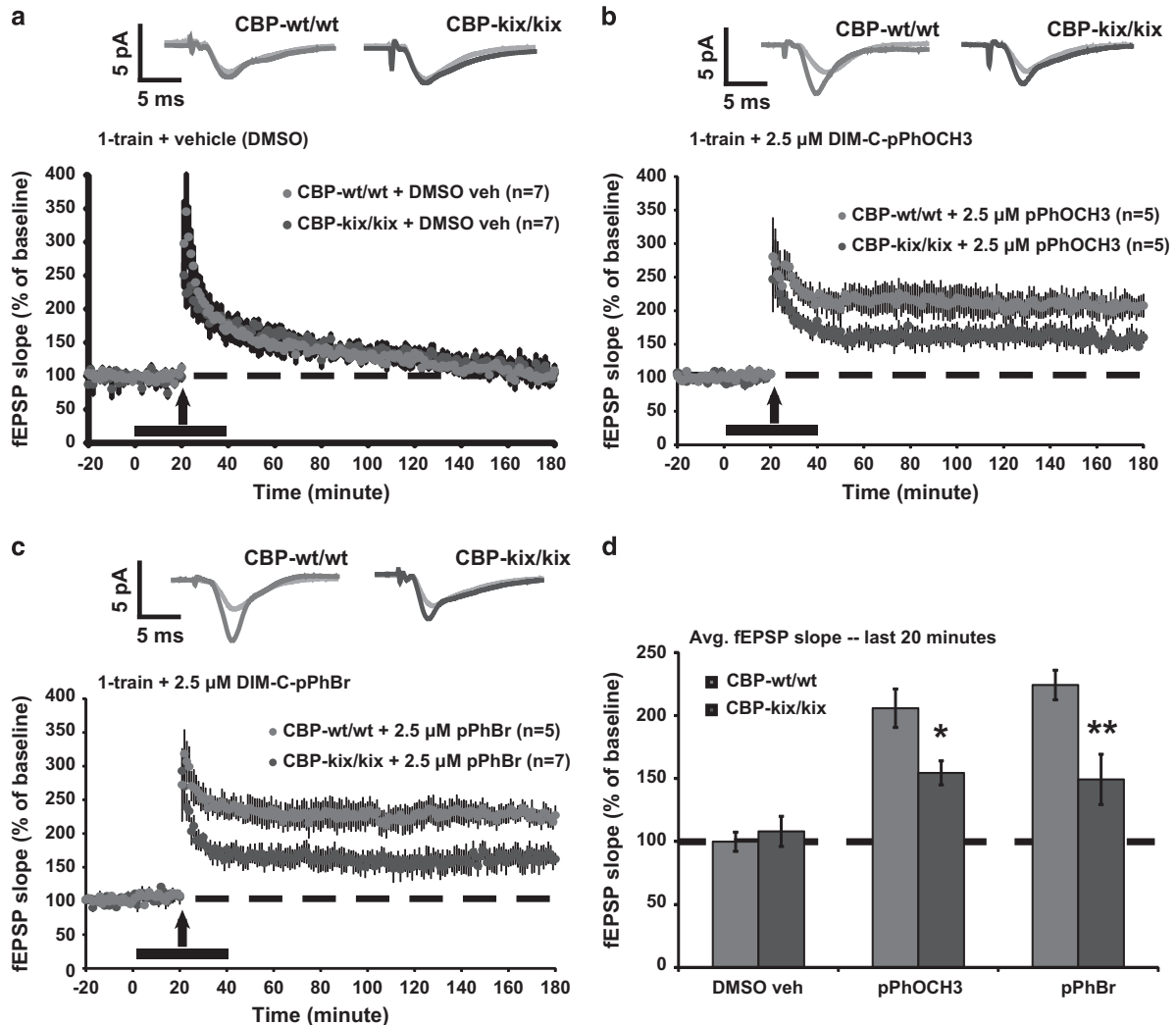
Figure 4 Disruption of the cAMP-response element binding (CREB)–CREB-binding protein (CBP) interaction domain reduces the enhancement of hippocampal LTP by C-DIM substituted compounds. (a) A single 1 s, 100 Hz train induces short-lived LTP in both CBP$^{kix/kix}$ and CBP$^{wt/wt}$ mice in the presence of DMSO vehicle. (b) Administration of 2.5 µM DIM-C-pPhOCH$_3$ around the time of LTP induction enhanced LTP in both CBP$^{kix/kix}$ and CBP-wt/wt mice relative to vehicle-treated slices, but potentiation in CBP$^{kix/kix}$ mice was still significantly lower than in wild-type control slices (repeated measures ANOVA: genotype (CBP$^{wt/wt}$ *vs* CBP$^{kix/kix}$), F = 3.7, $p = 0.069$; treatment (veh *vs* DIM-C-pPhOCH$_3$), F = 42.4, $p = 0.000002$; genotype × treatment, F = 6.3, $p = 0.021$. Tukey's *post-hoc*: CBP$^{wt/wt}$+pPhOCH$_3$ *vs* CBP$^{kix/kix}$+pPhOCH$_3$, $p = 0.0408$; CBP$^{wt/wt}$+veh *vs* CBP$^{wt/wt}$+pPhOCH$_3$, $p = 0.000187$; CBP$^{kix/kix}$+veh *vs* CBP$^{wt/wt}$+pPhOCH$_3$, $p = 0.000207$; CBP$^{kix/kix}$+veh *vs* CBP$^{kix/kix}$+pPhOCH$_3$, $p = 0.0197$; other comparisons, $p > 0.9$). (c) Administration of 2.5 µM DIM-C-pPhBr around the time of LTP induction enhances LTP in both CBP$^{kix/kix}$ and CBP$^{wt/wt}$ mice. Potentiation in CBP$^{kix/kix}$ mice was significantly lower than wt/wt mice (repeated measures ANOVA: genotype (CBP$^{wt/wt}$ *vs* CBP$^{kix/kix}$), F = 7.2, $p = 0.0133$; treatment (veh *vs* DIM-C-pPhBr), F = 42.1, $p = 0.000002$; genotype × treatment, F = 10.4, $p = 0.00388$. Tukey's *post-hoc*: CBP$^{wt/wt}$+pPhBr *vs* CBP$^{kix/kix}$+pPhBr, $p = 0.0032$; CBP$^{wt/wt}$+veh *vs* CBP$^{wt/wt}$+pPhBr, $p = 0.000167$; CBP$^{kix/kix}$+veh *vs* CBP$^{wt/wt}$+pPhBr, $p = 0.000169$; other comparisons, $p > 0.07$). (d) fEPSP slope averaged over the last 20 min for each experimental group (*$p < 0.05$, **$p < 0.01$, arrow indicates LTP induction, black bars indicate drug or vehicle treatment, representative traces show synaptic response during baseline and at end of recording).

(CBP$^{kix/kix}$+pPhBr, $n=7$, 149.3 ± 19.9%), potentiation was not enhanced compared with vehicle-treated CBP$^{kix/kix}$ mice, although a trend was observed (CBP$^{kix/kix}$+veh vs CBP$^{kix/kix}$+pPhBr, Tukey's post-hoc, $p=0.0767$), whereas it was significantly lower than in C-DIM-pPhBr-treated slices from CBP$^{wt/wt}$ controls (CBP$^{wt/wt}$+pPhBr vs CBP$^{kix/kix}$+pPhBr, Tukey's post-hoc, $p=0.0032$; Figure 4c and d). These data indicate that C-DIM administration enhances Schaffer-collateral LTP in hippocampal slices, in a manner requiring NR4A function. Interestingly, LTP enhancement by these ligands is at least partially dependent on activity-induced CREB–CBP interaction, which is required for the experience-dependent component of Nr4a gene expression (Figure 1) and enhancement of Nr4a gene expression by HDAC inhibition (Vecsey et al, 2007).

DISCUSSION

Histone acetylation regulates both long-term memory and LTP in the hippocampus, while HDAC inhibition enhances plastic processes and increases expression of many genes. However, we are only now discovering which acetylation-regulated genes mediate these effects. We have identified the Nr4a orphan nuclear receptors as candidate effectors, critical mediators of long-term memory consolidation and LTP, as well as their enhancement by pharmacological HDAC inhibition (Vecsey et al, 2007; Hawk et al, 2012; Bridi and Abel, 2013b). Here we found that hippocampal expression of Nr4a2 and Nr4a3 following contextual fear conditioning is attenuated by mutation of the CREB-interacting KIX domain of CBP, whereas baseline Nr4a expression is unaffected. Thus, activity-regulated CREB/CBP interaction is required for the learning-dependent increase in mRNA expression observed for these genes. We also found that post training upregulation of Nr4a mRNA coincides with a rapid increase in acetylation of histone H3 at the Nr4a2 promoter, which is blocked by disruption of CREB/CBP interaction. Further, we found that the C-DIM compounds, putative activators of NR4A, enhanced both in vivo long-term memory and ex vivo LTP when administered to wild-type mice. This LTP enhancement was eliminated by a dominant-negative mutation that blocks Nr4a function, supporting the specificity of this drug effect. In addition, the CBP-CREB interaction mutation that selectively eliminates experience-dependent Nr4a gene expression attenuated LTP enhancement by these ligands, suggesting that the activity-driven, CREB–CBP regulated increase of Nr4a expression is necessary for the memory- and plasticity-enhancing effects of the C-DIM compounds.

The three members of the Nr4a subfamily of nuclear receptor transcription factors have increased gene expression following hippocampus-dependent learning (Hawk et al, 2012; Poplawski et al, 2014). The increased acetylation of Nr4a gene promoters we observed after fear conditioning is consistent with their apparent role mediating the effects of HDAC inhibition on memory and LTP (Bridi and Abel, 2013b; Hawk et al, 2012), as well as their further upregulation after HDAC inhibitor treatment (Vecsey et al, 2007). Both Nr4a1 and Nr4a2 have been identified as CREB-regulated genes, whose expression after fear conditioning increases with HDAC inhibitor treatment (Vecsey et al, 2007; Impey

et al, 2004; Lemberger et al, 2008). It is therefore somewhat surprising that differential expression and promoter acetylation of Nr4a2 was found, but not of Nr4a1. One caveat is that our gene expression and promoter ChIP data were collected relatively soon after training, while changes in Nr4a expression after HDAC inhibitor administration can occur 2 h after training (Vecsey et al, 2007). It is possible that differences in H3 acetylation may still be seen at later timepoints. Alternatively, HDAC inhibitor treatment may upregulate Nr4a1 expression via a histone-independent mechanism such as acetylation of a transcription factor such as MEF2, which is known to regulate Nr4a gene expression (Lopez-Atalaya and Barco, 2014).

The Nr4a nuclear receptors hold potential as novel pharmacological targets in both basic and translational neuroscience research. Previous work with the C-DIM compounds has shown them to be well-tolerated in rodents and recent reports have found that C-DIMs can penetrate the blood–brain barrier and are effective in preventing neuronal loss in a mouse model of Parkinson's disease (Inamoto et al, 2008; De Miranda et al, 2013, 2015). Our contextual fear conditioning experiment took advantage of this property of the C-DIMs and demonstrated that these compounds can also be used to enhance long-term memory consolidation. The Nr4a transcription factors also have an important role in object recognition memory (McNulty et al, 2012), suggesting that this test would also be an excellent tool for future investigation into the effects of C-DIM administration and NR4A activation on hippocampal memory. The NR4A family represents a major component of the memory-promoting pathway downstream of HDAC inhibition. Some progress has been made linking specific NR4A proteins to different forms on memory (Colón-Cesario et al, 2006; Hawk et al, 2012; McNulty et al, 2012; Poplawski et al, 2014), but the identities of the plasticity-related genes downstream of NR4A and the mechanisms by which their post-learning expression is regulated remain elusive. Identification of these effector will improve our mechanistic understanding of plasticity and its regulation by epigenetic processes, and will provide valuable information necessary to validate the effects of these NR4A-activating drugs and to inform future research on their use as tools in basic research as well as in animal models of disease.

In conclusion, we report that the Nr4a genes are the targets of histone acetylation by the CREB/CBP memory-promoting pathway. Further, we found that pharmacological activation of NR4A proteins enhances both long-term memory and synaptic plasticity. These studies suggest that the Nr4a genes represent a critical node in the process of memory formation that may be amenable to safe and selective pharmacological enhancement.

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REFERENCES

Alarcón JM, Malleret G, Touzani K, Vronskaya S, Ishii S, Kandel ER et al (2004). Chromatin acetylation, memory, and LTP are impaired in CBP+/− mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. Neuron 42: 947–959.

Bridi MS, Abel T (2013a). Histone modifications in the nervous system and neuropsychiatric disorders. In: Sweatt JD, Meaney MJ, Nestler E, Akbarian S(eds). Epigenetic Regulation in the Nervous System. Elsevier: Waltham, pp 35–67.

Bridi MS, Abel T (2013b). The NR4A orphan nuclear receptors mediate transcription-dependent hippocampal synaptic plasticity. Neurobiol Learn Mem 105: 151–158.

Chintharlapalli S, Smith R, Samudio I, Zhang W, Safe S (2004). 1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl)methanes induce peroxisome proliferator-activated receptor gamma-mediated growth inhibition, transactivation, and differentiation markers in colon cancer cells. Cancer Res 64: 5994–6001.

Cho SD, Yoon K, Chintharlapalli S, Abdelrahim M, Lei P, Hamilton S et al (2007). Nur77 agonists induce proapoptotic genes and responses in colon cancer cells through nuclear receptor-dependent and nuclear receptor-independent pathways. Cancer Res 67: 674–683.

Colón-Cesario WI, Martínez-Montemayor MM, Morales S, Félix J, Cruz J, Adorno M et al (2006). Knockdown of Nurr1 in the rat hippocampus: implications to spatial discrimination learning and memory. Learn Mem 13: 734–744.

De Miranda BR, Miller JA, Hansen RJ, Lunghofer PJ, Safe S, Gustafson DL et al (2013). Neuroprotective efficacy and pharmacokinetic behavior of novel anti-inflammatory para-phenyl substituted diindolylmethanes in a mouse model of Parkinson's disease. J Pharmacol Exp Ther 345: 125–138.

De Miranda BR, Popichak KA, Hammond SL, Miller JA, Safe S, Tjalkens RB (2015). Novel para-phenyl substituted diindolyl-methanes protect against MPTP neurotoxicity and suppress glial activation in a mouse model of Parkinson's disease. Toxicol Sci 2: 360–373.

Dubois C, Hengerer B, Mattes H (2006). Identification of a potent agonist of the orphan nuclear receptor Nurr1. ChemMedChem 1: 955–958.

Fanselow MS (1980). Conditioned and unconditional components of post-shock freezing. Pavlov J Biol Sci 15: 177–182.

Guan J-S, Haggarty SJ, Giacometti E, Dannenberg J-H, Joseph N, Gao J et al (2009). HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459: 55–60.

Haettig J, Stefanko DP, Multani ML, Figueroa DX, McQuown SC, Wood MA (2011). HDAC inhibition modulates hippocampus-dependent long-term memory for object location in a CBP-dependent manner. Learn Mem 18: 71–79.

Hawk JD, Bookout AL, Poplawski SG, Bridi MS, Rao AJ, Sulewski ME et al (2012). NR4A nuclear receptors support memory enhancement by histone deacetylase inhibitors. J Clin Invest 122: 3593–3602.

Hawk JD, Florian C, Abel T (2011). Post-training intrahippocampal inhibition of class I histone deacetylases enhances long-term object-location memory. Learn Mem 18: 367–370.

Hebbes TR, Thorne AW (1988). A direct link between core histone acetylation and transcriptionally active chromatin. EMBO J 7: 1395–1402.

Hintermann S, Chiesi M, von Krosigk U, Mathé D, Felber R, Hengerer B (2007). Identification of a series of highly potent activators of the Nurr1 signaling pathway. Bioorg Med Chem Lett 17: 193–196.

Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM et al (2004). Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell 119: 1041–1054.

Inamoto T, Papineni S, Chintharlapalli S, Cho S-D, Safe S, Kamat AM (2008). 1,1-Bis(3'-indolyl)-1-(p-chlorophenyl) methane activates the orphan nuclear receptor Nurr1 and inhibits bladder cancer growth. Mol Cancer Ther 7: 3825–3833.

Johnson MM, Michelhaugh SK, Bouhamdan M, Schmidt CJ, Bannon MJ (2011). The transcription factor NURR1 exerts concentration-dependent effects on target genes mediating distinct biological processes. Front Neurosci 5: 135.

Kasper LH, Boussouar F, Ney PA, Jackson CW, Rehg J, van Deursen JM et al (2002). A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis. Nature 419: 738–743.

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

Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA et al (2002). Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, Protein Kinase A, and MAPK pathways. Mol Endocrinol 16: 1638–1651.

Lattal KM, Barrett RM, Wood MA (2007). Systemic or intrahippo-campal delivery of histone deacetylase inhibitors facilitates fear extinction. Behav Neurosci 121: 1125–1131.

Lee S-O, Li X, Hedrick E, Jin U-H, Tjalkens RB, Backos DS et al (2014). Diindolylmethane analogs bind NR4A1 and are NR4A1 antagonists in colon cancer cells. Mol Endocrinol 28: 1729–1739.

Lee SO, Chintharlapalli S, Liu S, Papineni S, Cho SD, Yoon K et al (2009). p21 expression is induced by activation of nuclear nerve growth factor-induced Balpha (Nur77) in pancreatic cancer cells. Mol Cancer Res 7: 1169–1178.

Lei P, Abdelrahim M, Cho SD, Liu S, Chintharlapalli S, Safe S (2008). 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes inhibit colon cancer cell and tumor growth through activation of c-jun N-terminal kinase. Carcinogenesis 29: 1139–1147.

Lemberger T, Parkitna JR, Chai M, Schütz G, Engblom D (2008). CREB has a context-dependent role in activity-regulated transcription and maintains neuronal cholesterol homeostasis. FASEB J 22: 2872–2879.

Levenson JM, O'Riordan KJ, Brown KD, Trinh MA, Molfese DL, Sweatt JD (2004). Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem 279: 40545–40559.

Li X, Lee S-O, Safe S (2012). Structure-dependent activation of NR4A2 (Nurr1) by 1,1-bis(3'-indolyl)-1-(aromatic)methane analogs in pancreatic cancer cells. Biochem Pharmacol 83: 1445–1455.

Lopez-Atalaya JP, Barco A (2014). Can changes in histone acetylation contribute to memory formation? Trends Genet 30: 529–539.

Malvaez M, McQuown SC, Rogge G a, Astarabadi M, Jacques V, Carreiro S et al (2013). HDAC3-selective inhibitor enhances extinction of cocaine-seeking behavior in a persistent manner. Proc Natl Acad Sci USA 110: 2647–2652.

Maren S, Quirk GJ (2004). Neuronal signalling of fear memory. *Nat Rev Neuroservice* 5: 844–852.

Maxwell MA, Muscat GEO (2006). The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl Recept Signal* 4: e002.

Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER (1996). Control of memory formation through regulated expression of a CaMKII transgene. *Science (80-)* 277: 1678–1683.

McNulty SE, Barrett RM, Vogel-Ciernia A, Malvaez M, Hernandez N, Davatolhagh MF *et al* (2012). Differential roles for Nr4a1 and Nr4a2 in object location vs. object recognition long-term memory. *Learn Mem* 19: 588–592.

Oike Y, Hata A, Mamiya T, Kaname T, Noda Y, Suzuki M *et al* (1999). Truncated CBP protein leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. *Hum Mol Genet* 8: 387–396.

Ordentlich P, Yan Y, Zhou S, Heyman RA (2003). Identification of the antineoplastic agent 6-mercaptopurine as an activator of the orphan nuclear hormone receptor Nurr1. *J Biol Chem* 278: 24791–24799.

Park AJ, Havekes R, Choi JHK, Luczak V, Nie T, Huang T *et al* (2014). A presynaptic role for PKA in synaptic tagging and memory. *Neurobiol Learn Mem* 114: 101–112.

Pearen MA, Muscat GEO (2010). Minireview: nuclear hormone receptor 4A signaling: Implications for metabolic disease. *Mol Endocrinol* 24: 1891–1903.

Peixoto L, Abel T (2013). The role of histone acetylation in memory formation and cognitive impairments. *Neuropsychopharmacology* 38: 62–76.

Poplawski SG, Schoch H, Wimmer ME, Hawk JD, Walsh JL, Giese KP *et al* (2014). Object-location training elicits an overlapping but temporally distinct transcriptional profile from contextual fear conditioning. *Neurobiol Learn Mem* 116: 90–95.

Robert NM, Martin LJ, Tremblay JJ (2006). The orphan nuclear receptor NR4A1 regulates insulin-like 3 gene transcription in Leydig cells. *Biol Reprod* 74: 322–330.

Rogge G a., Singh H, Dang R, Wood MA (2013). HDAC3 is a negative regulator of cocaine-context-associated memory formation. *J Neurosci* 33: 6623–6632.

Rojas P, Joodmardi E, Hong Y, Perlmann T, Ogren SO (2007). Adult mice with reduced Nurr1 expression: an animal model for schizophrenia. *Mol Psychiatry* 12: 756–766.

Stefanko DP, Barrett RM, Ly AR, Reolon GK, Wood MA (2009). Modulation of long-term memory for object recognition via HDAC inhibition. *Proc Natl Acad Sci USA* 106: 9447–9452.

Vecsey CG, Hawk JD, Lattal KM, Stein JM, Fabian SA, Attner MA *et al* (2007). Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. *J Neurosci* 27: 6128–6140.

Wansa KDSA, Harris JM, Yan G, Ordentlich P, Muscat GEO (2003). The AF-1 domain of the orphan nuclear receptor NOR-1 mediates trans-activation, coactivator recruitment, and activation by the purine anti-metabolite 6-mercaptopurine. *J Biol Chem* 278: 24776–24790.

Wood MA, Attner MA, Oliveira AMM, Brindle PK, Abel T (2006). A transcription factor-binding domain of the coactivator CBP is essential for long-term memory and the expression of specific target genes. *Learn Mem* 13: 609–617.

Wood MA, Kaplan MP, Park A, Blanchard EJ, Oliveira AMM, Lombardi TL *et al* (2005). Transgenic mice expressing a truncated form of CREB-binding protein (CBP) exhibit deficits in hippocampal synaptic plasticity and memory storage. *Learn Mem* 12: 111–119.

Zhan Y, Du X, Chen H, Liu J, Zhao B, Huang D *et al* (2008). Cytosporone B is an agonist for nuclear orphan receptor Nur77. *Nat Chem Biol* 4: 548–556.