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CRTC1 Nuclear Translocation Following Learning Modulates Memory Strength via Exchange of Chromatin Remodeling Complexes on the *Fgf1* Gene

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SUMMARY

Memory is formed by synapse-to-nucleus communication that leads to regulation of gene transcription, but the identity and organizational logic of signaling pathways involved in this communication remain unclear. Here we find that the transcription factor CRTC1 is a critical determinant of sustained gene transcription and memory strength in the hippocampus. Following associative learning, synaptically localized CRTC1 is translocated to the nucleus and regulates *Fgf1b* transcription in an activity-dependent manner. After both weak and strong training, the HDAC3–N-CoR corepressor complex leaves the *Fgf1b* promoter and a complex involving the translocated CRTC1, phosphorylated CREB and histone acetyltransferase CBP induces transient transcription. Strong training later substitutes KAT5 for CBP, a process that is dependent on

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AUTHOR CONTRIBUTIONS

S.U. and G.P.S. designed the experiments. S.U. performed the cell culture, molecular, biochemical, and behavioral experiments. B.J.W.T. and S.S.Z. performed electrophysiology. C.H. R.M.D. and K.H. performed the immunohistochemical analyses, in situ hybridization, and behavioral assay. A.K. and T. Shintaku performed the Q-PCR experiment. H.Y. and Y.W. provided research materials. P.J. and T. Suzuki designed and synthesized selective HDAC3 inhibitor. S.U and G.P.S. wrote the manuscript. All of the authors discussed the results and commented on the manuscript.

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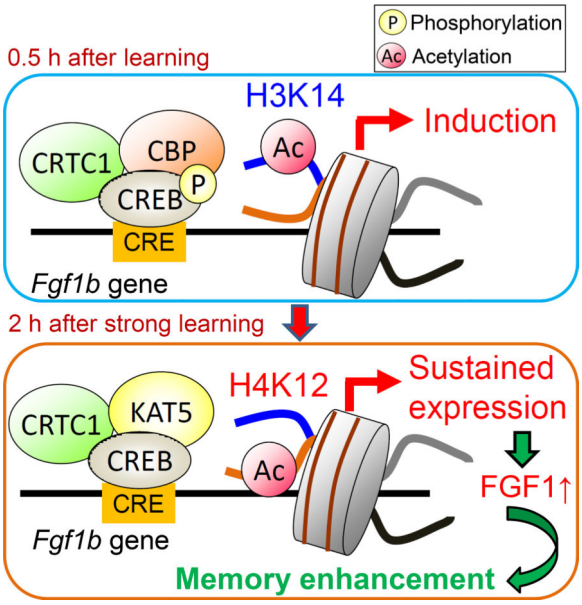
CRTC1, but not on CREB phosphorylation. This in turn leads to long-lasting *Fgf1b* transcription and memory enhancement. Thus, memory strength relies on activity-dependent changes in chromatin and temporal regulation of gene transcription on specific CREB/CRTC1 gene targets.

Graphical Abstract



Keywords

Memory enhancement; Long-term potentiation; Hippocampus; Nuclear transport; Epigenetics; FGF1; CRTC1; KAT5/Tip60; HDAC3

INTRODUCTION

Experience-dependent changes, such as those associated with long-term memory, require *de novo* gene transcription (Alberini, 2009; Mayford et al., 2012). To initiate stimulus-dependent gene transcription, signals must be relayed from active synapses to the nucleus (Greer and Greenberg, 2008) and the activity-dependent nuclear transport of synaptically localized transcriptional modulators represents a uniquely direct route to transmit this information (Ch'ng and Martin, 2011; Jordan and Kreutz, 2009).

Several critical for memory signaling pathways connecting synaptic inputs to gene transcription involve activation of the nuclear transcription factor cAMP-response element (CRE) binding protein (CREB), which induces transcription of CRE-containing genes and is required for synaptic plasticity and long-term memory (Benito and Barco, 2015; Kida et al., 2002). CREB mobilization is dependent on phosphorylation at its Ser133 site (phosphoCREB), which occurs via synaptically activated kinase pathways and includes association with the CREB-binding protein (CBP/p300). However, CREB-mediated transcriptional coactivators (CRTCs) may potentiate the interaction of CREB with CBP/p300 (Xu et al., 2007) and dramatically increase CREB transcriptional activity

independently of Ser133 phosphorylation (Conkright et al., 2003; Iourgenko et al., 2003). Recent studies suggest important roles for CRTC1 in synaptic plasticity (Kovacs et al., 2007; Zhou et al., 2006) and memory (Hirano et al., 2013; Nonaka et al., 2014; Parra-Damas et al., 2014; Sekeres et al., 2012). Although CRTC1 has been shown to move from the synapse/dendrite to the nucleus in response to neural activity and learning (Ch'ng et al., 2012; Nonaka et al., 2014; Parra-Damas et al., 2016), it remains unclear how CRTC1 acts during memory formation, what shuttling mechanisms are, and how CRTC1 activates target gene transcription independently of CREB phosphorylation.

Fibroblast growth factor (FGF) signaling has emerged as a key player in brain function and neuropsychiatric disorders (Bookout et al., 2013; Kang and Hebert, 2015; Turner et al., 2012). In mammals, the FGF family consists of 22 members, of which FGF1 is predominantly expressed in neurons (Elde et al., 1991). The brain-specific *Fgf1* gene promoter B, *Fgf1b*, is induced immediately following electroconvulsive stimulation in the mouse hippocampus (Ma et al., 2009), suggesting a role for activity-regulated FGF1 signaling in synaptic plasticity. Growing evidence indicates that epigenetic control of activity-dependent gene transcription is critical for synaptic plasticity, long-term memory and cognition (Day and Sweatt, 2011; Graff and Tsai, 2013) but whether epigenetic mechanisms are involved in *Fgf* transcription during these processes remains unknown.

Here we report that weak training in associative learning induces CRTC1 translocation from synapses to the nucleus, transiently activating *Fgf1b* transcription via phosphoCREB-CBP-mediated histone acetylation, a form of epigenetic regulation. In contrast to weak training, strong training substitutes histone acetyltransferase KAT5 (also referred to as Tip60) for CBP on the *Fgf1b* promoter in a CRTC1-dependent manner, but independently of CREB phosphorylation, and induces long-lasting *Fgf1b* transcription and stronger memory. Thus, we describe a molecular mechanism that links the intensity of associative learning via strength of synaptic activity to the level of gene transcription and consecutive memory strength.

RESULTS

Neuronal stimulation- and learning-dependent transcription of *Fgf1b* in the cornu ammonis region of the hippocampus

We measured mRNA levels of 17 *Fgfs*, including brain-specific *Fgf1b* (Alam et al., 1996) and kidney- and liver-enriched *Fgf1g* (Zhang et al., 2001) in primary hippocampal neuronal cultures treated with the $GABA_A$ receptor antagonist bicuculline to induce action potential bursting in the absence or presence of the NMDA receptor antagonist MK-801. Quantitative real-time PCR (Q-PCR) revealed that the bicuculline treatment significantly increased *Fgf1b*, *Fgf2*, and *Fgf14* mRNA transcription, which was abolished by MK-801 (Figure 1A). The levels of *Fgf4*, *Fgf6*, *Fgf8*, *Fgf15/19*, *Fgf21*, and *Fgf23* mRNAs were undetectable. Mice injected with bicuculline or potassium chloride (KCl) into the hippocampus showed enhanced *Fgf1b* and *Fgf2* expression in the cornu ammonis (CA) region (Figure 1B).

We examined whether *Fgf* expression is induced by hippocampus-dependent contextual fear conditioning (CFC) using five behavioral groups: home cage (HC), context only, immediate

shock, 1 shock (weak CFC training), and 3 shocks (strong CFC training) (Figure 1C). Contextual fear memory (CFM) 24 h after training was highest in mice that received strong training (Figure 1D). Q-PCR of the CA revealed increased *Fgf1b* expression 1 h after CFC with no significant differences between 1- and 3-shock groups (Figure 1E and S1A). Importantly, *Fgf1b* mRNA expression in CA returned to baseline 2 h following 1-shock training, while it was still elevated following 3-shock training (Figures 1F and 1G). No change in *Fgf1b* expression was found in the dentate gyrus (DG) (Figures 1H, S1B, and S1C). Western blotting revealed a significant increase in FGF1 protein levels 2 h following 3-shock but not 1-shock CFC (Figure 1I). We also examined *Fgf1b* expression in the CA in object location learning (OLL), a form of hippocampus-dependent associative recognition memory (Barker and Warburton, 2015)). *Fgf1b* mRNA was enhanced 2 h following 15 min (strong) training but not 3 min (weak) training (Figures S1D–S1G). No changes in *Fgf1b* expression were observed in the DG following either weak or strong training in OLL (Figure S1H). There were no significant differences in expression of immediate-early genes (IEGs), *c-fos* and *Arc*, between mice received 1- and 3-shock CFC (Figures 1J and 1K), but they were induced in the context-only or immediate-shock groups (Figures 1L and 1M). Thus, *Fgf1b* induction in the CA is directly correlated with strength of training and is specific to associative learning in our experimental settings.

Hippocampal FGF1 enhances maintenance of synaptic plasticity and improves associative memory

We tested the effects of the FGF receptor antagonist PD173074 on long-term potentiation (LTP) of synaptic transmission at excitatory synapses between CA3 and CA1 pyramidal neurons (CA3-CA1 synapses). Input-output curves and paired-pulse ratio (PPR) were comparable between PD173074- and vehicle-treated hippocampal slices (Figures 2A and 2B) suggesting that PD173074 does not change basal synaptic transmission and short-term synaptic facilitation. Strong high-frequency stimulation (3 × HFS) elicited robust LTP at CA3-CA1 synapses, which was significantly attenuated by PD173074 (Figure 2C). In reverse experiments we used the recombinant FGF1. It had no effect on input-output relationship or PPR (Figures 2D and 2E). However, the recombinant FGF1 enhanced and prolonged transient CA3-CA1 LTP induced by weak high-frequency stimulation (1 × HFS) (Figure 2F). LTP in vehicle-treated slices returned to the baseline on average within approximately 100 minutes after stimulation, whereas LTP in FGF1-treated slices lasted substantially longer. Thus, FGF1 signaling appears to be necessary for the transition from transient plasticity to sustained plasticity.

We investigated the role of hippocampal FGF1 in memory formation. Hippocampal injection of PD173074 1 h before strong (3-shock) CFC training did not alter short-term (0.5 h) memory but disrupted long-term (24 h) memory (Figures 2G, 2H, and S2A). Infusion 1 h but not 96 h after strong training also disrupted CFM (Figures 2I, 2J, and S2A). Conversely, recombinant FGF1 infusion 1 h after weak (1-shock) training enhanced long-term (24 h) CFM (Figures 2K, 2L, and S2A). In addition, hippocampal injection of recombinant FGF1 1 h after a weak (3 min) OLL session increased long-term object location memory (OLM) (Figures S2B and S2C).

To further explore the function of FGF1 in hippocampal CA subregion in memory formation, we bilaterally injected mice with the adeno-associated virus (AAV) vectors expressing an interfering short hairpin RNA (shRNA) targeting *Fgf1* (AAV-shFGF1) or shRNA-resistant *Fgf1* (AAV-FGF1res) (Figure 2M). Western blotting confirmed successful knockdown of FGF1 protein expression in mice injected with AAV-shFGF1 and elevated expression of FGF1res in mice injected with AAV-FGF1res (Figure 2N). Additionally, immunohistochemistry revealed that the markers mCherry (shFGF1) and GFP (FGF1res) are localized specifically in the CA region of the hippocampus (Figure 2O). Mice injected with AAV-shFGF1 exhibited significantly reduced long-term CFM, but unaltered short-term CFM, in response to strong 3-shock CFC training, and this CFM deficit was rescued by co-injection of AAV-FGF1res (Figures 2P and 2Q). Mice injected with AAV-shFGF1 also showed reduced long-term OLM, which again was rescued by FGF1res overexpression (Figures S2D and S2E). These results further support the notion that FGF1 signaling in CA is required for sustained synaptic plasticity and memory enhancement.

CRTC1 is required for learning-dependent induction of *Fgf1b*

We examined whether *Fgf1b* transcription is regulated by CREB because there are at least two putative CRE sites (CRE1 and CRE2) on the *Fgf1b* promoter (Figure 3A). A chromatin immunoprecipitation (ChIP) assay revealed that phospho-activated CREB (phosphorylated at Ser133, pCREB) occupancy at both CRE1 and CRE2 sites 2 h after CFC was comparable among mice receiving 1-shock or 3-shock training and home cage controls (Figure 3B). pCREB levels were induced similarly in the CA1 and CA3 subregions of mice receiving 1-shock or 3-shock CFC (Figures S3A and S3B). These results suggest that strong-training-induced sustained *Fgf1b* expression may be independent of CREB phosphorylation. Given that CRTCs enhance CREB-mediated transcriptional activity independently of CREB phosphorylation (Conkright et al., 2003; Iourgenko et al., 2003), we speculated that CRTC1 is required for sustained expression of *Fgf1b* following strong CFC training. Indeed, ChIP assay revealed significantly higher CREB and CRTC1 occupancies on the *Fgf1b* promoter CRE1 site 2 h after 3-shock CFC compared to home cage controls (Figures 3C and 3D). ChIP assays showed increased pCREB occupancy on the *Fgf1b* promoter 0.5 h after strong 3-shock training, but this binding was transient (Figure 3E), while CREB occupancy on the *Fgf1b* promoter was increased 2 h following strong 3-shock CFC training (Figure 3F). Weak training (1-shock CFC) elicited transient CRTC1 occupancy on the *Fgf1b* promoter, whereas 3-shock CFC induced sustained CRTC1 occupancy (Figure 3G). To validate our ChIP assay, we measured pCREB, CREB, and CRTC1 occupancy on the *Fgf1g* promoter, as *Fgf1g* expression was not affected by neuronal stimulation or CFC (Figures 1A, 1B, and 1E). Indeed, there were no significant effects of CFC on pCREB, CREB, and CRTC1 occupancies on the *Fgf1g* promoter (Figures 3H–3J). We also performed a ChIP assay to measure the occupancies of these molecules on the *c-fos* promoter. Occupancies of pCREB and CRTC1 were increased at 0.5 h but returned to baseline 1 h following CFC, and there were no significant differences in occupancy between mice receiving 1-shock and 3-shock CFC (Figures 3K–3M).

Are there differences in binding of CRTC1 to CREB between weak and strong learning? Immunohistochemistry revealed that CRTC1 and CREB are co-localized in CA1 and CA3

subregion (Figure S3C). Immunoprecipitation indicated increased binding of CRTC1 to CREB in CA following 1-shock CFC compared to home cage control mice and even greater binding following 3-shock CFC (Figure S3D). Although CFC also increased the binding of CRTC1 to pCREB, there was no significant difference between 1-shock and 3-shock CFC groups (Figure S3E). Western blotting also revealed no significant difference in pCREB levels between mice receiving 1-shock and 3-shock CFC (Figures S3F). Thus, strong-learning-induced enhancement of *Fgf1b* expression is independent of pCREB but requires CRTC1. Indeed, a luciferase reporter assay revealed that *Fgf1b* promoter activity in primary mouse hippocampal neurons stimulated with bicuculline and forskolin was enhanced by transfection of wild type CRTC1 vector (Figure S3G), suggesting a direct contribution of CRTC1 to *Fgf1b* transactivation.

CRTC1 is required for synaptic plasticity and memory enhancement

To determine if CRTC1 deficiency affects CA3-CA1 synaptic plasticity, we constructed AAV vectors to overexpress shRNA targeting *crtc1* (AAV-shCRTC1-GFP, Figure 3N). CRTC1, but not CRTC2, was successfully knocked down following injection of the shCRTC1 vector into the CA1 (Figures 3O and 3P). The robust LTP at CA3-CA1 synapses induced by strong stimulation (3 × HFS) in control mice was significantly attenuated by shCRTC1 overexpression (Figure 3Q).

Mice overexpressing shCRTC1 in CA1 exhibited normal short-term CFM but reduced long-term CFM (Figures 3R and S3H). Similarly, mice overexpressing shCRTC1 in CA3 showed normal short-term CFM but reduced long-term CFM (Figures S3I–S3K). Moreover, Q-PCR revealed that upregulation of *Fgf1b* 2 h after 3-shock CFC was prevented by shCRTC1 overexpression (Figure 3S). To further confirm that CRTC1 is necessary for memory enhancement, we overexpressed a dominant-negative mutant (dnCRTC1) consisting of the N-terminal 44 amino acids containing the CREB binding site but lacking the transactivation domain (Bittinger et al., 2004; Zhou et al., 2006) via AAV-mediated gene transfer (Figure 3T). Transfection of primary mouse hippocampal neurons with this dnCRTC1 vector abolished enhanced *Fgf1b* promoter-driven luciferase reporter activity induced by bicuculline and forskolin stimulation (Figure S3G). Western blotting and immunohistochemistry revealed successful overexpression of dnCRTC1-GFP in CA1 and CA3 (Figures 3U, S3L, and S3M). Mice overexpressing dnCRTC1 in CA1 (Figure 3V) or CA3 (Figure S3N) exhibited reduced long-term CFM following strong training, suggesting that CRTC1 is critical for sustained *Fgf1b* expression and memory enhancement.

Learning induces CRTC1 nuclear translocation

How can CRTC1, localized to dendrites and synapses in hippocampal neurons (Ch'ng et al., 2012), affect the nuclear transcriptional machinery? We examined whether CFC induces nuclear accumulation of CRTC1 in the mouse hippocampus. CRTC1 immunoreactivity was higher in CA1 and CA3 (but not DG) of mice received CFC compared to mice exposed to shock or context only and higher in mice receiving 3-shock CFC compared to 1-shock CFC (Figures S4A–S4L). We also found that strong training in OLL (15 min exposure, which induces sustained memory (Figures S1D–S1H)), led to an increase in CRTC1 immunoreactivity in CA, while weak training (3 min exposure) did not (Figures S4M and

S4N). Western blotting also showed that reduced CRTC1 expression in the postsynaptic density (PSD) fractions was greater in mice receiving 3-shock training compared to 1-shock training, while there was no difference in whole-cell CRTC1 levels between groups (Figures S5A and S5B). Q-PCR revealed no difference in CRTC1 mRNA levels among home cage controls, 1-shock training, and 3-shock training groups (Figure S4J). Thus, subcellular redistribution of CRTC1 is not due to altered expression of total mRNA or protein. Furthermore, administration of the protein synthesis inhibitor anisomycin did not affect the CFC-induced increase in nuclear CRTC1 (Figures S5D and S5E), while c-Fos induction was diminished following learning, confirming anisomycin efficacy (Figure S5D). Moreover, hippocampal injection of the proteasome inhibitor clasto-lactacystin β-lactone (LAC) did not affect the learning-induced reduction in synaptic CRTC1 (Figures S5F and S5G). These results suggest synapse-to-nucleus translocation of CRTC1 following learning.

Deficits in microtubule-mediated intracellular transport impair synaptic plasticity and memory formation (Shumyatsky et al., 2005; Uchida et al., 2014; Uchida and Shumyatsky, 2015), suggesting that nuclear translocation of CRTC1 may be dependent on microtubules. Injection of nocodazole, a microtubule destabilizer, into the hippocampus 1 h before 3-shock CFC blocked the increase in nuclear CRTC1 and the decrease in synaptic CRTC1 but did not change whole-cell CRTC1 levels, as measured 1 h following CFC (Figure S5H). Furthermore, nocodazole reduced long-term CFM (Figures S5I and S5J) and suppressed sustained *Fgf1b* expression 2 h following 3-shock CFC (Figure S5K). These results suggest that microtubule-mediated retrograde transport of CRTC1 from the synapse to the nucleus is required for *Fgf1b* expression and memory enhancement. The CFC-dependent nuclear translocation of CRTC1 occurred exclusively in excitatory neurons within CA1 and CA3 (Figure S5M).

Nuclear translocation of CRTC1 required for memory formation is regulated by calcineurin

Because nuclear-cytoplasmic redistribution of CRTCs is known to depend on their phosphorylation status (Altarejos and Montminy, 2011), we generated CRTC1 mutants in which Ser151 and/or Ser167 were mutated to Ala (CRTC1-S151A, CRTC1-S167A, CRTC1-S151A/S167A (CRTC1-2SA)). These mutant CRTC1s were primarily localized to the cytoplasm of unstimulated primary hippocampal neurons but showed nuclear localization similar to wild-type CRTC1 following KCl and forskolin stimulation (Figures 4A and 4B). One hour after KCl and forskolin removal (washout), nuclear wild-type and mutant CRTC1s CRTC1-S151A and CRTC1-S167A returned to basal levels, whereas CRTC1-2SA remained elevated in the nucleus (Figures 4A and 4B). We injected AAV vector expressing CRTC1-2SA (Figure 4C) into either CA1 or CA3 (Figures 4D, 4E, and S5N) and found that mice overexpressing CRTC1-2SA in CA1 (Figure 4F) or CA3 (Figure S5O) exhibited increased long-term CFM in response to weak training.

Nuclear translocation of CRTC1 in hippocampal neurons treated with bicuculline was also blocked by pretreatment with a calcineurin inhibitor (Figure S5P), so we constructed a CRTC1 mutant lacking two consensus calcineurin-binding motifs (PxIxIT) (Screaton et al., 2004). This mutant disrupted bicuculline-induced nuclear translocation of CRTC1 in hippocampal neurons (Figure 4G) and thus represents a CRTC1 with constitutive cytosolic

localization (CRTC1cyt). To provide additional evidence that CRTC1 nuclear translocation is necessary for memory enhancement, we injected AAVs expressing shCRTC1 or shControl together with AAVs expressing shRNA-resistant CRTC1 (CRTC1res), shRNA-resistant CRTC1cyt, or mCherry into the CA subregion (Figures 4H and 4I). Mice injected with both AAV-CRTC1res and AAV-shCRTC1 showed significantly greater freezing 24 h after CFC training compared to mice injected with AAV-shCRTC1 alone (previously shown to cause deficient CFM, Figure 3R). This rescue was not seen in mice injected with CRTC1cyt plus AAV-shCRTC1 (Figure 4J). In addition, the reduced long-term OLM in mice given AAV-shCRTC1 was rescued by CRTC1res, but not CRTC1cyt, overexpression (Figures S5Q and S5R). Furthermore, suppressed *Fgf1b* expression 2 h following 3-shock CFC in mice injected with AAV-shCRTC1 was rescued by CRTC1res but not by CRTC1cyt overexpression (Figure 4K). These results suggest a critical role for calcineurin-mediated CRTC1 nuclear translocation and resulting sustained *Fgf1b* expression in memory enhancement.

Epigenetic mechanisms for sustained *Fgf1b* expression

To examine the role of the CRTC1-CREB in *Fgf1b* expression following strong learning, we analyzed histone acetylation on lysine (K) residues at the *Fgf1b* promoter following 1- or 3-shock CFC. ChIP assay revealed that acetylation levels of H3K9 and H3K14, but not of H4K8 or H4K16, were significantly increased 0.5–1 h after both strong and weak CFC (Figures 5A and S6A–S6C). In contrast, acetylation of H4K5 was increased 1 h after CFC only in mice receiving 3-shock training (Figure 5B). There was also a sustained increase in H4K12 acetylation in mice receiving 3-shock CFC but not 1-shock training (Figure 5C). In contrast, there were no significant effects of strength of learning on histone acetylation at the *c-fos* promoter (Figures 5E–5G and S6D–S6F).

The histone acetyltransferase CBP regulates synaptic plasticity and memory formation (Alarcon et al., 2004; Wood et al., 2005). We speculated that the levels of CBP occupancy on the *Fgf1b* promoter would differ between mice receiving 1-shock and 3-shock CFC. Unexpectedly, however, ChIP assay showed a comparable increase in CBP recruitment to the *Fgf1b* in both groups (Figure 5D), which was similar in magnitude to CBP recruitment at the *c-fos* promoter (Figure 5H). Thus, CBP does not mediate the specific epigenetic modifications associated with strong learning.

Given that ChIP assay indicated enrichment of acetylated H4K5 and H4K12 at the *Fgf1b* promoter by strong but not weak CFC, we examined histone acetyltransferase KAT5, which is known to enhance H4K5 and H4K12 acetylation (Grezy et al., 2016; Kouzarides, 2007; Wee et al., 2014). ChIP assay revealed KAT5 recruitment to the *Fgf1b* promoter 2 h following 3-shock CFC but not 1-shock CFC (Figure 5I), while there was no difference in recruitment to the *c-fos* promoter (Figure 5J). Therefore, KAT5 recruitment to the *Fgf1b* promoter is associated with the specific increase in H4K12 acetylation following strong learning, leading to sustained *Fgf1b* transcription.

We also investigated the effects of learning on the recruitment of histone deacetylases (HDACs) to the *Fgf1b* promoter. ChIP assay revealed progressive dissociation of HDAC3 and corepressor N-CoR, which can interact with HDAC3, from the *Fgf1b* promoter

following 3-shock but not 1-shock CFC (Figures 5K and 5L). In contrast, we observed no changes in HDAC1 and HDAC2 occupancy on the promoter following CFC (Figures S6G and S6H). These results suggest that basal *Fgf1b* transcription is suppressed by recruitment of HDAC3-N-CoR to its promoter. Together with the data shown in Figures 3E–3G, recruitment of the CRTC1-pCREB-CBP complex to the promoter following training enhances H3K14 acetylation and transiently activates *Fgf1b* transcription (Figure 5M). Alternatively, following strong learning, KAT5 is recruited to the promoter independently of CREB phosphorylation and enhances H4K12 acetylation, leading to sustained *Fgf1b* transcription (Figure 5M). Strong learning does not recruit KAT5 to the *c-fos* promoter, which may explain the transient induction of *c-fos* transcription following both weak and strong training (Figure 5M).

HDAC3 inhibition leads to *Fgf1b* transactivation and memory enhancement

To test if HDAC3 removal from the *Fgf1b* promoter is required for *Fgf1b* induction and memory enhancement, we synthesized and injected T247, a potent and selective HDAC3 inhibitor (Figure 6A) (Suzuki et al., 2013) bilaterally into the hippocampus. T247 increased *Fgf1b* expression (Figure 6B) and enhanced H3K14 at the *Fgf1b* promoter (Figure 6C). T247 increased freezing 24 h after 1-shock CFC compared to vehicle-treated controls (Figure 6D). Similarly, mice bilaterally injected with AAV-shHDAC3 (Figures 6E and 6F) into CA exhibited reduced HDAC3 proteins (Figure 6G) and enhanced long-term CFM but not short-term CFM compared to mice injected with AAV-shControl (Figures 6H and 6I). This effect was reversed by overexpression of HDAC3res (Figure 6I) but not by overexpression of HDAC3-K25A (Figure 6I), a mutant lacking enzymatic activity (Sun et al., 2013). We also observed increased *Fgf1b* expression by HDAC3 knockdown, which was reversed by overexpression of shRNA-resistance *Hdac3* (HDAC3res) but not HDAC3-K25A (Figure 6J). These results suggest that HDAC3 is important for *Fgf1b* silencing and is a negative regulator of long-term memory.

KAT5 is critical for epigenetic enhancement of *Fgf1b* transcription, synaptic plasticity, and memory enhancement

We constructed an AAV vector expressing shRNA targeting *Kat5* (Figure 7A), injected it bilaterally into CA1 (Figures 7B and 7C) and measured LTP at CA3-CA1 synapses in acute hippocampal slices. Input-output curves and paired-pulse ratio (PPR) were comparable between slices from mice injected with AAV-shKAT5 or AAV-shControl (Figures 7D and 7E). LTP induced by strong 3 × HFS in slices from mice injected with AAV-shControl was significantly attenuated in slices from AAV-shKAT5-injected mice (Figure 7F).

Mice injected with AAV-shKAT5 showed normal short-term but reduced long-term CFM, and this long-term memory impairment was prevented by co-injection of an AAV vector encoding shRNA-resistance *Kat5* (AAV-KAT5res) (Figures 7G and 7H). At the molecular level, KAT5 knockdown suppressed both H4K12 acetylation at the *Fgf1b* promoter and *Fgf1b* expression 2 h after 3-shock CFC, effects prevented by KAT5res overexpression (Figures 7I and 7J). In contrast, there were no significant effects of KAT5 knockdown on H3K14 acetylation and *Fgf1b* expression 0.5 h after 3-shock CFC (Figures 7K and 7L). Furthermore, KAT5 knockdown had no effect on learning-induced enhancement of H3K14

acetylation at the *c-fos* promoter or *c-fos* expression following 3-shock CFC (Figures S7A and S7B). Together, these results suggest that KAT5-catalyzed histone acetylation leads to upregulation of specific genes associated with strong training-induced enduring memory.

We tested whether learning-induced nuclear translocation of CRTC1 is necessary for KAT5-mediated enhancement of *Fgf1b* transcription. We injected bilaterally into CA an AAV expressing shCRTC1 or shControl together with an AAV expressing CRTC1res, CRTC1cyt, or mCherry and quantified KAT5 occupancy at the *Fgf1b* promoter 2 h after 3-shock CFC. ChIP assay revealed that increased KAT5 recruitment to the *Fgf1b* promoter following learning was suppressed by shCRTC1, an effect rescued by overexpression of CRTC1res but not CRTC1cyt (Figure 7M). Concomitantly, the suppressed enhancement of H4K12 acetylation following 3-shock CFC in AAV-shCRTC1-injected mice was rescued by overexpression of CRTC1res but not CRTC1cyt (Figure 7N). These results suggest that learning-dependent nuclear translocation of CRTC1 is required for KAT5 recruitment and subsequent H4K12 acetylation at the *Fgf1b* promoter.

DISCUSSION

We show that associative learning induces calcineurin-dependent transport of CRTC1 along microtubules into the nucleus of excitatory neurons of the hippocampal CA subregion. This is followed by the removal of HDAC3-N-CoR corepressor complex from the *Fgf1b* promoter. Following weak training this leads to pCREB and CBP recruitment, H3K14 acetylation, and transient *Fgf1b* expression (0.5–1 h following training) (Figure S8). Strong training induces long-lasting *Fgf1b* transcription (2 h following training), due to CRTC1-mediated substitution of KAT5 for CBP independently of pCREB, leading to increased H4K12 acetylation (Figure S8) and memory enhancement. Disruption of this signaling pathway weakens LTP and reduces memory. Thus, nuclear translocation of CRTC1 and subsequent initiation of KAT5-dependent epigenetic modifications are critical for hippocampal synaptic plasticity and memory enhancement.

Learning-dependent nuclear translocation of CRTC1 is required for memory enhancement

We showed activity-dependent nuclear transport of CRTC1 in the hippocampal CA subregion using two types of hippocampus-dependent associative learning, confirming previous reports (Parra-Damas et al., 2016; Parra-Damas et al., 2014). We also found that strong, compared to weak, training induces greater nuclear translocation of CRTC1, suggesting that it may signal the strength of synaptic activity to the nucleus. Neuronal activity leads to calcium influx and calcium, in turn, activates calcineurin (Figure S8), which dephosphorylates CRTC1 at Ser151 leading to CRTC1 nuclear translocation (Bittinger et al., 2004; Ch'ng et al., 2012; Screaton et al., 2004). Our CRTC1 mutant lacking two calcineurin binding sites (CRTC1cyt) was confined to the cytoplasm following neuronal stimulation. The shRNA targeting CRTC1 disrupted CFM, which was rescued by a fully functional CRTC1, but not by CRTC1cyt. CRTC1 knockdown prevented KAT5 recruitment, H4K12 acetylation at the *Fgf1b* promoter, upregulation of *Fgf1b* transcription and memory strengthening; however, these processes were rescued by overexpression of functional shRNA-resistant CRTC1. These results suggest that calcineurin-dependent nuclear

translocation of CRTC1 is critical for KAT5-mediated histone acetylation at the *Fgf1b* promoter and strengthening of memory. Contrary to previous reports that phosphorylation at both Ser151 and Ser167 is required for CRTC1 nuclear translocation (Bittinger et al., 2004; Ch'ng et al., 2012; Sasaki et al., 2011) (Figure S8), our CRTC1-S151A/S167A (CRTC1-2SA) mutant was localized to the cytoplasm under basal conditions. This is in agreement with a report showing that the CRTC1-S151A mutant does not move to the nucleus (Ch'ng et al., 2012). Our CRTC1-2SA mutant was transported to the nucleus upon stimulation with KCl and forskolin and it remained in the nucleus following neuronal stimulation. Thus, it is plausible that these two phosphorylation sites are important for nuclear retention rather than import, which is in agreement with a report showing that the S151 and S245 phosphorylation sites contribute to nuclear import of CRTC1 (Nonaka et al., 2014).

The relationship between CREB and CRTC1 in memory formation

CRTCs may play a major role in CREB-mediated transcription (Conkright et al., 2003; Iourgenko et al., 2003) and support, independent of CREB phosphorylation at Ser133, synaptic plasticity and memory (Bonni et al., 1995; Lonze and Ginty, 2002; Rammes et al., 2000). We found that weak training leads to the induction of *Fgf1b* and *c-fos* via CREB phosphorylation and recruitment of CBP and CRTC1. However, the CREB phosphorylation was transient, and strong training, while maintaining CRTC1 and CREB at the *Fgf1b* promoter, also employed KAT5 and thus allowed for sustained *Fgf1b* transcriptional activity. Thus, CRTC1 acts as a switch allowing pCREB-independent gene expression and memory strengthening.

Epigenetic control of *Fgf1b* and memory formation

We found that *Fgf1b* is blocked under basal conditions by the HDAC3-N-CoR corepressor but pharmacological or virus-mediated acute HDAC3 downregulation increases *Fgf1b* transcription and long-term memory, confirming an earlier report that HDAC3 inhibits memory (McQuown et al., 2011). In contrast, a prolonged HDAC3 depletion reduces memory (Nott et al., 2016). The discrepancy between this and our study may be due to the duration of HDAC3 deficiency.

Our finding that weak training recruits CBP acetyltransferase and H3K14 acetylation is supported by the fact that CBP knockout in CA1 decreases H3K14, but not H4K12, acetylation (Barrett et al., 2011). We find that strong training employs KAT5 acetyltransferase and H4K12 acetylation, which is confirmed by the report that KAT5 mediates H4K5 and H4K12 acetylation (Grezy et al., 2016; Kouzarides, 2007; Wee et al., 2014). Thus, weak training induces H3K14 acetylation by CBP at the *Fgf1b* and *c-fos* promoters, whereas strong training induces H4K12 acetylation by KAT5 at the *Fgf1b* promoter. Therefore, a transfer from CBP to KAT5 on the *Fgf1b* promoter may be a central molecular switch underlying memory enhancement. In addition to our findings, dysregulation of H4K12 acetylation is involved in age-associated memory loss (Peleg et al., 2010). FGF1 may be involved in the pathophysiology of Alzheimer's disease (Lou et al., 2012; Tao et al., 2014; Yamagata et al., 2004) and recombinant FGFs have been used to treat

multiple disorders (Beenken and Mohammadi, 2009). Therefore, *Fgf1b* and the components of its transcriptional machinery may be a target for cognitive enhancement therapy.

EXPERIMENTAL PROCEDURES

Animals

Adult (8–9 weeks of age) male C57BL/6J mice were purchased from The Jackson Laboratory and Charles River Japan and were maintained under a 12 h/12 h light/dark cycle and provided with mouse chow and water *ad libitum*. The care and use of animals was reviewed and approved by the Rutgers University Institutional Animal Care and Use Committee, by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee, and by the Yamaguchi University Animal Care and Use Committee.

Statistical analysis

Analyses of the data were performed using an appropriate analysis of variance. Significant effects were determined using Fisher's *post hoc* test or Bonferroni's correction. Unpaired Student *t*-tests were used for two-group comparisons. In all cases, p values were two-tailed, and the comparisons were considered to be statistically significant when $p < 0.05$. All data are presented as the mean ± SEM.

Additional materials and methods are described in the Supplemental Experimental Procedures section. The information regarding the primary antibodies used in this study is summarized in Table S1. The primer sequences used for Q-PCR, ChIP assay, and cloning are shown in Table S2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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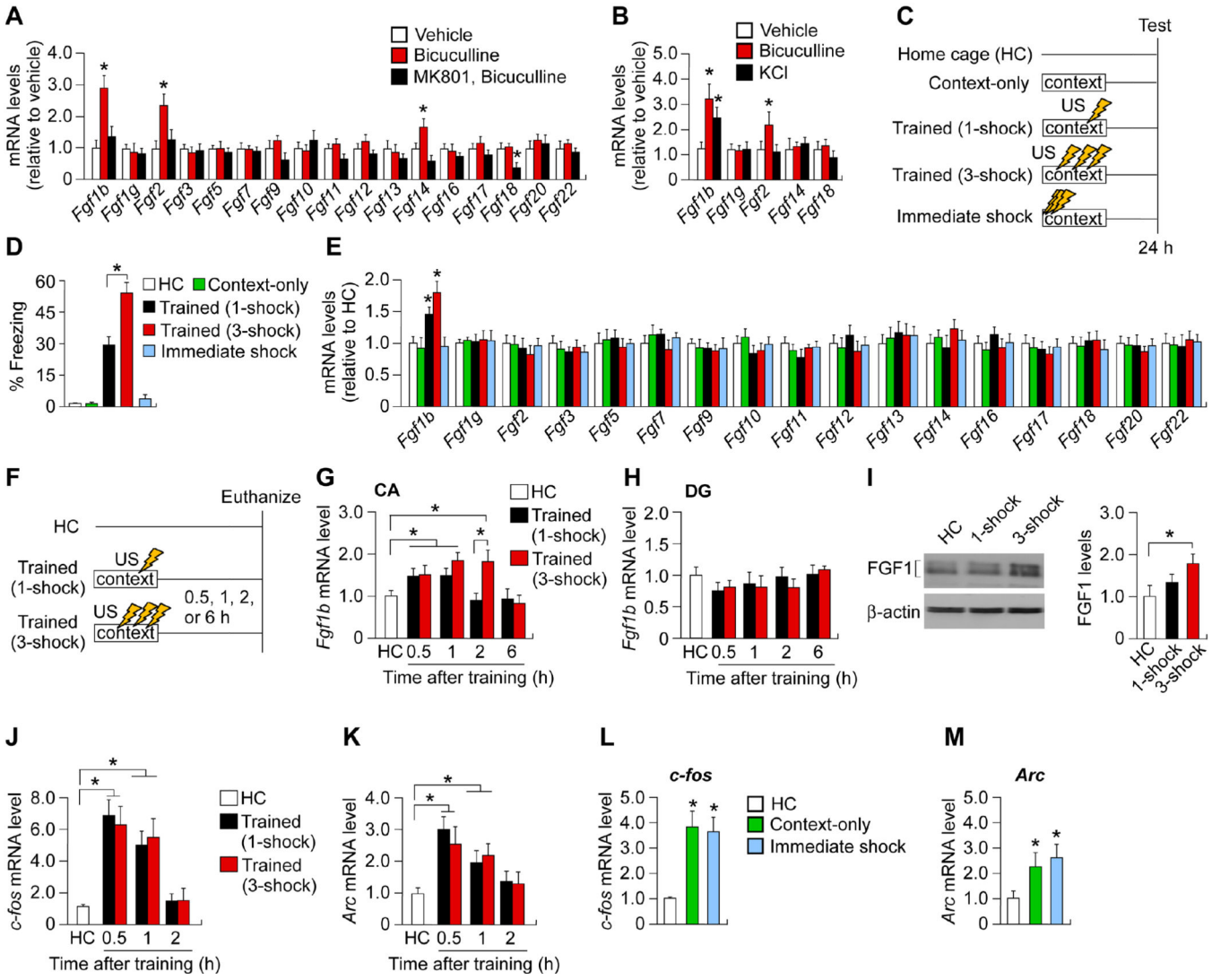

Figure 1. Neuronal activity- and training strength-dependent expression of *Fgf1b*

(A) Q-PCR analysis of the *Fgf* family mRNA levels in primary hippocampal neurons after bicuculline stimulation in the absence or presence of MK-801. $n = 4$ independent cultures. $*p < 0.05$ vs. vehicle.

(B) Q-PCR analysis of *Fgf* family mRNA levels in CA after bicuculline or potassium chloride (KCl) intra-hippocampal injections. $n = 6$ mice/group. $*p < 0.05$ vs. vehicle.

(C) A scheme for CFC. Following weak (1-shock) or strong (3-shocks) training fear memory (CFM) to context was assessed 24 h later. US, unconditioned stimulus (shock).

(D) Mice receiving 3-shock CFC exhibited greater freezing than mice receiving 1-shock CFC. $n = 10$ mice/group. $*p < 0.05$.

(E) Q-PCR analysis of *Fgf* family mRNAs in CA 1 h after CFC. $n = 6$ mice/group. $*p < 0.05$ vs. HC controls.

(F–H) Experimental design (F) for Q-PCR analysis of *Fgf* family mRNA levels over time in CA

(G) and DG (H) in mice after 1- or 3-shock CFC. $n = 6$ mice/group. $*p < 0.05$.

(I) Western blot of FGF1 levels in CA in mice 2 h after 1- or 3-shock CFC. $n = 6$ mice/group. $*p < 0.05$.
(J and K) Q-PCR analysis of *c-fos* (J) and *Arc* (K) mRNA expression in CA in mice subjected to 1- or 3-shock CFC. $n = 6$ mice/group. $*p < 0.05$.
(L and M) Q-PCR analysis of *c-fos* (L) and *Arc* (M) mRNA expression in CA in mice exposed to context alone or immediate shock. $n = 6$ mice/group. $*p < 0.05$ vs. HC controls. All data presented as the mean ± SEM. See also Figure S1.

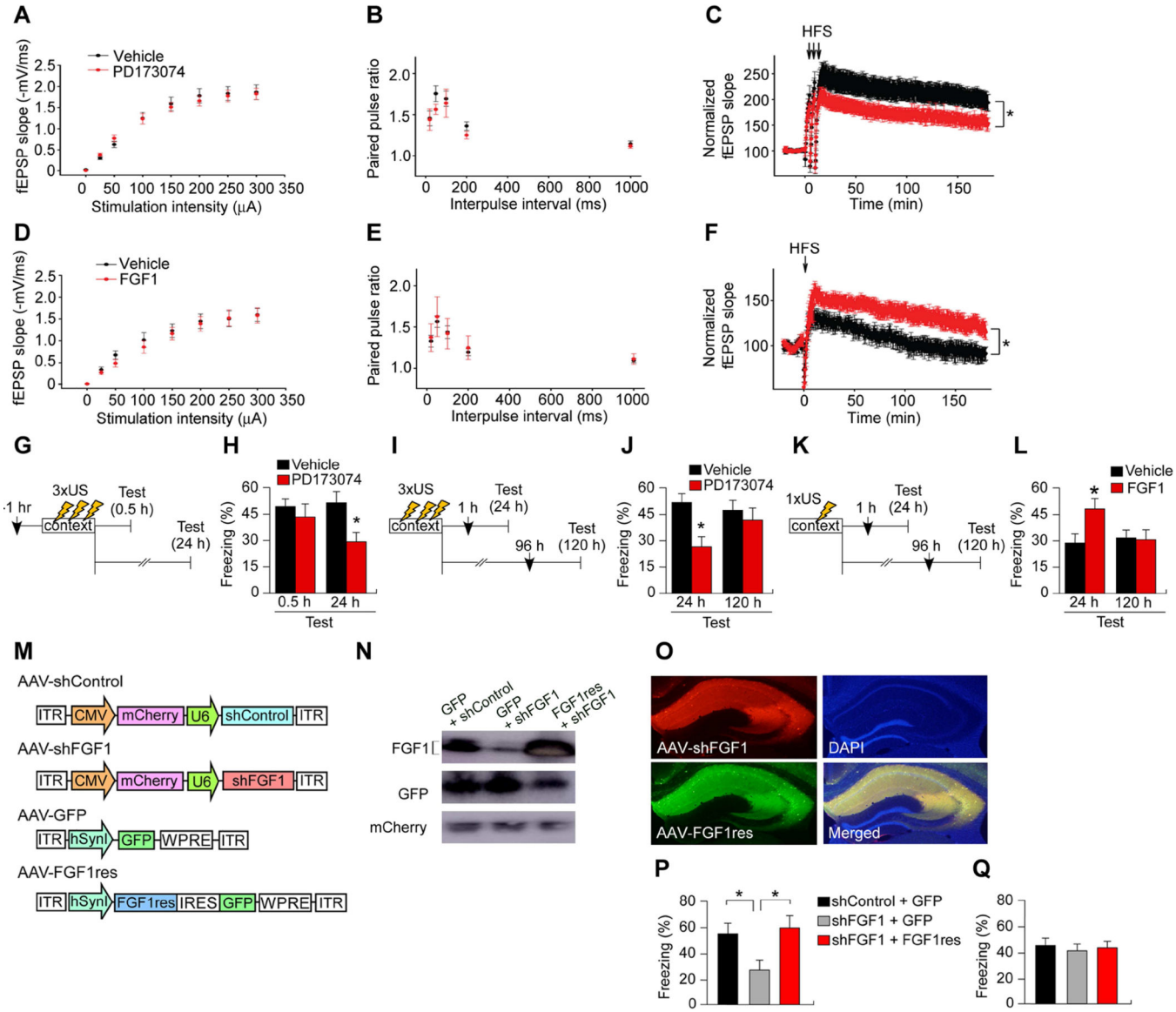

Figure 2. FGF1 is required for long-term potentiation and memory enhancement

(A–C) Effect of the FGF receptor antagonist PD173074 on (A) input-output relationship (fEPSP slope in response to 50 μA–300 μA synaptic stimulations), (B) paired pulse ratio, and (C) 3 × HFS-evoked LTP at CA3-CA1 synapses. HFS: high frequency stimulation. Vehicle, 26 slices; PD173074, 30 slices. *$p < 0.05$.

(D–F) Effect of recombinant FGF1 on (D) synaptic the input–output relation, (E) paired pulse ratio, and (F) weak stimulus (1 × HFS)-evoked LTP. Vehicle, 11 slices; FGF1, 9 slices. *$p < 0.05$.

(G and H) Effect of PD173074 pretreatment on contextual fear memory (CFM). $n = 13$ or 14 per group. *$p < 0.05$ vs. vehicle-treated group.

(I and J) Effect of PD173074 post-treatment on strong CFC training. $n = 13$ or 14 per group. *$p < 0.05$ vs. vehicle-treated group.

(K and L) Effect of recombinant FGF1 post-treatment on weak CFM training. $n = 12$ or 13 per group. $*p < 0.05$ vs. vehicle-treated group.

(M) AAV vectors engineered to overexpress shRNA targeting *Fgf1* (AAV-shFGF1), mock control (AAV-shControl), GFP (AAV-GFP), or shRNA-resistant *Fgf1* (AAV-FGF1res).

(N) Western blot showing knockdown of FGF1 by AAV-shFGF1 and rescue by AAV-FGF1res in CA.

(O) Successful transduction of mCherry and GFP in CA region by AAV vectors. Scale bar, 1 mm.

(P) Mice co-injected with AAV-shFGF1 and AAV-GFP showed decreased long-term (24 h) CFM following 3-shock CFC. This reduction was not observed in mice co-injected with AAV-shFGF1 and AAV-FGF1res. $n = 14–16$ per group. $*p < 0.05$.

(Q) Mice injected with the viruses described in panel (P) showed normal short-term (1 h) CFM following 3-shock CFC. $n = 10–13$ per group.

Data presented as mean ± SEM. See also Figure S2.

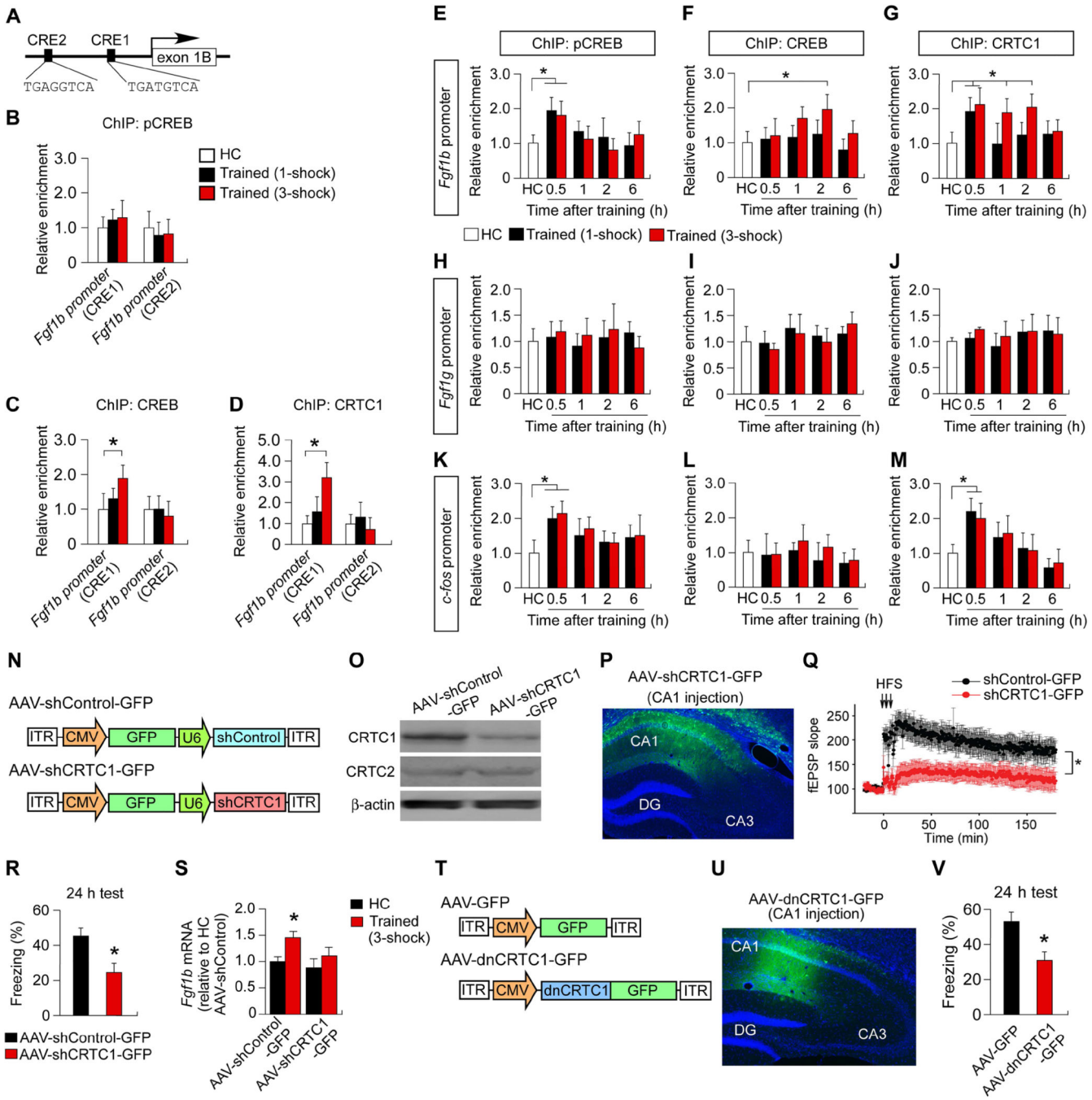

Figure 3. CRTC1 regulates *Fgf1b* expression, LTP, and memory

(A) Putative CRE sites within the mouse *Fgf1b* promoter. Arrows indicate major transcription start sites.

(B–D) ChIP assay showing recruitment of pCREB (B), CREB (C), or CRTC1 (D) to CRE1 and CRE2 sites following 1- or 3-shock CFC. $n = 6–8$ samples/group. $*p < 0.05$.

(E–M) ChIP assay showing recruitment of pCREB (E, H, K), CREB (F, I, L), or CRTC1 (G, J, M) to the *Fgf1b* (E–G), *Fgf1g* (H–J), or *c-fos* (K–M) promoter 0.5 h, 1 h, 2 h, and 6 h after 1- or 3-shock CFC. $n = 6–10$ samples/group. $*p < 0.05$.

(N) AAV vectors engineered to overexpress shRNA targeting *Crtc1* or a mock control under the U6 promoter. GFP is expressed under the CMV promoter.
(O) Western blot showing specific knockdown of CRTC1, but not CRTC2, in CA in mice injected with AAV-shCRTC1-GFP.
(P) GFP fluorescence following AAV-shCRTC1-GFP microinjection into CA1. Scale bar, 200 μm.
(Q) Effect of CRTC1 knockdown on CA3-CA1 LTP evoked by strong stimulation (3 × HFS) in hippocampal slices from mice injected with AAV-shCRTC1 (n=7) or AAV-shControl (n=8). $*p < 0.05$.
(R) Long-term CFM in mice injected with AAV-shCRTC1-GFP into CA1. $n = 13$ or 14 mice/group. $*p < 0.05$.
(S) Q-PCR analysis of *Fgf1b* mRNA expression in CA in mice injected with AAV-shCRTC1 or AAV-shControl 2 h after strong CFC. $n = 6$ mice/group. $*p < 0.05$ vs. HC AAV-shControl.
(T) AAV vectors overexpressing a dominant-negative CRTC1 (dnCRTC1) fused with GFP or GFP alone under the CMV promoter.
(U) GFP fluorescence after AAV-dnCRTC1-GFP microinjection into CA1. Scale bar, 200 μm.
(V) Long-term CFM in mice injected with AAV-dnCRTC1-GFP into CA1. $n = 13$ or 14 mice/group. $*p < 0.05$.
Data presented as mean ± SEM. See also Figure S3.

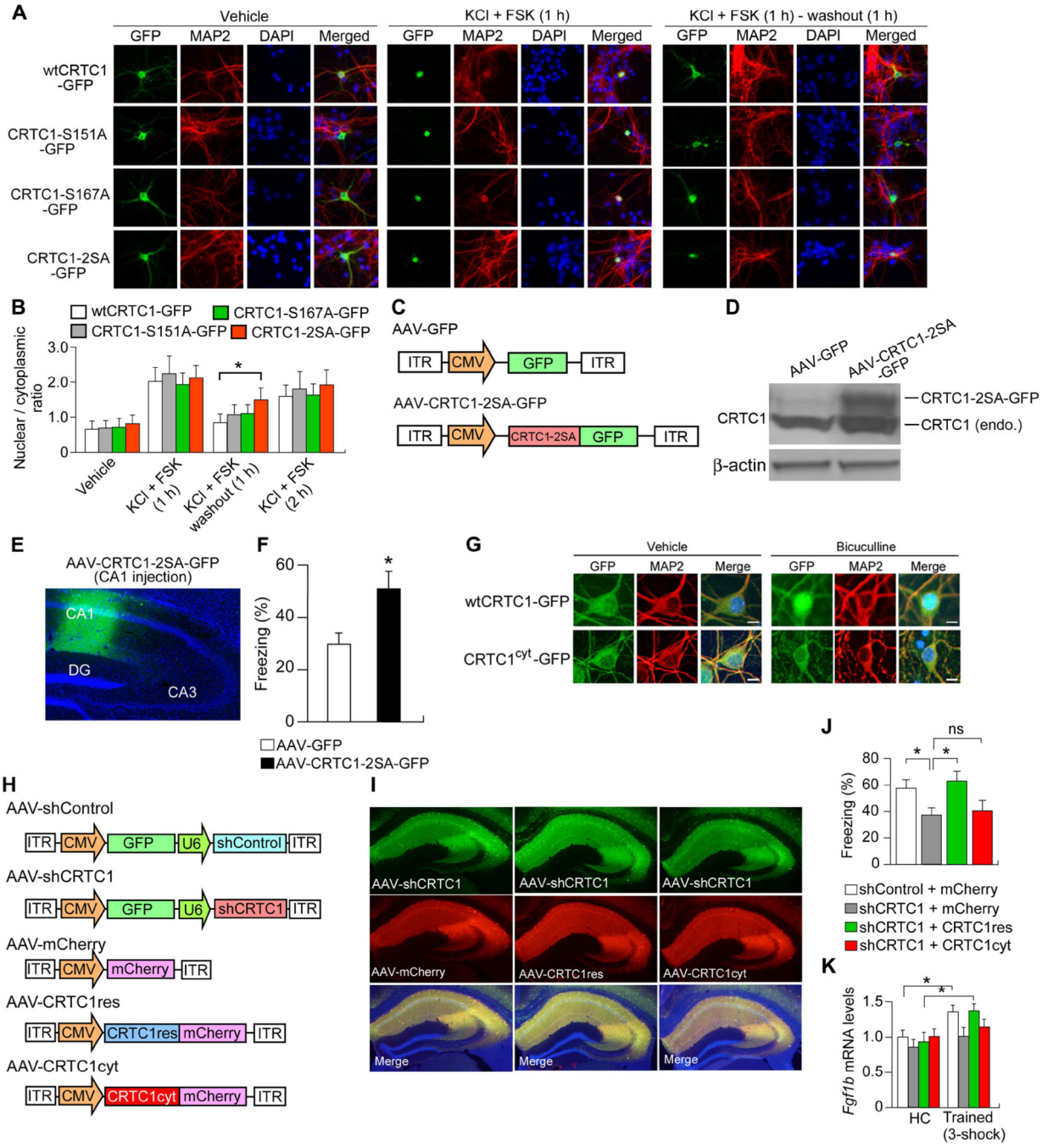

Figure 4. Calcineurin-dependent nuclear translocation of CRTC1 is required for memory enhancement

(A) Mouse primary hippocampal neurons were transiently transfected with either full-length CRTC1 (wtCRTC1) or a mutant CRTC1 [CRTC1-S151A, CRTC1-S167A, or CRTC1-S151A/S167A (CRTC1-2SA)] lacking the indicated serine phosphorylation sites. Each vector also encoded fused GFP. After 16 h, transfected neurons were incubated in 50 mM KCl and 20 μM forskolin for 1 h, followed by 1 h washout, fixation, and immunostaining using GFP (green) and MAP2 (red) antibodies and DAPI nuclear stain (blue). Scale bar, 100 μm.

(B) Nuclear-to-cytoplasmic ratio of GFP immunostaining from (A). *$p < 0.05$.
(C) AAV vector engineered to overexpress CRTC1-2SA-GFP.
(D) Western blot showing transduction of CRTC1-2SA-GFP.
(E) GFP fluorescence following AAV-CRTC1-2SA-GFP microinjection into CA1. Scale bar, 200 μm.
(F) Long-term CFM in mice injected with AAV-CRTC1-2SA-GFP into CA1. $n = 14$ or 15 mice/group. *$p < 0.05$.
(G) Mouse primary hippocampal neurons were transiently transfected with either wild-type CRTC1 (wtCRTC1) or mutant CRTC1cyt lacking calcineurin-binding motifs, each fused with GFP. After 16 h, transfected neurons were incubated in bicuculline for 1 h, fixed, and stained using GFP (green) and MAP2 (red) antibodies and DAPI (blue). Scale bar, 10 μm.
(H) AAV vectors overexpressing shRNA targeting *Crtc1*, mock control shRNA, mCherry, shRNA-resistant CRTC1, or shRNA-resistant CRTC1cyt.
(I) GFP and mCherry expression in CA following AAV microinjection. Scale bar, 1 mm.
(J) Overexpression of CRTC1res, but not CRTC1cyt, prevents shCRTC1-induced impairment of long-term CFM. n = 11–16 mice/group. *$p < 0.05$. n.s., not significant.
(K) Overexpression of CRTC1res, but not CRTC1cyt, rescues shCRTC1-induced suppression of *Fgf1b* expression following 3-shock CFC. n = 6–8 mice/group. *$p < 0.05$. Data presented as mean ± SEM. See also Figure S4 and S5.

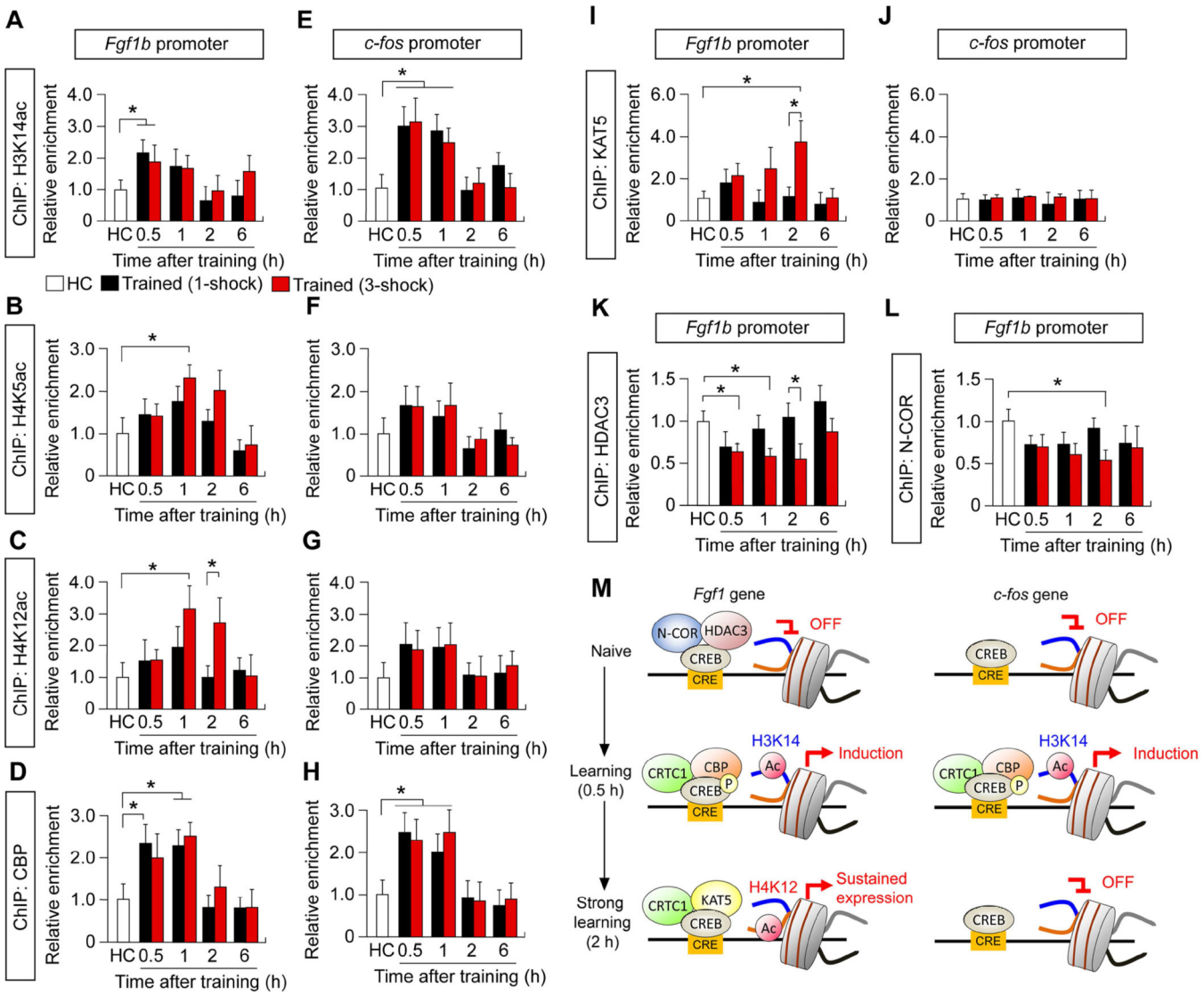

Figure 5. Learning-induced exchange of chromatin remodeling complexes at the *Fg1b* promoter

(A–L) ChIP assay showing H3K14ac (A, E), H4K5ac (B, F), H4K12ac (C, G), CBP (D, H), KAT5 (I, J), HDAC3 (K), and N-CoR (L) occupancy on the *Fgf1b* (A–D, I, K, L) and/or *c-fos* (E–H, J) promoter following 1- or 3-shock CFC. $n = 6–8$ samples/group. $*p < 0.05$.

(M) Cofactor occupancy on the *Fgf1b* promoter in response to learning. (Left) Under basal conditions, the HDAC3-N-CoR complex represses *Fgf1b* transcription. Learning promotes dissociation of this complex from the *Fgf1b* promoter, while nuclear CRTC1 can bind to p-CREB and CBP, enhancing H3K14 acetylation and subsequent transient *Fgf1b* transcription. Strong 3-shock CFC training maintains upregulation of *Fgf1b* transcription 2 h after learning by recruiting KAT5 to the promoter region independently of p-CREB, enhancing H4K12 acetylation. (Right) Strong training does not recruit KAT5 to the *c-fos* promoter.

Data presented as mean ± SEM. See also Figure S6.

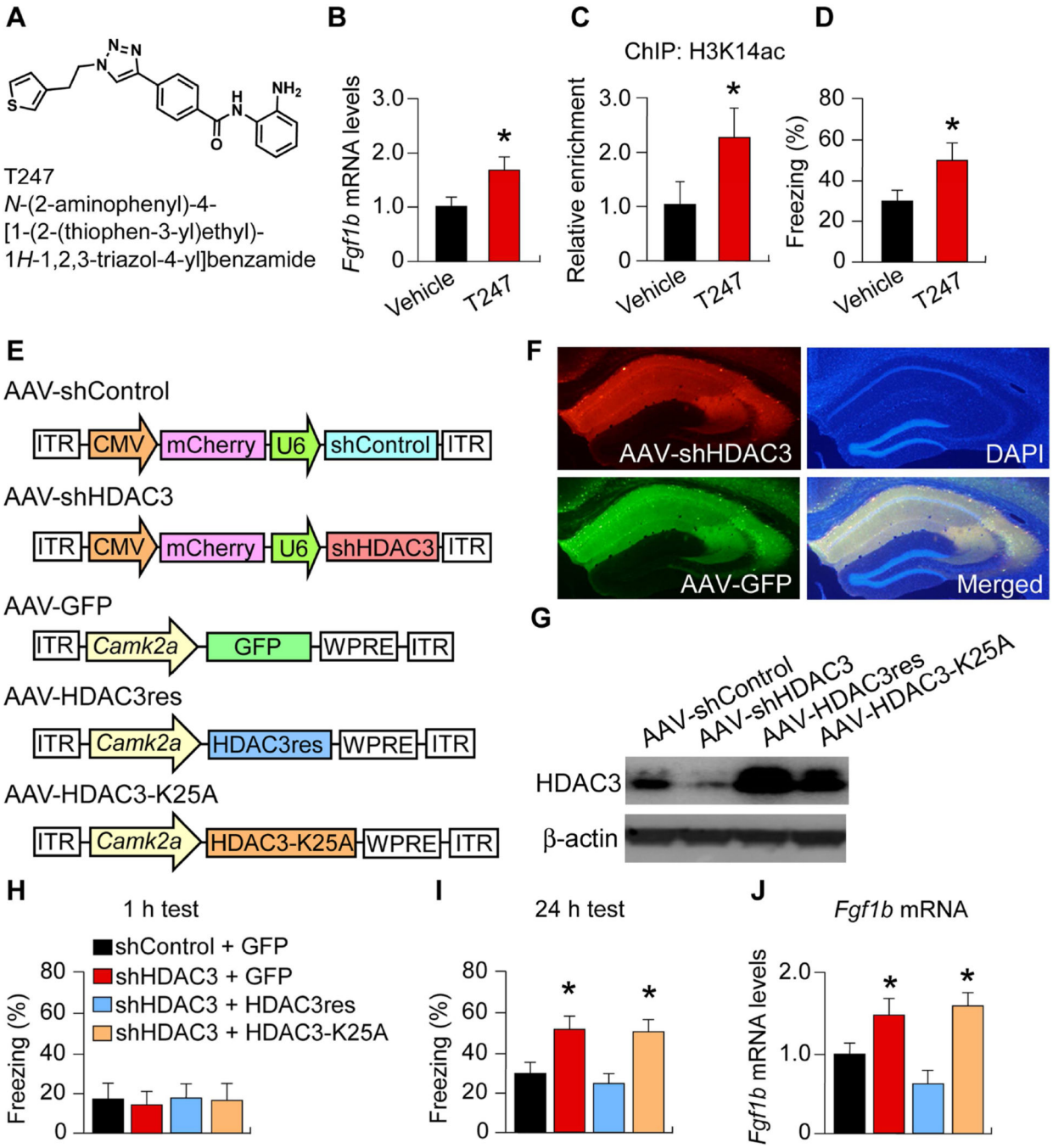

Figure 6. HDAC3 negatively regulates *Fgf1b* expression and memory formation

(A) Structure of T247, a potent and selective HDAC3 inhibitor.

(B) Intra-hippocampal injection of T247 enhances *Fgf1b* mRNA in the CA. n = 6 or 7 mice/group. *$p < 0.05$.

(C) ChIP assay showing H3K14ac occupancy on the *Fgf1b* promoter in mice receiving intra-hippocampal injection of T247 or vehicle. $n = 6–8$ samples/group. *$p < 0.05$.

(D) Increased long-term CFM in mice receiving intra-hippocampal injection of T247 compared to mice receiving vehicle. $n = 11$ or 12 mice/group. *$p < 0.05$.

(E) AAV vectors overexpressing shRNA targeting *Hdac3* (AAV-shHDAC3), mock control shRNA (AAV-shControl), GFP (AAV-GFP), shRNA-resistant HDAC3 (AAV-HDAC3res), or shRNA-resistant HDAC3-K25A lacking HDAC3 enzymatic activity (AAV-HDAC3-K-25A).
(F) mCherry and GFP expression in CA following AAV microinjection. Scale bar, 1 mm.
(G) Western blot showing knockdown of HDAC3 by AAV-shHDAC3 and overexpression of HDAC3res by AAV-HDAC3res, and HDAC3-K25A overexpression by AAV-HDAC3-K25A in CA1.
(H) Normal short-term CFM (1 h) in mice injected with AAV-shHDAC3. n = 11–13 mice/group.
(I) Enhanced long-term CFC (24 h memory, 1-shock training) in mice injected with AAV-shHDAC3. Enhancement was blocked by HDAC3res overexpression but not HDAC3-K25A overexpression. n = 11–13 mice/group. $*p < 0.05$.
(J) Q-PCR analysis of *Fgf1b* mRNA expression in CA of untrained mice injected with the indicated AAVs. $n = 6–8$ mice/group. $*p < 0.05$.
Data presented as mean ± SEM.

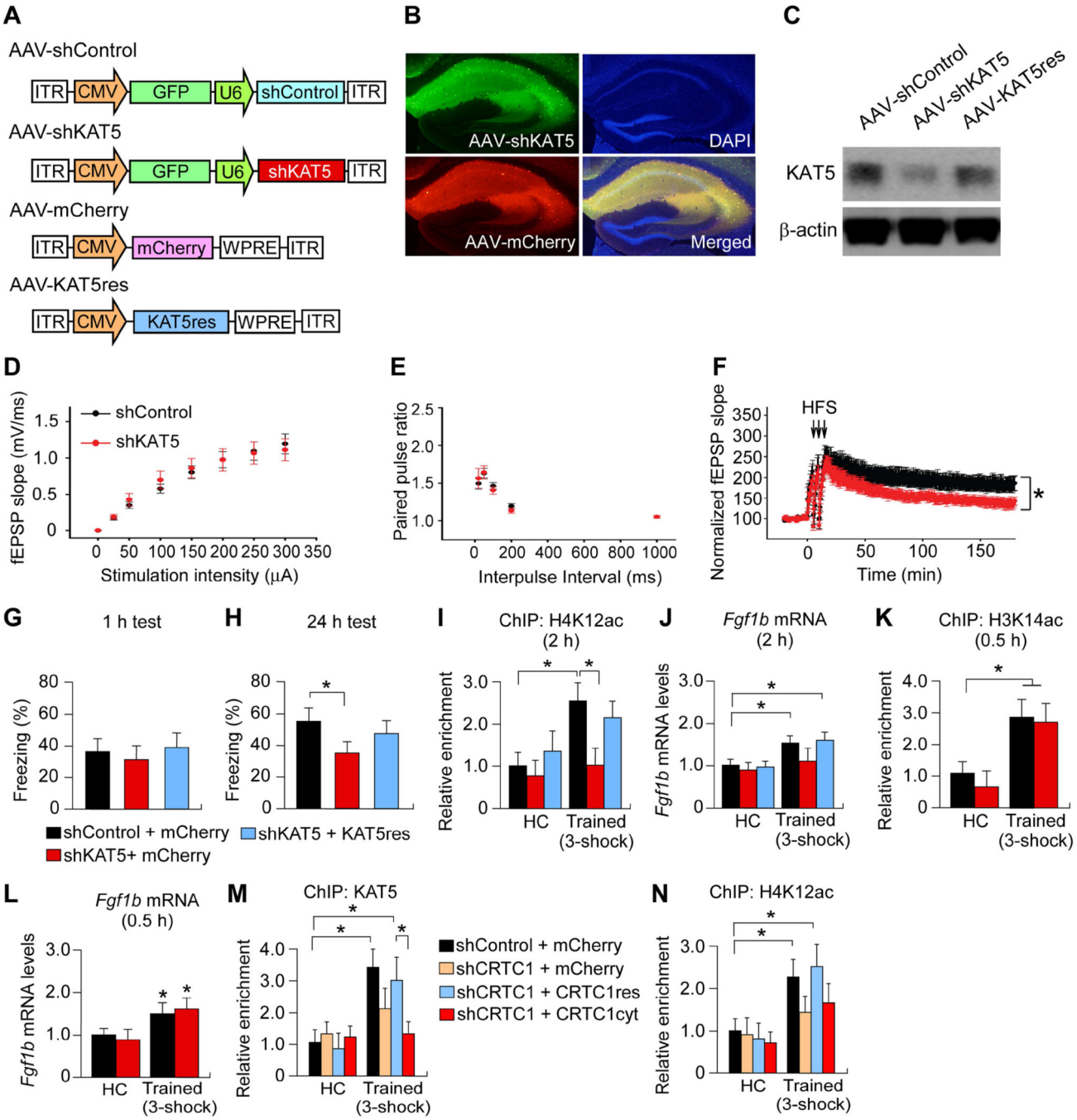

Figure 7. KAT5 is essential for *Fgf1b* transcription, synaptic plasticity, and memory

(A) AAV vectors overexpressing shRNA targeting *Kat5* (AAV-shKAT5), mock control (AAV-shControl), or shRNA-resistant KAT5 (AAV-KAT5res).

(B) GFP and mCherry expression in CA following AVV microinjection. Scale bar, 1 mm.

(C) Western blot showing KAT5 knockdown by AAV-shKAT5 and rescue by AAV-KAT5res.

(D–F) Effects of KAT5 knockdown on (D) input–output relationship, (E) paired-pulse ratio, and (F) LTP at CA3-CA1 synapses induced by strong stimulation. shControl, 20 slices; shKAT5, 17 slices. *$p < 0.05$.

(G) Normal short-term CFM (1 h after 3-shock training) in mice injected with AAV-shKAT5. n = 10–14 mice/group.
(H) Decreased long-term CFM (24 h after 3-shock training) in mice injected with AAV-shKAT5 and rescue by KAT5res overexpression. n = 10–14 mice/group. *$p < 0.05$.
(I) ChIP assay showing suppressed H4K12 acetylation at the *Fgf1b* promoter in mice receiving AAV-shKAT5 2 h after 3-shock CFC and reversal by AAV-KAT5res. $n = 6–10$ samples/group. *$p < 0.05$.
(J) Q-PCR analysis showing suppressed induction of *Fgf1b* mRNA in mice receiving AAV-shKAT5 2 h after 3-shock CFC and reversal by AAV-KAT5res. $n = 6–10$ mice/group. *$p < 0.05$.
(K) ChIP assay showing no effect of shKAT5 overexpression on H3K14 acetylation 0.5 h following 3-shock training. $n = 6–8$ samples/group. *$p < 0.05$.
(L) Q-PCR analysis showing no effect of shKAT5 overexpression on *Fgf1b* mRNA expression 0.5 h following 3-shock training. $n = 7–9$ mice/group. *$p < 0.05$.
(M) ChIP assay showing suppressed recruitment of KAT5 to the *Fgf1b* promoter 2 h following 3-shock training in mice given AAV-shCRTC1 and reversal by CRTC1res, but not CRTC1cyt. $n = 6–11$ samples/group. *$p < 0.05$.
(N) ChIP assay showing suppressed H4K12 acetylation at the *Fgf1b* promoter 2 h following 3-shock CFC training in mice receiving AAV-shCRTC1 and reversal by CRTC1res, but not CRTC1cyt. $n = 6–11$ samples/group. *$p < 0.05$.
Data presented as mean ± SEM. See also Figure S7.