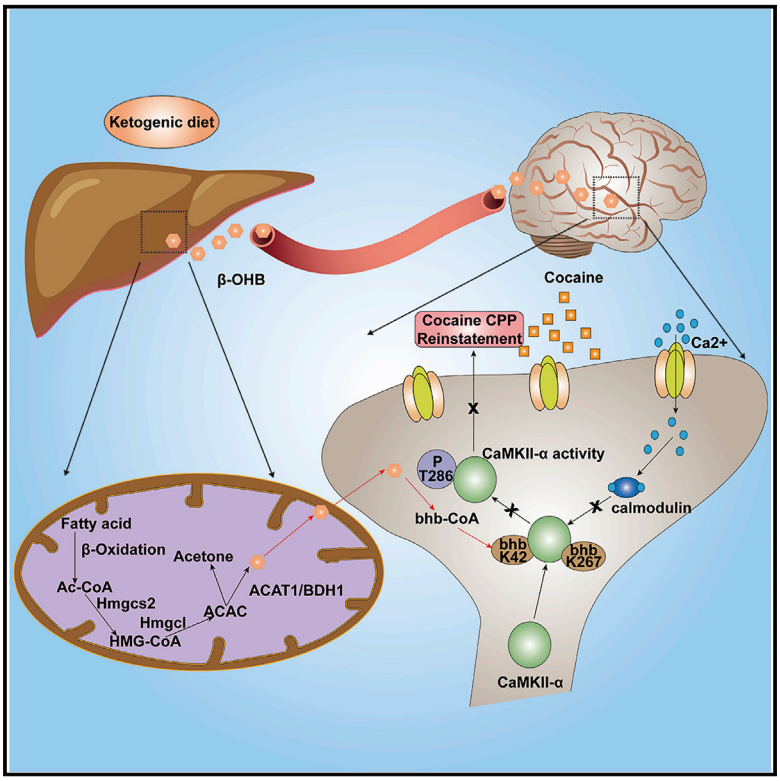
Cell Reports

Article

β-hydroxybutyrate reduces reinstatement of cocaine conditioned place preference through hippocampal CaMKII-α β-hydroxybutyrylation

Graphical abstract



Authors

Hongchun Li, Xuemei Wan, Zhixiang Wu, ..., Jingwei Tian, Yinglan Zhao, Xiaobo Cen

Correspondence

xbcen@scu.edu.cn

In brief

β-hydroxybutyrate (β-OHB) synthesized in liver mitochondria is carried to the brain through the bloodstream. β-OHB serves for protein lysine β-hydroxybutyrylation via bhb-CoA. Li et al. identify lysine β-hydroxybutyrylation of CaMKII-α in the hippocampus and show it underlies an inhibitory effect of β-OHB on cocaine conditional place preference reinstatement.

Highlights

- Ketogenic diet reduces cocaine-induced reinstatement in mice
- Reduced reinstatement is accompanied by increased β-hydroxybutyrate levels in hippocampus
- Proteomics identifies 105 sites of β-hydroxybutyrylation in 85 proteins in hippocampus
- Lysine β-hydroxybutyrylation of CaMKII-α inhibits its autophosphorylation and activity

Li et al., 2022, Cell Reports *41*, 111724
November 29, 2022
https://doi.org/10.1016/j.celrep.2022.111724

CellPress

Cell Reports

CellPress
OPEN ACCESS

Article

β-hydroxybutyrate reduces reinstatement of cocaine conditioned place preference through hippocampal CaMKII-α β-hydroxybutyrylation

Hongchun Li,[1,6] Xuemei Wan,[1,6] Zhixiang Wu,[2] Yuanyi Zhou,[1] Rong Chen,[1] Wei Xu,[1,3] Jiamei Zhang,[1] Zhen Yang,[4] Lin Bai,[4] Jie Zhang,[4] Feng Qin,[1] Liang Wang,[1] Yaxing Chen,[1] Linhong Jiang,[1] Yuman He,[1] Xiaojie Wang,[1] Qingfan Wei,[1] Shu Li,[1] Yanping Dai,[1] Yuanyuan Chen,[1] Yonghai Wang,[5] Hongbo Wang,[5] Jingwei Tian,[5] Yinglan Zhao,[1] and Xiaobo Cen[1,7,*]

[1]National Chengdu Center for Safety Evaluation of Drugs, State Key Laboratory of Biotherapy/Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China

[2]Faculty of Environmental and Life Science, Beijing University of Technology, Beijing 100124, China

[3]Shenzhen Key Laboratory of Drug Addiction, The Brain Cognition and Brain Disease Institute, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences (CAS), Shenzhen 518055, China

[4]Histology and Imaging Platform, Core Facilities of West China Hospital, Sichuan University, Chengdu 610041, China

[5]Ministry of Education, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, Yantai University, Yantai 264005, China

[6]These authors contributed equally

[7]Lead contact

*Correspondence: xbcen@scu.edu.cn

https://doi.org/10.1016/j.celrep.2022.111724

SUMMARY

Studies have shown the therapeutic effects of a ketogenic diet (KD) on epilepsy, but the effect of a KD on drug reinstatement is largely unclear. This study aims to investigate whether KD consumption possesses therapeutic potential for cocaine reinstatement and the molecular mechanism. We find that a KD significantly reduces cocaine-induced reinstatement in mice, which is accompanied by a markedly elevated level of β-hydroxybutyrate (β-OHB), the most abundant ketone body, in the hippocampus. The underlying mechanism is that β-OHB posttranslationally modifies CaMKII-α with β-hydroxybutyrylation, resulting in significant inhibition of T286 autophosphorylation and downregulation of CaMKII activity. Collectively, our results reveal that β-hydroxybutyrylation is a posttranslational modification of CaMKII-α that plays a critical role in mediating the effect of KD consumption in reducing cocaine reinstatement.

INTRODUCTION

Drug addiction is a chronic mental disease involving aberrant memory, which plays a critical role in promoting the rewarding effect of drugs and subsequent cravings and relapses (Torregrossa et al., 2011; Wolf, 2016). Successful treatment of drug addiction depends on long-term abstinence, making relapse prevention an essential therapeutic goal (Werner et al., 2020). However, drug relapse is always the greatest obstacle for the treatment of addictive disorders. Repeated exposure to environmental cues without reward or punishment stimuli reduces the behavioral response through a period of extinction. Disruption of the motivational impact of drug-associated cues during the extinction phase has long been proposed as an important strategy to prevent drug relapse (Taylor et al., 2009; Torregrossa et al., 2010).

A ketogenic diet (KD), which is a high-fat, low-carbohydrate, and adequate-protein formulation, has been applied to treat epilepsy (Lutas and Yellen, 2013), neurodegenerative diseases, and psychiatric disorders (Kraeuter et al., 2020). A decrease in carbohydrates reduces glucose utilization, leading to conversion of free fatty acids to ketone bodies within the liver (Hartman et al., 2007). Beta-hydroxybutyrate (β-OHB), acetoacetate, and acetone are the three primary ketone bodies that circulate among extrahepatic tissues as important energy sources during KD treatment (D'Andrea Meira et al., 2019). Recent studies have shown that administration of a KD diminishes behavioral responses to psychostimulants and alcohol in rodents and reduces withdrawal symptoms in humans, suggesting that KD consumption holds potential as a novel therapy for the treatment of psychostimulant addiction (Martinez et al., 2019; Wiers et al., 2021). However, the effect of KD consumption on drug relapse and the underlying mechanism remain largely unknown.

β-OHB, the most abundant ketone body, is synthesized in the liver and possesses diverse bioactive properties. KD administration is one way to deliver high levels of β-OHB for a prolonged time outside of a fasting or exercise context (Newman and Verdin, 2014), and the predominant fate of β-OHB is terminal oxidation as an energy substrate. Epigenetic refers to changes in gene-expression patterns not caused by an alteration in

DNA sequence. Currently, several classic epigenetic mechanisms have been extensively investigated, including modifications of DNA and histone posttranslational modification (PTM). Accumulated evidence has demonstrated that β-OHB is involved in cellular signaling, epigenetic control, and PTM of histone lysine (Puchalska and Crawford, 2017; Ruan and Crawford, 2018; Xie et al., 2016), indicating that β-OHB may serve as a regulator of epigenetics and cell signaling in addition to playing a role as a metabolite.

Lysine β-hydroxybutyrylation (kbhb) is a PTM mediated by β-OHB that plays a critical role in the regulation of target gene expression and protein activity (Huang et al., 2021; Xie et al., 2016; Zhang et al., 2020). For instance, H3K9 kbhb is associated with active gene expression of multiple metabolic pathways in response to starvation (Xie et al., 2016) and acts as an epigenetic regulator of memory development in the $CD8^+$ cells (Zhang et al., 2020). A recent study revealed that kbhb occurs not only on histone proteins but also on a large variety of non-histone proteins (Liu et al., 2019). In this process, β-OHB serves as the β-hydroxybutyryl donor, and the intermediate metabolite β-hydroxybutyryl-coenzyme A (CoA) is then synthesized, which is subsequently utilized by p300 as a cofactor to generate kbhb (Huang et al., 2021; Kaczmarska et al., 2017). However, the role of protein kbhb in the regulation of enzyme activity, as well as downstream signal transduction in drug addiction, is largely unknown.

CaMKII-α is extremely abundant in the hippocampus (Hanson and Schulman, 1992), and its activity is essential for synaptic plasticity and memory formation (Yamagata et al., 2009). CaMKII-α activity is strongly regulated by CaMKII-α autophosphorylation at a critical residue, threonine 286 (T286) (Irvine et al., 2006), and dysregulation of CaMKII-α T286 is closely involved in the pathogeneses of depressive disorder, schizophrenia, epilepsy, and neurodevelopmental disorders (Robison, 2014). Activation of CaMKII by autophosphorylation at T286, particularly the α-isoform, plays an important role in the development of drug addiction (Mijakowska et al., 2017; Muller et al., 2016).

In this study, through proteome-wide kbhb analysis, we discovered that kbhb is a PTM of CaMKII-α. β-OHB treatment during the extinction period of cocaine conditional place preference (CPP) markedly enhanced kbhb of hippocampal CaMKII-α at the K42 and K267 residues and then suppressed CaMKII-α T286 autophosphorylation and activity, which is necessary for reducing cocaine-induced reinstatement. Our findings reveal that KD consumption or β-OHB treatment holds therapeutic potential for cocaine relapse, broadening the scope of diet therapy for neuropsychiatric diseases.

RESULTS

KD consumption disrupts cocaine CPP reinstatement

CPP is a model used to evaluate the rewarding effects of psychostimulant drugs. In this model, mice show a preference for a drug-associated context that can be extinguished following a drug-free period, but the drug-associated memories can be reinstated by exposure to the drug (Bavley et al., 2020; Sanchis-Segura and Spanagel, 2006). We first explored the effect of a KD on cocaine-induced reinstatement in mice. In the conditioning, mice received injections of cocaine (10 mg/kg, intraperitoneally [i.p.]) or saline and were confined to alternative sides of the CPP apparatus daily for 4 consecutive days. During extinction, the mice were maintained on a KD or a control diet (CD) and placed in the CPP apparatus daily for 3 weeks. After extinction, the mice received a priming dose of cocaine (7.5 mg/kg, i.p.) and underwent a reinstatement test to assess relapse behavior (Figure 1A). We found that the mice fed a KD showed a lower CPP score than the mice fed a CD (Figure 1B; treatment: $F_{(3,\ 42)} = 8.884$, $p < 0.001$; time: $F_{(2.149,\ 90.24)} = 6.893$, $p < 0.05$; interaction: $F_{(9,\ 126)} = 3.874$, $p < 0.001$), indicating that KD consumption may disrupt cocaine-induced reinstatement in mice.

By ultra-high-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) analysis, we first measured the levels of β-OHB in the brain and peripheral blood of mice at the end of reinstatement test. The fragment ion peak and standard curve of β-OHB measurements are shown in Figures S1A and S1B. The results showed that the plasma concentration of β-OHB was higher in the KD groups than in the CD groups (Figure 1C; $F_{(3,\ 20)} = 69.88$, $p < 0.0001$). Since the hippocampus is a crucial brain region for learning, cognitive function, and drug relapse (Deschaux et al., 2014), we quantitatively measured the hippocampal β-OHB level. As expected, KD feeding increased the level of β-OHB in the hippocampus (Figure 1D; $F_{(3,\ 20)} = 46.63$, $p < 0.0001$); nevertheless, the KD failed to increase the level of β-OHB in the prefrontal cortex, nucleus accumbens, or striatum (Figures S1C–S1E). In addition, KD-treated mice weighed less than CD mice (Figure 1E; $F_{(3,\ 42)} = 42.32$, $p < 0.0001$).

β-OHB acts as an endogenous blocker of histone deacetylase (HDAC) and reduces drug relapse by suppressing HDAC activity (Malvaez et al., 2013) or enhancing histone acetylation (Shimazu et al., 2013). To investigate whether the KD reduced cocaine-induced reinstatement through these mechanisms, we quantitatively measured HDAC activity in the hippocampus. However, KD treatment did not affect HDAC activity (Figure 1F). As H3K9 and H3K14 acetylation are involved in the pathogenesis of drug addiction (Heller et al., 2016; Zeng et al., 2019), we next detected the acetylation levels of these two residues in the hippocampus, prefrontal cortex, nucleus accumbens, and striatum. Similarly, KD treatment showed no effect on H3K9 and H3K14 acetylation in these brain regions (Figures 1G and S2A–S2C). Collectively, these data suggest that the inhibitory effect of KD consumption on cocaine-induced reinstatement may not rely on the modulation of hippocampal HDAC activity or H3K9 and H3K14 acetylation.

Considering that β-OHB is not only a KD metabolite for energy supply but also a β-hydroxybutyryl donor for kbhb (Kaczmarska et al., 2017; Liu et al., 2019; Xie et al., 2016; Zhang et al., 2020), we explored whether kbhb (Pan-kbhb) of proteins occurred in the hippocampus. Interestingly, CD and KD feeding induced Pan-kbhb protein modification in the hippocampus, nucleus accumbens, and striatum of mice (Figures 1H, S2D, and S2E).

β-OHB treatment suppresses cocaine reinstatement

To investigate whether β-OHB directly affected cocaine reinstatement, mice were i.p. injected with different doses of β-OHB during extinction of the cocaine CPP and then subjected

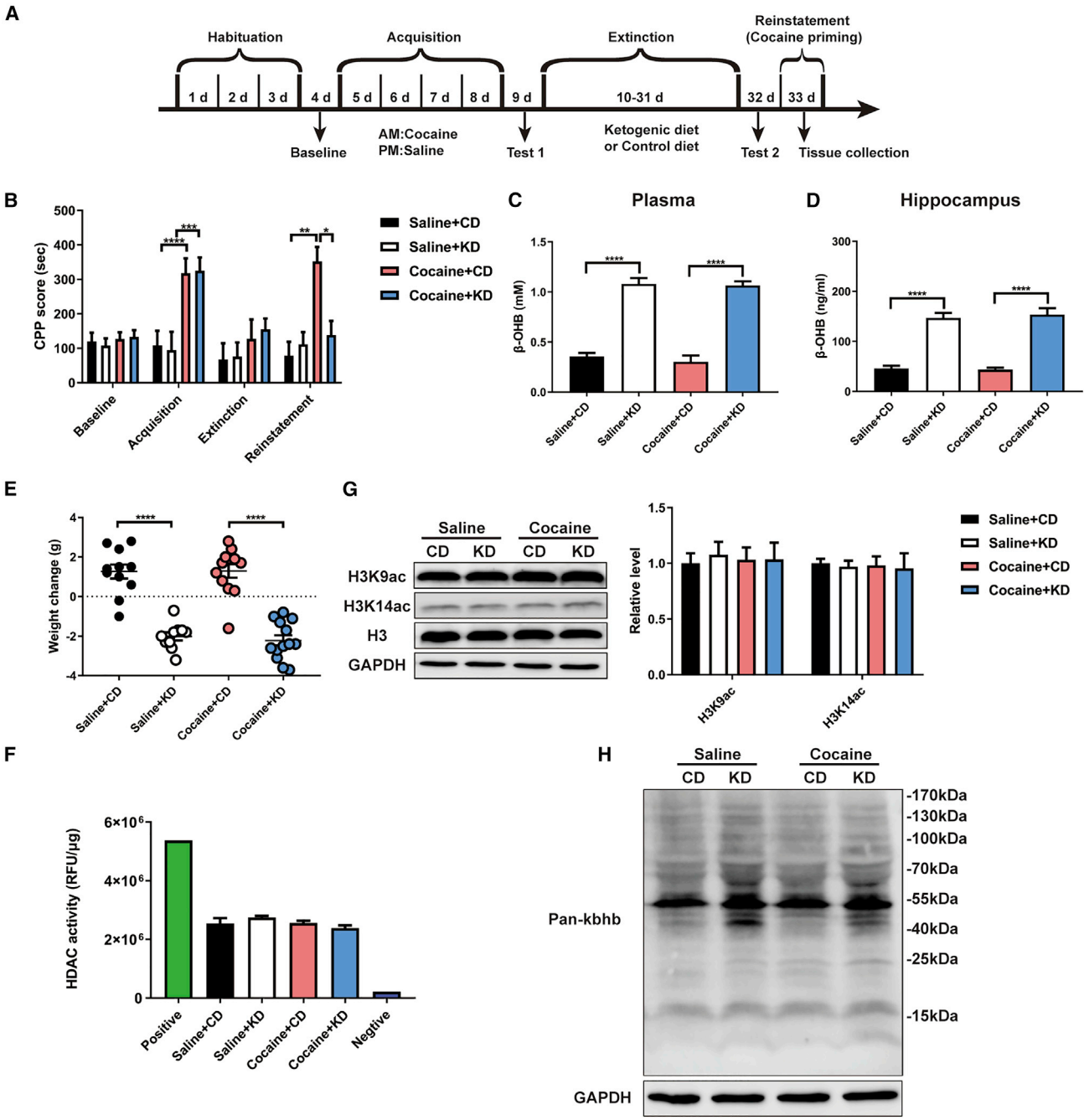


Figure 1. Ketogenic diet disrupts cocaine CPP reinstatement

(A) Schematic diagram of the cocaine CPP reinstatement experiment.

(B) KD treatment disrupts cocaine-induced reinstatement (n = 11 for saline + CD group, n = 10 for saline + KD group, n = 12 for cocaine + CD group, n = 13 for cocaine + KD group).

(C) KD treatment induced a high concentration of β-OHB in the plasma (n = 6 for per group).

(D) KD treatment induced a high concentration of β-OHB in the hippocampus (n = 7 for per group).

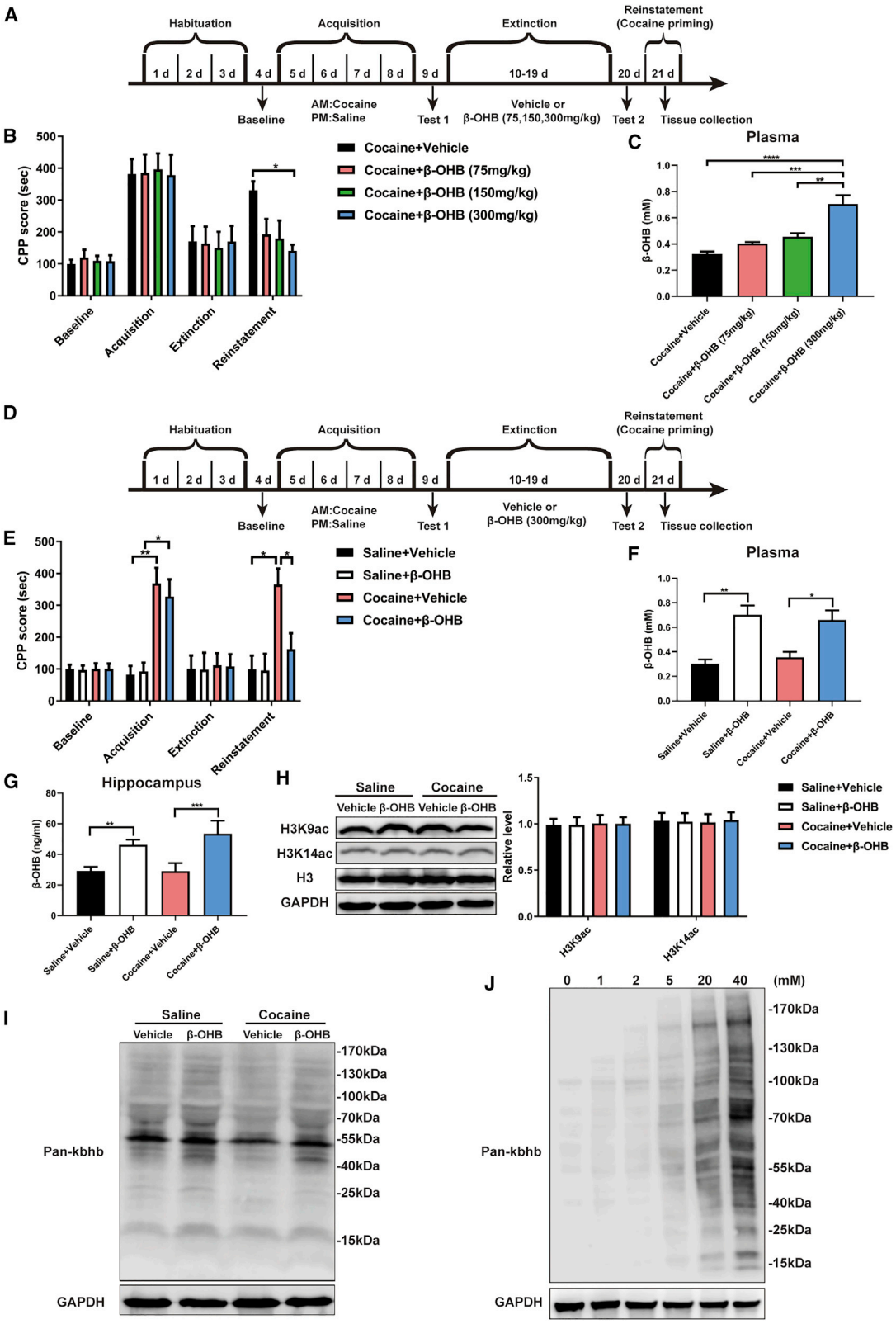
(E) KD treatment decreased the weight of mice (n = 11 for saline + CD group, n = 10 for saline + KD group, n = 12 for cocaine + CD group, n = 13 for cocaine + KD group).

(F) KD treatment did not alter the HDAC activity in the hippocampus (n = 4 per group).

(G) Western blot analysis showed the levels of H3K9ac and H3K14ac in the hippocampus (n = 4 per group).

(H) Western blot analysis showed the level of Pan-kbhb in the hippocampus (n = 4 per group).

Data are the means ± SEM. One-way or two-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.



(legend on next page)

to behavioral tests (Figure 2A). The results showed that injection of β-OHB (300 mg/kg) blocked cocaine-induced reinstatement; however, injections of 150 and 75 mg/kg β-OHB showed no obvious effects (Figure 2B; treatment: $F_{(3,\ 29)} = 0.6036$, $p = 0.618$; time: $F_{(2.426,\ 70.36)} = 34.61$, $p < 0.0001$; interaction: $F_{(9,\ 87)} = 1.105$, $p = 0.368$). In addition, the blood concentration of β-OHB dose dependently increased, although the increase was significant only after the injection of 300 mg/kg β-OHB (Figure 2C; $F_{(3,\ 12)} = 18.98$, $p < 0.0001$). We thus selected 300 mg/kg as the dose for β-OHB treatment in the subsequent behavioral experiments.

After identifying the ideal dose of β-OHB, we measured the effect of β-OHB on CPP reinstatement using the full experimental model that included saline controls. Compared with the saline groups, β-OHB (300 mg/kg, i.p.) treatment in the extinction phase significantly decreased the cocaine CPP score in the reinstatement test (Figures 2D and 2E; treatment: $F_{(3,\ 39)} = 8.600$, $p < 0.001$; time: $F_{(2.704,\ 105.5)} = 9.751$, $p < 0.0001$; interaction: $F_{(9,\ 117)} = 4.844$, $p < 0.0001$), which was accompanied by elevated β-OHB concentrations both in the plasma and hippocampus (Figure 2F; $F_{(3,\ 12)} = 11.09$, $p < 0.001$; Figure 2G; $F_{(3,\ 12)} = 20.65$, $p < 0.0001$). In addition, β-OHB failed to affect the acetylation levels of H3K9 and H3K14 (Figure 2H) but induced Pan-kbhb modification in the hippocampus (Figure 2I). To validate these results, we treated N2a cells with different concentrations of β-OHB (1, 2, 5, 20, and 40 mM) *in vitro* for 24 h. Consistently, β-OHB induced the level of Pan-kbhb modification in the N2a cells (Figure 2J), indicating that β-OHB is capable of directly driving protein kbhb *in vitro*. Collectively, our results reveal a link between β-OHB and protein kbhb both *in vivo* and *in vitro*.

β-OHB significantly modifies the landscape of protein kbhb

Using an affinity-directed MS approach, we performed hippocampal proteomics analysis of kbhb based on affinity enrichment of kbhb peptides with a specific anti-kbhb antibody. A total of 105 kbhb sites in 85 proteins were identified in the hippocampus of β-OHB-treated mice (Table S1). Among these identified proteins, the levels of 46 proteins were upregulated, and the levels of 39 proteins were downregulated, compared with the levels in the control group. In addition, among all the altered kbhb sites, the levels at 53 sites were increased, while those at 52 sites were decreased in the β-OHB-treated group compared with the control group (Figure S3A). Moreover, 35 sites were altered only in the control group, 40 sites were altered only in the β-OHB-treated group, and 30 sites overlapped between the two groups (Figure S3B).

Cellular localization and biological function analyses showed that over 50% of kbhb-modified proteins are localized to the cytoplasm (Figure S3C) and participate in cellular biological regulation as well as metabolic processes (Figure S3D). Interestingly, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that kbhb modification occurred among a few kinases and receptor channels that are critical for synaptic signaling and function, such as Glul, CaMKII, and Gnb1 (Figure S3E). To determine the connection of each β-hydroxybutyrylated protein, an interaction network was generated and visualized using Cytoscape software based on the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. Through functional analysis, we divided these proteins into several highly related categories, such as Glud1, Gls, and Gnbl. Interestingly, interactions of these kbhb-modified proteins are mainly involved in signaling and metabolic pathways. For instance, Maob regulates histidine metabolism, and CaMKII-α as well as Gnb1 are involved in synaptic signaling (Figure S3F).

CaMKII-α is highly β-hydroxybutyrylated at the K42 and K267 sites

Considering that CaMKII-α is a critical regulatory protein for learning and memory (Rossetti et al., 2017; Yamagata et al., 2009) and participates in various aspects of drug addiction (Easton et al., 2014; Liu et al., 2014; Loweth et al., 2013; Salling et al., 2016), we focused on the potential role of CaMKII-α kbhb in modulating cocaine-induced reinstatement. To clarify whether CaMKII-α is a target of kbhb, we enriched CaMKII-α proteins from β-OHB-treated or untreated cells by immunoprecipitation and then detected kbhb of CaMKII-α with a Pan-kbhb antibody. We investigated whether or not the level of CaMKII-α kbhb increased in response to β-OHB *in vivo*. After the mice were administered β-OHB (300 mg/kg, i.p.) for 10 days continuously, the level of CaMKII-α kbhb was markedly increased in the hippocampus, whereas the total CaMKII-α level was not altered (Figure 3A; $t_{(4)} = 4.539$, $p < 0.05$). In addition, western blot analysis showed that the levels of CaMKII-α kbhb were significantly increased in β-OHB-treated cells; nevertheless, total CaMKII-α expression remained unchanged (Figure 3B; $t_{(4)} = 3.985$,

Figure 2. β-OHB administration disrupts cocaine CPP reinstatement

(A) Timeline of cocaine CPP experimental procedure and β-OHB injection.

(B) Mice were intraperitoneally injected with different doses of β-OHB (75, 150, and 300 mg/kg) during the extinction period of cocaine CPP (n = 8 for cocaine + vehicle group, n = 8 for cocaine + β-OHB [75 mg/kg] group, n = 8 for cocaine + β-OHB [150 mg/kg] group, n = 9 for cocaine + β-OHB [300 mg/kg] group).

(C) β-OHB treatment increased the level of plasma β-OHB dose dependently during the extinction period of cocaine CPP (n = 4 per group).

(D) Schematic diagram shows the experimental procedure of cocaine CPP reinstatement and β-OHB injection.

(E) β-OHB (300 mg/kg) administration disrupts the cocaine-induced reinstatement (n = 11 for saline + vehicle group, n = 11 for saline + β-OHB group, n = 10 for cocaine + vehicle group, n = 11 for cocaine + β-OHB group).

(F) β-OHB (300 mg/kg) treatment induced a high concentration of β-OHB in the plasma of mice (n = 4 per group).

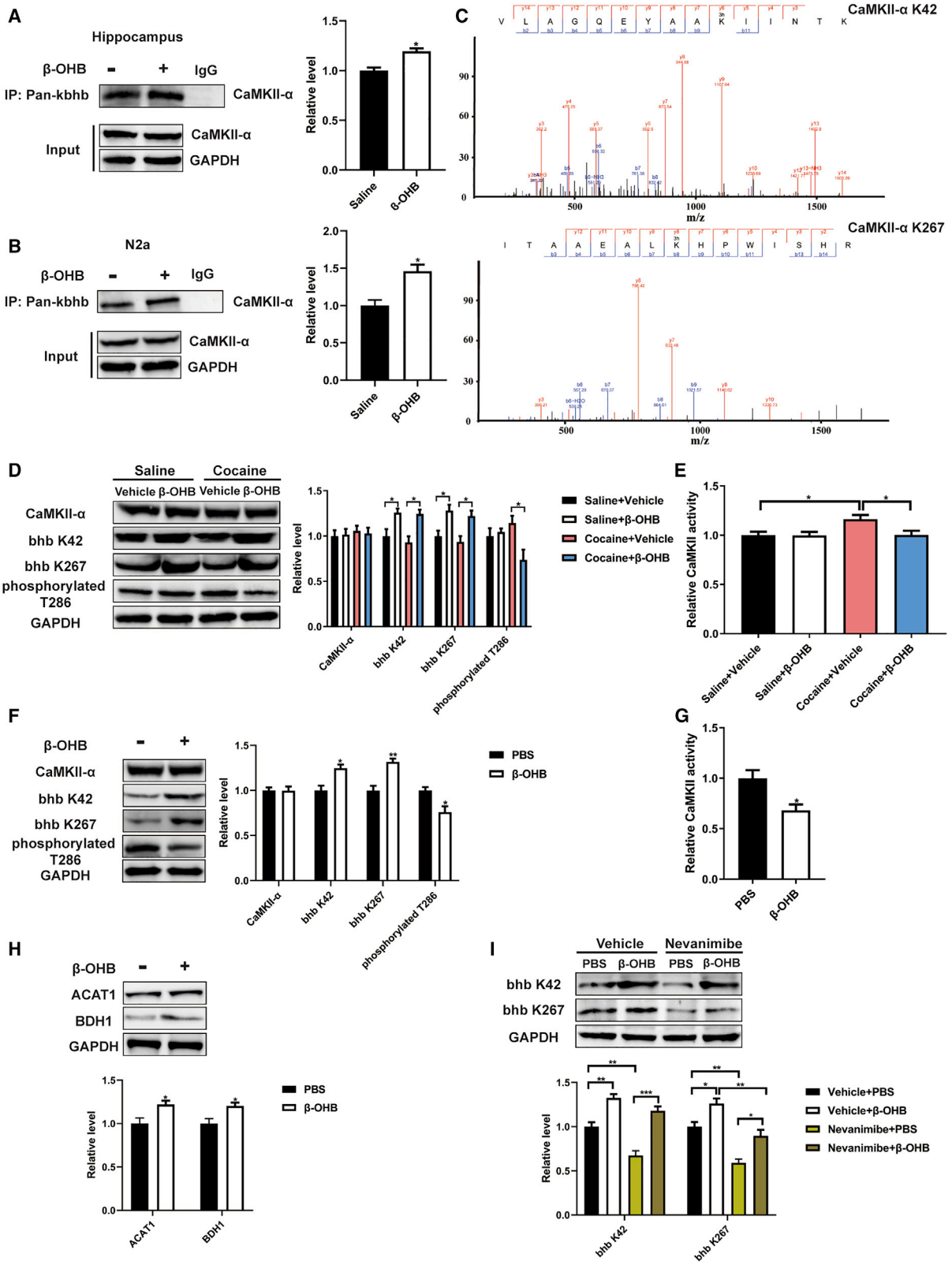
(G) β-OHB (300 mg/kg) treatment induced a high concentration of β-OHB in the hippocampus (n = 4 per group).

(H) Immunoblotting analysis showed H3K9ac and H3K14ac levels in the hippocampus (n = 6 per group).

(I) Western blot showed Pan-kbhb level in the hippocampus (n = 4 per group).

(J) Western blot showed Pan-kbhb level in N2a cells (n = 3 per group).

Data are the means ± SEM. One-way or two-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.



(legend on next page)

$p < 0.05$). Proteomic quantification of kbhb revealed that CaMKII-α K42 and K267 were highly β-hydroxybutyrylated. Notably, CaMKII-α kbhb on K42 was uniquely detected in β-OHB-treated mice, and the level of CaMKII-α kbhb on K267 was 1.489 times higher than that in the control mice (Figure S3G). The MS data supported this finding, showing a mass shift at the K42 and K267 residues of CaMKII-α (Figure 3C). To further validate the sites of CaMKII-α kbhb, specific antibodies targeting the K42 and K267 kbhb sites of CaMKII-α were generated and validated (Figures S4A–S4J). We transfected N2a cells with a lentivirus (LV) expressing Flag-tagged CaMKII-α or mutated CaMKII-α (K42 mutated to M42; K267 mutated to A267); an LV expressing Flag-tagged CaMKII-α was used as a control. Importantly, the expression of kbhb at CaMKII-α K24 and K267 was significantly increased by β-OHB treatment at a final concentration of 40 mM for 24 h. However, this effect completely disappeared when K42 and K267 of CaMKII-α were mutated (Figures S4E and S4J). Collectively, these results demonstrate that β-OHB indeed enhances CaMKII-α kbhb both *in vivo* and *in vitro*.

β-OHB acts as a donor of CaMKII-α kbhb

We first measured the effect of β-OHB on the levels of CaMKII-α kbhb and phosphorylation in the hippocampus after cocaine CPP reinstatement. Notably, β-OHB increased kbhb levels at K42 and K267 of CaMKII-α but reduced the phosphorylation level at T286 (Figure 3D; treatment: $F_{(3, 12)} = 3.745$, $p < 0.05$; protein: $F_{(2.604, 31.24)} = 3.535$, $p < 0.05$; interaction: $F_{(9, 36)} = 4.998$, $p < 0.01$). Consistently, a quantitative assay of CaMKII activity revealed that hippocampal CaMKII activity was decreased by β-OHB treatment (Figure 3E; $F_{(3, 24)} = 4.300$, $p < 0.05$). Furthermore, β-OHB treatment (40 mM) for 24 h upregulated the kbhb levels at CaMKII-α K42 and K267 in the N2a cells, whereas both the phosphorylation levels at T286 and CaMKII activity were downregulated (Figure 3F; treatment: $F_{(1, 4)} = 22.69$, $p < 0.01$; protein: $F_{(3, 12)} = 11.90$, $p < 0.001$; interaction: $F_{(3, 12)} = 11.90$, $p < 0.01$; Figure 3G; $t(6) = 3.199$, $p < 0.05$). These data suggest that β-OHB-induced kbhb of CaMKII-α may preferentially disrupt T286 phosphorylation, thus inhibiting CaMKII-α activity.

We further detected the expressions of mitochondrial acetyl-CoA acetyltransferase 1 (ACAT1) and 3-OHB dehydrogenase 1 (BDH1), two key enzymes in ketogenesis. Interestingly, the protein expression levels of ACAT1 and BDH1 were also upregulated, suggesting active ketogenesis in N2a cells in response to β-OHB exposure (Figure 3H; treatment: $F_{(1, 4)} = 44.69$, $p < 0.01$; protein: $F_{(1, 4)} = 0.01726$, $p = 0.9018$; interaction: $F_{(1, 4)} = 0.01726$, $p = 0.9018$). Unlike BDH1, ACAT1 is an upstream ketogenesis-regulating enzyme that has an important role in regulating β-OHB production. To further verify the involvement of exogenous β-OHB in CaMKII-α kbhb, we first diminished endogenous β-OHB in N2a cells with nevanimibe, a specific inhibitor of ACAT1, and then treated the cells with β-OHB. Importantly, nevanimibe treatment (10 μM) for 24 h decreased the levels of K42 and K67 kbhb of CaMKII-α; however, these decreases were reversed by the addition of 40 mM β-OHB (Figure 3I; treatment: $F_{(3, 8)} = 48.22$, $p < 0.0001$; protein: $F_{(1, 8)} = 9.383$, $p < 0.05$; interaction: $F_{(3, 8)} = 3.082$, $p = 0.0902$), indicating that exogenous β-OHB is able to act as a donor of kbhb and directly promote CaMKII-α kbhb.

β-OHB specifically β-hydroxybutyrylates CaMKII-α K42 and K267 but reduces T286 phosphorylation

N2a cells were transfected with LVs expressing CaMKII-α or mutant CaMKII-α and then treated with 40 mM β-OHB for 24 h. We found that the levels of K42 and K67 kbhb of CaMKII-α were significantly increased in the cells overexpressing CaMKII-α but that the phosphorylation level of T286 was obviously decreased (Figure 4A). Moreover, kbhb modifications and T286 phosphorylation were not altered in the cells expressing mutant CaMKII-α (Figure 4A; treatment: $F_{(3, 8)} = 1.316$, $p = 0.3350$; protein: $F_{(1.427, 11.42)} = 4.923$, $p < 0.05$; interaction: $F_{(6, 16)} = 10.27$, $p < 0.0001$). We further detected the specific CaMKII-α kbhb in the N2a cells by using FLAG as a tag protein to represent exogenous CaMKII-α or mutant CaMKII-α. The results showed that the levels of K42 and K67 kbhb of CaMKII-α were increased by β-OHB treatment in the N2a cells expressing CaMKII-α, while this effect completely disappeared when CaMKII-α was mutated, regardless of the presence of β-OHB (Figures 4B, S6E, and S6J). Furthermore, the level of T286 phosphorylation of exogenously expressed CaMKII-α was decreased by β-OHB treatment, whereas this decrease was not observed in the N2a cells expressing mutant CaMKII-α (Figure 4B; treatment: $F_{(3, 8)} = 262.8$, $p < 0.0001$; protein: $F_{(1.549, 12.39)} = 113.1$, $p < 0.0001$; interaction: $F_{(6, 16)} = 115.9$, $p < 0.0001$). We next investigated the effect of kbhb on the activity of exogenous CaMKII in the N2a cells. As expected, β-OHB treatment (40 mM) for 24 h suppressed exogenous CaMKII activity, but not exogenous mutant CaMKII activity (Figure 4C; $F_{(3, 20)} = 23.80$, $p < 0.0001$), demonstrating a direct action of β-OHB on CaMKII-α activity.

Figure 3. CaMKII-α is highly β-hydroxybutyrylated at K42 and K267 sites

(A) Kbhb modification of endogenous CaMKII-α in the hippocampus of mice treated with β-OHB (n = 3 per group).
(B) Kbhb modification of endogenous CaMKII-α in the N2a cells treated with β-OHB (n = 3 per group).
(C) Mass spectrometry analysis identified CaMKII-α-derived peptides containing bhb K42 and K267.
(D) Western blot showed the levels of CaMKII-α kbhb at K42 and K267 as well as phosphorylated T286 in the hippocampus of mice treated with β-OHB during the extinction of cocaine CPP (n = 4 per group).
(E) β-OHB administration decreased hippocampal CaMKII activity (n = 7 per group).
(F) Immunoblot analysis showed the levels of CaMKII-α kbhb (K42 and K267) and phosphorylated T286 in N2a cells (n = 3 per group).
(G) β-OHB treatment suppressed the CaMKII activity of N2a cells (n = 4 per group).
(H) Immunoblot analysis showed the levels of ACAT1 and BDH1 in N2a cells (n = 3 per group).
(I) Western blot analysis showed the levels of CaMKII-α kbhb at K42 and K267 in N2a cells treated with nevanimibe (n = 3 per group).
Data are the means ± SEM. One-way, two-way ANOVA, or unpaired t test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

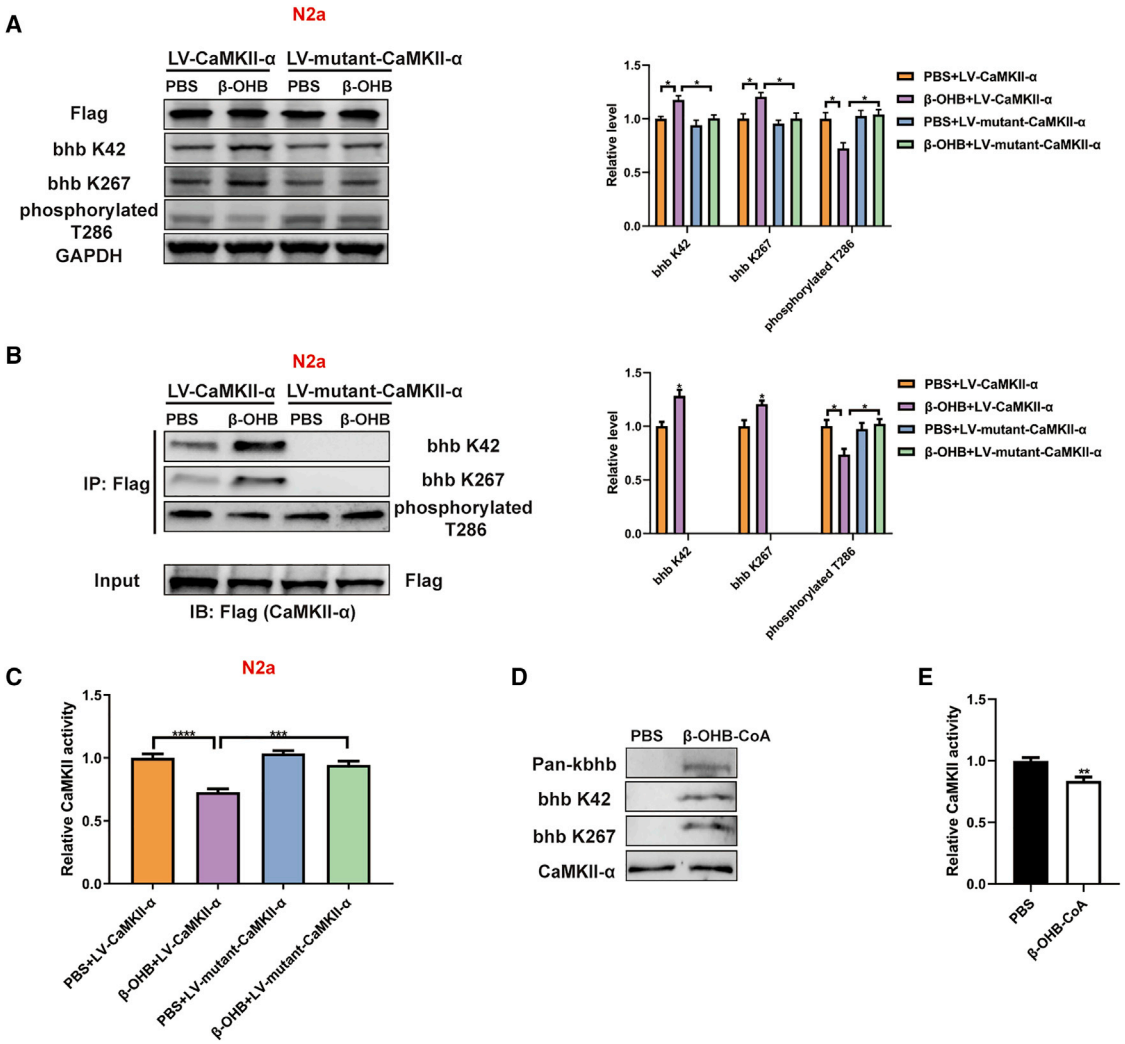


Figure 4. Kbhb of CaMKII-α K42 and K267 inhibits CaMKII activity *in vitro*

(A) Western blot analysis showed the levels of Flag, CaMKII-α, CaMKII-α kbhb (K42, K267), and phosphorylated T286 in N2a cells transfected with WT or mutant CaMKII-α (n = 3 per group).

(B) Immunoblot analysis showed the levels of CaMKII-α kbhb (K42, K267) and phosphorylated T286 of exogenous WT and mutant CaMKII-α in N2a cells (n = 3 per group).

(C) β-OHB treatment decreased the activity of exogenous CaMKII in N2a cells (n = 6 per group).

(D) Western bolt analysis showed the levels of Pan-kbhb, bhb K42, and bhb K267 of recombinant CaMKII-α protein after β-OHB-CoA treatment.

(E) β-OHB-CoA treatment decreased the activity of recombinant CaMKII protein (n = 6 per group; unpaired t test).

Data are the means ± SEM. One-way, two-way ANOVA, or unpaired t test, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Finally, to further demonstrate the direct contribution of β-OHB to CaMKII-α modification and activity, recombinant CaMKII-α protein was incubated with β-hydroxybutyryl-CoA at 37°C for 2 h, and formation of CaMKII-α kbhb was monitored by western blot analysis. Notably, β-hydroxybutyryl-CoA not only enhanced CaMKII-α kbhb at the K42 and K267 sites in the absence of any potential acylating enzymes (Figure 4D) but also significantly reduced CaMKII activity (Figure 4E; $t_{(10)} = 3.929$, $p < 0.01$). Taken together, the findings suggest that β-OHB is able to induce CaMKII-α kbhb and inhibit T286 phosphorylation, which may contribute to the decrease of CaMKII activity.

Hippocampal CaMKII-α kbhb inhibits cocaine reinstatement in mice

The hippocampal CA3 region plays an important role in drug relapse, and treatment targeting CA3 may be effective in managing relapse in the drug addiction (Kesner et al., 2016; Luo et al., 2011). To explore the effect of hippocampus-specific K42 and K67 kbhb of CaMKII-α on cocaine-induced reinstatement, a doxycycline-inducible AAV2/9 expressing CaMKII-α and mutant CaMKII-α (K42 mutated to M42; K267 mutated to A267) were infused into the hippocampal CA3 regions of mice before the cocaine CPP paradigm. After cocaine CPP training was

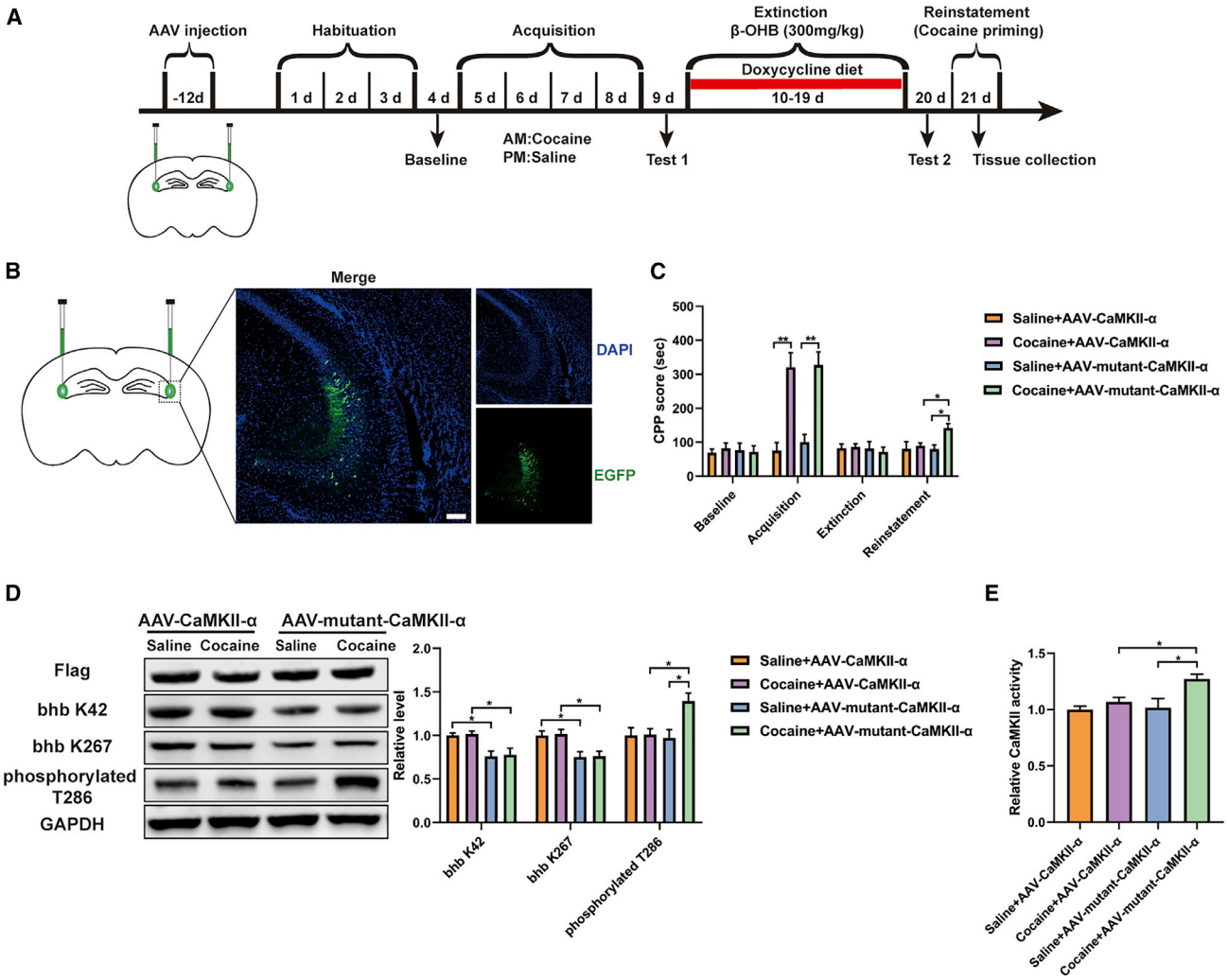


Figure 5. Hippocampal CaMKII-α kbhb reduces cocaine CPP reinstatement

(A) Schematic diagram of cocaine CPP experiment and β-OHB injection.

(B) Representative images of CaMKII-α expression in the hippocampus CA3 region (green); DAPI, nucleus (blue); scale bar: 25 μm.

(C) Increased CaMKII-α kbhb suppressed cocaine-induced reinstatement (n = 7 for saline + AAV-CaMKII-α group; n = 9 for cocaine + AAV-CaMKII-α group; n = 9 for saline + AAV-mutant-CaMKII-α group; n = 9 for cocaine + AAV-mutant-CaMKII-α group).

(D) Immunoblot analysis showed the levels of CaMKII-α kbhb (K42, K267) and phosphorylated T286 in the hippocampus CA3 region (n = 6 per group).

(E) β-OHB treatment decreased the CaMKII activity of hippocampus CA3 region (n = 6 per group).

Data are the means ± SEM, One-way or two-way ANOVA, *p < 0.05 and **p < 0.01.

completed, mice were fed doxycycline (0.02% in 5% sucrose drinking water) to switch on the expression of the exogenous CaMKII-α and mutant CaMKII-α gene and injected with β-OHB (300 mg/kg, i.p.) for 10 consecutive days in the extinction phase (Figures 5A and 5B). Importantly, β-OHB treatment significantly reduced the cocaine CPP score in the CaMKII-α-overexpressing mice compared with mutant CaMKII-α overexpressing mice (Figure 5C; treatment: $F_{(3, 30)} = 17.48$, $p < 0.0001$; time: $F_{(1.912, 57.36)} = 28.78$, $p < 0.0001$; interaction: $F_{(9, 90)} = 8.465$, $p < 0.0001$); moreover, the levels of bhb K42 and K67 were increased by β-OHB, but the level of T286 phosphorylation and CaMKII activity in the hippocampal CA3 region were reduced (Figure 5D; treatment: $F_{(3, 20)} = 5.589$, $p < 0.01$; protein: $F_{(1.916, 38.32)} = 12.06$, $p < 0.001$; interaction: $F_{(6, 40)} = 6.153$, $p < 0.001$; Figure 5E; $F_{(3, 20)} = 5.763$, $p < 0.01$). Overall, these results indicate that β-OHB-induced bhb at K42 and K67 residues of CaMKII-α inhibit T286 phosphorylation and CaMKII activity, which is involved in the inhibitory effect of β-OHB on cocaine-induced reinstatement.

To further illustrate whether K42 and K67 kbhb of CaMKII-α would affect the initial place preference learning of mice, mice were fed doxycycline to switch on the expression of the exogenous CaMKII-α or mutant CaMKII-α gene, followed by β-OHB injection (300 mg/kg, i.p.) for 4 consecutive days in the acquisition phase of CPP (Figure S4K). The results showed that bhb K42 and K67 of CaMKII-α failed to alter the acquisition of cocaine CPP (Figure S4L; treatment: $F_{(3, 32)} = 3.927$, $p < 0.05$;

time: $F_{(1, 32)} = 45.55$, $p < 0.0001$; interaction: $F_{(3, 32)} = 6.967$, $p < 0.01$) in spite of the fact that the levels of bhb K42 and K67 of CaMKII-α were increased in the hippocampal CA3 region (Figure S4M; treatment: $F_{(3, 20)} = 13.62$, $p < 0.0001$; protein: $F_{(1, 20)} = 0.0939$, $p = 0.7624$; interaction: $F_{(3, 20)} = 0.09854$, $p = 0.9599$). Collectively, kbhb of CaMKII-α may play an important role in the cocaine-induced reinstatement.

DISCUSSION

The effect of KD consumption in regulating drug relapse and the underlying mechanism are largely unknown (Martinez et al., 2019; Wiers et al., 2021). Here, we found that administration of a KD or β-OHB during the extinction phase significantly inhibited cocaine-induced reinstatement in mice. This effect was mainly attributed to the enhancement of CaMKII-α kbhb and subsequent decrease in CaMKII-α activity in the hippocampus. In this process, ketogenesis-generated β-OHB contributed directly to CaMKII-α kbhb and inhibited CaMKII-α T286 phosphorylation as well as its activity. These findings provide insight into how a KD affects cocaine relapse, revealing a link between β-OHB-mediated kbhb on CaMKII-α and drug addiction.

β-OHB promotes protein kbhb modification

β-OHB is a non-volatile and stable compound; once synthesized, it is released into the bloodstream, and most β-OHB in the brain is supplied by the liver via the bloodstream (Achanta and Rae, 2017). Recent studies have shown that β-OHB plays a signaling role by inducing kbhb, a type of PTM, on histone or non-histone proteins (Koronowski et al., 2021; Liu et al., 2019; Xie et al., 2016). Of note, numerous kbhb sites have been identified in enzymes compared with in non-enzymes, suggesting that kbhb can regulate enzymatic activity (Koronowski et al., 2021; Liu et al., 2019; Zhang et al., 2020). For example, kbhb modification of AHCY, a rate-limiting enzyme that hydrolyses the conversion of S-adenosylhomocysteine to homocysteine and adenosine, decreases AHCY enzymatic activity (Koronowski et al., 2021). As the potential substrates for kbhb remain largely unknown, we first performed a proteomic characterization of kbhb substrates in the hippocampus of mice treated with β-OHB. Notably, we acquired a kbhb proteome dataset containing 85 proteins with 105 kbhb sites, most of which were highly enriched in the cytoplasm. This feature of kbhb is quite different from lysine-succinylated (ksucc) proteins, which are prevalently located in mitochondria (Ou et al., 2020), suggesting that different types of PTMs may possess unique substrate profiles. Indeed, kbhb is enriched on proteins associated with biological regulation processes, whereas ksucc is highly enriched on proteins involved in diverse metabolic pathways (Huang et al., 2021).

What is the driving force for protein kbhb, and how are kbhb pathways regulated in cells? Previous studies have shown that the CoA-bound form of β-OHB may serve as the substrate for kbhb (Xie et al., 2016), and the local concentration of short-chain fatty acids is an important factor influencing lysine acylation. Intriguingly, a recent work has shown that locally generated succinyl-CoA is also used by lysine acetyltransferase 2A (KAT2A; also known as GCN5) for histone ksucc (Wang et al., 2017). Indeed, we also found that β-hydroxybutyryl-CoA greatly promoted kbhb modification of recombinant CaMKII-α, suggesting that dynamic changes in short-chain CoAs and corresponding short-chain fatty acids may drive the kbhb process. Protein kbhb functions as a mechanism by which ketone bodies regulate cellular physiology, supporting a model in which cellular utilization of metabolite sources can regulate enzyme activity through metabolite-directed PTM.

β-OHB reduces cocaine reinstatement through hippocampal CaMKII-α kbhb

A few studies have suggested that high-fat dietary therapy protects against the rewarding effects of drugs by disrupting neuronal excitability or balancing excitatory and inhibitory neurotransmission (Davis et al., 2008; Wellman et al., 2007). However, little is known about the mechanism by which KD consumption modulates neuronal excitability and cocaine-induced reinstatement. CaMKII-α is a central node of several signaling pathways implicated in drug-induced synaptic plasticity and addictive behaviors (Muller et al., 2016; Robison, 2014). Moreover, CaMKII-α seems to be a "memory molecule" that is important for the maintenance of drug-contextual memory (Bayer and Schulman, 2019). In this study, inhibition of CaMKII-α activity via β-OHB treatment obviously reduced cocaine-induced reinstatement, further supporting the notion that CaMKII-α plays a key role in maintaining the hypersensitivity of the hippocampus to drug cues in the reinstatement test. Through PTM analysis, we discovered CaMKII-α as one of the targets of kbhb, and further studies demonstrated that CaMKII-α was modified by kbhb at the K42 and K267 sites in the presence of β-OHB, which suppressed its activity. Similarly, β-OHB-mediated p53 kbhb is a mechanism for the regulation of p53 activity, providing a promising therapeutic target for cancer (Liu et al., 2019).

The enzymes participating directly in CaMKII-α kbhb are currently unclear. Previous studies have suggested that CREB-binding protein (CBP) is a flexible enzyme that may play an important role in p53 kbhb (Liu et al., 2019). Acetyltransferase p300 has been found to bind an array of acyl-CoAs (Kaczmarska et al., 2017) and facilitate histone kbhb in a cell-free assay (Huang et al., 2021). Further study is needed to clarify the enzymes responsible for CaMKII-α kbhb in the hippocampus of mice treated with a KD or β-OHB.

Histone hyperacetylation promotes cocaine reinstatement through inhibition of HDAC (Shimazu et al., 2013). Interestingly, a few studies have shown that β-OHB acts as an endogenous inhibitor of HDAC, suggesting that β-OHB may disrupt cocaine reinstatement by inhibiting HDAC directly and enhancing histone acetylation (Shimazu et al., 2013). However, the acetylation levels of H3K9 and H3K14 in the hippocampus were not altered in the current study, indicating that KD or β-OHB treatment disrupts cocaine-induced reinstatement by promoting CaMKII-α kbhb. We infer that β-OHB may be a preferred substrate for kbhb modification rather than an HDAC inhibitor for modulation of histone acetylation.

CaMKII-α kbhb suppresses cocaine-induced autophosphorylation

CaMKII-α is activated by Ca^{2+} influx through NMDA receptors or voltage-gated Ca^{2+} channels, resulting in its autophosphorylation

at T286. The dynamics of CaMKII-α activity and its autophosphorylation suggest a role in learning and memory (Irvine et al., 2006). Increase of T286 autophosphorylation may be a mechanism by which naringin improves long-term cognitive function in the Alzheimer's disease transgenic mouse model (Wang et al., 2013). Importantly, CaMKII-α autophosphorylation at T286 is a primary molecular mechanism of drug addiction (Easton et al., 2013; Salling et al., 2017). Therefore, inhibition of T286 autophosphorylation is proposed to be a therapeutic approach for drug addiction and relapse. A key finding in this study was that CaMKII-α kbhb at K42 and K267 sites suppressed CaMKII-α T286 autophosphorylation as well as CaMKII-α activity in the hippocampus of mice treated with the KD or β-OHB during the extinction period. This finding suggests that KD therapy is a therapeutic strategy for the treatment of cocaine reinstatement. Similarly, conditioned reinforcing properties that induce T286 autophosphorylation of CaMKII-α promote alcohol reinstatement in mice, and inhibition of T286 autophosphorylation is a useful medical management technique for alcohol relapse (Salling et al., 2017). In addition, CaMKII-α autophosphorylation controls remodeling of glutamatergic synapses in the reward system, which is a plausible mechanism for alcohol-addiction-related behavior (Mijakowska et al., 2017).

The role of CaMKII-α T286 autophosphorylation in maintaining drug-related neuroplasticity and rewarding effects seems to be cocaine specific and may be not specifically required for the development of morphine addition (Andersen et al., 2020). Mice without the capacity for CaMKII-α autophosphorylation (T286A) fail to establish a preference for cocaine (Easton et al., 2014). Nevertheless, morphine induces a similar CPP in mice lacking CaMKII-α autophosphorylation (T286A) as in wild-type mice (Andersen et al., 2020), indicating that CaMKII-α T286 autophosphorylation is not specifically required for the development of CPP induced by opioids. Emerging evidence has shown that environmental and non-pharmacological factors determine the differences in the final pharmacological effects observed among different types of addictive drugs (Ahmed et al., 2020). The results of these studies, together with our findings, indicate that CaMKII-α T286 autophosphorylation induced by different drugs mediates distinct neuropsychological mechanisms.

In summary, the present study broadens the therapeutic scope for metabolic therapies and generates significant interest in the link between β-OHB-mediated protein PTM and drug addiction. Notably, we have discovered a mechanism by which β-OHB acts as a β-hydroxybutyryl donor, modifies CaMKII-α kbhb, and then suppresses CaMKII-α activity, which is necessary for disruption of cocaine relapse. Further studies are needed to explore whether CaMKII-α kbhb affects synaptic plasticity or NMDA-dependent long-term potentiation (LTP) and to determine whether KD therapy indeed has clinical applications.

Limitations of the study

In this study, we only measured HDAC H3K9 and H3K14 acetylation as well as its overall deacetylase activity. We admit that more histone acetylation sites potentially induced by KD or β-OHB need to be investigated. In addition, it is likely that certain HDACs or Sirtuins may regulate histone acetylation or other type of modifications, which may also inhibit cocaine-induced reinstatement. Therefore, we cannot completely exclude other mechanisms by which KD or β-OHB disrupts cocaine-induced reinstatement.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Animals
  - Cell lines
- METHOD DETAILS
  - Cocaine conditioned place preference (CPP)
  - Apparatus
  - Experimental procedure
  - Pre-conditioning (habituation)
  - CPP training (acquisition)
  - Extinction
  - Reinstatement
  - Tissue isolation
  - Western blot
  - Measurement of β-hydroxybutyrate (β-OHB) levels
  - Assay of HDAC activity
  - Lentiviral vector construction and cell transfection
  - Co-immunoprecipitation analysis
  - Western blot analysis of CaMKII-α kbhb
  - Protein purification
  - CaMKII-α β-hydroxybutyrylation *in vitro*
  - CaMKII activity assay
  - Adeno-associated virus injection
  - Immunohistochemistry
  - Dot blots
  - LC-MS/MS analysis of protein β-hydroxybutyrylation
  - LC-MS/MS analysis
  - Database search
  - Bioinformatics methods
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111724.

ACKNOWLEDGMENTS

We thank PTM Biolabs Co., Ltd. for kbhb proteomics analysis and Yuanhong Deng and his colleagues (PTM Biolabs Co., Ltd. Hangzhou, China) for the MS analysis of CaMKII-α β-hydroxybutyrylation. This work was partially supported by National Natural Science Foundation of China (Grants 82071494, 81871043, 81272459) and "1 · 3·5 Project for Disciplines of Excellence, West China Hospital, Sichuan University".

AUTHOR CONTRIBUTIONS

H.L. and X.W., conception and design, performed the experiments, acquisition of data, as well as analysis and interpretation of data, and wrote the manuscript; Z.W., R.C., W.X., Jiamei Zhang, Z.Y., L.B., Jie Zhang, F.Q., L.W., L.J., Y.H., Y.W., Q.W., S.L, and Yaxing Chen., acquisition of data, as well as analysis and interpretation of data; Y. Zhou, Yuanyuan Chen., Y.D., and Y.W., analysis and interpretation of behavior data; H.W., J.T., and Y. Zhao., help designing experiments and provided experimental reagents; X.C., conceived and supervised this research, as well as drafted and revised the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 18, 2022
Revised: August 29, 2022
Accepted: October 3, 2022
Published: November 29, 2022

REFERENCES

Achanta, L.B., and Rae, C.D. (2017). Beta-hydroxybutyrate in the brain: one molecule, multiple mechanisms. Neurochem. Res. *42*, 35–49.

Ahmed, S.H., Badiani, A., Miczek, K.A., and Muller, C.P. (2020). Non-pharmacological factors that determine drug use and addiction. Neurosci. Biobehav. Rev. *110*, 3–27.

Andersen, J.M., Opdal, S.H., Muller, C.P., and Boix, F. (2020). CaMKII is activated in opioid induced conditioned place preference, but alphaCaMKII Thr286 autophosphorylation is not necessary for its establishment. Behav. Brain Res. *390*, 112676.

Bavley, C.C., Fetcho, R.N., Burgdorf, C.E., Walsh, A.P., Fischer, D.K., Hall, B.S., Sayles, N.M., Contoreggi, N.H., Hackett, J.E., Antigua, S.A., et al. (2020). Cocaine- and stress-primed reinstatement of drug-associated memories elicit differential behavioral and frontostriatal circuit activity patterns via recruitment of L-type Ca(2+) channels. Mol. Psychiatr. *25*, 2373–2391.

Bayer, K.U., and Schulman, H. (2019). CaM kinase: still inspiring at 40. Neuron *103*, 380–394.

D'Andrea Meira, I., Romão, T.T., Pires do Prado, H.J., Krüger, L.T., Pires, M.E.P., and da Conceição, P.O. (2019). Ketogenic diet and epilepsy: what we know so far. Front. Neurosci. *13*.

Davis, J.F., Tracy, A.L., Schurdak, J.D., Tschop, M.H., Lipton, J.W., Clegg, D.J., and Benoit, S.C. (2008). Exposure to elevated levels of dietary fat attenuates psychostimulant reward and mesolimbic dopamine turnover in the rat. Behav. Neurosci. *122*, 1257–1263.

Deschaux, O., Vendruscolo, L.F., Schlosburg, J.E., Diaz-Aguilar, L., Yuan, C.J., Sobieraj, J.C., George, O., Koob, G.F., and Mandyam, C.D. (2014). Hippocampal neurogenesis protects against cocaine-primed relapse. Addict. Biol. *19*, 562–574.

Easton, A.C., Lourdusamy, A., Havranek, M., Mizuno, K., Solati, J., Golub, Y., Clarke, T.K., Vallada, H., Laranjeira, R., Desrivieres, S., et al. (2014). alpha-CaMKII controls the establishment of cocaine's reinforcing effects in mice and humans. Transl. Psychiatry *4*, e457.

Easton, A.C., Lucchesi, W., Mizuno, K., Fernandes, C., Schumann, G., Giese, K.P., and Muller, C.P. (2013). alphaCaMKII autophosphorylation controls the establishment of alcohol-induced conditioned place preference in mice. Behav. Brain Res. *252*, 72–76.

Engblom, D., Bilbao, A., Sanchis-Segura, C., Dahan, L., Perreau-Lenz, S., Balland, B., Parkitna, J.R., Lujan, R., Halbout, B., Mameli, M., et al. (2008). Glutamate receptors on dopamine neurons control the persistence of cocaine seeking. Neuron *59*, 497–508.

Hanson, P.I., and Schulman, H. (1992). Neuronal Ca2+/calmodulin-dependent protein kinases. Annu. Rev. Biochem. *61*, 559–601.

Hartman, A., Gasior, M., Vining, E., and Rogawski, M.J.P.n. (2007). The neuropharmacology of the ketogenic diet. Pediatr. Neurol. *36*, 281–292.

Heller, E.A., Hamilton, P.J., Burek, D.D., Lombroso, S.I., Pena, C.J., Neve, R.L., and Nestler, E.J. (2016). Targeted epigenetic remodeling of the Cdk5 gene in nucleus accumbens regulates cocaine- and stress-evoked behavior. J. Neurosci. *36*, 4690–4697.

Huang, H., Zhang, D., Weng, Y., Delaney, K., Tang, Z., Yan, C., Qi, S., Peng, C., Cole, P.A., Roeder, R.G., et al. (2021). The regulatory enzymes and protein substrates for the lysine β-hydroxybutyrylation pathway. Sci. Adv. *7*.

Irvine, E.E., von Hertzen, L.S., Plattner, F., and Giese, K.P. (2006). alphaCaMKII autophosphorylation: a fast track to memory. Trends Neurosci. *29*, 459–465.

Kaczmarska, Z., Ortega, E., Goudarzi, A., Huang, H., Kim, S., Marquez, J.A., Zhao, Y., Khochbin, S., and Panne, D. (2017). Structure of p300 in complex with acyl-CoA variants. Nat. Chem. Biol. *13*, 21–29.

Kesner, R.P., Kirk, R.A., Clark, J.K., Moore, A., and Keefe, K. (2016). Naloxone injections into CA3 disrupt pattern completion associated with relapse from cocaine seeking. Hippocampus *26*, 892–898.

Koronowski, K.B., Greco, C.M., Huang, H., Kim, J.K., Fribourgh, J.L., Crosby, P., Mathur, L., Ren, X., Partch, C.L., Jang, C., et al. (2021). Ketogenesis impact on liver metabolism revealed by proteomics of lysine beta-hydroxybutyrylation. Cell Rep. *36*, 109487.

Kraeuter, A.-K., Phillips, R., and Sarnyai, Z. (2020). Ketogenic therapy in neurodegenerative and psychiatric disorders: from mice to men. Prog. Neuro Psychopharmacol. Biol. Psychiatr. *101*.

Liu, K., Li, F., Sun, Q., Lin, N., Han, H., You, K., Tian, F., Mao, Z., Li, T., Tong, T., et al. (2019). p53 beta-hydroxybutyrylation attenuates p53 activity. Cell Death Dis. *10*, 243.

Liu, X., Liu, Y., Zhong, P., Wilkinson, B., Qi, J., Olsen, C.M., Bayer, K.U., and Liu, Q.S. (2014). CaMKII activity in the ventral tegmental area gates cocaine-induced synaptic plasticity in the nucleus accumbens. Neuropsychopharmacology *39*, 989–999.

Loweth, J.A., Li, D., Cortright, J.J., Wilke, G., Jeyifous, O., Neve, R.L., Bayer, K.U., and Vezina, P. (2013). Persistent reversal of enhanced amphetamine intake by transient CaMKII inhibition. J. Neurosci. *33*, 1411–1416.

Luo, A.H., Tahsili-Fahadan, P., Wise, R.A., Lupica, C.R., and Aston-Jones, G. (2011). Linking context with reward: a functional circuit from hippocampal CA3 to ventral tegmental area. Science *333*, 353–357.

Lutas, A., and Yellen, G. (2013). The ketogenic diet: metabolic influences on brain excitability and epilepsy. Trends Neurosci. *36*, 32–40.

Malvaez, M., McQuown, S.C., Rogge, G.A., Astarabadi, M., Jacques, V., Carreiro, S., Rusche, J.R., and Wood, M.A. (2013). HDAC3-selective inhibitor enhances extinction of cocaine-seeking behavior in a persistent manner. Proc. Natl. Acad. Sci. USA *110*, 2647–2652.

Martinez, L.A., Lees, M.E., Ruskin, D.N., and Masino, S.A. (2019). A ketogenic diet diminishes behavioral responses to cocaine in young adult male and female rats. Neuropharmacology *149*, 27–34.

Mijakowska, Z., Lukasiewicz, K., Ziolkowska, M., Lipinski, M., Trabczynska, A., Matuszek, Z., Leski, S., and Radwanska, K. (2017). Autophosphorylation of alpha isoform of calcium/calmodulin-dependent kinase II regulates alcohol addiction-related behaviors. Addict. Biol. *22*, 331–341.

Muller, C.P., Quednow, B.B., Lourdusamy, A., Kornhuber, J., Schumann, G., and Giese, K.P. (2016). CaM kinases: from memories to addiction. Trends Pharmacol. Sci. *37*, 153–166.

Newman, J.C., and Verdin, E. (2014). Ketone bodies as signaling metabolites. Trends Endocrinol. Metabol. *25*, 42–52.

Ou, T., Yang, W., Li, W., Lu, Y., Dong, Z., Zhu, H., Sun, X., Dong, Z., Weng, X., Chang, S., et al. (2020). SIRT5 deficiency enhances the proliferative and therapeutic capacities of adipose-derived mesenchymal stem cells via metabolic switching. Clin. Transl. Med. *10*, e172.

Perez-Riverol, Y., Bai, J., Bandla, C., Garcia-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., Kundu, D.J., Prakash, A., Frericks-Zipper, A., Eisenacher, M., et al. (2022). The PRIDE database resources in 2022: a hub for

mass spectrometry-based proteomics evidences. Nucleic Acids Res. *50*, D543–D552.

Puchalska, P., and Crawford, P.A. (2017). Multi-dimensional roles of ketone bodies in fuel metabolism, signaling, and therapeutics. Cell Metabol. *25*, 262–284.

Robison, A.J. (2014). Emerging role of CaMKII in neuropsychiatric disease. Trends Neurosci. *37*, 653–662.

Rossetti, T., Banerjee, S., Kim, C., Leubner, M., Lamar, C., Gupta, P., Lee, B., Neve, R., and Lisman, J. (2017). Memory erasure experiments indicate a critical role of CaMKII in memory storage. Neuron *96*, 207–216.e2.

Ruan, H.B., and Crawford, P.A. (2018). Ketone bodies as epigenetic modifiers. Curr. Opin. Clin. Nutr. Metab. Care *21*, 260–266.

Salling, M.C., Faccidomo, S.P., Li, C., Psilos, K., Galunas, C., Spanos, M., Agoglia, A.E., Kash, T.L., and Hodge, C.W. (2016). Moderate alcohol drinking and the Amygdala proteome: identification and validation of calcium/calmodulin dependent kinase II and AMPA receptor activity as novel molecular mechanisms of the positive reinforcing effects of alcohol. Biol. Psychiatr. *79*, 430–442.

Salling, M.C., Hodge, C.J., Psilos, K.E., Eastman, V.R., Faccidomo, S.P., and Hodge, C.W. (2017). Cue-induced reinstatement of alcohol-seeking behavior is associated with increased CaMKII T286 phosphorylation in the reward pathway of mice. Pharmacol. Biochem. Behav. *163*, 20–29.

Sanchis-Segura, C., and Spanagel, R.J.A.b. (2006). Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. Addict. Biol. *11*, 2–38.

Shimazu, T., Hirschey, M.D., Newman, J., He, W., Shirakawa, K., Le Moan, N., Grueter, C.A., Lim, H., Saunders, L.R., Stevens, R.D., et al. (2013). Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor. Science *339*, 211–214.

Taylor, J.R., Olausson, P., Quinn, J.J., and Torregrossa, M.M. (2009). Targeting extinction and reconsolidation mechanisms to combat the impact of drug cues on addiction. Neuropharmacology *56* (*Suppl 1*), 186–195.

Torregrossa, M.M., Corlett, P.R., and Taylor, J.R. (2011). Aberrant learning and memory in addiction. Neurobiol. Learn. Mem. *96*, 609–623.

Torregrossa, M.M., Sanchez, H., and Taylor, J.R. (2010). D-cycloserine reduces the context specificity of pavlovian extinction of cocaine cues through actions in the nucleus accumbens. J. Neurosci. *30*, 10526–10533.

Wagatsuma, A., Okuyama, T., Sun, C., Smith, L.M., Abe, K., and Tonegawa, S. (2018). Locus coeruleus input to hippocampal CA3 drives single-trial learning of a novel context. Proc. Natl. Acad. Sci. USA *115*, E310–E316.

Wang, Y., Guo, Y.R., Liu, K., Yin, Z., Liu, R., Xia, Y., Tan, L., Yang, P., Lee, J.H., Li, X.J., et al. (2017). KAT2A coupled with the alpha-KGDH complex acts as a histone H3 succinyltransferase. Nature *552*, 273–277.

Wang, D.M., Yang, Y.J., Zhang, L., Zhang, X., Guan, F.F., and Zhang, L.F. (2013). Naringin enhances CaMKII activity and improves long-term memory in a mouse model of Alzheimer's disease. Int. J. Mol. Sci. *14*, 5576–5586.

Wellman, P.J., Nation, J.R., and Davis, K.W. (2007). Impairment of acquisition of cocaine self-administration in rats maintained on a high-fat diet. Pharmacol. Biochem. Behav. *88*, 89–93.

Werner, C.T., Altshuler, R.D., Shaham, Y., and Li, X. (2020). Epigenetic mechanisms in drug relapse. Biol. Psychiatr *89*, 331–338.

Wiers, C.E., Vendruscolo, L.F., van der Veen, J.W., Manza, P., Shokri-Kojori, E., Kroll, D.S., Feldman, D.E., McPherson, K.L., Biesecker, C.L., Zhang, R., et al. (2021). Ketogenic diet reduces alcohol withdrawal symptoms in humans and alcohol intake in rodents. Sci. Adv. *7*, eabf6780.

Wolf, M.J.N.r.N. (2016). Synaptic mechanisms underlying persistent cocaine craving. Nat. Rev. Neurosci. *17*, 351–365.

Xie, Z., Zhang, D., Chung, D., Tang, Z., Huang, H., Dai, L., Qi, S., Li, J., Colak, G., Chen, Y., et al. (2016). Metabolic regulation of gene expression by histone lysine β-hydroxybutyrylation. Mol. Cell *62*, 194–206.

Yamagata, Y., Kobayashi, S., Umeda, T., Inoue, A., Sakagami, H., Fukaya, M., Watanabe, M., Hatanaka, N., Totsuka, M., Yagi, T., et al. (2009). Kinase-dead knock-in mouse reveals an essential role of kinase activity of Ca2+/calmodulin-dependent protein kinase IIalpha in dendritic spine enlargement, long-term potentiation, and learning. J. Neurosci. *29*, 7607–7618.

Zeng, K., Xie, A., Dong, X., Jiang, J., Hao, W., Jiang, M., and Liu, X. (2019). GABA-Aalpha5 might Be involved in learning-memory dysfunction in the offsprings of chronic ethanol-treated rats via GABA-Aalpha5 histone H3K9 acetylation. Front. Neurosci. *13*, 1076.

Zhang, H., Tang, K., Ma, J., Zhou, L., Liu, J., Zeng, L., Zhu, L., Xu, P., Chen, J., Wei, K., et al. (2020). Ketogenesis-generated beta-hydroxybutyrate is an epigenetic regulator of CD8(+) T-cell memory development. Nat. Cell Biol. *22*, 18–25.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-H3K9ac (1:1000)	Abcam	Cat# ab 4441
Rabbit anti-H3K14ac (1:1000)	Abclonal	Cat# A7254
Rabbit anti-H3 (1:1000)	Abcam	Cat# ab 12079
Mouse anti-GAPDH (1:1000)	Abcam	Cat# ab 8245
Rabbit anti-Pan-kbhb (1:1000)	PTM biolabs	Cat# PTM-1201
Mouse anti-CaMKII-α (1:1000)	Invitrogen	Cat# 13-7300
Rabbit anti-kbhb K42 (1:1000)	PTM biolabs	N/A
Rabbit anti-kbhb K267 (1:1000)	PTM biolabs	N/A
Rabbit anti-p T286 (1:1000)	Cell signaling	Cat# 12716
Rabbit anti-ACAT1 (1:1000)	Proteintech	Cat# 16215-1-AP
Rabbit anti-BDH1 (1:1000)	Proteintech	Cat# 15417-1-AP
Mouse anti-Flag (1:1000)	Sigma	Cat# F1084
Bacterial and virus strains		
AAV2/9-CaMKII-α-EGFP-Flag	Vigene Biotechnology	N/A
AAV2/9-mutant CaMKII-α-EGFP-Flag	Vigene Biotechnology	N/A
pLenti-EF1a-CMV-CaMKII-α-Flag-GFP-2A-Puro	Vigene Biotechnology	N/A
pLenti-EF1a-CMV-mutant CaMKII-α-Flag-GFP-2A-Puro	Vigene Biotechnology	N/A
Chemicals, peptides, and recombinant proteins		
Cocaine	National Institute for the Control of Pharmaceutical and Biological Products	N/A
β-hydroxybutyrate sodium	Sigma-Aldrich	Cat# 54965
Doxycycline	TCI	Cat# D4116
Nevanimibe	MCE	Cat# HY-10039A
3-Hydroxybutyric acid	Sigma-Aldrich	Cat# 166898
DMEM/High glucose Medium	Gibco	Cat# C1195500BT
Fetal bovine serum	Gibco	Cat# 10099-141
Antibiotic-Antimycotic	Gibco	Cat# 15240-062
Ketone diet	Bio-Serv	Cat# F3666
PBS	Hyclone	Cat# 10010049
Recombinant human CaMKII-α Protein	Abcam	Cat# ab 60899
Chemiluminescence substrate	Millipore	Cat# WBKLS0500
Protein A/G magnetic beads	Bimake	Cat# B23201
Anti-Flag beads	Bimake	Cat# B26101
Flag peptide	Bimake	Cat# B23111
DL-β-Hydroxybutyryl coenzyme A lithium salt	Sigma-Aldrich	Cat#166898
Critical commercial assays		
HDAC Activity Fluorometric Assay Kit	Biovision	Cat# K330-100
Mammalian Cell & Tissue Extraction Kit	Biovision	Cat# K269-500
Bradford assay kit	Beyotime	Cat# P0006
β-Hydroxybutyrate Colorimetric Assay Kit	Cayman	Cat# 700190

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pierce™ Crosslink Magnetic IP/co-IP Kit	Thermo	Cat# 88805
CaMKII enzymatic activity assay kits	Jingmei biotechnology	Cat: # JM-11610M1
Nuclear Extraction Kit	Abcam	Cat# ab 113474
Deposited data		
Identified Kbhb peptides (see also Table S1)	ProteomeXchange	PXD037321
Experimental models: Cell lines		
Mouse neuroblastoma 2a cells	ATCC	Cat# CCL-131
Experimental models: Organisms/strains		
Mouse: Adult C57BL6/J	Charles River	N/A
Software and algorithms		
UniProt-GOA database	UniPort	http://www.ebi.ac.uk/GOA/
UniProt database	UniPort	http://www.uniprot.org
GraphPad Prism 7.0	GraphPad Software	http://www.graphpad.com/
Chemi Analysis software	CLINX	N/A
Nis-Elements	Nikon	www.microscope.healthcare.nikon.com
CELLO	N/A	http://cello.life.nctu.edu.tw/
MoMo	N/A	http://motif-x.med.harvard.edu/
Wolfpsort	WoLF PSORT	https://wolfpsort.hgc.jp/
STRING database (version 10.1)	STRING	https://cn.string-db.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiaobo Cen (xbcen@scu.edu.cn).

Materials availability

Our study did not generate any new unique reagents.

Data and code availability

- The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier ProteomeXchange: PXD037321 and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. The hippocampal proteomics analysis of kbhb data are also available in Table S1.
- This paper does not report original code
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Male C57BL/6J wild-type (WT) mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China), and 8–12 weeks old mice were used in this study. All mice were housed in the animal room on a standard 12-h light/12-h dark cycle with a constant temperature and food and water available *ad libitum*. All experimental procedures and use of the animals were conducted in accordance with the guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care and the Institutional Animal Care and Use Committee of Sichuan University. All efforts were made to minimize the suffering of the mice.

Cell lines

Mouse neuroblastoma 2a (N2a) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, C1195500BT, Gibco) containing 10% fetal bovine serum (10,099-141, Gibco) and grown in a 5% CO_2 atmosphere at 37°C.

METHOD DETAILS

Cocaine conditioned place preference (CPP)

Cocaine CPP-induced reinstatement test was performed as previously described, with slight modification (Engblom et al., 2008). The modification experimental methods are as fellow:

Apparatus

The CPP test was conducted using a standard three-chambered apparatus equipped with two large conditioning compartments (black and white) that differed in their flooring (bar and grid) and a small middle chamber (gray, smooth PVC floor) that connected the two large compartments.

Experimental procedure

The paradigm of CPP reinstatement was consisted of the following phases: pre-conditioning (habituation), CPP training (acquisition), post-conditioning (test 1), extinction (test 2) and reinstatement (Figure 1A).

Pre-conditioning (habituation)

All mice were placed in the CPP chamber without receiving any injection and allowed to freely access to two chambers for 10 min for once per day for 3 days. On day 4, baseline preference was assessed by placing the mice in the middle chamber and allowing them to habituate the entire chambers freely for 15 min; an initial measurement of baseline preference ($Time_{baseline)}$ was assessed by recording the time spent in each chamber. Animals were excluded from the test if they showed a strong unconditioned preference for either side chamber (chamber bias >300 s).

CPP training (acquisition)

During the condition training, mice were randomly assigned to saline and cocaine group and received an injection of saline or cocaine (10 mg/kg, i.p.). Cocaine-treated mice were immediately placed into the cocaine-paired chamber for 30 min after cocaine injection in the morning. In the afternoon, the mice received a saline injection and were immediately placed into the opposite chamber for 30 min for 4 consecutive days. Saline-treated mice received saline and then were placed into the saline-paired chamber for 30 min in the morning and with repeated saline injection in the other chamber in the afternoon. On the test day, the animals were placed in the middle compartment and the time of spent in the two compartments was recorded for 15 min. $Time_{test1}$ was defined as the time spent in the cocaine-paired chamber minus time spent in the cocaine-unpaired chamber. The CPP scores were calculated by $Time_{test1}$ minus $Time_{baseline}$ (CPP score = $Time_{test1}$-$Time_{baseline}$). Generally, cocaine strongly reverses preference and results in a positive CPP score, and the CPP score was defined as the extent of the shift in preference after the cocaine injection.

Extinction

After post-conditioning preference test, ketone diet (KD) group mice were fed with KD (F3666, Bio-Serv, 10% protein and 90% fat), and the control diet (CD) group mice were given an equal volume of CD (10% protein,13% fat and 77% carbohydrates) for 21 days, which was conducted as previously reported (Koronowski et al., 2021). The previously studies have shown that mice fed with KD could induced a high level of β-OHB in peripheral blood. All the food was provided *ad libitum* at all the time.

For β-OHB treatment, 5 min before extinction training, mice were injected β-OHB (75, 150 and 300 mg/kg) twice a day (*i.p.*) for 10 successive days during extinction phase. While control mice were injected with same volume of saline.

In the extinction training, mice were placed into middle chamber and allowed to explore both chambers freely for 15 min. The mice received extinction training daily for 21 days (KD treatment) or 10 day (β-OHB treatment), and cocaine injections were not given during this extinction period. On the test day, the animals were placed in the middle compartment and the time of spent in the two compartments was recorded for 15 min. $Time_{test2}$ was defined as the time spent in the cocaine-paired chamber minus time spent in the cocaine-unpaired chamber. CPP scores were calculated by $Time_{test2}$ minus $Time_{baseline}$ (CPP score = $Time_{test2}$ - $Time_{baseline}$).

Reinstatement

After the extinction training, the mice were treated with a priming injection of cocaine (7.5 mg/kg, *i.p.*), and then placed into CPP apparatus to examine the reinstatement of cocaine CPP. During this reinstatement test, mice were allowed to freely access to the entire apparatus for 15 min, and the time spent in the cocaine-paired chamber minus time spent in the cocaine-unpaired chamber as $Time_{test}$. CPP scores were calculated by $Time_{test}$ minus $Time_{baseline}$ (CPP score = $Time_{test}$ - $Time_{baseline}$).

Tissue isolation

Mice were sacrificed by rapid decapitation at the end of the behavioral tests. Tissues of hippocampus, prefrontal cortex, nucleus accumbens, striatum or hippocampus CA3 (separated from hippocampus) were separately removed from the brain, dry ice snap-frozen, and stored at −80°C until assay.

Western blot

Brain tissues and cells were lysed, and proteins were extracted using a mammalian cell and tissue extraction kit (K269-500, Biovision) containing phosphatase inhibitors (4,906,845,001, Roche). Total protein concentration was detected with a bradford assay kit (P0006, Beyotime). Protein (20 μg) was loaded and separated with the 10 or 12.5% sodium dodecyl sulfatepolyacrylamide gel. After loading, the gels were then transferred to the polyvinylidene difluoride (PVDF) membrane. Membrane was blocked in TBST buffer containing 5% non-fat dry milk for 1 h at room temperature, and then incubated and gently shaken overnight with primary antibody at 4°C. After incubation with secondary antibody at room temperature for 2 h, protein immunoreactivity was visualized using a chemiluminescence substrate (WBKLS0500, Millipore) on chemiluminescence imagine system (CLINX, Shanghai, China). Band optical density was quantified using Chemi Analysis software (CLINX, Shanghai, China). GAPDH level was used to normalize Western blot.

Measurement of β-hydroxybutyrate (β-OHB) levels

Plasma of mice was separated by centrifugation at 1500 *g* at 4°C for 15 min and frozen at −80°C until thawed for assay. β-OHB level of plasma was detected using the β-OHB assay kit (CAY-700190, Cayman Chemical), and brain tissues β-OHB level was measured using Ultra Performance Liquid Chromatography (UPLC). Briefly, the cold methanol and Methyl tert-butyl ether (MTBE) (1:3, V/V) were added to the hippocampus tissue and mixed gently. After samples were sonicated on ice, the organic phase and the aqueous phase were separated by adding 300 μL 25% methanol. After centrifugation at 14,000 *g* at 4°C for 10 min, the sample were clearly divided into three layers. The upper layer was the organic phase (lipid), the middle layer was the aqueous phase (metabolite), and the lower layer was the protein. The middle aqueous phase (metabolite) was collected and stored at −80°C. The standard β-hydroxybutyric acid was formulated into different concentration gradients of 0, 20, 50, 100, 200, 300 ng/mL for establishing a standard curve. The extracted metabolites were transferred to clear insert pipes, and 4 μL volumes sample was injected into the UPLC system (LC-30A, Shimadzu) and equipped with a C18 column (100 mm × 2.1 mm×1.8 μm, Thermo) maintained at 40°C by gradient elution with a mobile phase flow rate of 0.3 mL/min. Gradient elution mobile phase was consisted of A (0.1% formic acid water) and B (methanol).

Assay of HDAC activity

Nuclear proteins of hippocampus tissue were extracted using Nuclear Extraction Kit (ab113474, Abcam). HDAC enzyme activity of nuclear protein were determined using HDAC Activity Fluorometric Assay Kit (K330, Biovision). Measurements were performed according to the manufacturer's instructions.

Lentiviral vector construction and cell transfection

The lentiviral vectors (LV) pLenti-EF1a-CMV-CaMKII-α-Flag-GFP-2A-Puro and pLenti-EF1a-CMV-mutant CaMKII-α-Flag-GFP-2A-Puro (Vigene Biotechnology Co.Ltd, China) were constructed for cell studies. DMEM was replaced with serum-free DMEM and incubated for 2 h before transfection, followed by incubations with the lentiviral vector containing mouse CaMKII-α or mutant CaMKII-α. Antibiotic-resistant clones were selected with 2.5 μg/mL puromycin and cultured in DMEM medium containing puromycin. The purified Flag-CaMKII-α or Flag-mutant CaMKII-α was extracted from the transfected N2a cells with Flag beads (B26101, Bimake). Western blot analysis was performed with antibodies specific for β-hydroxybutyrylated K42 and K267 residues.

Co-immunoprecipitation analysis

To clarify whether CaMKII-α was a target of β-hydroxybutyrylation, cells or tissues were harvested for the co-immunoprecipitation (co-IP) analysis using the simplified and reliable Pierce Crosslink Magnetic IP/co-IP Kit (88,805, Thermo Scientific). Briefly, the CaMKII-α primary antibody was bound to 50 μL of Protein A/G magnetic beads (B23201, Bimake) for 15 min and washed three times. The protein was extracted using the mammalian cell and tissue extraction kit (K269-500, Biovision), and the protein supernatant from each sample was incubated with the antibody-crosslinked beads overnight at 4°C. On the next day, beads were washed two times with IP lysis/wash buffer and one time with ultrapure water. The supernatants were collected for Western blot analysis. Band optical density was quantified using Chemi Analysis software (CLINX, Shanghai, China). CaMKII-α level was used as a control to quantify CaMKII-α kbhb level.

Western blot analysis of CaMKII-α kbhb

The hippocampus tissues and cells were lysed with mammalian cell and tissue extraction kit (K269-500, Biovision). CaMKII-α kbhb were purified by IP with CaMKII-α primary antibody, and kbhb were detected by Western blots using the pan β-hydroxybutyrylation-lysine antibodies (Pan-kbhb, PTM-1201, PTM Bio).

Protein purification

CaMKII-α and mutant CaMKII-α overexpression resistant cells were harvested and lysed proteins were extracted using a mammalian cell and tissue extraction kit (K269-500, Biovision). Anti-Flag beads (B26101, Bimake) were incubated with the cell lysates overnight at 4°C. On the next day, the beads were washed with PBST buffer three times, and purified proteins were competitively eluted with Flag peptide (B23111, Bimake). Purified proteins were collected for Western blot or enzyme activity analysis.

CaMKII-α β-hydroxybutyrylation *in vitro*

A vitro assay to induce β-hydroxybutyrylation of CaMKII-α was performed as described previously (Kaczmarska et al., 2017; Koronowski et al., 2021). Reactions were set up in reaction buffer (25 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM DTT, 100 μM EDTA, 10% glycerol, supplemented with Protease Inhibitor Cocktail, 10 mM nicotinamide and 100 ng/mL TSA), with 2 μg recombinant CaMKII-α (ab60899, Abcam) and 10 mM DL-β-hydroxybutyryl-CoA lithium salt (H0261, Sigma) for 2 h in a 37°C water bath. Reactions were stopped with addition of SDS loading buffer for Western blot or loaded directly into CaMKII activity assay wells for measurement.

CaMKII activity assay

CaMKII activity was measured using CaMKII enzymatic activity assay kits (JM-11610M1, JINGMEI BIOTECHNOLOGY). Measurements were performed according to the manufacturer's instructions.

Adeno-associated virus injection

Doxycycline-inducible adeno-associated virus (AAV), AAV2/9-CaMKII-α-EGFP-Flag and AAV2/9-mutant CaMKII-α-EGFP-Flag were constructed (Vigene Biotechnology Co. Ltd, China). Point mutations of lysine 42 to methionine and lysine 267 to alanine were synthesized in the murine full-length CaMKII-α amino acid sequence. Mice (8-12 weeks old) were anesthetized with sodium pentobarbital (60 mg/kg) and fixed on a stereotaxic apparatus. Small craniotomy holes were drilled with a skull rotor (RWD Life Science) for virus injection. AAV2/9-CaMKII-α-EGFP-Flag (1μL, 0.1μL/min) and AAV2/9-mutant CaMKII-α-EGFP-Flag (1μL, 0.1μL/min) were infused into the bilateral hippocampus CA3 region (AP, −2.0 mm; ML, ±2.1 mm; DV, −2.0 mm) (Wagatsuma et al., 2018). Mice were recovered for at least 2 weeks before behavioral tests. At the end of the induction session, all mice were fed doxycycline (0.02% in 5% sucrose drinking water) to switch on exogenous gene expression during the extinction period.

Immunohistochemistry

To illustrate the AAV injection sites of hippocampus, mice were anesthetized with sodium pentobarbital (60 mg/kg) and perfused intracardially with PBS, followed by ice-cold 4% paraformaldehyde in 0.1M PBS (pH 7.4). Brains were carefully extracted from the skull, fixed in 4% PFA for overnight, and then dehydrated with 30% sucrose at 4°C. The brain sections were washed in TBS for 3 times and covered on Anti-fade Mounting Medium with Dapi (H-1200, Vector). Confocal images were acquired with a laser confocal microscope (Nikon, Japan).

Dot blots

The purified peptides were prepared by PTM biolabs and then provided to PTM biolabs for generation of rabbit polyclonal antibodies (PTM Biolabs Co., Ltd. China). For peptide dot blots, peptides were dotted as progressive protein concentrations (1, 4, 16 and 64 ng, respectively) on a nitrocellulose membrane. Membranes were left to dry at room temperature for 1 h and then blocked in 5% milk/PBST for 1 h. Immunoreactivity was visualized using a chemiluminescence substrate (WBKLS0500, Millipore) on chemiluminescence imagine system (CLINX, Shanghai, China).

LC-MS/MS analysis of protein β-hydroxybutyrylation

Protein extraction

Hippocampal tissues were transferred to a 5 mL centrifuge tube. Lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail) was added to the tubes, followed by sonication 3 times on ice using a high intensity ultrasonic processor. The remaining debris was removed by centrifugation at 12,000 *g* at 4°C for 10 min. The supernatant was collected and the protein concentration was determined with BCA kit.

Trypsin digestion

Protein was reduced with 5 mM dithiothreitol for 30 min at 56°C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM TEAB to urea concentration less than 2M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion.

Peptide fractionation and immunoaffinity enrichment

Enrichment of kbhb peptides from different fractions by immunoprecipitation with Pan anti-kbhb antibody was carried out as described previously (Huang et al., 2021). Briefly, the peptides were first dissolved in NETN buffer [100 mM NaCl, 50 mM tris-HCl, 1 mM EDTA, and 0.5% NP-40 (pH 8.0)] and incubated with pan anti-kbhb beads at 4°C overnight. Then, the beads were washed three times with NETN buffer and twice with ddH_2O. The combined peptides were eluted with 0.1% (v/v) TFA. The isolated kbhb peptides were dried in SpeedVac.

LC-MS/MS analysis

The tryptic peptides were dissolved in 0.1% formic acid (solvent A) and then directly loaded onto a home-made reversed-phase analytical column (15cm length, 75 μm i.d.). The gradient was comprised of an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23%–35% in 8 min and climbing to 80% in 3 min then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350–1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15s dynamic exclusion. Automatic gain control (AGC) was set at 5×10^4. Fixed first mass was set as 100 m/z.

Database search

The resulting MS/MS data were processed using Maxquant search engine (v1.6.6.0). Tandem mass spectra were searched against Human uniprot database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 4 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in first search and 5 ppm in main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and acetylation modification and oxidation on Met were specified as variable modifications. FDR was adjusted to <1% and minimum score for modified peptides was set >40.

Bioinformatics methods

Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). The identified Kbhb sites were matched with the recorded binding and mutation sites extracted from the UniProt database (www.uniprot.org). We used wolfpsort, a subcellular localization predication soft, to predict subcellular localization. For protokaryon species, subcellular localization prediction soft CELLO was used. Soft MoMo (motif-x algorithm) was used to analysis the model of sequences constituted with amino acids in specific positions of modify-21-mers (10 amino acids upstream and downstream of the site) in all protein sequences. All differentially expressed modified protein database accession or sequence were searched against the STRING database version 10.1 for protein-protein interactions. STRING defines a metric called ''confidence score'' to define interaction confidence. We fetched all interactions that had a confidence score ≥ 0.7 (high confidence). The interaction network identified using STRING was visualized with Cytoscape.

QUANTIFICATION AND STATISTICAL ANALYSIS

The number of mice per group and experimental repeats are annotated in each of the corresponding figure legends. All data were analyzed with GraphPad Prism 7 software, presented as means ± SEM, and subjected to the Kolmogorov-Smirnov test to assess the normality of the distribution. For simple comparisons, an unpaired two-tailed Student's t test was used. For multiple comparisons, one-way ANOVA or two-way ANOVA, with a repeated-measures factor when necessary, followed by Bonferroni's post hoc tests was utilized for each experiment. In all cases, *n* refers to the number of animals. For all results, statistical significance was defined as $p < 0.05$.