Behavioral/Cognitive

Extinction of Contextual Cocaine Memories Requires Ca$_v$1.2 within D1R-Expressing Cells and Recruits Hippocampal Ca$_v$1.2-Dependent Signaling Mechanisms

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Exposure to cocaine-associated contextual cues contributes significantly to relapse. Extinction of these contextual associations, which involves a new form of learning, reduces cocaine-seeking behavior; however, the molecular mechanisms underlying this process remain largely unknown. We report that extinction, but not acquisition, of cocaine conditioned place preference (CPP) in male mice increased Ca$_v$1.2 L-type Ca^{2+} channel mRNA and protein in postsynaptic density (PSD) fractions of the hippocampus, a brain region involved in drug– context associations. Moreover, viral-mediated deletion of Ca$_v$1.2 in the dorsal hippocampus attenuated extinction of cocaine CPP. Molecular studies examining downstream Ca$_v$1.2 targets revealed that extinction recruited calcium/calmodulin (Ca^{2+}/CaMK)-dependent protein kinase II (CaMKII) to the hippocampal PSD. This occurred in parallel with an increase in phosphorylation of the AMPA GluA1 receptor subunit at serine 831 (S831), a CaMKII site, along with an increase in total PSD GluA1. The necessity of S831 GluA1 was further demonstrated by the lack of extinction in S831A GluA1 phosphomutant mice. Of note hippocampal GluA1 levels remained unaltered at the PSD, but were reduced near the PSD and at perisynaptic sites of dendritic spines in extinction-resistant S831A mutant mice. Finally, conditional knock-out of Ca$_v$1.2 in dopamine D1 receptor (D1R)-expressing cells resulted in attenuation of cocaine CPP extinction and lack of extinction-dependent changes in hippocampal PSD CaMKII expression and S831 GluA1 phosphorylation. In summary, we demonstrate an essential role for the hippocampal Ca$_v$1.2/CaMKII/S831 GluA1 pathway in cocaine CPP extinction, with data supporting contribution of hippocampal D1R-expressing cells in this process. These findings demonstrate a novel role for Ca$_v$1.2 channels in extinction of contextual cocaine-associated memories.

Key words: CaMKII; Ca$_v$1.2; cocaine; dopamine D1 receptor; extinction; GluA1

Significance Statement

Continued drug-seeking behavior, a defining characteristic of cocaine addiction, can be precipitated by contextual cues, yet the molecular mechanisms required for extinction of these context-specific memories remain poorly understood. Here, we have uncovered a novel and selective role of the Ca$_v$1.2 L-type Ca^{2+} channel and its downstream signaling pathway in the hippocampus that mediate extinction of cocaine conditioned place preference (CPP). We additionally provide evidence that supports a role of Ca$_v$1.2 within dopamine D1 receptor-expressing cells of the hippocampus for extinction of cocaine CPP. Therefore, these findings reveal a previously unknown role of Ca$_v$1.2 channels within the hippocampus and in D1 receptor-expressing cells in extinction of cocaine-associated memories, providing a framework for further exploration of mechanisms underlying extinction of cocaine-seeking behavior.

Introduction

Addiction to drugs of abuse such as cocaine results in part from exceptionally strong, maladaptive associations formed between contextual cues and the rewarding properties of the drug (Kelley, 2004; Koob and Volkow, 2010; Perry et al., 2014). These drug-associated contextual memories are long lasting, highly resistant

to extinction, and contribute to the high rate of relapse in cocaine-dependent individuals, as well as the reinstatement of cocaine-seeking behavior in rodents (Marchant et al., 2015; Venniro et al., 2016). Therefore, identifying mechanisms that can facilitate extinction and diminish relapse may aid in treating addiction. Human and rodent studies implicate the hippocampus, a critical substrate of learning and memory, as a key brain region for the development and maintenance of cocaine–context associations (Koob and Volkow, 2010; Kutlu and Gould, 2016). Studies modeling extinction training in rodents have revealed that extinction recruits a new learning process (Bouton, 2004; Vianna et al., 2004; Lattal et al., 2006) at the level of synaptic and molecular changes within brain reward regions (Nic Dhonnchadha et al., 2013) including the hippocampus, which is particularly involved in the extinction of contextual aspects of drug-associated memories (Chambers and Self, 2002; Szalay et al., 2013). However, the molecular mechanisms within the hippocampus that mediate contextual extinction remain largely unknown.

L-type Ca^{2+} channels (LTCCs) are well known for their role in hippocampal-dependent learning and memory (Moosmang et al., 2005; Krug et al., 2014; Temme et al., 2016; Kabir et al., 2017a). In particular, LTCCs have been implicated in mediating cocaine-associated behaviors (Licata and Pierce, 2003; Chartoff et al., 2006; Anderson et al., 2008; Giordano et al., 2010; Schierberl et al., 2011; Berger and Bartsch, 2014; Martinez-Rivera et al., 2017) and, recently, extinction of cocaine conditioned place preference (CPP) (Degoulet et al., 2016).

LTCCs are key mediators of activity-dependent molecular processes with activation and synaptic localization of the calcium/calmodulin (Ca^{2+}/CaMK)-dependent protein kinase II (CaMKII) subunits α and β representing its central downstream targets (Rose et al., 2009; Ma et al., 2012; Murphy et al., 2014; Striessnig et al., 2014). CaMKIIα/β are critically important for hippocampal-dependent contextual learning (Shonesy et al., 2014) and contribute to the molecular mechanisms of cocaine's action (Robison, 2014). CaMKIIα promotes glutamatergic signaling via phosphorylation at serine 831 (S831) on the GluA1 subunit of AMPA receptors (Mammen et al., 1997), a posttranslational modification that modulates channel conductance (Derkach et al., 1999; Kristensen et al., 2011). AMPA receptors play a central role in mediating cocaine-induced synaptic and behavioral plasticity (Pierce and Wolf, 2013; Wolf, 2016), including extinction of cocaine-associated memories, within brain reward regions including the nucleus accumbens (NAc) and prefrontal cortex (PFC) (Self et al., 2004; Peters et al., 2009; Rothwell et al., 2011; Nic Dhonnchadha et al., 2013); however, the role of hippocampal AMPA receptors in the extinction of cocaine contextual learning remains unknown.

One mechanism that regulates LTCC channel activity and signaling involves the activation of dopamine D1 receptors (D1Rs) (Surmeier et al., 1995; Liu and Graybiel, 1996; Giordano et al., 2010; Navakkode et al., 2012). Growing evidence indicates that D1Rs contribute to extinction of learned behaviors (Abraham et al., 2014; Singewald et al., 2015), including extinction of cocaine

CPP (Brenhouse et al., 2010; Fricks-Gleason et al., 2012; Brenhouse et al., 2015; Abraham et al., 2016).

Together, these findings suggest that the extinction of cocaine contextual memories may involve activation of a Ca$_v$1.2-regulated signaling pathway within hippocampal D1R-expressing cells. To test this hypothesis, a combination of genetic, molecular, and pharmacological approaches were used to assess the role and cell-type specificity of Ca$_v$1.2 channels in CaMKII and GluA1 levels in hippocampal PSD fractions after cocaine CPP extinction. We provide evidence for a selective role of a Ca$_v$1.2 channel-mediated mechanism in the hippocampus in extinction of cocaine CPP, with data supporting that this occurs via Ca$_v$1.2 signaling in D1R-expressing cells of the hippocampus.

Materials and Methods

Animals
Adult (>postnatal day 60) male wild-type (WT) mice (C57BL/6J; The Jackson Laboratory) as well as transgenic and mutant mice on the C57BL/6J background were used for all experiments. Viral-vector-mediated deletion of Ca$_v$1.2 was achieved using homozygous *cacna1c* floxed (Ca$_v$1.2$^{fl/fl}$) mice (Moosmang et al., 2005). Mice expressing a serine to alanine substitution at position 831 of GluA1 (S831A mutants) were generated as described previously (Lee et al., 2010) and bred in the Weill Cornell Medicine facilities. Mice with conditional knock-out of Ca$_v$1.2 in CaMKII-expressing cells (CaMK2^{cre}, Ca$_v$1.2$^{fl/fl}$) and their WT littermates (CaMK2^{cre}, Ca$_v$1.2$^{+/+}$) were generated by crossing Ca$_v$1.2$^{fl/fl}$ mice with mice expressing Cre recombinase under the transcriptional control of the CaMK2a promoter (Camk2a-Cre T29-1; The Jackson Laboratory). Mice with conditional knock-out of Ca$_v$1.2 in D1R-expressing cells (D1^{cre}, Ca$_v$1.2$^{fl/fl}$) and their WT littermates (D1^{cre}, Ca$_v$1.2$^{+/+}$) were generated by crossing Ca$_v$1.2$^{fl/fl}$ mice with mice expressing Cre recombinase under the transcriptional control of the D1R promoter [D1cre, Tg(Drd1-cre)EY262Gsat/Mmucd, GENSAT]. For each mouse line, male and female Cre$^{+/-}$, Ca$_v$1.2$^{fl/+}$ mice were bred to generate Cre$^{+/-}$, Ca$_v$1.2$^{+/+}$ and Cre$^{+/-}$, Ca$_v$1.2$^{fl/fl}$ experimental mice (herein referred to as CaMK2^{cre}, Ca$_v$1.2$^{+/+}$, CaMK2^{cre}, Ca$_v$1.2$^{fl/fl}$ and D1^{cre}, Ca$_v$1.2$^{+/+}$, D1^{cre}, Ca$_v$1.2$^{fl/fl}$). Mutant mice were indistinguishable from WT mice in weight, development, and general health. Mice were maintained under climate-controlled conditions on a 12 h light/dark cycle with *ad libitum* access to food and water. All procedures were approved by the Weill Cornell Medicine Institutional Animal Care and Use Committee and conducted in accordance with the 2011 Eighth Edition of the National Institutes of Health's *Guidelines for the Care and Use of Laboratory Animals*.

Drugs
Cocaine hydrochloride (Sigma-Aldrich) was dissolved in 0.9% saline at a concentration of 1 mg/ml and injected intraperitoneally at 0.01 ml/g body weight for a final dose of 10 mg/kg. Ca^{2+}/calmodulin-dependent protein kinase II inhibitor N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamidephosphate salt (KN93; Sigma-Aldrich) was dissolved in 0.9% saline containing 1.5% DMSO and 1.5% Tween 80 at a concentration of 15 μg/μl and microinjected into the dorsal hippocampus at a volume of 0.2 μl/hemisphere.

Stereotaxic viral vector delivery
Bilateral brain-region-specific deletion of *cacna1c* was achieved using stereotaxic surgical procedures as described previously (Lee et al., 2012a). Adeno-associated viral vectors expressing either GFP (AAV-GFP) or GFP and Cre recombinase (AAV-Cre) were delivered into the respective target regions of *cacna1c* floxed mice using coordinates adopted from the Mouse Brain Atlas (Paxinos and Franklin, 2004). To target the dorsal hippocampus, either AAV2/2-GFP or AAV2/2-Cre was microinjected at a volume of 0.2 μl/hemisphere 1.4 mm posterior, 2.0 mm ventral, and ±1.1 mm lateral to bregma at a 10° angle. To target the ventral hippocampus, either AAV2/5-GFP or AAV2/5-Cre was microinjected at a volume of 0.5 μl/hemisphere 3.5 mm posterior, 4.2 mm ventral, and ±2.8 mm lateral to bregma at a 4° angle. For each microinjection site, the

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needle was left in place for an additional 5 min after the infusion to ensure complete delivery of the viral vector. Behavioral experiments began after a minimum of 3 weeks to allow for maximal gene deletion.

Guide cannulae implantation

For delivery of the CaMKII inhibitor KN93 into the dorsal hippocampus, bilateral guide cannulae were implanted as described previously (Schierberl et al., 2011). Briefly, chronic indwelling, intracranial stainless-steel guide cannulae (5 mm in length; CMA/Microdialysis, model CMA/7) were implanted stereotaxically in mice under anesthesia (ketamine, 100 mg/ml; xylazine, 20 mg/ml) over the dorsal hippocampus at coordinates 1.9 mm posterior, 0.8 mm ventral, and $\pm$1.3 mm lateral to bregma (Paxinos and Franklin, 2004). Cannulae were secured to the skull with dental acrylic resin. Sham cannulae (33 gauge, 5 mm stainless-steel stylets) were cut flush with the 24 gauge cannulae, inserted until the time of drug microinfusions, and replaced after microinjections. After surgery, mice were allowed a minimum of 7 d recovery before beginning behavioral experiments. Microinfusions were delivered into the dorsal hippocampus using microinjectors (33 gauge) that extended 1 mm beyond the cannulae, connected by flexible polyethylene tubing to a microinjection system, and mounted with a 5 μl Hamilton syringe. KN93 (0.2 μl/hemisphere) was microinjected bilaterally into the dorsal hippocampus immediately before each extinction session. Accurate bilateral cannulae placement was confirmed in 50 μm paraformaldehyde-perfused sections by cresyl violet staining. Mice with inaccurate targeting on either side of the dorsal hippocampus were eliminated from the study.

CPP behavioral protocol

Cocaine CPP was performed as described previously (Tropea et al., 2008) using a biased protocol. Cocaine preference was assessed using a three-chamber place preference apparatus (Med Associates Inc.). The behavioral protocols implemented in this study are outlined in Figure 1A. During the first preconditioning testing session (baseline test, day 1), mice were initially placed in the central gray chamber for a 1 min acclimation period, followed by free access to all three chambers for 20 min. Time spent in each chamber was recorded. During the acquisition training period (days 2–4), in the morning session, each mouse was given a systemic injection of cocaine (10 mg/kg, i.p.) and confined for 20 min to the chamber that the mouse preferred less during the baseline test, after which it was returned to its home cage. Six hours later (afternoon session), each mouse was given a systemic injection of saline (0.01ml/g body weight) and confined to the opposite chamber for 20 min and then returned to its home cage. For experiments involving a saline control group, mice were injected with saline in both chambers during the morning and afternoon sessions. For the acquisition test session (day 5), mice were placed in the central chamber without drug treatment for a 1 min acclimation period before being allowed free access to all three chambers. Cocaine preference for all testing sessions was calculated as time spent in the cocaine-paired chamber minus time spent in the saline-paired chamber and is reported as a preference score (in seconds). Mice were defined as having acquired cocaine CPP when the average cocaine preference score of the cocaine-treated group was significantly higher than the preference score at baseline. To assess extinction ("CPP extinction" group in Fig. 1A), mice were allowed free access to all three chambers (beginning on day 6) without drug treatment, and cocaine preference was evaluated as above. This extinction protocol was repeated every 24 h until the average preference score of mice within the cocaine extinction control group was significantly lower than the preference score on the acquisition test day. To achieve consistent extinction across experiments and within 4 d of extinction training, a second separate investigator not previously exposed to the mice during cocaine pairing and acquisition conducted the extinction training sessions. For the "CPP no extinction" group (Fig. 1A), mice remained in their home cage for 4 d after acquisition testing. To test maintenance of cocaine CPP, mice were given free access to all three chambers of the cocaine CPP apparatus. For molecular studies with the "CPP no extinction" group, mice were killed after 4 d of home cage exposure without reexposure to the CPP chamber.

Fluorescence immunohistochemistry

Fluorescence immunohistochemistry was performed to confirm injection placement of the AAV as described previously (Lee et al., 2012a). After the final cocaine CPP extinction session, mice were anesthetized with an injection of euthasol (150 mg/kg, i.p.) before transcardial perfusion with 4% paraformaldehyde (PFA). Brains were dissected and postfixed overnight in 4% PFA, followed by cryoprotection in 30% sucrose in 0.1 M phosphate buffer (PB) at 4°C for 72 h. Forty-micrometer sections spanning the injection site region were obtained using a sliding microtome. Sections were incubated in anti-chicken GFP (1:5000; Aves Laboratories) for 18 h at 4°C, rinsed in PB, and incubated with donkey Alexa Fluor 488 (1:300; Thermo Fisher Scientific) antibody for 1 h at room temperature before mounting. Sections were imaged using an epifluorescence microscope (Leica DM550B with Leica Application Suite Advanced Fluorescence 3.0.0 build 8134 software).

Subcellular fractionation

Subcellular fractionation was performed using a previously established protocol (Knackstedt et al., 2010) and as we have described previously (Bavley et al., 2017; Kabir et al., 2017c). One hour after the final extinction trial, mice were rapidly decapitated and whole brains were quickly removed. Fresh brains were sectioned in the coronal plane using a brain block (Zivic Instruments). For each mouse, the hippocampus was bilaterally dissected with microforceps and the PFC and NAc were bilaterally dissected with a 17-gauge stainless steel stylet. Tissue was immediately frozen on dry ice and subsequent processing steps were performed at 4°C. Once all tissue samples were collected, a region was homogenized using a Teflon pestle (Pyrex) in cold sucrose buffer containing protease and phosphatase inhibitors. After centrifuging the homogenate at 1000 $\times$ g for 10 min, the supernatant was centrifuged at high speed (12,000 $\times$ g) for 20 min. The pellet was resuspended in HEPES/EDTA buffer and centrifuged at high speed for 20 min. The supernatant was saved as the cytoplasmic fraction. To obtain the synaptosomal fraction, the pellet was resuspended in 1% SDS buffer and saved. For generation of the PSD fraction, the synaptosomal pellet was resuspended in 0.5% Triton X-100 and incubated for 15 min before being centrifuged at 12,000 $\times$ g. The pellet, containing the Triton X-100-insoluble PSD fraction, was further resuspended in 1% Triton X-100 plus 1% SDS buffer and incubated for 1 h. This solution was centrifuged at 10,000 $\times$ g for 15 min and the supernatant was saved as the Triton X-100-insoluble PSD fraction.

Western immunoblotting

Protein concentration was determined by a BCA assay (Pierce) and absorbance was read on a plate reader at 570nm (iMark Absorbance Microplate Reader; Bio-Rad Laboratories). Twenty to 30 μg of protein was separated by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% nonfat dry milk and then incubated 12–24 h at 4°C in a primary antibody.

Blots were incubated with the following primary antibodies at a dilution of 1:1000; Ca$_v$1.2 (Alomone Laboratories, catalog #AGP-001, RRID: AB_11219156), pan-CaMKII (Cell Signaling Technology, catalog #3362, RRID:AB_2067938), phospho-CaMKII at threonine 286/287 (P-CaMKII T286/T287; Cell Signaling Technology, catalog #3361S, RRID:AB_2275070), GluA1 (Millipore, catalog #AB1504, RRID:AB_2113602), phospho-GluA1 at serine 831 (P-GluA1 S831; Millipore, catalog #AB5847, RRID: AB_92077), or 1:10,000;GAPDH (Abcam, catalog #ab22555, RRID: AB_447153). The Ca$_v$1.2 antibody used has been validated for its specificity using full-brain Ca$_v$1.2 knock-out mice in our recent study (Bavley et al., 2017). Two bands surrounding the predicted 250 kDa full-length Ca$_v$1.2 protein were identified as Ca$_v$1.2-specific variants. Proteins were quantified at the following molecular weights: Ca$_v$1.2 at 250 kDa, CaMKIIβ and P-CaMKIIβ at 60 kDa, CaMKIIα and P-CaMKIIα at 50 kDa, GluA1 and P-GluA1 S831 at 100 kDa, and GAPDH at 36 kDa. Membranes were incubated in horseradish peroxidase-linked IgG conjugated secondary antibody at 1:5000 at room temperature for 1 h before development using chemiluminescence solution (Western Lighting; PerkinElmer Life Science). Prestained standards (Bio-Rad) were used for protein size determination. Blots were processed on a medical film processor (Konica Minolta Medical and Graphic,
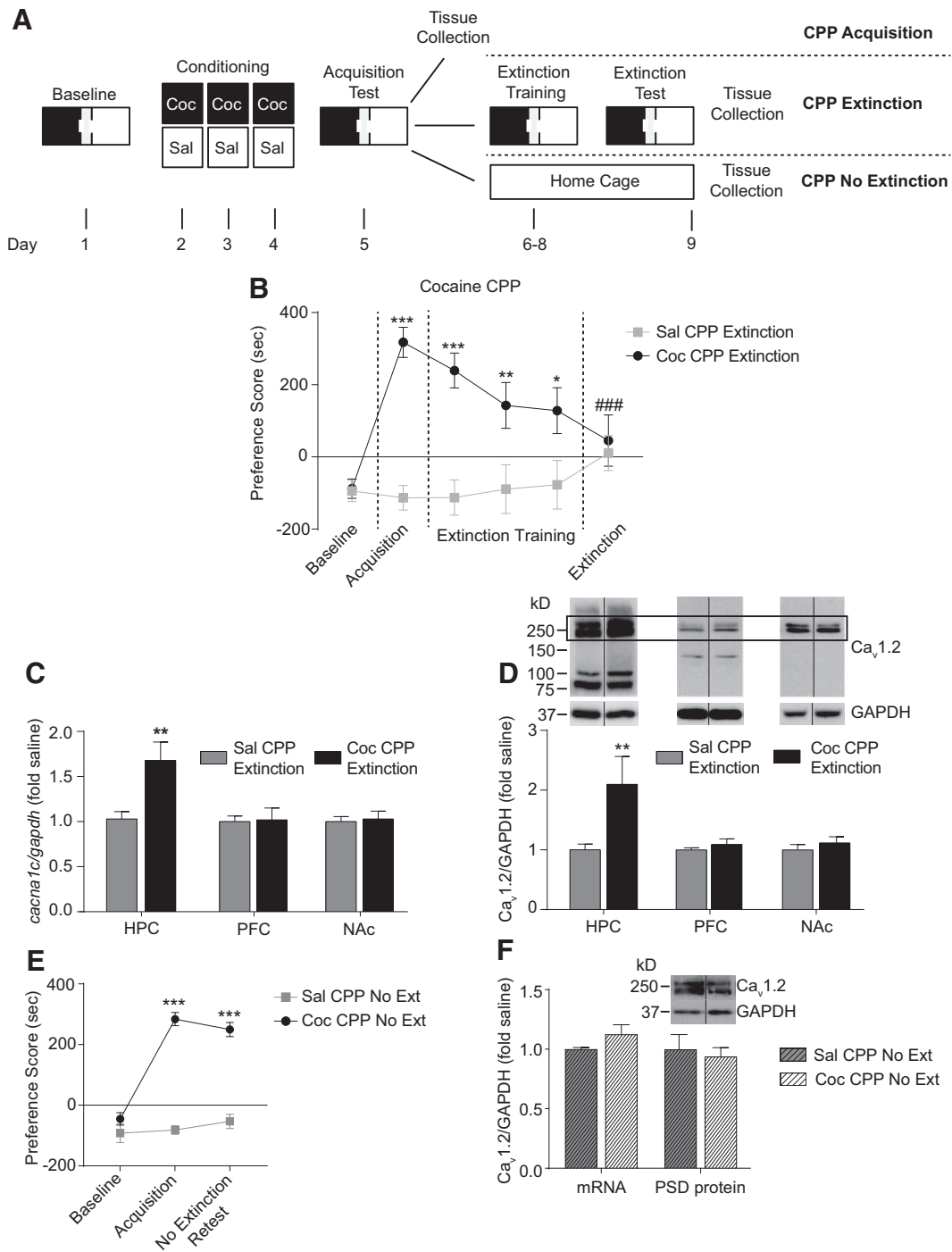
Figure 1. Extinction of cocaine CPP increases Ca$_v$1.2 mRNA and protein in the hippocampus. *A*, Experimental timeline of the CPP protocol indicating the "CPP acquisition," "CPP extinction," and "CPP no extinction" groups used for behavioral and molecular studies. *B*, Cocaine-treated WT mice demonstrate robust acquisition of cocaine CPP as shown by significantly higher preference scores on acquisition versus baseline testing. After extinction training, these mice exhibit significantly lower preference scores on the extinction test day compared with the acquisition test day, indicating extinction of cocaine CPP. Control mice treated with saline did not show any changes in preference score when tested after acquisition and extinction training. *p < 0.05, **p < 0.01, ***p < 0.001 compared with baseline test; ###p < 0.001 compared with acquisition test. *C*, Extinction of cocaine CPP increased Ca$_v$1.2 (*cacna1c*) mRNA significantly in the hippocampus, but not in the PFC or NAc, compared with saline control mice. **p < 0.01. *D*, Extinction of cocaine CPP increased Ca$_v$1.2 protein levels significantly in the PSD-enriched fraction from the hippocampus, but not the PFC or NAc compared with saline controls. For quantitation of Ca$_v$1.2 protein, the doublet surrounding the predicted full-length 250 kDa protein was used. **p < 0.01. *E*, Cocaine-treated mice demonstrate robust CPP acquisition and sustained preference score in the absence of extinction training when retested 4 d later compared with baseline. Control mice treated with saline did not show any change in preference scores after testing on acquisition and a retest 4 d later. ***p < 0.001 compared with baseline test. *F*, Ca$_v$1.2 mRNA and PSD protein were unaltered in mice that underwent cocaine CPP acquisition after 4 d of home cage exposure without extinction training. Data are presented as mean $\pm$ SEM.

model SRX-101A) and films were scanned with a Xerox DocuMate 510 scanner. Protein bands were quantified as mean optical intensity using ImageJ software and normalized to GAPDH. Normalized optical density values from each sample were used to calculate the percentage fold change for each drug condition compared with the control group (set to 1.0).

Quantitative real-time PCR (qPCR)
To examine mRNA levels in mice after cocaine CPP, we performed qPCR as described previously (Schierberl et al., 2011). Mice were decapitated and regions were dissected and immediately frozen as performed for cellular subfractionation described above. mRNA was extracted from

one hemisphere of the hippocampus with the RNeasy Mini Kit (Qiagen). cDNA was synthesized from resulting purified RNA using the High-Capacity RNA-to-cDNA kit (Applied Biosystems). *Cacna1c* levels were examined using the mRNA-specific primers (QuantiTect Primer assay QT00150752; Qiagen) on an ABI PRISM 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). Amplification was performed for 40 cycles (95°C for 15 s, 60°C for 30 s, and 72°C for 30 s), followed by an extension period (72°C for 10 min). Cycle threshold (Ct) values for target genes were normalized to the housekeeping gene *gapdh* (QuantiTect Primer assay QT01658692; Qiagen). Each experiment was performed in triplicate and values were averaged. For mRNA data analysis, the ΔCt method was used (Livak and Schmittgen, 2001). Briefly, experimental Ct values were normalized to *gapdh* values using the following formula: ΔCt = Ct (*cacna1c*) − Ct (*gapdh*). Expression levels were calculated relative to controls using the following formula: ΔΔCt = ΔCt (treated) − ΔCt (control average). The final expression levels were obtained using the formula $2^{-\Delta\Delta Ct}$.

Electron microscopic immunocytochemistry
Immunocytochemical studies were performed in S831A phosphomutant mice to examine the subcellular distribution of GluA1 in dendritic spines after cocaine CPP extinction. Mice were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and transcardially perfused with normal saline containing 2% heparin, followed by 30 ml of 3.75% acrolein and 2% PFA in PB (Milner et al., 2011). Brains were removed and postfixed for 30 min in 1.85% acrolein and 2% PFA in PB. Forty-micrometer sections containing the dorsal hippocampus (1.10 mm posterior to bregma through 2.06 mm posterior to bregma) were sectioned on a vibratome (VT1000 S; Leica) and stored in cryoprotectant solution (30% sucrose and 30% ethylene glycol in PB) at −20°C.

Before immunocytochemistry, sections were rinsed in PB. To ensure identical labeling conditions between experimental groups for quantitative immunocytochemistry, the sections were coded with hole punches, pooled in single containers, and then processed together through all immunocytochemical procedures (Pierce et al., 1999). The sections were processed for immunocytochemistry using a preembedding protocol as described previously (Milner et al., 2011). To remove excess aldehydes, sections were incubated in 1% sodium borohydride for 30 min. Sections were then sequentially rinsed in PB and 0.1 M Tris-saline (TS), followed by a 30 min incubation in a blocking solution of 0.5% bovine serum albumin (BSA) in TS. Next, sections were rinsed in TS and then incubated in rabbit anti-GluA1 (1:150; Millipore, catalog #AB1504, RRID: AB_2113602) antisera for 24 h at room temperature and 24 h at 4°C. Sections were then processed for immunogold labeling of GluA1 as described previously (Beckerman et al., 2013). Sections were rinsed in PB with 0.001% gelatin and 0.08% BSA and then incubated overnight at 4°C in an anti-rabbit secondary antiserum conjugated to 1 nm colloidal gold particles (1:50; Electron Microscopy Sciences). The nanogold-conjugated IgG was fixed by incubating sections in 2% glutaraldehyde and the gold particles were enhanced using a Silver IntenSEM kit (RPN491; GE Healthcare) for 7 min.

Sections were postfixed in 2% osmium tetroxide for 1 h, dehydrated, and flat embedded in Embed-812 (Electron Microscopy Sciences) between two sheets of Aclar plastic (Milner et al., 2011). Ultrathin sections (70 nm) through the dorsal hippocampus were cut with a diamond knife (Electron Microscopy Sciences) on a Leica EM UC6 ultratome and collected on 400-mesh, thin-bar copper grids (Electron Microscopy Sciences). Grids were then counterstained with uranyl acetate and Reynold's lead citrate (Milner et al., 2011). Sections were examined on a CM10 electron microscope (FEI).

Ultrastructural data analysis
An investigator blinded to the experimental conditions collected and analyzed all electron microscopic data. Sections were first examined at low magnification (3400×) to select fields having good morphological preservation and the presence of silver-intensified immunogold (SIG) particle labeling for GluA1, which were visible as black, electron-dense particles. From these fields, images were randomly photographed at 13,500×. These images were obtained exclusively at the plastic–tissue interface to ensure even antibody tissue penetration (Milner et al., 2011). Profiles were identified by defined morphological criteria (Peters et al., 1991). Dendritic profiles contained regular arrays of microtubules and were generally found postsynaptic to axon terminals. Dendritic spines were postsynaptic to an axon terminal, exhibited postsynaptic densities, sometimes contained spine apparati, and were devoid of mitochondria.

Single-labeled dendritic spines in the dorsal hippocampus at least 55 μm distal to the stratum radiatum region of CA1 or within the dentate gyrus (n = 50, area examined for each type of profile in each subregion per mouse) were collected from three animals per experimental condition using established methods (Milner et al., 2011). The subcellular localization of GluA1 SIG particles was defined as either on the plasmalemma (touching the membrane), near the plasmalemma (within 50 nm, but not touching the plasma membrane), or cytoplasmic. The distribution of GluA1 was assessed in synaptic (PSD), perisynaptic (within 50 nm of the PSD), and nonsynaptic (>70 nm from the PSD and perisynaptic regions) areas of dendritic spines (Marques-Lopes et al., 2017). The numbers of SIG particles in each subcellular compartment were compared statistically by genotype.

Statistics
All CPP behavioral data were analyzed by two-way repeated-measures (RM) ANOVA using a within-subjects factorial design followed by the Bonferroni–Dunn *post hoc* test to compare main effects of drug condition and testing session. For all mRNA and Western analyses, data were first analyzed for normality using the Shapiro–Wilk normality test. All normally distributed experimental data were analyzed by a parametric independent-samples *t* test. Non-normally distributed data were analyzed with a nonparametric test (Mann–Whitney). Statistical analyses were conducted using GraphPad Prism version 6.0 for Mac and were considered to be statistically significant for values of $p \le 0.05$. Details of each statistical analysis are listed below.

Experimental procedures
Experiment 1. Effect of acquisition of cocaine CPP on Ca$_v$1.2 mRNA and protein levels in the hippocampus. A factorial design that included the within-subject factors of day (baseline and acquisition) and drug condition (saline or cocaine) was used to assess the acquisition of cocaine CPP following the behavioral protocol outlined in Figure 1A ("CPP acquisition" group). After behavior, a between-subject factor of drug condition (saline or cocaine) was used to assess the effect of cocaine CPP acquisition on levels of Ca$_v$1.2 mRNA or PSD protein within the hippocampus. The following numbers of mice were used for analysis of behavior, mRNA levels, and protein levels: n = 7 saline and n = 9 cocaine.

Experiment 2. Effect of extinction of cocaine CPP on Ca$_v$1.2 mRNA and PSD protein levels in the hippocampus, PFC, and NAc. A factorial design that included the within-subject factors of day (baseline, acquisition, and extinction test) and drug condition (saline or cocaine) was used to assess extinction after acquisition of cocaine CPP (Fig. 1A: "CPP extinction" group, n = 16 saline- and n = 16 cocaine-treated mice). After behavior, a between-subject factor of drug condition (saline or cocaine) was used to assess the effect of cocaine CPP extinction on levels of Ca$_v$1.2 mRNA or PSD protein in the hippocampus, PFC, and NAc. The following numbers of mice were used for molecular studies: hippocampus mRNA: saline, n = 9 and cocaine, n = 8; hippocampus protein: saline, n = 12 and cocaine, n = 13; PFC mRNA: saline, n = 9 and cocaine, n = 7; PFC protein: saline, n = 5 and cocaine, n = 9; NAc mRNA: saline, n = 10 and cocaine, n = 6; and NAc protein: saline, n = 8 and cocaine, n = 9.

Experiment 3. Effect of home cage exposure without extinction training on maintenance of cocaine CPP. A factorial design that included the within-subject factors of day (baseline, acquisition, and no extinction retest) and drug condition (saline or cocaine) was used to assess acquisition and maintenance of CPP after a home cage exposure period of 4 d without extinction training. The following numbers of mice were used for behavior: saline, n = 8 and cocaine, n = 10.

Experiment 4. Effect of home cage exposure without extinction training on Ca$_v$1.2 mRNA and PSD protein levels in the hippocampus. A factorial design that included the within-subject factors of day (baseline and ac-

quisition) and drug condition (saline or cocaine) was used to assess acquisition of cocaine CPP (Fig. 1A: "CPP no extinction" group; saline, $n = 7$ and cocaine, $n = 8$). A between-subject factor of drug condition (saline or cocaine) was used to assess the effect of 4 d of home cage exposure without extinction training on levels of Ca$_v$1.2 mRNA and PSD protein in the hippocampus. The following numbers of mice per drug condition were used for molecular analyses: saline, $n = 6$ and cocaine, $n = 8$ for mRNA analysis; and saline, $n = 6$ and cocaine, $n = 8$ for protein analysis.

Experiment 5. Effect of deleting Ca$_v$1.2 in the dorsal hippocampus or ventral hippocampus on extinction of cocaine CPP behavior. Ca$_v$1.2 deletion in the dorsal hippocampus and ventral hippocampus was achieved with site-specific microinjection of AAV2/2 (dorsal hippocampus) or AAV2/5 (ventral hippocampus) expressing Cre-GFP or GFP (control) in *cacna1c* floxed mice. In this experiment, a factorial design that included the within-subject factors of day (baseline, acquisition, and extinction test) and viral treatment (AAV-GFP or AAV-Cre) was used to assess the effect of Ca$_v$1.2 knock-down on acquisition and extinction of cocaine CPP. The following numbers of mice were used for behavioral analysis for each region: dorsal hippocampus: AAV-GFP, $n = 9$ and AAV-Cre, $n = 10$; ventral hippocampus: AAV-GFP, $n = 6$, AAV-Cre, $n = 7$.

Experiment 6. Effect of deleting Ca$_v$1.2 in the dorsal hippocampus or ventral hippocampus on maintenance of cocaine CPP without extinction training. In this experiment, a factorial design that included the within-subject factors of day (baseline, acquisition, and no extinction retest) and viral treatment (AAV-GFP or AAV-Cre) was used to assess the effect of Ca$_v$1.2 knock-down within the dorsal hippocampus or ventral hippocampus on acquisition of cocaine CPP and maintenance of CPP behavior without extinction training. The following numbers of mice were used for behavioral analysis for each region: dorsal hippocampus: AAV-GFP, $n = 8$ and AAV-Cre, $n = 6$; ventral hippocampus: AAV-GFP, $n = 7$ and AAV-Cre, $n = 8$.

Experiment 7. Effect of extinction of cocaine CPP on total and phosphorylated CaMKIIα/β protein levels in the hippocampus. Levels of CaMKIIα, P-CaMKIIα T286, CaMKIIβ, and P-CaMKIIβ T287 were examined in saline- and cocaine-extinguished mice. In this experiment, a between-subject factor of drug condition (saline or cocaine) was used to assess the effect of cocaine CPP extinction on PSD protein within the hippocampus. The following numbers of mice was used for analysis of each protein: P-CaMKIIα and CaMKIIα: saline $n = 16$, cocaine $n = 16$; CaMKIIβ: saline $n = 6$, cocaine $n = 8$; and P-CaMKIIβ: saline $n = 8$, cocaine $n = 10$.

Experiment 8. Effect of home cage exposure without extinction training on total and phosphorylated CaMKIIα/β protein levels in the hippocampus. A between-subject factor of drug condition (saline or cocaine) was used to assess the effect of home cage exposure on levels of CaMKIIα, P-CaMKIIα T286, and P-CaMKIIβ T287 PSD protein in the hippocampus. The following numbers of mice per drug condition was used for analysis of each protein: P-CaMKIIα and P-CaMKIIβ: saline, $n = 6$ and cocaine, $n = 8$; CaMKIIα: saline, $n = 6$ and cocaine, $n = 7$.

Experiment 9. Effect of pharmacological inhibition of CaMKIIα/β in the dorsal hippocampus on extinction of cocaine CPP. The necessity of CaMKII activity within the dorsal hippocampus was tested by local infusion of KN93, a CaMKIIα/β inhibitor, or vehicle into the dorsal hippocampus of C57BL/6J mice during extinction training. In this experiment, a within-subject factor of day (baseline and acquisition) and treatment condition (vehicle or KN93) was used to confirm that both groups designated to the treatment conditions acquired cocaine CPP, followed by a between-subject factor of treatment condition (vehicle or KN93) to assess the effect of CaMKIIα/β inhibition in the dorsal hippocampus on CPP behavior on the final extinction test. The following numbers of mice were used for behavioral analysis: vehicle, $n = 7$ and KN93, $n = 7$.

Experiment 10. Effect of extinction of cocaine CPP on protein levels of total and phosphorylated GluA1 at site S831 in the hippocampus. Levels of GluA1 and phosphorylated GluA1 at S831 (P-GluA1 S831) in hippocampal PSD fractions and GluA1 in hippocampal cytoplasmic fractions were first examined in saline- and cocaine-extinguished mice. In this experiment, a between-subject factor of drug condition (saline or cocaine) was used to assess the effect of cocaine CPP extinction on levels of protein in the PSD (saline, $n = 13$ and cocaine, $n = 13$–14) and cytoplasmic (saline,

$n = 8$ and cocaine, $n = 6$) fraction in addition to mRNA levels (saline, $n = 9$ and cocaine, $n = 10$).

Experiment 11. Effect of home cage exposure without extinction training on P-GluA1 S831 protein levels in the hippocampus. A between-subject factor of drug condition (saline or cocaine) was used to assess the effect of home cage exposure on levels of PSD P-GluA1 S831 protein in the hippocampus. The following numbers of mice per drug condition were used for behavioral analyses to confirm acquisition of cocaine CPP: saline, $n = 6$; cocaine, $n = 8$. The following numbers of mice per drug condition were used for analysis of protein levels: saline, $n = 6$; cocaine, $n = 8$.

Experiment 12. Effect of loss of phosphorylation of GluA1 at site S831 on extinction of cocaine CPP and GluA1 levels in the PSD of the hippocampus. The necessity of S831 phosphorylation for cocaine CPP extinction was tested in S831A phosphomutant mice. In this experiment, the within-subject factors of day (baseline, acquisition, and extinction test) and genotype (WT or S831A mutants) were used to assess the effect of disruption of the S831 phosphorylation site on cocaine CPP extinction (WT, $n = 13$ and S831A, $n = 15$). After extinction, GluA1 levels were measured in hippocampal PSD and synaptosomal fractions. In this experiment, a between-subject factor of drug condition (saline or cocaine) was used to assess the effect of loss of GluA1 S831 phosphorylation on GluA1 levels in PSD and synaptosomal fractions (WT, $n = 13$; S831A, $n = 13$–14).

Experiment 13. Effect of loss of phosphorylation of GluA1 at site S831 on subcellular localization of GluA1 protein in the CA1 and dentate gyrus of the hippocampus. After the final cocaine CPP extinction test, S831A GluA1 mutant mice and WT littermates were killed and their brains were processed for analysis by electron microscopy to measure GluA1 levels in the CA1 and the dentate gyrus (on and near the membrane of synaptic, perisynaptic, and nonsynaptic sites). In this experiment, a between-subject factor of genotype (WT or S831A mutant) was used to assess the effect of disruption of the S831 phosphorylation site on subcellular localization of GluA1 for each subcellular compartment within the hippocampal CA1 and dentate gyrus after cocaine CPP extinction (WT: $n = 3$, 50 fields examined per mouse; S831A: $n = 3$, 50 fields examined per mouse).

Experiment 14. Effects of knock-out of Ca$_v$1.2 in CaMKII-expressing cells on acquisition and extinction of cocaine CPP. CaMK2^{cre}, Ca$_v$1.2$^{fl/fl}$ mice and CaMK2^{cre}, Ca$_v$1.2$^{+/+}$ littermates were tested in cocaine CPP extinction behavior. In this experiment, within-subject factor of day (baseline, acquisition, and extinction test) and genotype (CaMK2^{cre}, Ca$_v$1.2$^{+/+}$ or CaMK2^{cre}, Ca$_v$1.2$^{fl/fl}$) was used to assess the effect of knock-out of Ca$_v$1.2 in CaMKII-expressing cells on cocaine CPP acquisition and extinction behavior (CaMK2^{cre}, Ca$_v$1.2$^{+/+}$ mice, $n = 13$; CaMK2^{cre}, Ca$_v$1.2$^{fl/fl}$ mice, $n = 14$).

Experiment 15. Effects of knock-out of Ca$_v$1.2 in D1R-expressing cells on acquisition and extinction of cocaine CPP in addition to changes in hippocampal protein levels of Ca$_v$1.2-regulated targets. D1^{cre}, Ca$_v$1.2$^{+/+}$ mice and D1^{cre}, Ca$_v$1.2$^{fl/fl}$ littermates were tested in cocaine CPP extinction behavior using within-subject factors of day (baseline, acquisition, and extinction test) and genotype (D1^{cre}, Ca$_v$1.2$^{+/+}$ or D1^{cre}, Ca$_v$1.2$^{fl/fl}$) to assess the effect of knock-out of Ca$_v$1.2 in D1R-expressing cells on cocaine CPP acquisition and extinction behavior (D1^{cre}, Ca$_v$1.2$^{+/+}$ mice, $n = 13$; D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice, $n = 7$). After the final cocaine CPP extinction session, PSD fractions of the hippocampus and NAc were used to measure protein levels of CaMKIIα, P-CaMKIIα T286, CaMKIIβ, P-CaMKIIβ T287, GluA1 and P-GluA1 S831. In this experiment, a between-subject factor of genotype (D1^{cre}, Ca$_v$1.2$^{+/+}$ or D1^{cre}, Ca$_v$1.2$^{fl/fl}$) was used to assess the effect of knock-out of Ca$_v$1.2 in D1R-expressing cells on levels of proteins in the hippocampus and NAc PSD (D1^{cre}, Ca$_v$1.2$^{+/+}$ mice, $n = 9$–17; D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice, $n = 10$) was used for the treatment groups.

Results

Extinction but not acquisition of cocaine CPP increases Ca$_v$1.2 in the hippocampus

Mice were trained and tested for cocaine CPP acquisition as outlined in Figure 1A ("CPP acquisition" group). Cocaine-conditioned mice exhibited robust acquisition of cocaine CPP,

represented by a significantly higher preference score for the cocaine-associated context during the acquisition test compared with the baseline test (data not shown; two-way RM-ANOVA, significant interaction test day × drug condition, $F_{(1,13)} = 27.93$, $p = 0.0001$). Hippocampal Ca$_v$1.2 mRNA and Ca$_v$1.2 protein levels in PSD fractions were measured in brain tissue collected 1 h after the acquisition session to assess gene expression and synaptic Ca$_v$1.2, respectively. After acqusition of cocaine CPP, no significant difference in Ca$_v$1.2 mRNA (saline CPP: 1.00 ± 0.09 vs cocaine CPP: 0.995 ± 0.04, $t_{0.1100} = 14$; $p = 0.9140$, unpaired t test) or Ca$_v$1.2 PSD protein levels (saline CPP: 1.00 ± 0.07 vs cocaine CPP: 0.94 ± 0.06, $t_{(0.6549)} = 13$; $p = 0.5240$, unpaired t test) was observed in the hippocampus.

In a second cohort, mice were trained and tested for cocaine CPP acquisition, followed by 4 d of extinction training (Fig. 1A: "CPP extinction" group) until they demonstrated significantly lower preference for the cocaine-associated context compared with that on the acquisition test day (Fig. 1B; two-way RM-ANOVA, significant interaction, test day × drug condition, $F_{(5,150)} = 6.998$, $p < 0.0001$). Brain tissue was collected 1 h after the last extinction session. After extinction, there were significantly higher levels of Ca$_v$1.2 mRNA (Fig. 1C; $t_{(2.421)} = 12$; $p = 0.0085$, unpaired t test) and PSD Ca$_v$1.2 protein (Fig. 1D; $p = 0.0080$, $U = 30.0$, Mann–Whitney) in the hippocampus.

Next to determine whether the increase in Ca$_v$1.2 mRNA and protein is specific to extinction, we examined levels of Ca$_v$1.2 in the hippocampus of mice that underwent home cage exposure without extinction training for 4 d after the acquisition of cocaine CPP, a time period equivalent to that of extinction training. First, we confirmed that mice maintain CPP at this time point (Fig. 1E; two-way RM-ANOVA, significant interaction, test day × drug condition, $F_{(2,32)} = 38.73$, $p < 0.0001$). Examination of Ca$_v$1.2 levels in a separate cohort (Fig. 1A:"CPP no extinction" group) that acquired cocaine CPP (cocaine CPP: baseline = 48.32 ± 48.36 s, acquisition = 572.6 ± 59.01 s vs saline CPP: baseline = −38.6 ± 61.35 s, acquisition = 83.14 ± 72.77 s, two-way RM-ANOVA, significant interaction, test day × drug condition, $F_{(1,13)} = 21.97$, $p = 0.0004$) and returned to their home cage environment for 4 d without extinction training did not exhibit altered levels of Ca$_v$1.2 mRNA (Fig. 1F; $t_{(1.354)} = 12$; $p = 0.1583$, unpaired t test) or Ca$_v$1.2 PSD protein levels (Fig. 1F; $t_{(0.4459)} = 12$; $p = 0.6636$, unpaired t test) compared with saline control mice. These results indicate that extinction training increases hippocampal Ca$_v$1.2 mRNA levels selectively, with subsequent increases in Ca$_v$1.2 at synaptic sites in the hippocampus after extinction.

In addition, the increase in Ca$_v$1.2 mRNA and protein after extinction training was specific to the hippocampus. After extinction of cocaine CPP, no difference in levels of Ca$_v$1.2 were seen in the PFC or NAc (Fig. 1C: PFC mRNA, $t_{(0.1486)} = 14$; $p = 0.8840$, NAc mRNA, $t_{(0.3038)} = 14$; $p = 0.7658$; Fig. 1D: PFC protein, $t_{(1.099)} = 12$; $p = 0.2932$, NAc protein, $t_{(0.8456)} = 15$; $p = 0.4111$, unpaired t test), brain regions implicated in extinction of cocaine seeking behavior. Therefore, extinction training of cocaine CPP increases hippocampal Ca$_v$1.2 mRNA and Ca$_v$1.2 protein selectively within PSD fractions.

Spatiotemporal deletion of Ca$_v$1.2 in the dorsal hippocampus results in a deficit in extinction of cocaine CPP
Because we observed an increase in Ca$_v$1.2 protein in the hippocampus after extinction, we next tested the necessity of hippocampal Ca$_v$1.2 channels for extinction of cocaine CPP by deleting Ca$_v$1.2 locally. We targeted the dorsal hippocampus

because it has been shown to be necessary for aspects of cocaine-associated memories (Meyers et al., 2003; Meyers et al., 2006; Ramirez et al., 2009), including extinction of cocaine-seeking behavior (Szalay et al., 2013). In addition, we targeted the ventral hippocampus because it has been shown to be required for extinction of cocaine self-administration (Chambers and Self, 2002). We generated focal deletion of Ca$_v$1.2 in the dorsal hippocampus, targeted within the CA1 subregion, by bilateral stereotactic delivery of AAV-Cre in adult Ca$_v$1.2$^{fl/fl}$ mice (Fig. 2A). Virus expression typically spread from 1.2 mm through 1.7 mm posterior to bregma (Fig. 2A). This resulted in significantly lower levels of Ca$_v$1.2 mRNA in CA1 compared with control AAV-GFP-injected mice (mRNA fold change compared with controls: AAV-GFP, $n = 7$, $1.0 ± 0.11$ vs AAV-Cre, $n = 4$, $0.65 ± 0.09$: $t_{(2.325)} = 10$; $p = 0.0424$, unpaired t test). Deleting Ca$_v$1.2 in the dorsal hippocampus had no effect on acquisition of cocaine CPP. Both AAV-GFP- and AAV-Cre-injected mice demonstrated significantly higher preference for the cocaine-associated context compared with the baseline test day (Fig. 2B). However, AAV-Cre injected mice showed a significant deficit in the extinction of cocaine CPP compared with AAV-GFP injected controls (Fig. 2B; two-way RM-ANOVA, significant interaction, test day × viral injection, $F_{(2,34)} = 9.074$, $p = 0.0007$), demonstrating that Ca$_v$1.2 in the dorsal hippocampus is not required for the acquisition of cocaine CPP, but is necessary for extinction of cocaine CPP. This deficit was not due to alterations in locomotor activity because viral vector injections had no effect on basal locomotion (AAV-GFP, $n = 10$, $5273 ± 181$ cm vs AAV-Cre, $n = 11$, $4842 ± 379$ cm, $F_{(1,19)} = 0.9930$, $p = 0.3332$, unpaired t test). In addition, the deletion of Ca$_v$1.2 in the dorsal hippocampus had no effect on maintenance of CPP behavior because a separate cohort of mice demonstrated sustained CPP scores when tested again 4 d after acquisition of cocaine CPP but without receiving extinction training (Fig. 2C; two-way RM-ANOVA, main effect of day, $F_{(2,24)} = 98$, $p < 0.0001$; no main effect of viral treatment, $F_{(1,12)} = 0.0020$, $p = 0.9649$).

Contrary to the dorsal hippocampus, bilateral deletion of Ca$_v$1.2 in the ventral hippocampus, which resulted in AAV-Cre expression in the ventral CA1, CA3, and DG from 3.1 mm through 3.8 mm posterior to bregma (Fig. 2D), had no effect on CPP acquisition or extinction (Fig. 2E; two-way RM-ANOVA, main effect of day, $F_{(2,22)} = 17.01$, $p < 0.0001$; no main effect of viral treatment, $F_{(1,11)} = 0.0195$, $p = 0.8916$). Similar to the dorsal hippocampus, deletion of Ca$_v$1.2 in the ventral hippocampus had no effect on maintenance of CPP behavior without extinction (Fig. 2F; two-way RM-ANOVA, main effect of day, $F_{(2,26)} = 95.2$, $p < 0.0001$; no main effect of viral treatment, $F_{(1,13)} = 1.062$, $p = 0.3215$).

Together, these results demonstrate that Ca$_v$1.2 channels, specifically in the dorsal hippocampus, and not the ventral hippocampus, are essential for extinction of cocaine CPP, whereas Ca$_v$1.2 channels in neither of these subregions are necessary for the acquisition of cocaine CPP or maintenance of CPP tested 4 d later.

CaMKII α/β activity in the dorsal hippocampus is essential for extinction of cocaine CPP
Due to its localized positioning at the synapse (Di Biase et al., 2008; Tippens et al., 2008; Kabir et al., 2017b), Ca$_v$1.2 initiates a multitude of intracellular signaling cascades after channel activation (Schierberl et al., 2011; Simms and Zamponi, 2014; Mullins et al., 2016; Kabir et al., 2017a). One key downstream target of Ca$_v$1.2 channels is CaMKII (Wu et al., 2001; Schierberl et al., 2011; Wheeler et al., 2012), which is involved in extinction learning (Szapiro et al., 2003; Kimura et al., 2008; Myskiw et al., 2014;
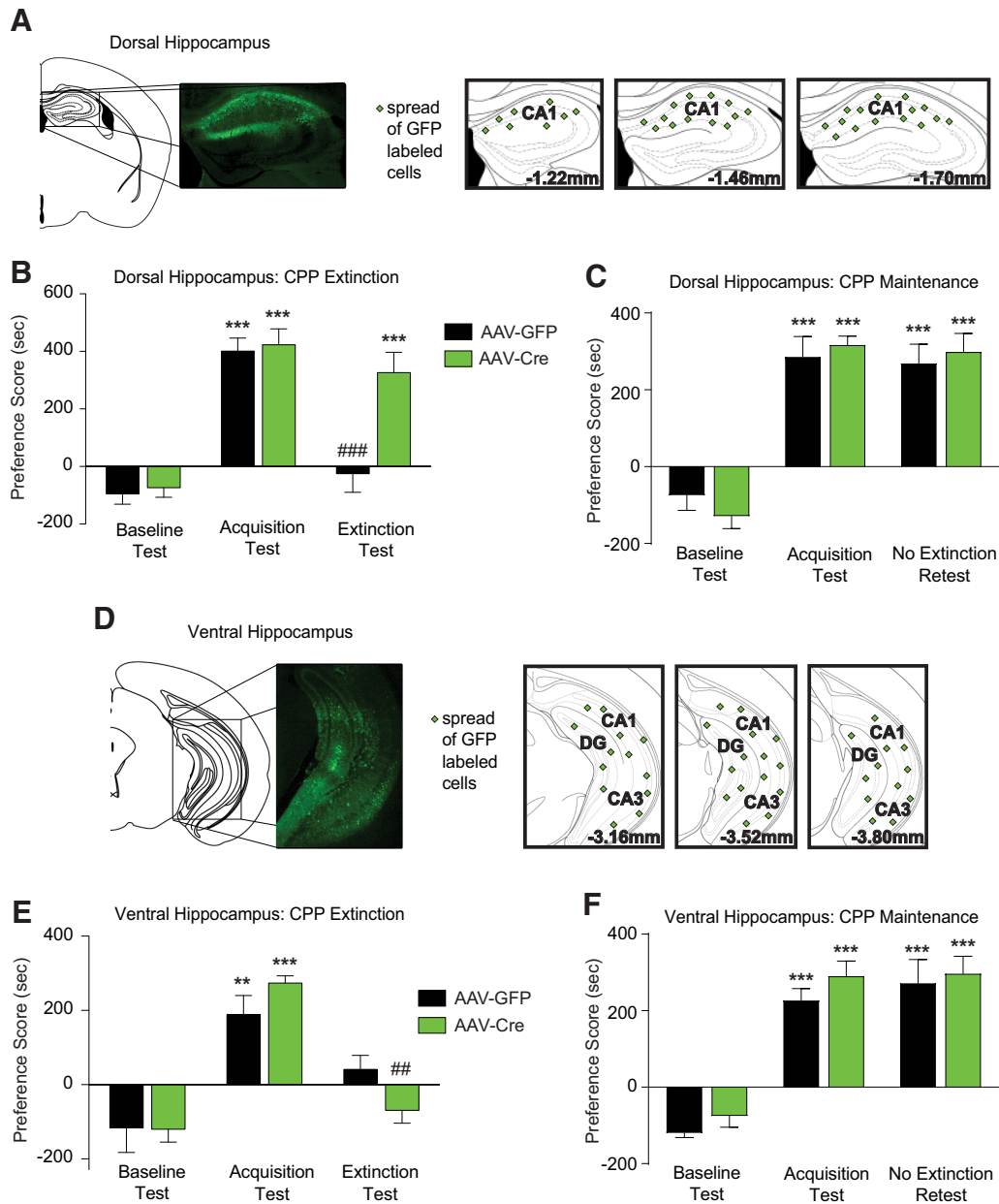
Figure 2. Focal deletion of Ca$_v$1.2 in the dorsal hippocampus induces a deficit in extinction of cocaine CPP. ***A*, *D***, Representative images of green fluorescent protein (GFP)-positive cells and representations of coronal sections showing spread of AAV expression, as indicated by green symbol labeling, resulting from AAV bilaterally microinjected into dorsal hippocampus (***A***) or ventral hippocampus (***D***) of *cacna1c*$^{fl/fl}$ mice. ***B***, Deletion of Ca$_v$1.2 in the dorsal hippocampus had no effect on acquisition of cocaine CPP, but attenuated extinction significantly compared with mice injected with AAV-GFP. ***C***, Deletion of Ca$_v$1.2 in the dorsal hippocampus had no effect on maintenance of cocaine CPP when tested 4 d after the acquisition test. ***E***, Deletion of Ca$_v$1.2 in the ventral hippocampus had no effect on acquisition or extinction of cocaine CPP. ***F***, Deletion of Ca$_v$1.2 in the ventral hippocampus had no effect on maintenance of cocaine CPP. ***p < 0.001 compared with baseline test; #p < 0.05, ###p < 0.001 compared with acquisition test. Data are presented as mean ± SEM.

de Carvalho Myskiw et al., 2014). We first examined whether CaMKII levels were altered at synaptic locations in the hippocampus after extinction of cocaine CPP. Protein levels of the α and β CaMKII isoforms, as well as their phosphorylated active forms, were measured in hippocampal PSD fractions. We found that total CaMKIIα, as well as the phosphorylated form at the T286 site, were increased in PSD fractions (Fig. 3A; CaMKIIα: $t_{(2.341)} = 30$; $p = 0.0261$, unpaired t test; P-CaMKIIα T286: $t_{(3.371)} = 18$; $p = 0.0014$, unpaired t test). Examination of CaMKIIβ protein revealed a trending increase in total protein and significantly higher phosphorylated CaMKIIβ at site T287 compared with saline controls (Fig. 3A; total CaMKIIβ: $t_{(1.936)} = 12$; $p = 0.0768$, unpaired t test; P-CaMKIIβ T287: $t_{(2.756)} = 16$; $p = 0.014$, un-

paired t test). This increase in CaMKII protein within the PSD fraction of the hippocampus appeared to be dependent on extinction training because mice that acquired cocaine CPP followed by 4 d of home cage exposure without extinction training ("CPP no extinction" group outlined in Fig. 1A) had lower levels of CaMKIIα protein ($t_{(2.691)} = 11$; $p = 0.0210$, unpaired t test), P-CaMKIIα protein ($t_{(2.405)} = 12$; $p = 0.0332$, unpaired t test), and trending lower levels of P-CaMKIIβ T287 ($t_{(1.813)} = 12$; $p = 0.0950$, unpaired t test) compared with saline control mice (Fig. 3B).

Next, to test directly whether CaMKII activity is essential for extinction of cocaine CPP, mice were bilaterally infused with the CaMKIIα/β inhibitor KN93 (Sumi et al., 1991) in the dorsal
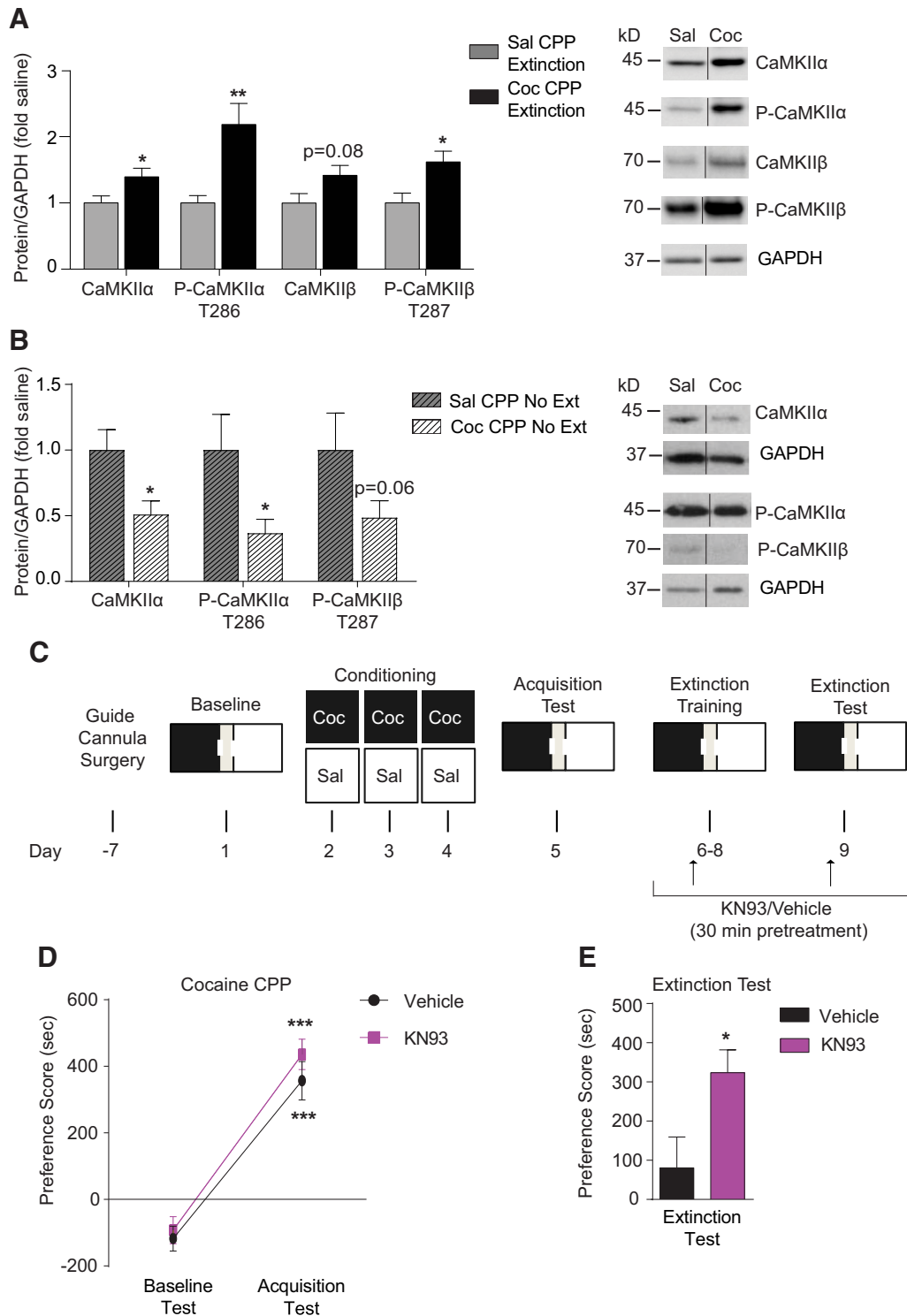
Figure 3. Pharmacological inhibition of CaMKIIα/β in the dorsal hippocampus induces a deficit in extinction of cocaine CPP. **A**, Total and phosphorylated CaMKIIα, as well as phosphorylated CaMKIIβ, in the PSD-enriched fraction from the hippocampus were significantly higher after extinction of cocaine CPP compared with saline control mice. Total CaMKIIβ trended higher but did not reach statistical significance. **B**, Total and phosphorylated CaMKIIα in the PSD-enriched fraction from the hippocampus were significantly lower in mice that acquired cocaine CPP followed by home cage exposure without extinction training compared with saline control mice. Phosphorylated CaMKIIβ trended lower but did not reach statistical significance. *$p < 0.05$, **$p < 0.01$ compared with saline group. **C**, Timeline of surgery, CPP conditioning, acquisition test, and microinjection of the CaMKII α/β inhibitor KN93 or vehicle before extinction training. **D**, Similar acquisition of cocaine CPP was observed within each designated group before treatment during extinction training. ***$p < 0.001$ compared with baseline test. **E**, Infusion of KN93 into the dorsal hippocampus of mice ($n = 7$) before the each extinction training session attenuated extinction compared with vehicle-infused mice ($n = 7$). *$p < 0.05$ compared with vehicle group. Data are presented as mean ± SEM.
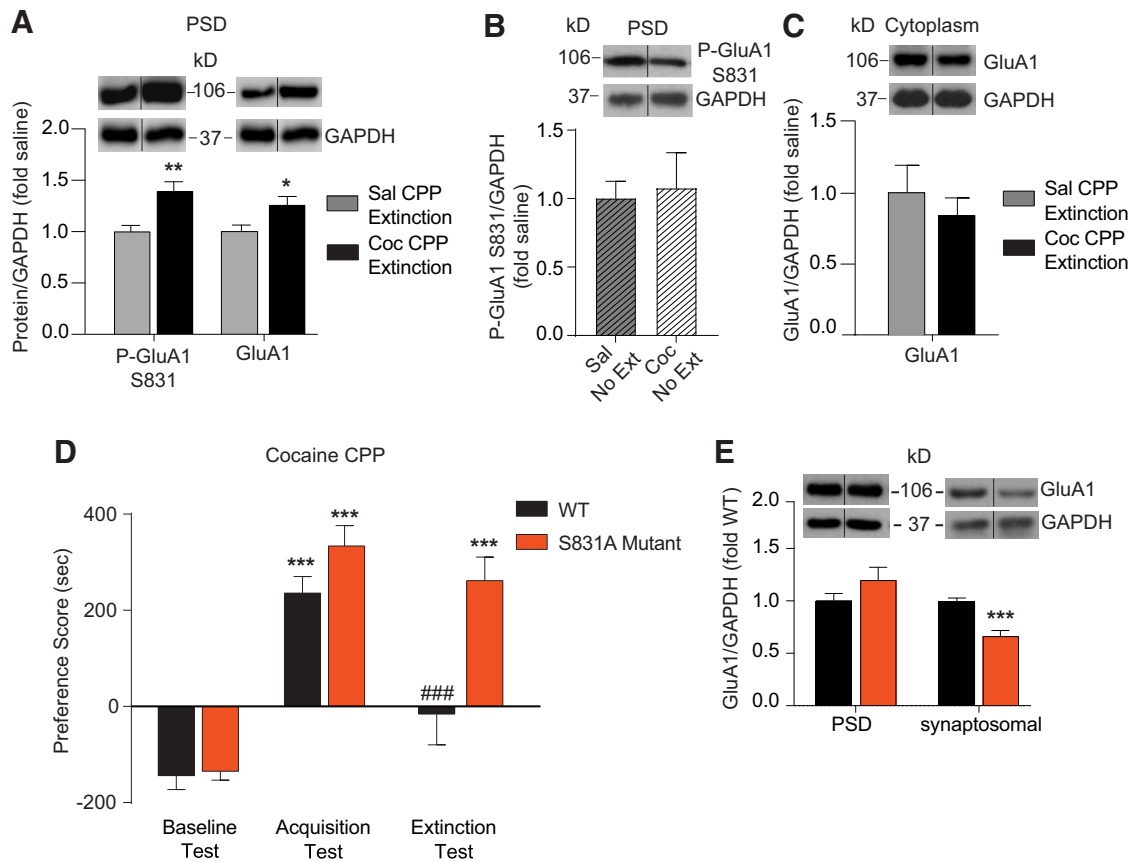
Figure 4. S831A GluA1 mutant mice have a deficit in extinction of cocaine CPP and lower levels of GluA1 in synaptosomal fractions but not at the hippocampal PSD. **A**, Total and phosphorylated GluA1 at S831 were significantly higher in the PSD-enriched fraction of the hippocampus after extinction of cocaine CPP compared with saline control mice. *$p < 0.05$, **$p < 0.01$. **B**, Phosphorylated GluA1 at S831 was not altered in the PSD-enriched fraction of the hippocampus in mice that underwent cocaine CPP acquisition followed by home cage exposure without extinction training compared with saline control mice. **C**, Total GluA1 was not altered in the cytoplasmic fraction of the hippocampus after extinction of cocaine CPP compared with saline control mice. **D**, S831A mutant mice displayed no difference in acquisition of cocaine CPP, but demonstrated an extinction deficit compared with WT mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with baseline test; #$p < 0.05$ compared with acquisition test. **E**, After extinction, there was no difference in levels of GluA1 in PSD fractions generated from the hippocampus of S831A mutant mice compared with WT mice, whereas synaptosomal fractions had significantly lower levels of GluA1 in mutants compared with WT mice. ***$p < 0.001$. Data are presented as mean ± SEM.

hippocampus during extinction training, as outlined in Figure 3C. Mice were split randomly into two treatment groups that would subsequently receive vehicle or KN93 during extinction. Each group was then trained to acquire cocaine CPP. Both groups demonstrated increased preference scores during the acquisition test compared with baseline test (Fig. 3D; two-way RM-ANOVA, main effect of day $F_{(1,12)} = 81.42$, $p < 0.0001$; no main effect of treatment assignment, $F_{(1,12)} = 2.394$, $p = 0.1477$). After acquisition, vehicle or KN93 was infused into the dorsal hippocampus during extinction training (Fig. 3C). KN93 attenuated extinction significantly compared with vehicle treated mice (Fig. 3E; $t_{(2.4740)} = 12$; $p = 0.0293$, unpaired t test), demonstrating the necessity of CaMKIIα/β within the dorsal hippocampus for extinction of cocaine CPP.

Phosphorylation of GluA1 at S831 is increased in the hippocampus and is essential for extinction of cocaine CPP
One key target of active CaMKII is phosphorylation of the AMPA receptor GluA1 subunit at S831 (Mammen et al., 1997), a key post-translational modification necessary for hippocampal-dependent learning and memory (Olivito et al., 2016). Using a phosphospecific antibody, we found increased levels of phosphorylated GluA1 at S831 (P-GluA1 S831) in the hippocampal PSD of cocaine-extinguished WT mice (Fig. 4A; $t_{(3.416)} = 25$; $p = 0.0022$, unpaired t test). This increase in P-GluA1 S831 was dependent on

extinction training because mice that acquired cocaine CPP followed by 4 d of home cage exposure without extinction training ("CPP no extinction" group outlined in Fig. 1A) demonstrated no difference in P-GluA1 S831 protein compared with saline control mice (Fig. 4B; $t_{(0.23)} = 12$; $p = 0.8220$, unpaired t test). We also examined levels of total GluA1 because changes in cell surface levels of this protein have been observed after cocaine-associated behavior (Wolf, 2016). Extinction increased total GluA1 protein levels significantly in hippocampal PSD fractions compared with saline controls (Fig. 4A; $t_{(2.376)} = 24$; $p = 0.0258$, unpaired t test). No difference in hippocampal GluA1 mRNA levels (saline extinction, $n = 9$, 1.00 ± 0.14 vs cocaine extinction, $n = 10$, 1.05 ± 0.36, $t_{(0.1241)} = 17$; $p = 0.9027$, unpaired t test) and no difference in GluA1 protein levels in the cytoplasmic fraction of the hippocampus was observed (Fig. 4C; $t_{(0.6552)} = 12$; $p = 0.5247$, unpaired t test), suggesting that the observed increase in PSD GluA1 levels after extinction are not due to increased gene expression and could involve changes in GluA1 subcellular localization.

To further investigate the necessity of GluA1 S831 phosphorylation for extinction of cocaine context learning, mice harboring a serine to alanine substitution at the S831 site of GluA1 (Lee et al., 2010) and their WT littermates were trained and tested for acquisition and extinction of cocaine CPP. Although S831A mutant and WT mice had similar CPP acquisition scores, S831A

mutant mice differed from WT mice in their sustained high preference for the cocaine-associated context during extinction testing (Fig. 4D; two-way RM-ANOVA, significant interaction, test day × genotype, $F_{(2,52)} = 6.475$, $p = 0.0031$). Therefore, S831 GluA1 phosphorylation is necessary for cocaine CPP extinction, but is not required for the acquisition of the initial cocaine context-associated memory.

Next, to determine whether phosphorylation of GluA1 S831 influences the increase in hippocampal PSD GluA1 levels observed after extinction (Fig. 4A), we measured levels of GluA1 in PSD fractions from S831A GluA1 mutants after the last extinction session. We found no difference in levels of PSD GluA1 protein between S831A mutant and WT littermates (Fig. 4E), suggesting that the increase in GluA1 at the hippocampal PSD is not necessary for extinction of cocaine CPP.

In addition to being located at the PSD, GluA1 is also present within other compartments, including the plasma membrane of perisynaptic and nonsynaptic sites (Richmond et al., 1996; Passafaro et al., 2001). Therefore, we probed synaptosomal fractions containing these components to determine whether GluA1 levels were affected in S831A GluA1 mutants after cocaine CPP extinction. S831A mutants had significantly lower levels of GluA1 protein within synaptosomal fractions compared with WT mice after extinction training (Fig. 4E, $t_{(5.048)} = 10$; $p = 0.0005$, unpaired t test), demonstrating that GluA1 is decreased outside the PSD after CPP extinction in S831A mutants. No difference in basal GluA1 levels was observed in hippocampal synaptosomal fractions from S831A mutants compared with WT littermates (data not shown), which is supported by previous findings demonstrating no difference in GluA1 levels in crude membrane fractions (Lee et al., 2010).

S831A mutation is associated with decreases in perisynaptic GluA1 in dendritic spines of hippocampal CA1 neurons

The reduction in synaptosomal GluA1 after extinction prompted us to further probe the subcellular localization of GluA1 in dendritic spines of hippocampal pyramidal neurons. We used high-resolution immunoelectron microscopy to detect the presence of GluA1 on and near select compartments within dendritic spines, as indicated in Figure 5B, of the stratum radiatum of the CA1 subregion and the dentate gyrus of the dorsal hippocampus (Fig. 5A).

Consistent with the Western blot analysis (Fig. 4D), electron microscopy revealed that there was no difference in levels of GluA1 SIG particles on the PSD of spines in the CA1 of S831A mutants compared with WT littermates after CPP extinction (Fig. 5C). However, there were significantly lower levels of GluA1 SIG particles near the PSD (Fig. 5C, $t_{(2.1683)} = 298$; $p = 0.0309$, unpaired t test), as well as both on and near the plasma membrane at perisynaptic sites of S831A mutants compared with WT littermates (Fig. 5C, on: $t_{(3.0791)} = 298$; $p = 0.0023$, unpaired t test; near: $t_{(3.2915)} = 298$; $p = 0.0011$, unpaired t test). There were no differences in levels of GluA1 SIG particles on or near the plasma membrane of nonsynaptic sites of dendritic spines between the two genotypes (Fig. 5C). The altered levels of GluA1 in S831A mutants was specific to the CA1 because examination of spines of the dentate gyrus revealed no difference in levels of GluA1 particles both on and near the PSD, as well as on and near perisynaptic or nonsynaptic sites (Fig. 5D). These results indicate that GluA1 is reduced near the PSD and at perisynaptic areas of dendritic spines of CA1 neurons in S831 mutant mice with impaired cocaine CPP extinction.

Conditional knock-out of Ca$_v$1.2 in D1R-expressing but not glutamatergic neurons results in a deficit in extinction of cocaine CPP with corresponding decreases in PSD CaMKII and P-GluA1 S831 in the hippocampus

To identify potential cell-type-specific contributions of Ca$_v$1.2 to extinction of cocaine CPP, we first examined the role of Ca$_v$1.2 in glutamatergic neurons, a cell type in which Ca$_v$1.2 is highly expressed at postsynaptic sites (Di Biase et al., 2008; Kabir et al., 2017b). We generated the CaMK2^{cre}, Ca$_v$1.2$^{fl/fl}$ mouse strain in which Ca$_v$1.2 is conditionally deleted in glutamatergic neurons of the forebrain, resulting in an ~70% decrease in Ca$_v$1.2 mRNA in the hippocampus and other forebrain regions (Lee et al., 2012b). CaMK2^{cre}, Ca$_v$1.2$^{fl/fl}$ mice and CaMK2^{cre}, Ca$_v$1.2$^{+/+}$ littermates were tested in the acquisition and extinction of cocaine CPP. Interestingly, knock-out of Ca$_v$1.2 in glutamatergic neurons had no effect on acquisition or extinction of cocaine CPP because CaMK2^{cre}, Ca$_v$1.2$^{fl/fl}$ mice demonstrated similar acquisition and extinction as that seen in CaMK2^{cre}, Cav1.2$^{+/+}$ littermates (Fig. 6A; two-way RM-ANOVA, main effect of day, $F_{(2,50)} = 42.45$, $p < 0.0001$, no main effect of genotype, $F_{(1,25)} = 0.0073$, $p = 0.9326$).

Because recent studies have highlighted a role for D1Rs in the extinction of cocaine CPP (Fricks-Gleason et al., 2012; Abraham et al., 2014, 2016), particularly within the hippocampus for memory of contextual cocaine-paired stimuli (Xie et al., 2014), we next generated the D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mouse strain in which Ca$_v$1.2 is conditionally deleted in D1R-expressing cells. The efficacy of knock-out in the hippocampus was revealed by lower levels of Ca$_v$1.2 protein in D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice compared with D1^{cre}, Ca$_v$1.2$^{+/+}$ mice (Fig. 6B, $t_{(3.537)} = 24$; $p = 0.0017$, unpaired t test). D1^{cre}, Ca$_v$1.2$^{fl/fl}$ and D1^{cre}, Ca$_v$1.2$^{+/+}$ mice were trained and tested for CPP acquisition and extinction. Although D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice acquired cocaine CPP to the same degree as D1^{cre}, Ca$_v$1.2$^{+/+}$ mice, D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice were unable to extinguish cocaine CPP (Fig. 6C; two-way RM-ANOVA, significant interaction, test day × genotype, $F_{(2,36)} = 5.191$, $p = 0.0105$). These results demonstrate that Ca$_v$1.2 channels in D1R-expressing cells are required for extinction of cocaine-associated memories.

Next, to investigate whether the molecular changes observed in the hippocampus after extinction (Figs. 3A, 4A) are mediated by Ca$_v$1.2 channels in D1R-expressing cells, we measured levels of CaMKII and GluA1 proteins in hippocampal PSD fractions of D1^{cre}, Ca$_v$1.2$^{+/+}$ mice and D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice after cocaine CPP extinction training. D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice had significantly lower levels of total CaMKIIα (Fig. 6D, $t_{(6.788)} = 24$; $p < 0.0001$, unpaired t test) and a trend toward lower levels of CaMKIIβ (Fig. 6D, $t_{(2.027)} = 12$; $p = 0.0655$, unpaired t test). Interestingly, no difference in P-CaMKIIα T286 or P-CaMKIIβ T287 between the genotypes was observed. Examination of GluA1 in hippocampal PSD fractions revealed significantly lower P-GluA1 S831 in D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice ($t_{(6.062)} = 23$; $p < 0.0001$, unpaired t test), but no difference in total GluA1 levels (Fig. 6D, $t_{(0.9258)} = 24$; $p = 0.3638$, unpaired t test). Therefore, the above results demonstrated that Ca$_v$1.2 channels in D1R-expressing cells are necessary for hippocampal GluA1 phosphorylation at S831 after extinction, but do not appear to mediate the increase in levels of total GluA1 at the PSD, further supporting the finding in S831A GluA1 phosphomutant mice that S831 phosphorylation does not mediate the increase in PSD GluA1 levels in the hippocampus after extinction (Fig. 4C). There was no difference in basal levels of any of the proteins tested in PSD fractions of the hippocampus between genotypes (CaMKIIα, P-CaMKIIα T286, CaMKIIβ, P-CaMKIIβ T287, GluA1, and P-GluA1 S831; data not shown).
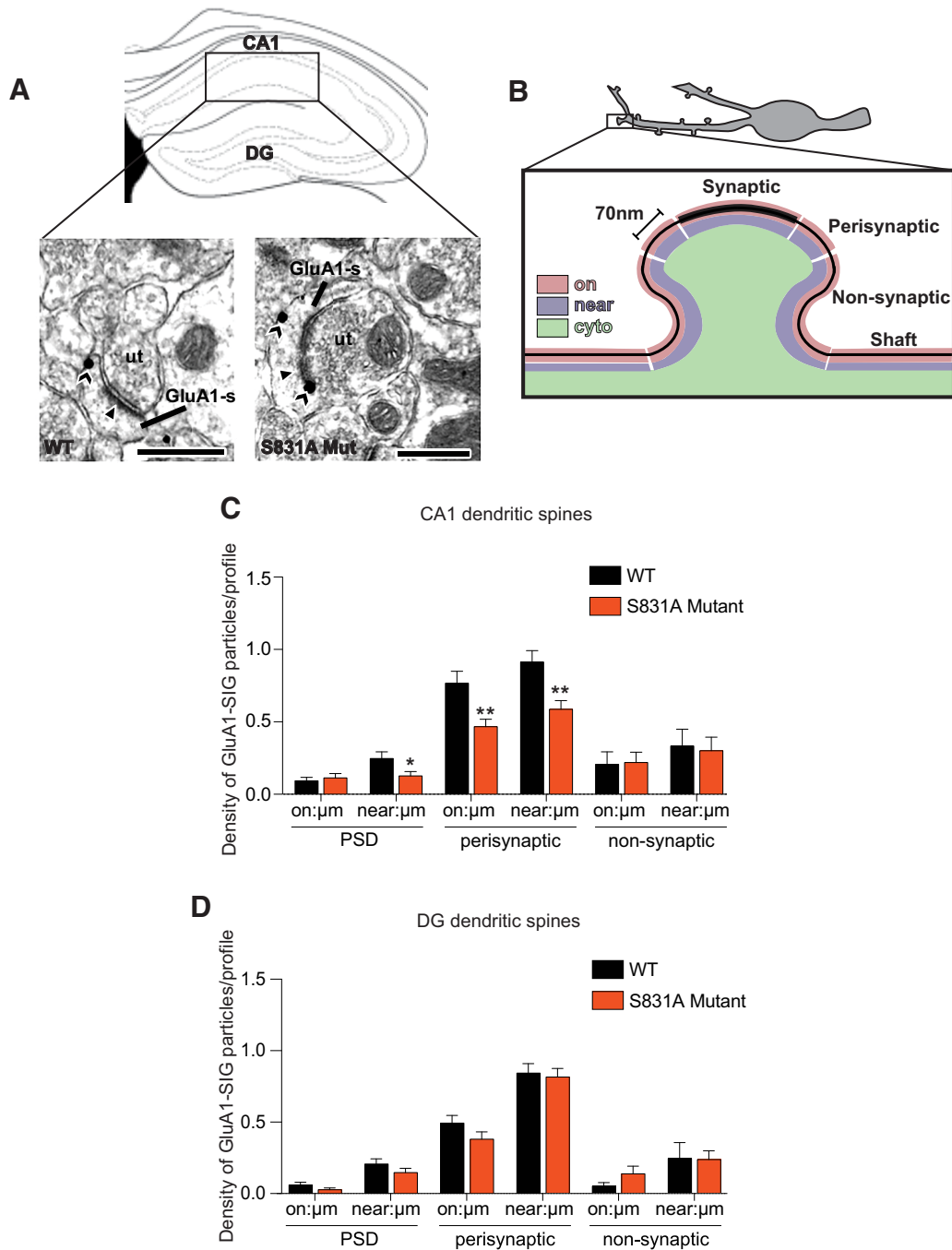
Figure 5. S831A GluA1 mutant mice have lower levels of GluA1 near the PSD and on and near the plasma membrane of perisynaptic sites of dendritic spines in the hippocampal CA1 subregion after impaired cocaine CPP extinction. **A**, Representative electron micrographs from stratum radiatum of CA1 of WT and S831A mutant mice after cocaine CPP extinction showing GluA1 SIG particle labeling in spines (sp) contacted by unlabeled axon terminals (ut). Scale bars, 500nm. **B**, Schematic figure demonstrating the criteria used to apportion SIG particles when quantifying GluA1 localization in dendritic spines by electron microscopy. **C**, In dendritic spines, significantly lower densities of GluA1 SIG particles were seen near, but not on the plasma membrane of the PSD in S831A mice compared with WT mice. Moreover, significantly lower densities of GluA1 SIG particles were observed on and near the plasma membrane of perisynaptic sites in S831A mice compared with WT mice. No difference in density of GluA1 SIG particles was observed on or near the plasma membrane of nonsynaptic sites. **D**, No difference in densities of GluA1 SIG particles were observed at synaptic or perisynaptic sites of the dentate gyrus of S831A mice and WT mice *$p < 0.05$, **$p < 0.01$. Data are presented as mean ± SEM.

Because D1R-expressing cells in the NAc are key mediators of important behavioral effects of cocaine (Smith et al., 2013), including extinction of cocaine CPP (Calipari et al., 2016), we investigated the effect of loss of Ca$_v$1.2 in D1R-expressing cells in the NAc on CaMKII and GluA1 levels. Western blot confirmed the efficacy of Ca$_v$1.2 knock-out in the NAc with lower Ca$_v$1.2 protein in the PSD fraction of D1^{cre}, Ca$_v$1.2$^{fl/fl}$ mice compared with D1^{cre}, Ca$_v$1.2$^{+/+}$ mice (Fig. 6B; $t_{(3.093)} = 15$;

$p = 0.0074$, unpaired t test). As opposed to the hippocampus, there was no significant difference in levels of CaMKIIα, CaMKIIβ, GluA1, or their phosphorylated forms in PSD fractions isolated from the NAc (Fig. 6D). Therefore, together, the above data suggest that Ca$_v$1.2-mediated changes in levels of CaMKII and P-GluA1 S831 within D1R-expressing cells of the hippocampus are necessary for extinction of cocaine CPP.
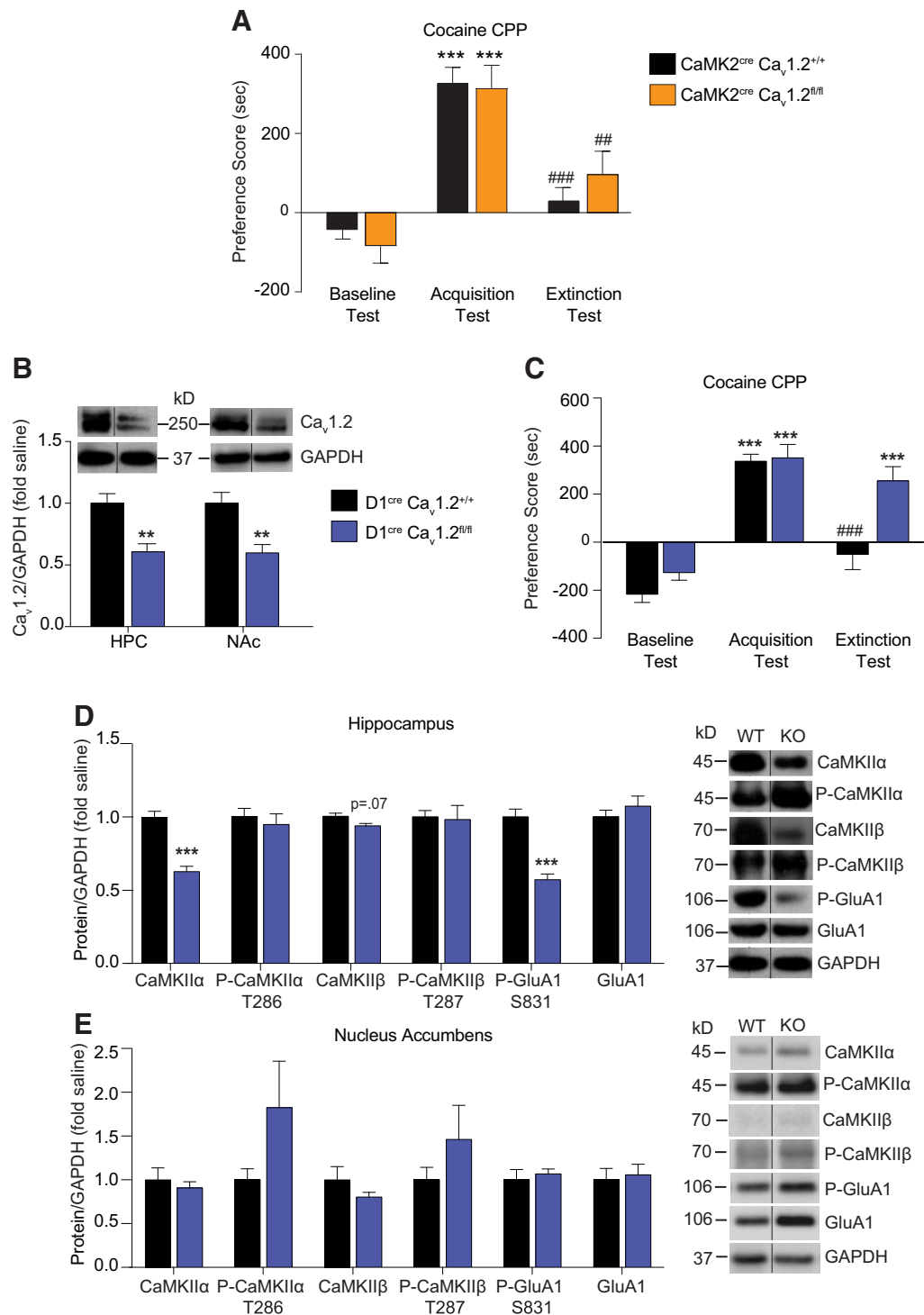
Figure 6. Mice with selective deletion of Ca$_v$1.2 in D1R-expressing cells have a deficit in extinction of cocaine CPP and lower levels of CaMKIIα and P-GluA1 S831, but not total GluA1 at the hippocampal PSD. ***A***, CaMK2 ^{cre}, Ca$_v$1.2 $^{+/+}$ mice and CaMK2 ^{cre}, Ca$_v$1.2 $^{fl/fl}$ mice displayed similar acquisition and extinction of cocaine CPP. ***p < 0.001 compared with baseline; #p < 0.05, ###p < 0.001 compared with acquisition. ***B***, Validation of the D1 ^{cre}, Ca$_v$1.2 $^{fl/fl}$ mouse line. Ca$_v$1.2 protein levels in hippocampal and NAc PSD fractions were significantly lower in D1 ^{cre}, Ca$_v$1.2 $^{fl/fl}$ mice compared with D1 ^{cre}, Ca$_v$1.2 $^{+/+}$ mice. **p < 0.01. ***C***, Compared with D1 ^{cre}, Ca$_v$1.2 $^{+/+}$ mice, D1 ^{cre}, Ca$_v$1.2 $^{fl/fl}$ mice displayed similar acquisition of cocaine CPP, but demonstrated a deficit in extinction of cocaine CPP. ***p < 0.001 compared with baseline test; ###p < 0.001 compared with acquisition test. ***D***, After cocaine CPP extinction test, D1 ^{cre}, Ca$_v$1.2 $^{fl/fl}$ mice had significantly lower protein levels of CaMKIIα and P-GluA1 S831 in the PSD-enriched fraction of the hippocampus compared with D1 ^{cre}, Ca$_v$1.2 $^{+/+}$ mice, with no difference in GluA1 levels. ***p < 0.001. ***E***, After the cocaine CPP extinction test, there were no significant changes in the proteins examined in NAc PSD fractions of D1 ^{cre}, Ca$_v$1.2 $^{fl/fl}$ mice compared with D1 ^{cre}, Ca$_v$1.2 $^{+/+}$ mice. Data are presented as mean ± SEM.

Discussion

Here, we describe a previously unknown role for Ca$_v$1.2 channels in the dorsal hippocampus in extinction of cocaine-associated memories. In addition, we show that Ca$_v$1.2/CaMKII-mediated

phosphorylation of GluA1 at S831 in the hippocampus is essential for extinction of cocaine CPP. By conditionally deleting Ca$_v$1.2 in D1R-expressing cells, we further demonstrate a cell-type-specific role for Ca$_v$1.2 channels in extinction of cocaine CPP, hippocam-

pal CaMKII expression, and phosphorylation of GluA1 at S831. These results provide the first illustration that a signaling pathway in the hippocampus involving Ca_v1.2 channels, CaMKII, and S831 GluA1 phosphorylation plays an essential role in extinguishing contextual cocaine-associated memories, with data supporting the contribution of hippocampal D1R-expressing cells.

Ca_v1.2 channels, dorsal hippocampus, and extinction of cocaine-associated memories

Extinction learning is thought to involve new learning that overrides, as opposed to erases, original memories (Bouton, 2004). Extinction of cocaine memories, as well as extinction of fear memories, which share overlapping mechanisms (Peters et al., 2009; Tipps et al., 2014), requires activation of new gene expression and protein synthesis (Cammarota et al., 2003; Vianna et al., 2004; Lattal et al., 2006; Szalay et al., 2013). We found extinction-specific increases in hippocampal Ca_v1.2 mRNA and PSD protein with no change in levels after acquisition, suggesting that newly synthesized Ca_v1.2 contributes to extinction learning. In addition, viral-mediated deletion of Ca_v1.2 in the dorsal hippocampus attenuates extinction of cocaine CPP with no effect on acquisition. The lack of a role for dorsal hippocampal Ca_v1.2 in acquisition of cocaine CPP is intriguing because Ca_v1.2 channels are highly involved in hippocampal-dependent learning and memory (Moosmang et al., 2005; Krug et al., 2014; Temme et al., 2016; Kabir et al., 2017a), suggesting that Ca_v1.2 may play a differential role in cocaine-associated versus other forms of hippocampal-dependent learning. Our data further suggest that distinct hippocampal mechanisms are most likely recruited for the acquisition versus extinction of cocaine-associated contextual memories.

Although studies examining hippocampal mechanisms involved in acquisition versus extinction of cocaine CPP are limited, few studies have suggested that acquisition and extinction recruit distinct transcriptional mechanisms (Quirk and Mueller, 2008). For example, a recent study on the role of the transcriptional regulator histone deacetylase 3 (HDAC3) in cocaine CPP revealed that dorsal hippocampal HDAC3 plays a selective role in extinction of cocaine CPP, but has no role in acquisition (Alaghband et al., 2017). Similarly, a study examining changes in microRNAs (miRNAs) that are involved in posttranscriptional regulation of gene expression via silencing mRNAs, has found that miRNA profiles in the hippocampus of rats after extinction of cocaine CPP differs compared with that seen after acquisition of cocaine CPP (Chen et al., 2013), suggesting that these phases of CPP are recruiting distinct molecular pathways. Although the regulatory relationship between these particular HDAC3 and miRNA-mediated mechanisms and Ca_v1.2 is unknown, these prior studies are nonetheless consistent with the present finding that distinct signaling pathways contribute to the extinction and acquisition of cocaine CPP.

Our finding that Ca_v1.2 channels within the dorsal, but not ventral, hippocampus are required for extinction of cocaine CPP is supported by previous results showing the necessity of dorsal hippocampus activity for extinction of cocaine-associated memories (Szalay et al., 2011; Szalay et al., 2013; Castilla-Ortega et al., 2016; McGlinchey and Aston-Jones, 2017) and extinction of fear memories (Corcoran and Maren, 2004; Corcoran et al., 2005; Ji and Maren, 2005). Furthermore, LTCCs within the dorsal hippocampus are particularly required for extinction of contextual fear memories (de Carvalho Myskiw et al., 2014). The observed lack of a role for Ca_v1.2 channels in the ventral hippocampus for extinction of cocaine CPP, a region required for extinction of

cocaine self-administration (Chambers and Self, 2002), may reflect disparate circuitry involved in classical conditioning versus instrumental behavior.

Ca_v1.2, CaMKII, S831 GluA1 phosphorylation, and extinction of cocaine contextual memories

CaMKIIα/β serve as key downstream LTCC targets (Ma et al., 2012; Li et al., 2016) that transduce activity-dependent signals critical for various aspects of learning/memory (Hell, 2014; Shonesy et al., 2014), cocaine extinction (Rich et al., 2016), and fear extinction learning (Szapiro et al., 2003; Kimura et al., 2008; Radwanska et al., 2011). Here, we find that cocaine CPP extinction increases total and phosphorylated CaMKIIα/β protein in hippocampal PSD fractions and pharmacological inhibition of CaMKIIα/β in the dorsal hippocampus attenuates extinction. Interestingly, the increase in T286 phospho-CaMKIIα, the most characterized active form of CaMKIIα, is not attenuated in the hippocampus of D1^cre, Ca_v1.2^fl/fl mice, potentially a reflection of the limited population of D1R-expressing cells within the hippocampus (discussed below) (Gangarossa et al., 2012). Alternatively, CaMKII activity may be modulated by other phosphorylation sites such as S331, which was shown recently to mediate extinction of cocaine self-administration in the NAc (Rich et al., 2016).

CaMKIIα activity increases phosphorylation of GluA1 at site S831 (Barria et al., 1997; Mammen et al., 1997), resulting in potentiation of single-channel conductance of GluA1 homomeric and heteromeric AMPA receptors (Derkach et al., 1999; Oh and Derkach, 2005; Kristensen et al., 2011), a process implicated in synaptic activity associated with hippocampal-dependent learning (Kessels and Malinow, 2009). Phosphorylation of S831 has been linked to learning and memory (Whitlock et al., 2006; Crombag et al., 2008; Lee et al., 2013) and cocaine-related behaviors (Anderson et al., 2008; Kim et al., 2009; Schierberl et al., 2011). Here, we show that extinction of cocaine CPP is associated with an increase in phosphorylated S831 in hippocampal PSD fractions and demonstrate the necessity of S831 for extinction, with no role in acquisition of cocaine CPP using phosphomutant mice. In addition, lower levels of phosphorylated S831 in the hippocampus, but not the NAc, of extinction-deficient D1^cre, Ca_v1.2^fl/fl mice suggests that hippocampal Ca_v1.2-induced phosphorylation of GluA1 at S831 may be involved selectively in extinction of contextual cocaine memories.

Phosphorylation of S831 has been suggested to increase trafficking of GluA1 to the cell surface (Shepherd and Huganir, 2007). Increase in cell surface GluA1, particularly in the NAc, has been shown by several groups of investigators to be a critical step in mediating cocaine's long-term effects, particularly via formation of Ca^2+-permeable GluA1 homomeric AMPARs (Wolf, 2016), a mechanism that we have demonstrated to require S831 (Martinez-Rivera et al., 2017). Although no studies on hippocampal GluA1 trafficking in extinction of cocaine behaviors exist, the necessity of GluA1 trafficking to the cell surface in the NAc has been demonstrated for extinction of cocaine self-administration behavior (Sutton et al., 2003). Here, using Western blots and electron microscopy, we found that S831 is not necessary for the increase in GluA1 at the hippocampal PSD, which is consistent with a previous in vitro study showing that the S831A mutation does not alter CaMKII-dependent or activity-dependent synaptic delivery of GluA1 (Hayashi et al., 2000). However, using electron microscopy, we found that S831 is necessary for trafficking of GluA1 near the PSD and near or on the plasma membrane of perisynaptic sites, suggesting that these particular pools of GluA1 may be essential for extinction of cocaine CPP. This is supported by stud-

ies demonstrating that perisynaptically localized GluA1 is important for expression of hippocampal LTP and modulation of synaptic strength (Guire et al., 2008; Yang et al., 2010), cellular processes known to be associated with extinction of cocaine-associated memories (del Olmo et al., 2006). Future studies will address the necessity of nonsynaptic GluA1 in the hippocampus for extinction of cocaine CPP and the role of Ca$_v$1.2 channels therein.

Hippocampal D1Rs, Ca$_v$1.2, and extinction of cocaine contextual memories

Growing evidence suggests that dopamine signaling via D1Rs plays a key role in extinction learning (El-Ghundi et al., 2007; Singewald et al., 2015), including extinction of cocaine-associated memories (Fricks-Gleason et al., 2012; Abraham et al., 2016). Although D1Rs are expressed throughout the brain, dorsal hippocampal D1Rs have been shown to play a role in extinction of contextual fear memories (Fiorenza et al., 2012; Menezes et al., 2015), in addition to drug context-induced reinstatement of self-administration (Xie et al., 2014). D1R-expressing cells are found within the hippocampus, located most densely within the stratum radiatum of CA1 and CA3 and the granule layer of the dentate gyrus (Gangarossa et al., 2012), and are suggested to be part of an extinction circuit, with mechanisms initiated within the CA1 subregion (Sil'kis, 2008). Our electron microscopy findings in S831A GluA1 phosphomutant mice support a role for CA1 in Ca$_v$1.2-mediated extinction of cocaine CPP.

We found that deleting Ca$_v$1.2 in D1R-expressing cells of the brain results in an impairment in the extinction of cocaine CPP. We also showed that CaMKII and phosphorylation of GluA1 at S831 are lower in the hippocampus, but not the NAc, of extinction-deficient D1R-cell-specific Ca$_v$1.2 knock-out mice. Although our molecular data suggest that the Ca$_v$1.2/CaMKII/S831 GluA1 signaling pathway in D1R-expressing cells of the hippocampus may mediate extinction of cocaine CPP, Ca$_v$1.2 is lacking in all D1R-expressing cells in the mouse model used, allowing for the possibility that Ca$_v$1.2 in D1R cells within other D1R-rich regions are involved in this behavior.

Previous studies support the interaction of D1Rs, CaMKII, and GluA1 activity because pharmacological activation of D1R produces a CaMKII-mediated increase in surface expression of GluA1 (Gao et al., 2006; Anderson et al., 2008; Schierberl et al., 2011) and total S831 phosphorylation (Anderson et al., 2008; Schierberl et al., 2011). Although D1Rs (Gangarossa et al., 2012) and Ca$_v$1.2 (Hell et al., 1993) are both expressed in hippocampal glutamatergic neurons, the precise cell type within the hippocampus that contributes to extinction remains to be addressed, given that a recent study has classified D1R-expressing cells as primarily GABAergic interneurons (Puighermanal et al., 2017). Ca$_v$1.2 is also present in several GABAergic interneuron subtypes within the hippocampus (Vinet and Sík, 2006; Dedic et al., 2017); however, low or undetectable levels of CaMKIIα within inhibitory neurons of the hippocampus (Jones et al., 1994; Liu et al., 2014) raise the possibility that both glutamatergic and GABAergic cell types within the hippocampus may be recruited during extinction of cocaine CPP, a subject for future studies.

In summary, this study is the first to report that Ca$_v$1.2 channels play a critical role in the dorsal hippocampus for the extinction of cocaine-associated memories, with molecular data supporting a contribution of Ca$_v$1.2-activated CaMKII/S831 GluA1 within D1R-expressing cells of the hippocampus. Therefore, this study sheds light on a new role of Ca$_v$1.2 channels in extinction of contextual cocaine-associated memory and advances our understanding of a dopamine- and Ca^{2+}-dependent molecular pathway mediating extinction learning. These findings merit further exploration to better understand hippocampal mechanisms that contribute to addictive behavior as studied using animal models.

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