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$Ca_v1.2$ L-Type Ca^{2+} Channels Mediate Cocaine-Induced GluA1 Trafficking in the Nucleus Accumbens, a Long-Term Adaptation Dependent on Ventral Tegmental Area $Ca_v1.3$ Channels

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AMPA receptor (AMPAR) plasticity at glutamatergic synapses in the mesoaccumbal dopaminergic pathway has been implicated in persistent cocaine-induced behavioral responses; however, the precise mechanism underlying these changes remains unknown. Utilizing cocaine psychomotor sensitization, we have examined phosphorylation of GluA1 at key residues serine 845 (S845) and S831, as well as GluA1 cell surface levels in the nucleus accumbens (NAc) of cocaine-preexposed mice and the role of brain-specific $Ca_v1.2$ and $Ca_v1.3$ L-type Ca^{2+} channels (LTCCs), therein. We found higher basal levels of S845 phospho-GluA1 (P-GluA1) and cell surface GluA1 in the NAc following protracted withdrawal from cocaine exposure, changes that occur independently of LTCCs. In contrast, we found that a cocaine challenge that elicits expression of the cocaine-sensitized response increases S831 P-GluA1 that further increases surface GluA1 beyond the higher basal levels. Intra-NAc pharmacological manipulations indicate that the $Ca_v1.2$-activated CaM kinase II (CaMKII) mediates cocaine-induced increase in S831 P-GluA1 and that both $Ca_v1.2$-activated CaMKII and extracellular signal-regulated kinase 2 (ERK2) mediate the increase in GluA1 cell surface levels specific to the sensitized response. Experiments using adenoassociated viral vectors expressing $Ca_v1.3$ and ERK2 siRNA further indicate that recruitment of the $Ca_v1.2$ pathway in the NAc is dependent on ventral tegmental area $Ca_v1.3$ LTCCs and ERK2. Together, these results identify candidate pathways that mediate cocaine-induced AMPAR plasticity in the NAc and provide a mechanism linking LTCCs and GluA1 plasticity to cocaine-induced persistent behavioral changes.

Introduction

Repeated cocaine exposure causes persistent adaptations within the mesoaccumbal dopamine pathway that extends from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), and these changes are believed in part to underlie compulsive drug-seeking behavior and drug-induced relapse, despite extended drug-free periods (Berke and Hyman, 2000; Nestler, 2001). A commonly used model of behavioral plasticity that has aided in our understanding of the actions of cocaine actions is psychomotor sensitization (Kalivas and Stewart, 1991; Robinson and Berridge, 1993). This model is composed of two phases, development and expression (Pierce and Kalivas, 1997b; Vanderschuren and Kalivas, 2000). Development of sensitization is a progressive increase in psychomotor activity following repeated cocaine treatment that has been shown to involve activation of molecular mechanisms in the VTA. Expression of sensitization is a persistently elevated drug challenge-induced locomotor response observed following an extended withdrawal period, and several lines of evidence have found that adaptations in the NAc mediate this long-term sensitized response.

Recent studies using cocaine psychomotor sensitization have found alterations in AMPA receptors (AMPARs) within the NAc following extended drug-free periods (Bowers et al., 2010; Schmidt and Pierce, 2010; Wolf and Ferrario, 2010). Specifically, regulation of trafficking of the GluA1 subunit of AMPARs (Boudreau and Wolf, 2005; Mameli et al., 2009; Ferrario et al., 2010), potentially via changes in phosphorylation (Chao et al., 2002a,b), and alterations in AMPAR function (Kourrich et al., 2007; Mameli et al., 2009) have been observed. Consistent with this,

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Table 1. $Ca_v1.3$-specific shRNA oligonucleotides

$Ca_v1.3$ shDNA molecules	Sequence	Mouse nucleotide position	Rat nucleotide position	Knockdown siRNA versus AAV empty vector (%)
siRNA 1	GGATCTCTCTCCCAAAGAA	248–270	586–604	88 ± 0.95
siRNA 2	GTAGGATTGTTTAGTGTAA	635–657	973–991	91 ± 0.52
siRNA 3	GAACCACTCTAGTGGCAAA	688–710	1026–1044	74 ± 15.6
siRNA 4	GTACAAAGCTATTGATTCA	3397–3419	—	60 ± 3.5
siRNA 5	GGAGCAAGCTAATGAAGAA	4774–4796	5184–5201	64 ± 2.5
siRNA 6	GAACAGGAGTATTTCAGTA	5594–5616	—	70 ± 1.5
sC (control)	GTAGAGTTGTTATGTGTAA	—	—	3 ± 1.5

activity-dependent AMPAR cell surface trafficking and AMPAR synaptic function is regulated via changes in phosphorylation of the GluA1 subunit (Kessels and Malinow, 2009). The GluA1 subunit possesses multiple phosphorylation sites on its intracellular C terminus, including serine 845 (S845), a protein kinase A (PKA) site (Roche et al., 1996), and S831, a Ca^{2+}/calmodulin kinase II (CaMKII) site (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997), kinases regulated by cocaine.

While cocaine-induced adaptations in AMPAR function are evident, the mechanism by which cocaine causes these changes remains unknown. The brain-specific L-type Ca^{2+} channel (LTCC) isoforms, $Ca_v1.2$ and $Ca_v1.3$, are intriguing candidates for mediating these phenomena as they mediate cocaine-elicited behaviors (Rajadhyaksha and Kosofsky, 2005; Anderson et al., 2008; Giordano et al., 2010), downstream signaling in response to dopamine D_1 receptor activation (Surmeier et al., 1995; Liu and Graybiel, 1996; Giordano et al., 2010), and stimulate Ca^{2+}-activated kinases that phosphorylate GluA1 and regulate its trafficking (Deisseroth et al., 2003; Rajadhyaksha and Kosofsky, 2005).

Utilizing cocaine psychomotor sensitization, we recently reported that $Ca_v1.3$ channels mediate the development of sensitization, whereas $Ca_v1.2$ channels mediate expression (Giordano et al., 2010). However, the precise anatomical sites of action of these two LTCC isoforms during these phases of sensitization and their roles in cocaine-induced modifications of GluA1 in the NAc following withdrawal remain unknown. Thus, we have used a combination of genetic mutant mice and site-specific manipulations to elucidate the precise mechanism by which $Ca_v1.2$ and $Ca_v1.3$ channels in the VTA and NAc mediate these molecular and behavioral adaptations.

Materials and Methods

Animals

Male C57BL/6 mice (Charles River Laboratories), $Ca_v1.3$ wild-type (WT) and knock-out mice (KO) (Platzer et al., 2000), $Ca_v1.2$ dihydropyridine (DHP)-insensitive mice (Sinnegger-Brauns et al., 2004), and CNS-specific $Ca_v1.2$ WT and KO mice (Moosmang et al., 2005) generated on the C57BL/6 background were 9–10 weeks of age at the start of the experiments. Heterozygote ($Ca_v1.3^{+/-}$ and $Ca_v1.2DHP^{+/-}$) mice were bred to generate F_2 male ($Ca_v1.3^{+/+}$, $Ca_v1.3^{-/-}$, and $Ca_v1.2DHP^{-/-}$) mice as previously described (Platzer et al., 2000; Giordano et al., 2010). In $Ca_v1.2$DHP-insensitive mice, the $Ca_v1.2$ LTCC subunit has been mutated at the DHP binding site (tyrosine to threonine at position 1066 in helix IIIS5 of exon 24) (Sinnegger-Brauns et al., 2004; Giordano et al., 2010) such that the high sensitivity for DHPs, such as nifedipine, which was used in this study, is eliminated while their function is completely preserved. Thus, in mice homozygous for the mutant $Ca_v1.2$ gene, treatment with DHPs selectively targets the $Ca_v1.3$ subunit. To generate CNS-specific $Ca_v1.2$ KO mice and WT littermate control mice, heterozygous floxed $Ca_v1.2$ mice ($Ca_v1.2^{fl/+}$) were bred with mice expressing Cre recombinase under the control of the nestin promoter ($Nestin^{Cre/+}$, $Ca_v1.2^{fl/+}$) (Moosmang et al., 2005). For AAV-Cre experiments, homozygous $Ca_v1.2$ floxed mice (Moosmang et al., 2005) were generated from this same line. Mice were provided food and water *ad libitum*. Animals were maintained on a 12 h light/dark cycle (from 7:00 A.M. to 7:00 P.M.). All procedures were conducted in accordance with the Massachusetts General Hospital Subcommittee on Research Animal Care rules and the Weill Cornell Medical College Institutional Animal Care and Use Committee rules.

Reagents

Cocaine HCl, CaM kinase (CaMK) inhibitor *N*-[2-[*N*-(4-chlorocinnamyl)-*N*-methylaminomethyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzene-sulfonamide phosphate salt (KN93), LTCC inhibitor nifedipine, and the MEK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) were obtained from Sigma-Aldrich. Anti-rabbit Ser831 and Ser845 phospho-GluA1 antibodies were obtained from Abcam. Anti-rabbit GluA1 and GluA2 antibodies was obtained from Millipore. Anti-rabbit Thr286 P-CaMKII, CaMKII, Thr183/Tyr185 P-ERK1/2, and ERK1/2 antibodies were obtained from Cell Signaling. Anti-mouse $Ca_v1.3$ antibody was obtained from University of California Davis/NINDS/NIMH NeuroMab facility care of Antibodies, Inc. and anti-mouse β-actin antibody was from Millipore Bioscience Research Reagents. For immunohistochemistry, donkey Cy3 anti-mouse and Cy2 anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. For immunoblot analyses, goat anti-rabbit and horse anti-mouse secondary antibodies were obtained from Vector Laboratories.

Design, screening, and packaging of recombinant adenoassociated viral $Ca_v1.3$ siRNA

Design and packaging of shRNAs was performed as previously reported by Garraway et al. (2007). Briefly, sequence data for the mouse $Ca_v1.3$ subunit was obtained from GenBank (accession number AB086123), and six candidate siRNAs (Table 1) were identified using a computational program developed by the Whitehead Institute for Biomedical Research (http://jura.wi.mit.edu/siRNAext/). Similarity searching of candidate siRNA sequences against the rat and mouse UNIGene database was used to avoid selection of siRNAs that match related mRNA sequences such as the $Ca_v1.2$ L-type subunit, as well as other family members of voltage-gated Ca^{2+} channels (Ertel et al., 2000). A control scrambled siRNA sequence (sC) was designed by scrambling base pairs within a 19 bp region of the $Ca_v1.3$ gene (Table 1). Candidate siRNA and control sequences were then converted into short hairpin DNAs (shDNAs) with the addition of a loop sequence (TTCAAGAGA) (Brummelkamp et al., 2002), an antisense sequence, BglII and XbaI restriction sites to the 5′ and 3′ ends, respectively, to allow for vector cloning and six Ts on the 3′ end (the Pol III promoter termination signal). shDNAs were commercially synthesized (Sigma-Genosys) and cloned into the recombinant adenoassociated viral (rAAV)-2 vector downstream of the H1 promoter. The rAAV vector also contains sequences for EGFP (enhanced green fluorescent protein) under the control of the β-actin promoter. Insertion of target shDNA was confirmed by DNA sequencing (Cornell University Core Laboratories Center, Ithaca, NY).

The psiCHECK Dual Luciferase Reporter Assay (Promega) was used to screen for efficiency of siRNAs in mediating $Ca_v1.3$-mRNA knockdown (KD) *in vitro*, as described by Garraway et al. (2007). HEK293 cells were cotransfected with a psiCHECK plasmid that contained $Ca_v1.3$ cDNA, downstream of *Renilla* luciferase (hRluc), and rAAV plasmids containing candidate shRNA sequences. Cells were harvested 24 h after transfection and screened for $Ca_v1.3$-silencing activity based on the rel-

ative intensity of hRluc chemiluminescence. Based on *in vitro* $Ca_v1.3$-silencing activity, siRNA-1 and siRNA-2 were identified as the most effective silencer sequences of $Ca_v1.3$ mRNA (88 and 91%, respectively; Table 1). The scrambled siRNA control (sC) did not significantly affect levels of $Ca_v1.3$ cDNA (Table 1), confirming that it was an effective sequence control. Most importantly, siRNA 1 or siRNA 2 did not alter levels of $Ca_v1.2$ mRNA (data not shown), when tested in the psiCHECK assay using the $Ca_v1.2$ cDNA plasmid, confirming specificity of siRNA-1 and siRNA-2 for targeting $Ca_v1.3$ mRNA.

For viral packaging, HEK293 cells were transfected with rAAV-siRNA-1, rAAV-siRNA-2, or rAAV-sC siRNA along with a helper plasmid encoding essential genes involved in adenoviral replication as described previously (Garraway et al., 2007). Cells were harvested and lysed 72 h after transfection and rAAV was purified and concentrated using a HiTrap heparin column (GE Healthcare). The genomic titer was determined by real-time PCR to be between 10^{12} and 10^{13} genome copies per milliliter.

Generation of rAAV ERK2 siRNA

ERK2 siRNA was identified using an identical approach as that described for the $Ca_v1.3$ siRNAs described above and is published in the study by Xu et al. (2008). The siRNA sequence used for this study was 5′-GGAGCAGTATTATGACCCA-3′. The sequence was reversed to produce a control sequence, 5′-ACCCAGTATTATGACGAGG-3′.

Delivery of rAAV into the ventral tegmental area

All experimental procedures were conducted in accordance with the guidelines set by the Weill Cornell Medical College Institutional Animal Care and Use Committee. Before surgery, mice were anesthetized with a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml), and mounted to a stereotaxic surgical unit (David Kopf Instruments). A midline incision was made atop the scalp, skin was retracted using bulldog clips, and holes were formed through the skull using an electric drill between −3.4 and −3.5 mm A/P relative to bregma and ±0.53 mm M/L relative to the sagittal suture. All stereotaxic coordinates for the VTA were adopted from the Mouse Brain Atlas (Paxinos and Franklin, 2004). A 26s ga Hamilton syringe (series 600/700–5 μl; Hamilton Company) was inserted through the incision site in the left hemisphere (−5.13 mm D/V) to deliver 0.5 μl of virus (siRNA-rAAV or sC-rAAV in Figs. 3, 4, and 7) into the left VTA (0.1 μl/min). The syringe needle was left in place for 8 min, withdrawn, and the injection was repeated in the right VTA. The skull was then sealed with bone wax and the scalp was sutured. Mice were housed individually after administration of virus.

Fluorescent dual-label immunohistochemistry was used to confirm injection placement. Upon completion of behavioral testing, mice were killed and perfused with 4% paraformaldehyde (PFA) and postfixed overnight in 4% PFA. Forty micrometer sections spanning the VTA were obtained using a vibratome and incubated in anti-mouse tyrosine hydroxylase (1:500) and anti-rabbit GFP (1:500) primary antibodies for 24 h at 4°C. The sections were rinsed in 0.1 M PBS and incubated with donkey anti-mouse Cy2 (1:500) and donkey anti-rabbit Cy3 (1:500) secondary antibodies for 2 h at room temperature. GFP and TH colabeling was visualized to confirm spatial distribution of rAAV-infected VTA dopaminergic neurons (see Fig. 1*E*). Animals with improper bilateral injection placement were excluded from behavioral data analysis. GFP labeling revealed equal infectivity of rAAV-siRNA-1 and rAAV-siRNA-2; thus, for simplicity, all behavioral experiments described in this study were performed using rAAV-siRNA-2.

Delivery of rAAV into the nucleus accumbens

NAc coordinates for 9- to 10-week-old male C57BL/6 mice were +1.6 mm A/P, ±1.52 mm M/L, and −4.55 mm D/V at an angle of 10° relative to bregma (Paxinos and Franklin, 2004). To confirm injection placement in the NAc, 300 μm sections were obtained from freshly dissected brain. GFP fluorescence was used to confirm accurate bilateral targeting of the NAc. Mice with inaccurate targeting on either side of the NAc were eliminated from the study.

Guide cannula implantation in the NAc

For delivery of pharmacological drugs into the NAc, guide cannulae were implanted bilaterally in adult male mice under ketamine (100 mg/ml) and xylazine (20 mg/ml) anesthesia in a stereotaxic frame. Coordinates used were +1.6 mm A/P, ±1.52 mm M/L, and −4.55 mm D/V at an angle of 15° relative to bregma (Paxinos and Franklin, 2004). The tip of the cannula was positioned 1.5 mm above the NAc. Stainless-steel guide cannula (5 mm in length; CMA/Microdialysis; model CMA/7) was secured to the skull with dental acrylic resin and 5 mm stainless-steel stylets were inserted until the time of experiments. Following surgery, mice were individually housed with *ad libitum* access to food and water and allowed a minimum of 7 d recovery before all experiments. Pharmacological agents were delivered into the NAc using an injection cannula connected by flexible polyethylene tubing to a microinjection system, mounted with a 5 ml Hamilton syringe. Accurate bilateral cannulae placement was confirmed in a 50 μM paraformaldehyde-perfused section by cresyl violet Nissl staining. Mice with inaccurate targeting on either side of the NAc were eliminated from the study. The CaM kinase inhibitor KN93 (3 μg in 0.2 μl) or MEK inhibitor U0126 (80 ng in 0.2 μl) was microinjected into the NAc 30 min before challenge with cocaine. KN93 and U0126 doses were chosen following an initial dose–response with 1, 3, and 5 μg/0.2 μl and 60, 80, and 100 ng/μl, respectively. Both drugs were resuspended and dissolved in 0.9% saline containing 1.5% DMSO and 1.5% Tween 80 used as vehicle.

Psychomotor sensitization protocol

Cocaine psychomotor sensitization was performed as previously described by Giordano et al. (2010). Briefly, mice were habituated to open-field locomotor activity chambers (MED Associates) for 30 min. Mice were then administered saline or cocaine (15 mg/kg, i.p.) once a day for 5 d (days 1–5), and locomotor activity was measured for 30 min on each testing day. Following a 21 d drug-free period, mice were challenged with saline or 15 mg/kg intraperitoneal cocaine, and locomotor activity measured for 30 min. For pharmacological experiments, nifedipine, KN93, and U0126 were administered 30 min before cocaine treatment. Cocaine and 3-allyl-6-chloro-1-phenyl-1,2,4,5-tetrahydro-3-benzazepine-7,8-diol (SKF82958) were dissolved in 0.9% saline. Nifedipine, KN93, and U0126 were dissolved in 0.9% saline containing 1.5% DMSO and 1.5% Tween 80.

Immunoblot analysis

Immunoblotting was performed as previously described by Giordano et al. (2010). Briefly, mice were decapitated 30 min following behavioral testing and brains were rapidly dissected and frozen in isopentane at −40°C. To obtain NAc tissue, brains were sectioned in the coronal plane on a cryostat to the rostral end of the NAc, and 0.5 mm bilateral tissue punches (containing NAc shell and core), spanning approximately +1.7 to +1.2 mm A/P relative to bregma (Paxinos and Franklin, 2004), were isolated with a 17 ga stainless-steel stylet. To obtain VTA tissue, brains were sectioned to the rostral end of the VTA, and an approximate 0.5 mm unilateral punch [spanning −3.16 to −3.64 A/P relative to bregma (Paxinos and Franklin, 2004)] was isolated with a 17 ga stylet. Tissue was processed as previously described by Giordano et al. (2010). Twenty to 40 μg of protein was loaded on 12% SDS-polyacrylamide gels and run at 200 V constant voltage. Blots were probed with anti-rabbit (1:850 Ser845 P-GluA1, Ser831 P-GluA1, GluA2, 1:1000 GluA1, P-ERK1/2, ERK1/2, Thr286 P-CaMKII, and CaMKII) or anti-mouse (1:30,000 β-actin and 1:1000 $Ca_v1.3$) primary antibodies overnight at 4°C. Blots were then incubated with goat anti-rabbit (1:5000 for all anti-rabbit primary antibodies) or horse anti-mouse (1:40,000 for actin and 1:5000 for $Ca_v1.3$) horseradish peroxidase-linked IgG. Protein bands were visualized by chemiluminescence. Kaleidoscope prestained standards (Bio-Rad) were used for protein size determination. For quantitation, β-actin was used as a loading control as we have found that repeated cocaine does not significantly alter total protein levels in the NAc, compared with repeated saline-treated mice (saline, 100 ± 10%, vs cocaine, 97 ± 7%). Blots were scanned with an HP Scanjet 7400c scanner (Hewlett Packard). Intensity of the protein bands was measured as optical density, using the NIH ImageJ program.

Surface GluA1 and GluA2 detection using BS^3 cross-linking

Experiments were performed using a modified protocol of that published in Boudreau and Wolf (2005). A single NAc tissue punch [spanning +1.7

to +1.2 mm relative to bregma (Paxinos and Franklin, 2004)] was rapidly dissected using a stainless-steel stylet (15 ga) from 0.5 mm coronal section placed on an ice-cold surface, obtained from a mouse brain matrix. NAc tissue was pooled from three mice. Tissue was incubated in ice-cold artificial CSF (aCSF) containing 2 mM BS^3 (Pierce) and incubated for 30 min at 4°C on a rotator. Cross-linking reaction was quenched with 100 mM glycine in aCSF for 10 min at 4°C on a rotator. Samples were centrifuged for 2 min at 4°C. Supernatants were discarded and pellets washed once with aCSF. Samples were recentrifuged, supernatants were discarded, and pellets were sonicated in ice-cold lysis buffer [0.1% NP-40 buffer in Tris-EDTA, pH 7.4, containing 1× protease inhibitor mixture (Sigma-Aldrich), 5 mM NaF, and 1× phosphatase inhibitor mixture (Sigma-Aldrich)]. Protein concentration was determined by BCA assay and 15 μg of protein was loaded on a 4–15% gradient Tris-HCl gel (Bio-Rad) and run at 100 V constant voltage. Gels were processed for GluA1 and GluA2 immunoblot analysis as described above and in the study by Tropea et al. (2008). Blots were probed with anti-rabbit GluA1 (1:850) and GluA2 (1:1000) and goat anti-rabbit horseradish peroxidase-linked IgG (1:5000; Vector Laboratories).

Quantitative real-time PCR

For quantitation of $Ca_v1.3$ knockdown in the VTA of rAAV-injected mice, brains were freshly dissected and a single 330 μm section spanning the VTA was obtained on a vibratome. The VTA was dissected and processed for RNA using the RNeasy Mini Kit (QIAGEN). cDNA was synthesized from purified RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) and quantified by quantitative real-time PCR (qPCR) using $Ca_v1.3$-specific primers (sequence accession NM_001083616.1): forward primer (nucleotide position 552–572), 5′-CCATGCGAACGAGGCAAACTA-3′, and reverse primer (nucleotide position 736–756), 5′-TTGCTGACGTTT TCTTTGGGA-3′, on an ABI PRISM 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). $Ca_v1.2$ mRNA was quantified using RNA isolated from NAc and dorsal striatum [spanning ~1.7–1.2 mm relative to bregma (Paxinos and Franklin, 2004)], and tissue punches were obtained as described for immunoblot analysis. Amplification was performed for 40 cycles (95°C for 15 s, 60°C for 30 s, 72°C for 30 s; extension, 72°C for 10 min). For $Ca_v1.2$ mRNA detection, $Ca_v1.2$-specific primers (QuantiTect Primer assay QT00150752; QIAGEN) were used. Cycle threshold (Ct) values for all target genes were normalized to the housekeeping gene β-actin [sequences as published in the study by Giordano et al. (2006)]. β-Actin was used as a loading control, as β-actin mRNA levels were unaltered following repeated cocaine treatment (saline, 100 ± 6%, vs cocaine, 102 ± 8%) as revealed by using the standard curve method with known concentrations (in nanograms) of first-strand cDNA, as we have previously reported in the study by Giordano et al. (2006). 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 β-actin values using the formula: $\Delta Ct = Ct(Ca_v1.3) - Ct(\beta\text{-actin})$. Expression levels were calculated relative to controls using the formula: $\Delta\Delta Ct = \Delta Ct(\text{treated}) - \Delta Ct(\text{control average})$. The final expression levels were obtained using the formula $2^{-\Delta\Delta Ct}$. For $Ca_v1.2$ mRNA levels (in nanograms), a standard curve generated with Ct and known cDNA concentration values was used as published in the study by Giordano et al. (2006).

Experiment procedures

Experiment 1. The effect of genetic deletion of $Ca_v1.3$ on expression of cocaine-induced psychomotor sensitization, NAc S845 P-GluA1, NAc S831 P-GluA1, NAc surface GluA1, and NAc surface GluA2 levels was examined in $Ca_v1.3$ WT and $Ca_v1.3$ KO mice. In this experiment (see Fig. 1), a factorial design that included the between-subject factors of treatment (S-S, C-S, S-C, C-C) and genotype (WT, KO) was used to assess the effect of loss of $Ca_v1.3$ on cocaine-induced locomotor activity on day 26 (to evaluate expression of psychomotor sensitization; see Fig. 1*A*), NAc S845 P-GluA1 (see Fig. 1*B*), NAc S831 P-GluA1 (see Fig. 1*C*), NAc surface GluA1 levels (see Fig. 1*D*), and NAc surface GluA2 levels (see Fig. 1*E*). $N = 8–12$ WT and KO mice were used for the treatment groups in Figure 1*A–C*, and $n = 6–7$ for WT and KO mice were used for the treatment groups in Figure 1, *D* and *E*.

Experiment 2. The effect of systemic pretreatment of the LTCC antagonist, nifedipine, during the development of cocaine psychomotor sensitization on NAc S831 P-GluA1, NAc surface GluA1, and NAc S845 P-GluA1 21 d later was examined in Cav1.2DHP $^{-/-}$ mutant mice. In this experiment (see Fig. 2*B–D*), a factorial design that included the between-subject factors of pretreatment (vehicle, nifedipine) and preexposure (saline, cocaine) was used to assess the effect of blocking $Ca_v1.3$ channels during development on cocaine-induced NAc S831 P-GluA1 (see Fig. 2*B*), S/I GluA1 (see Fig. 2*C*), and S845 P-GluA1 (see Fig. 2*D*). $N = 6$ mice were used for s-s, v-s, and n-s groups, and $n = 7$ for the v-c and n-c groups.

Experiment 3. The effect of systemic pretreatment of the LTCC antagonist nifedipine administered immediately before cocaine challenge 21 d following development of psychomotor sensitization on NAc S831 P-GluA1, NAc surface GluA1, and NAc S845 P-GluA1, was examined in $Ca_v1.2$DHP-insensitive mutant mice. In this experiment (see Fig. 2*F–H*), a factorial design that included the between-subject factors of pretreatment (vehicle, nifedipine) and challenge (saline, cocaine) was used to assess the effect of blocking $Ca_v1.3$ channels at expression on cocaine-induced S831 P-GluA1 (see Fig. 2*F*), S/I GluA1 (see Fig. 2*G*), and S845 P-GluA1 (see Fig. 2*H*). $N = 6$ mice were used for s-s, v-s, and n-s groups, and $n = 7$ for the v-c and n-c groups.

Experiment 4. The effect of KD of $Ca_v1.3$ in the VTA on the development of psychomotor sensitization, on the subsequent expression of cocaine sensitization examined 21 d later, and on NAc S831 P-GluA1 was assessed in control and $Ca_v1.3$ siRNA-injected mice. $Ca_v1.3$ KD in the VTA was achieved with site-specific microinjection of rAAV expressing $Ca_v1.3$ siRNA before the start of the sensitization regimen. In this experiment (see Fig. 3*E*,*F*), between-subject factors of viral treatment (control siRNA, $Ca_v1.3$ siRNA) and day (1, 5 or 26) was used to assess the effect of VTA $Ca_v1.3$ KD on development and expression of sensitization (see Fig. 3*E*) and a between-subject factor of viral treatment (control siRNA, $Ca_v1.3$ siRNA) was used to assess the effect of VTA $Ca_v1.3$ KD on NAc S831 P-GluA1 (see Fig. 3*F*) ($N = 12$ and 14 for control and $Ca_v1.3$ siRNA, respectively, in Fig. 3*E*; $n = 10$ for control and $Ca_v1.3$ siRNA in Fig. 3*F*).

Experiment 5. The effect of KD of $Ca_v1.3$ in the NAc on the development of psychomotor sensitization, on the subsequent expression of cocaine sensitization examined 21 d later, and on NAc S831 P-GluA1 was assessed in control and $Ca_v1.3$ siRNA-injected mice. $Ca_v1.3$ KD in the NAc was achieved with site-specific microinjection of rAAV expressing $Ca_v1.3$ siRNA before the start of the sensitization regimen. In this experiment (see Fig. 3*H*,*I*), a between-subject factor of viral treatment (control siRNA, $Ca_v1.3$ siRNA) and day (1, 5 or 26) was used to assess the effect of NAc $Ca_v1.3$ KD on development and expression of sensitization (see Fig. 3*H*), and a between-subject factor of viral treatment (control siRNA, $Ca_v1.3$ siRNA) was used to assess the effect of NAc $Ca_v1.3$ KD on NAc S831 P-GluA1 (see Fig. 3*I*) ($N = 10$ mice for control and $Ca_v1.3$ siRNA groups).

Experiment 6. The effect of KD of ERK2 in the VTA during the development of psychomotor sensitization on the subsequent expression of cocaine sensitization 21 d later and on NAc S831 P-GluA1 was assessed in mice microinjected with rAAV expressing ERK2 siRNA. ERK2 KD in the VTA was achieved before the start of development. In this experiment (see Fig. 4*C*,*D*), a between-subject factor of viral treatment (control siRNA, ERK2 siRNA) was used to assess the effect of VTA ERK2 KD on development and expression of sensitization (see Fig. 4*C*) and on NAc S831 P-GluA1 (see Fig. 4*D*) ($N = 10$ and 12 for control and $Ca_v1.3$ siRNA, respectively, in Fig. 4*C*; $n = 10$ for control and $Ca_v1.3$ siRNA in Fig. 4*D*).

Experiment 7. The effect of brain-specific neuronal genetic deletion of $Ca_v1.2$ on the development and expression of cocaine psychomotor sensitization and NAc S831 P-GluA1 was examined in CNS-specific $Ca_v1.2$ conditional knock-out (Cav1.2^{CNSKO}) mice. In this experiment (see Fig. 5), a factorial design that included the between-subject factors of day (1, 5, and 26) and genotype (WT, KO) was used to assess the effect of loss of neuronal $Ca_v1.2$ on development and expression of cocaine sensitization (see Fig. 5*A*). A between-subject factor of genotype ($Ca_v1.2^{CNSWT}$, $Ca_v1.2^{CNSKO}$) was used to assess S831 P-GluA1 on day 26 (see Fig. 5*B*)

($N = 8$ and 12 mice for $Ca_v1.2^{CNSWT}$ and $Ca_v1.2^{CNSKO}$, respectively in Fig. 5*A*; $n = 8$ for both genotypes in Fig. 5*B*).

Experiment 8. The effect of KD of $Ca_v1.2$ in the NAc on cocaine-induced expression of sensitization and S831 P-GluA1 was assessed in $Ca_v1.2$ floxed mice microinjected in the NAc with AAV-Cre. Twenty-four hours after the development of cocaine sensitization, AAV-Cre-GFP or control AAV-GFP was microinjected into the NAc. Fourteen days later, mice were tested for expression of cocaine sensitization and NAc S831 P-GluA1 levels. Fourteen days of withdrawal was chosen as maximal KD is achieved at this time point using AAV-Cre. In this experiment (see Fig. 5*E*,*F*), a between-subject factor of viral treatment (control AAV-GFP, AAV-Cre-GFP) was used to assess the effect of NAc $Ca_v1.2$ KD on expression of sensitization (see Fig. 5*E*) and on NAc S831 P-GluA1 (see Fig. 5*F*) ($N = 8$ mice for control and $n = 10$ mice for AAV-Cre-GFP groups).

Experiment 9. The effects of intra-NAc administration of the CaMK inhibitor KN93 and the MEK inhibitor U0126 on cocaine-induced expression of sensitization and S831 P-GluA1 were examined in cocaine-sensitized C57BL/6 mice. In this experiment (see Fig. 6*F*,*G*), the between-subject factor of pretreatment (vehicle, KN93; or vehicle, U0126) was used to assess the effect of blocking CaM kinases or ERK on expression of cocaine sensitization (see Fig. 6*F*) and on S831 P-GluA1 (see Fig. 6*G*). KN93 (3 μg in 0.2 μl) and U0126 (80 ng in 0.2 μl) were microinjected into the NAc 30 min before cocaine challenge. $N = 12$ and 14 mice were used for vehicle and KN93 treatment groups, respectively, and $n = 13$ and 14 for vehicle and U0126 treatment groups, respectively.

Experiment 10. The effect of systemic pretreatment of the MEK inhibitor U0126 administered immediately before cocaine challenge on NAc surface GluA1 was examined in cocaine-sensitized C57BL/6 mice. In this experiment (see Fig. 6*H*), a between-subject factor of pretreatment (vehicle, U0126) was used to assess the effect of blocking ERK on surface GluA1 levels. U0126 (80 ng in 0.2 μl) was microinjected into the NAc 30 min before cocaine challenge. $N = 6$ mice were used for vehicle and U0126 groups.

Experiment 11. The effect of KD of VTA $Ca_v1.3$ and ERK2 during the development of cocaine sensitization, on NAc $Ca_v1.2$ mRNA 21 d later, was assessed in C57BL/6 mice microinjected with rAAV expressing the respective siRNA. $Ca_v1.3$ and ERK2 KD were achieved before the start of development of sensitization. In this experiment (see Fig. 7*C*,*D*), a between-subject factor of viral injection (control siRNA, $Ca_v1.3$ siRNA; or control siRNA, ERK2 siRNA) was used to assess the effect of VTA $Ca_v1.3$ KD (see Fig. 7*C*) and VTA ERK2 KD (see Fig. 7*D*) on NAc $Ca_v1.2$ mRNA levels ($N = 10$ mice for control and $Ca_v1.3$ siRNA in Fig. 7*C*; $n = 10$ and 12 for control and ERK2 siRNA, respectively).

Statistical analyses

For psychomotor sensitization, total distance traveled was analyzed by two-way ANOVA followed by the Bonferroni-Dunn *post hoc* test. For immunoblot analysis, normalized optical density values were used to calculate percentage fold change for each treatment group compared with control group (set to 100%). Surface (S)/intracellular (I) GluA1 ratios were calculated using optical density for each band within the same immunoblot. Data were analyzed by either a one-way or two-way ANOVA followed by Bonferroni-Dunn *post hoc* test. Statview 4.5 software (SAS Institute) was used for all statistics.

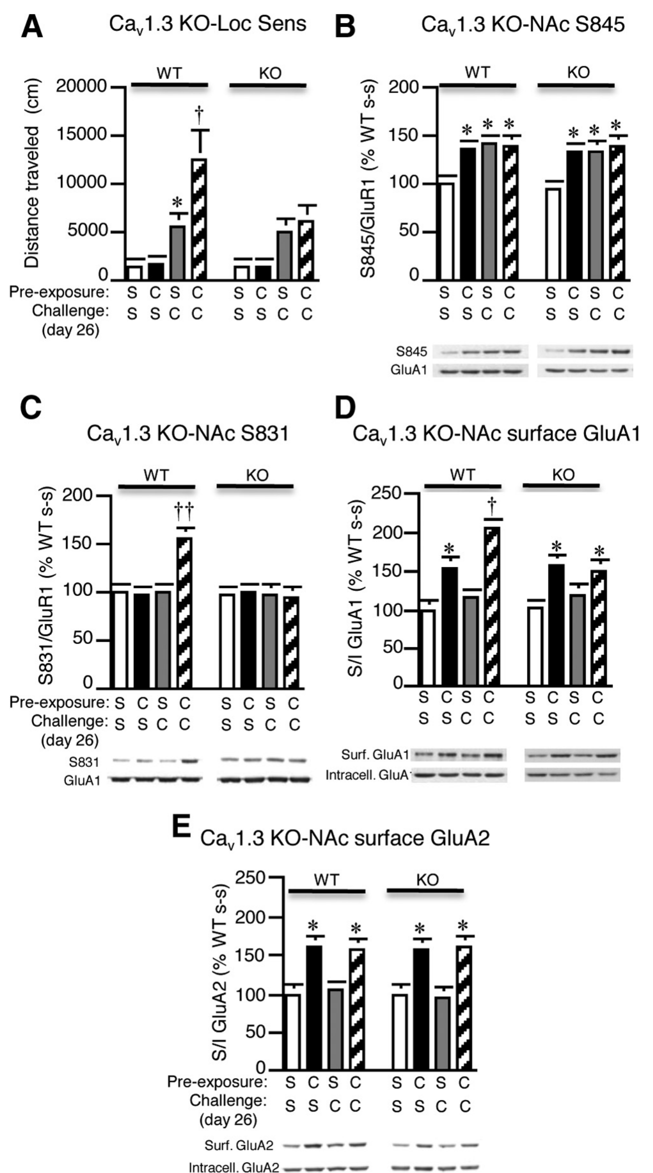


Figure 1. Cocaine-induced increase in Ser831 P-GluA1 and GluA1 cell surface levels in the NAc following 21 d of withdrawal is dependent on $Ca_v1.3$ channels. ***A***, Cocaine-preexposed $Ca_v1.3$ WT but not KO mice exhibit expression of psychomotor sensitization. A cocaine challenge significantly increased locomotor activity in cocaine-preexposed $Ca_v1.3$ WT but not KO mice when challenged 21 d following cocaine withdrawal. ***B***, ***D***, Cocaine-preexposed $Ca_v1.3$ WT and KO mice had significantly higher basal S845 (***B***) and surface GluA1 (***D***) levels. A cocaine challenge had no effect on either measures (***B***, ***D***) in both genotypes. ***C***, ***D***, Cocaine challenge significantly increased S831 (***C***) and surface (S)/intracellular (I) GluA1 (***D***) in $Ca_v1.3$ WT but not KO mice. ***E***, Cocaine preexposure resulted in significantly higher basal S/I GluA2 levels in $Ca_v1.3$ WT and KO mice with no effect of a cocaine challenge. $^*p < 0.05$ versus s-s. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus s-c. s, Saline; c, cocaine. Data represent the mean ± SEM.

Results

Cocaine-induced increase in Ser831 and cell surface GluA1 levels in the NAc underlie expression of psychomotor sensitization and are dependent on $Ca_v1.3$ channels

We have previously reported that $Ca_v1.3$ KO mice do not exhibit expression of cocaine psychomotor sensitization (Giordano et al., 2010). Consistent with this finding, cocaine significantly increased locomotor activity in cocaine-preexposed (15 mg/kg, i.p., cocaine, once a day for 5 d) $Ca_v1.3$ WT but not KO mice when challenged 21 d later (significant interaction between treatment and genotype, $F_{(3,72)} = 7.90$, $p < 0.05$). Following behavioral testing, the NAc of mice was examined for changes in basal and cocaine-induced S845 and S831 GluA1 phosphorylation and their relationship to GluA1 and GluA2 cell surface levels. Examination of basal changes revealed that cocaine-preexposed mice had significantly higher S845 P-GluA1 (Fig. 1*B*; WT, c-s vs s-s) that paralleled higher levels of basal GluA1 cell surface levels (Fig. 1*D*; c-s vs s-s). Both of these adaptations occurred independently of $Ca_v1.3$ channels (Fig. 1*B*,*D*; WT vs KO, main effect of treatment, $F_{(3,72)} = 26.17$, $p < 0.0001$, but not genotype, $F_{(1,72)} = 0.6143$, $p > 0.05$). No changes were seen in basal phosphorylation at S831 in either genotype (Fig. 1*C*; WT and KO, c-s vs s-s). In contrast, following a cocaine challenge that elicited expression of

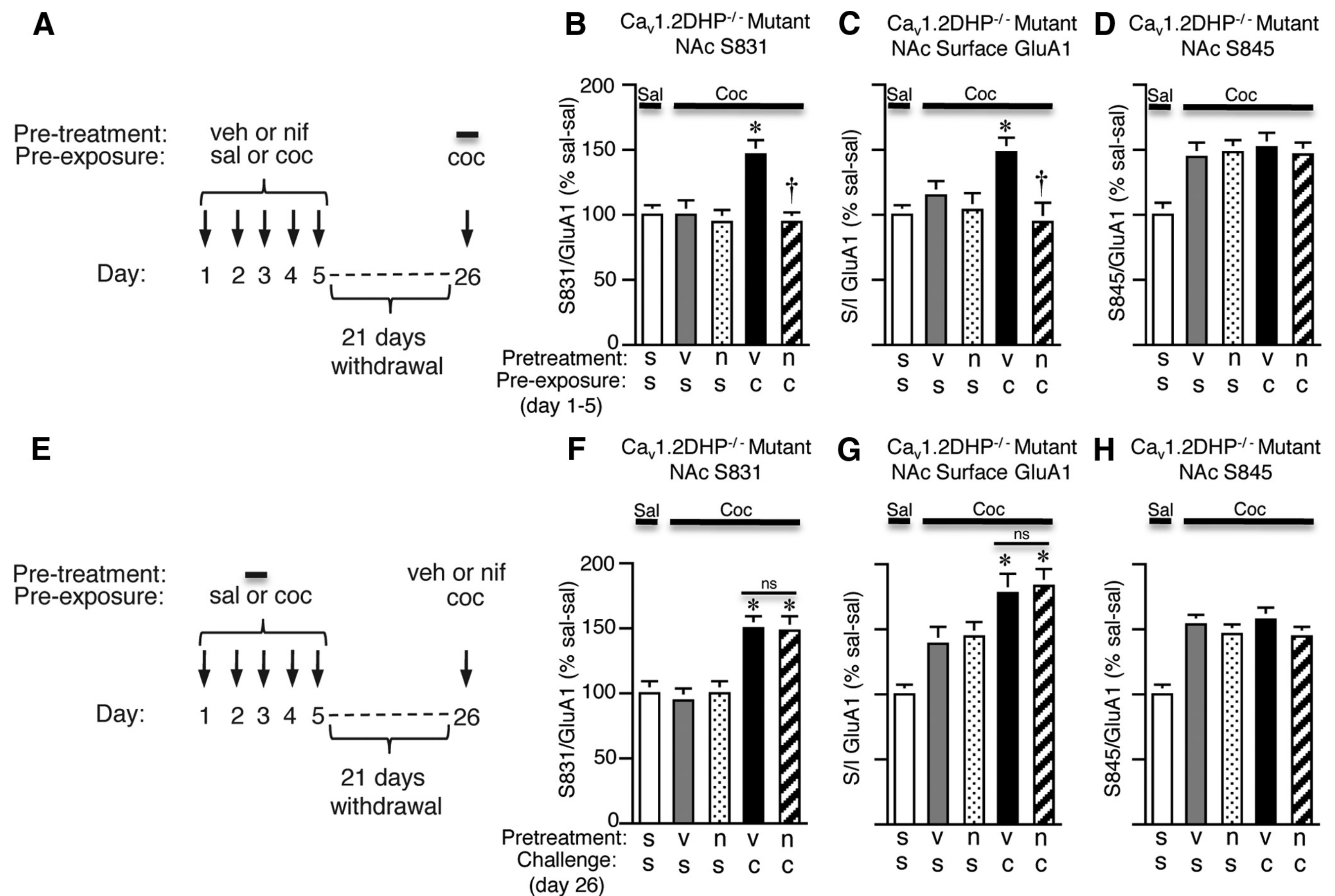


Figure 2. $Ca_v1.3$ channels during development and not at expression are essential for cocaine-induced increase in S831 P-GluA1 and GluA1 cell surface levels 21 d later. ***A***, Experimental timeline to test the role of $Ca_v1.3$ channels during development of cocaine psychomotor sensitization. ***B–D***, $Ca_v1.2DHP^{-/-}$ mutant mice were pretreated with either vehicle (v) or nifedipine (n) (25 mg/kg, i.p.) before each saline (s) or cocaine (c) injection during the development of cocaine sensitization (15 mg/kg, i.p., cocaine once a day for 5 d), generating four treatment groups, v-s, n-s, v-c, and n-c. Twenty-one days later, mice were challenged with cocaine and NAc tissue was used to examine S831 (***B***), S/I (***C***), and S845 (***D***) GluA1 levels. A saline control group was included. Nifedipine significantly lowered S831 P-GluA1 (***B***) and surface GluA1 (***C***) levels compared with vehicle-treated mice. Nifedipine had no effect on S845 P-GluA1 levels (***D***). ***E***, Experimental timeline to test the role of $Ca_v1.3$ channels at expression of cocaine psychomotor sensitization. ***F–H***, Twenty-one days following cocaine preexposure (15 mg/kg, i.p., cocaine once a day for 5 d), $Ca_v1.2DHP^{-/-}$ mutant mice were pretreated with nifedipine 30 min before a cocaine challenge. Following behavioral testing, NAc tissue was examined for S831 (***F***), S/I GluA1 (***G***), and S845 (***H***). $^*p < 0.001$ versus s-s. $^\dagger p < 0.01$ versus v-c. Data represent mean ± SEM.

cocaine psychomotor sensitization, S845 P-GluA1 levels were not altered (Fig. 1*B*; WT, c-c vs c-s), but there was a significant increase in phosphorylation at S831 (Fig. 1*C*; WT, c-c vs c-s) and a further increase in surface levels of GluA1 beyond that seen in cocaine-preexposed mice challenged with saline (Fig. 1*D*; WT, c-c vs c-s). In $Ca_v1.3$ KO mice that did not exhibit expression of cocaine sensitization (Fig. 1*A*; KO, c-c vs s-c), the cocaine-induced increase in S831 was eliminated and cell surface GluA1 levels specific to the sensitized response were reduced to levels observed in cocaine-preexposed mice challenged with saline (Fig. 1*C*,*D*; c-c vs c-s, WT vs KO, significant treatment by genotype interaction, $F_{(3,72)} = 33.78$, $p < 0.0001$). Examination of cell surface GluA2 levels revealed significantly higher basal levels in cocaine-preexposed mice compared with saline-preexposed mice (Fig. 1*E*; WT, c-s vs s-s) with no change following a cocaine challenge (Fig. 1*E*; WT, c-c vs c-s, $F_{(3,72)} = 33.78$, $p < 0.0001$). Cocaine had no effect on total GluA1 or GluA2 protein levels following any treatment (data not shown).

We have previously shown by using $Ca_v1.2$DHP-insensitive mice, a pharmacological $Ca_v1.3$ KO mouse line, that $Ca_v1.3$ channels primarily exert their long-term behavioral and molecular effects during the development of sensitization with no role at expression (Giordano et al., 2010). Using this same mouse line, we found that the cocaine-induced increase in S831 phosphorylation and cell surface GluA1 levels in the NAc were solely dependent on $Ca_v1.3$ channels during the development of sensitization and not at expression (Fig. 2). In this experiment, we first systemically treated $Ca_v1.2$DHP mutant mice with nifedipine before each cocaine injection during the development of sensitization (Fig. 2*A*), which specifically blocks $Ca_v1.3$ channels during this phase of sensitization. We then examined NAc S831 P-GluA1 and S/I GluA1 levels 21 d later. We found that nifedipine pretreatment during development blocked cocaine-induced increase in S831 P-GluA1 (Fig. 2*B*) and surface GluA1 (Fig. 2*C*) levels 21 d later as revealed by a significant interaction between nifedipine pretreatment and cocaine challenge (Fig. 2*B*, S831 P-GluA1, $F_{(1,26)} = 42.88$, $p < 0.0001$; *C*, surface GluA1, $F_{(1,28)} = 23.48$, $p < 0.0001$). To test the role of $Ca_v1.3$ channels at expression, $Ca_v1.2$DHP-insensitive mutant mice that had been sensitized to cocaine (once a day for 5 d) were systemically treated with nifedipine 30 min before the cocaine challenge 21 d later (Fig. 2*E*), blocking $Ca_v1.3$ channels only at this time point. There was no effect of nifedipine on cocaine-induced increase in S831 P-GluA1 (Fig. 2*F*) or surface GluA1 (Fig. 2*G*). We found that nifedipine on

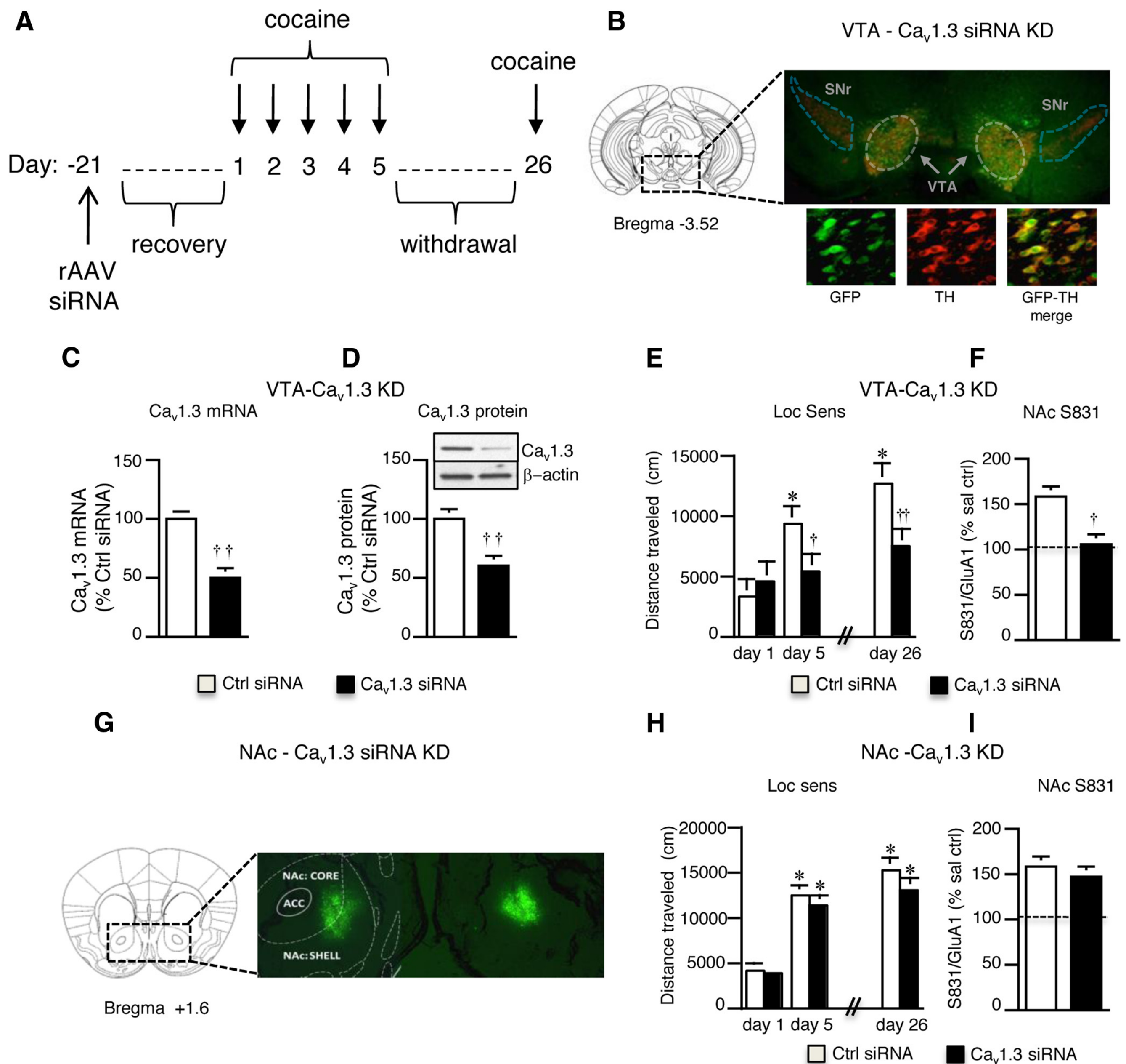


Figure 3. Cocaine-induced expression of psychomotor sensitization and increase in S831 GluA1 phosphorylation in the NAc following 21 d of withdrawal is dependent on VTA and not NAc $Ca_v1.3$ channels. ***A***, Experimental timeline of rAAV siRNA microinjection and cocaine psychomotor sensitization treatment regimen. ***B***, GFP (green) and TH (red) dual-labeled cells in mouse VTA. ***C***, ***D***, Intra-VTA stereotaxic delivery of $Ca_v1.3$ siRNA significantly decreased $Ca_v1.3$ mRNA (***C***) and protein (***D***) levels. ***E***, ***F***, Knockdown of $Ca_v1.3$ in the VTA attenuated the development (day 5: $Ca_v1.3$ siRNA vs Ctrl siRNA) and expression (day 26: $Ca_v1.3$ siRNA vs Ctrl siRNA) of psychomotor sensitization (***E***) and increase in S831 in NAc (***F***). ***G***, Neurons microinjected with rAAV-GFP in the mouse NAc. ***H***, ***I***, Knockdown of $Ca_v1.3$ in the NAc had no effect on development or expression of sensitization (***H***) or increase in S831 in NAc (***I***). $^{\dagger}p < 0.01$, $^{\dagger\dagger}p < 0.001$ versus Ctrl siRNA; $^{*}p < 0.001$ versus day 1. Ctrl siRNA, Control scrambled siRNA. Dashed line, Saline control. Data represent the mean ± SEM.

its own had no effect on S831 P-GluA1 (Fig. 2*B*,*F*) or surface GluA1 levels (Fig. 2*C*,*G*). Examination of S845 P-GluA1 revealed that nifedipine pretreatment during development or at expression had no effect on S845 phosphorylation levels in any of the treatment groups (Fig. 2*D*,*H*).

Cocaine-induced increase in S831 GluA1 phosphorylation in the NAc is dependent on VTA and not NAc $Ca_v1.3$ channels

Next, to identify the neuroanatomical site of the actions of $Ca_v1.3$, we targeted the VTA as this region has been implicated in initiating mechanisms during the development of sensitization that are essential for long-term expression of psychomotor sensitization following withdrawal (Vanderschuren and Kalivas, 2000). Additionally, $Ca_v1.3$ is highly expressed in VTA DA neurons (Rajadhyaksha et al., 2004) and activation of LTCCs in this region is sufficient to induce long-term expression of cocaine sensitization (Licata et al., 2000). As no $Ca_v1.3$-specific pharmacological agents currently exist, we generated neuron-specific rAAV expressing $Ca_v1.3$ siRNA (described in Materials and Methods). rAAV-$Ca_v1.3$ siRNA or rAAV-Ctrl siRNA was stereotaxically delivered bilaterally to the VTA 3 weeks before the start of the cocaine sensitization regimen (Fig. 3*A*,*B*). $Ca_v1.3$ siRNA generated a 50% (±10%) KD of $Ca_v1.3$ mRNA (Fig. 3*C*; $F_{(1,14)} = 12.757$, $p < 0.01$) as demonstrated by qPCR and 55% (±8%) KD

of $Ca_v1.3$ protein (Fig. 3*D*; $F_{(1,14)} = 8.232$, $p < 0.01$) using immunoblot analysis. We found that KD of VTA $Ca_v1.3$ channels attenuated development of cocaine sensitization and expression of the sensitized response when examined 21 d later (Fig. 3*E*; significant day by viral treatment interaction, $F_{(2,72)} = 32.68$, $p < 0.001$). Locomotor activity was significantly lower in $Ca_v1.3$ siRNA-injected mice compared with control siRNA injected at days 5 and 26 (Fig. 3*E*). KD of VTA $Ca_v1.3$ had no effect on acute cocaine-induced locomotor response (Fig. 3*E*, day 1). Examination of S831 P-GluA1 levels revealed significantly lower levels in $Ca_v1.3$ siRNA-injected mice compared with control mice (Fig. 3*F*; $F_{(1,18)} = 8.373$, $p < 0.01$). No change in phosphorylation was seen at S845 (data not shown). In contrast, KD of $Ca_v1.3$ in the NAc, which was also achieved before the start of the sensitization regimen, had no effect on development or expression of sensitization (Fig. 3*G–I*). There was no difference in locomotor activity between NAc $Ca_v1.3$ siRNA- and control siRNA-microinjected mice at day 5 or 26 or following acute cocaine treatment on day 1 (Fig. 3*H*), nor was there a difference in NAc S831 P-GluA1 levels examined at day 26 (Fig. 3*I*).

$Ca_v1.3$-activated ERK2 in the VTA is essential for cocaine-induced increase in Ser831 GluA1 in the NAc

Next, we examined the role of VTA ERK2, as we have shown previously that the LTCC-activated ERK pathway is involved in psychostimulant-mediated changes in the VTA (Rajadhyaksha et al., 2004). By using $Ca_v1.3$ KO mice, we found that repeated cocaine treatment increased ERK2 phosphorylation in the VTA via $Ca_v1.3$ channels. Cocaine-induced P-ERK2 was significantly higher in $Ca_v1.3$ WT mice but not KO mice (Fig. 4*A*; significant treatment by genotype interaction, $F_{(1,20)} = 5.832$, $p < 0.05$). To specifically test the causal role of ERK2 in the VTA on behavior and on S831 P-GluA1 in the NAc, we used a previously characterized rAAV expressing ERK2 siRNA (Xu et al., 2008), as this is one of two existing strategies for targeting ERK2 *in vivo*. For this experiment, rAAV-ERK2 siRNA or rAAV-Ctrl siRNA was stereotaxically delivered into the VTA 3 weeks before the start of the cocaine sensitization regimen as shown in Figure 3*A*. ERK2 siRNA generated a 55% (±13%) KD of ERK2 protein in the VTA (Fig. 4*B*; $F_{(1,14)} = 7.235$, $p < 0.01$). Knockdown of VTA ERK2 attenuated both the development and cocaine-induced expression of sensitization (Fig. 4*C*; significant day by viral treatment interaction, $F_{(2,60)} = 20.50$, $p < 0.0001$). Locomotor activity was significantly lower in ERK2 siRNA injected mice compared with control siRNA injected at days 5 and 26 (Fig. 4*C*). KD of VTA ERK2 had no effect on acute cocaine-induced locomotor response (Fig. 4*C*, day 1). KD of VTA ERK2 also blocked the increase in phosphorylation at S831 in the NAc (Fig. 4*D*; $F_{(1,18)} = 16.348$, $p < 0.0001$), when examined 21 d later. VTA ERK2 KD did not affect levels of phosphorylated S845 (data not shown).

$Ca_v1.2$ channels mediate cocaine-induced expression of psychomotor sensitization and increase in cocaine and dopamine D_1-induced Ser831 GluA1 in the NAc

The above experiments that used rAAV $Ca_v1.3$ siRNA and $Ca_v1.2$DHP mutant mice established that $Ca_v1.3$ channels in the VTA mediate the effects of cocaine during the development of sensitization and that VTA $Ca_v1.3$ channels are necessary for the cocaine-induced increase in S831 P-GluA1 observed 21 d later. Utilizing $Ca_v1.2$DHP-insensitive mice, we also showed that $Ca_v1.3$ channels do not play a role in mediating cocaine-induced increase in S831 P-GluA1 at expression. Thus, to elucidate the mechanism regulating the cocaine-induced increase in S831

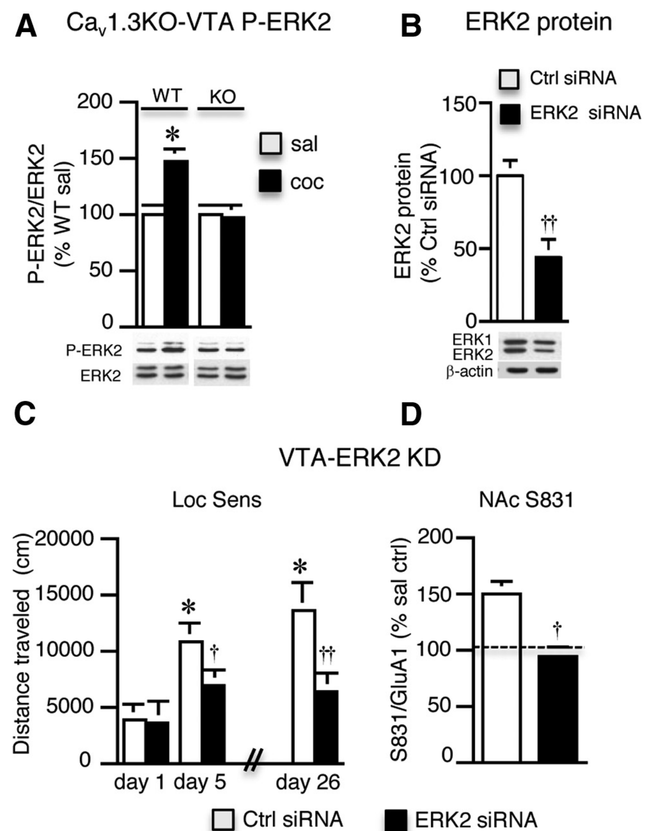


Figure 4. Cocaine-induced increase in S831 GluA1 phosphorylation in the NAc following 21 d of withdrawal is dependent on VTA ERK2. ***A***, $Ca_v1.3$ WT and KO mice were treated with cocaine (15 mg/kg, i.p., once a day for 5 d). Thirty minutes following the last injection, mice were killed and VTA tissue was used for P-ERK1/2 immunoblot analysis. Repeated cocaine significantly increased P-ERK2 in the VTA of $Ca_v1.3$ WT but not KO mice compared with saline-preexposed mice. *$p < 0.01$ versus saline. $n = 6$/genotype and treatment. ***B***, Intra-VTA stereotaxic delivery of ERK2 siRNA significantly decreased ERK2 protein levels. $^{††}p < 0.001$ versus Ctrl siRNA. ***C***, ***D***, KD of VTA ERK2 significantly decreased cocaine-induced development (day 5: ERK2 siRNA vs Ctrl siRNA) and expression (day 26: ERK2 siRNA vs Ctrl siRNA) of sensitization (***C***) and increase in S831 in NAc (***D***). *$p < 0.001$ versus day 1. $^{†}p < 0.05$, $^{††}p < 0.01$ versus Ctrl siRNA. Data represent the mean ± SEM.

GluA1 in the NAc, we explored the role of $Ca_v1.2$ channels as our recent data using $Ca_v1.2$DHP-insensitive mice has suggested that $Ca_v1.2$ channels mediate expression (Giordano et al., 2010). In the present study, we performed experiments in CNS-specific $Ca_v1.2$ conditional KO mice (Moosmang et al., 2005). $Ca_v1.2$ channels were found to mediate cocaine-induced expression of psychomotor sensitization with no role during development (Fig. 5*A*; significant day by genotype interaction, $F_{(1,63)} = 14.12$, $p < 0.0001$). Locomotor activity was significantly lower in $Ca_v1.2^{CNSKO}$ mice compared with $Ca_v1.2^{CNSWT}$ mice on day 26 but not day 5 or day 1 (Fig. 5*A*). Examination of S831 P-GluA1 levels in the NAc following behavioral testing revealed significantly lower levels in $Ca_v1.2$ KO mice compared with WT mice (Fig. 5*B*; $F_{(1,14)} = 11.859$, $p < 0.01$) with no difference in S845 P-GluA1 levels (data not shown). Next, to directly test the role of $Ca_v1.2$ channels in the NAc, we used AAV-Cre to knockdown $Ca_v1.2$ in the NAc of $Ca_v1.2$ floxed mice (Fig. 3*C–F*). For this experiment, mice were sensitized to cocaine (once a day for 5 d; Fig. 5*C*). Twenty-four hours later (day 6), mice were bilaterally microinjected in the NAc with AAV-Cre-GFP or AAV-GFP (Fig. 5*C*). When examined 14 d later, a time point that results in maximal knockdown, 55% (±10%) KD of $Ca_v1.2$ mRNA was

achieved in AAV-Cre-GFP-microinjected mice compared with AAV-GFP-microinjected mice (Fig. 5*D*). Following cocaine challenge, locomotor activity was significantly attenuated in NAc AAV-Cre-microinjected mice compared with AAV-GFP-microinjected mice that displayed expression of cocaine sensitization (Fig. 5*E*; significant day by viral treatment interaction, $F_{(2,48)} = 26.57, p < 0.001$). Cocaine-induced increase in NAc S831 P-GluA1 was also significantly lower in AAV-Cre-microinjected mice (Fig. 5*F*; $F_{(1,16)} = 4.612, p < 0.05$), demonstrating that NAc $Ca_v1.2$ channels mediate cocaine-induced increase in S831 P-GluA1 that underlies expression of cocaine sensitization. Additionally, we found that treating cocaine-sensitized $Ca_v1.2$ WT but not KO mice with the dopamine D_1 agonist SKF82958 significantly increased NAc phosphorylation of S831 (Fig. 5*G*; skf vs vehicle in WT but not KO mice, significant treatment by genotype interaction, $F_{(1,28)} = 26.51, p < 0.0001$).

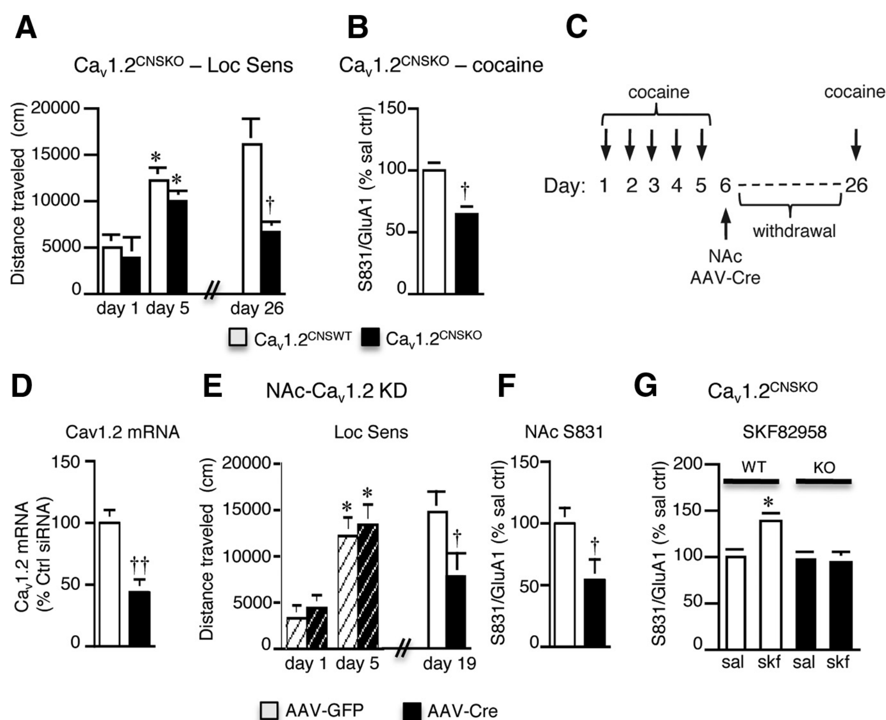


Figure 5. Cocaine-induced expression of psychomotor sensitization and increase in cocaine- and D_1-induced Ser831 P-GluA1 in the NAc is mediated by $Ca_v1.2$ channels. ***A***, CNS-specific $Ca_v1.2$ wild-type ($Ca_v1.2^{CNSWT}$) and knock-out ($Ca_v1.2^{CNSKO}$) littermate control mice were treated with cocaine on days 1–5 and then challenged 21 d later with cocaine. $Ca_v1.2^{CNSWT}$ and $Ca_v1.2^{CNSKO}$ mice exhibited development but not expression of cocaine psychomotor sensitization. On day 5, there was no difference in locomotor activity between $Ca_v1.2^{CNSWT}$ and $Ca_v1.2^{CNSKO}$ mice. On day 26, $Ca_v1.2^{CNSKO}$ mice exhibited significantly lower locomotor activity than $Ca_v1.2^{CNSWT}$ mice. $^*p < 0.001$ versus day 1; $^{\dagger}p < 0.0001$ versus $Ca_v1.2^{CNSWT}$. ***B***, Cocaine significantly increased NAc S831 P-GluA1 in $Ca_v1.2^{CNSWT}$ but not $Ca_v1.2^{CNSKO}$ mice. ***C***, Experimental timeline of rAAV siRNA microinjection into the NAc to test the role of $Ca_v1.2$ channels on expression of cocaine psychomotor sensitization. $Ca_v1.2$ floxed mice were treated with cocaine on days 1–5 and then microinjected with AAV-GFP or AAV-Cre-GFP on day 6. Mice were then challenged 14 d later with cocaine. ***D***, Stereotaxic delivery of AAV-Cre into the NAc of $Ca_v1.2$ floxed mice significantly decreased $Ca_v1.2$ mRNA. $^{\dagger\dagger}p < 0.001$ versus AAV-GFP. ***E***, On day 26, mice treated with AAV-Cre-GFP exhibited significantly lower locomotor activity than AAV-GFP injected mice. $^{\dagger}p < 0.001$ versus AAV-GFP. ***F***, Cocaine significantly increased NAc S831 P-GluA1 in AAV-GFP but not AAV-Cre-GFP injected mice. $^{\dagger}p < 0.001$ versus AAV-GFP. ***G***, D_1 agonist SKF82958 (1 mg/kg, i.p.) significantly increased NAc S831 P-GluA1 in $Ca_v1.2^{CNSWT}$ but not $Ca_v1.2^{CNSKO}$ mice. skf, SKF82958. $^*p < 0.001$. Data represent the mean ± SEM.

Cocaine-induced increase in Ser831 GluA1 and cell surface levels in the NAc is mediated by $Ca_v1.2$-activated CaM kinase II and ERK2

As LTCC-mediated activation of CaMKII and ERK in the NAc contributes to psychostimulant-induced behaviors (Pierce et al., 1998; Anderson et al., 2008; Giordano et al., 2010), we next tested the role of CaMKII and ERK2 in mediating the cocaine-induced increase in Ser831 that underlies expression of cocaine psychomotor sensitization. Western blots revealed that $Ca_v1.2$ channels mediated cocaine-induced increase in Thr286 P-CaMKII and Thr202/Tyr204 P-ERK2 in the NAc when examined 21 d following withdrawal. Cocaine significantly increased P-CaMKII (Fig. 6*A*; significant treatment by genotype interaction, $F_{(1,18)} = 4.839, p < 0.05$) and P-ERK2 (Fig. 6*B*; significant treatment by genotype interaction, $F_{(1,18)} = 5.944, p < 0.05$) in $Ca_v1.2$ WT but not KO mice. To directly test the role of NAc CaMKs and the ERK pathway on expression of cocaine sensitization and S831 P-GluA1, we next pharmacologically inhibited these two kinases in the NAc 30 min before the cocaine challenge on day 26 (Fig. 6*C*). Intra-NAc administration of KN93 was used to inhibit CaMKs and ERK was inhibited by U0126 (Fig. 6*D*,*E*). However, it should be noted that KN93 has additionally been found to inhibit LTCCs *in vitro* (Gao et al., 2006). Administration of KN93 attenuated both expression of sensitization (Fig. 6*F*; $F_{(1,24)} = 4.567, p < 0.05$) and levels of phosphorylated S831 in the NAc (Fig. 6*G*; $F_{(1,24)} = 4.851, p < 0.05$). Contrary to this, U0126 attenuated expression (Fig. 6*F*; $F_{(1,25)} = 5.166, p < 0.05$) but not S831 P-GluA1 (Fig. 6*G*) or S845 P-GluA1 (data not shown). However, U0126 blocked the cocaine-induced increase in NAc cell surface GluA1 levels (Fig. 6*H*; $F_{(1,12)} = 4.159, p < 0.05$), suggesting that cocaine-induced GluA1 trafficking to the cell surface requires both a CaMKII-dependent S831 phosphorylation event and an ERK2-dependent event. Together, the above experiments demonstrated that D1/$Ca_v1.2$-activated P-CaMKII and P-ERK2 mediate cocaine-induced increase in NAc S831 GluA1 phosphorylation following protracted withdrawal.

Repeated cocaine-induced increase in $Ca_v1.2$ mRNA in the NAc following 21 d of withdrawal is a correlate of expression of sensitization and is dependent on VTA $Ca_v1.3$ and ERK2

As activation of new gene expression underlies cocaine-induced long-term plasticity and NAc $Ca_v1.2$ mRNA is upregulated by repeated cocaine treatment (Renthal et al., 2009) and repeated treatment with the psychostimulant amphetamine (Rajadhyaksha et al., 2004), we next examined NAc $Ca_v1.2$ mRNA levels 21 d following cocaine preexposure and the role of VTA $Ca_v1.3$ and ERK2 therein. We first examined $Ca_v1.2$ mRNA levels in $Ca_v1.3$ WT and KO mice (Fig. 7*A*). We found higher levels in the NAc of WT but not KO mice (Fig. 7*A*; significant interaction, cocaine preexposure by genotype, $F_{(1,22)} = 20.43, p < 0.0001$), a change not observed in the dorsal striatum, an adjacent brain region (Fig. 7*A*). $Ca_v1.2$ mRNA levels positively correlated with expression of psychomotor sensitization (Fig. 7*B*; $r = 0.78, p < 0.01$, Pearson's correlation). We next tested the role of VTA $Ca_v1.3$ channels and ERK2 during the development of sensitization on the increase in NAc $Ca_v1.2$ mRNA observed 21 d later. Knockdown of VTA $Ca_v1.3$ (Fig. 7*C*) and ERK2 (Fig. 7*D*) was achieved before the start

of the sensitization protocol and 21 d following development $Ca_v1.2$ mRNA levels were measured in the NAc. Both VTA $Ca_v1.3$ KD and VTA ERK2 KD significantly attenuated the increase in NAc $Ca_v1.2$ mRNA (Fig. 7*C*, $F_{(1,18)} = 10.126$, $p < 0.01$; *D*, $F_{(1,20)} = 12.321$, $p < 0.01$).

Discussion

In this study, we found that following extended withdrawal from repeated cocaine there is an increase in phosphorylation of GluA1 at S845 in the NAc with a parallel increase in cell surface GluA1 that occurs independently of $Ca_v1.2$ or $Ca_v1.3$ channels. We found that a challenge injection of cocaine that elicits expression of the sensitized response further increases surface GluA1 via both a D_1/$Ca_v1.2$-mediated increase in GluA1 phosphorylation at S831 by CaMKII and by an ERK2-dependent mechanism. We further demonstrate that this long-term change in the NAc is dependent on the $Ca_v1.3$/ERK2 pathway in the VTA during the development of cocaine sensitization (Fig. 8).

$Ca_v1.2$, dopamine D_1, and cocaine-induced AMPA receptor plasticity in the NAc

We found that protracted cocaine withdrawal increases basal GluA1 and GluA2 cell surface levels consistent with the findings of others (Boudreau and Wolf, 2005; Boudreau et al., 2007; Ghasemzadeh et al., 2009), suggesting an increase in cell surface GluA1/A2 heteromers. However, these results conflict with findings of increased GluA1 homomers (Mameli et al., 2009), possibly due to the use of younger animals and/or greater withdrawal times.

We have uncovered a role for NAc $Ca_v1.2$ signaling at expression in accordance with increased $Ca_v1.2$ gene expression. This is consistent with upregulation of NAc $Ca_v1.2$ mRNA following repeated cocaine treatment in a microarray study (Renthal et al., 2009) and our own finding that repeated amphetamine increases $Ca_v1.2$ (Rajadhyaksha et al., 2004).

Our findings of a cocaine challenge-induced increase in cell surface GluA1 and not GluA2 suggests that cocaine-induced expression of sensitization may involve an increase in Ca^{2+}-permeable GluA2-lacking AMPAR channels. This is consistent with cocaine-induced behavioral responses involving enhanced NAc AMPAR neurotransmission (Bell and Kalivas, 1996; Pierce et al., 1996; Reid and Berger, 1996; Bachtell et al., 2008). However, our finding is inconsistent with that of Ferrario et al. (2010), who found no change in GluA1 cell

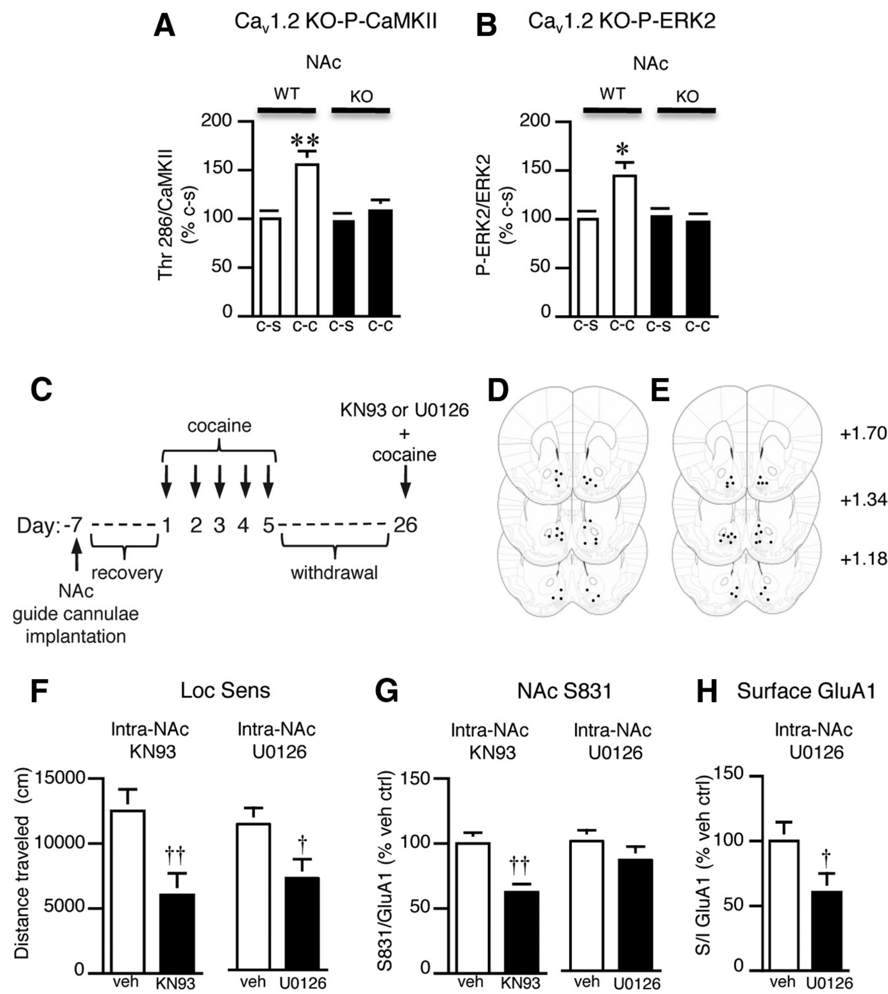


Figure 6. Cocaine-induced increase in Ser831 GluA1 in the NAc is mediated by $Ca_v1.2$-activated CaM kinase II and ERK2. ***A***, ***B***, Cocaine-preexposed CNS-specific $Ca_v1.2^{CNSWT}$ and $Ca_v1.2^{CNSKO}$ littermate control mice were challenged 21 d later with saline (s) or cocaine (c). Thirty minutes following behavioral testing, mice were killed and NAc tissue was used for Thr286 P-CaMKII and P-ERK1/2 immunoblot analysis. A cocaine challenge significantly increased Thr286 P-CaMKII (***A***) and P-ERK2 (***B***) in $Ca_v1.2^{CNSWT}$ but not $Ca_v1.2^{CNSKO}$ mice. $n = 5$–6/treatment. ***C***, Experimental timeline of NAc guide cannulae implantation, psychomotor sensitization, and pharmacological blocker treatment regimen. ***D***, ***E***, Probe placement in the NAc. Coronal sections indicating injection sites for intra-NAc KN93 (***D***) and U0126 (***E***) microinjection experiments are shown. ***F***, ***G***, Intra-NAc KN93 and U0126 significantly attenuated cocaine-induced expression of sensitization (***F***), and intra-NAc KN93 but not U0126 attenuated the increase in S831 (***G***). ***H***, Intra-NAc U0126 significantly attenuated S/I GluA1 levels. $^{*}p < 0.01$ versus c-s, $^{**}p < 0.001$ versus c-s; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus veh. Data represent the mean ± SEM.

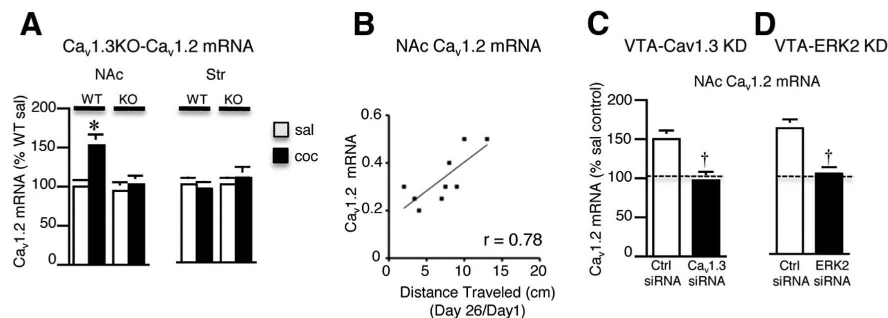


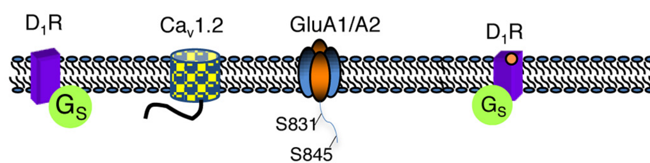
Figure 7. Repeated cocaine-induced increase in $Ca_v1.2$ mRNA in the NAc following 21 d of withdrawal is a correlate of expression of sensitization and is dependent on VTA $Ca_v1.3$ and ERK2. ***A***, Repeated cocaine significantly increased $Ca_v1.2$ mRNA in the NAc of $Ca_v1.3$ WT but not KO mice 21 d following withdrawal from cocaine. No change was seen in the dorsal striatum. $n = 8$–9/treatment. ***B***, NAc $Ca_v1.2$ mRNA levels correlate with expression of sensitization. $n = 9$. ***C***, ***D***, Knockdown of VTA $Ca_v1.3$ (***C***) and ERK2 (***D***) significantly decreased levels of NAc $Ca_v1.2$ mRNA. $^{*}p < 0.001$ versus saline; $^{\dagger}p < 0.01$ versus Ctrl siRNA. Data represent the mean ± SEM.

surface levels 30 min after cocaine challenge. This could be due to their use of a 14 d withdrawal period and/or rats. Consistent with our findings, a systemic cocaine challenge that reinstates cocaine seeking results in increased surface GluA1 (Anderson et al., 2008). Although these represent two different addiction models, cocaine-induced reinstatement was shown to involve an LTCC-activated CaMKII-mediated increase in surface GluA1 and S831 levels.

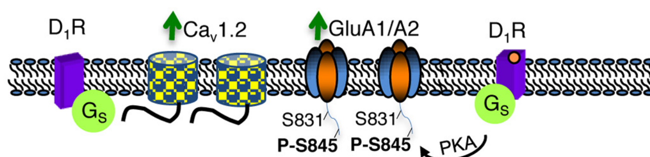
At the signaling level, we found that $Ca_v1.2$-activated CaMKII and ERK2 in the NAc mediate expression of sensitization and are involved in the cocaine-induced increase in surface GluA1. A role for CaMKII is consistent with its involvement in expression of sensitization (Pierce et al., 1998), CaMKII being a direct downstream target of the LTCCs (Wheeler et al., 2008), and CaMKII-mediated S831 phosphorylation of GluA1 increasing surface GluA1 (Hayashi et al., 2000). Additionally, CaMKII phosphorylation of S831 has been implicated in recruitment of GluA1 to synaptic sites (Derkach et al., 1999; Poncer et al., 2002) via NMDA receptors (Shi et al., 1999; Zhu and Malinow, 2002), following an initial exocytosis of GluA1 onto extrasynaptic sites via phosphorylation at S845 (Oh et al., 2006; Wolf, 2010; Wolf and Ferrario, 2010). This model is consistent with our findings and those of others (Boudreau and Wolf, 2005; Ferrario et al., 2010, 2011; Edwards et al., 2011) that protracted withdrawal from cocaine increases basal S845 P-GluA1 and GluA1 cell surface levels in the NAc. Moreover, we show that this occurs independently of LTCCs and that, following a cocaine challenge, $Ca_v1.2$-activated CaMKII further increases GluA1 cell surface levels. As LTCCs mediate NMDA receptor signaling (Liu and Graybiel, 1996; Rajadhyaksha et al., 1999), it is plausible that $Ca_v1.2$ activation may mediate both cocaine- and NMDA-induced trafficking of GluA1 from extrasynaptic to synaptic sites. This hypothesis is supported by work demonstrating that activity-dependent synaptic clustering of GluA1 involves NMDA receptors, LTCCs, and CaMKII activation (Rose et al., 2009). We also found that $Ca_v1.2$-activated ERK2 increases cocaine-induced GluA1 trafficking. This finding is consistent with reports that ERK, in addition to CaMKII, modulates postsynaptic AMPA receptor trafficking (Zhu and Malinow, 2002; Kim et al., 2005; Patterson et al., 2010).

We also found that $Ca_v1.2$ mediates D_1-induced increase in S831 P-GluA1, suggesting that cocaine-induced recruitment of $Ca_v1.2$ occurs via D_1 activation. Accordingly, D_1-activated PKA has been shown to regulate $Ca_v1.2$ LTCCs (Surmeier et al., 1995). Additionally, D_1-containing medium spiny neurons in the NAc are the primary cell type in which cocaine-induced increase in dendritic spines, a correlate of sensitized behavior (Robinson and Kolb, 2004), is maintained (Lee et al., 2006).

A Cocaine naïve NAc neuron



B Molecular adaptations in the NAc following 21 days of withdrawal from repeated cocaine



C Cocaine-induced molecular response that underlies the expression of cocaine sensitization (21-days withdrawal)

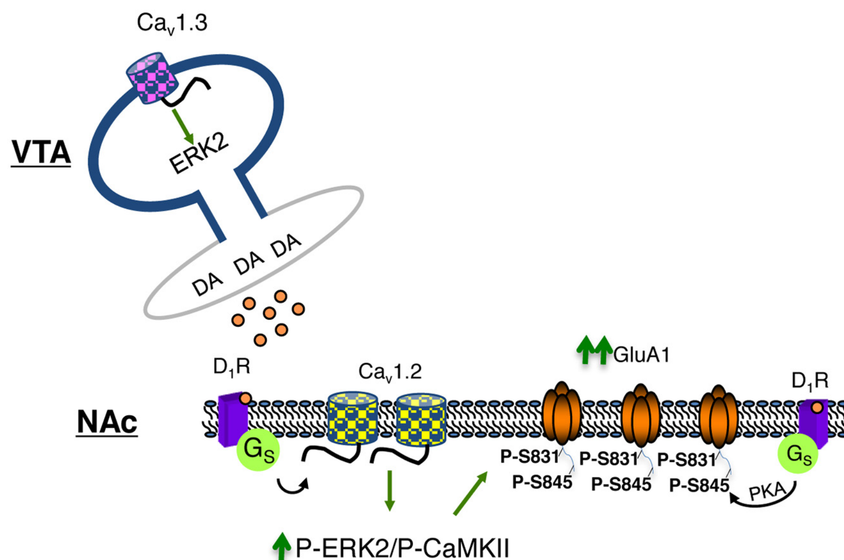


Figure 8. Schematic representation of the role of $Ca_v1.2$ and $Ca_v1.3$ LTCCs in the persistent NAc molecular adaptations following extended withdrawal from repeated cocaine exposure. ***A***, The cocaine naive dopamine D_1-containing NAc neuron expresses AMPARs and $Ca_v1.2$ channels (Rajadhyaksha et al., 2004). AMPARs exist primarily as GluA1/GluA2 tetramers (Reimers et al., 2011) on the cell surface, consisting of two molecules of each subunit (Greger and Esteban, 2007). ***B***, Twenty-one days following withdrawal from repeated cocaine treatment, we found an increase in phosphorylation of GluA1 at S845 in the NAc, a PKA site, with a parallel increase in cell surface GluA1 levels and also GluA2 levels. We additionally found higher levels of $Ca_v1.2$ mRNA. ***C***, A cocaine challenge that elicits expression of cocaine psychomotor sensitization involves dopamine D_1 receptors and $Ca_v1.2$-activated CaMKII that increases GluA1 phosphorylation at S831 and $Ca_v1.2$-activated ERK2, which further increases cell surface GluA1 over that seen in ***B***. This long-term adaptation is dependent on $Ca_v1.3$ channels and ERK2 in the VTA during the development of sensitization.

VTA $Ca_v1.3$ channels and long-term AMPA receptor plasticity in the NAc

By using rAAV expressing $Ca_v1.3$-specific siRNA to knockdown $Ca_v1.3$ in a brain region-specific manner, we demonstrate that VTA and not NAc $Ca_v1.3$ channels are essential for mediating development of cocaine psychomotor sensitization and for the recruitment of the $Ca_v1.2$ pathway in the NAc following extended withdrawal from cocaine. This finding extends our previous work in which we used $Ca_v1.3$ genetic mutant mice to show that $Ca_v1.3$ channels play an important role during development

(Giordano et al., 2010). Additionally, here we show that $Ca_v1.3$-activated mechanisms in the VTA during development are critical for the long-term expression of cocaine sensitization. This is consistent with the finding that activation of LTCCs in the VTA, with the activator BayK8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid, methyl ester), is sufficient for mediating expression of cocaine sensitization following withdrawal (Licata and Pierce, 2003; Licata et al., 2004).

At the signaling level, we found that the $Ca_v1.3$-activated ERK pathway in the VTA during the development of sensitization, as observed by an increase in phosphorylation of ERK2, plays a critical role in mediating the long-term molecular and behavioral changes following repeated cocaine. This is consistent with ERK being a downstream target of the LTCCs as we have shown in the VTA (Rajadhyaksha et al., 2004) and as has been found in other brain regions (Dolmetsch et al., 2001; Wu et al., 2001; Giordano et al., 2010). By using rAAV expressing ERK2 siRNA to KD ERK2 in the VTA, we directly demonstrate that VTA ERK2 is essential for the development of cocaine sensitization with no effect on the acute locomotor response. This is in line with the finding that inhibiting the ERK pathway in the VTA with the antagonist PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one] blocked development of cocaine sensitization with no effect on the acute locomotor response (Pierce et al., 1999). The involvement of the $Ca_v1.3$/ERK2 pathway in the VTA during development is consistent with an important role of LTCCs in mediating cocaine-activated Ca^{2+} pathways in this region (Licata and Pierce, 2003; Rajadhyaksha and Kosofsky, 2005) and of LTCC- and ERK-activated gene expression in long-term neuronal plasticity (Deisseroth et al., 2003; Thomas and Huganir, 2004; Rajadhyaksha and Kosofsky, 2005; Girault et al., 2007), a critical mechanism in the VTA for the transition from development to expression of sensitization (Sorg and Ulibarri, 1995). However, the specific VTA cell type (Lüscher and Malenka, 2011) in which $Ca_v1.3$ signaling pathways are activated by cocaine, and which contribute to the development of cocaine psychomotor sensitization, remains to be further explored.

While the precise mechanisms underlying the transition from VTA-relevant signaling that occurs during the development of sensitization to the subsequent recruitment of the NAc at expression of sensitization remains unknown, one potential mechanism could be via an increase in $Ca_v1.2$ channels in VTA dopamine neurons. As opposed to the transient molecular and physiological changes reported in the VTA following repeated cocaine (Zhang et al., 1997; Kauer and Malenka, 2007; Chen et al., 2008; Lüscher and Malenka, 2011), we have previously reported that repeated treatment with the psychostimulant amphetamine increases $Ca_v1.2$ mRNA and protein in VTA dopamine neurons (Rajadhyaksha et al., 2004) that persists up to 14 d following drug treatment, the time point tested in this study. We have found that this increase in $Ca_v1.2$ is mediated by VTA $Ca_v1.3$ channels and by ERK2 (our unpublished data). The functional significance of $Ca_v1.2$ in VTA DA neurons remains to be identified. However, as pharmacological studies using intra-NAc administration of the LTCC blocker diltiazem have suggested a role for LTCCs in augmented dopamine release (Pierce and Kalivas, 1997a), it is plausible that $Ca_v1.2$ channels could play a role in enhanced cocaine-induced dopamine release at presynaptic dopamine terminals. Although $Ca_v1.2$ channels have been reported to function primarily postsynaptically, a role for LTCCs in neurotransmitter release has been reported (Watanabe et al., 1998; Evans and Pocock, 1999; Okita et al., 2000). Additionally, a recent study has identified the presence of $Ca_v1.2$ channels on axons terminals in the hippocampus (Tippens et al., 2008). $Ca_v1.2$ channels serving as a potential molecular link in the transition from VTA to the NAc is also in line with the requirement of new protein synthesis in the VTA ($Ca_v1.3$-activated $Ca_v1.2$ gene expression in this case) for cocaine sensitization (Sorg and Ulibarri, 1995). However, additional experiments are required to further confirm this hypothesis.

In conclusion, the present study elucidates how $Ca_v1.2$ and $Ca_v1.3$ LTCCs in temporally and anatomically specific manners mediate cocaine sensitization-specific adaptations in GluA1 trafficking in the NAc, findings that can be explored in other preclinical rodent models of addiction.

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