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Enhancing VTA Ca_v1.3 L-type Ca²⁺ channel activity promotes cocaine and mood - related behaviors via overlapping AMPA receptor mechanisms in the nucleus accumbens

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Abstract

Genetic factors significantly influence susceptibility for substance abuse and mood disorders. Rodent studies have begun to elucidate a role of Ca_v1.3 L-type Ca²⁺ channels in neuropsychiatric-related behaviors, such as addictive and depressive-like behaviors. Human studies have also linked the *CACNA1D* gene, which codes for the Ca_v1.3 protein, with bipolar disorder (BD). However, the neurocircuitry and the molecular mechanisms underlying the role of Ca_v1.3 in neuropsychiatric phenotypes are not well established. In the present study, we directly manipulated Ca_v1.3 channels in Ca_v1.2 dihydropyridine (DHP) insensitive mutant mice and found that VTA

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Conflict of Interest

None

Author Contributions

AM-R, JH, TFT, TPG, NAA and AMR contributed to the overall experimental design, and data interpretation. AM-R, NAA and AMR wrote the manuscript. AL, RLH, and JS provided reagents and edited the manuscript. MK and RCR performed research, analyzed data and edited the paper. SH contributed to the design and performed the GWAS and wrote the manuscript.

Ca_v1.3 channels mediate cocaine-related and depressive-like behavior through a common nucleus accumbens (NAc) shell calcium permeable AMPA receptor (CP-AMPA) mechanism that requires GluA1 phosphorylation at S831. Selective activation of VTA Ca_v1.3 with (±)-BayK-8644 (BayK) enhanced cocaine conditioned place preference (CPP) and cocaine psychomotor activity while inducing depressive-like behavior, an effect not observed in S831A phospho-mutant mice. Infusion of the CP-AMPA-specific blocker Naspm into the NAc shell reversed the cocaine and depressive-like phenotypes. In addition, activation of VTA Ca_v1.3 channels resulted in social behavioral deficits. In contrast to the cocaine- and depression-related phenotypes, GluA1/A2 AMPARs in the NAc core mediated social deficits, independent of S831-GluA1 phosphorylation. Using a candidate gene analysis approach, we also identified single nucleotide polymorphisms in the *CACNA1D* gene associated with cocaine dependence (CD) in human subjects. Together, our findings reveal novel, overlapping mechanisms through which VTA Ca_v1.3 mediates cocaine-related, depressive-like and social phenotypes suggesting that Ca_v1.3 may serve as a target for the treatment of neuropsychiatric symptoms.

INTRODUCTION

Altered reward brain circuitry and reward processing is associated with multiple psychiatric disorders¹. In particular, substance abuse disorders are often co-morbid with mood disorders particularly bipolar disorder (BD)² and major depressive disorder³. Common genetic risk factors^{4–5}, overlapping neurocircuitry^{6, 7} and convergence of cellular and molecular mechanisms^{7, 8} have been suggested to underlie this co-morbidity. In particular, recent findings provide evidence that human variants in the *CACNA1D* gene, which codes for the Ca_v1.3 subunit of L-type Ca²⁺ channels (LTCCs), can confer risk for the development of neuropsychiatric disorders^{9, 10} including BD^{11, 12}. Emerging data on impact of human mutations on Ca_v1.3 physiology¹³ and from animal studies^{14–16} suggests that enhanced Ca_v1.3 activity may contribute to neuropsychiatric disorders. This is in agreement with a crucial role of this channel in neuronal signaling^{10, 17, 18} underlying drug taking and emotional behaviors. However, as for other risk genes, the precise Ca_v1.3-dependent mechanisms and neurocircuits that contribute to the neurobiology of neuropsychiatric-related behavioral phenotypes remain unknown.

The VTA-NAc pathway plays a central role in mediating the effects of cocaine¹⁹, depressive-like^{20, 21} and social behavior²². LTCCs in the VTA regulate DA burst firing activity^{23, 24}, a property known to mediate reward behaviors²⁵, depressive-like behaviors²⁶, social interaction²² and responses to stress^{20, 21}. We and others have shown that Ca_v1.3 that is expressed in VTA dopamine neurons^{27, 28} is required for the short- and the long-term effects of cocaine^{14–16}. Acute activation of Ca_v1.3 by systemic injection of BayK, has also been shown to induce depressive-like behavior²⁸. Collectively, these findings suggest a role of Ca_v1.3 channels in addictive behavior and mood disorders.

Emerging evidence is establishing neuropsychiatric disorders as synaptic diseases. Several studies have reported enhanced glutamatergic AMPAR transmission, in particular increase in synaptic GluA2-lacking Ca²⁺ permeable AMPARs (CP-AMPA herein) in medium spiny neurons of the NAc, as a key mechanism underlying cocaine-induced long-lasting behavioral

plasticity^{29–35}. Enhanced AMPAR transmission in the NAc has also been reported to drive depressive-like and social behavior³⁶, suggesting that modulation of AMPAR transmission could represent one common mechanism linking addiction and mood disorders.

Given the evidence of enhanced VTA-NAc activity in drug taking and mood-related behaviors^{19, 21, 22}, Ca_v1.3 channel properties in VTA dopamine cells²⁴ and Ca_v1.3 gain of function mutations in neurological disease¹⁰, we hypothesized that enhanced activity of VTA Ca_v1.3 channels would drive cocaine-related behaviors and mood-related phenotypes such as depressive-like, anxiety-like and social behavior. To this end, we utilized Ca_v1.2 DHP insensitive (Ca_v1.2 DHP^{-/-}) mutant mice. A single point mutation in the Ca_v1.2 α 1-subunit of these mice does not affect Ca_v1.2 function and expression, but renders them insensitive to (\pm)-BayK-8644 and strongly reduces their sensitivity to DHP Ca²⁺ channel blockers, such as nifedipine^{28, 37}. As successfully demonstrated in previous studies^{15, 38} this allows us to specifically pharmacologically manipulate Ca_v1.3 channels in the VTA in these mice. Subsequently, we pharmacologically assessed AMPARs in the NAc and utilized AMPAR subunit GluA1 phosphorylation deficient mutant mice to assess the necessity of GluA1 phosphorylation in cocaine and mood-related behaviors. Finally, we tested genetic variants within and around *CACNA1D* for association with cocaine dependence (CD; as defined by DSM-IV criteria) using a genome-wide association dataset.

MATERIALS AND METHODS

Detailed methods are provided in Supplementary Information.

Animals

All experimental procedures were conducted in accordance with Weill Cornell Medicine IACUC guidelines. Male C57BL/6 (Jackson Laboratories, Bar Harbor, Maine), Ca_v1.3 knockout (KO)³⁹, Ca_v1.2 DHP-insensitive²⁸ and GluA1 S831A phospho-mutant mice⁴⁰ all on C57BL/6J background, were 8–10 weeks old at the start of the experiments. Animals were maintained on a 12-hr light/dark cycle (from 7A.M. to 7P.M.).

Drugs and antibodies

Cocaine HCl, nifedipine (Nif), (\pm)-BayK-8644 (BayK), and Nasp_m were obtained from Sigma (St. Louis, MO). NBQX was obtained from Tocris Bioscience (Minneapolis, MN). Nif and BayK were dissolved in 0.9% saline containing 1.5% DMSO and 1.5% Tween80. Nasp_m and NBQX were dissolved in 0.9% saline. Antibodies listed in Supplementary Table 1.

Subcellular fractionation

Mice were euthanized by rapid decapitation and bilateral VTA (18-gauge stainless-steel stylet), NAc (18-gauge stainless-steel stylet) or PFC (17-gauge stainless-steel stylet) tissue was obtained. Total protein lysates were isolated as previously described⁴¹. For postsynaptic density (PSD) fractions, subcellular fractionation was performed as published before⁴².

Coimmunoprecipitation assay

Crude membrane fractions were generated from bilateral VTA tissue pooled from four mice. Co-immunoprecipitation was performed as published in Calin-Jageman et al. (2007)⁴³.

Immunoblot Analysis

Immunoblotting and quantitation was performed as previously described in Schierberl et al. (2011)¹⁵. Tubulin was used as a loading control for all PSD immunoblots.

Guide cannula surgery

For delivery of pharmacological drugs into the VTA or the NAc, guide cannula were implanted bilaterally in adult male mice as previously published¹⁵. Coordinates and drug concentrations are included in Supplementary Table 2.

Delivery of viral vectors into the VTA or NAc

AAV stereotaxic surgery and delivery of the AAV (Supplementary Table 3) was performed as previously published¹⁵.

Cocaine conditioned place preference test

A three-chamber place preference protocol (Med Associates Inc., St. Albans, VT, USA) was used as previously described in Tropea et al. 2008⁴⁴. On Day 1 mice were allowed to freely explore all three chambers (20 min). On Day 2–4 (conditioning sessions), a biased procedure was used wherein mice were paired with cocaine (10 mg/kg, i.p.) for 20 min in the morning on the less preferred side, and paired with saline for 20 min in the afternoon on the opposite side. On Day 5 (WD1) mice were allowed to freely explore the CPP box for 20 min and time spent in cocaine-paired side (sec) was recorded. For some experiments, mice were re-tested on day 34 (WD30).

Sucrose Preference Test (SPT)

Individually housed mice were habituated to two 50 ml bottles, one containing water and the other 1% sucrose for one day. For two days, body weight and mass of water and sucrose consumed was monitored. Results are presented as sucrose preference calculated as (sucrose consumed (g)/(sucrose consumed (g) + water consumed (g)))*100.

Forced Swim Test (FST)

Mice were placed in a 2 liter beaker filled with 1600 ml of water between 25–26°C for 10 minutes. Immobility time was scored using the computer-assisted software ButtonBox v5.1 (Behavioral Research Solutions, Dallas Texas).

Three-Chambered Social Interaction test

Social approach was performed as published in Inan et al. 2016⁴⁵. Following a 10 min habituation, mice were allowed to freely explore the apparatus containing an inanimate object and a stranger mouse and time spent within a predefined contact zone was recorded.

Elevated Plus Maze (EPM) test

EPM test was performed as previously reported by Lee et al. (2012)⁴⁶. Data is reported as the percent of time spent in the open arms which was calculated as: (time in open arms (s)/total time (s))*100.

Human Genetics

To examine whether genetic variants within and around *CACNA1D* contribute to CD susceptibility in humans, we conducted a candidate gene association analysis for *CACNA1D* with CD using a genome-wide association study (GWAS) data set from the Study of Addiction: Genetics and Environment (SAGE), previously described in detail⁴⁷ and as detailed in the Supplementary Methods.

Statistical Analyses

All statistical analyses were conducted using Graphpad Prism 6 (GraphPad Software, La Jolla, CA). A two-tailed unpaired t-test was performed after confirmation of normal distribution of the data using the Shapiro-Wilk test. If data were not normally distributed, the Mann Whitney nonparametric test was performed as indicated in the text. For experiments with four groups, a two-way analyses of variance (ANOVA) for main effect of experimental variables was performed followed by the Bonferroni-Dunn *post hoc* test. Statistical tests are specified in the results or in the figure legends and data were considered to be statistically significant for values of $P < 0.05$.

RESULTS

Ca_v1.3 forms functional channels at the VTA postsynaptic density

Ca_v1.3 mRNA is highly expressed in rat VTA dopamine neurons²⁷. Ca_v1.3 protein is enriched in PSD fractions prepared from mouse VTA (Figure 1a) and associates with the PDZ scaffolding protein PSD-95 and Shank3 (Figure 1b, c). This interaction through its C-terminal class I PDZ binding domain (ITTL) is necessary for activity-dependent signaling in VTA slices, as revealed by lack of phosphorylation of the transcription factor CREB, a downstream target of Ca_v1.3 channels³⁸, in the presence of a dominant-negative peptide to ITTL (Figure 1d). Thus, Ca_v1.3 protein is present in functional signaling complexes in the mouse VTA.

VTA Ca_v1.3 channel activation enhances cocaine-related behaviors

We have reported that blocking VTA Ca_v1.3 channels with nifedipine blocks cocaine psychomotor sensitization¹⁵. Similarly, VTA nifedipine infusions in Ca_v1.2 DHP^{-/-} mice (Figure 1e–f, Supplementary Figure 1a) attenuated cocaine CPP, a measure of cocaine reward, twenty-four hours (WD1 herein) and 30 days (WD30 herein) after the last conditioning session (Figure 1g). These mice also exhibited a lower cocaine-induced locomotor response (Figure 1h), an effect replicated by VTA Ca_v1.3shRNA-mediated Ca_v1.3 knockdown (Figure 1e, i–k). Of note, blocking (Supplementary Figure 1b–d) or knocking down (Supplementary Figure 1e–f) Ca_v1.3 channels in the NAc had no effect on cocaine CPP.

To test if pharmacological activation of VTA Ca_v1.3 channels enhanced cocaine-induced behaviors, Ca_v1.2 DHP^{-/-} mice received BayK infusions into the VTA (Figure 1e, 1l), which further increased cocaine CPP (Figure 1m) and cocaine-induced locomotor activity (Figure 1n). Thus, cocaine-induced behaviors require VTA Ca_v1.3 channels and VTA Ca_v1.3 activation is capable of enhancing cocaine-mediated behaviors.

VTA Ca_v1.3 channels are necessary for a GluA1 S831 phosphorylation-dependent increase in CP-AMPA receptors in the NAc shell

Increase in synaptic AMPAR transmission by redistribution of AMPAR subunits, GluA1 and GluA2 has been observed in the NAc following cocaine treatment and behaviors³³. We measured levels of GluA1 and GluA2 in PSD fractions, indicative of synaptic levels, from NAc (shell and core combined) at WD1 and WD30 and role of VTA Ca_v1.3 channels, therein (Figure 2a). Cocaine CPP increased PSD GluA1 but not GluA2 levels in the NAc at WD30 (Figure 2b) but not WD1 (Supplementary Figure 2c–d). The GluA1 increase was blocked in VTA nifedipine (Figure 2b) and VTA Ca_v1.3shRNA (Supplementary Figure 2a) pretreated mice. Cocaine had no effect on PSD GluA1 levels in the PFC, a region that also receives VTA projections (Figure 2a, c and Supplementary Figure 2b).

The increase in GluA1 and not GluA2 at WD30 suggested the possibility of accumulation of GluA2-lacking CP-AMPA receptors. Thus, we infused the CP-AMPA receptor blocker, Naspm into the NAc shell 20 min before the WD30 CPP test (Figure 2d, e) and observed an attenuation of cocaine CPP (Figure 2f). Similarly, Naspm in the NAc shell blocked the expression of cocaine psychomotor sensitization on WD30 (Figure 2g). Thus, cocaine results in upregulation of CP-AMPA receptors within the NAc shell following extended withdrawal, an adaptation that requires activation of VTA Ca_v1.3 channels.

Since GluA1 synaptic function and trafficking is regulated by phosphorylation at Ser 831 (S831) via CaMKinase IIα (CaMKIIα), and Ser 845 (S845) via Protein Kinase A (PKA)⁴⁸, we measured levels of these proteins in NAc PSD fractions at WD30. Cocaine treated mice had higher CaMKIIα, P-CaMKIIα and P-S831 GluA1, an effect that was absent in VTA nifedipine pretreated mice (Figure 2h). No differences in levels of S845 or PKA were seen (Figure 2h). To test the causal role of S831-GluA1 phosphorylation in inducing the cocaine behaviors, we utilized S831 phosphorylation deficient mutant mice (S831A)⁴⁰. WT and S831A mice exhibited similar levels of cocaine preference at WD1 (Figure 2i) and similar psychomotor sensitization on cocaine Day 5 (Figure 2j). However, at WD30, S831A mice had a blunted CPP response (Figure 2i) and a blunted psychomotor response (Figure 2j). Collectively, the above experiments demonstrated that activation of VTA Ca_v1.3 channels are necessary for mediating the protracted accumulation of CP-AMPA receptors in the NAc shell via upregulation of CaMKIIα and S831-GluA1 phosphorylation.

Activation of Ca_v1.3 channels in the VTA promotes depressive-like behavior, cocaine-related behavior, and induces deficits in social behavior

To determine whether VTA Ca_v1.3 channel manipulation is sufficient to alter mood-related and social behavior we used a repeated Ca_v1.3 channel activation protocol (BayK, once a day for 3 days, Figure 3a) in the VTA of Ca_v1.2DHP^{-/-} mice, which has previously been

shown to induce long-term behavioral changes in response to cocaine¹⁴. Repeated VTA-BayK infusion induced anhedonia in the sucrose preference test (SPT) and increased immobility in the forced swim test (FST), when tested 30 days after the last BayK infusion (Figure 3b, c). In a separate cohort, we also observed that BayK effects emerged as early as 24 hours after the last BayK infusion (Supplementary Figure 3a–b). VTA-BayK treatment also decreased social approach behavior in the three-chamber social interaction test (Figure 3d, Supplementary Figure 3c). As previously reported¹⁴, VTA-BayK also potentiated cocaine-induced locomotor activity when measured 30 days after the last BayK infusion (Supplementary Figure 3g) but not 24 hours later (Supplementary Figure 3d). VTA-BayK infusions had no effect on anxiety-like behavior or basal locomotor activity at either time point (Supplementary Figure 3e–f, h–i). Of note, repeated systemic BayK administration in $Ca_v1.2DHP^{-/-}$ mice had a similar effect on SPT, FST, social and cocaine-related behaviors, with no effect on EPM and basal locomotor activity (Supplementary Figure 3j–o). Thus, activation of $Ca_v1.3$ channels in the VTA is sufficient to induce mood-related and cocaine behaviors that are long lasting. In contrast to VTA $Ca_v1.3$ activation, inhibition of $Ca_v1.3$ with repeated nifedipine had no effect on depressive-like, social or cocaine induced-locomotion when measured 24 hours after the last nifedipine infusion (Supplementary Figure 4a–d) and had a milder effect 30 days later (Supplementary Figure 4g–j) with no effect on anxiety-like behavior or basal locomotion (Supplementary Figure 4e–f, k–l).

CP-AMPARs in the NAc shell mediate BayK-induced cocaine and depressive-like behavior

Formation of CP-AMPARs in the NAc has been reported in models of chronic stress³⁶ and chronic neuropathic pain-induced depressive-like behaviors⁴⁹. Thus, we measured levels of GluA1 and GluA2 in NAc (shell and core) PSD fractions 30 days following VTA-BayK infusions and both were higher in BayK infused mice (Figure 3e). Interestingly, even though BayK induced depressive-like and social behavioral deficits 24 hours after the last BayK administration, neither GluA1 nor GluA2 were significantly altered at 24 hours (Supplementary Figure 5a). To test the potential role of CP-AMPARs in VTA BayK-induced behaviors at the 30d time point, given overlapping mechanisms⁸, Naspnm was administered into the NAc shell 30 mins prior to behavioral testing (Figure 3f), a manipulation that blocked VTA BayK-induced decrease in SPT (Figure 3g) and increase in immobility during FST (Figure 3h) (no effect on vehicle treated mice). Naspnm additionally attenuated enhanced cocaine-induced locomotor activity in BayK-treated mice (Figure 3i). Interestingly, Naspnm in the NAc shell had no effect on social approach behavior (Figure 3j) nor in the NAc core (Figure 3k), whereas blocking GluA1/A2 AMPARs with NBQX in the NAc core (Figure 3l, Supplementary Figure 5b) but not in the NAc shell (Figure 3m), reversed the deficit in social approach. Thus, long-term effect of repeated VTA-BayK on depressive behavior results from increase in CP-AMPARs in the NAc shell, whereas its effect on social behavior results from increase in GluA1/A2 AMPARs in the NAc core.

Next, we tested if repeated VTA-BayK alters protein kinase and GluA1 phosphorylation levels in the NAc. Similar to cocaine exposure (Figure 2h), we found that VTA-BayK infusion increased levels of NAc CaMKII α , and S831-GluA1 30 days following BayK infusion (Figure 4a). To test the causal role of S831-GluA1 phosphorylation on behaviors, we utilized S831A-GluA1 phospho-mutant mice. At baseline, S831A did not differ from

WT mice in the SPT (Supplementary Figure 6a), FST (Supplementary Figure 6b), social behavior (Supplementary Figure 6c), EPM (Supplementary Figure 6d), or basal locomotor activity (Supplementary Figure 6e). In contrast, VTA BayK-induced anhedonia (Figure 4b), increased immobility (Figure 4c), and enhanced cocaine-induced locomotor activity (Figure 4e) were not observed in S831A mice. Interestingly, WT and mutant mice exhibited similar social approach behavior (Figure 4d), demonstrating a causal role of S831-GluA1 phosphorylation in the NAc for VTA BayK-induced depressive-like and cocaine-related behaviors, but not social behavior. VTA-BayK infusions had no effect on anxiety-like behavior or basal locomotor activity in either genotype (Supplementary Figure 6f–g).

Significant association of *CACNA1D* SNPs with cocaine dependence

Based on our findings of a role for $Ca_v1.3$ channels in rodent cocaine behaviors, we tested whether variants within and around *CACNA1D* were associated with CD using the genomewide association study (GWAS data set from the Study of Addiction: Genetics and Environment (SAGE)). To examine as many variants as possible, we imputed the 1000 Genomes Project variants into *CACNA1D*. Specifically, we tested 947 single nucleotide polymorphism (SNPs) within and around *CACNA1D* in European-Americans (EA; 493 cases and 1,058 controls) and followed analyses of any significant findings in African-Americans (AA; 521 cases and 369 controls). We performed an association test in a logistic regression framework including sex, age and top three principle components as covariates. We found the strongest association with CD for intronic SNP rs4687735 in EAs ($p = 1.3 \times 10^{-5}$, OR = 2.46, 95% Confidence Interval (CI) = 1.65–3.67), an imputed intronic SNP (imputation quality score = 0.82) with a risk allele ‘T’ frequency of 6.5% in cases and 3.5% in controls, which remains significant after Bonferroni correction ($p = 0.012$). In addition, another two imputed SNPs, rs2926559 and rs898407, which are in high linkage disequilibrium (LD) with rs4687735, also showed strong association with CD (rs2926559, $p=0.018$ and rs898407, $p=0.019$ after Bonferroni correction). Figure 5 illustrates the association test results for 947 SNPs within and around *CACNA1D* in EAs.

We observed a trend association in the same direction as in EAs for rs4687735 in AAs ($p = 0.14$, OR = 2.62, 95% CI = 0.68–10.15), with risk allele ‘T’ frequency of 1.1% in cases and 0.45% in controls (imputation quality score = 0.88). Meta-analysis of the EA and AA samples showed stronger association between rs4687735 and CD ($p=4.0 \times 10^{-6}$, OR = 2.47, 95% CI = 1.68–3.63). We also observed trend associations for the rs2926559 ($p = 0.13$, OR = 2.42, 95% CI = 0.72–8.07) and rs898407 ($p = 0.14$, OR = 2.51, 95% CI = 0.68–9.23) in AAs. Supplementary Table 5 shows association results for EAs, AAs and meta-analysis for 778 SNPs that exist in both EAs and AAs.

To evaluate the association evidence at the gene level, we further computed a gene-based p-value for the EA and AA samples using GATES, a gene-based association test using extended Simes procedure⁵⁰. We found significant association evidence in both EA ($p=0.003$) and AA ($p=0.008$).

DISCUSSION

Here, we demonstrate that VTA $Ca_v1.3$ channel activation robustly mediates cocaine-related, depression-related and social behavior via distinct NAc AMPAR mechanisms with no effect on anxiety-like behavior. These findings provide novel insight into VTA $Ca_v1.3$ to NAc mechanisms that likely underlie behavioral phenotypes in multiple neuropsychiatric illnesses. We propose that $Ca_v1.3$ activation may mediate co-morbid phenotypes in addiction and mood disorders. Our identification of *CACNA1D* SNPs associated with cocaine dependence, in combination with previous human genetic data linking *CACNA1D* with BD^{11, 12}, suggests a potential role for VTA *CACNA1D* in mediating co-morbid behavioral endophenotypes in addiction and BD. The data also support further investigation of $Ca_v1.3$ as a potential therapeutic target for neuropsychiatric disorders with substance abuse, depression or social deficit-related phenotypes.

The current findings add to the emerging evidence for convergent mesolimbic dopamine pathway mechanisms mediating behavioral deficits in reward-related and mood disorders^{2, 7, 51}. Our study is the first to examine $Ca_v1.3$ mechanisms across drug-related, depression-related and social-related behaviors and the first to link $Ca_v1.3$ animal studies to human GWAS data. The ability of VTA $Ca_v1.3$ activation to enhance cocaine behaviors after extended withdrawal is consistent with previous demonstration of VTA LTCC activation potentiating cocaine-induced psychomotor response¹⁴ and with a critical role of VTA $Ca_v1.3$ LTCCs in cocaine behaviors^{15, 16}. The observed depressive-like effects of VTA $Ca_v1.3$ activation are consistent with our previous demonstration where global $Ca_v1.3$ activation resulted in depressive-like behavior²⁸, while $Ca_v1.3^{-/-}$ mice showed an antidepressant-like response⁵². We additionally show for the first time that VTA $Ca_v1.3$ channel activation results in social behavioral deficits. The novelty of the present finding is our identification of the VTA as a critical brain locus mediating these $Ca_v1.3$ -dependent depressive-like and social behavioral phenotypes in parallel with cocaine-induced phenotypes. Thus, we demonstrate for the first time that enhancing VTA $Ca_v1.3$ activation is a common mechanism that can induce behavioral alterations associated with substance abuse and mood-related psychiatric illnesses. Indeed, multiple investigations over the last several years have begun to elucidate specific VTA mechanisms that are activated by stress and by drugs of abuse^{6, 8, 51}.

Importantly, our findings provide novel pathway-specific molecular insights regarding the mechanisms underlying these reward, mood, and social interaction-related behaviors. Our data show that activation of VTA $Ca_v1.3$ channels is sufficient to increase CP-AMPA receptors in the NAc shell; their activation, in turn, is necessary for cocaine CPP and enhanced locomotor response measured after greater than 1 month, but not 1 day, of withdrawal. These findings are generally consistent with a role for cocaine-evoked CP-AMPA receptors in the NAc shell in certain models of addictive behavior, although most such evidence has been obtained after cocaine self-administration^{31–35, 53–55}. Cocaine-evoked CP-AMPA receptors in NAc core also mediate cocaine seeking after cocaine self-administration and prolonged withdrawal^{30, 54–56}. It will be interesting in future studies to further examine NAc core versus shell AMPAR mechanisms in the model used in this study.

We further demonstrate that similar to cocaine, VTA Ca_v1.3-induced depressive-like behavior results from increase in CP-AMPARs in the NAc shell. While the role of CP-AMPARs in depressive-like behavior has not been clearly delineated in the literature, recent studies have revealed changes in CP-AMPAR expression that are associated with exposure to a stress protocol that induces anhedonia³⁶. Vialou V. et al. 2010⁵⁷ also showed that mice susceptible to social defeat stress show higher levels of GluA1 expression in the NAc, with no change in GluA2 suggestive of an increase in CP-AMPARs. Additionally, CP-AMPARs in the NAc have been found to emerge in a chronic neuropathic pain-induced depression model⁴⁹. Collectively these studies support the conclusion that VTA Ca_v1.3 activation promotes cocaine and depressive-like behavior via enhancing synaptic CP-AMPARs. One mechanism suggested to contribute to increases in CP-AMPAR levels at the synapse is S831-GluA1 phosphorylation by CaMKIIα⁴⁸. We demonstrate the necessity of S831-GluA1 phosphorylation for VTA Ca_v1.3-mediated enhancement of cocaine and depressive-like behaviors, highlighting VTA Ca_v1.3 mechanistic convergence. The ability of VTA Ca_v1.3 activation to increase NAc CaMKIIα activity also argues for a converging CaMKIIα mechanism. Previous studies have found an increase in CaMKIIα in the NAc of rats following 14 days of withdrawal from non-contingent cocaine administration⁵⁸ and in the NAc of cocaine-dependent humans⁵⁸, as well as following social defeat stress⁵⁹. Additionally, a SNP in the *CaMK2A* gene has been linked to faster transition to severe cocaine use in humans⁶⁰. A direct link between CaMKIIα and formation of CP-AMPARs remains to be established, however concurrent increase in phosphorylated CaMKII and CP-AMPARs at the NAc PSD following extended withdrawal from cocaine self-administration has been observed⁶¹.

We additionally demonstrate that activation of VTA Ca_v1.3 channels also promotes social deficits however via enhancing GluA1/A2 AMPARs in the NAc consistent with enhanced AMPAR transmission associated with social deficits⁶² including in the NAc core⁶³. To the best of our knowledge, this is the first time that Ca_v1.3 channels have been associated with social behavior. The recruitment of AMPARs in the NAc core versus CP-AMPARs in the NAc shell in our models of cocaine and depressive-like phenotypes suggests the possibility of recruitment of different VTA-NAc neurons mediating diverse behavioral phenotypes. This is not surprising given the highly heterogeneous nature of the VTA afferents to the NAc and other regions of the brain^{26, 64}, a question that will be addressed in future studies. Additionally, the higher but not significant levels of S880 P-GluA2 in the PSD of NAc (core +shell) is intriguing given that this phosphorylation event has been suggested to endocytose GluA2 subunits^{65, 66}. However, States et al. (2008)⁶⁷ have reported that S880 phosphorylation can stabilize GluA2 at the PSD, that could be the case in the NAc core where we find higher GluA1/GluA2 AMPARs. In future studies, it will be interesting to further explore the role of S880 GluA2 phosphorylation in GluA2 trafficking and social behavior.

The precise VTA Ca_v1.3 mechanisms that promote cocaine and depressive-like behaviors remain to be answered. Preliminary findings from our laboratory suggest that Ca_v1.3/CaMKII/ERK2 signaling within the VTA mediates long-term molecular changes in the NAc via a CREB-dependent mechanism. CREB in the VTA has been suggested to play a role in cocaine-induced plasticity⁶⁸, an area of research that remains underexplored. One potential

candidate downstream of the $Ca_v1.3$ /CREB pathway in the VTA that may mediate the transition from the VTA to NAc is the neurotrophic factor BDNF, a downstream target of LTCCs^{69, 70} and of CREB^{71, 72} that regulates both cocaine⁷³ and depressive-like behavior⁷⁴. Phasic firing of VTA dopamine neurons promotes cocaine²⁵ and depressive behaviors²⁶, a dopamine neuron property that also facilitates BDNF release in the NAc⁸. BDNF in the NAc via its postsynaptic TrkB receptor, regulates CaMKII-mediated S831 GluA1 phosphorylation⁷⁵, AMPAR trafficking^{76, 77} and cocaine and depressive behaviors^{73, 74, 78}. Thus, BDNF produced in the VTA and released in the NAc serves as a promising candidate in mediating the effect of VTA $Ca_v1.3$ channels. Our unpublished data supports this hypothesis as we find that VTA $Ca_v1.3$ channel activation increases *Bdnf* transcription in the VTA. Future studies will address VTA $Ca_v1.3$ -mediated *Bdnf* release and postsynaptic effects in the NAc on AMPAR signaling and cocaine and mood-related behaviors.

Candidate gene association analysis in humans supports the role of genetic variations within *CACNA1D* in CD. To the best of knowledge, this is the first report describing a genetic association between *CACNA1D* and CD in two independent human samples, which corroborates the functional aspects of $Ca_v1.3$ in the mice models of cocaine behavior. Considering the fact that *CACNA1D* SNPs can confer risk for BD^{11, 12} as well as the association of human *CACNA1D* gain of function mutations associated with neurological disorders¹⁰, including autism¹³, characterized by significant mood dysregulation, an important follow-up investigation would be to examine via targeted sequencing whether rare variants within *CACNA1D* are also associated with CD. Further fine-mapping of this gene may contribute to the identification of functional causal variants underlying CD.

In summary, findings of this study support enhanced $Ca_v1.3$ channel activity within the VTA-NAc pathway as one potential mechanism underlying drug abuse and mood disorders, with implications for co-morbid neuropsychiatric-related behavioral phenotypes, in particular those resulting from disease-associated *CACNA1D* SNPs. The study provides a novel framework to further understand the pathophysiology of mental illness that can aid in designing therapeutic studies using available $Ca_v1.3$ -selective LTCC blockers⁷⁹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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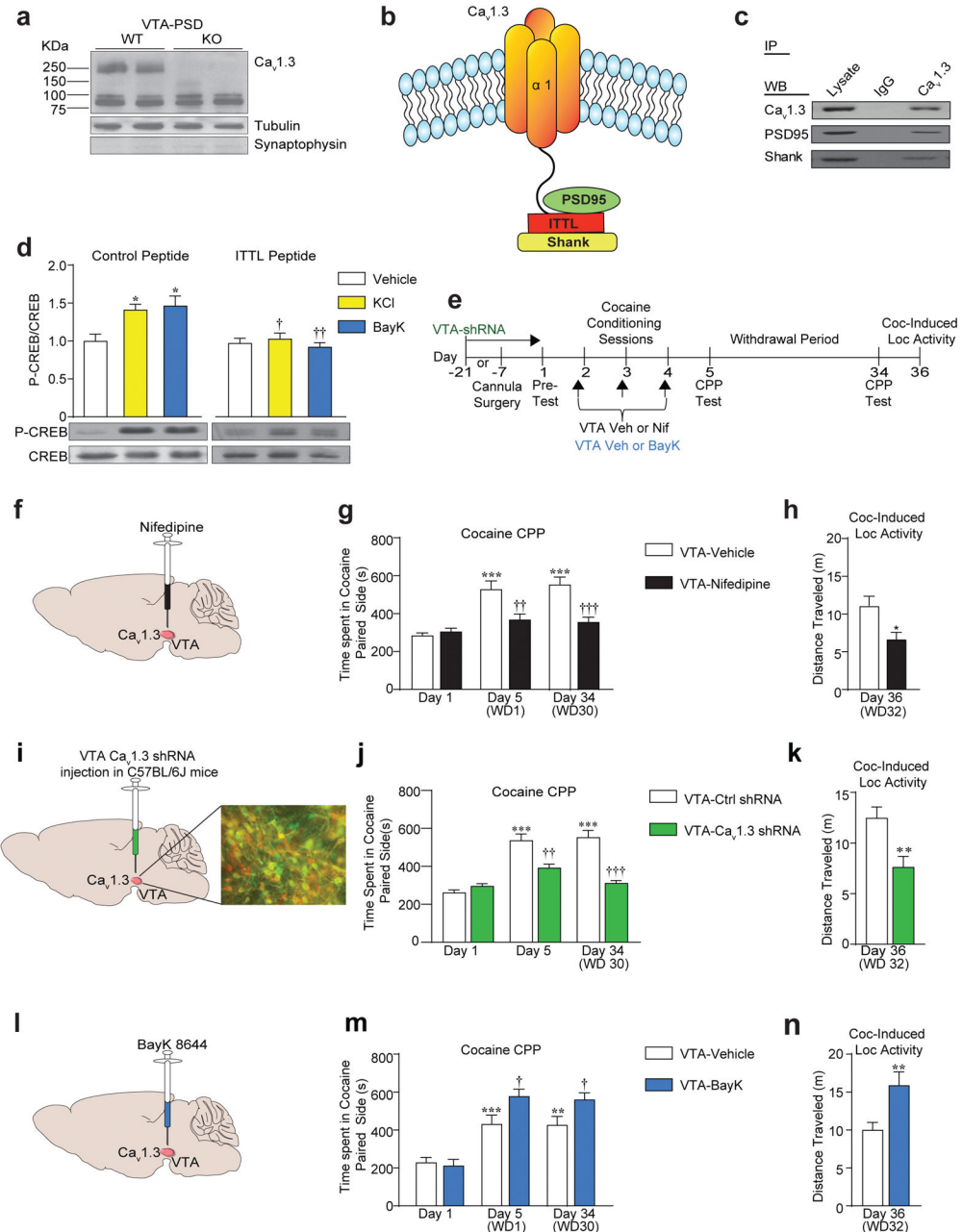
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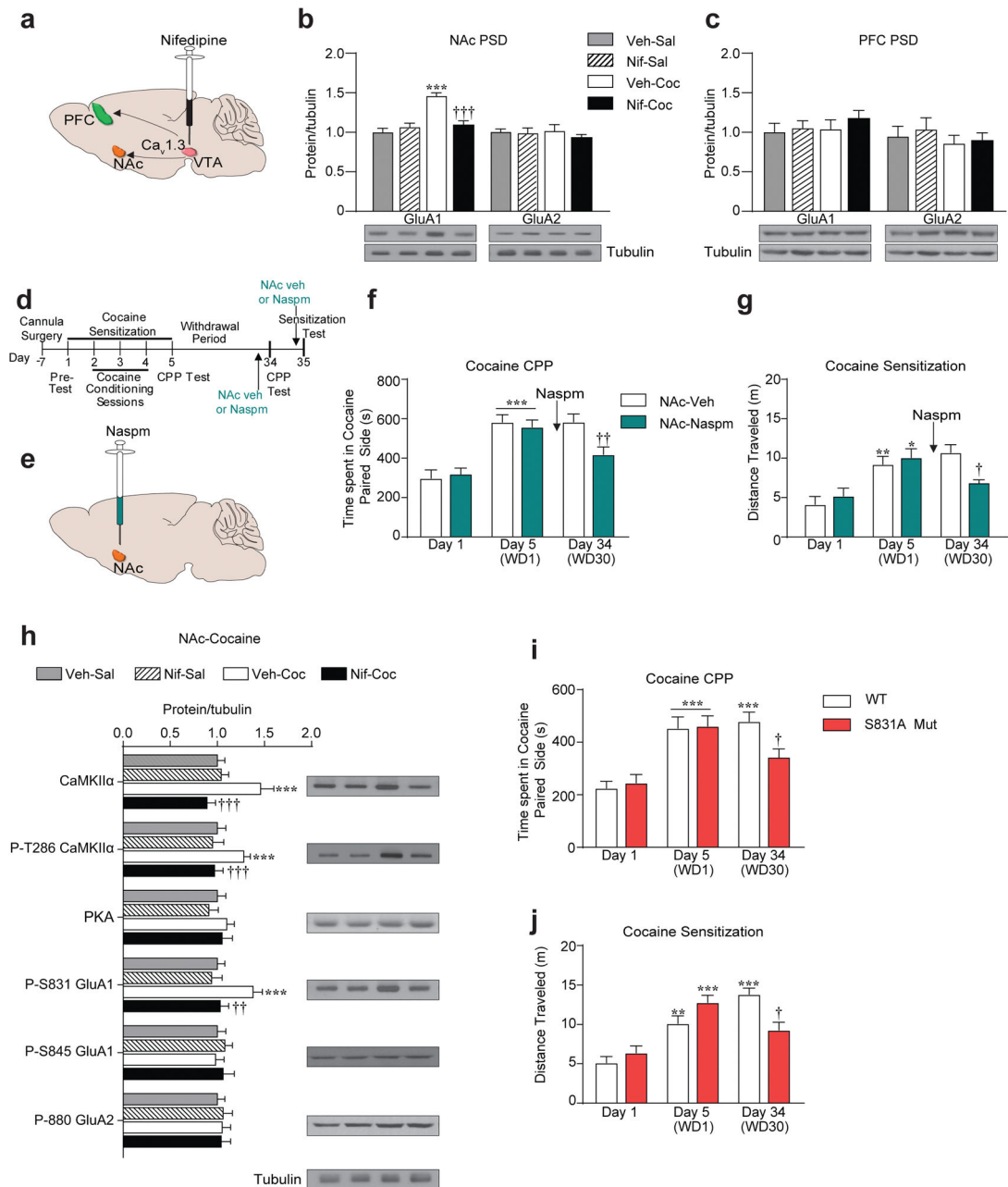
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**Figure 1.**

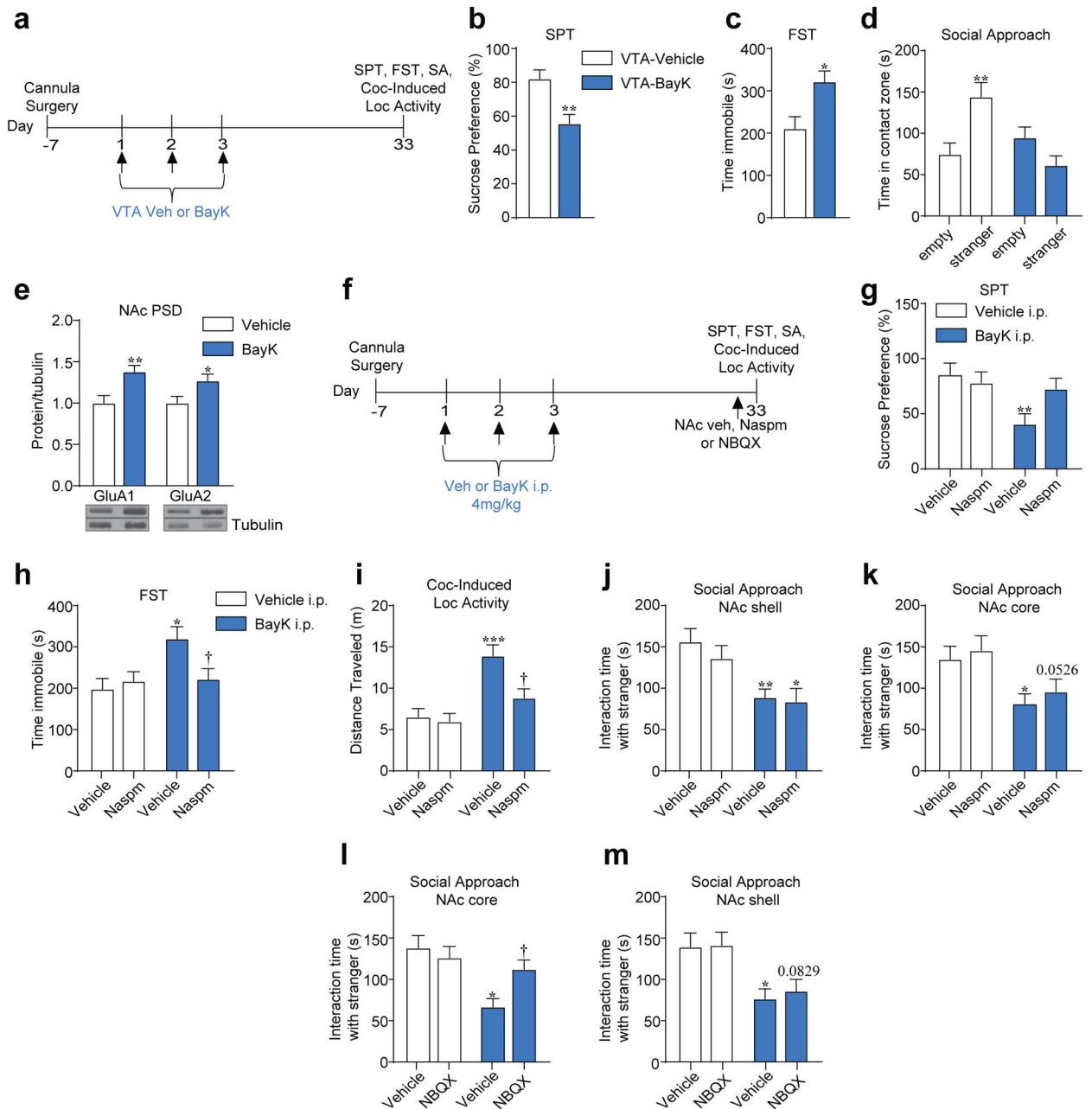
Pharmacological inhibition and activation of VTA Ca_v1.3 channels oppositely regulates cocaine behaviors. **(a)** Western blot showing the presence of Ca_v1.3 protein in VTA PSD fractions from wildtype (WT) and Ca_v1.3 knockout (KO) mice. **(b)** Schematic of Ca_v1.3 showing its ITTL in the C-terminal cytoplasmic domain and its interaction with PSD-95 and Shank. **(c)** Western blots showing immunoprecipitated PSD95 and Shank with Ca_v1.3 antibody in VTA protein lysates. **(d)** Disruption of Ca_v1.3 PDZ domain ITTL with an inhibitory peptide blocks KCl- (Two-way ANOVA, [Peptide x KCl, $F_{(1,20)} = 4.831$, $P = 0.0399$]; Bonferroni *post hoc*, Control Peptide: Vehicle vs KCl * $P < 0.05$, KCl: Control

Peptide vs ITTL Peptide $\dagger P < 0.05$) and BayK- (Two-way ANOVA, [Peptide x BayK, $F_{(1, 20)} = 7.593$, $P = 0.0122$]; Bonferroni *post hoc* test: Control Peptide: Vehicle vs BayK $* P < 0.05$, BayK: Control Peptide vs ITTL Peptide $\dagger\dagger P < 0.01$) induced Ser 133 P-CREB phosphorylation in VTA slices from $Ca_v1.2$ DHP^{-/-} mice. For all groups, $n = 6$. **(e, f, i, l)** Schematic timeline of behavioral protocol and VTA infusion of either Veh or Nif, Veh or BayK in $Ca_v1.2$ DHP^{-/-} mice, or $Ca_v1.3$ shRNA in C57BL/6 mice. **(g-h)** Intra-VTA microinjection of Nif in $Ca_v1.2$ DHP^{-/-} mice administered prior to each cocaine conditioning session attenuated **(g)** expression of CPP on Day 5 (WD1) and Day 34 (WD30) (Two-way ANOVA, [VTA infusion x day, $F_{(2,60)} = 7.595$, $P = 0.0011$]; Bonferroni *post hoc* test: Veh: Day 1 vs Day 5 $*** P < 0.001$, Veh: Day 1 vs Day 34 $*** P < 0.001$, Day 5: Veh vs Nif $\dagger\dagger P < 0.01$, Day 34: Veh vs Nif $\dagger\dagger\dagger P < 0.001$. Veh $n = 12$, Nif $n = 10$), and **(h)** the cocaine-induced locomotor activity measured on day 36 ($t_{(17)} = 2.86$, $* P = 0.0108$. Veh $n = 10$, Nif $n = 9$). **(i)** Inset, image shows green fluorescent protein (GFP-green), tyrosine hydroxylase (TH-red) and dual-labeled (yellow) cells. **(j, k)** Intra-VTA stereotaxic delivery of $Ca_v1.3$ shRNA (21 days before the start of CPP) attenuated **(j)** the expression of CPP tested on day 5 and 34 (Two-Way ANOVA, [VTA injection x day, $F_{(2,51)} = 14.09$, $P < 0.0011$]; Bonferroni *post hoc* test: Ctrl shRNA: Day 1 vs Day 5 $*** P < 0.001$, Ctrl shRNA Day 1 vs Day 34 $*** P < 0.001$, Day 5: Ctrl shRNA vs $Ca_v1.3$ shRNA $\dagger\dagger P < 0.01$, Day 34: Ctrl shRNA vs $Ca_v1.3$ shRNA $\dagger\dagger\dagger P < 0.001$. Ctrl shRNA $n = 10$, $Ca_v1.3$ shRNA $n = 9$), and **(k)** cocaine-induced locomotor activity measured on day 36 (t -test, $t_{(17)} = 3.066$, $** P = 0.0070$. Ctrl shRNA $n = 10$, $Ca_v1.3$ shRNA $n = 9$). **(m, n)** Intra-VTA infusion of BayK prior to each cocaine conditioning session enhanced **(m)** expression of cocaine CPP on day 5 and 34 (Two-way ANOVA, [VTA infusion x day $F_{(2,48)} = 3.206$, $P = 0.0493$]; Bonferroni *post hoc* test: Veh: Day 1 vs Day 5 $*** P < 0.001$, Veh: Day 1 vs Day 34 $** P < 0.01$, Day 5: Veh vs BayK $\dagger P < 0.05$, Day 34: Veh vs BayK $\dagger P < 0.05$. Veh $n = 9$, BayK $n = 9$), and **(n)** enhanced cocaine-induced locomotor response on day 36 ($t_{(16)} = 3.955$, $** P = 0.0011$. Veh $n = 9$, BayK $n = 9$). Error bars represent \pm s.e.m.

**Figure 2.**

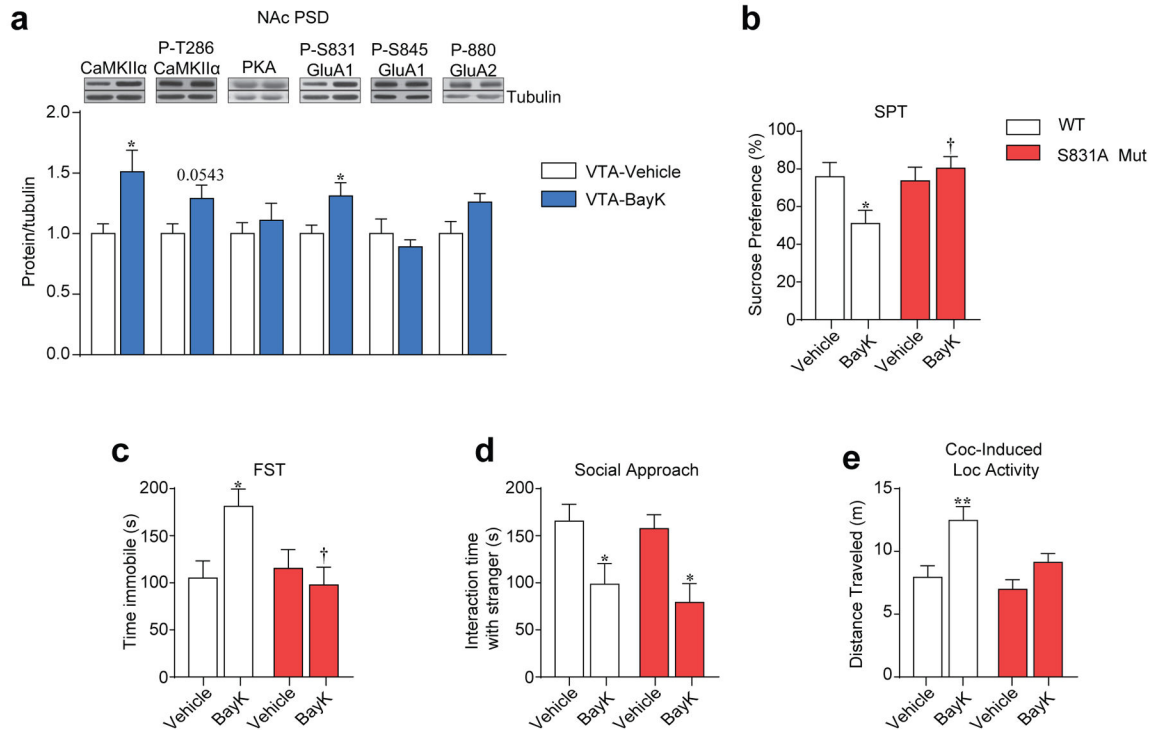
VTA $Ca_v1.3$ channels mediate long-term increase in CP-AMPA receptors at the NAc PSD of cocaine-exposed mice. **(a)** Schematic representation of VTA projection to the NAc and PFC. **(b)** Cocaine administration increased GluA1 but not GluA2 protein levels at the NAc PSD at WD30 that was blocked by Nif pretreatment (GluA1: Two-way ANOVA, [pre-treatment \times post-treatment, $F_{(1,22)} = 18.82$, $P < 0.0003$]. Bonferroni *post hoc* test: Veh-Sal vs Veh-coc $***P < 0.001$, Veh-coc vs Nif-coc $\dagger\dagger\dagger P < 0.001$; Veh-Sal $n = 7$, Nif-Sal $n = 5$, Veh-Coc $n = 7$, Nif-Coc $n = 8$). **(c)** No difference in GluA1 or GluA2 levels was seen in the PFC PSD (Veh-Sal $n = 7$, Nif-Sal, $n = 5$, Veh-Coc, $n = 7$, Nif-Coc, $n = 8$). $***P < 0.0001$, $\dagger\dagger\dagger P < 0.001$. **(d)**

Experimental timeline of Nasp^m microinjection. **(e)** Schematic of Nasp^m infusion in the NAc shell. **(f-g)** Nasp^m infusion in the NAc shell prior to **(f)** cocaine CPP test on day 34 attenuated expression of cocaine CPP (Day 1 and 5, Two-way ANOVA, [day, $F_{(1,36)} = 51.05$, $P < 0.001$]; Bonferroni *post hoc* test: Veh and Nasp^m groups: Day 5 vs Day 1 *** $P < 0.001$; Day 34, *t*-test, $t_{(18)} = 2.881$, $^{\dagger\dagger}P = 0.0099$. Veh $n = 8$, Nasp^m $n = 12$) and **(g)** cocaine-induced locomotor activity test on day 34 attenuated expression of psychomotor sensitization (Day 1 and 5, Two-way ANOVA, [day, $F_{(1,28)} = 23.76$, $P < 0.001$]; Bonferroni *post hoc* test: Veh group: Day 5 vs Day 1 ** $P < 0.01$, Nasp^m group: Day 1 vs. Day 5 * $P < 0.05$; Day 34, *t*-test, $t_{(14)} = 2.767$, $^{\dagger}P = 0.0151$. Veh $n = 8$, Nasp^m $n = 12$). **(h)** VTA Nif pretreatment in Ca_v1.2DHP^{-/-} mice blocked cocaine-induced increase in CaMKII α , P-T286 CaMKII α and P-S831 GluA1 in the NAc of cocaine exposed mice examined 30 days later (Two-way ANOVA, [CaMKII α : pretreatment x posttreatment, $F_{(1,23)} = 9.091$, $P = 0.0062$]; Bonferroni *post hoc* test: Veh-Sal vs Veh-Coc *** $P < 0.001$, Veh-Coc vs Nif-Coc $^{\dagger\dagger\dagger}P < 0.001$; [P-T286 CaMKII α : $F_{(1,23)} = 13.11$, $P = 0.0014$]; Veh-Sal vs Veh-Coc *** $P < 0.001$, Veh-Coc vs Nif-Coc $^{\dagger\dagger\dagger}P < 0.001$; [P-S831 GluA1: $F_{(1,23)} = 4.247$, $P = 0.05$]; Veh-Sal vs Veh-Coc *** $P < 0.001$, Veh-Coc vs Nif-Coc $^{\dagger\dagger}P < 0.01$. Veh-Sal $n = 7$, Nif-Sal $n = 6$, Veh-Coc $n = 7$, Nif-Coc $n = 7$). **(i)** S831A GluA1 phospho-mutant mice presented a blunted cocaine CPP response (Two-Way ANOVA, [day x genotype, $F_{(2,50)} = 3.211$, $P = 0.0488$]; Bonferroni *post hoc* test: WT and S831A: Day1 vs Day 5 *** $P < 0.001$, WT: Day1 vs Day 34 *** $P < 0.001$, Day 34: WT vs. S831A $^{\dagger}P < 0.05$. WT $n = 10$, S831A $n = 9$), and **(j)** blunted cocaine-induced locomotor activity on Day 34 (Two-Way ANOVA, [day x genotype, $F_{(2,48)} = 8.863$, $P = 0.0005$]; Bonferroni *post hoc* test: WT: Day1 vs Day 5 ** $P < 0.01$, S831A: Day1 vs Day 5 *** $P < 0.001$, WT: Day 1 vs Day 34 *** $P < 0.001$, Day 34: WT vs S831A $^{\dagger}P < 0.05$. WT $n = 8$, S831A $n = 10$). Error bars represent \pm s.e.m.

**Figure 3.**

Repeated VTA BayK 8644 treatment results in depressive-like behavior, social interaction deficits and enhanced cocaine psychomotor activity. **(a)** Experimental timeline of VTA-BayK infusion and behavioral testing. **(b–d)** Repeated administration of BayK in the VTA decreased sucrose preference **(b)**, $t_{(18)} = 3.486$, $**P=0.0026$. Veh $n = 10$, BayK $n = 10$), increased immobility time in FST **(c)**, $t_{(12)} = 2.725$, $*P=0.0184$. Veh $n = 7$, BayK $n = 7$), and impaired social approach behavior **(d)**, Two-Way ANOVA, [genotype \times contact zone, $F_{(1,36)} = 13.56$, $P=0.0008$]; Veh: stranger vs empty cup $**P<0.01$. Veh $n = 10$, BayK $n = 10$). **(e)** BayK administration in the VTA increased GluA1 and GluA2 protein in the NAc PSD when

tested 30 days later (GluA1: $t_{(17)} = 3.247$, $**P=0.0047$. Veh $n = 9$, BayK: $n = 10$; GluA2: $t_{(17)} = 2.366$, $*P=0.0301$. Veh $n = 9$, BayK $n = 10$). **(f)** Experimental timeline. **(g-i)** Nasp_m infusion in the NAc shell prior to **(g)** SPT (Two-Way ANOVA, [pre-treatment (Veh or BayK): $F_{(1,34)} = 6.351$, $P=0.016$]; Bonferroni *post hoc* test: Veh-Veh vs. BayK-Veh $**P=0.0079$. Veh-Veh $n = 8$, Veh-Nasp_m, $n = 9$, BayK-Veh $n = 10$, BayK-Nasp_m $n = 11$) or **(h)** FST (Two-Way ANOVA, [pretreatment x posttreatment, $F_{(1,34)} = 4.903$, $P=0.0336$]; Bonferroni *post hoc* test: Veh-Veh vs BayK-Veh $*P<0.05$, BayK-Veh vs BayK-Nasp_m $†P<0.05$. Veh-Veh $n = 8$, Veh-Nasp_m $n = 9$, BayK-Veh $n = 10$, BayK-Nasp_m $n = 11$), rescued depressive-like behavior and **(i)** decreased the enhanced cocaine-induced locomotor response (Two-Way ANOVA, [pre-treatment (Veh or BayK): $F_{(1,34)} = 19.96$, $P<0.0001$]; post-treatment (Veh or Nasp_m): $F_{(1,34)} = 6.117$, $P=0.0185$]; Bonferroni *post hoc* test: Veh-Veh vs BayK-Veh $***P<0.001$, BayK-Veh vs BayK-Nasp_m $†P<0.05$. Veh-Veh $n = 8$, Veh-Nasp_m $n = 9$, BayK-Veh $n = 10$, BayK-Nasp_m $n = 11$), observed in BayK treated mice. **(j-m)** Nasp_m infusion in the **(j)** NAc shell (Two-way ANOVA, [pre-treatment (Veh or BayK): $F_{(1,34)} = 16.83$, $P< 0.0002$]; Bonferroni *post hoc* test: Veh-Veh vs BayK-Veh $**P<0.01$, Veh-Nasp_m vs BayK-Nasp_m $*P<0.05$. Veh-Veh $n = 9$; Veh-Nasp_m $n = 9$, BayK-Veh $n = 10$, BayK-Nasp_m $n = 10$), or **(k)** in the NAc core (Two-way ANOVA, [pre-treatment (Veh or BayK): $F_{(1,32)} = 11.67$, $P< 0.0017$]; Bonferroni *post hoc* test: Veh-Veh vs BayK-Veh $*P<0.05$, Veh-Nasp_m vs BayK-Nasp_m $P=0.0526$. Veh-Veh $n = 8$, Veh-Nasp_m $n = 8$, BayK-Veh $n = 10$, BayK-Nasp_m $n = 10$) had no effect on BayK-induced social approach deficit, whereas NBQX infusion in **(l)** the NAc core (Two-Way ANOVA, [pretreatment x posttreatment, $F_{(1,35)} = 5.403$, $P=0.0260$]; Bonferroni *post hoc* test: Veh-Veh vs BayK-Veh $*P<0.01$, BayK-Veh vs BayK-NBQX $†P<0.05$. Veh-Veh $n = 9$, Veh-NBQX $n = 9$, BayK-Veh $n = 10$, BayK-NBQX $n = 11$), but not in **(m)** the NAc shell [pre-treatment (BayK or Veh), $F_{(1,28)} = 15.74$, $P=0.0005$]; Post-treatment (NBQX or Veh), $F_{(1,28)} = 0.1436$, $P = 0.7076$]; Bonferroni *post hoc* test: Veh-Veh vs BayK-Veh $*P<0.05$. Veh-NBQX vs BayK-NBQX $P=0.0829$. Veh-Veh $n = 8$, Veh-NBQX $n = 8$, BayK-Veh $n = 8$, BayK-NBQX $n = 8$), rescued the BayK- induced social approach deficit. Error bars represent \pm s.e.m.

**Figure 4.**

Repeated BayK-induced depressive-like and cocaine behavior is dependent on phosphorylation of GluA1 at S831. **(a)** Repeated BayK infusion in the VTA increased CaMKII α and P-S831 GluA1 phosphorylation in the NAc examined 30 days later (CaMKII α : $t_{(12)} = 2.589$, $*P=0.0237$; P-T286 CaMKII α : $t_{(12)} = 2.132$, $P=0.0543$; P-S831 GluA1: $t_{(12)} = 2.378$, $*P=0.0349$. VTA-Veh $n = 7$, VTA-BayK $n = 7$). **(b, c)** Repeated BayK infusion in the VTA of WT mice but not S831A mice resulted in depressive-like behavior as revealed in **(b)** the SPT (Two-Way ANOVA, [genotype x pretreatment, $F_{(1,32)} = 4.983$, $P=0.0327$]; Bonferroni *post hoc* test: WT: Veh vs BayK $*P<0.05$, BayK: WT vs S831A $\dagger P<0.05$. WT-Veh $n = 9$, WT-BayK $n = 10$, S831A-Veh $n = 8$, S831A-BayK $n = 9$) and **(c)** FST (Two-Way ANOVA, [genotype x pretreatment, $F_{(1,32)} = 6.145$, $P=0.0186$]; Bonferroni *post hoc* test: WT: Veh vs BayK $*P<0.05$, BayK: WT vs S831A Mut $\dagger P<0.05$. WT-Veh $n = 9$, WT-BayK $n = 10$, S831A-Veh $n = 8$, S831A-BayK $n = 9$). **(d)** Repeated BayK infusion in the VTA of WT and S831A mutant mice induced social approach deficits in both genotypes (Two way ANOVA, [pretreatment, $F_{(1,32)} = 14.10$, $P=0.0007$]; Bonferroni *post hoc* test: WT: Veh vs BayK $*P<0.05$, S831A: Veh vs BayK $*P<0.05$. WT-Veh $n = 9$, WT-BayK $n = 10$, S831A-Veh: $n = 8$, S831A-BayK $n = 9$). **(e)** Repeated BayK treatment in the VTA resulted in higher cocaine-induced locomotor activity in WT but not S831A mutant mice (Two-Way ANOVA, [genotype $F_{(1,32)} = 13.58$, $P=0.0008$], Bonferroni *post hoc* test: WT: Veh vs BayK $**P<0.01$. WT-Veh $n = 9$, WT-BayK $n = 10$, S831A-Veh $n = 8$, S831A-BayK $n = 9$). Error bars represent \pm s.e.m.

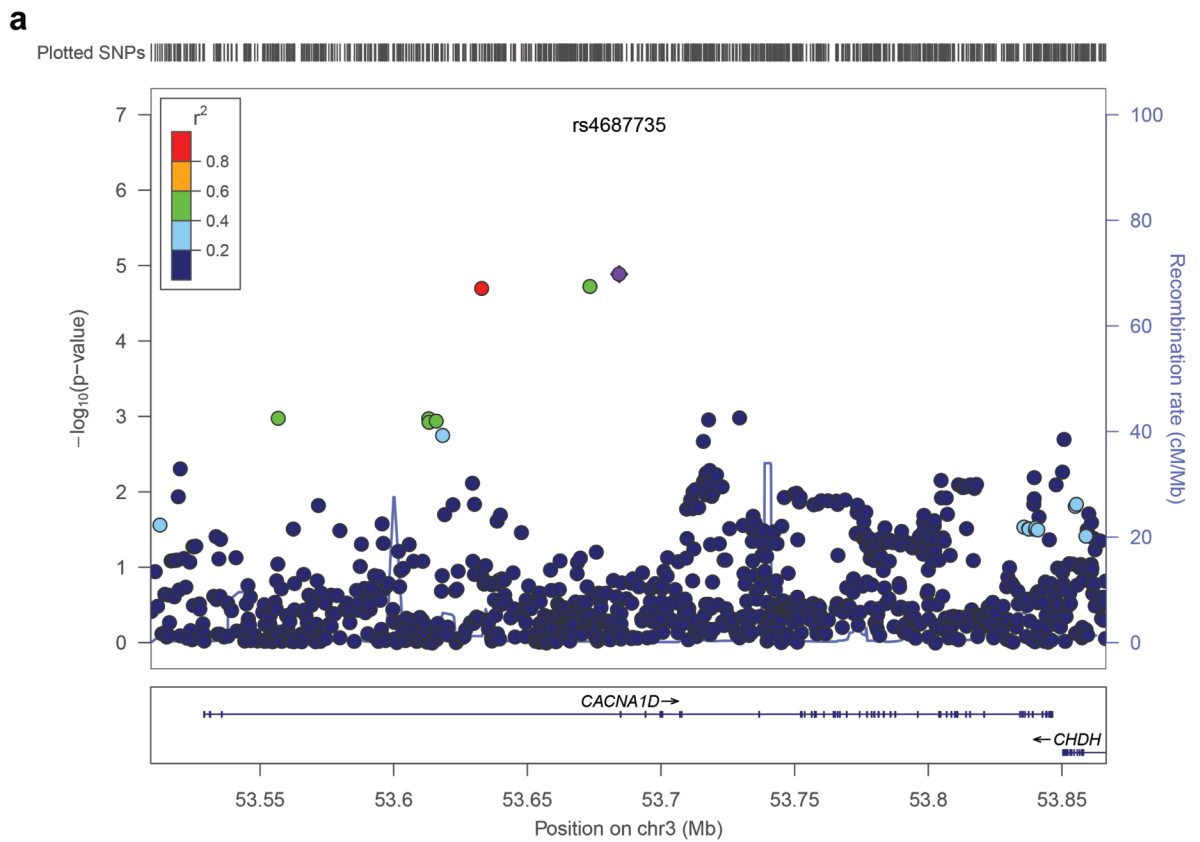


Figure 5.

Regional association plot of SNPs in and around *CACNA1D*. (a) SNPs are plotted with their $-\log_{10}(p\text{-value})$ on the y-axis along with their physical position (NCBI build 36) on the x-axis. The SNPs are color coded according to their correlations (r^2) with the most significant SNP rs4687735 shown in purple. The light blue line and right y-axis indicates the observed recombination rates in the HapMap CEU samples.