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Reduced levels of *Cacna1c* attenuate mesolimbic dopamine system function

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Abstract

Genetic variation in *CACNA1C*, which codes for the L-type calcium channel (LTCC) $Ca_v1.2$, is associated with clinical diagnoses of bipolar disorder, depression, and schizophrenia. Dysregulation of the mesolimbic dopamine (DA) system is linked to these syndromes and LTCCs are required for normal DAergic neurotransmission between the ventral tegmental area (VTA) and nucleus accumbens (NAc). It is unclear, however, how variations in *CACNA1C* genotype, and potential subsequent changes in expression levels in these regions, modify risk. Using constitutive and conditional knockout mice, and treatment with the LTCC antagonist nimodipine, we examined the role of *Cacna1c* in DA-mediated behaviors elicited by psychomotor stimulants. Using fast-scan cyclic voltammetry (FSCV), DA release and reuptake in the NAc were measured. We find that subsecond DA release in *Cacna1c* haploinsufficient mice lacks normal sensitivity to inhibition of the DA transporter (DAT). Constitutive haploinsufficiency of *Cacna1c* led to attenuation of hyperlocomotion following acute administration of stimulants specific to DAT, and locomotor sensitization of these mice to the DAT antagonist GBR12909 did not reach the same level as wild type mice. The maintenance of sensitization to GBR12909 was attenuated by administration of nimodipine. Sensitization to GBR12909 was attenuated in mice with reduced *Cacna1c* selectively in the VTA but not in the NAc. Our findings reveal that *Cacna1c* is crucial for normal behavioral responses to DA stimulants and that its activity in the VTA is required for behavioral sensitization. *Cacna1c* likely exerts these effects through modifications to presynaptic mesolimbic DA system function.

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INTRODUCTION

A genetic component substantially influences risk for developing mood disorders. Genome wide association studies (GWAS) have identified genetic variants in *CACNA1C* as a risk factor for the development of bipolar disorder and depression, as well as schizophrenia (Ripke *et al.*, 2011, Sklar *et al.*, 2011, Smoller *et al.*, 2013, Ripke *et al.*, 2014, Bhat *et al.*, 2012, Ferreira *et al.*, 2008, Green *et al.*, 2010, Green *et al.*, 2012, Hamshere *et al.*, 2013, Liu *et al.*, 2011, Moskvina *et al.*, 2009, Nyegaard *et al.*, 2010, Ripke *et al.*, 2013, Sklar *et al.*, 2008). Despite the significance of this human genetic finding, the mechanism by which genetic variants in *CACNA1C* modify the risk of developing a psychiatric illness remains largely unknown. *CACNA1C* codes for the α1C subunit of the $Ca_v1.2$ channel, which contains the voltage sensor, the conduction pore, and is a primary target for both drugs and second messengers acting on L-type calcium channels (LTCCs) (Catterall *et al.*, 2005). As the identified single nucleotide polymorphisms are found in an intronic (non protein-coding) region of *CACNA1C*, mechanisms whereby genetic changes modify risk are likely via altered levels of *CACNA1C* in specific regions of the brain. Studies have supported this, showing that the *CACNA1C* risk allele is associated with increased mRNA expression of *CACNA1C* in the human postmortem dorsolateral prefrontal cortex and in induced human neurons (Bigos *et al.*, 2010, Yoshimizu *et al.*, 2014). Decreased expression of *CACNA1C* was also identified in the human postmortem cerebellum and parietal cortex samples (Gershon *et al.*, 2014) and in an analysis of human brain available from multiple sources (Roussos *et al.*, 2014).

There is evidence that *Cacna1c* is involved in regulation of mesolimbic-dopamine ML-DA system mediated behaviors in rodents, including reinstatement of cocaine seeking after LTCC activation in the NAc (Anderson *et al.*, 2008), and LTCC mediated changes in calcium currents in the NAc following repeated cocaine administration (Zhang *et al.*, 2002). In rats, sensitization to amphetamine is associated with an increase in *Cacna1c* mRNA and protein in the VTA (Rajadhyaksha *et al.*, 2004). Although there is mounting evidence that normal *Cacna1c* function is important in ML-DA system mediated behaviors, the specific neural substrates through which it acts are largely unknown. ML-DA system function modulation by $Ca_v1.2$ may be related to the pathophysiology of psychiatric disorders linked to genetic changes in *CACNA1C*. Dysregulation of the ML-DA system is implicated in the expression of endophenotypes of bipolar mania and schizophrenia, as well as depression (Basar *et al.*, 2010, Grace, 2016, Nestler & Carlezon, 2006, Ryding *et al.*, 2008, Salamone *et al.*, 2016, Whitton *et al.*, 2015). Drugs that acutely increase release, or reduce reuptake of DA result in mania and psychosis phenotypes in humans (Anand *et al.*, 2000, Drevets *et al.*, 2001, Leyton *et al.*, 2002, Lieberman *et al.*, 1987, Murphy *et al.*, 1971). Antipsychotics, as well as other treatments that impede DAergic neurotransmission, diminish mania, as well as psychotic symptoms, in humans (Creese *et al.*, 1976, Mctavish *et al.*, 2001, Perlis *et al.*, 2006).

We previously found that *d*-amphetamine-induced hyperlocomotion was attenuated in mice lacking one copy of *Cacna1c* (Dao *et al.*, 2010). Here, we further characterized the role of that decreased *Cacna1c* function on ML-DA system-mediated behaviors and hypothesized that stimulant-induced DA release and/or reuptake would be modulated by *Cacna1c* levels. Using rodent models of reduced *Cacna1c* function both globally and in specific brain

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regions, we show that *Cacna1c* modulates DA dependent stimulant-induced locomotor activity and sensitization. Additionally, we used fast-scan cyclic voltammetry (FSCV) to directly examine the role of *Cacna1c* in subsecond DA release and reuptake, finding that reduced *Cacna1c* leads to an attenuated response to DA reuptake blockers following stimulant administration. Therefore we show that *Cacna1c* modulates DA reuptake and that its function in the VTA is necessary for the development of stimulant-induced sensitization.

MATERIALS AND METHODS

Animals

Male and female *Cacna1c* haploinsufficient (*Cacna1c*$^{+/-}$) founder mice were obtained from Jackson Laboratories (Bar Harbor, ME) as previously described (Dao *et al.*, 2010). *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ mice were the product of in-house breeding of *Cacna1c*$^{+/-}$ males generated in our own colony and WT C57BL/6 females obtained from Jackson Laboratories and were backcrossed for at least 10 generations (Dao *et al.*, 2010). We previously showed that *Cacna1c*$^{+/-}$ mice have ~50% decreased $Ca_v1.2$ protein levels and ~30% decrease in mRNA levels in the hippocampus, as well as decreased L-VGCC current density in CA1 compared to their wild-type littermates (Dao *et al.*, 2010, Zanos *et al.*, 2015). We previously found that there were baseline differences in locomotor activity in female, but not male, haploinsufficient mice (Dao *et al.*, 2010), consistent with an overall decrease in locomotor activity observed in Cacna1c haploinsufficient mice across 30 inbred mouse strains (Sittig *et al.*, 2016). We therefore only used male animals for experiments, as it would have been difficult to interpret any results obtained using female haploinsufficient mice, due to the baseline difference in locomotor activity. In the conditional *Cacna1c* knockout mouse line, exons 14 and 15 of *Cacna1c* are excised in the presence of Cre, leading to a premature stop codon and removing all known functional significance of the resulting protein (Jeon *et al.*, 2010). A line of conditional *Cacna1c* knockout mice (Jeon *et al.*, 2010) were also bred on a C57BL/6 background and maintained as homozygous for the floxed allele. All mice used were group housed males between 8–20 weeks of age at the time of behavioral testing and between 10–14 weeks at the time of testing in FSCV and tissue collection for immunoblotting. *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ mice used in experiments were littermates, and likewise AAV-Cre-GFP and AAV-Cre injected mice from the conditional *Cacna1c* knockout mouse line were littermates. Mice were tested during the light phase. All experimental procedures were approved by the University of Maryland Animal Care and Use Committee and were conducted in full accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Fast-Scan Cyclic Voltammetry

Carbon fiber microelectrodes were prepared as previously described (Cheer *et al.*, 2004). Stimulation electrodes were made by twisting electrodes (Plastics One) and cutting ends evenly. Ag/AgCl reference electrodes were made from 0.5 mm Ag wire (Sigma-Aldrich, St. Louis, MO, USA) through electrolysis in HCl (Sigma-Aldrich).

Voltammetry experiments were performed based on a protocol previously described (Loewinger *et al.*, 2012). Animals were anesthetized with urethane (1.5 g/kg, i.p.) and placed

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in a stereotaxic apparatus. Holes were drilled for the carbon fiber microelectrode to access the NAc (+1.2 AP, +1.1 ML from bregma), for a bipolar stimulating electrode to access the VTA (−3.1 AP; +0.7 ML from bregma), and for an Ag/AgCl reference electrode placed in the contralateral hemisphere. Once positioned, the carbon fiber electrode was held at −0.4 V in reference to the Ag/AgCl electrode. Cyclic voltammograms were collected at 10 Hz by ramping up to +1.3 V and back in a triangular fashion at 400 V/s. Stimulation, voltage, and data collection were controlled through the Tarheel Echem suite (University of North Carolina, Chapel Hill, NC, USA). After experimentation, changes in electrical current were converted to DA concentration changes via post-calibration of the carbon fiber electrodes to a known concentration of DA.

To determine DA release and reuptake following dopamine transporter (DAT) blockade, peak amplitude and decay rates following a 1-second, 60Hz, 300 μA stimulation were recorded following a 5 ml/kg i.p. saline injection and at 21 minutes following an i.p. injection of GBR12909 (16mg/kg) (Sigma-Aldrich). To determine decay rate, % of peak DA concentration vs. time plots were exported to Prism version 5 (Graph Pad, La Jolla, CA, USA). One-phase decay regression lines were fit to the data and software generated values were determined for the decay constant tau. Tau indicates the time at which DA levels have dropped to 37% of the maximum, and was used as a measure of DA reuptake (Yorgason *et al.*, 2011).

Western Blots

Western blots were performed using a previously published protocol (Gould *et al.*, 2004). Immediately following decapitation the brain was sectioned into 1.0mm slices using a matrix (ASI Instruments, Warren, MI, USA) and the NAc was obtained using a 1.5mm punch (Miltex, Inc., York, PA, USA). Nucleus accumbens tissue samples from homozygous dopamine transporter knock-out ($DAT^{-/-}$) and wild-type mice ($DAT^{+/+}$) (Giros *et al.*, 1996) were obtained from Dr. Sara Jones. Samples were homogenized in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). The homogenates were centrifuged at 12000g for 20 minutes at 4°C. Protein concentrations were determined using a BCA assay (Peirce Biotechnology, Inc., Rockford, IL, USA). For immunoblotting, 1ug of each sample was loaded on to a 4–12% Bis-Tris gel (Life Technologies, Grand Island, NY, USA) and transferred onto PVDF membranes (Life Technologies). Membranes were incubated overnight at 4°C with rat anti-DAT at a 1:4 000 dilution (Millipore MAB369) and rabbit anti-GAPDH at a 1:40 000 dilution (Millipore #5174) (EMD Millipore, Billerica, MA, USA). Membranes were washed and incubated with HRP-tagged anti-rat (Cell Signaling Technology Inc, Danvers, MA) and anti-rabbit (KPL, Inc., Gaithersburg, MD, USA) secondary antibodies, visualized using a chemiluminescence reaction (Clarity™ Western ECL Substrate, Bio-Rad Laboratories, Inc., Hercules, CA, USA), and quantified using densitometry (Image J (Schneider *et al.*, 2012)). Results are expressed as relative optical density with DAT values normalized to GAPDH.

Virus Injections

Mice were anesthetized with isoflurane and stereotaxically received an injection of 0.7μl AAV-CMV-Cre-GFP or AAV-CMV-GFP (UNC Vector Core, Chapel Hill, NC, USA)

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bilaterally into the NAc (+1.6 anterior/posterior, +1.5 lateral, and −4.4 dorsal/ventral, 10° angle) or the VTA (−3.2 anterior/posterior, +1.0 lateral, and −4.6 dorsal/ventral, 7° angle). Injections were performed at a rate of 0.1ul/minute and the needle was left in place for 10 minutes prior to being removed. Following injections, a two-week recovery period was given prior to experiments. Following experiments, mice were euthanized. Immediately following decapitation the brain was sectioned into 1.0 mm slices using a matrix (ASI Instruments), and placed in cold PBS. Brain slices were then visualized through a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). Mice that did not show fluorescence bilaterally in the targeted structures were excluded from the results. In the NAc injected group, this included 9 mice, and in the VTA injected group this included 6 mice.

qPCR and Immunohistochemistry

mRNA was extracted from 1.5mm tissue punches from AAV-injected mice. Tissue samples were homogenized in RNAzol RT (Sigma-Aldrich) using BashingBead lysis tubes (Zymo Research Corporation, Irvine, CA, USA) in a disrupter genie (Scientific Industries, Bohemia, NY, USA) for 10 minutes at 3000RPM. mRNA was isolated and DNAse treated using the Directzol RNA mini prep kit, according to manufacturer directions (Zymo Research). Using an iScript cDNA Synthesis Kit (Bio-Rad), total RNA was reverse transcribed into cDNA. Real-time RT-PCR was conducted using a SensiFast SYBER Lo-ROX Kit (Bioline, Taunton, MA, USA) in a 15μl reaction. A primer pair for the target gene, *Cacna1c* (5′-GTGCTGAGATGTGTGCGGTTG-3′, 5′-GCACTGAGTTCAGCAAGGATGC-3′), as well as the control genes Tfrc (5′-TGCTAATCCAATTGCTGTCTCT-3′, 5′-TGGATAAAGTTGTCCTTGGTACT-3′), and Rplp0 (5′-GCACAGTGACCTCACACG-3′, 5′-AGAAACTGCTGCCTCACATC-3′) were used (Integrated DNA Technologies, Coralville, IA, USA). The PCR reactions were run on a ViiA 7 Real-Time PCR System (Life Technologies) with a reaction volume of 15μl and an annealing temperature of 60°C. ViiA 7 software (Life Technologies) was used to determine Ct values. *Cacna1c* levels were normalized to the mean of Tfrc and Rplp0, and fold difference was determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Three mice that had *Cacna1c* expression levels that were two standard deviations from the mean in the NAc were excluded from the results.

Microscopy

30μm coronal sections of paraformaldehyde perfused brains were cut in a cryostat and placed in 1x PBS. Sections were then blocked in 20% Triton X-100 (Sigma-Aldrich) for 30 minutes and incubated overnight with primary antibody (Chicken anti-GFP, 1:4 000, Aves Labs, Inc., Tigard, OR, USA) at room temperature. Sections were then washed and incubated in secondary antibody for two hours at room temperature (Donkey anti-Chicken Alexa-488 Green, 1:1 000, Life Technologies), mounted and cover slipped. After drying, sections were visualized under a confocal microscope (Olympus Fluoview) and images were obtained.

Acute locomotor response to stimulants

Mice were habituated to an open field (50x50cm; illuminated at 30 lux) for 30 minutes, after which they received an injection of *d*-amphetamine (2 mg/kg i.p.), cocaine (10 mg/kg s.c.),

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GBR12909 (16 mg/kg i.p.), or MK-801 (0.3 mg/kg i.p.) (Sigma-Aldrich, St. Louis, MO) and were returned to the open field for an additional 45 (*d*-amphetamine and cocaine) or 90 (GBR12909 and MK-801) minutes. Doses and route of administration were based on those established previously in the literature as resulting in a moderate hyperlocomotor response, in order to allow a further increase to be possible without inducing stereotypic behaviors. (Hirabayashi *et al.*, 1991, Liljequist *et al.*, 1991, Mcnamara *et al.*, 2006, Young *et al.*, 2010). All compounds were dissolved in 0.9% saline on the day of testing. Distance travelled was assessed using TopScan tracking software (CleverSys, Inc., Reston, VA, USA).

Sensitization to GBR12909

GBR12909, unlike amphetamine and cocaine, works specifically through blockade of the DAT (Andersen, 1989, Heikkila & Manzino, 1984). GBR12909 was therefore used to specifically evaluate activity at the DAT, eliminating confounding effects of drugs such as amphetamine or cocaine that affect other monoamine transporters. Mice were tested for GBR12909-induced locomotor sensitization in an open field (50 cm x 50 cm) illuminated at 30 lux. During the first three days of testing, mice were habituated to the open field following saline injections. Mice were then administered 16 mg/kg GBR 12909 (Sigma-Aldrich) over six consecutive sensitization days. In experiments where nimodipine was used, mice were habituated to the open field following saline injections for three (4 mg/kg) or four (6 mg/kg) days and on the fourth (or fifth) day were given a nimodipine (Alexis Biochemicals, San Diego, CA, USA) injection (4mg/kg or 6mg/kg, suspended in 20% DMSO (Sigma-Aldrich), 1.5% Tween-80 (Sigma-Aldrich) and saline) or vehicle. Over six consecutive sensitization days mice received 4mg/kg or 6 mg/kg nimodipine or vehicle once per day, were returned to the home cage, and after 30 minutes received 16 mg/kg GBR12909 and were placed in the open field. In a protocol based on that used by Giordano *et al.* 2010, the mice in the experiment using 4mg/kg nimodipine were tested again at 1 week, 3 weeks, and 4 weeks after the last sensitization day During the 4-week test, half of the mice previously treated with nimodipine received a vehicle injection and half of the mice previously treated with vehicle received 4 mg/kg nimodipine prior to administration of GBR12909. The low volume of DMSO included in the nimodipine vehicle did not lead to any locomotor changes in control experiments. All sessions were one hour, and distance travelled was analyzed using CleverSys tracking software (CleverSys, Inc.).

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Software). The statistics used were two-tailed *t* test or repeated measure two-way ANOVA, either paired or unpaired depending on the experimental design, and *post hoc* comparisons utilized the Bonferroni method. The results of final (4 week) challenge using 4 mg/kg nimodipine were analyzed with a planned comparison *t* test. Data are reported as mean ± SEM and $p < 0.05$ was considered significant.

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RESULTS

DA release and reuptake in Cacna1c$^{+/+}$ and Cacna1c$^{+/-}$ mice

Using FSCV, we assessed DA release and reuptake in the NAc following electrical stimulation in the VTA of *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ mice following saline and GBR12909 administration (Figure 1A). Administration of GBR12909 led to increased extracellular DA concentrations (Figure 1A, B) and slowed DA reuptake compared to saline administration (Figure 1A, C). This is indicated by an overall significant increase in DA following a 300 μA stimulation (Figure 1B; $F(1,10) = 11.82$, $p < 0.01$) and increased decay rate (tau; Figure 1C; $F(1,10) = 8.71$, $p < 0.05$). Additionally, there was a significant overall effect of genotype on tau following GBR12909 administration (Figure 1C; $F(1, 10) = 5.65$, $p < 0.05$). There was no effect of genotype on DA levels ($F(1, 10) = 0.36$, $p = 0.56$), and no significant genotype by drug interaction for increase in DA (Figure 1B; $F(1, 10) = 0.31$, $p = 0.59$) or increase in tau (Figure 1C; $F(1, 10) = 1.60$, $p = 0.23$). While *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ mice had similar levels of DA reuptake following saline administration, an exploratory Bonferroni analysis indicated that *Cacna1c*$^{+/+}$ mice had a significantly higher tau value compared to *Cacna1c*$^{+/-}$ mice only following GBR12909 administration ($p < 0.05$), indicating that *Cacna1c* haploinsufficiency is associated with attenuated effects of GBR12909 on DA reuptake (Figure 1C).

DAT protein levels in Cacna1c$^{+/+}$ and Cacna1c$^{+/-}$ mice

Both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ mice had similar levels of DAT protein in the NAc as assessed by western blot (Figure 1D, E; $t(14) = 1.01$, $p = .33$).

Response to acute psychostimulant administration in Cacna1c$^{+/+}$ and Cacna1c$^{+/-}$ mice

Reduction of sensitivity to DAT blockade in *Cacna1c*$^{+/-}$ mice predicts an effect of *Cacna1c* haploinsufficiency on stimulant-induced behaviors. Acute psychostimulant-induced hyperlocomotion was assessed in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ mice. There were no significant baseline differences between genotypes during a 30-minute habituation in the open field (Figure 2 A–D). Compared with *Cacna1c*$^{+/+}$ mice, *Cacna1c*$^{+/-}$ mice displayed a significant decrease in hyperlocomotor response following administration of *d*-amphetamine (Figure 2A; $F(1,14) = 8.12$, $p < 0.05$) and cocaine (Figure 2B; $F(1,29) = 8.31$, $p < 0.01$). As both of these stimulants have non-specific actions on multiple monoamine neurotransmitters, we also assessed hyperlocomotion following administration of the specific DAT inhibitor GBR12909. There was a significant difference in hyperlocomotor response to GBR12909 in *Cacna1c*$^{+/-}$ mice compared to that observed in *Cacna1c*$^{+/+}$ mice (Figure 2C; $F(1,14) = 4.60$, $p < 0.05$). In contrast, there was no significant difference in hyperlocomotor activity between genotypes following administration of the NMDA receptor antagonist MK-801 (Figure 2D; $F(1,13) = 0.92$, $p = 0.3546$). Following administration of all psychostimulants, two-way ANOVAs further revealed there was a significant overall effect of day (*d*-amphetamine (Figure 2A) $F(8, 112) = 4.99$, $p < 0.05$; cocaine (Figure 2B) $F(8, 232) = 2.96$, $p < 0.01$; GBR12909 (Figure 2C) $F(17, 238) = 2.54$, $p < 0.001$; MK-801 (Figure 2D) $F(17, 221) = 68.52$, $p < 0.0001$) and a significant interaction between time and genotype following cocaine (Figure 2B; $F(8, 232) = 12.68$, $p < 0.0001$) and GBR12909 (Figure 2C; $F(17, 238) = 2.01$, $p < 0.05$) administration. There was no significant interaction following *d*-

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amphetamine (Figure 2A; $F(8, 112) = 0.72$, $p = 0.67$) or MK-801 (Figure 2D; $F(17, 221) = 0.49$, $p = 0.96$) administration.

Sensitization to GBR12909 in Cacna1c$^{+/+}$ and Cacna1c$^{+/-}$ mice

Compared to *Cacna1c*$^{+/+}$ mice, *Cacna1c*$^{+/-}$ mice displayed a different hyperlocomotor activity in response to the specific DAT inhibitor GBR12909, which was maintained after repeated administration. There was no significant difference in baseline locomotor activity during the habituation period (Figure 3A; Day 1–3). However, *Cacna1c*$^{+/-}$ mice displayed a reduced hyperlocomotor response to GBR12909 during the sensitization period. A repeated measures two-way ANOVA revealed a significant overall effect of day (Figure 3A; $F(5, 105) = 26.11$, $p < 0.0001$) and genotype (Figure 3A; $F(8,21) = 3.32$, $p < 0.01$), with no significant interaction ($F(5, 105) = .29$, $p = 0.92$) . The reduced response to GBR12909 in *Cacna1c*$^{+/-}$ mice is evident at the beginning of treatment, and is most apparent on the second through sixth day of sensitization (Figure 3A; Day 5–9).

Locomotor responses to GBR12909 in mice with a pharmacological blockade of LTCCs

The LTCC antagonist nimodipine (6 or 4 mg/kg) was administered 30 minutes prior to administration of GBR12909 during the sensitization period. There were no baseline differences during habituation to the open field between groups (Figure 3B; Day 1–4; Figure 3C; Day 1–3) or following 6 mg/kg or 4 mg/kg nimodipine alone (Figure 3B, Day 5; Figure 3C; Day 4). Two-way repeated measures ANOVA revealed that there was a significant overall effect of GBR12909 treatment over days ($F(5, 100) = 23.2$, $p < 0.0001$), an overall effect of nimodipine treatment ($F(1, 20) = 19.41$, $p < 0.001$), and an no significant interaction ($F(5, 100) = 0.69$, $p = 0.63$) in mice treated with vehicle or 6 mg/kg nimodipine prior to GBR12909. In mice treated with vehicle or 4 mg/kg nimodipine prior to GBR12909 a two-way repeated measures ANOVA revealed a significant overall effect of GBR12909 treatment over days ($F(7, 154) = 28.59$, $p < 0.0001$), a significant overall effect of nimodipine treatment ($F(1, 22) = 6.40$, $p < 0.05$) and a significant interaction ($F(7, 154) = 7.48$, $p < 0.0001$). One week following the last sensitization day, mice were challenged with GBR12909 following treatment with vehicle or 4 mg/kg nimodipine. Bonferroni post-hoc tests revealed that mice treated with 4 mg/kg nimodipine manifested an attenuated sensitization to GBR12909 compared to vehicle treated mice (Figure 3C, $p < 0.0001$). At the three-week challenge, mice treated with 4 mg/kg nimodipine again manifested attenuated sensitization (Figure 3C, $p < 0.0001$).

At four weeks following the initial sensitization procedure half of the mice previously treated with nimodipine received a vehicle injection and half of the mice previously treated with vehicle received 4 mg/kg nimodipine prior to administration of GBR12909. The other half received the previously assigned treatment. As outlined in Table 1, planned comparison *t*-tests revealed that mice that were sensitized to GBR12909 with treatment of 4 mg/kg nimodipine, but were given vehicle prior to the four week GBR12909 challenge dose displayed no significant difference in locomotor activity from mice that received only vehicle during sensitization and challenge and displayed a significant difference from mice that received nimodipine both during sensitization and prior to the 4-week challenge. Mice that had been treated previously with vehicle, but that received 4 mg/kg nimodipine prior to

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GBR12909 on the challenge day showed no significant difference in locomotor activity compared to mice that received only nimodipine injections throughout the study, but manifested attenuated sensitization to GBR12909 compared to mice that received only vehicle during sensitization and challenge, Mice that were treated with nimodipine both during sensitization and prior to the 4-week challenge manifested significantly attenuated locomotor activity compared to mice given only vehicle injections. Furthermore, mice that were sensitized to GBR12909 with treatment of 4 mg/kg nimodipine, but were given vehicle prior to the four week GBR12909 challenge dose manifested attenuated locomoter activity compared to mice that were treated with nimodipine during sensitization but were given a vehicle injection prior to the 4-week GBR-12909 challenge. These findings suggest that pharmacological blockade of LTCCs at a higher dose leads to an attenuation of locomotor response to GBR12909 similar to that seen in *Cacna1c*$^{+/-}$ mice (Figure 3B); however a lower dose does not result in the acute reduction of GBR12909 induced locoomotor activity. Instead, similar to findings of Giordano *et al.* 2010, this dose attenuates the maintenance of sensitization, indicating an effect of $Ca_v1.2$ blockade on sensitization to psychostimulants even in the absence of an acute effect (Figure 3C).

Locomotor responses to GBR12909 in mice with a knock-down of Cacna1c in the NAc

To determine if the attenuated response to GBR12909 was mediated by *Cacna1c* in the NAc, sensitization to GBR12909 in mice with virally mediated knock-down of *Cacna1c* in the NAc was measured. AAV-Cre-GFP was expressed in the NAc of mice containing a floxed *Cacna1c* allele. To control for any non-specific effects of the viral injection, control mice received a GFP-only expressing virus. Expression of GFP-tagged Cre in the NAc was confirmed by the use of fluorescence microscopy to visualize GFP expression in neurons in the NAc (Figure 4A). Injection of Cre-GFP significantly reduced the level of *Cacna1c* expression, as measured by qPCR, in the NAc by ~ 40% ($t(17) = 4.43$, $p < 0.001$).

In NAc-injected mice, Cre-GFP injection did not lead to baseline differences in locomotor activity during the habituation period compared to GFP-only injected mice (Figure 4B).

During the sensitization period, there was a significant effect of repeated GBR12909 administration ($F(8, 96) = 78.44$, $p < 0.0001$), however there was no overall effect of *Cacna1c* knockdown in the NAc ($F(1,12) = 0.054$, $p = 0.82$) and no significant interaction ($F(8, 96) = 0.43$, $p = 0.90$ (Figure 4B).

Locomotor responses to GBR12909 in mice with a knock down of Cacna1c in the VTA

Expression of GFP- tagged Cre in the VTA was indicated by the use of fluorescence microscopy to visualize GFP expression in neuronal cells in the VTA (Figure 4C). While each injection was visually evaluated, we note that an unavailability of VTA RNA from these samples prevented qPCR analysis of the level of *Cacna1c* knockdown as had been performed with the NAc samples. Compared to GFP-only injected mice, mice with a VTA knockdown of *Cacna1c* displayed a significant difference in sensitization to GBR12909, as revealed by a significant interaction between day and injection type (Figure 4D; $F(8,13) = 3.97$, $p < 0.001$). There was an overall significant effect of day ($F(5, 65) = 8.54$, $p < 0.0001$, but no overall effect of *Cacna1c* knockdown in the VTA ($F(1, 13) = 3.41$, $p = 0.09$).

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DISCUSSION

We have shown that *Cacna1c* haploinsufficient mice manifest an attenuated response to the specific DAT blocker GBR12909, indicating that *Cacna1c* critically regulates DA terminal function. We also demonstrate that mice with one functional allele of the *Cacna1c* gene manifest a diminished locomotor response to acute and chronic administration of DA elevating psychostimulants, an effect that is replicated with pharmacological blockade of LTCCs. A lower dose of pharmacological LTCC blockade results in attenuation of a maintained locomotor sensitization response, without modifying acute responses to GBR12909. Moreover, reduced levels of *Cacna1c* in the VTA, but not the NAc, led to attenuation of sensitization to GBR12909. Overall, the data in this study indicate that *Cacna1c* modulates ML-DA dependent behavior and DA terminal dynamics, potentially through altered DA reuptake by VTA neurons.

Stimulant-induced blockade of DAT in VTA DA synaptic terminals within the NAc leads to elevated DA levels, slowed DA reuptake, and hyperlocomotion in rodents. The hyperlocomotor response induced by acute psychostimulants and sensitization, as well as changes in DA release and reuptake, are widely used to model aspects of mania and psychosis in rodents (Einat & Manji, 2006, Nestler & Hyman, 2010, O'donnell & Gould, 2007). In this study, we show that *Cacna1c* has a role in subsecond DA reuptake kinetics, in particular DA reuptake in VTA neurons projecting to the NAc. While *Cacna1c* is not expressed exclusively in neurons (*Cacna1c* expression has also been identified in astrocytic cells (D'ascenzo *et al.*, 2004, Latour *et al.*, 2003)) DA reuptake through the DAT in response to stimulant administration occurs primarily through neuronal, and not astrocyte expressed DAT (Takeda *et al.*, 2002). GBR12909, unlike amphetamine and cocaine, works specifically through blockade of the DAT (Andersen, 1989, Heikkila & Manzino, 1984), giving increased specificity for interpreting the results of DAT inhibition on DA terminal dynamics. Therefore, GBR12909 was used to evaluate specific activity at the DAT, eliminating confounding effects of drugs such as amphetamine or cocaine that affect other monoamine transporters. GBR12909 administration produced the expected decrease in reuptake in wild-type mice; however, in mice with genetically reduced levels of *Cacna1c* this effect was attenuated.

One mechanism by which the behavioral effects of DAT blockade may be attenuated is through altered levels of the DAT protein. Animal models in which DAT expression level is changed have an altered response to psychostimulants. Amphetamine and cocaine-induced hyperlocomotion is absent in mice with a mutation in a DAT gene that leads to deletion of DAT (Giros *et al.*, 1996) and cocaine does not slow DA reuptake in DAT knockout mice (Budygin *et al.*, 2002). Additionally, overexpression of DAT leads to an increase in amphetamine-induced response in mice (Salahpour *et al.*, 2008). Our western blot results indicate that the overall level of DAT is not significantly altered in *Cacna1c*$^{+/-}$ mice, however the finding that GBR12909 does not block reuptake of DA in *Cacna1c*$^{+/-}$ mice to the same degree as it does in *Cacna1c*$^{+/+}$ mice indicates that DAT function is likely altered by *Cacna1c* haploinsufficiency in some way other than regulation of total DAT protein levels. Further studies are needed to determine how *Cacna1c*, which is localized mainly somatodendritically (Hell *et al.*, 1993, Leitch *et al.*, 2009), may alter terminal DAT function.

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Reduced stimulant-induced slowed DA reuptake in *Cacna1c* haploinsufficient mice indicates that there may be an effect of reduced *Cacna1c* levels on stimulant-induced locomotor activity. While stimulant-induced locomotion is a rudimentary model of mania in rodents, it has been commonly used to assess mania related behaviors due to a lack of alternative measures (Einat & Manji, 2006, Nestler & Hyman, 2010, O'donnell & Gould, 2007). We have previously shown that male *Cacna1c* haploinsufficient mice manifest a reduced hyperlocomotor response to acute and chronic amphetamine administration. While female *Cacna1c* haploinsufficient mice also manifest reduced stimulant induced hyperlocomotion, baseline locomotor differences would confound our capacity to interpret results (Dao *et al.*, 2010). In the present study we replicate our previous finding in male mice, as well as show that *Cacna1c* haploinsufficiency leads to an attenuated hyperlocomotor response to both acute and repeated administration of several psychostimulants. This suggests that reduced levels of *Cacna1c* likely represent a protective phenotype against mania related behavior. While the dose of psychostimulants used in this study were selected to produce a moderate effect based on what has been published previously in the literature (Hirabayashi *et al.*, 1991, Liljequist *et al.*, 1991, Mcnamara *et al.*, 2006, Young *et al.*, 2010), the use of only one dose of psychostimulant is a limitation. Previous studies have indicated that calcium influx through brain LTCCs is necessary for mediating psychostimulant-induced behavior and *Cacna1c* is particularly important in sensitization to psychostimulants. In rats, the calcium channel blocker flunarizine attenuated the increased locomotor response induced by chronic cocaine administration (Mills *et al.*, 2007) and nifedipine blocks expression of amphetamine or cocaine induced sensitization (Giordano *et al.*, 2010). Following sensitization to psychostimulants, LTCC dependent calcium uptake increases (Mills *et al.*, 2007) and signaling pathways downstream of *Cacna1c* are activated in the NAc (Giordano *et al.*, 2010).

The locomotor response to psychostimulants is largely mediated through the ML-DA pathway, although in the NAc, both DAergic and glutamatergic inputs are important for the psychostimulant induced response (Vanderschuren & Kalivas, 2000, Wolf & Khansa, 1991). Our finding that there was no attenuation of the hyperlocomotor response induced by the NMDA receptor antagonist MK-801 in *Cacna1c*$^{+/-}$ mice indicates that DA, rather than glutamate, system function is likely altered as a result of reduced *Cacan1c*. Previous studies support this conclusion, showing that LTCCs mediate cocaine-induced elevations of monoamine levels in terminal regions (Mills *et al.*, 2007, Okita *et al.*, 2000).

In the present study, we further identified a role for *Cacna1c* in regions of the ML-DA circuit that underlie sensitization to GBR12909. While reduction of *Cacna1c* in the NAc was not sufficient to attenuate sensitization to GBR12909, we found that when *Cacna1c* was reduced in the VTA, sensitization above the initial acute response to GBR12909 was completely absent. This result suggests that *Cacna1c* in the VTA is essential for normal psychostimulant induced sensitization. This finding is consistent with previous research, which has found that pharmacological blockade of LTCCs directly in the VTA blocks sensitization (Licata & Pierce, 2003), while activation of LTCCs augments sensitization (Licata *et al.*, 2000). Furthermore, sensitization leads to increased expression of *Cacna1c* mRNA and $Ca_V1.2$ protein in the VTA (Rajadhyaksha *et al.*, 2004). While *Cacna1c* does influence the downstream effects of psychostimulant exposure in the NAc, such as through increases in calcium uptake increases and activation of signaling pathways (Mills, Ansah et al. 2007,

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Giordano, Tropea et al. 2010), blocking LTCCs exclusively in the NAc does not lead to attenuation of the psychostimulant induced hyperlocomotion (Pierce, Quick et al. 1998). Our findings, combined with those of previous studies and our own FSCV results, indicate that *Cacna1c* in the VTA may have a significant role in presynaptic regulation of the response to psychostimulants.

Our finding that selective reduction of *Cacna1c* levels in only the VTA or NAc did not modify acute locomotor responses to GBR12909 is consistent with previous studies that have found no acute locomotor response to cocaine following administration of an LTCC antagonist to the NAc (Karler *et al.*, 1991, Pierce *et al.*, 1998). It is of interest to note the difference in the response to chronic psychostimulant administration in haploinsufficient mice compared to mice with conditional knockdown of *Cacna1c*. In *Cacna1c*$^{+/-}$ mice, sensitization was reduced, but not completely blocked, as opposed to the complete lack of sensitized response when *Cacna1c* was selectively knocked down in the VTA. In *Cacna1c*$^{+/-}$ mice, the different outcome could be due to the involvement of reduced *Cacna1c* in additional circuits contributing to psychostimulant induced behavior, or the constitutive nature of the knock out in *Cacna1c*$^{+/-}$ mice leading to compensatory mechanisms that modulate aspects of this behavior. In support of this, a low dose of nimodipine is sufficient to selectively block the maintenance of sensitization to GBR12909; however when a higher level of LTCC blockade is present, the locomotor response is attenuated both acutely and during sensitization.

The results from this study demonstrate that when levels of *Cacna1c* are reduced, a potential protective phenotype against mania- or psychosis-related behavior emerges. In addition, these studies indicate that the attenuation of mania- or psychosis-related behavior following reduction of *Cacna1c* levels is due at least in part to altered terminal DA reuptake. As dysregulation of the DAergic system contributes to the etiology of mood disorders, the knowledge that *Cacna1c* is important for normal function of the ML-DA system has considerable implications for our understanding of how *Cacna1c* may confer risk. Additional studies are needed to further understand how DA reuptake is altered following sensitization to GBR12909, as well as the specific mechanism through which *Cacna1c* modifies DA reuptake. As the risk associated SNPs identified in *CACNA1C* likely influence risk through altered levels of expression of *CACNA1C* (Bigos *et al.*, 2010, Eckart *et al.*, 2016, Gershon *et al.*, 2014, Roussos *et al.*, 2014, Yoshimizu *et al.*, 2014), these findings are important steps toward understanding the ramifications of altered expression in brain regions particularly relevant to psychiatric disorders.

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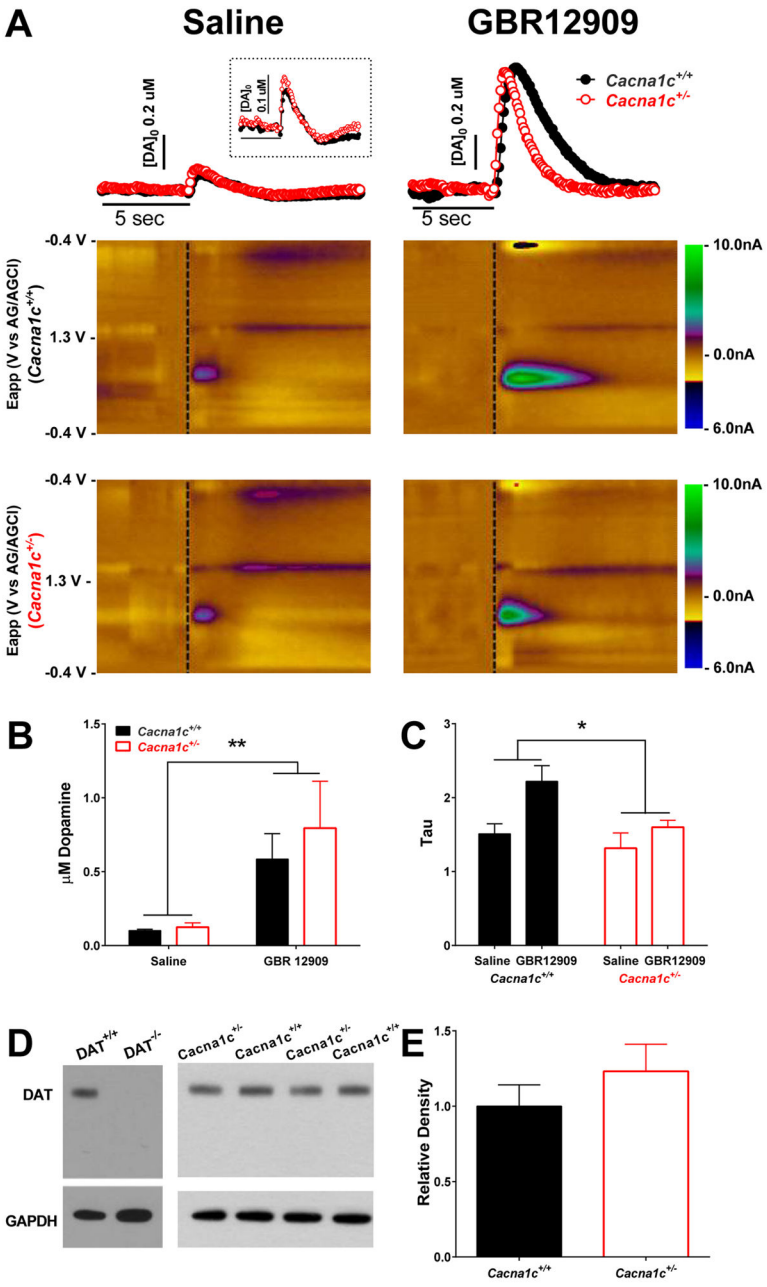

Figure 1. Dopamine release and reuptake following saline and GBR12909 administration

(A) Representative concentration trace over time (top) and color plots (middle, bottom) showing voltammetric current (z-axis) against applied scan potential (y-axis) and time (x-axis) of dopamine (DA) release in *Cacna1c*$^{+/+}$ (center) and *Cacna1c*$^{+/-}$ (bottom) mice following saline (top left) and GBR12909 (top right) administration. (B) Administration of GBR12909 (n=6/group) led to an overall significant increase in DA release in both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ mice (p<0.01). (C) Administration of GBR12909 led to an overall significant increase in tau (p<0.05), and an overall significant effect of genotype on DA reuptake (p<0.05). (D, E) There was no significant effect of genotype on dopamine

transporter (DAT) protein levels in the nucleus accumbens (p=.33) as measured by immunoblot. n=8/group.

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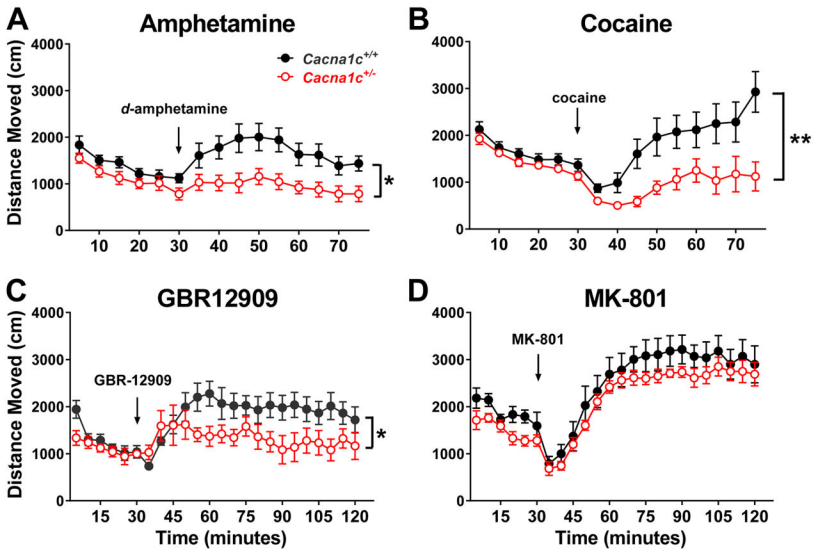

Figure 2. Altered hyperlocomotor response to dopamine-acting stimulants in *Cacna1c*$^{+/-}$ mice

Cacna1c$^{+/+}$ and *Cacna1c*$^{+/-}$ mice were habituated to the open field for 30 minutes and then received either (A) *d*-amphetamine (2 mg/kg i.p., n=8/group), (B) cocaine (10 mg/kg s.c., n=15–16/group), (C) GBR12909 (16 mg/kg i.p., n=8/group), or (D) MK-801 (0.3 mg/kg i.p., n=7–8/group). * indicates an overall significant effect of genotype during the time following stimulant administration. *p<0.05, **p<0.01.

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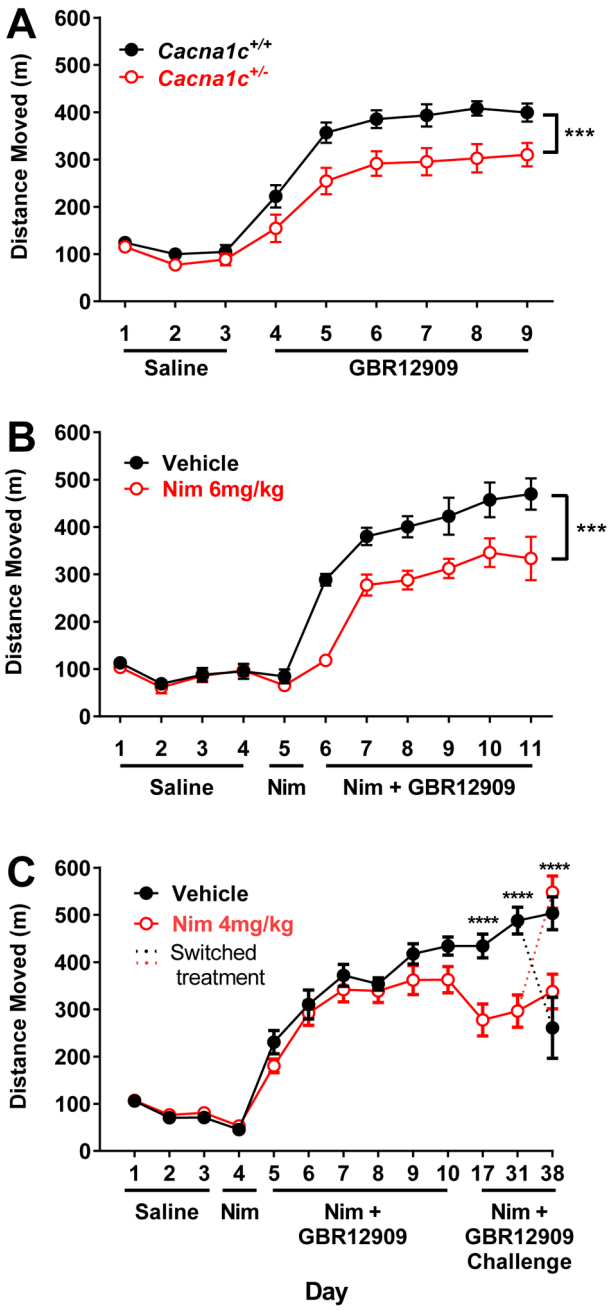

Figure 3. Altered locomotor sensitization to GBR12909 in *Cacna1c*$^{+/-}$ mice and following L-type calcium channel blockade

Locomotor activity was measured during habituation to saline injections and sensitization to GBR12909 injections. (A) In *Cacna1c*$^{+/+}$ compared to *Cacna1c*$^{+/-}$ mice there is a significant genotype and day interaction on locomotor sensitization to GBR12909 (p<0.001). *Post-hoc* tests revealed a significant difference in locomotor activity on the second through sixth days of GBR12909 administration. (B) There is a significant overall effect of 6 mg/kg nimodipine (Nim) on locomotor sensitization to GBR12909 (p<0.0001). *Post-hoc* tests revealed a significant difference in locomotor activity on the first through sixth days of GBR12909

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administration. (C) There is a significant overall effect of 4 mg/kg nimodipine on locomotor sensitization to GBR12909 ($p<0.0001$). Bonferroni post-hoc tests revealed a significant difference in locomotor activity during 1 week and 3 week challenges. On week 4, planned comparisons *t*-tests revealed that mice whose treatment was switched to nimodipine from vehicle or vehicle from nimodipine displayed an attenuation of sensitization or normal sensitization, respectively. * indicates a significant effect of genotype or nimodipine. *$p<0.05$, **$p<0.01$, ***$p<0.001$,****p<0.0001 n= 11–12/group.

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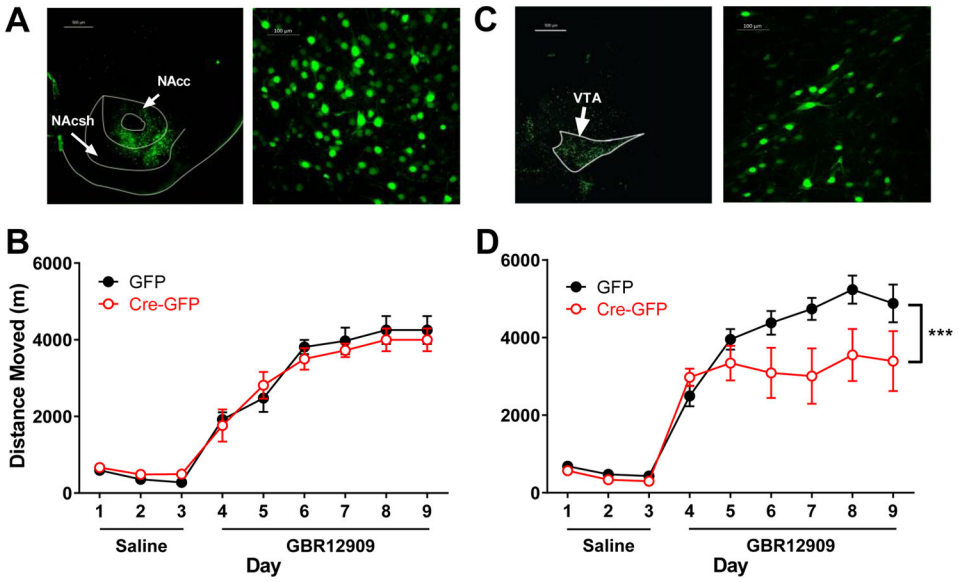

Figure 4. Attenuated sensitization to GBR12909 following knock-out of *Cacna1c* in the ventral tegmental area

Conditional *Cacna1c* knockout mice received AAV-CMV-Cre-GFP or AAV-CMV-GFP bilaterally in the nucleus accumbens (NAc) or ventral tegmental area (VTA). (A) Representative image of GFP fluorescent tag indicates injection region (left) and cell specificity (right) in the NAc. (B) In NAc injected mice, there was no effect of Cre injection on sensitization. (C) Representative image of GFP fluorescent tag indicates injection region (left) and cell specificity (right) in the VTA. (D) In VTA Cre-injected mice, there was a significant interaction of day and injection during sensitization ($p<0.001$). *** indicates the significant interaction, n=7/GFP group, 8/Cre-GFP group.

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Table 1

Effects of nimodipine on the maintenance of sensitization to GBR12909

Mice were sensitized to GBR12909 following administration of either vehicle (Veh) or 4 mg/kg nimodipine (Nim). At four weeks following the initial sensitization procedure half of the mice previously treated with nimodipine received a vehicle injection and half of the mice previously treated with vehicle received 4 mg/kg nimodipine prior to administration of GBR12909. Planned comparisons *t*-tests revealed that mice whose treatment was switched to nimodipine from vehicle, or vehicle from nimodipine displayed an attenuation of sensitization or normal sensitization, respectively. For all comparisons, df = 10 and n = 6/group.

	Veh-Veh	**Nim-Nim**	**Veh-Nim**	**Nim-Veh**
Veh-Veh		t=3.29, p=0.01	t=2.99, p=0.01	t=0.92, p=0.38
Nim-Nim	t=3.29, p=0.01		t=0.50, p=0.63	t=3.29, p=0.01
Veh-Nim	t=2.99, p=0.01	t=0.50, p=0.63		t=3.67, p=0.004
Nim-Veh	t=0.92, p=0.38	t=3.29, p=0.01	t=3.67, p=0.004	