Molecular Switch from L-Type $\text{Ca}_v1.3$ to $\text{Ca}_v1.2$ Ca^{2+} Channel Signaling Underlies Long-Term Psychostimulant-Induced Behavioral and Molecular Plasticity

Thomas P. Giordano,^{1,3} Thomas F. Tropea,¹ Shirish S. Satpute,³ Martina J. Sinnegger-Brauns,⁴ Joerg Striessnig,⁴ Barry E. Kosofsky,^{1,2,3} and Anjali M. Rajadhyaksha^{1,2,3}

¹Department of Pediatrics, Division of Pediatric Neurology, New York Presbyterian Hospital, ²Department of Neurology and Neuroscience, Weill Medical College of Cornell University, New York, New York 10065, ³Laboratory of Molecular and Developmental Neuroscience and Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129, and ⁴Department of Pharmacology and Toxicology, Institute of Pharmacy, and Center of Molecular Biosciences Innsbruck, University of Innsbruck, A-6020 Innsbruck, Austria

L-type Ca^{2+} channel (LTCC)-activated signaling cascades contribute significantly to psychostimulant-induced locomotor sensitization; however, the precise contribution of the two brain-specific subunits $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ remains mostly unknown. In this study, by using amphetamine and cocaine locomotor sensitization in mutant mice expressing dihydropyridine (DHP)-insensitive $\text{Ca}_v1.2$ LTCCs ($\text{Ca}_v1.2\text{DHP}^{-/-}$), we find that, as opposed to a previously identified role of the $\text{Ca}_v1.3$ subunit of LTCCs in development of sensitization, the $\text{Ca}_v1.2$ subunit mediates expression of amphetamine and cocaine sensitization when examined after a 14 d drug-free period. Molecular studies to further elucidate the role of $\text{Ca}_v1.2$ versus $\text{Ca}_v1.3$ LTCCs in activating signaling pathways in the nucleus accumbens (NAc) of drug-naïve versus drug-preexposed mice examined 14 d later revealed that an acute amphetamine and cocaine challenge in drug-naïve mice increases Ser133 cAMP response element-binding protein (CREB) phosphorylation in the NAc via $\text{Ca}_v1.3$ channels and via a dopamine D_1 -dependent mechanism, independent of the extracellular signal-regulated kinase (ERK) pathway, an important mediator of psychostimulant-induced plasticity. In contrast, in amphetamine- and cocaine-preexposed mice, an amphetamine or cocaine challenge no longer activates CREB unless $\text{Ca}_v1.2$ LTCCs are blocked. This $\text{Ca}_v1.2$ -dependent blunting of CREB activation that underlies expression of locomotor sensitization occurs only after extended drug-free periods and involves recruitment of D_1 receptors and the ERK pathway. Thus, our results demonstrate that specific LTCC subunits are required for the development ($\text{Ca}_v1.3$) versus expression ($\text{Ca}_v1.2$) of psychostimulant sensitization and that subunit-specific signaling pathways recruited by psychostimulants underlies long-term drug-induced behavioral responses.

Introduction

Repeated exposure to the psychostimulants amphetamine and cocaine results in long-lasting molecular adaptations that are hypothesized to underlie persistent drug-induced relapse to drug use, despite extended drug-free periods (Berke and Hyman, 2000; Nestler, 2001). A useful rodent model used to study drug-induced long-term plasticity is locomotor sensitization that includes the development of psychomotor sensitization, a progressive increase in locomotor activity with repeated drug administration and the expression of psychomotor sensitization, an enduring augmented locomotor response observed after a subsequent drug challenge (Kalivas and Stewart, 1991; Robinson and

Berridge, 1993). Psychomotor sensitization results, in part, from neuronal adaptations in the dopamine (cAMP) signal transduction pathway within the mesoaccumbens dopamine circuitry as well as recruitment of Ca^{2+} signaling cascades (Berke and Hyman, 2000; Nestler, 2001). Pharmacological studies using psychomotor sensitization have implicated Ca^{2+} influx via L-type Ca^{2+} channels (LTCCs) in the development and the expression of locomotor sensitization with no role in acute locomotor responses (Karler et al., 1991; Pierce and Kalivas, 1997; Pierce et al., 1998; Licata et al., 2001). Additionally, LTCCs mediate dopamine signaling (Surmeier et al., 1995; Liu and Graybiel, 1996; Rajadhyaksha et al., 1999) and activate several downstream molecular targets that are common to those activated by psychostimulants (Berke and Hyman, 2000; Rajadhyaksha and Kosofsky, 2005), thus supporting a role for LTCCs as a molecular link between recurrent psychostimulant exposure and long-lasting behavioral changes.

LTCCs are composed of the brain-specific subunits $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ (Hell et al., 1993; Sinnegger-Brauns et al., 2004) that couple synaptic activity to the activation of intracellular Ca^{2+} cascades that conduct signals to the nucleus (Deisseroth et al., 2003).

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Correspondence should be addressed to Anjali M. Rajadhyaksha, Weill Cornell Medical College, 1300 York Avenue, Box 91, New York, NY 10065. E-mail: amr2011@med.cornell.edu.

T. F. Tropea's present address: University of New England College of Osteopathic Medicine, Biddeford, ME 04005. DOI:10.1523/JNEUROSCI.2255-10.2010

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A primary intracellular LTCC target is cAMP response element-binding protein (CREB), an important mediator of psychostimulant-activated gene expression (McClung and Nestler, 2003; Renthall et al., 2009) in the nucleus accumbens (NAc), a major anatomical site responsible for psychostimulant-induced responses (Berke and Hyman, 2000; Nestler, 2001). In addition, CREB in the NAc has been found to directly alter behavioral responses to cocaine (McClung and Nestler, 2003; Carlezon et al., 2005). Another downstream LTCC target is extracellular signal-regulated kinase (ERK) (Wu et al., 2001b), a mediator of psychostimulant-induced CREB activation in the NAc (Brami-Cherrier et al., 2005) and several behavioral effects of cocaine, including conditioned place preference, and psychomotor sensitization (Lu et al., 2006; Schumann and Yaka, 2009). To date, because of the lack of Ca_v subunit-specific pharmacological agents, the functional specificity of $\text{Ca}_v1.2$ versus $\text{Ca}_v1.3$ that mediates psychostimulant-induced CREB activation remains unknown. However, the differential anatomical distribution of these subunits (Hell et al., 1993; Rajadhyaksha et al., 2004), their distinct biophysical properties (Lipscombe, 2002), and the distinct signaling molecules they associate with (Rajadhyaksha and Kosofsky, 2005; Calin-Jageman and Lee, 2008), suggest that they may contribute differentially to psychostimulant-activated changes.

In this study, we used amphetamine- and cocaine-induced locomotor sensitization and a pharmacological knock-out mouse (Fig. 1) (Sinnegger-Brauns et al., 2004) to characterize the distinct contribution of $\text{Ca}_v1.2$ versus $\text{Ca}_v1.3$ LTCCs to the development versus the long-term expression of locomotor sensitization. Additionally, we performed experiments to identify $\text{Ca}_v1.2$ versus $\text{Ca}_v1.3$ LTCC mechanisms that mediate psychostimulant activation of CREB in the drug-naïve versus the drug-preexposed NAc, a region that expresses both LTCC subunits (Rajadhyaksha et al., 2004).

Materials and Methods

Animals

For all experiments, we used 10- to 12-week-old male C57BL/6 mice (Charles River Laboratories) and $\text{Ca}_v1.2$ DHP-insensitive mice generated on the C57BL/6 background (Sinnegger-Brauns et al., 2004). Mice were group-housed, provided food and water *ad libitum*, and maintained on a 12 h light/dark cycle (from 7:00 A.M. to 7:00 P.M.). All procedures were conducted in accordance with the rules of the Weill Cornell Medical College Institutional Animal Care and Use Committee.

To generate F_2 $\text{Ca}_v1.2$ DHP-insensitive wild-type ($\text{Ca}_v1.2$ DHP $^{+/+}$) and mutant ($\text{Ca}_v1.2$ DHP $^{-/-}$) experimental mice, heterozygote ($\text{Ca}_v1.2$ DHP $^{+/-}$) male and female mice were bred. In these mutant mice, the $\text{Ca}_v1.2$ LTCC subunit has been mutated by substitution of the amino acid tyrosine for threonine at position 1066 in helix IIIS5 of exon 24 (Sinnegger-Brauns et al., 2004). Thus, the high affinity of $\text{Ca}_v1.2$ LTCCs for dihydropyridines (DHPs) is eliminated while their function is completely preserved. In wild-type mice ($\text{Ca}_v1.2$ DHP $^{+/+}$) that contain the normal $\text{Ca}_v1.2$ gene, DHPs, such as nifedipine used in this study, target both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ LTCCs (see Fig. 1A) that are the primary isoforms expressed in the brain (Hell et al., 1993; Sinnegger-Brauns et al., 2004, 2009). In mice homozygous for the mutant $\text{Ca}_v1.2$ gene ($\text{Ca}_v1.2$ DHP $^{-/-}$) that lack DHP sensitivity, treatment with DHPs specifically target the $\text{Ca}_v1.3$ subunit leaving the $\text{Ca}_v1.2$ subunit fully functional (see Fig. 1B), hence allowing for the isolation of $\text{Ca}_v1.3$ -dependent versus $\text{Ca}_v1.2$ -dependent processes. These mutant mice have normal $\text{Ca}_v1.2$ levels and channel function in both the brain and heart (Sinnegger-Brauns et al., 2004), exhibit normal basal locomotor activity (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), and are indistinguishable from wild-type littermates unless administered DHPs. Utilizing this line of mutant mice, we have per-

formed baseline experiments that have confirmed our previously published findings in $\text{Ca}_v1.3$ knock-out mice (Giordano et al., 2006) that $\text{Ca}_v1.3$ LTCCs do not play a role in acute amphetamine-induced locomotor responses but are essential for the development of amphetamine sensitization (supplemental Fig. 2A, available at www.jneurosci.org as supplemental material), observations we have now extended to include the development of cocaine sensitization (supplemental Fig. 2B, available at www.jneurosci.org as supplemental material).

Drugs

D-Amphetamine sulfate, cocaine hydrochloride, nifedipine, α -[amino [(4-aminophenyl)thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile (SL327), and 3-allyl-6-chloro-1-phenyl-1,2,4,5-tetrahydro-3-benzazepine-7,8-diol (SKF82958) were obtained from Sigma-Aldrich. D-Amphetamine, cocaine, and SKF82958 were dissolved in 0.9% saline. Nifedipine and SL327 were dissolved in 0.9% saline containing 1.5% DMSO and 1.5% Tween 80.

Amphetamine and cocaine locomotor sensitization

Locomotor sensitization was conducted as previously published (Giordano et al., 2006). Briefly, locomotor activity was measured in 27.3×27.3 cm open-field locomotor activity chambers using open-field activity software (MED Associates). Development of amphetamine and cocaine sensitization was induced in mice by repeated treatment with the respective psychostimulant, once a day for 5 d (days 1–5). On each day, mice were habituated for 30 min to the chambers followed by intraperitoneal injections of saline or psychostimulant (2 mg/kg, i.p., amphetamine, or 15 mg/kg, i.p., cocaine). Locomotor activity was measured as total distance traveled (in centimeters) for 30 min on each day. Fourteen days later (day 19), mice were tested for expression of the sensitized response after a challenge with the same psychostimulant (2 mg/kg, i.p., amphetamine, or 15 mg/kg, i.p., cocaine) or saline, and locomotor activity was measured for 30 min. The doses of amphetamine and cocaine were selected after a dose–response with 2, 4, and 6 mg/kg, intraperitoneal, amphetamine, and 10, 15, and 20 mg/kg, intraperitoneal, cocaine, respectively (data not shown).

Immunoblot analysis

To examine protein phosphorylation changes, immediately after behavioral testing, mice were killed by rapid decapitation and brains were rapidly dissected and frozen in isopentane at -40°C . Brains were sectioned in a cryostat in the coronal plane up to the rostral end of the NAc and 0.5-mm-deep bilateral tissue punches (NAc shell and core), spanning approximately $+1.7$ to $+1.2$ mm relative to bregma (Paxinos and Franklin, 2004), were obtained with a 17 gauge stainless-steel stylet. Tissue was sonicated in 1% SDS buffer in Tris-EDTA, pH 7.4, containing $1 \times$ protease inhibitor mixture (Sigma-Aldrich), 5 mM NaF, and $1 \times$ phosphatase inhibitor mixture (Sigma-Aldrich). Samples were boiled for 5 min and centrifuged at $16,100 \times g$ for 10 min. Supernatants were collected, and protein concentration was determined by BCA assay (Pierce). Twenty-five to 30 μg of protein was loaded on a 12% SDS polyacrylamide gel for both Ser133 P-CREB and Thr183/Tyr185 P-ERK1/2 immunoblots, transferred to polyvinylidene difluoride membrane, and blocked in blocking buffer (5% nonfat dried milk in 0.25 M Tris-HCl, pH 7.6, 1.37 M NaCl, 0.1% Tween) for 60 min. Membranes were probed with primary antibody to P-CREB, CREB, P-ERK1/2, and ERK, all at a dilution of 1:1000, overnight at 4°C . All antibodies were obtained from Cell Signaling Technology. Membranes were washed four times for 5 min each in blocking buffer and then incubated with goat anti-rabbit horseradish peroxidase-linked IgG (Vector Laboratories) at a dilution of 1:5000, for 1 h at room temperature. Blots were washed once for 15 min and then four times for 5 min each in Tris-buffered saline containing 0.1% Tween 20. Protein bands were detected by chemiluminescence (Western Lightning; PerkinElmer Life Sciences) and exposed to X-Omat Blue autoradiographic film (Kodak). Kaleidoscope-prestained standards (Bio-Rad) were used for protein size determination. ERK and P-ERK1 were detected at 44 kDa, and P-CREB, CREB, ERK2, and P-ERK2 at 42 kDa. For quantitation, films were scanned with an HP ScanJet 7400c scanner (Hewlett Packard) and densitometry was used. Intensity of the protein bands was measured as optical density using the NIH Image program (National Institutes of Health, Bethesda, MD). P-CREB and P-ERK2

levels were normalized to total CREB and ERK2 (which were unaltered after any of the treatments) (quantitative data not shown) levels, respectively, and calculated as a percentage of the respective control groups. We quantified P-ERK2 bands because they showed dynamic regulation of phosphorylation compared with P-ERK1.

Experiment procedures

Experiment 1. The effect of systemic pretreatment of the LTCC antagonist nifedipine on expression of amphetamine- and cocaine-induced locomotor sensitization was examined in $\text{Ca}_v1.2\text{DHP}^{+/+}$ wild-type and $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant mice. In this experiment (see Fig. 2), a factorial design that included the between-subject factors of preexposure (saline, amphetamine, or cocaine), pretreatment (vehicle, nifedipine), and challenge (saline, psychostimulant) was used to assess the effect of nifedipine on psychostimulant-induced locomotor activity. Nifedipine was used at a dose of 25 mg/kg, intraperitoneal, based on results from a dose-response with 15, 20, and 25 mg/kg, intraperitoneal, nifedipine, consistent with previously published results (Sinnegger-Brauns et al., 2004). Nifedipine was administered 20 min before psychostimulant treatment. $N = 7$ –8 mice were used for the veh-sal and nif-sal challenge groups, and $n = 10$ –12 for the veh-psychostimulant and nif-psychostimulant groups.

Experiment 2. The effect of nifedipine on amphetamine- and cocaine-induced P-CREB and P-ERK2 was examined in the NAc of mice that were behaviorally tested in Figure 2. For this experiment (see Figs. 3, 6), for each preexposure group (saline, amphetamine, or cocaine), a factorial design that included the between-subject factors of pretreatment (vehicle, nifedipine) and challenge (saline, psychostimulant) was used to assess the effect of nifedipine on psychostimulant-induced P-CREB (see Fig. 3) and P-ERK2 (see Fig. 6). $N = 7$ –8 mice were used for the veh-sal and nif-sal challenge groups, and $n = 10$ –12 were used for the veh-psychostimulant and nif-psychostimulant groups.

Experiment 3. The effect of systemic pretreatment of the ERK inhibitor SL327 on expression of amphetamine- and cocaine-induced locomotor sensitization was examined in C57BL/6 mice. For this experiment (see Fig. 8A), a factorial design that included the between-subject factors of preexposure (saline, amphetamine, or cocaine), pretreatment (vehicle, SL327), and challenge (saline, psychostimulant) was used to assess the effect of SL327 on psychostimulant-induced locomotor activity. SL327 was used at a dose of 40 mg/kg, intraperitoneal, based on results published by Ferguson et al. (2006). SL327 was administered 20 min before psychostimulant treatment. $N = 6$ –7 mice for the veh-sal and SL327-sal challenge groups, and $n = 8$ –10 for the veh-psychostimulant and SL327-psychostimulant challenge groups.

Experiment 4. The effect of SL327 on amphetamine- and cocaine-induced P-CREB in the NAc was measured in mice that were behaviorally tested in Figure 8A. In this experiment (see Fig. 8B), for each preexposure group (saline, amphetamine, or cocaine), a factorial design that included the between-subject factors of pretreatment (vehicle, SL327) and challenge (saline, psychostimulant) were used to assess the effect of SL327 on psychostimulant-induced P-CREB. $N = 6$ –7 mice for the veh-sal and SL327-sal challenge groups, and $n = 8$ –10 for the veh-psychostimulant and SL327-psychostimulant challenge groups.

Experiment 5. The effect of nifedipine on D_1 agonist SKF82958-induced P-CREB in the NAc was measured in $\text{Ca}_v1.2\text{DHP}^{+/+}$ wild-type and $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant mice. In this experiment (see Fig. 9), for each preexposure group (saline, amphetamine, or cocaine), a factorial design that included the between-subject factors of pretreatment (vehicle, nifedipine) and challenge (saline, SKF82958) were used to assess the effect of nifedipine on SKF82958-induced P-CREB. SKF82958 was used at a dose of 1 mg/kg, intraperitoneal, based on results from an initial dose-response with 0.5, 1, 1.5, and 2 mg/kg, intraperitoneal. $N = 7$ –8 mice were used for the veh-sal challenge groups, and $n = 9$ –10 were used for the veh-SKF82958 and nif-SKF82958 challenge groups.

Experiment 6. The effect of nifedipine pretreatment during development of locomotor sensitization on the subsequent expression of amphetamine- and cocaine-induced locomotor sensitization was assessed in $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant mice. In this experiment (see Fig. 10A),

a factorial design that included the between-subject factors of pretreatment (vehicle, nifedipine), preexposure (saline, amphetamine, or cocaine), and challenge (saline, psychostimulant) were used to assess the effect of nifedipine pretreatment during development on psychostimulant-induced locomotor activity at expression of sensitization. $N = 8$ –10 mice were used for each treatment group.

Experiment 7. The effect of nifedipine pretreatment during the development of locomotor sensitization on amphetamine- and cocaine-induced P-CREB in the NAc at expression was examined in mice that were behaviorally tested in Figure 10A. In this experiment (see Fig. 10B), for each psychostimulant (amphetamine or cocaine) preexposure group a factorial design that included the between-subject factors of pretreatment (vehicle, nifedipine) and preexposure (saline, psychostimulant) were used to assess the effect of nifedipine pretreatment during development on psychostimulant-induced P-CREB at expression. $N = 8$ –10 mice were used for each treatment group.

Data and statistical analyses

For locomotor sensitization, total distance traveled was analyzed by a three-way ANOVA with factors of preexposure, pretreatment, and challenge. For immunoblots, normalized P-CREB and P-ERK2 optical density data obtained by densitometry was used to calculate percentage fold change in protein levels for each treatment group compared with saline control group (set to 100%) within the same blot. Two controls were included on each blot. Fold induction value for each control sample was calculated relative to the average of the two controls included in the same blot. Fold induction values for all control and treatment groups were pooled across multiple blots. Error bars for all treatment groups (including controls) represent $\pm$ SEM of fold induction values. Statistical analyses were performed using fold change values across several blots by a two-way ANOVA with factors of preexposure and challenge. Isolated comparisons between all treatment groups for both locomotor sensitization and immunoblots were determined by the Bonferroni–Dunn *post hoc* tests. Statistical analyses were performed separately for behavioral and molecular data for amphetamine and cocaine treatment groups using Statview software (SAS Institute).

Results

$\text{Ca}_v1.2$ LTCCs, not $\text{Ca}_v1.3$ LTCCs, selectively mediate the expression of locomotor sensitization to amphetamine and cocaine

Previous pharmacological studies using DHPs that block both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ LTCCs have demonstrated that LTCCs mediate expression of amphetamine and cocaine sensitization (Karler et al., 1991; Pierce et al., 1998). We have confirmed this finding in wild-type ($\text{Ca}_v1.2\text{DHP}^{+/+}$) mice preexposed (days 1–5) to saline or psychostimulant (amphetamine or cocaine), and challenged 14 d later with either vehicle or nifedipine before saline or psychostimulant treatment (see Fig. 2A). Nifedipine blocked expression of amphetamine and cocaine locomotor sensitization, as indicated by a significant interaction between nifedipine pretreatment and psychostimulant challenge (amphetamine, $F_{(1,47)} = 4.694$, $p < 0.05$; cocaine, $F_{(1,40)} = 7.078$, $p < 0.05$). Next, we tested whether this effect of nifedipine on expression of locomotor sensitization was mediated by $\text{Ca}_v1.3$ LTCCs by repeating the identical treatment regimen followed in Figure 1A, in $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant mice (Fig. 2B). In contrast to the observation in wild-type mice, nifedipine had no effect on expression of either amphetamine or cocaine locomotor sensitization as indicated by no significant interaction between nifedipine pretreatment and psychostimulant challenge (Fig. 2B), demonstrating that the effect of nifedipine on the expression of amphetamine and cocaine sensitization seen in wild-type mice (Fig. 2A) was entirely attributable to $\text{Ca}_v1.2$ LTCCs.

To control for the possibility that the effect of nifedipine is attributable to action at targets in addition to the LTCCs, as

Figure 1. A mouse model to study $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ L-type Ca^{2+} channels. **A**, **B**, A schematic diagram of $\text{Ca}_v1.2$ DHP (dihydropyridine)-sensitive $+/+$ (wild-type) (**A**) and $\text{Ca}_v1.2$ DHP-insensitive $-/-$ (mutant) (**B**) mice. A mutation in one of the residues critical for DHP binding in the III5S transmembrane helix (T1066Y) eliminates high sensitivity of $\text{Ca}_v1.2$ LTCCs for DHP LTCC blockers (such as nifedipine used in this study) without affecting $\text{Ca}_v1.2$ function. In the absence of DHPs, both wild-type and mutant mice are identical and have functional $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ LTCCs. In the presence of nifedipine, $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels are blocked in wild-type mice (**A**), whereas in mutant mice (**B**), nifedipine selectively blocks $\text{Ca}_v1.3$ LTCCs.

suggested by a recent report finding that nifedipine binds to nicotinic receptors *in vitro* (Wheeler et al., 2006), mice were pretreated with the nicotinic receptor antagonist mecamylamine before the psychostimulant challenge (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), which had no effect on expression of amphetamine or cocaine sensitization.

$\text{Ca}_v1.2$ channels mediate the blunting of psychostimulant-induced CREB activation in the nucleus accumbens of psychostimulant-preexposed, but not drug-naïve mice at expression

To further explore the long-term molecular adaptations that underlie the expression of psychostimulant sensitization and the role of $\text{Ca}_v1.2$ channels therein, we examined CREB phosphorylation at Ser133 (P-CREB) in the NAc of wild-type and mutant mice immediately after the behavioral tests reported in Figure 1 (i.e., 30 min after psychostimulant challenge). Examination of P-CREB in saline-preexposed wild-type (Fig. 3A) and mutant (Fig. 3B) mice revealed that $\text{Ca}_v1.3$ and not $\text{Ca}_v1.2$ LTCCs are the primary LTCC isoform that mediate increase in acute amphetamine and cocaine-induced P-CREB, as indicated by a significant interaction between nifedipine pretreatment and amphetamine (wild type, $F_{(1,36)} = 4.681$, $p < 0.05$; mutant, $F_{(1,36)} = 6.233$, $p < 0.05$) and cocaine (wild type, $F_{(1,33)} = 4.966$, $p < 0.05$; mutant, $F_{(1,32)} = 4.854$, $p < 0.05$) challenge, in both genotypes. In contrast, in wild-type mice previously exposed to amphetamine or cocaine (Fig. 3A), a psychostimulant challenge did not increase P-CREB levels unless mice were pretreated with nifedipine [sig-

Figure 2. $\text{Ca}_v1.2$ LTCCs mediate expression of amphetamine and cocaine locomotor sensitization. **A**, **B**, Nifedipine had no effect on acute amphetamine or cocaine-induced locomotor activity in wild-type (**A**) or mutant (**B**) mice. Nifedipine blocked the expression of amphetamine and cocaine locomotor sensitization in $\text{Ca}_v1.2$ DHP $+/+$ wild-type (**A**) but not $\text{Ca}_v1.2$ DHP $-/-$ mutant (**B**) mice as seen in psychostimulant-preexposed mice. * $p < 0.05$ versus veh-sal; # $p < 0.01$ versus Sal-preexposed: veh-psychostimulant; + $p < 0.01$ versus veh-amp and nif-coc versus veh-coc. Data represent the mean $\pm$ SEM.

nificant interaction of nifedipine pretreatment and amphetamine ($F_{(1,40)} = 4.612$; $p < 0.05$) and cocaine ($F_{(1,32)} = 7.634$; $p < 0.01$) challenge]. The effect of nifedipine was mediated by $\text{Ca}_v1.2$ channels, as nifedipine had no effect on the blunted P-CREB response in amphetamine- or cocaine-preexposed mutant mice (Fig. 3B). Of note, neither psychostimulant preexposure nor nifedipine by itself altered basal NAc P-CREB levels in either genotype. Additional experiments revealed that the blunted amphetamine-induced P-CREB response in the NAc of amphetamine-preexposed mice occurs only after extended drug-free periods (Fig. 4) and occurs via $\text{Ca}_v1.2$ channels as identified above (Fig. 3). In contrast, $\text{Ca}_v1.3$ is the primary LTCC subunit that mediates psychostimulant-induced P-CREB in the NAc during the development of amphetamine and cocaine locomotor sensitization (Fig. 5A) and after short periods of withdrawal thereafter (Fig. 5B).

Figure 3. $\text{Ca}_v1.2$ LTCCs mediate blunting of amphetamine- and cocaine-induced P-CREB in the NAC of psychostimulant-preexposed mice. **A, B.** In saline-preexposed $\text{Ca}_v1.2\text{DHP}^{+/+}$ wild-type (**A**) and $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant (**B**) mice, amphetamine and cocaine challenge significantly increased P-CREB levels that were blocked by nifedipine in both genotypes. In psychostimulant-preexposed wild-type (**A**) and mutant (**B**) mice, amphetamine and cocaine challenge did not increase P-CREB levels. Nifedipine pretreatment significantly increased amphetamine and cocaine-induced P-CREB in $\text{Ca}_v1.2\text{DHP}^{+/+}$ (**A**) but not $\text{Ca}_v1.2\text{DHP}^{-/-}$ mice (**B**). * $p < 0.05$ versus veh-sal; † $p < 0.05$ versus veh-psychostimulant. Data represent the mean $\pm$ SEM.

$\text{Ca}_v1.2$ L-type Ca^{2+} channels mediate psychostimulant-induced ERK activation in the NAC of psychostimulant-preexposed but not drug-naïve mice at expression

We next examined the extent to which ERK, an activator of P-CREB (Brami-Cherrier et al., 2005), was phosphorylated at Thr183 or Tyr185 (P-ERK1/2) in the NAC of wild-type and mutant mice, in the identical samples used for P-CREB analysis in Figure 3. Examination of P-ERK2 levels in saline-preexposed wild-type and mutant mice revealed that neither $\text{Ca}_v1.2$ nor $\text{Ca}_v1.3$ play a role in acute psychostimulant-induced P-ERK2 in the NAC. Nifedipine pretreatment had no effect on acute amphetamine- or cocaine-induced P-ERK2 (Fig. 6A). Examination

Figure 4. Blunting of amphetamine-induced P-CREB in the NAC occurs only after extended withdrawal from amphetamine. C57BL/6 mice were killed for P-CREB immunoblot analysis at the time points indicated. Amphetamine significantly increased P-CREB levels in the NAC after acute amphetamine treatment and after repeated amphetamine treatment at 15 min, 1 d, and 5 d, but not 14 and 21 d of withdrawal compared with saline control mice. * $p < 0.05$, Amp versus Sal. Data represent the mean $\pm$ SEM, and $n = 4-6$ /time point.

tion of P-ERK2 levels in amphetamine- and cocaine-preexposed wild-type (Fig. 6A) and mutant (Fig. 6B) mice revealed that psychostimulant-induced P-ERK2 is mediated by $\text{Ca}_v1.2$ channels as indicated by a significant interaction of nifedipine pretreatment and amphetamine ($F_{(1,26)} = 4.574$; $p < 0.05$) and cocaine ($F_{(1,26)} = 4.345$; $p < 0.05$) challenge in wild-type (Fig. 6A) but not mutant mice (Fig. 6B). Additional experiments in $\text{Ca}_v1.2\text{DHP}$ mutant and $\text{Ca}_v1.3$ knock-out mice revealed that $\text{Ca}_v1.2$ channels mediate amphetamine-induced ERK activation only after extended drug-free periods (Fig. 7A,B), whereas $\text{Ca}_v1.3$ serves as the primary LTCC subunit that mediates amphetamine-induced P-ERK2 in the NAC during development of locomotor sensitization (Fig. 7C) and after short periods of withdrawal thereafter (Fig. 7A,B,D).

The blunting of psychostimulant-induced CREB activation in the NAC of psychostimulant-preexposed mice at expression is mediated by ERK

As the previous experiments revealed that amphetamine and cocaine challenge increases P-ERK2, but not P-CREB, in the NAC of psychostimulant-preexposed mice, the next experiment was aimed at directly testing whether the ERK pathway is involved in the psychostimulant-induced blunted P-CREB response. We used the MAP (mitogen-activated protein kinase)/ERK kinase (MEK) inhibitor SL327, which inhibits the ERK pathway by blocking ERK1/2 phosphorylation. Repeated (days 1–5) saline or psychostimulant-preexposed C57BL/6 mice were challenged 14 d later with either vehicle or SL327 pretreatment before saline or psychostimulant treatment (Fig. 8). Locomotor activity and P-CREB levels were measured. SL327 had no effect on acute amphetamine- or cocaine-induced locomotor activity (Fig. 8A). However, SL327 blocked expression of amphetamine and cocaine sensitization as indicated by a significant interaction between SL327 pretreatment and amphetamine ($F_{(1,46)} = 5.689$; $p < 0.05$) and cocaine ($F_{(1,46)} = 7.698$; $p < 0.05$) challenge. Examination of P-CREB levels in saline-preexposed mice revealed

Figure 5. $\text{Ca}_v1.3$ LTCCs mediate psychostimulant-induced P-CREB in the NAC after acute treatment, during development of locomotor sensitization and after short withdrawal. **A**, $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant mice pretreated (days 1–5) with nifedipine (Nif) 20 min before each dose of saline (Sal), amphetamine (Amp), or cocaine (Coc) showed reduced psychostimulant-induced P-CREB in the NAC compared with vehicle-pretreated mice. * $p < 0.01$ versus Veh-Sal; † $p < 0.05$, Nif-Amp versus Veh-Amp and Nif-Coc versus Veh-Coc. $n = 6–8$ /group. **B**, Amphetamine-induced P-CREB was significantly lower in $\text{Ca}_v1.3$ knock-out (KO) mice compared with wild-type (WT) mice after acute treatment and 15 min, 1 d, and 5 d of withdrawal from repeated amphetamine. * $p < 0.01$ versus $\text{Ca}_v1.3$ WT. Data represent the mean $\pm$ SEM, and $n = 4–6$ /genotype and time point.

that the ERK pathway is necessary for acute amphetamine- and cocaine-induced P-CREB as indicated by a significant interaction of SL327 pretreatment and amphetamine ($F_{(1,28)} = 5.192$; $p < 0.05$) and cocaine ($F_{(1,28)} = 4.345$; $p < 0.05$) challenge (Fig. 8B). In psychostimulant-preexposed mice, the blunted amphetamine and cocaine-induced P-CREB response was relieved by blocking the ERK pathway as indicated by a significant interaction between SL327 pretreatment and amphetamine ($F_{(1,43)} = 4.233$; $p < 0.05$) or cocaine ($F_{(1,32)} = 6.546$; $p < 0.05$) challenge (Fig. 8B).

Figure 6. $\text{Ca}_v1.2$ LTCCs mediate amphetamine- and cocaine-induced P-ERK2 in the NAC of psychostimulant-preexposed mice. **A**, **B**, In saline-preexposed $\text{Ca}_v1.2\text{DHP}^{+/+}$ wild-type (**A**) and $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant (**B**) mice, nifedipine had no effect on amphetamine- or cocaine-induced P-ERK2. In amphetamine- and cocaine-preexposed wild-type (**A**) but not mutant mice (**B**), nifedipine significantly blocked psychostimulant-induced P-ERK2. * $p < 0.05$ versus veh-sal; † $p < 0.05$ versus veh-psychostimulant. Data represent the mean $\pm$ SEM.

Dopamine D_1 receptor-mediated CREB activation is blunted in the NAC of psychostimulant-preexposed mice via $\text{Ca}_v1.2$ LTCCs

As adaptation in dopamine D_1 signaling has been reported to be one of the long-term changes that occurs after recurrent psychostimulant exposure (Berke and Hyman, 2000), we next tested the role of $\text{Ca}_v1.2$ versus $\text{Ca}_v1.3$ LTCCs in D_1 -induced CREB phosphorylation in the NAC of psychostimulant-naïve and psychostimulant-preexposed wild-type and mutant mice using the D_1 agonist SKF82958. Repeated (days 1–5) saline- or

Figure 7. $\text{Ca}_v1.3$ LTCCs mediate amphetamine- and cocaine-induced P-ERK2 during the development of locomotor sensitization and after short withdrawal, whereas $\text{Ca}_v1.2$ LTCCs mediate psychostimulant-induced P-ERK2 only after extended withdrawal. **A, B,** Nifedipine had no effect on acute amphetamine-induced P-ERK2 in the NAC of $\text{Ca}_v1.2\text{DHP}^{+/+}$ wild-type (**A**) and $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant (**B**) mice. Nifedipine blocked amphetamine-induced P-ERK2 in wild-type (**A**) and mutant (**B**) mice, 15 min after the last dose of repeated amphetamine and 1 and 5 d of withdrawal. At 14 and 21 d of withdrawal from amphetamine, nifedipine blocked amphetamine-induced P-ERK2 in wild-type but not mutant mice. * $p < 0.01$ versus veh-amp. Data represent the mean $\pm$ SEM, and $n = 4$ –6/group and time point. **C,** Nifedipine pretreatment in $\text{Ca}_v1.2\text{DHP}^{-/-}$ mice during development of amphetamine and cocaine locomotor sensitization blocked increase in P-ERK2 in the NAC after a psychostimulant challenge. * $p < 0.01$ versus Veh-Sal; † $p < 0.01$ for Nif-Amp versus Veh-Amp and Nif-Coc versus Veh-Coc. $n = 6$ –8/group. **D,** There was no difference in amphetamine-induced P-ERK2 between $\text{Ca}_v1.3$ wild-type (WT) and knock-out (KO) mice after acute treatment. Amphetamine-induced P-ERK2 was significantly lower in KO versus WT mice, 15 min after the last dose of repeated amphetamine and 1 and 5 d of withdrawal. * $p < 0.01$ versus $\text{Ca}_v1.3$ WT. $n = 4$ –6/genotype and time point. Data represent the mean $\pm$ SEM.

psychostimulant-preexposed wild-type (Fig. 9A) and mutant (Fig. 9B) mice were challenged 14 d later with either vehicle or nifedipine pretreatment before treatment with SKF82958. Similar to previous experiments, all drug treatments were administered in the behavioral testing chambers, and P-CREB levels were measured in the NAC 30 min later. Examination of P-CREB levels in saline-preexposed $\text{Ca}_v1.2\text{DHP}^{+/+}$ wild-type (Fig. 9A) and $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant (Fig. 9B) mice revealed that acute D_1 -induced CREB phosphorylation is mediated by $\text{Ca}_v1.3$ LTCCs as indicated by a significant interaction of nifedipine pretreatment and SKF82958 challenge (wild type, $F_{(1,23)} = 4.530$, $p < 0.05$; mutant, $F_{(1,23)} = 6.317$, $p < 0.05$) in both genotypes. This finding was consistent with results we ob-

tained in $\text{Ca}_v1.3$ knock-out mice (supplemental Fig. 4A, available at www.jneurosci.org as supplemental material) and in cultured striatal neurons from $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant mice (supplemental Fig. 4B, available at www.jneurosci.org as supplemental material). Additionally, pharmacological experiments revealed that D_1 -induced P-CREB is mediated by CaM kinases and not ERK (supplemental Fig. 4B, available at www.jneurosci.org as supplemental material). Examination of P-CREB levels in amphetamine- or cocaine-preexposed wild-type (Fig. 9A) and mutant (Fig. 9B) mice revealed that the P-CREB response was blunted after a D_1 challenge similar to that seen after a cocaine challenge (Fig. 3) and that the blunting was mediated by $\text{Ca}_v1.2$ channels as indicated by a significant interaction between nifedipine pretreatment and SKF82958 challenge (amphetamine, $F_{(1,24)} = 9.623$, $p < 0.01$; cocaine, $F_{(1,24)} = 4.577$, $p < 0.05$) in wild-type (Fig. 9A) but not mutant mice (Fig. 9B).

Recruitment of the psychostimulant-activated $\text{Ca}_v1.2$ pathway in the NAC of psychostimulant-preexposed mice at expression is dependent on functional $\text{Ca}_v1.3$ channels during the development of sensitization

The above experiments demonstrated that the blunting of psychostimulant-induced CREB activation via $\text{Ca}_v1.2$ LTCCs represents a long-term adaptation associated with expression of the sensitized response. As we have found that $\text{Ca}_v1.3$ LTCCs are critical for mediating the development of amphetamine and cocaine locomotor sensitization (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) (Giordano et al., 2006), we next tested whether blocking $\text{Ca}_v1.3$ LTCCs during the development of sensitization would prevent the subsequent expression of locomotor sensitization and the associated drug-induced blunting of CREB activation (Fig. 10). For this experiment, $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant mice pretreated during develop-

ment with either vehicle or nifedipine before each amphetamine or cocaine injection were challenged 14 d later with amphetamine or cocaine. Locomotor activity was measured for 30 min (Fig. 10A), and P-CREB levels were measured in the NAC immediately after behavioral testing (Fig. 10B). We found that $\text{Ca}_v1.3$ channels during the development of amphetamine and cocaine locomotor sensitization are necessary for the subsequent expression of psychostimulant locomotor sensitization, as revealed by a significant interaction between nifedipine pretreatment and amphetamine ($F_{(1,32)} = 6.882$; $p < 0.05$) and cocaine ($F_{(1,32)} = 5.416$; $p < 0.05$) preexposure (Fig. 10A). Examination of P-CREB levels revealed that blocking $\text{Ca}_v1.3$ channels with nifedipine during development abolishes the blunted psychostimulant-induced P-CREB response observed at expression

Figure 8. The ERK pathway mediates the expression of amphetamine- and cocaine-induced locomotor sensitization and blunts the P-CREB response. **A**, SL327, a selective MEK inhibitor, had no effect on acute amphetamine- or cocaine-induced locomotor activity. SL327 blocked the expression of amphetamine and cocaine locomotor sensitization. * $p < 0.05$ versus veh-sal; † $p < 0.01$ versus Sal-preexposed: veh-psychostimulant; ‡ $p < 0.05$ versus SL-psychostimulant. **B**, SL327 blocked the increase in acute amphetamine- and cocaine-induced P-CREB. SL327 pretreatment relieved the blunted amphetamine- and cocaine-induced P-CREB seen in amphetamine- and cocaine-preexposed mice. * $p < 0.01$ versus veh-sal; † $p < 0.05$ SL-psychostimulant versus veh-psychostimulant. Data represent the mean $\pm$ SEM.

as indicated by a significant interaction between nifedipine pretreatment and amphetamine ($F_{(1,32)} = 9.210$; $p < 0.01$) and cocaine ($F_{(1,32)} = 8.980$; $p < 0.01$) preexposure. Using $\text{Ca}_v1.3$ heterozygous knock-out mice, we additionally found that, even though a single $\text{Ca}_v1.3$ gene was sufficient for mediating the development of amphetamine locomotor sensitization, these mice did not exhibit expression of sensitization (main effect of genotype, $F_{(2,38)} = 7.386$; $p < 0.01$) (Fig. 11A), indicating that both $\text{Ca}_v1.3$ genes are necessary for the transition from development to long-term expression of psychostimulant sensitization. Furthermore, using a single-amphetamine injection sensitization protocol, we found that blocking $\text{Ca}_v1.3$ channels in $\text{Ca}_v1.2\text{DHP}^{-/-}$ mice by pretreatment with nifedipine before administration of a single amphetamine dose was sufficient to block expression of amphetamine sensitization ex-

Figure 9. $\text{Ca}_v1.3$ channels mediate dopamine D_1 -induced increase in CREB phosphorylation in the NAC of psychostimulant-naive mice, whereas $\text{Ca}_v1.2$ LTCCs mediate blunting of D_1 -induced CREB phosphorylation in psychostimulant-preexposed mice. **A**, **B**, In saline-preexposed $\text{Ca}_v1.2\text{DHP}^{+/+}$ wild-type (**A**) and $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant (**B**) mice, SKF82958, a D_1 -specific agonist, significantly increased P-CREB that was blocked by nifedipine in both genotypes. In psychostimulant-preexposed mice, SKF82958 did not increase P-CREB and this blunted response was relieved by nifedipine in wild-type (**A**) but not mutant (**B**) mice. * $p < 0.01$ versus veh-sal; † $p < 0.05$, nif-skf versus veh-skf. Data represent the mean $\pm$ SEM.

amined 14 d later (significant interaction of nifedipine pretreatment and amphetamine preexposure, $F_{(1,46)} = 4.092$; $p < 0.05$) (Fig. 11B).

Discussion

In this study, using locomotor sensitization as a model of psychostimulant-induced long-term plasticity, we find an important, but distinct role for the brain-specific LTCC subunits $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in mediating the persistent versus early

Figure 10. $\text{Ca}_v1.3$ LTCCs during the development of amphetamine and cocaine locomotor sensitization are essential for the subsequent expression of psychostimulant-induced locomotor sensitization and mediate the blunted P-CREB response in the NAc. **A**, Nifedipine pretreatment blocked expression of amphetamine and cocaine locomotor sensitization in $\text{Ca}_v1.2\text{DHP}^{-/-}$ mice. **B**, Nifedipine-pretreated mice had higher amphetamine- and cocaine-induced P-CREB levels compared with vehicle-pretreated mice. * $p < 0.01$ versus Veh-Sal; † $p < 0.05$ versus veh-psychostimulant. Data represent the mean $\pm$ SEM.

psychostimulant-induced changes (Fig. 12). We find that $\text{Ca}_v1.3$ LTCCs mediate development of sensitization, whereas $\text{Ca}_v1.2$ LTCCs mediate expression of the psychostimulant-induced sensitized response. At the molecular level, we find that acute psychostimulant treatment activates a $\text{D}_1/\text{Ca}_v1.3/\text{CREB}$ pathway in the NAc (Fig. 12A), and during development the ERK pathway is additionally recruited (Fig. 12B). In contrast, after extended withdrawal from drug, we find that psychostimulant-induced expression of the sensitized response is associated with activation of a $\text{D}_1/\text{Ca}_v1.2/\text{ERK}$ pathway that blunts CREB activation (Fig. 12C). Thus, we propose that this molecular switch from $\text{Ca}_v1.3$ to $\text{Ca}_v1.2$ channels in mediating the early versus persistent changes represents a mechanism that may be one of the

Figure 11. $\text{Ca}_v1.3$ heterozygous knock-out mice do not exhibit expression of amphetamine locomotor sensitization. **A**, Repeated amphetamine (Amp) treated $\text{Ca}_v1.3$ wild-type ($\text{Ca}_v1.3^{+/+}$) and heterozygous ($\text{Ca}_v1.3^{+/-}$), but not homozygous knock-out ($\text{Ca}_v1.3^{-/-}$) mice exhibit significantly greater locomotor on day 5 compared with day 1. On day 19, $\text{Ca}_v1.3^{+/+}$ and $\text{Ca}_v1.3^{+/-}$ mice exhibit significantly lower locomotor activity compared with $\text{Ca}_v1.3^{-/-}$ mice. # $p < 0.05$, day 5 versus day 1; † $p < 0.05$ versus $\text{Ca}_v1.3^{+/+}$ Amp. $n = 5-6$ for Sal groups and $n = 8-10$ for Amp groups. **B**, Nifedipine pretreatment in $\text{Ca}_v1.2\text{DHP}^{-/-}$ mutant mice blocked expression of single amphetamine-induced expression of locomotor sensitization. * $p < 0.001$ versus Veh-Sal and † $p < 0.01$ versus Veh-Amp. $n = 10$ for Veh-Sal and Nif-Sal groups, and $n = 15$ for Veh-Amp and Nif-Amp groups. Data represent the mean $\pm$ SEM.

determinants of the transition from the drug-naïve to the drug-dependent state.

$\text{Ca}_v1.3$, dopamine D_1 receptors, and psychostimulant-induced plasticity

We find that $\text{Ca}_v1.3$ LTCCs are necessary for psychostimulant-induced development of sensitization, but they do not play a role in mediating the psychostimulant-induced expression of sensitization. Additionally, we find that activation of $\text{Ca}_v1.3$ channels after a single psychostimulant injection is sufficient to mediate long-term expression of sensitization. We also find that $\text{Ca}_v1.3$ LTCCs mediate acute psychostimulant- and dopamine D_1 -induced CREB activation in the NAc, but they do not play a role

A Psychostimulant response in drug naïve NAc **B** Psychostimulant response during development **C** Psychostimulant response at expression

Figure 12. A working model illustrating the molecular switch from the $\text{Ca}_v1.3$ to the $\text{Ca}_v1.2$ signaling cascade in the NAc as a persistent adaptation after psychostimulant exposure. D_1R , Dopamine D_1 receptor; G_s , G-protein-coupled receptor.

in mediating psychostimulant-induced CREB regulation at expression. These behavioral and molecular findings strongly suggest that $\text{Ca}_v1.3$ activation in the NAc during early psychostimulant exposure activates a Ca^{2+} /CREB signal transduction pathway via D_1 receptors that trigger molecular events essential for long-term expression of sensitization. This conclusion is in line with a critical role of LTCCs in coupling changes in synaptic activity at the neuronal membrane, where D_1 receptors reside, to activation of gene expression (Wu et al., 2001a; Deisseroth et al., 2003). Moreover, LTCC-activated CREB-dependent gene expression is critically involved in mechanisms of neuronal plasticity (Deisseroth et al., 2003), consistent with an important role of NAc CREB-mediated gene expression in psychostimulant-induced behavioral plasticity (McClung and Nestler, 2003). D_1 receptor-containing medium spiny neurons (MSNs) in the NAc have been found to be the primary cell type in which psycho-

stimulants activate signaling pathways (Lee et al., 2006) and in which psychostimulant-induced long-term adaptations, such as changes in dendritic spines, a correlate of sensitized behavior (Robinson and Kolb, 2004), are maintained (Lee et al., 2006). A role for $\text{Ca}_v1.3$ channels in mediating psychostimulant-induced synaptic changes is further supported by the fact that $\text{Ca}_v1.3$ channels couple to the synaptic scaffolding protein Shank (Olson et al., 2005), a molecule critical for the coupling $\text{Ca}_v1.3$ channels to CREB activation (Zhang et al., 2005), for synapse formation, and long-term behavioral plasticity (Hung et al., 2008). Findings in this study also reveal that $\text{Ca}_v1.3$ channels do not mediate acute psychostimulant-induced locomotor responses; however, $\text{Ca}_v1.3$ channels are essential for single psychostimulant injection-induced long-term expression of sensitization. Thus, $\text{Ca}_v1.3$ -activated Ca^{2+} mechanisms after an acute drug exposure, such as activation of CREB in the NAc, may be sufficient for initiating long-term psychostimulant-induced changes. This hypothesis is consistent with the finding that activation of new protein synthesis (a hallmark of LTCCs) (Deisseroth et al., 2003) immediately after drug exposure (i.e., within the first 8 h) is sufficient for converting initial drug exposure to long-lasting molecular and behavioral changes (Valjent et al., 2009). In addition to a role of NAc $\text{Ca}_v1.3$ channels in sensitization, $\text{Ca}_v1.3$ in the ventral tegmental area (VTA) may also contribute to the development of sensitization and for initiating mechanisms in the VTA necessary for subsequent expression of sensitization. We have previously shown that $\text{Ca}_v1.3$ is the primary LTCC subunit expressed in VTA dopamine neurons and that VTA LTCCs regulate repeated amphetamine-induced ERK activation (Rajadhyaksha et al., 2004). Furthermore, activation of LTCCs directly in the VTA has been shown to be sufficient for mediating expression of cocaine sensitization (Licata et al., 2000).

Interestingly, our study finds that $\text{Ca}_v1.3$ channels do not mediate acute psychostimulant-induced ERK activation in the NAc but activate ERK after repeated psychostimulant exposure. This is consistent with the role of LTCCs (specifically $\text{Ca}_v1.3$ as we show here) and ERK in the development of psychostimulant-induced locomotor sensitization and not in the acute locomotor response (Karler et al., 1991; Pierce et al., 1999; results from this study). ERK is activated by psychostimulants via D_1 and NMDA receptors in D_1 receptor-expressing MSNs of the NAc (Brami-Cherrier et al., 2005) and plays a role in the development of sensitization (Girault et al., 2007). Hence, based on our findings and those of others, we conclude that psychostimulant-activated D_1 receptors recruit both the $\text{Ca}_v1.3$ channel- and NMDA receptor-activated ERK pathway during the development of locomotor sensitization and these pathways lead to activation of CREB-mediated gene expression in the NAc that contributes to the persistent neuronal changes that underlie long-term drug-induced behavioral responses.

This study has established an important role of $\text{Ca}_v1.3$ L-type channels during early psychostimulant exposure. However, additional studies directed toward identifying the specific cell types within the NAc where $\text{Ca}_v1.3$ channels exert their effects, along with identifying the precise genes activated downstream of the $\text{Ca}_v1.3$ /CREB pathway in the NAc, are necessary to further our understanding of $\text{Ca}_v1.3$ channel mechanisms and psychostimulant-induced behavioral plasticity.

$\text{Ca}_v1.2$, D_1 receptors, and long-term behavioral responses induced by psychostimulants

Our study finds that $\text{Ca}_v1.2$ channels mediate expression of psychostimulant sensitization after extended withdrawal, with no

apparent role of these channels in mediating behavioral or molecular changes that result from acute drug exposure, during the development of sensitization, or after short withdrawal. To our knowledge, this is the first report demonstrating that a molecule that is not necessary for the development of sensitization mediates the long-term expression of psychostimulant sensitization. The emergence of a role for $\text{Ca}_v1.2$ in long-term expression of amphetamine and cocaine sensitization is consistent with our previous findings that repeated amphetamine increases NAC $\text{Ca}_v1.2$ mRNA and that this increase is maintained after extended withdrawal from drug (Rajadhyaksha et al., 2004). Similarly, Renthal et al. (2009) have found in a gene expression microarray study that $\text{Ca}_v1.2$ mRNA is upregulated in the NAC after repeated cocaine treatment via CREB activation. Thus, it is plausible that $\text{Ca}_v1.2$ may be one of the genes activated downstream of the psychostimulant-induced $\text{Ca}_v1.3$ /CREB pathway discussed above; however, this possibility needs to be further explored.

NMDA receptors have long been identified as a Ca^{2+} source for mediating psychostimulant sensitization (Wolf, 1998). NMDA receptors have been found to be critical for development of sensitization (Wolf, 1998; Vanderschuren and Kalivas, 2000); however, systemic injection of the NMDA receptor antagonist MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] has no effect on expression of cocaine sensitization (Valjent et al., 2009) even though an increase in NMDA receptor subunit protein levels in the NAC after extended withdrawal has been reported (Schumann and Yaka, 2009). Similarly, ERK has been found to be necessary for development but not expression of cocaine and amphetamine locomotor sensitization (Ferguson et al., 2006; Valjent et al., 2009), contrary to our pharmacological findings in this study that the ERK pathway mediates expression of amphetamine and cocaine locomotor sensitization. In support of our findings of a role of ERK at expression, enhanced P-ERK2 levels have been observed in the NAC of cocaine locomotor sensitized rats after withdrawal (Boudreau et al., 2007; Schumann and Yaka, 2009). Thus, together, our study supports a role of $\text{Ca}_v1.2$ -activated ERK pathway in expression of psychostimulant locomotor sensitization via D_1 -mediated activation as discussed below. Future experiments with site-specific pharmacological manipulations are necessary to identify the role of NAC NMDA, $\text{Ca}_v1.2$, and ERK in expression of psychostimulant locomotor sensitization.

Our molecular studies find that expression of psychostimulant sensitization is associated with a $\text{Ca}_v1.2$ -mediated blunting of CREB activation. Although the direct effect of manipulating NAC CREB or phosphorylated CREB levels on expression of psychostimulant sensitization is lacking, viral-mediated expression of a mutant CREB form in the NAC that decreases levels of P-CREB (Carlezon et al., 1998) has been shown to enhance the rewarding effects of cocaine. A similar effect has also been seen in CREB knock-out mice (Walters and Blendy, 2001), which suggests that a lack of CREB activation may enhance psychostimulant-induced behavioral responses. However, additional studies such as site-specific manipulation of $\text{Ca}_v1.2$ and CREB are necessary to investigate the precise role of NAC $\text{Ca}_v1.2$ /CREB in the expression of sensitization. Nevertheless, our studies demonstrate for the first time that a psychostimulant challenge recruits the $\text{Ca}_v1.2$ channel that blunts CREB activation in the NAC, an adaptation associated with expression of sensitization. We also show that D_1 receptors no longer activate CREB in the psychostimulant-preexposed NAC and the blunted D_1 -induced CREB response occurs via $\text{Ca}_v1.2$ channels, similar to that observed after psychostimulant challenge, suggesting that the

psychostimulant-induced recruitment of $\text{Ca}_v1.2$ channels is mediated by D_1 receptors. D_1 -activated Gs proteins and PKA (protein kinase A) have been shown to regulate $\text{Ca}_v1.2$ LTCCs (Surmeier et al., 1995; Davare et al., 2001). However, the precise mechanism by which D_1 receptors activate a $\text{Ca}_v1.2$ pathway that blunts CREB activation in the psychostimulant-preexposed NAC as opposed to that seen in the drug-naïve NAC remains unknown and requires additional investigation.

In conclusion, the present study sheds light on how Ca^{2+} via distinct LTCCs can contribute to the long-term molecular and behavioral adaptations that result as a consequence of psychostimulant exposure, findings that can be explored in other rodent models of addiction.

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