L-Type Ca^{2+} Channels Mediate Adaptation of Extracellular Signal-Regulated Kinase 1/2 Phosphorylation in the Ventral Tegmental Area after Chronic Amphetamine Treatment

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L-type Ca^{2+} channels (LTCCs) play an important role in chronic psychostimulant-induced behaviors. However, the Ca^{2+} second messenger pathways activated by LTCCs after acute and recurrent psychostimulant administration that contribute to drug-induced molecular adaptations are poorly understood. Using a chronic amphetamine treatment paradigm in rats, we have examined the role of LTCCs in activating the mitogen-activated protein (MAP) kinase pathway in the ventral tegmental area (VTA), a primary target for the reinforcing properties of psychostimulants. Using immunoblot and immunohistochemical analyses, we find that in chronic saline-treated rats a challenge injection of amphetamine increases phosphorylation of MAP [extracellular signal-regulated kinase 1/2 (ERK1/2)] kinase in the VTA that is independent of LTCCs. However, in chronic amphetamine-treated rats there is no increase in amphetamine-mediated ERK1/2 phosphorylation unless LTCCs are blocked, in which case there is robust phosphorylation in VTA dopamine neurons. Examination of the expression of phosphatases reveals an increase in calcineurin [protein phosphatase 2B (PP2B)] and MAP kinase phosphatase-1 (MKP-1) in the VTA. Using *in situ* hybridization histochemistry and immunoblot analyses, we further examined the mRNA and protein expression of the LTCC subtypes $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in VTA dopamine neurons in drug-naïve animals and in rats after chronic amphetamine treatment. We found an increase in $\text{Ca}_v1.2$ mRNA and protein levels, with no change in $\text{Ca}_v1.3$. Together, our results suggest that one aspect of LTCC-induced changes in second messenger pathways after chronic amphetamine exposure involves activation of the MAP kinase phosphatase pathway by upregulation of $\text{Ca}_v1.2$ in VTA dopaminergic neurons.

Key words: amphetamine; L-type Ca^{2+} channels; VTA; ERK1/2 phosphorylation; $\text{Ca}_v1.2$; addiction

Introduction

Repeated exposure to the psychostimulants amphetamine and cocaine causes long-lasting neuronal adaptations that underlie compulsive behavior (Hyman and Malenka, 2001; Nestler, 2001b; Robinson and Berridge, 2003). The ventral tegmental area (VTA) serves as a primary neural site that initiates the molecular mechanisms that underlie such behaviors (Vezina and Stewart, 1990; Vezina et al., 2002; Bonci et al., 2003). Amphetamine and cocaine activate intracellular signaling pathways in the VTA that

cause downstream changes in protein phosphorylation and gene expression (White and Kalivas, 1998; Wolf, 1998; Licata and Pierce, 2003). In addition to the pivotal role of Ca^{2+} signaling via the NMDA subtype of the glutamate receptor, Ca^{2+} influx via voltage-gated L-type Ca^{2+} channels (LTCCs) is necessary for psychostimulant-induced behavioral and neurochemical changes (Karler et al., 1991; Pani et al., 1991; Kuzmin et al., 1992; Suzuki et al., 1992; Martellotta et al., 1994; Pierce and Kalivas, 1997; Pierce et al., 1998; Licata et al., 2000, 2001; Pliakas and Carlezon, 2001). However, their respective contribution to psychostimulant-induced changes remains unknown. Based on pharmacological studies, LTCCs appear to play a downstream role in NMDA and psychostimulant-induced sensitization. NMDA blockers inhibit both acute and chronic psychostimulant-induced behaviors (Karler et al., 1989; Vezina and Queen, 2000), whereas LTCC blockers inhibit only chronic, but not acute, psychostimulant-induced neurochemical and behavioral changes (Karler et al., 1991; Pierce and Kalivas, 1997; Pierce et al., 1998).

LTCCs play an important role in long-term neuronal plasticity (Bito et al., 1996; Deisseroth et al., 2003). They activate both

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Ca^{2+} -mediated kinase and phosphatase pathways (Deisseroth et al., 2003; Groth et al., 2003). LTCCs are heteromeric complexes and form channels that contain either the $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ Ca^{2+} pore-forming subunit (Catterall, 2000; Ertel et al., 2000; Stotz and Zamponi, 2001). Both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ are activated and blocked by the same drugs and hence traditionally are believed to have similar properties. However, there is growing evidence that $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ have different neuronal distributions (Hell et al., 1993; Furuyashiki et al., 2002) and different physiological characteristics (Koschak et al., 2001; Xu and Lipscombe, 2001; Lipscombe, 2002). $\text{Ca}_v1.2$ -containing channels open at high voltages, are present in signaling complexes (Davare et al., 1999, 2000, 2001), and activate Ca^{2+} -mediated second messenger pathways such as the mitogen-activated protein (MAP) kinase pathway (Dolmetsch et al., 2001; Weick et al., 2003). In contrast, the $\text{Ca}_v1.3$ -containing LTCCs activate at low voltages (Platzer et al., 2000; Koschak et al., 2001; Xu and Lipscombe, 2001) and are implicated in more rapid neuronal responses.

Here we present data for the role of LTCCs and their subtypes $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in chronic amphetamine-mediated molecular changes in the VTA. We have used an amphetamine sensitization paradigm known to induce robust, long-lasting locomotor sensitization in rats (Wolf and Jeziorski, 1993; Wolf et al., 1994). We have examined the MAP kinase pathway, a mediator of psychostimulant-induced plasticity (Pierce et al., 1999; Valjent et al., 2000), and its regulation after acute and chronic amphetamine treatment.

Materials and Methods

Animals. Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 200–225 gm at the start of the experiment were used in these studies. Rats were group housed and provided food and water *ad libitum*. A 12 hr light/dark cycle was used (from 7 A.M. to 7 P.M.). All procedures were conducted in accordance with the Massachusetts General Hospital Subcommittee on Research Animal Care rules and regulations.

Drugs and antibodies. D-Amphetamine sulfate and diltiazem were obtained from Sigma (St. Louis, MO). Anti-mouse phospho-extracellular signal-regulated kinase 1/2 (ERK1/2 kinase), calcineurin-A, and tyrosine hydroxylase (TH) antibodies were obtained from Sigma and anti-rabbit ERK1/2 kinase antibody from Cell Signaling Technology (Beverly, MA). Anti-rabbit $\text{Ca}_v1.2$, $\text{Ca}_v1.3$, TH, and anti-mouse actin antibodies were from Chemicon (Temecula, CA). Anti-rabbit MAP kinase phosphatase 1 (MKP-1) and anti-goat MAP kinase phosphatase 3 (MKP-3) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Protease and phosphatase inhibitor mixtures were from Sigma.

Amphetamine treatment paradigm. The amphetamine treatment regimen chosen has been shown to induce robust induction of locomotor sensitization (Wolf and Jeziorski, 1993; Wolf et al., 1994). Rats were handled for 3–4 d. Rats were given intraperitoneal injections of saline or 5 mg/kg D-amphetamine sulfate once per day for 5 d. For the ERK1/2 kinase phosphorylation (P-ERK1/2) studies, on day 6 the rats were divided into acute and chronic treatment groups as outlined in Table 2. The acute group that was treated with saline on days 1–5 was challenged with saline or amphetamine with or without diltiazem pretreatment on day 6. The chronic amphetamine group was treated chronically with amphetamine on days 1–5 and challenged with saline or amphetamine with or without diltiazem pretreatment on day 6. For immunoblot analysis the rats were decapitated 15 min after the last injection, and for immunohistochemical analysis the rats were perfused 15 min after the last injection. For immunoblot analysis of all other proteins the rats were decapitated on day 6, 1 d after the last saline or amphetamine injection. For the *in situ* hybridization histochemistry the rats were decapitated 1, 3, or 14 d after the last injection.

Immunoblots. For the P-ERK1/2 immunoblots brains were dissected rapidly and frozen in isopentane at -40°C . Brains were sectioned in a

cryostat to the rostral end of the nucleus accumbens (NAc), and a 1 mm section was cut with a razor blade. A 17 gauge steel bore was used to punch out both sides of the NAc. The same brain was sectioned to the rostral end of the VTA. A 1 mm section was cut, and an 18 gauge steel bore was used to get a midline punch of the VTA. Tissue samples were sonicated in SDS sample buffer [1% SDS in Tris-EDTA, pH 7.4, containing $1\times$ protease inhibitor mixture [containing 1 mM AEBF and (in μM) 0.08 aprotinin, 21 leupeptin, 36 bestatin, 15 pepstatin A, and 14 E-64], 5 mM sodium fluoride (NaF), 0.1 mg/ml benzamidin, and $1\times$ phosphatase inhibitor mixture (containing cantharidin, bromotetramisole, microcystin-LR, sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole)]. Samples were boiled for 10 min and centrifuged at 14,000 rpm for 10 min. Supernatants were collected, and protein concentration was determined by the BCA assay (Pierce, Rockford, IL). Then 50 μg of protein was loaded on a 12% SDS-polyacrylamide gel. For $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ immunoblots the tissue was sonicated in TE, pH 7.4, containing the above protease and phosphatases inhibitors and also containing 8 $\mu\text{g}/\text{ml}$ calpain inhibitor peptide; samples were separated on a 10% SDS-polyacrylamide gel. Protein was transferred to polyvinylidene fluoride (PVDF) membrane (Immuno-Blot PVDF, 0.2 μm , Bio-Rad, Hercules, CA) 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. Blots were probed with primary antibody (1:10,000, P-ERK1/2; 1:40,000, ERK1/2; and 1:1000, $\text{Ca}_v1.2$, $\text{Ca}_v1.3$, and actin) overnight at 4°C . The next day the blots were washed four times for 5 min each in blocking buffer. Blots were incubated with horse anti-mouse (1:15,000 for P-ERK1/2 and 1:10,000 for actin), goat anti-rabbit (1:20,000 for ERK1/2 and 1:10,000 for $\text{Ca}_v1.2$), or horse anti-goat (1:10,000 for $\text{Ca}_v1.3$) horseradish peroxidase-linked IgG (Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. Blots were washed once for 15 min and four times for 5 min each. Protein bands were detected by chemiluminescence (Western Lightning, PerkinElmer Life Sciences, Boston, MA) and exposed to X-OMAT Blue autoradiographic film (Kodak, Rochester, NY). Kaleidoscope-prestained standards (Bio-Rad) were used for protein size determination. ERK1 and P-ERK1 were detected at 44 kDa; ERK2, P-ERK2, and MKP-3 at 42 kDa; MKP-1 at 38 kDa; protein phosphatase 2B (PP2B) at 61 kDa; and $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ at 210 kDa. For quantitation the blots were scanned with an HP ScanJet 7400c scanner (Hewlett Packard, Palo Alto, CA). Intensity of the protein bands was measured as optical density, using the NIH Image program (National Institutes of Health, Bethesda, MD).

Immunohistochemistry. At 15 min after the last injection the rats were perfused transcardially with 20 ml of 0.9% NaCl plus 0.1 mM NaF, followed by 300 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; 0.1 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.5) with 0.1 mM NaF. Brains were removed and postfixed in 4% PFA for 2 hr and then placed in 15% sucrose in 0.1 M PB, pH 7.5, and 0.1 mM NaF at 4°C . Before sectioning, the brains were frozen in -40°C isopentane, and coronal sections (20 μm) were cut in a cryostat and kept in phosphate buffer, pH 7.5, with 0.1 mM NaF at 4°C . Sections were collected between -5.20 and -6.04 mm relative to bregma (Paxinos and Watson, 1997). Free-floating sections were rinsed in TBS (0.25 M Tris and 0.5 M NaCl, pH 7.5), incubated for 20 min at 4°C in methanol with 1% hydrogen peroxide, and then rinsed three times for 10 min each in TBS. Sections were incubated for 30 min in 3% horse serum and 0.2% Triton X-100 in TBS, followed by incubation in 1:500 phospho-ERK1/2 antibody for 24 hr at 4°C . The next day the sections were washed three times in TBS and incubated for 2 hr at room temperature with 1:200 biotinylated horse anti-mouse IgG (Vector Laboratories). Sections were incubated for 1 hr at room temperature in avidin–biotin–peroxidase complex solution (ABC solution, Vector Laboratories). Sections then were washed three times with TBS for 10 min each and developed for 12 min with 2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma). For quantitation, P-ERK1/2-positive neurons were counted bilaterally at a $20\times$ magnification over a 0.25 mm^2 area of the VTA.

For P-ERK1/2 and TH fluorescence double-labeling immunohistochemistry the sections were processed as above and incubated with 1:500 anti-mouse phospho-ERK1/2 and 1:1000 anti-rabbit TH antibody for 24 hr at 4°C . Sections were visualized with 1:200 anti-mouse Cy3 and anti-

Table 1. PCR templates

Target	Bases	Upper (5' to 3')	Lower (5' to 3')	cRNA size
Ca _v 1.2, M67516	3112–3507	GAAACAGGAGGTGATGGAGA	GCCAGGGAGATGCTACTGAG	395 bp
Ca _v 1.3, M76558	2863–3172	AATAAAAGAACAACAACCC	AGATGTGGTGGTTGATGAGC	309 bp
TH, NM–012740	553–1040	TCACCAAGTTTGACCTTGAT	GGGAGAACTGGGCAATGTG	507 bp
DARPP-32	213–600	TCCGCCACGAGAACCTCAG	GTGTTGCTCTGCCTCCCT	407 bp

rabbit Cy2 secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and mounted with Vectashield (Vector Laboratories). For the Ca_v1.2 and TH double-labeling experiments the sections were permeabilized with methanol and 1% H₂O₂ for 15 min. Sections were incubated at room temperature for 20 min with 5% donkey serum and 0.1% Triton X-100 in 0.1 M PB and then for 40 min with 5% bovine serum albumin in 0.1 M PB. Sections were incubated with 1:1000 anti-rabbit Ca_v1.2 and 1:1000 anti-mouse TH antibodies. For the P-ERK1/2 and Ca_v1.2 double labeling the sections were incubated with 1:500 anti-mouse P-ERK1/2 and 1:1000 anti-rabbit Ca_v1.2 antibodies. Sections were visualized with 1:200 anti-rabbit Cy3 and anti-mouse Cy2 secondary antibodies. Images were captured with the Zeiss Axiovert 200 fluorescence microscope and the LSM 5 Pascal software (Carl Zeiss, Oberkochen, Germany). Digital images were obtained at magnifications of 40× and 63×, using fluorescent filters appropriate for the specific emission wavelengths at which the secondary antibodies are visualized optimally.

In situ hybridization histochemistry. Rats treated with chronic saline or chronic amphetamine (5 mg/kg, once per day for 5 d) were decapitated 1, 3, and 14 d after the last saline or amphetamine injection. Brains were frozen in −40°C isopentane and stored at −80°C. For complementary RNA (cRNA) generation, DNA templates for synthesizing Ca_v1.2 and Ca_v1.3 cRNA were generated from hippocampal RNA, using a PCR strategy as described previously (Kerner et al., 1998). cRNAs were targeted to a region that shows no sequence similarity between the Ca_v1.2 and Ca_v1.3 genes. Total RNA was isolated with the TRI-reagent (Sigma), and first-strand cDNA was synthesized by using the cDNA synthesis system from Invitrogen (Carlsbad, CA). Ca_v1.2 and Ca_v1.3 primers were designed by using the Oligo 4.0 software (National Biosciences, Plymouth, MN). The first-strand cDNA was used in a first round of PCR to isolate a PCR template (735 bp) that spanned both Ca_v1.2 and Ca_v1.3 sequences, using a 5' upper primer, 5'-CTTCATCATCCTCTTCATTT-3' (nucleotides 2950–2969 of Ca_v1.2, M67516; nucleotides 2703–2722 of Ca_v1.3, M76558), and 3' lower primer, 5'-GCACTGGATTGAATCCCCAA-3' (nucleotides 3704–3723 of Ca_v1.2 and 3418–3437 of Ca_v1.3). To isolate PCR templates specific for Ca_v1.2 and Ca_v1.3, we designed 40-mer inner primers (Table 1), 20 bases specific for the target sequence and 20 bases specific for the RNA polymerases SP6 (5'-GGGATTTAGGTGACACTATAGAA3'-) or T7 (5'-CTGTAATACGACTCACTATAGGG-3'). SP6 RNA polymerase was used to generate the antisense cRNA and T7 for the sense cRNA. For double ISHH in the VTA, dopamine (DA) neurons were identified by labeling TH RNA. PCR template for synthesis of the TH cRNA was generated from a TH cDNA plasmid (Table 1). In the striatum and NAc the medium spiny neurons representing the predominant cell type were identified by labeling DARPP-32 mRNA. PCR template for synthesis of the DARPP-32 cRNA was generated from striatal RNA as described above. A previously published DARPP-32 cDNA sequence (Ehrlich et al., 1990) was used for designing DARPP-32-specific PCR primers (Table 1). All primers were obtained from the Massachusetts General Hospital (MGH) DNA Oligonucleotide synthesis facility (Boston, MA). All PCR templates were sequenced by the MGH DNA Sequencing Core, Boston, MA) to confirm their identity.

Radioactive ³⁵S-labeled Ca_v1.2 and Ca_v1.3 cRNAs were synthesized by *in vitro* transcription, using SP6 or T7 RNA polymerase (Promega, Madison, WI). For double *in situ* hybridization histochemistry (ISHH) digoxigenin (DIG)-labeled TH and DARPP-32 antisense cRNAs were synthesized with SP6 RNA polymerase and DIG 11-UTP (DIG RNA labeling mixture, Boehringer Mannheim, Indianapolis, IN).

Hybridization was performed as previously described (Kerner et al., 1998). Briefly, rats were decapitated rapidly, and brains were frozen in

chilled isopentane and stored at −70°C. Then 12 μm sections were cut on a cryostat and fixed in 4% paraformaldehyde in 0.1 M PB, pH 7.4 (10 min), three washes in 0.1 M PBS, pH 7.4 (5 min each), acetylated in 0.1 M triethanolamine, pH 8.0, with 0.25% acetic anhydride (10 min), rinsed in PBS (5 min), dehydrated through 70, 80, 90, and 100% ethanol (2 min each), and delipidated in chloroform. For single ISHH ³⁵S-labeled Ca_v1.2 or Ca_v1.3 the cRNAs were combined in a hybridization buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris, pH 8.0, 5 mM EDTA, 10% dextran sulfate, 1× Denhardt's solution, 100 mM DTT, 0.1% SDS, 0.1% sodium thiosulfate, 100 μg/ml salmon sperm DNA, 250 μg/ml yeast tRNA, and 250 μg/ml yeast total RNA. For double ISHH, ³⁵S-labeled Ca_v1.2 or Ca_v1.3 cRNA and DIG-labeled TH cRNA or DARPP-32 cRNA were combined in the above hybridization buffer. Hybridization was performed at 50°C for 4 hr. After hybridization the sections were washed in 2× SSC at room temperature. For film autoradiograms the sections were dried and exposed to Hyperfilm β-Max film (Amersham Biosciences, Piscataway, NJ) for ~7 d. For double ISHH the DIG-labeled TH or DARPP-32 was detected by using rabbit anti-digoxigenin antisera coupled to alkaline phosphatase (1:2000 dilution for 5 hr at room temperature; Roche Bioscience, Palo Alto, CA). Slides then were incubated in a substrate solution of nitroblue tetrazolium (0.34 mg/ml; Roche Bioscience) and bromo-chloro-indolyl-phosphate (0.18 mg/ml; Roche Bioscience) in (in mM) 100 Tris-HCl, 100 NaCl, and 50 MgCl₂, pH 9.5, for 4–6 hr at room temperature. Slides were rinsed briefly in water and 70% ethanol and then dried. Slides were exposed to β-Max film (Amersham Biosciences) for ~7 d to obtain film autoradiograms. To obtain emulsion autoradiograms, we dipped slides in Ilford K5 emulsion (Ilford Imaging, Moberley, Cheshire, UK) diluted 1:1 with distilled water and developed after ~3 weeks.

Quantitative analysis was performed on emulsion autoradiograms for VTA as described previously (Landwehrmeyer et al., 1995; Testa et al., 1995), using a computer-assisted M1 image analysis system (Imaging Research, St. Catherine's, Ontario, Canada). DIG-labeled neurons were visualized under bright-field optics with a 100× water immersion lens. The soma of each labeled neuron was outlined, and the overlying grains were counted by the image analysis system. Both the area of the neuronal soma, in μm², and the number of grains present were recorded. The intensity of labeling of each neuron was computed as grains/1000 μm². Three to four animals were used for each probe and each time point, and 25–50 neurons were counted per animal. Sections corresponding to −5.20 to −6.04 mm relative to bregma (Paxinos and Watson, 1997) were used for the analysis.

For the striatum and NAc sections the film autoradiograms were quantitated by using the M1 image analysis system calibrated with Kodak density step standards. Brain sections between 1.2 and 1.7 mm relative to bregma (Paxinos and Watson, 1997) were captured with a camera and digitized; the hybridization signal was measured as optical density (OD). Ca_v1.2 and Ca_v1.3 mRNA hybridization signal was measured in two subregions of the NAc (NAc core and NAc shell) and four striatal subregions (D, dorsal; DL, dorsolateral; DM, dorsomedial; VL, ventrolateral) as shown in Figure 6. The OD was measured in the anterior commissure and used as background level for the NAc. The OD in the corpus callosum was used as background for the striatum. Averages of the two sides for each subregion were calculated after subtraction of the background values. Four to six sections were used per animal, with three to four animals per probe and per time point.

Statistical analyses. For immunoblot, immunohistochemical analyses, and film autoradiography the data were analyzed by one-way ANOVA with *post hoc* comparisons (Fisher's probability of least significant difference; PLSD) between treatment groups and control. For double ISHH

Table 2. Amphetamine-treatment groups for P-ERK1/2 analyses

Group	Days 1–5	Day 6	
	Pretreatment	First injection	Second injection
Acute amphetamine group			
Saline (Chr SAL, SAL)	Saline	Saline	Saline
Acute DILT (Chr SAL, DILT)	Saline	Diltiazem	Saline
Acute AMPH (Chr SAL, AMPH)	Saline	Saline	Amphetamine
DILT plus acute AMPH (Chr SAL, DILT + AMPH)	Saline	Diltiazem	Amphetamine
Chronic amphetamine group			
Saline (Chr AMPH, SAL)	Amphetamine	Saline	Saline
AMPH (Chr AMPH, AMPH)	Amphetamine	Saline	Amphetamine
DILT plus AMPH (Chr AMPH, DILT + AMPH)	Amphetamine	Diltiazem	Amphetamine
DILT plus SAL (Chr AMPH, DILT + SAL)	Amphetamine	Diltiazem	Saline

Rats from all groups were given intraperitoneal injections of saline or amphetamine on days 1–5. On day 6, injection 1 was given 20 min before injection 2. AMPH, d-amphetamine sulfate, 5 mg/kg salt; DILT, diltiazem, 30 or 60 mg/kg.

Figure 1. Acute amphetamine induces ERK1/2 phosphorylation (P-ERK1/2) in the VTA independent of L-type calcium channels. *A*, Representative immunoblots of P-ERK1/2 with rat VTA tissue from saline (SAL), acute amphetamine (5 mg/kg; Chr SAL, AMPH), L-type Ca^{2+} channel antagonist diltiazem (60 mg/kg; Chr SAL, DILT), and diltiazem plus acute amphetamine (Chr SAL, DILT + AMPH) treatment groups (see Table 2). Rats were decapitated 15 min after injection, and VTA tissue was isolated and analyzed by immunoblot analysis. P-ERK1/2 was examined with an antibody that detects phosphorylated ERK1 (P-ERK1) at 44 kDa and phosphorylated ERK2 (P-ERK2) at 42 kDa. Acute amphetamine increased P-ERK1/2, an effect that was not blocked by pretreatment with diltiazem. Diltiazem alone had no effect. To confirm equal protein loading, we stripped and reprobbed blots with an antiserum that detects total ERK1 and ERK2. *B*, Quantitative analysis of P-ERK2 immunoreactivity. P-ERK2 showed a consistent increase in phosphorylation when compared with P-ERK1 in all treatment groups and was used for quantitation. P-ERK2 bands were normalized to total ERK2 and are represented as a percentage of saline control. There was a significant increase in P-ERK2 after amphetamine (A) treatment compared with saline (S) treatment (A vs S; $p < 0.05$), with no significant change with diltiazem pretreatment (D + A vs A). DILT + A was significantly different from S ($p < 0.05$). Error bars represent $\pm$ SEM; $n = 6$ per group.

experiments grains/1000 μm^2 for each treatment group were compared with the saline control by using ANOVA with repeated measures analysis and *post hoc* comparisons (Fisher's PLSD).

Results

Acute amphetamine induces MAP kinase (ERK1/2) phosphorylation in the VTA independent of L-type Ca^{2+} channels

Chronic saline-treated rats were injected with saline (SAL), the LTCC antagonist diltiazem (30 and 60 mg/kg; DILT), amphetamine (5 mg/kg; AMPH), or diltiazem 20 min before an amphetamine injection (DILT + AMPH) (Table 2). Rats were decapitated 15 min after injection, and VTA tissue was isolated.

Phosphorylation of ERK1/2 (P-ERK1/2) was examined by immunoblot analysis. An antibody that specifically recognizes the dually phosphorylated MAP kinase subtypes, ERK1 (P-ERK1) and ERK2 (P-ERK2) at Thr¹⁸³ and Tyr¹⁸⁵, respectively, was used. ERK1 was detected at 44 kDa and ERK2 at 42 kDa. Acute injection of amphetamine increased P-ERK1/2 in the VTA (Fig. 1A, AMPH vs SAL). The increase in P-ERK1/2 was not blocked by diltiazem (60 mg/kg) (Fig. 1A, DILT + AMPH vs AMPH). Diltiazem on its own had no effect (Fig. 1A, DILT). Similar results were obtained with 30 mg/kg diltiazem (data not shown). Blots were stripped and reprobbed with an antibody that recognizes total ERK1/2. No difference in total ERK1/2 was detected across all groups. P-ERK2 bands were used for quantitation because they showed a consistent increase in phosphorylation compared with P-ERK1 in all treatment groups. P-ERK2 was normalized to total ERK2 protein and is represented as a percentage of saline control. There was a significant increase in P-ERK2 after an amphetamine challenge (Fig. 1B, A vs S; $p < 0.05$), with no significant change with diltiazem pretreatment (Fig. 1B, D + A vs A; $p < 0.05$ for D + A vs S).

Acute amphetamine does not induce ERK1/2 phosphorylation in chronic amphetamine-treated rats unless L-type Ca^{2+} channels are blocked

Chronic saline-treated rats were injected with saline (Chr SAL, SAL) or amphetamine (Chr SAL, AMPH), and chronic amphetamine-treated rats were injected with amphetamine (Chr AMPH, AMPH) or diltiazem 20 min before an amphetamine injection (Chr AMPH, DILT + AMPH) (Table 2; Fig. 2A, top). VTA tissue was isolated 15 min after injection and used for P-ERK1/2 immunoblot analysis. Compared with the P-ERK1/2 increase in chronic saline-treated rats challenged with amphetamine (Chr SAL, A vs S), there was no increase in P-ERK1/2 in chronic amphetamine-treated rats challenged with amphetamine on day 6 (Fig. 2A, Chr AMPH, A vs Chr SAL, S). Blocking LTCCs with diltiazem (60 mg/kg) 20 min before the challenge amphetamine injection showed a significant increase in P-ERK1/2 compared with saline-treated rats ($p < 0.001$; Chr AMPH, D + A, vs Chr SAL, S). Similar results were obtained with 30 mg/kg diltiazem. Chronic amphetamine-treated rats injected with either saline (Fig. 3A, Chr AMPH, SAL) or diltiazem alone (Chr AMPH, DILT + SAL) had no effect on P-ERK1/2 and looked similar to chronic SAL-treated rats (data not shown). Examination of P-ERK1/2 in the NAC, a site that receives projections from VTA dopamine neurons, revealed a significant increase ($p < 0.01$) in P-ERK1/2 in chronic saline- and chronic amphetamine-treated

rats after an amphetamine challenge (Fig. 2*B*, Chr SAL, A, and Chr AMPH, A, vs Chr SAL, S), as has been shown previously for chronic cocaine (Valjent et al., 2000). Unlike the VTA, pretreatment with diltiazem had no effect on P-ERK1/2 in the NAc (Fig. 2*B*, Chr AMPH, D+A vs A).

Neuroanatomic confirmation of ERK1/2 phosphorylation in the VTA

The above results were confirmed by immunohistochemical analysis with rat coronal sections containing VTA. Rats treated chronically with saline and amphetamine were challenged on day 6 as outlined in Table 2, perfused 15 min after injection, and probed with the P-ERK1/2 antibody (Fig. 3*A*). P-ERK1/2-positive neurons in the VTA from sections corresponding to bregma -5.20 to -6.04 mm (Paxinos and Watson, 1997) were counted (Fig. 3*B*). Similar to the results from immunoblot analysis, acute amphetamine significantly increased P-ERK1/2 in the VTA ($**p < 0.01$) (Fig. 3*A,C*, Chr SAL, A vs S), with no effect of diltiazem pretreatment ($*p < 0.05$) (Fig. 3*A,C*, Chr SAL, D+A vs A). There was no increase in basal P-ERK1/2 in chronic amphetamine-treated rats (Fig. 3*A,C*, Chr AMPH, S, vs Chr SAL, S). A challenge injection of amphetamine did not increase P-ERK1/2 in chronic amphetamine-pretreated rats (Chr AMPH, A, vs Chr SAL, S). Pretreatment with diltiazem (60 mg/kg) before the challenge amphetamine injection induced a significant increase in P-ERK1/2 ($***p < 0.001$; Chr AMPH, D+A, vs Chr SAL, S).

To determine whether P-ERK1/2 was expressed in VTA dopamine neurons, we performed double-label immunohistochemistry of TH and P-ERK1/2 in the Chr SAL, AMPH, and Chr AMPH, DILT+AMPH, groups (Fig. 3*D*). In the Chr SAL, AMPH, group P-ERK1/2-positive cells were distinct from the TH neurons (Fig. 3*D*, red and green arrows, respectively), whereas in the Chr AMPH, DILT+AMPH, group there was an increase in the number of P-ERK1/2-positive cells that colocalized with TH neurons (Fig. 3*D*, yellow arrow). In addition, a small number of P-ERK1/2-positive non-TH cells was evident (Fig. 3*D*, red arrow). P-ERK1/2-positive cells also were observed in the substantia nigra pars compacta (SNc) of these two groups, which did not colocalize with TH-positive neurons (data not shown).

Expression of protein phosphatases in the VTA after chronic amphetamine treatment

A decrease in P-ERK1/2 in the VTA after chronic amphetamine treatment suggested either an inactivation of the MAP kinase pathway or activation of a phosphatase pathway. We explored the second possibility by examining levels of the protein phosphatases MKP-1, MKP-3, and calcineurin (PP2B). VTA tissue was isolated from chronic saline- or amphetamine-treated (5 mg/kg) rats on day 6, 24 hr after the last injection, and was used for immunoblot analysis. There was a significant increase in MKP-1 ($**p < 0.01$) and PP2B ($*p < 0.05$) protein in chronic amphetamine-treated rats com-

A. VTA

B. NAc

Figure 2. Acute amphetamine does not increase ERK1/2 phosphorylation in the VTA of chronic amphetamine-treated rats unless LTCCs are blocked. Shown are representative immunoblots of P-ERK1/2 with rat VTA (*A*) and NAc (*B*) tissue. Rats chronically treated with saline or amphetamine on days 1–5 were challenged on day 6 as outlined in Table 2. Rats were decapitated 15 min after the last injection; VTA and NAc tissue was isolated and analyzed for P-ERK1/2 by immunoblot analysis. Blots were stripped and reprobed with an antiserum that detects total ERK1 and ERK2 for equal protein loading. P-ERK2 bands were normalized to total ERK2 and were used for quantitation. *A*, In chronic saline-treated rats, amphetamine (5 mg/kg) significantly increased P-ERK1/2 in the VTA (Chr SAL, A vs S; $**p < 0.01$). In chronic amphetamine-treated rats, there was no increase in P-ERK1/2 with a challenge amphetamine injection. Pretreatment with diltiazem (60 mg/kg) before the challenge amphetamine injection showed an increase in P-ERK1/2 (Chr AMPH, D+A, vs Chr SAL, S; $***p < 0.001$). Error bars represent $\pm$ SEM; $n = 4–7$ per group. *B*, In the NAc, there was a significant increase in P-ERK1/2 after a challenge injection of amphetamine in chronic saline- and amphetamine-treated rats (Chr SAL, A vs S, and Chr AMPH, A, vs Chr SAL, S; $**p < 0.01$). Pretreatment with diltiazem (60 mg/kg) before the challenge amphetamine injection in chronic amphetamine-treated rats had no effect on P-ERK1/2 (Chr AMPH, D+A, vs Chr SAL, S; $**p < 0.01$). Error bars represent $\pm$ SEM; $n = 7–8$ per group.

pared with chronic saline-treated rats (Fig. 4). There was no change in MKP-3 protein levels (Fig. 4).

Ca_v1.2 mRNA is expressed at relatively low levels in the VTA of drug-naïve rats compared with Ca_v1.3 mRNA, which is expressed robustly

The ability of diltiazem to increase amphetamine-mediated ERK1/2 phosphorylation in chronic amphetamine-treated rats suggested that Ca²⁺ signaling via LTCCs was altered in these animals and that expression of one or both of the LTCC subtypes, Ca_v1.2 or Ca_v1.3, may underlie this phenomenon. To test this hypothesis, we pursued experiments to establish the normative pattern of Ca_v1.2 and Ca_v1.3 expression in the VTA, NAc, and striatum and investigated whether there were regional changes in the expression of these subtypes as a consequence of chronic amphetamine treatment.

The mRNA and protein distributions of Ca_v1.2 and Ca_v1.3 have been examined in several brain regions (Tanaka et al., 1995; Ludwig et al., 1997; Takada et al., 2001); however, the distribution in the VTA, NAc, and striatum have not been well characterized (Tanaka et al., 1995; Ludwig et al., 1997). We first examined expression in the VTA, NAc, and striatum of drug-naïve rats by using *in situ* hybridization histochemistry (ISHH). The nucleotide sequences of Ca_v1.2 and Ca_v1.3 were compared and found to have a high degree of sequence similarity as previously reported (Ertel et al., 2000). The sequences differ over a small region that corresponds to the variable region between the second and third transmembrane domains of the Ca²⁺ pore-forming Ca_v subunit (Fig. 5*A*). cRNA probes specific for Ca_v1.2 and Ca_v1.3

Figure 3. Immunohistochemical analysis of P-ERK1/2 in the VTA. Rats chronically treated with saline or amphetamine on days 1–5 were challenged on day 6 as outlined in Table 2. Coronal sections through the VTA were used for P-ERK1/2 immunohistochemistry. *A*, Representative P-ERK1/2-immunopositive sections. SAL, Saline; Chr SAL, DILT, chronic saline-treated rats challenged with diltiazem (60 mg/kg); Chr SAL, AMPH, chronic saline-treated rats challenged with amphetamine (5 mg/kg); Chr SAL, DILT + AMPH, chronic saline-treated rats challenged with diltiazem, followed by amphetamine; Chr AMPH, AMPH, chronic amphetamine-treated rats challenged with amphetamine; Chr AMPH, DILT + AMPH, chronic amphetamine-treated rats challenged with diltiazem, followed by amphetamine. *B*, Representative coronal levels (with the Paxinos atlas-based stereotaxic positions identified) of DIG-labeled TH mRNA sections used to identify the VTA for quantitation of P-ERK1/2-positive neurons. SNC, Substantia nigra pars compacta. *C*, Quantitative analysis of P-ERK1/2-positive neurons. P-ERK1/2-positive neurons were counted in the VTA from sections corresponding to -5.20 to -6.04 mm relative to bregma (Paxinos and Watson, 1997). In chronic saline-treated rats, there was a significant increase in P-ERK1/2-positive neurons after an amphetamine challenge (Chr SAL, A vs S; $**p < 0.01$), an effect that was not blocked by pretreatment with diltiazem (60 mg/kg; Chr SAL, D + A vs S; $*p < 0.05$). In chronic amphetamine-treated rats, there was no increase in P-ERK1/2 immunoreactivity after a challenge amphetamine injection (Chr AMPH, A vs Chr SAL, S). Pretreatment with diltiazem before the challenge amphetamine injection showed an increase in P-ERK1/2 immunoreactivity (Chr AMPH, D + A, vs Chr SAL, S; $***p < 0.001$). Error bars represent $\pm$ SEM; $n = 5$ per group. *D*, TH and P-ERK1/2 double-label immunohistochemistry in Chr SAL, AMPH (left), and Chr AMPH, DILT + AMPH (right), treatment groups. TH neurons were labeled with a green fluorescent label and P-ERK1/2 with a red fluorescent label and were visualized at $40\times$ magnification. P-ERK1/2-positive cells (red arrows) were distinct from TH neurons (green arrows) in the Chr SAL, AMPH, group. In the Chr AMPH, DILT + AMPH, there was an increase in the number of P-ERK1/2-positive cells that colocalized with TH neurons (yellow arrows). In addition, a small number of P-ERK1/2-positive non-TH-containing cells was evident (red arrows).

were generated corresponding to this region and used for ISHH. ISHH was performed with 35 S-labeled $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ cRNAs. In the VTA these two mRNAs were expressed differentially (Fig. 5B). $\text{Ca}_v1.2$ mRNA was present at low levels, whereas $\text{Ca}_v1.3$ mRNA was expressed strongly (Fig. 5B, top). Strong expression of both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ was seen in the hippocampus, as previously reported (Tanaka et al., 1995; Ludwig et al., 1997). No hybridization signal was present with a sense $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ cRNA (Fig. 5B, bottom). Double ISHH was performed to examine the expression of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in VTA dopamine neurons. Dopamine neurons were identified with a digoxigenin

(DIG)-labeled TH cRNA. Rat VTA-containing sections were hybridized with 35 S-labeled $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ probes and the DIG-labeled TH cRNA. $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ mRNA was visualized as silver grains over TH-positive dopamine neurons by emulsion autoradiography. In agreement with the results from film autoradiograms, $\text{Ca}_v1.2$ mRNA was present only at low levels in VTA dopamine neurons, whereas $\text{Ca}_v1.3$ was expressed strongly (Fig. 5C).

In the NAc and striatum $\text{Ca}_v1.2$ mRNA and $\text{Ca}_v1.3$ mRNA were expressed moderately strongly, as seen by film autoradiography (Fig. 6A) and by emulsion autoradiography (Fig. 6B). A DIG-labeled DARPP-32 cRNA, a marker of medium spiny neurons of the NAc and striatum, was used to label the predominant cell type in the NAc and striatum. $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ grains were present over DARPP-32-positive cell bodies (Fig. 6B, bottom). No hybridization signal was present with the sense probes (Fig. 6A, bottom). Autoradiograms were quantitated to obtain the relative distributions of the two subtypes in subregions of the NAc and dorsal striatum. There was significantly higher expression of $\text{Ca}_v1.2$ mRNA in the NAc shell ($**p < 0.01$) compared with the core and the dorsal (STR D), dorsolateral (STR DL), dorsomedial (DM), and ventrolateral (VL) striatum (Fig. 6C, left). $\text{Ca}_v1.3$ was significantly higher in the NAc core ($**p < 0.01$) and shell ($***p < 0.001$) compared with the striatum (Fig. 6C, right).

Chronic amphetamine increases $\text{Ca}_v1.2$ mRNA and protein levels in the VTA, with no change in $\text{Ca}_v1.3$ mRNA or protein

To examine the expression of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ mRNA after chronic amphetamine treatment, we decapitated chronic saline- and amphetamine-treated rats on day 6, 24 hr after the last amphetamine injection, and the brains were used for double ISHH. The density of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ grains over TH-positive cell bodies was quantitated and is represented as grains/1000 μm^2 . There was a significant increase in $\text{Ca}_v1.2$ mRNA in the VTA ($***p < 0.001$)

of amphetamine-treated rats compared with saline-treated rats (Fig. 7A). There was no significant difference in $\text{Ca}_v1.3$ mRNA between the two groups (Fig. 7C). Quantitation of grains over TH-positive neurons of the SNC showed no change in $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ mRNA (Fig. 7D,E).

$\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ protein levels were examined in the VTA of chronic saline- and amphetamine-treated rats by immunoblot and immunohistochemical analyses. VTA tissue was isolated on day 6, 24 hr after the last injection. A significant increase in $\text{Ca}_v1.2$ protein was seen in chronic amphetamine-treated rats ($*p < 0.0001$) compared with saline-treated rats, with no change in

$\text{Ca}_v1.3$ protein (Fig. 8A). To determine whether the increase in $\text{Ca}_v1.2$ protein was present in TH-positive neurons, we used double labeling. TH neurons were labeled with a green fluorescent label and $\text{Ca}_v1.2$ with a red fluorescent label. An increase in $\text{Ca}_v1.2$ protein was seen over TH-positive neurons after chronic amphetamine treatment (Fig. 8B, compare left and right) although such induction of $\text{Ca}_v1.2$ protein was additionally evident in non-TH-containing neurons (Fig. 8B, right, white arrow).

To examine further whether the increase in P-ERK1/2 seen in the Chr AMPH, DILT+AMPH, group (Fig. 3A,D) was present in neurons that exhibited an increase in $\text{Ca}_v1.2$ protein, we used double labeling (Fig. 8C). P-ERK1/2 was labeled with a green fluorescent label and $\text{Ca}_v1.2$ with a red fluorescent label. P-ERK1/2-positive neurons colocalized with the neurons that had an increase in $\text{Ca}_v1.2$ protein [compare P-ERK1/2 alone with green labeling (left), $\text{Ca}_v1.2$ alone with red labeling (middle), and the merged image of P-ERK1/2 and $\text{Ca}_v1.2$ (right)].

Increase in $\text{Ca}_v1.2$ mRNA expression is maintained in the VTA at 3 and 14 d of withdrawal

Chronic amphetamine treatment results in molecular and behavioral changes that are maintained even after long periods of withdrawal (Wolf, 1998). Hence we examined the expression of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ at 3 and 14 d after the last amphetamine injection in VTA dopamine neurons. $\text{Ca}_v1.2$ mRNA levels were significantly higher in chronic amphetamine-treated compared with chronic saline-treated rats at 3 and 14 d of withdrawal (Fig. 9). However, there was a trend toward a decrease in the fold induction of $\text{Ca}_v1.2$ mRNA levels from 1 to 3 to 14 d of withdrawal (Figs. 7A, 9A). There was no change in $\text{Ca}_v1.3$ mRNA at 3 and 14 d of withdrawal (Fig. 9B).

$\text{Ca}_v1.2$ mRNA is increased in the nucleus accumbens for 1, but not for 3 or 14, d after chronic amphetamine treatment

Autoradiograms from ISHH with chronic saline- and amphetamine-treated rat sections containing the NAc and striatum (between 1.0 and 2.0 mm anterior to bregma; Paxinos and Watson, 1997) at 1, 3, and 14 d were used for quantitation. There was a significant increase ($*p < 0.05$) in $\text{Ca}_v1.2$ mRNA in the NAc shell 1 d after chronic amphetamine treatment that returned to basal levels at 3 and 14 d of withdrawal (Fig. 10A). There was no change in $\text{Ca}_v1.2$ mRNA expression in the NAc core or of $\text{Ca}_v1.3$ mRNA in the NAc shell or core (Fig. 10A,B). No change in $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ mRNA was

Figure 4. Expression of phosphatases in the VTA after chronic amphetamine. Shown are representative immunoblots of MAP kinase phosphatases 1 (MKP-1), 3 (MKP-3), and calcineurin-A (PP2B). Rats were treated chronically with saline or amphetamine (5 mg/kg) on days 1–5. On day 6, the rats were decapitated, and VTA tissue was isolated and analyzed via immunoblot analysis by using antibodies specific for MKP-1, MKP-3, and the α -subunit of PP2B. Blots were stripped and reprobed with actin to control for equal protein loading. Phosphatase bands were normalized to actin and used for quantitation. A significant increase in MKP-1 ($**p < 0.01$) and PP2B ($*p < 0.05$) was seen after chronic amphetamine treatment. No change in MKP-3 protein was observed. MKP-1 was detected at 38, MKP-3 at 42, and PP2B at 61 kDa, respectively; actin was detected at 42 kDa. Error bars represent $\pm$ SEM; $n = 10$ per group for the MKP-1 Westerns and $n = 8$ per group for the MKP-3 and PP2B Westerns.

Figure 5. L-type Ca^{2+} channel $\text{Ca}_v1.2$ mRNA is expressed at relatively low levels in the VTA of naive rat brain compared with $\text{Ca}_v1.3$ mRNA, which is expressed moderately strongly. *A*, Schematic diagram of the Ca_v subunit of voltage-gated Ca^{2+} channels. The L-type Ca^{2+} channel consists of two neuronal subtypes, $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, which share a high degree of nucleotide sequence similarity. cRNAs corresponding to the variable nucleotide region between the second and third transmembrane-spanning domains were used for ISHH. *B*, ISHH was performed on coronal sections from drug-naïve rat brain with $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ cRNAs. Representative autoradiograms of sections through the VTA hybridized with ^{35}S -labeled $\text{Ca}_v1.2$ (left) and $\text{Ca}_v1.3$ (right) antisense (top) and sense (bottom) cRNAs are shown. Relatively low levels of $\text{Ca}_v1.2$ mRNA were detectable in the VTA, whereas $\text{Ca}_v1.3$ mRNA demonstrated more robust expression. No signal was detectable with sense cRNAs. *C*, Double ISHH was performed with either a ^{35}S -labeled $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ cRNA and a DIG-labeled TH cRNA used to label dopamine neurons. Top, TH-positive neurons in the VTA and SNc are identified. Bottom, Clusters of grains corresponding to ^{35}S -labeled $\text{Ca}_v1.3$, but not $\text{Ca}_v1.2$, mRNA are seen colocalized with TH-positive VTA cell bodies.

present in the subregions of the striatum after chronic amphetamine treatment (data not shown).

Discussion

We report here for the first time a study that has examined the role of LTCCs in amphetamine-mediated molecular changes in the VTA. We find that LTCCs do not play a role in acute

Figure 6. Distribution of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in the nucleus accumbens and striatum. *A*, ISHH was performed on coronal sections through the NAc and striatum from drug-naïve rat brains with $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ cRNAs. Representative autoradiograms with ^{35}S -labeled $\text{Ca}_v1.2$ (left) and $\text{Ca}_v1.3$ (right) antisense (top) and sense (bottom) cRNAs are shown. $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ mRNAs were expressed moderately strongly. No signal was detectable with sense cRNAs. *B*, Clusters of grains corresponding to ^{35}S -labeled $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ mRNA are apparent over DARPP-32-positive cell bodies of NAc and striatal neurons. *C*, Autoradiograms were used for quantitation of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ mRNA in the subregions of the NAc (core and shell) and striatum (D, dorsal; DL, dorsolateral; DM, dorsomedial; VL, ventrolateral). There was significantly higher expression of $\text{Ca}_v1.2$ mRNA in the NAc shell (** $p < 0.01$) compared with the NAc core and the subregions of the striatum. $\text{Ca}_v1.3$ was significantly higher in the NAc core (** $p < 0.01$) and shell (***) compared with the subregions of the striatum. Error bars represent $\pm$ SEM; $n = 4$ per group.

amphetamine-mediated ERK1/2 phosphorylation in the VTA. In chronic amphetamine-treated rats we find no increase in amphetamine-mediated ERK1/2 phosphorylation unless LTCCs are blocked, in which case there is robust phosphorylation of ERK1/2 in dopaminergic VTA neurons. We also find that chronic amphetamine treatment is accompanied by an increase in the LTCC subtype $\text{Ca}_v1.2$ mRNA and protein in dopaminergic VTA neurons and in the phosphatases calcineurin (PP2B) and MKP-1. Together, our studies identify that chronic amphetamine up-regulates $\text{Ca}_v1.2$ -containing LTCCs, which activate a phosphatase pathway that results in decreased ERK1/2 phosphorylation in the dopaminergic neurons of the VTA (Fig. 11).

ERK1/2 pathway activation in the VTA after acute versus chronic psychostimulants

Our finding of an increase in ERK1/2 phosphorylation after acute amphetamine-treated, but not after chronic amphetamine-treated, rats was unexpected because the opposite has been reported for cocaine (Berhow et al., 1996). The difference in our results may be attributable to the different drugs that were used or to the different time points that were examined. For their acute experiments Berhow et al. (1996) measured ERK1/2 activity 2 hr after cocaine injection, a time by which phosphorylation already may have returned to basal levels. Our studies were performed 15 min after amphetamine injection.

In their chronic studies Berhow et al. (1996) reported an increase in basal ERK1/2 activity in the VTA 10 hr after chronic cocaine administration, although we found no basal P-ERK1/2 or amphetamine-induced P-ERK1/2 when the VTA was examined 24 hr after the last amphetamine injection. Another reason for the discrepant results may be attributable to the different assays used; Berhow et al. (1996) studied ERK1/2 activity by using biochemical assays, whereas we detected ERK1/2 phosphorylation by immunoblotting and immunohistochemistry.

Two possible routes of ERK1/2 phosphorylation in the VTA are via the NMDA receptor or the neurotrophic factor neurotrophin-3. NMDA receptors are essential for psychostimulant-induced Ca^{2+} signaling (Kalivas and Alesdatter, 1993; Churchill et al., 1999; Pierce et al., 1999; Vezina and Queen, 2000; Ungless et al., 2001; Suto et al., 2003) and activate ERK1/2 phosphorylation (Sweatt, 2001). Neurotrophin-3 via the TrkC receptors also activates the ERK1/2 pathway in the VTA (Pierce et al., 1999). Consistent with this is the fact that acute amphetamine has been found to increase calmodulin (CaM) mRNA and protein in the VTA (Michelhaugh and Gnegy, 2000) that would activate the ERK1/2 pathway.

The role of $\text{Ca}_v1.2$ in ERK1/2 pathway activation in the VTA after acute versus chronic psychostimulants

The absence of a role of LTCCs in acute amphetamine-mediated ERK1/2 correlated with the presence of very low levels of the LTCC subtype $\text{Ca}_v1.2$ mRNA and protein in the VTA of drug-naïve rats. The involvement of LTCCs in regulating chronic amphetamine-mediated ERK1/2 phosphorylation corresponds with an increase in $\text{Ca}_v1.2$ mRNA and protein. The $\text{Ca}_v1.2$ gene contains the upstream regulatory sites CRE and AP1, which bind CREB and the Fos family of proteins, respectively (Liu et al., 2000). These regulatory sites and their transcription factors are activated by psychostimulants and are involved in psychostimulant-induced changes (Berke and Hyman, 2000; Nestler, 2001a). Because chronic amphetamine-induced long-lasting changes require new protein synthesis (Karler et al., 1993; Sorg and Ulibarri, 1995), the increase in $\text{Ca}_v1.2$ mRNA may contribute to psychostimulant-induced neuroplasticity. Chronic nicotine (Katsura et al., 2002), ethanol (Walter and Messing, 1999; Walter et al., 2000), and norepinephrine (Maki et al., 1996; Golden et al., 2002) also have been found to increase the expression of $\text{Ca}_v1.2$.

Activation of phosphatases in the VTA after acute versus chronic psychostimulants

In chronic amphetamine-treated rats we find that LTCCs down-regulate ERK1/2 phosphorylation. LTCCs are a major source of activation of Ca^{2+} /CaM kinases but also have been found to activate the phosphatase pathway (Groth et al., 2003). We find that in chronic amphetamine-treated rats there is an increase in

Figure 7. $\text{Ca}_v1.2$ mRNA is induced in VTA dopamine neurons after chronic amphetamine treatment, with no change in $\text{Ca}_v1.3$ mRNA levels. Shown is quantitative analysis of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ mRNA expression in the VTA from rats treated chronically with saline or amphetamine (5 mg/kg) on days 1–5 and decapitated on day 6, 24 hr after the last saline or amphetamine injection. $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ grains over VTA and SNc TH-positive neurons were counted in sections corresponding to -5.20 to -6.04 mm relative to bregma (Paxinos and Watson, 1997). Data reflect the mean density of grains overlying TH-positive neurons (grains/1000 μm^2). **A**, Representative images of ^{35}S -labeled $\text{Ca}_v1.2$ grains over TH-positive VTA neurons from chronic saline-treated (Chr SAL) and chronic amphetamine-treated (Chr AMPH) rats. **B**, There was a significant increase in $\text{Ca}_v1.2$ mRNA in TH-positive neurons after chronic amphetamine treatment compared with chronic saline treatment ($***p < 0.001$). **C**, There was no change in $\text{Ca}_v1.3$ mRNA after chronic amphetamine. In the SNc, there was no change in $\text{Ca}_v1.2$ (**D**) or $\text{Ca}_v1.3$ (**E**) mRNA between chronic saline- and chronic amphetamine-treated rats. Error bars represent $\pm$ SEM; $n = 4$ per group.

the phosphatase calcineurin (PP2B). This is in line with other studies that have shown an increase in calcineurin activity via LTCCs (Graef et al., 1999; Chang and Berg, 2001; Snyder et al., 2003). In addition, acute amphetamine has been found to increase PP2B expression (Wang and Uhl, 1998).

We also find an increase in MKP-1, a phosphatase that directly dephosphorylates ERK1/2 at the Tyr and Thr residues. It has been found that ERK1/2 and MKP functions are tightly linked. P-ERK1/2 has been found to phosphorylate MKP, rendering the protein more resistant to degradation (Brondello et al., 1999) and thus creating a negative feedback loop for its own phosphorylation (Bhalla et al., 2002). In addition, PP2B has been found to increase MKP-1 mRNA expression (Lim et al., 2001). In response to psychostimulants, MKP-1 mRNA expression has been found to increase after acute cocaine and methamphetamine (Berke et

Figure 8. Chronic amphetamine increases $\text{Ca}_v1.2$ protein levels in the VTA, with no change in $\text{Ca}_v1.3$ protein. **A**, Representative immunoblots of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ protein expression in the VTA from rats chronically treated with saline or amphetamine (5 mg/kg) on days 1–5 and decapitated on day 6, 24 hr after the last saline or amphetamine injection. VTA tissue was isolated and analyzed for $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ protein by immunoblot analysis. Blots were stripped and reprobed with actin to control for equal protein loading. $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ bands were normalized to actin and used for quantitation. A significant increase in $\text{Ca}_v1.2$ protein was observed in chronic amphetamine-treated compared with chronic saline-treated rats ($*p < 0.0001$). There was no change in $\text{Ca}_v1.3$ protein after chronic amphetamine treatment. $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ were detected at ~ 210 kDa. Error bars represent $\pm$ SEM; $n = 5$ for the chronic saline group and $n = 8$ for the chronic amphetamine group. **B**, $\text{Ca}_v1.2$ and TH double-label immunohistochemistry in chronic saline-treated (Chr SAL, left) and chronic amphetamine-treated (Chr AMPH, right) VTA. TH neurons were labeled with a green fluorescent label and $\text{Ca}_v1.2$ with a red fluorescent label and were visualized at $63\times$ magnification. An increase in $\text{Ca}_v1.2$ was seen over TH-positive neurons after chronic amphetamine treatment. **C**, P-ERK1/2 and $\text{Ca}_v1.2$ double-label immunohistochemistry in the VTA of the Chr AMPH, DILT+AMPH, treatment group. P-ERK1/2 was labeled with a green fluorescent label (left) and $\text{Ca}_v1.2$ with a red fluorescent label (middle) and were visualized at $40\times$ magnification. The P-ERK1/2 cells colocalized with $\text{Ca}_v1.2$ -containing cells in the VTA (right, yellow cells).

al., 1998; Takaki et al., 2001; Ujike et al., 2002) and to persist after chronic methamphetamine treatment (Takaki et al., 2001; Ujike et al., 2002). Our results suggest that acute amphetamine increases MKP-1 expression via an increase in ERK1/2 phosphorylation and in PP2B expression. The increased levels of MKP-1 and PP2B then would downregulate the phosphorylation of ERK1/2.

Figure 9. Ca_v1.2 mRNA remains upregulated in VTA dopamine neurons 3 and 14 d after withdrawal from chronic amphetamine treatment. Shown is quantitative analysis of Ca_v1.2 (*A*) and Ca_v1.3 (*B*) mRNA expression in the VTA from rats treated chronically with saline or amphetamine (5 mg/kg) on days 1–5 and decapitated 3 and 14 d after the last saline or amphetamine injection. Ca_v1.2 and Ca_v1.3 grains over VTA TH-positive neurons were counted in sections corresponding to –5.20 to –6.04 mm relative to bregma (Paxinos and Watson, 1997). Data reflect the mean density of grains overlying TH-positive neurons (grains/1000 μm²). There was a significant increase in Ca_v1.2 mRNA in TH-positive neurons in chronic amphetamine-treated compared with chronic saline-treated rats at 3 and 14 d of withdrawal (***p* < 0.01). There was no change in Ca_v1.3 mRNA in chronic amphetamine-treated compared with chronic saline-treated rats at 3 and 14 d of withdrawal. Error bars represent ± SEM; *n* = 4 per group.

Figure 10. Quantitation of Ca_v1.2 and Ca_v1.3 mRNA in the NAC at 1, 3, and 14 d of withdrawal. ISHH was performed on coronal sections through the NAC from drug-naïve rat brains with Ca_v1.2 (*A*) and Ca_v1.3 (*B*) cRNAs. Film autoradiograms were used for quantitation of Ca_v1.2 and Ca_v1.3 mRNA in the NAC core and shell from rats treated chronically with saline or amphetamine (5 mg/kg) and decapitated 1, 3, and 14 d of withdrawal. There was significantly higher expression of Ca_v1.2 mRNA in the NAC shell (**p* < 0.05) 1 d after chronic amphetamine treatment, which returned to basal levels after 3 and 14 d of withdrawal. There was no change in Ca_v1.2 mRNA expression in the NAC core or Ca_v1.3 mRNA in the NAC core or shell of chronic amphetamine-treated rats. Error bars represent ± SEM; *n* = 3–4 per group.

The role of LTCCs in mediating psychostimulant-induced neuroadaptations

A lack for a role of LTCCs in ERK1/2 phosphorylation in the NAC was surprising. LTCCs mediate dopamine and glutamate signaling in the NAC and striatum (Surmeier et al., 1995; Liu and Gray-

biel, 1996, 1998; Cepeda et al., 1998; Rajadhyaksha et al., 1999). One possible explanation could be a role for LTCCs in long-term ERK1/2 phosphorylation similar to what has been seen in the hippocampal and cortical cultures (Bito et al., 1996; Dolmetsch et al., 2001). This would be consistent with a role of LTCCs and the MAP kinase pathway in activation of long-term P-ERK1/2 and gene expression (Dolmetsch et al., 2001; Wu et al., 2001a,b), which may contribute to long-term psychostimulant-induced neuronal plasticity.

The LTCC-mediated decrease in ERK1/2 phosphorylation may represent a homeostatic adaptation after chronic amphetamine exposure. The MAP kinase pathway specifically via activation of MKPs has been suggested as one route that neurons use in adaptive responses to chronic stimuli (Bhalla et al., 2002). A decrease in ERK1/2 phosphorylation has been reported after chronic ethanol exposure, which becomes even more robust with an increase in withdrawal times (Sanna et al., 2002). Further examination of the state of ERK1/2 phosphorylation after longer periods of withdrawal will address this question.

The role of LTCCs in mediating long-term potentiation

Recent studies have shown that a single exposure to amphetamine or cocaine induces long-term potentiation (LTP) at the glutamatergic synapse on VTA dopamine neurons (Ungless et al., 2001; Saal et al., 2003) and is believed by some investigators to be a physiological correlate of psychostimulant-induced sensitized behavior. The same synapse has been shown to express voltage-gated Ca²⁺ channel-dependent long-term depression (LTD) that is inhibited by amphetamine (Jones et al., 2000). LTCCs contribute to an NMDA-independent form of LTP (Johnston et al., 1992), and in light of our current findings we hypothesize that Ca²⁺ influx from Ca_v1.3-containing LTCCs that are expressed predominantly in VTA dopamine neurons may contribute to LTD and that the increase in Ca_v1.2 expression after amphetamine treatment may mediate an LTCC-dependent LTP. This is in line with the localization of Ca_v1.2-containing LTCCs at synapses (Hell et al., 1993; Furuyashiki et al., 2002) and several reports showing a role for LTCCs in neuroadaptive changes in the brain (Weisskopf et al., 1999; Bauer et al., 2002; Deisseroth et al., 2003).

The influence of LTCC on the ERK1/2 pathway and psychostimulant-induced behavioral sensitization

Our finding of a role for LTCCs in chronic, but not acute, amphetamine-mediated ERK1/2 phosphorylation also agrees with psychostimulant-induced behavioral data. It has been reported that LTCC blockers do not block the acute effects of amphetamine and cocaine but block chronic amphetamine- and cocaine-induced locomotor activity (Karler et al., 1991; Pierce and Kalivas, 1997; Pierce et al., 1998). It is interesting that inhibiting the MAP kinase pathway does not block acute cocaine-induced locomotor activity but inhibits such effects after chronic cocaine administration (Pierce et al., 1999). This suggests that the LTCC-activated MAPK pathway may contribute to the adaptations specific to chronic psychostimulant exposure. In addition, we find that Cav1.2 mRNA is upregulated up to 14 d of withdrawal. LTCCs have been suggested to play a role in the augmented dopamine release observed during the expression of sensitized behaviors (Pierce and Kalivas, 1997). Although the neuronal distribution and physiology of Cav1.2 do not support a role in neurotransmitter release, LTCCs have been found to be involved in neurotransmitter release (Watanabe et al., 1998; Evans and Pocock, 1999; Okita et al., 2000), and Cav1.2 recently

has been suggested to play this role in fear-mediated behavior (Shinnick-Gallagher et al., 2003). Additional experiments are required to explore further the relevance of this mechanism in contributing to long-term changes in neurotransmitter release in forebrain targets innervated by VTA neurons, which may contribute to recurrent psychostimulant-induced behaviors.

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Figure 11. Schematic representation of molecular changes from acute to chronic amphetamine exposure in VTA dopaminergic (DA) neurons. *A*, Acute amphetamine increases somatodendritic dopamine release in the VTA. Glutamate is released via activation of presynaptic D1 receptors. Glutamate activates the AMPA receptor and depolarizes the VTA neuron, releasing the Mg^{2+} block on the NMDA receptor. The NMDA receptor activates the Ca^{2+} second messenger pathway via calmodulin (CaM), which leads to activation of the CaM kinase (CaMK) and MAP kinase (MAPK) pathways. The CaMK pathway phosphorylates CREB (P-CREB), which activates gene expression in the nucleus. The CaMK pathway also activates the MAPK pathway that leads to ERK1/2 phosphorylation via activation of MAP kinase kinase kinase (MAPKKK or Raf) and MAP kinase kinase (MEK). P-ERK1/2 phosphorylates P-CREB via activation of Rsk-2. Another route of ERK1/2 phosphorylation in the VTA is via activation of the neurotrophic factor-signaling pathway. Activation of these pathways by amphetamine leads to activation of gene expression, including the $\text{Ca}_v1.2$ gene. The upstream transcriptional region of the $\text{Ca}_v1.2$ gene contains CRE and AP1 binding sites that bind CREB and the Fos family of transcription factors, activated by amphetamine. *B*, In chronic amphetamine-exposed VTA, the newly expressed high-voltage-activated $\text{Ca}_v1.2$ -containing LTCCs activate a phosphatase pathway. Chronic amphetamine increases calcineurin (PP2B) expression, which increases levels of MKP-1 and also alters downstream gene expression. P-ERK1/2 also induces expression of the phosphatase MKP-1 that directly dephosphorylates P-ERK1/2. *C*, In the presence of diltiazem, there is an inhibition of the LTCC-activated phosphatase pathway that leads to activation of an amphetamine-mediated kinase pathway and an increase in ERK1/2 phosphorylation.

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