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# Repeated Cocaine Exposure Decreases Dopamine $D_2$-Like Receptor Modulation of $Ca^{2+}$ Homeostasis in Rat Nucleus Accumbens Neurons

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## Abstract

The nucleus accumbens (NAc) is a limbic structure in the forebrain that plays a critical role in cognitive function and addiction. Dopamine modulates activity of medium spiny neurons (MSNs) in the NAc. Both dopamine $D_1$-like and $D_2$-like receptors (including D1R or $D_{1,5}$R and D2R or $D_{2,3,4}$R, respectively) are thought to play critical roles in cocaine addiction. Our previous studies demonstrated that repeated cocaine exposure (which alters dopamine transmission) decreases excitability of NAc MSNs in cocaine-sensitized, withdrawn rats. This decrease is characterized by a reduction in voltage-sensitive $Na^+$ currents and high voltage-activated $Ca^{2+}$ currents, along with increased voltage-gated $K^+$ currents. These changes are associated with enhanced activity in the D1R/cAMP/PKA/protein phosphatase 1 pathway and diminished calcineurin function. Although D1R-mediated signaling is enhanced by repeated cocaine exposure, little is known whether and how the D2R is implicated in the cocaine-induced NAc dysfunction. Here, we performed a combined electrophysiological, biochemical, and neuroimaging study that reveals the cocaine-induced dysregulation of $Ca^{2+}$ homeostasis with involvement of D2R. Our novel findings reveal that D2R stimulation reduced $Ca^{2+}$ influx preferentially via the L-type $Ca^{2+}$ channels and evoked intracellular $Ca^{2+}$ release, likely via inhibiting the cAMP/PKA cascade, in the NAc MSNs of drug-free rats. However, repeated cocaine exposure abolished the $D_2$R effects on modulating $Ca^{2+}$ homeostasis with enhanced PKA activity and led to a decrease in whole-cell $Ca^{2+}$ influx. These adaptations, which persisted for 21 days during cocaine abstinence, may contribute to the mechanism of cocaine withdrawal.

### Keywords

withdrawal; L-type $Ca^{2+}$ channel; PKA; calcineurin; patch clamp



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## INTRODUCTION

The nucleus accumbens (NAc) is a forebrain structure that regulates cognitive function and drug-motivated behaviors in humans and animals (Hyman et al., 2006; Robbins and Everitt, 2002). Dopamine innervation from the midbrain mediates function of medium spiny neurons (MSNs) in the NAc by activating the D1R ($D_{1,5}R$) and D2R ($D_{2,3,4}R$). D2R modulates cellular activity via numerous signaling pathways (Beaulieu et al., 2007; Beom et al., 2004; Pedrosa et al., 2004; Senogles, 2000; Zhang et al., 2004). In striatal MSNs, D2R stimulation facilitates $G_{\beta\gamma}$/phospholypase $C_{\beta 1}$ coupling (Hernandez-Lopez et al., 2000) and phosphorylation of inositol-1,4,5-trisphosphate receptors ($IP_3R$) by PKA (Hu et al., 2005a). Such actions increase intracellular $Ca^{2+}$ release and decrease activity of L-type $Ca^{2+}$ channels, respectively (Bonci and Hopf, 2005; Greengard, 2001). Both the D2R effects activate calcineurin, which dephosphorylates L-channels and $IP_3Rs$, thereby decreasing $Ca^{2+}$ influx (Day et al., 2002; Hernandez-Lopez et al., 2000) but facilitating intracellular $Ca^{2+}$ release (Bultynck et al., 2003; Groth et al., 2003).

Repeated cocaine exposure disturbs $Ca^{2+}$ signaling in MSNs by enhancing and prolonging D1R/D2R stimulation. These changes result in part from reduced $Ca^{2+}$ influx and calcineurin function (Hu et al., 2004, 2005b; Zhang et al., 2002), leading to a reduction in NAc excitability (see Hu, 2007 for review). Alterations in $Ca^{2+}$ channel activity vary, depending upon subtypes of the channel. For instance, D1R stimulation increases L-channel activity with reinstatement of cocaine-seeking behavior in rats (Anderson et al., 2008; Self et al., 1998), whereas repeated cocaine exposure reduces $Ca^{2+}$ currents ($I_{Ca}$) via N- and R-type $Ca^{2+}$ channels in NAc MSNs (Zhang et al., 2002). Such differences can be attributed in part to enhanced phosphorylation of L-channels by PKA (Hernandez-Lopez et al., 1997) and dephosphorylation of N-/R-channels by protein phosphatase 1, respectively (Surmeier et al., 1995; Zhang et al., 2002). However, it is unknown whether and how D2R-mediated $Ca^{2+}$ homeostasis in NAc MSNs is altered after repeated cocaine exposure. Repeated D2R stimulation reduces D2R-coupled $G_i/G_o$ protein levels in the NAc (Nestler, 2004; Nestler et al., 1990) and induces desensitization and internalization of D2R after phosphorylation (Gainetdinov et al., 2004). These findings strongly suggest that dysregulated $Ca^{2+}$ homeostasis and signaling are associated with D2R downregulation in striatal MSNs.

Cocaine-induced D2R dysfunction plays a critical role in neuroadaptation of $Ca^{2+}$ influx and related signaling. Thus, chronic cocaine exposure reduces the a-subunits of D2R-coupled $G_{i/o}$ protein (Nestler et al., 1990), $Ca^{2+}$ influx (Hu et al., 2004; Zhang et al., 2002), and activity of the $G_{i/o}$/adenylate cyclase/cAMP/PKA/$Ca^{2+}$/ calcineurin pathway in NAc MSNs (Hu et al., 2005b). Cocaine abuse also decreases D2R availability in the striatum of abstinent humans (Volkow et al., 1999). On the basis of these findings, we hypothesized that D2R modulation of $Ca^{2+}$ homeostasis and signaling is decreased in NAc MSNs in cocaine-sensitized, withdrawn rats. This study was performed to determine whether (1) D2R modulates $Ca^{2+}$ channel function and intracellular $Ca^{2+}$ release in NAc MSNs of drug-free rats, and (2) repeated cocaine exposure decreases D2R modulation of $Ca^{2+}$ channel activity and $Ca^{2+}$ release via the D2R-coupled $Ca^{2+}$/calcineurin pathways.

## MATERIALS AND METHODS

### Animals and pretreatments

Adolescent male Sprague-Dawley rats at 4–5 weeks of age (Spear, 2000), which were more vulnerable to the development of drug addiction (Badanich et al., 2006, 2008; Schramm-Sapyta et al., 2006), were used in this study. They were group housed in a temperature and humidity-controlled vivarium under a 12-h light/dark cycle. Food and water were freely available. After 3 days acclimation to the vivarium, rats were randomly assigned to two

groups and received daily repeated intraperitoneal injections of saline (0.9% NaCl) or cocaine HCl (15 mg/kg) for 5 consecutive days. All rats used in this study received repeated injections of saline or cocaine in their home cage. All experiments were performed after a short-(3-day) or long-term (21-day) withdrawal from pre-treatments.

### Whole-cell recordings in brain slices

All procedures were in strict accordance with the National Research Council Guide for the Care and use of Laboratory Animals (NIH Publication No. 85-23, 1996) and were approved by our Institutional Animal Care and Use Committee. Rats were decapitated under halothane anesthesia. Brain was immediately excised and immersed in ice-cold artificial cerebrospinal fluid containing (in mM): NaCl 124, KCl 2.5, $NaHCO_3$ 26, $MgCl_2$ 2, $CaCl_2$ 2, and glucose 10; pH 7.4; 310 mosM/L. Coronal slices (300 μm) containing the NAc were cut with a vibratome (Leica VT1000S, Bannockburn, IL) and incubated in oxygenated (95% $O_2$/5% $CO_2$) artificial cerebrospinal fluid for 1 h at room temperature before recording. Slices were anchored in a recording chamber and perfused with gravity-fed oxygenated artificial cerebrospinal fluid (34°C) at a flow rate of 2–3 ml/min. Patch recording pipettes (3–5 MΩ) were pulled from Corning 7056 glass capillaries (Corning, NY) with a horizontal pipette puller (Flaming/Brawn P-97, Sutter Instruments, Novato, CA) and filled with internal recording solution (in mM): CsOH 130, HEPES 10, $MgCl_2$ 2, $Na_2$-phosphocreatine 10, $Na_2$ATP 3, and NaGTP 0.3, pH 7.3 with gluconic acid (50%), 280 mosM/l. To avoid influence of $Ca^{2+}$ chelation that alters dynamic D2R modulation of ion channel activity, $Ca^{2+}$ chelators were not included in the pipette solution (Hu et al., 2005a,b). Recordings were initiated in visually identified MSNs within the core region of the NAc using differential interference contrast microscopy and an amplifier (Axopatch 200B, Axon Instruments, Union City, CA).

After whole-cell configuration was formed, voltage-clamp mode was converted to current-clamp mode. Voltage signals were recorded, amplified in a bridge mode, and digitized by an interface (DigiData 1322A Series) into a computer running analysis software (pCLAMP 9) (Axon Instruments). To prevent influences of synaptic activities on the membrane potential ($V_m$), glutamate receptors and $GABA_A$ receptors were blocked during recording. $Na^+$ and $K^+$ channel were also blocked to separate the voltage-gated $Ca^{2+}$ channels (see Drug Application below for detail). High voltage-activated $Ca^{2+}$ plateau potentials were generated by injecting step depolarizing current pulses starting from 0 nA with 0.05 nA increments and 40 ms duration, which were delivered at 10 s intervals. The recording period in each episode was 3 s. Stabilized $Ca^{2+}$ potentials were recorded before application of agonists, antagonists, or blockers as control. Characteristics of $Ca^{2+}$ potentials were obtained from the initial $Ca^{2+}$ spike evoked by the minimal depolarizing current (rheobase). MSNs with stable resting membrane potential were recorded and used for analysis. Resting membrane potential was held at –80 mV (near the mean of –78 mV) during drug application and recording. This gave each NAc MSN the same basal potential level; thus, the results obtained from different cells would be comparable (Hu et al., 2004). The amplitude of $Ca^{2+}$ potentials was measured from the spike threshold to its peak. The half-amplitude duration of $Ca^{2+}$ potentials was measured at the amplitude level at which one-half of the spike peak was reached. The integrated area (size) of $Ca^{2+}$ potentials was defined and measured under the curve, which initiated at the rising part of the spike from resting membrane potential and ended with the recording period.

Separate subgroups of NAc MSNs were recorded with application of different drugs and ion channel blockers that were added in artificial cerebrospinal fluid immediately before use. Selective blockers/inhibitors for $Na^+$ channels (tetrodotoxin, TTX, 1 μM), $K^+$ channels (tetraethylammonium, TEA, 20 mM), gluta-mate receptors (kynurenic acid, 2.5 mM), and $GABA_A$ receptors [SR-95531 or 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-

pyridazinium bromide, 4 mM] were applied in bath in all experiments. Quinpirole, a selective D2R agonist (1–10 μM), was used to assess D2R modulation of $Ca^{2+}$ spikes and release. The selective D2R antagonist eticlopride (10 μM) was used to block the effects of quinpirole. Selective $Ca^{2+}$ channel blockers for L-type (nifedipine, 5 μM), N- (ω-conotoxin GVIA, 1 μM), and P/Q-type (ω-conotoxin MVIIC, 2 μM) channels were also applied in the bath to assess the role of non-L-type $Ca^{2+}$ channels. Active calcineurin (100 U) was added in the internal solution and applied directly in cytosol. Cyclosporin A (20 μM), a selective inhibitor for calcineurin, was applied externally.

## PKA assays

The NAc and motor cortex from saline- or cocaine-pretreated rats, with a short- or long-term withdrawal, were dissected and lysed in hypotonic buffer [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 μM (–)-*p*-bromotetramisole oxalate, 5 μM cantharidin, 5 μM microcystin-LF, and 5 μM cyclosporin A] and were supplemented with Complete Protease Inhibitor tablets (Roche Diagnostics, Indianapolis, IN). PKA activity in 2 μg of each sample was determined by the PepTag PKA assay (Promega, Madison, WI) (Dong et al., 2005). Positive controls contained 10 ng of purified PKA catalytic subunit (Promega), whereas the negative controls contained no PKA. The PepTag assay used the Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) peptide substrate tagged with a UV-fluorescent dye. The PKA activity was detected by the amount of phosphorylated substrate migrating toward the anode. Quantification was performed by calculating luminence intensity using TotalLab software (Nonlinear Dynamics, Durham, NC).

## Two-photon calcium imaging

Live-cell $Ca^{2+}$ imaging of individual neurons in thick brain slice preparations was performed using a custom-made video-rate two-photon imaging system based on an Olympus BX51 microscope frame (Nguyen et al., 2001), which is the gold standard technique currently available. Relative changes in $[Ca^{2+}]_{in}$ were readily measured and compared within and between samples. Although the absolute $[Ca^{2+}]_{in}$ could not be measured, a relative change from this baseline and estimated concentrations based on established baseline values were made. The resting $Ca^{2+}$ levels were interpreted to be similar based on similar fluorescence intensity values at the same laser intensity and power levels, and similar depths within the tissue slice. In addition, other $Ca^{2+}$-dependent effects that would affect passive and active membrane properties were also not different between groups, which were consistent with the observed fluorescent levels. Individual MSNs were filled with the $Ca^{2+}$ indicator fura-2 (50 μM) via a patch pipette as described (Stutzmann et al., 2003). Excitation was provided by trains (80 MHz) of ~100 fs pulses at 780 nm from a Ti:sapphire laser (Mai Tai Broadband, Spectra-Physics, Mountain View, CA). The laser beam was scanned at 30 fps using a custom-built scanner and focused through a 40× water-immersion objective (NA = 0.8). Emitted fluorescence light was detected by a wide-field photomultiplier (Electron Tubes, Rockaway, NJ) to derive a video signal that was captured and analyzed by Video Savant 5.0 software (IO Industries, ON, Canada). Further analysis of background-corrected images was performed using Metamorph software. For clarity, images and traces of fura-2 fluorescence are expressed as inverse pseudo-ratios: $F_0/\Delta F$ ($F_0$ is the average resting fluorescence before stimulation, and $\Delta F$ is the decrease of fura-2 fluorescence resulting from increased $[Ca^{2+}]$ when excited at 780 nM), so that increases in $[Ca^{2+}]$ correspond to increasing ratios.

## Statistical analysis

Student's *t*-test was used for comparison of drug effects on the membrane properties, characteristics of $Ca^{2+}$ plateau potentials, PKA activity, and intracellular $Ca^{2+}$ release between control and drug-treated NAc cells in saline- or cocaine-pretreated rats. Repeated-

measures ANOVA was used for comparison of the quinpirole-induced changes in the dose-response curves between saline- and cocaine-withdrawn groups. Newman-Keuls test was carried out for post hoc comparisons.

## RESULTS

### D2R stimulation reduces the size of evoked $Ca^{2+}$ plateau potentials in NAc MSNs

All medium spiny NAc cells were recorded in the core region. Blockade of $K^+$ channels (TEA in the bath and cesium in cytosol) depolarized resting membrane potential in NAc MSNs (SAL group = –61.1 ± 2.2, COC group = –60.7 ± 2.0 mV) when compared with that without such blockade (~–79 mV) (Zhang et al., 1998), which effectively stabilized generation of $Ca^{2+}$ potentials (Hu et al., 2004; Nasif et al., 2005a). This membrane depolarization was attributed mainly to blockade of the outflowing $K^+$ currents that were activated at the resting status of cells to maintain resting membrane potential at hyperpolarized levels. This change in resting membrane potential was not significantly affected by D2R stimulation (data not shown). $Ca^{2+}$ plateau potentials were evoked by depolarizing current pulses with blockade of $Na^+$ channels and $K^+$ channels (Fig. 1A). Bath-applied quinpirole (1–10 μM) decreased $Ca^{2+}$ channel activity in a dose-dependent manner (Figs. 1B and 1C). Because 10 μM of quinpirole was effective in producing a significant reduction in the size of evoked $Ca^{2+}$ plateau potentials ($n = 40$ cells, one-way ANOVA, $F_{(4,39)} = 5.07$, $^*P < 0.03$), it was used in the rest of experiments of this study. D2R-mediated decrease in $Ca^{2+}$ influx was reflected by a reduction in the size of evoked $Ca^{2+}$ spikes (control vs. quinpirole: 57,196 ± 8353 vs. 39,055 ± 5629 mV × ms; $n = 15$ cells, paired *t-test,* $t_{(1,14)} = 5.61$, $^*P < 0.05$), and the duration of $Ca^{2+}$ potentials (measured at the half-amplitude level (control vs. quinpirole: 336.3 ± 23.8 vs. 213 ± 23.4 ms and 47.5 ± 2.7 vs. 38.5 ± 2.9 mV, respectively; $n = 15$ cells/group, paired *t*-test, $t = 5.20$ and $t = 5.21$, all $^*P < 0.05$) (Fig. 2A–D). Rheobase and firing threshold of evoked $Ca^{2+}$ spikes were not significantly affected by quinpirole (SAL vs. COC: 0.7 ± 0.05 vs. 0.7 ± 0.05 nA, and 213.6 ± 1.7 vs. 212.4 ± 1.5 mV, respectively). The effects of D2R stimulation on $Ca^{2+}$ influx were washed out by fresh medium ($n = 15$, paired *t*-test, $P > 0.05$) (Figs. 2A–2D) or blocked by the selective D2R antagonist eticlopride (10 μM) (control vs. eticlopride + quinpirole: area, 34,479 ± 1969 vs. 35,450 ± 2709 mV × ms; amplitude: 38.3 ± 4.1 vs. 41.2 ± 2.3 mV; duration: 235.1 ± 15.8 vs. 255.3 ± 24.5 ms; $n = 10$ cells, paired *t*-test, $P > 0.05$) (Fig. 2E–H).

### Selective blockade of L-type, but not N- and P/Q-type $Ca^{2+}$ channel, mimics and occludes D2R-mediated reduction in $Ca^{2+}$ spikes

Given the fact that generation of $Ca^{2+}$ spikes in striatal cells of young rats depends primarily on activation of high voltage-activated $Ca^{2+}$ channels (Hu et al., 2004; Surmeier et al., 1995; Zhang et al., 2002), we evaluated whether and how the activity and which subtype of high voltage-activated $Ca^{2+}$ channels was modulated by the D2R. Bath application of nifedipine (5 μM), a specific L-channel blocker, significantly reduced the amplitude, duration, and size of evoked $Ca^{2+}$ spikes (control vs. nifedipine: 51.0 ± 2.9 vs. 42.5 ± 4.4 mV; 319.6 ± 32.6 vs. 251.7 ± 35.7 ms, and 50,816 ± 5800 vs. 39,811 ± 4425 mV × ms, respectively; $n = 10$ cells, paired *t*-test, $t = 3.51$, $t = 4.22$, and $t = 3.07$, all $^*P < 0.05$) (Fig. 3A). With blockade of L-channels, quinpirole (10 μM) was no longer able to suppress $Ca^{2+}$ potentials (nifedipine vs. nifedipine + quinpirole: 39,811 ± 4425 vs. 37,802 ± 4539 mV × ms, 42.5 ± 4.4 vs. 39.8 ± 4.7 mV, and 251.7 ± 35.7 vs. 248 ± 37.9 ms, respectively; $n = 10$ cells, paired *t*-test, all $P > 0.05$) (Fig. 3A). Because the size of $Ca^{2+}$ spikes was affected by the duration and amplitude, it was measured and used as the primary result and was compared among all experimental groups throughout this study.

In contrast, blockade of other types of high voltage-activated $Ca^{2+}$ channels did not eliminate D2R-mediated inhibition in $Ca^{2+}$ spikes. Bath-applied ω-conotoxin GVIA (5 μM), a specific N-type $Ca^{2+}$ channel blocker, significantly reduced the area of $Ca^{2+}$ spikes (control vs. ω-conotoxin GVIA: 58,290 ± 1117 vs. 48,208 ± 10079 mV × ms, $n = 10$ cells, paired $t$-test, $t = 3.55$, $^*P < 0.05$) (Fig. 3B). The N-channel blocker-induced reduction in $Ca^{2+}$ spikes was enhanced by concurrent application of quinpirole, which produced an additive decrease in the size of $Ca^{2+}$ spikes (ω-conotoxin GVIA vs. ω-conotoxin GVIA + quinpirole: 43,393 ± 8605 vs. 27,773 ± 5891 mV × ms, $n = 10$ cells, paired $t$-test; $t = 4.42$, $^*P < 0.05$) (Fig. 3B). ω-Conotoxin MVIIC (2 μM), a specific blocker for P/Q-type $Ca^{2+}$ channels (Brown and Randall, 2005; Phillips and Stamford, 2000), also significantly diminished the area of $Ca^{2+}$ spikes (control vs. ω-conotoxin MVIIC: 43,245 ± 4962 vs. 33,042 ± 2309 mV × ms, $n = 10$ cells, paired $t$-test, $t = 2.77$, $^*P < 0.05$) (Fig. 3C). Application of quinpirole with the P/Q-channel blocker also produced an additional reduction in the size of $Ca^{2+}$ spike (ω-conotoxin MVIIC vs. ω-conotoxin MVIIC + quinpirole: 33,042 ± 2309 vs. 21,383 ± 5609 mV × ms, $n = 10$ cells, paired $t$-test, $t = 4.45$, $^*P < 0.05$) (Fig. 3C).

## Calcineurin suppresses $Ca^{2+}$ plateau potentials and occludes D2R-mediated inhibition of $Ca^{2+}$ spikes

To determine whether and how $Ca^{2+}$ channel activity is modulated by the D2R-coupled $Ca^{2+}$/calcineurin pathway, we evaluated the interaction of calcineurin and D2R stimulation. We found that cytosolic application of active calcineurin (100 U) mimicked the D2R-mediated reduction in $Ca^{2+}$ spikes (control vs. calcineurin: 54,460 ± 3059 vs. 32,722 ± 2141 mV × ms, $n = 10$ cells, $t$-test, $t = 5.84$, $^*P < 0.05$). However, concurrent application of quinpirole with calcineurin produced no further reduction; and the calcineurin-induced decrease in the size of $Ca^{2+}$ potentials was not significantly affected by quinpirole (calcineurin vs. calcineurin + quinpirole: 32,722 ± 2141 vs. 33,823 ± 2397 mV × ms, $n = 10$ cells, paired $t$-test; $P > 0.05$; control vs. calcineurin + quinpirole: 54,460 ± 3059 vs. 33,823 ± 2397 mV × ms; paired $t$-test, $t = 5.06$, $^*P < 0.05$) (Fig. 4A–B).

To further investigate the calcineurin effects on $Ca^{2+}$ channel activity, we assessed if inhibition of calcineurin activity could induce an opposite responsiveness in $Ca^{2+}$ spikes. In contrast to calcineurin, bath application of cyclosporin A (20 μM) (Hu et al., 2005a), a selective inhibitor for calcineurin, significantly increased the duration and size of $Ca^{2+}$ potentials in NAc MSNs (control vs. cyclosporin A: 367.9 ± 24 vs. 404.9 ± 33.1 ms and 48,980 ± 2686 vs. 55,988 ± 3773 mV × ms, $n = 10$ cells, respectively; paired $t$-test, $t = 3.28$ and $t = 5.43$, respectively, both $^*P < 0.05$) (Fig. 4C and D). This effect of cyclosporin A on $Ca^{2+}$ potentials was not affected by concurrent application of quinpirole (cyclosporin A + vs. cyclosporin A quinpirole: 55,988 ± 3773 vs. 54,596 ± 3874 mV × ms, $n = 10$ cells, paired $t$-test, $P > 0.05$; control vs. cyclosporin A + quinpirole: 48,980 ± 2686 vs. 54,596 ± 3874 mV × ms, paired $t$-test, $t = 3.55$, $^*P < 0.05$) (Fig. 4D).

## Repeated cocaine exposure and withdrawal decreases $Ca^{2+}$ channel function and abolishes D2R-mediated inhibition of $Ca^{2+}$ spikes

In this study, we extended our earlier research regarding the decreased activity of high voltage-activated $Ca^{2+}$ channels in medium spiny NAc neurons of rats after a 3-day withdrawal (Hu et al., 2004) by investigating two additional questions: (1) does the decreased $Ca^{2+}$ channel activity persist after longer withdrawal? And (2) are the effects of D2R on modulating high voltage-activated $Ca^{2+}$ channel activity in NAc MSNs of cocaine-pretreated rats altered after cocaine withdrawal? To study the first question, we compared the integrated area of $Ca^{2+}$ spikes in NAc MSN from saline- and cocaine-pretreated rats. The size of $Ca^{2+}$ potentials was significantly reduced in cocaine-pretreated NAc neurons

either after a 3-day or a 21-day abstinence (3-day/withdrawl: SAL vs. COC = 53,138 ± 7742 vs. 35,285 ± 1707 mV × ms; 21-day/withdrawl: SAL vs. COC = 46,062 ± 4595 vs. 32,380 ± 2808 mV × ms; $n = 14$ cells/each group, paired *t*-test, $t = 2.99$ and $t = 2.63$, respectively, both $^*P < 0.05$) (Fig. 5A–D). This change resulted from reduced duration (3-day/withdrawl: SAL vs. COC = 323.5 ± 18.5 vs. 262.4 ± 13.5 ms; 21-day/withdrawl: SAL vs. COC = 233.1 ± 16.6 vs. 174.6 ± 11.2 ms; $t = 2.16$ and $t = 2.33$; all $^*P < 0.05$). Rheobase was also increased in cocaine-pretreated cells (3-day/withdrawl: SAL vs. COC = 0.66 ± 0.04 vs. 1.0 ± 0.06 nA; 21-day/withdrawl: SAL vs. COC = 0.7 ± 0.08 vs. 1.1 ± 0.11 nA; *t*-test, $t = 3.5$ and $t = 2.4$, respectively; all $^*P < 0.05$) (Fig. 5E). These changes indicate that the intrinsic excitability of medium spiny NAc neurons was remarkably reduced by downregulating high voltage-activated $Ca^{2+}$ channel function in cocaine-sensitized, withdrawn rats.

The second question was studied by comparing the spike area of medium spiny NAc neurons between saline- and cocaine-pretreated rats. Associated with cocaine-induced decrease in $Ca^{2+}$ influx, the D2R effects on reducing the duration of evoked $Ca^{2+}$ spikes were diminished in cocaine-pretreated NAc neurons after withdrawal. Under this circumstance, quinpirole was no longer able to suppress $Ca^{2+}$ spikes in cocaine-withdrawn NAc neurons, even at a higher concentration (3-day/withdrawl group: SAL vs. COC: $n = 14$ vs. 13 cells, and 21-day/withdrawl group: SAL vs. COC, $n = 12$ vs. 11 cells; two-way ANOVA with repeated measures, $F_{(2,46)} = 4.16$, $^*P < 0.05$, and $F_{(2,40)} = 14.2$, $^*P < 0.05$, respectively, both compared to quinpirole 0 μM; control) (Fig. 5E–H).

## Repeated cocaine administration persistently increases PKA activity in the NAc after a 3-day or a 21-day withdrawal

Previous findings indicated that repeated cocaine treatment increases PKA activity in various brain regions, including NAc (Edwards et al., 2007; Hope et al., 2005; Scheggi et al., 2007). To determine if the persistent dysregulation of $Ca^{2+}$ channels in NAc cells (see above) was associated with this change, we assessed PKA activity in the NAc using a highly specific fluorescent peptide substrate (kemptide) after a 3-day or a 21-day withdrawal. The net levels of this peptide were changed from positive to negative, depending upon the extent of phosphorylation of the peptide by PKA, which allowed electrophoretic separation and quantification of the phosphorylated substrate (Dong et al., 2005; Ford et al., 2009). We found that PKA activity was significantly increased in NAc cells after a 3-day (SAL vs. COC: 100% ± 11.8% vs. 137.2% ± 13.8%, $n = 20/22$ rats; *t*-test, $t = 2.026$, $^*P < 0.05$) (Fig. 6A) or a 21-day cocaine withdrawal (SAL vs. COC: 100% ± 9.0% vs. 134.5% ± 11.6%, $n = 22/22$ rats; $t = 2.370$, $^*P < 0.03$). However, there was no significant change of PKA activity in the motor cortex (3-day/withdrawl: 100% ± 6.2% vs. 102.2% ± 7.6%, $n = 17/19$ rats; 21-day/withdrawl: 100% ± 6.4% vs. 97.5% ± 5.7%, $n = 27/27$ rats; both $P > 0.05$) (Fig. 6B), indicating that the cocaine-induced changes in PKA activity were region specific.

## Repeated exposure to cocaine decreases D2R modulation of cytosolic $Ca^{2+}$ release in NAc MSNs after a 3-day or a 21-day withdrawal

It has been suggested that D2R stimulation increases $Ca^{2+}$ release in striatal cells (Greengard, 2001). Stimulation of D2R also elevates $[Ca^{2+}]_{in}$ in cortical astrocytes (Khan et al., 2001). However, little is known whether and how D2R could dynamically modulate this activity in medium spiny NAc neurons. Here, we used a two-photon laser scanning $Ca^{2+}$ imaging technology to evaluate cytosolic $Ca^{2+}$ release in medium spiny NAc neurons in slice preparations from saline- or cocaine-withdrawn rats. The basal levels of resting $Ca^{2+}$ level, as indicated by relative fluorescent intensity of the calcium indicator fura-2, were imaged for 3 min in both saline- and cocaine-pretreated rats. There was no significant difference in the relative baseline levels of fluorescent intensity between NAc neurons in saline- and cocaine-pretreated rats after a 3-day or a 21-day withdrawal (both $P > 0.05$) (Fig.

7A). D2Rs localized on the cell membrane of medium spiny NAc neurons were stimulated with bath application of quinpirole (10 μM) for 5 min, and then the changes in $[Ca^{2+}]_{in}$ after D2R stimulation were recorded and compared to the baseline. D2R stimulation by quinpirole evoked a marked relative increase in the somatic levels of free $Ca^{2+}$ in NAc MSNs in saline-pretreated rats ($F_0/\Delta F$ = 0.62% ± 0.21% or 162% ± 20.8% of the predrug baseline measurement, $n$ = 8 cells in four rats) (Fig. 7A and C). This D2R effect lasted ~5 min and was washed out completely with fresh artificial cerebrospinal fluid, as indicated in the raw two-photon images (Fig. 7A). The relative changes in $Ca^{2+}$ release in saline- or cocaine-pretreated NAc cells in response to D2R stimulation are presented with pseudocolored images (Fig. 7B).

Repeated cocaine exposure abolished the D2R-mediated intracellular $Ca^{2+}$ release in medium spiny NAc neurons. There was no detected $Ca^{2+}$ release in the presence of quinpirole in cocaine-pretreated NAc cells when compared with that in saline-pretreated cells after either a 3-day withdrawal ($F_0/\Delta F$ = –0.02% ± 0.1% or 98.3% ± 7.1% of baseline predrug response; $n$ = 9 cells, $t_{(1,16)}$ = 3.02; $^{**}P < 0.001$) (Fig. 7A and C) or a 21-day withdrawal (SAL- vs. COC-pretreated: $n$ = 10/9 cells, four rats in each group; $F_0/\Delta F$ = 0.37 ± 0.16 vs. –0.02 ± 0.02, respectively, $t_{(1,17)}$ = 2.29; $^{*}P < 0.05$) (Fig. 7C, right panel). Given the fact that the quinpirole-induced increase in $[Ca^{2+}]_{in}$ was observed without activation of voltage-gated $Ca^{2+}$ channels and with blockade of ionotropic glutamate receptors, these findings indicate that D2R-mediated increase in $[Ca^{2+}]_{in}$ resulted from intracellular stores.

### Protein levels of L-type $Ca^{2+}$ channels and $IP_3R$ are not significantly altered in the rat NAc after chronic cocaine treatment and withdrawal

$Ca^{2+}$ influx via high voltage-activated $Ca^{2+}$ channels and $Ca^{2+}$ release from intracellular stores depend not only on the activity but also on the number of these $Ca^{2+}$ channels and $IP_3$ receptors, respectively. Thus, we also evaluated if the decrease of $Ca^{2+}$ influx and $Ca^{2+}$ release in cocaine-pretreated NAc cells was affected by reduced protein levels (reflecting a decrease in the number) of the L-channels and/or $IP_3$Rs. Specific antibodies were used to measure the levels of L-channels and $IP_3$Rs in the rat NAc. The total protein levels of α1 subunit (pore-forming and ligand-binding protein) of L-channel and $IP_3$R were not significantly affected in the rat NAc after repeated cocaine treatment and withdrawal ($n$ = 18–31/group, all $P > 0.05$; data not shown). Whether the surface expression (a.k.a. trafficking) of the L-channels and $IP_3$Rs was altered by repeated cocaine exposure and withdrawal remains to be determined in future investigations.

## DISCUSSION

This study determined that D2R ($D_{2,3,4}$R) stimulation modulates $Ca^{2+}$ homeostasis and related signaling in rat NAc MSNs in the core region by decreasing $Ca^{2+}$ influx preferentially via the L-channels and increasing intracellular $Ca^{2+}$ release. Repeated cocaine exposure increased PKA activity and abolished the D2R modulation. These changes are found after a 3-day or a 21-day cocaine abstinence, indicating enduring neuroadaptations of NAc function in cocaine-sensitized, withdrawn rats.

### D2R stimulation decreases $Ca^{2+}$ influx by preferentially reducing L-channel activity: implications of $Ca^{2+}$ release and calcineurin activation

D2R-mediated decrease in $Ca^{2+}$ influx was reflected by reduced “size” of $Ca^{2+}$ potentials, which was reversible, receptor specific, and dose dependent. This decrease was mimicked and occluded by blockade of the L-, but not N- and P/Q-type $Ca^{2+}$ channels, suggesting that D2R modulation preferentially reduced L-channel activity in NAc MSNs. Intracellular $Ca^{2+}$ release and downstream calcineurin activation were implicated in the mechanisms, which

may underlie the D2R-modulated decrease of L-channel activity. We previously revealed that D2R stimulation facilitates $Na^+$ channel activity, most likely via elevating intracellular $[Ca^{2+}]_{in}$ in rat NAc MSNs (Hu et al., 2005a). Nevertheless, such $Ca^{2+}$ release has never been proven by real-time $Ca^{2+}$ imaging study. This study demonstrates that selective D2R stimulation increased $[Ca^{2+}]_{in}$ in the absence of membrane depolarization with blockade of ionotropic glutamate receptors, indicating a dynamic intracellular $Ca^{2+}$ release in rat NAc MSNs. Such increased $Ca^{2+}$ release could activate calcineurin and dephosphorylate L-channels, thereby subsequently reducing $Ca^{2+}$ influx via the L-channel (Day et al., 2002; Groth et al., 2003). Moreover, we also found that calcineurin mimicked and occluded D2R-mediated suppression of $Ca^{2+}$ spikes, and inhibition of calcineurin activity by cyclosporin A not only prolonged the duration but also prevented D2R suppression of $Ca^{2+}$ spikes. These findings suggest that via a D2R-coupled, cAMP/PKA/$IP_3$R/$Ca^{2+}$-mediated pathway (Hu et al., 2005a), and likely the others (Hernandez-Lopez et al., 2000), D2R stimulation activates calcineurin in a converged common path that may facilitate dephosphorylation of L-channels and therefore reduce $Ca^{2+}$ influx in NAc MSNs (Fig. 8A).

## Repeated cocaine exposure decreases $Ca^{2+}$ influx with enhanced PKA activity

Another major finding of this study is that the cocaine-induced decrease in $Ca^{2+}$ influx was associated with enhanced PKA activity in the NAc. Given the fact that the MSNs constitute 95% cell population in this brain region (Pasik, 1979), we could reasonably assume that the cocaine-induced increase of PKA activity in the NAc occurred mainly in MSNs. The mechanism underlying the decreased $Ca^{2+}$ influx has been related to enhanced D1R signaling and reduced D2R function with increased phosphorylation and diminished dephosphorylation of $Ca^{2+}$ channels, respectively (see Hu, 2007; Nestler, 2004 for review). Compelling evidence shows that cocaine-induced neuroadaptation in ion channels is attributable, at least in part, to facilitation of the cAMP/PKA cascade. For instance, upregulated D1R signaling (e.g., increase of $G_s$-coupled cAMP formation and PKA activity) is found in NAc cells after repeated cocaine treatment (Hope et al., 2005; Self et al., 1995; Terwilliger et al., 1991). With enhanced PKA/DARPP-32 activity and reduced calcineurin function (Hu et al., 2005b), both the $Ca^{2+}$ influx via high voltage-activated $Ca^{2+}$ channels and the evoked $Ca^{2+}$ spikes are significantly diminished in cocaine-pretreated NAc MSNs (Hu et al., 2004; Zhang et al., 2002).

Cocaine-induced decrease in $Ca^{2+}$ influx in NAc MSNs could result from enhanced PKA activity via a direct and indirect manner. Although direct phosphorylation of L-channels by PKA can facilitate activity of the channel, whole-cell $Ca^{2+}$ influx is actually reduced because of decreased $I_{Ca}$ through N- and R-type $Ca^{2+}$ channels in cocaine-withdrawn NAc MSNs (Zhang et al., 2002). This decrease in $I_{Ca}$ is most likely related to an indirect PKA action by which protein phosphatase 1 is activated and non-L-type high voltage-activated $Ca^{2+}$ channels are dephosphorylated (Surmeier et al., 1995; Zhang et al., 2002). It is worth noting that $I_{Ca}$ via the L-channels contribute to only ~30% of whole-cell $Ca^{2+}$ conductance, whereas the combined N- and R-type $Ca^{2+}$ currents consist of about 50% of $Ca^{2+}$ influx in control, drug-free NAc MSNs (Zhang et al., 2002). Even though repeated cocaine exposure tended to increase $Ca^{2+}$ influx via L-channels, such change was not statistically significant (at least not in the soma). Thus, we suggest that the reduced amount of $I_{Ca}$ across N- and R-type $Ca^{2+}$ channels was greater than the probably increased $Ca^{2+}$ currents via the L-channels and therefore led to suppression of $Ca^{2+}$ potentials in cocaine-preexposed NAc MSNs (Fig. 8B).

### Repeated cocaine treatment abolishes D2R-modulated $Ca^{2+}$ release and inhibition of L-channel activity

This study also reveals an intracellular $Ca^{2+}$ release induced by D2R stimulation. This novel finding provides the first evidence for dynamic D2R modulation of $Ca^{2+}$ mobilization in rat NAc MSNs. However, repeated cocaine exposure decreased the D2R effect. This decrease could be attributed in part to a reduction in D2R/$G_{i/o}$ coupling and $IP_3R$ activity, but not to a decrease in $IP_3R$ levels. Such conclusion results from the following facts: First, PKA phosphorylation inhibits $IP_3R$ activity and diminishes $Ca^{2+}$ release from endoplasmic reticulum (Cameron et al., 1995; Ferris et al., 1991; Quinton and Dean, 1992; Tertyshnikova and Fein, 1998). Second, D2R-coupled $Ca^{2+}$ modulation of ion channel activity involves inhibition of PKA activity and disinhibition of $IP_3R$ (Hu et al., 2005b). Third, D2R-mediated suppression of $Ca^{2+}$ spikes relies on reduced L-channel activity via dephosphorylation of the channel by calcineurin (Day et al., 2002; Hernandez-Lopez et al., 2000). Fourth, repeated cocaine exposure increases D1R/$G_s$ coupling (Terwilliger et al., 1991) and decreases D2R/$G_i$ coupling (Nestler et al., 1990) as well as calcineurin levels and efficacy in NAc MSNs (Hu et al., 2005b). Fifth, the $IP_3R$ protein levels are not changed by repeated cocaine exposure. Thus, reduced D2R modulation of intracellular $Ca^{2+}$ release by cocaine provides an informative knowledge for us to better understand the mechanism of cocaine withdrawal. Although the exact subtypes of D2R implicated in the cocaine-induced changes remain unknown (all $D_{2,3,4}R$ are found in the NAc, but may or may not be in the same cells), these findings suggest that diminished D2R signaling plays an important role in dysregulating $Ca^{2+}$ homeostasis in NAc MSNs of cocaine-preexposed, abstinent rats.

### Cocaine-induced adaptations in NAc MSNs persist during withdrawal

The reduced $Ca^{2+}$ influx, enhanced PKA activity, decreased intracellular $Ca^{2+}$ release, and abolished inhibition of L-channel activity mediated by D2R persisted for at least 21 days after cocaine withdrawal. These findings are consistent with earlier in vivo studies, showing that repeated cocaine exposure enduringly increases D1R-modulated inhibition of NAc activity in cocaine-withdrawn rats (Henry and White, 1991; Henry et al., 1989). They are also correlated with cocaine-induced behavioral sensitization and neuroadaptations in synaptic activity of NAc cells, which may increase cocaine craving and/or relapse in response to cocaine and cocaine-related cues (Conrad et al., 2008; Kourrich et al., 2007). These findings are in agreement with the perspective that cocaine-induced neuroadaptations in dopamine signaling pathways and ion channel function decrease the intrinsic excitability (Dong et al., 2006; Zhang et al., 1998) and activity of NAc MSNs (Hu, 2007; Kalivas and Hu, 2006). Such decrease in NAc activity provides a support for the reduced basal activity in the reward circuitry revealed by brain imaging study in cocaine-abstinent human (Kufahl et al., 2005; Volkow et al., 2003b), which may contribute to the neuropathophysiology of cocaine addiction.

It is worth noting that cocaine-induced changes in the L-channels, voltage-gated K+ channels, and PKA activity also occur in the medial prefrontal cortex (mPFC) (Dong et al., 2005; Ford et al., 2009; Nasif et al., 2005a,b), suggesting that cocaine-induced neuroadaptation preferentially affects function of the mesocorticolimbic dopamine system (a.k.a. the reward pathway). Given that both NAc and mPFC play critical roles in regulating cognitive function and addiction, these findings suggest that cocaine-induced neuroadaptation in neuronal activity may primarily or initially occur in the two brain regions.

### Functional implications

Reduced D2R modulation of $Ca^{2+}$ homeostasis in MSNs reveals dysregulation of the NAc after withdrawal from repeated cocaine exposure. These results are in agreement with

previous findings, showing involvement of D2R dysfunction in the mechanisms of cocaine withdrawal. For instance, brain imaging studies in cocaine-abstinent humans indicate decreased D2R availability in striatal cells (Volkow et al., 2003b). This decrease (likely via phosphorylation-induced internalization) is correlated with reduced glucose metabolism and oxygen consumption in the orbital and medial PFC during cocaine abstinence (known as PFC hypoactivity) (Volkow et al., 2003a, 2007). Such changes reflect a decreased basal activity in the orbital-mPFC and reduced excitatory outputs from these cortical regions to the NAc (Kalivas and Hu, 2006) and therefore may contribute to the neuro-pathogenesis of cocaine withdrawal symptoms, including but not limited to depression, apathy, anhedonia, and drug seeking (Hu, 2007). Our novel findings provide support for the perspective that cocaine-induced behavioral changes are fundamentally based upon neuroadaptations in ion channel function and dopamine/$Ca^{2+}$ signaling in the NAc and mPFC, in which D2R dysregulation, along with D1R dysfunction, plays a crucial role. Determining the altered D2R function in the NAc of cocaine-preexposed rats extends our knowledge to better understand the mechanisms of cocaine addiction, which may eventually help us to develop more effective therapeutic treatments for drug addiction.

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## Abbreviations

| | |
|---|---|
| **cAMP** | cyclic adenosine monophosphate |
| **D1R** | dopamine $D_1$-like receptors |
| **D2R** | dopamine $D_2$-like receptors |
| **$IP_3R$** | inositol-1,4,5-trisphosphate receptor |
| **MSNs** | medium spiny neurons |
| **NAc** | nucleus accumbens |
| **PKA** | protein kinase A |

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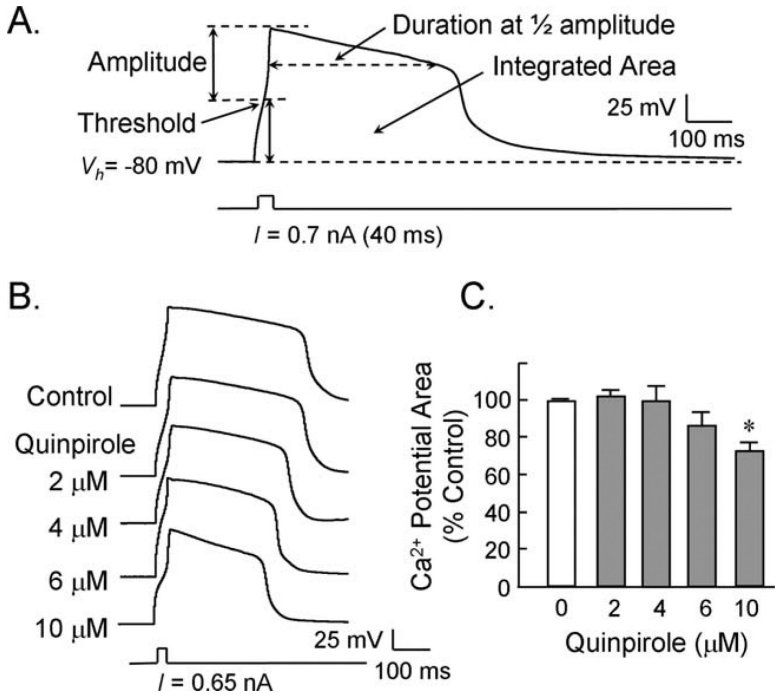


**Fig. 1.**

D2R stimulation reduced the size of evoked $Ca^{2+}$ plateau potentials in NAc MSNs of control rats. **A**: Representative trace shows an evoked $Ca^{2+}$ potential. The amplitude of $Ca^{2+}$ spikes was measured from the spike threshold to its peak. The half-amplitude duration of $Ca^{2+}$ potentials was measured at the amplitude level at which one-half of the spike peak was reached. The integrated area of $Ca^{2+}$ potentials was defined and measured under the trace which initiates at the beginning of the evoked potential from the resting membrane potential (held at –80 mV; the horizontal dash line) and ends at the end of the recording period (3 s). **B**: D2R stimulation by quinpirole (2, 4, 6, and 10 μM) reduced the duration of $Ca^{2+}$ spikes in a concentration-dependent fashion. **C**: Bar graphs show a signifi-cant decrease in the size of $Ca^{2+}$ spikes with 10 μM quinpirole (means ± S.E, $n$ = 10 cells; paired $t$-test, $*P < 0.05$).

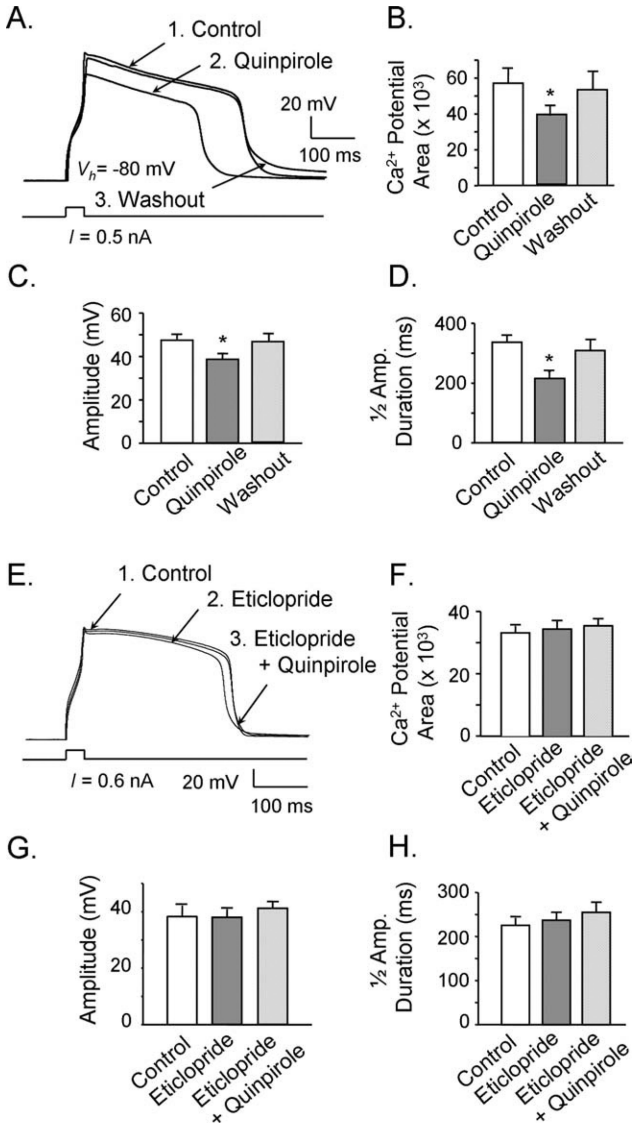


**Fig. 2.**

D2R-modulated inhibition in $Ca^{2+}$ potentials was reversible and prevented by D2R antagonist. **A**: Representative recording traces showing that D2R-modulated inhibition of $Ca^{2+}$ spikes was reversible. **B–D**: Bar graphs indicate that the significant reductions in the size, amplitude, and duration of $Ca^{2+}$ spikes induced by quinpirole (10 μM) were reversed following washout ($n = 10$ NAc cells; paired $t$-test, $^*P < 0.05$). Short bars represent means ± S.E. **E**: Concurrent application of the selective D2R antagonist eticlopride (10 μM) blocked the quinpirole-induced reduction in the size of $Ca^{2+}$ potentials. **F–H**: There was no significant difference in the area, amplitude, and duration of $Ca^{2+}$ spikes between control and eticlopride + quinpirole-treated cells. Bath-applied eticlopride produced no significant change in $Ca^{2+}$ potentials (means ± S.E., $n = 10$ cells; paired $t$-test, all $P > 0.05$).

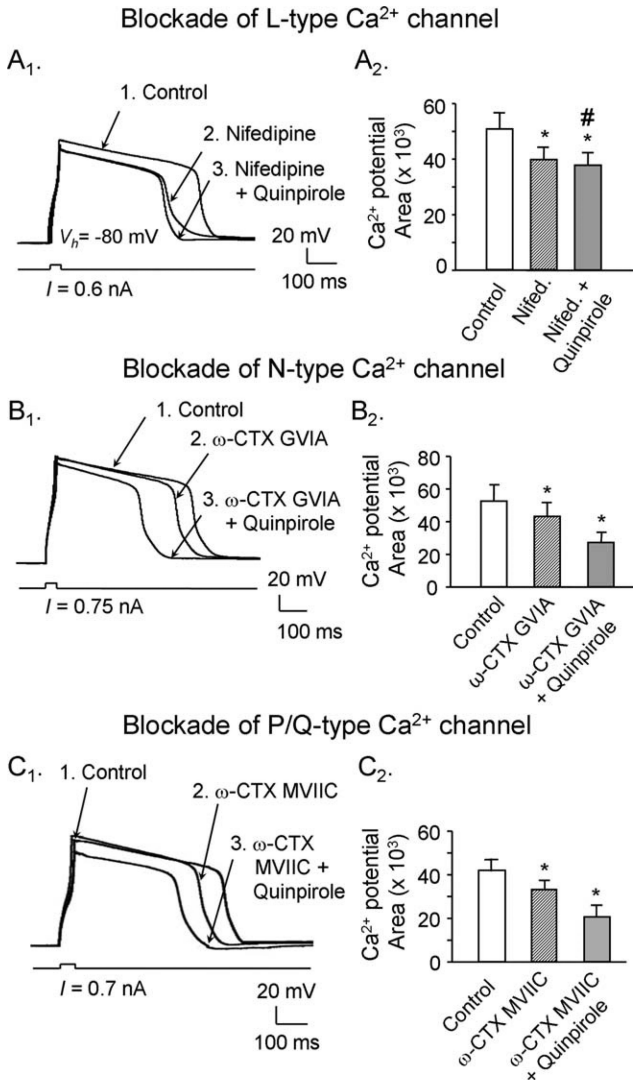


**Fig. 3.**

Blockade of L-, but not N- or P/Q-type $Ca^{2+}$ channels, mimicked and occluded D2R-modulated inhibition of $Ca^{2+}$ spikes. **$A_1$**: Representative traces showing that selective blockade of L-channel by nifedipine (5 μM) reduced the duration and amplitude of $Ca^{2+}$ spikes, whereas the D2R-modulated inhibition of $Ca^{2+}$ potentials was occluded by blockade of L-channel. **$A_2$**: Bar graphs indicate a significant decrease in the $Ca^{2+}$ spike area with L-channel blockade ($n$ = 10 cells, respectively; means ± S.E., paired $t$-test, both $^*P < 0.05$). There was no significant difference in the $Ca^{2+}$ spike between nifedipine-treated and nifedipine plus quinpirole-treated cells ($n$ = 10 cells; paired $t$-test, $^{\#}P > 0.05$). **$B_1$**: Selective blockade of N-type $Ca^{2+}$ channels with ω-conotoxin GVIA (1 μM) reduced the duration of $Ca^{2+}$ potentials, and this effect was further enhanced by concurrent application of quinpirole. **$B_2$**: There was a significant decrease in the $Ca^{2+}$ spike area between control cells and that with application of ω-conotoxin GVIA or ω-conotoxin GVIA plus quinpirole and between cells treated with ω-conotoxin GVIA and ω-conotoxin GVIA + quinpirole ($n$ = 10 cells; paired $t$-test, all $^*P < 0.05$). **$C_1$**: Selective blockade of P/Q-type channels with ω-conotoxin MVIIC (2 μM) reduced the $Ca^{2+}$ spike area, which was also enhanced by quinpirole. **$C_2$**: There was a significant difference in the spike area between control cells and that with ω-conotoxin MVIIC or ω-conotoxin MVIIC + quinpirole and between cells treated

with ω-conotoxin MVIIC and ω-conotoxin MVIIC + quinpirole ($n = 10$ cells; paired $t$-test, all $^*P < 0.05$).

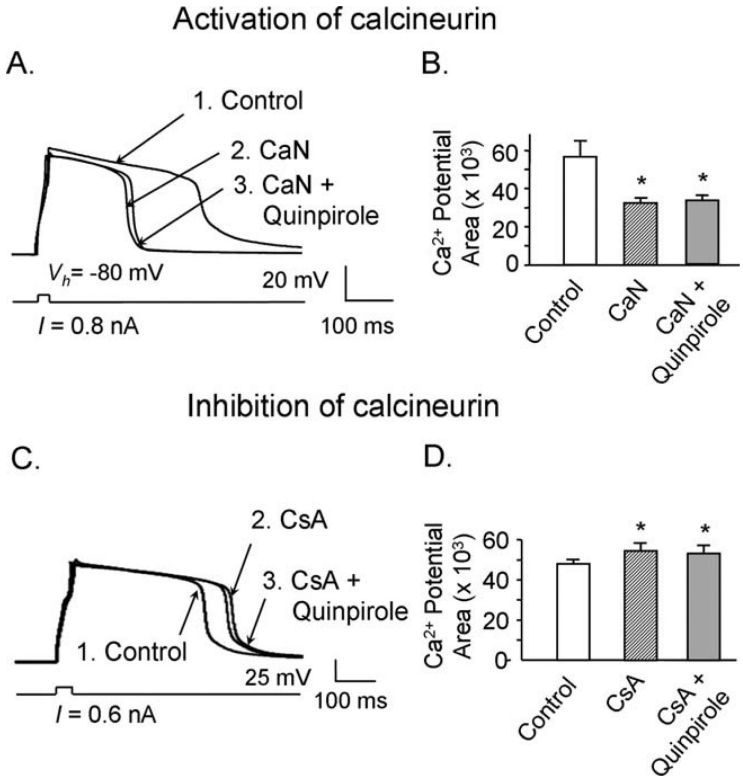


**Fig. 4.**

D2R/calcineurin-modulated inhibition of $Ca^{2+}$ spikes. **A**: Representative traces showing that cytosolic application of exogenous calcineurin reduced the duration of $Ca^{2+}$ potentials that occluded the effects of quinpirole on reducing $Ca^{2+}$ spikes. **B**: Calcineurin (100 U) significantly decreased the $Ca^{2+}$ spike area, whereas concurrent application of quinpirole produced no further effects on reducing $Ca^{2+}$ potentials ($n = 10$ cells; paired $t$-test, $^*P < 0.05$ and $P > 0.05$, respectively). Bars represent means ± S.E. **C**: Representative traces show that inhibition of calcineurin activity by cyclosporin A (20 μM) increased the area of $Ca^{2+}$ potentials and prevented the inhibitory effects of quinpirole on reducing the area of $Ca^{2+}$ spikes. **D**: There was a significant difference in the area of $Ca^{2+}$ potentials between control cells and that treated with cyclosporin A or cyclosporin A + quinpirole ($n = 10$ cells; paired $t$-test, both $^*P < 0.05$). Coapplication of quinpirole did not produce any significant change in the area of $Ca^{2+}$ spikes in NAc neurons when compared with cyclosporin A group ($n = 10$ cells; paired $t$-test, $P > 0.05$).

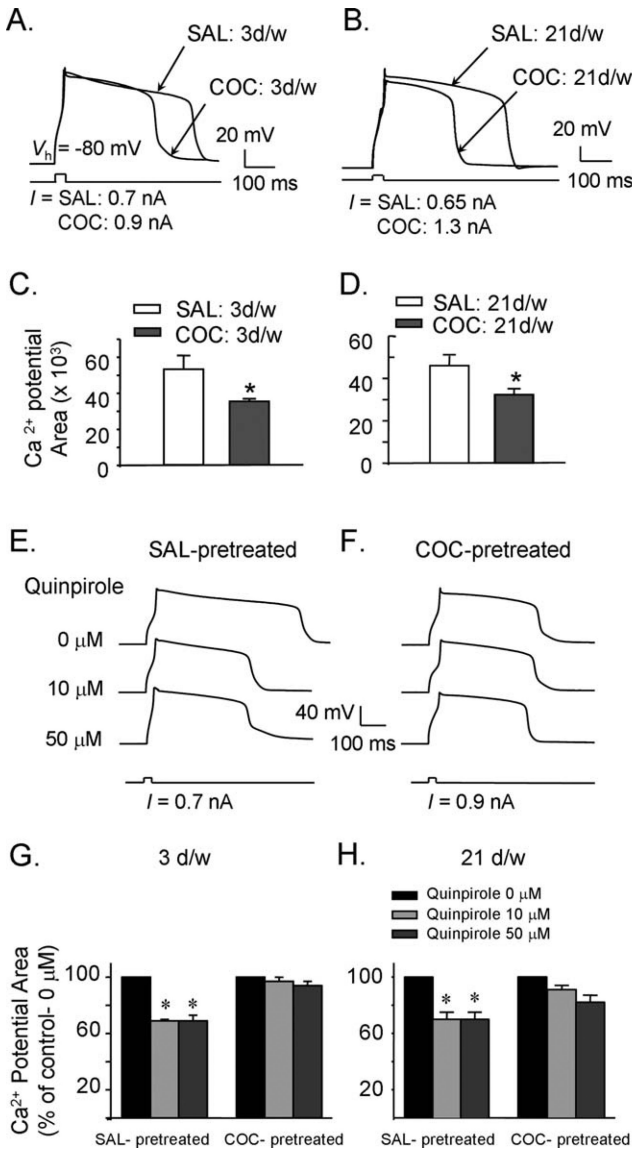


**Fig. 5.**

Repeated cocaine exposure attenuated both $Ca^{2+}$ channel function and D2R-modulated inhibition of $Ca^{2+}$ spikes in NAc MSNs after a short- or long-term withdrawal. **A, B**: Representative traces show that the duration of $Ca^{2+}$ potentials was reduced in NAc MSNs after a 3-day (3-day/withdrawl) or a 21-day (21-day/withdrawl) cocaine withdrawal. A greater rheobase was also needed in generating $Ca^{2+}$ spikes in NAc MSNs of cocaine-withdrawn rats. **C, D**: Bar graphs indicate a significant reduction in the $Ca^{2+}$ spike size in cocaine (COC)-pretreated MSNs when compared with saline (SAL)-pretreated controls after a 3-day or a 21-day withdrawal ($n$ = 14 cells/each group, unpaired $t$-test, both $*P < 0.05$). The bars represent mean ± S.E. **E**: D2R stimulation with quinpirole (10 μM) reduced the $Ca^{2+}$ spike area in SAL-pretreated NAc neurons after a 3-day withdrawal. **F**: Repeated cocaine administration reduced the $Ca^{2+}$ spike duration in NAc MSNs after a 3-day withdrawal (comparing the two top traces in 5E and 5F). Quinpirole failed to induce any significant change in the size of $Ca^{2+}$ potentials in cocaine-pretreated MSNs. **G, H**: Bar graphs show that quinpirole induced a significant reduction in the size of $Ca^{2+}$ spikes in saline-pretreated rats after either a 3-day withdrawal or a 21-day withdrawal (SAL 3-day/

withdrawl and SAL 21-day/withdrawl, $n = 14$ and 12 cells, respectively, paired $t$-test, $*P < 0.05$). However, this effect was abolished in cocaine-pretreated rats after a 3-day or a 21-day withdrawal (COC 3-day/withdrawl and 21-day/withdrawl, $n = 13$ and11 cells, respectively; two-way ANOVA with repeated measures, both $*P < 0.05$). Bars represent the means and the vertical lines indicate ± S.E.

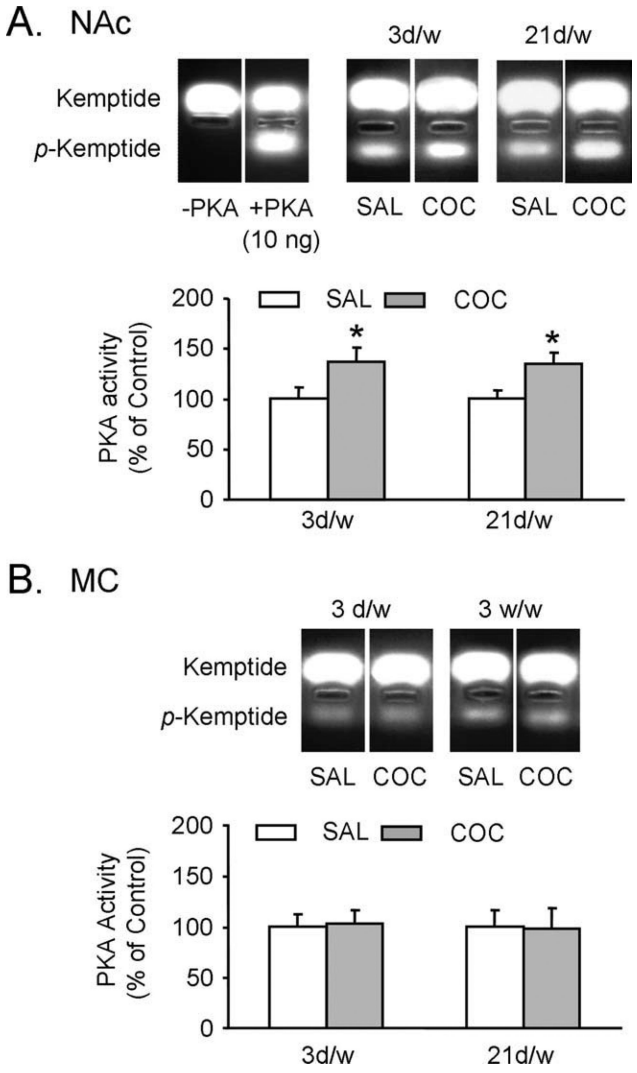


**Fig. 6.**

Repeated cocaine exposure enhanced PKA activity in the NAc but not motor cortex. **A**: Examples of gels show phosphorylation of peptide substrates resulting from PKA activation in the tissues from the NAc obtained from SAL- and COC-pretreated rats. PKA activity was increased after the short- or long-term cocaine withdrawal (3-day/withdrawl or 21-day/withdrawl, respectively). There was a significant difference in PKA activity in the NAc between SAL- and COC-pretreated rats after both short- and long-term withdrawal (SAL 3-day/withdrawl vs. COC 3-day/withdrawl, $n = 20$ vs. 22 rats, unpaired $t$-test, $^*P < 0.05$; SAL 21-day/withdrawl vs. COC 21-day/withdrawl, $n = 14$ vs. 14 rats, unpaired $t$-test, $^*P < 0.05$). The bars and vertical lines represent the mean ± S.E. **B**: There was no significant change in PKA activity in the motor cortex of SAL-pretreated rats when compared with COC-pretreated rats.

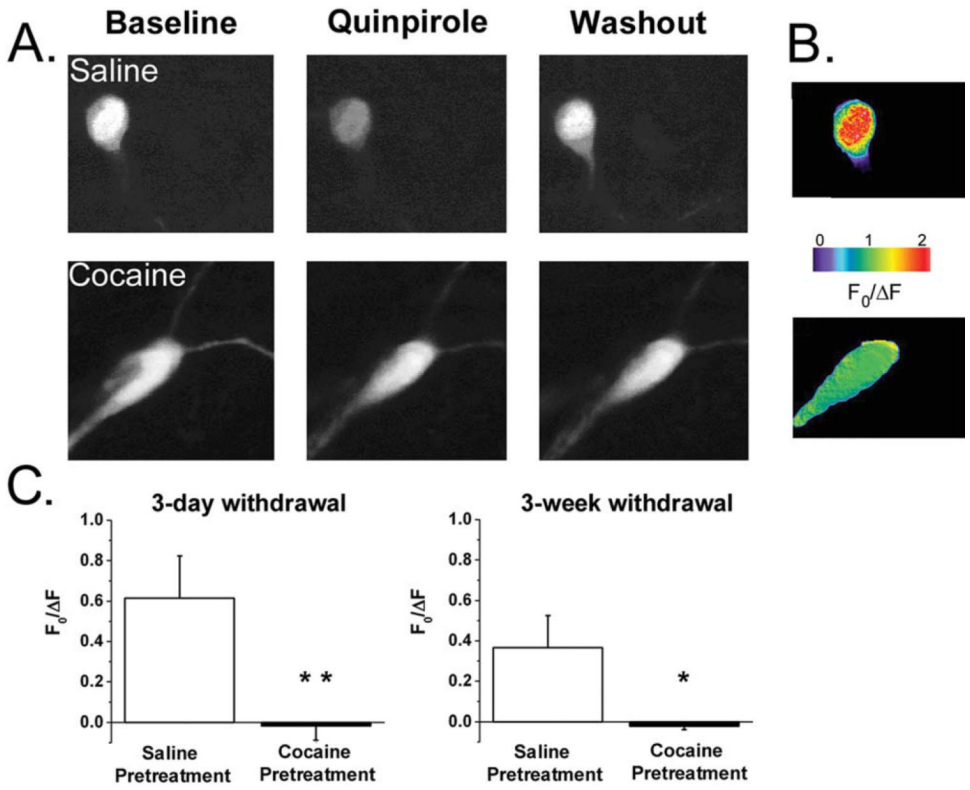


**Fig. 7.**

D2R modulation of intracellular $Ca^{2+}$ release was abolished in NAc MSNs in cocaine-pretreated rats after a short- or long-term withdrawal. **A**: Background-subtracted two-photon $Ca^{2+}$ images of quinpirole-stimulated $Ca^{2+}$ release in saline (top)- and cocaine-pretreated (bottom) NAc MSNs after a 3-day withdrawal. Representative images show averaged fluorescent intensity before application of quinpirole (left), with peak $Ca^{2+}$ response during quinpirole (middle), and after quinpirole was washed out (right). Similar responses were observed in NAc MSNs from 21-day withdrawn rats (not shown). Note that with the $Ca^{2+}$ indicator fura-2 at 780 nm two-photon excitation (which corresponds to greater fluorescence emission with calcium in the unbound state), an increase in $Ca^{2+}$ results in a decrease in fluorescence. Therefore, a dimmer image indicates greater $Ca^{2+}$ release. **B**: Psuedocolored images with color scale indicate the $F_0/\Delta F$ levels during the maximal $Ca^{2+}$ response induced by D2R stimulation with quinpirole in NAc MSNs from saline (top)- vs. cocaine (bottom)-withdrawn rats after a 3-day withdrawal. **C**: Bar graphs indicate the relative changes in the $Ca^{2+}$ levels ($F_0/\Delta F$) evoked by quinpirole in NAc MSNs recorded in saline-(open) and cocaine-(filled) pretreated rats with a 3-day or a 21-day withdrawal (left and right, respectively). Asterisks indicates a significant difference between saline- and cocaine-pretreated cells (unpaired *t*-test, $*P < 0.05$, $**P < 0.01$).

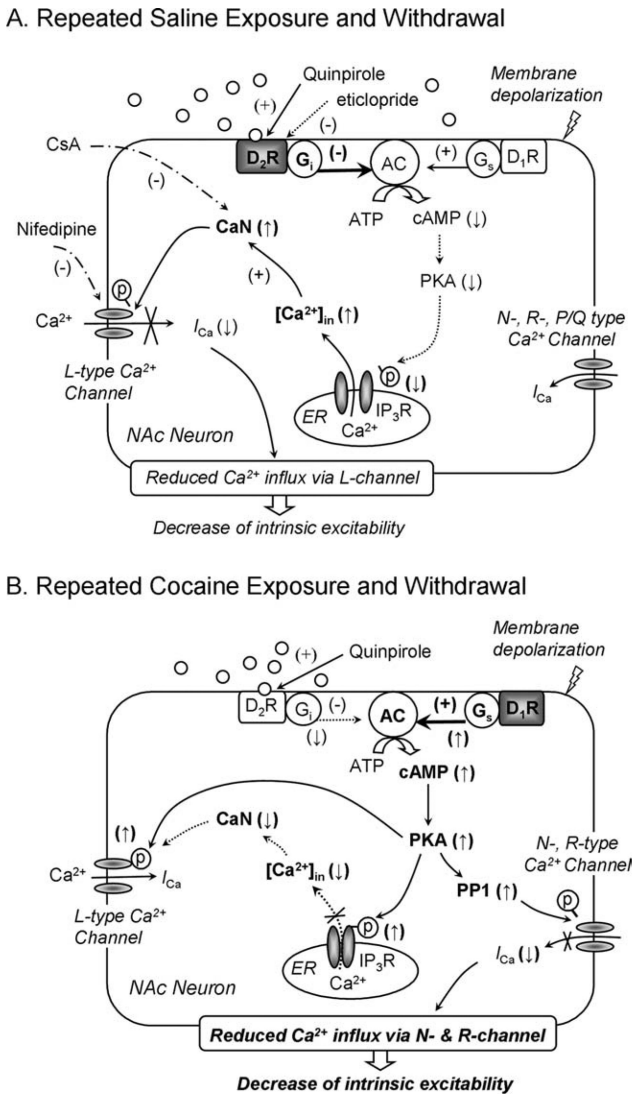


**Fig. 8.**

D2R modulation of L-type $Ca^{2+}$ channel activity is reduced after chronic exposure to cocaine: alterations in the D2R/AC/PKA/$IP_3$R/$Ca^{2+}$/calcineurin pathway. On the basis of the findings of this study and previous results, we propose here a working model that will help us to better understand the mechanisms that may underlie D2R modulation of $Ca^{2+}$ homeostasis in rat NAc MSNs, either under physiological condition or after repeated cocaine exposure and withdrawal. **A**: D2R modulation of $Ca^{2+}$ homeostasis in control NAc MSNs. D2R stimulation with quinpirole inhibits the cAMP/PKA cascade (by decreasing PKA activity) that reduces phosphorylation of $IP_3$Rs localized on endoplasmic reticulum. This D2R action triggers intracellular $Ca^{2+}$ release, which subsequently elevates $[Ca^{2+}]_{in}$ and activates $Ca^{2+}$/calmodulin-dependent calcineurin (CaN). Calcineurin-induced dephosphorylation of L-channels diminishes $I_{Ca}$ via the L-channels and therefore suppresses evoked $Ca^{2+}$ spikes. These changes lead to a decrease in the intrinsic excitability of NAs neurons. The D2R effects on suppressing $Ca^{2+}$ spikes are blocked by selective inhibition of D2R (eticlopride), mimicked (and occluded) by cytosolic application of calcineurin, eliminated by inhibition of calcineurin activity (cyclosporin A), and abolished by specific blockade of L-channel (nifedipine). However, inhibition of D2R did not significantly affect function of non-L-type high voltage-activated $Ca^{2+}$ channels. **B**: Repeated cocaine exposure

and withdrawal dysregulates D2R-modulated $Ca^{2+}$ homeostasis in NAc MSNs, leading to a significant decrease in D2R function with increased PKA activity. Such changes facilitate $IP_3R$ phosphorylation, reduce $Ca^{2+}$ release, decrease $[Ca^{2+}]_{in}$, and diminish calcineurin function in NAc MSNs. Although attenuated dephosphorylation of L-channels by calcineurin intends to increase $I_{Ca}$ via the channel, it is overcome by a significant reduction in $I_{Ca}$ via N- and R-type $Ca^{2+}$ channels. As described above, activity of N- and R-type $Ca^{2+}$ channels is decreased in cocaine-pretreated NAc MSNs in drug-withdrawn rats, likely via enhanced dephosphorylation of these channels by protein phosphatase 1, which is activated by PKA. Together, these findings indicate that neuroadaptation of D2R-mediated $Ca^{2+}$ homeostasis by repeated cocaine exposure dysregulates NAc MSNs, causing a persistent decrease in the intrinsic excitability of these cells.