Neuropsychopharmacology (2005) 30, 916–926

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Repeated Cocaine Administration Decreases Calcineurin (PP2B) but Enhances DARPP-32 Modulation of Sodium Currents in Rat Nucleus Accumbens Neurons

Xiu-Ti Hu*[,1], Kerstin Ford[1] and Francis J White[1]

[1]*Department of Cellular and Molecular Pharmacology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL, USA*

Our previous studies have demonstrated that repeated cocaine (COC) administration reduces voltage-sensitive sodium and calcium currents (I_{Na} or VSSCs and I_{Ca} or VSCCs, respectively) in medium spiny nucleus accumbens (NAc) neurons of rats. The present findings further indicate that chronic COC-induced I_{Na} reduction in NAc neurons is regulated by decreased dephosphorylation and enhanced phosphorylation of Na^+ channels. Whole-cell voltage-clamp recordings revealed that dephosphorylation of Na^+ channels by calcineurin (CaN) enhanced I_{Na}, while inhibition of protein phosphatase 1 (PP1) by phosphorylated dopamine- and cAMP-regulated phosphoprotein ($M_r = 32$ kDa) (DARPP-32) at the site of threonine 34 (p-Thr.34-DARPP-32) suppressed I_{Na}, in freshly dissociated NAc neurons of saline-pretreated rats. However, the effects of CaN on enhancing I_{Na} were significantly attenuated, and the action of p-Thr.34-DARPP-32 to decrease I_{Na} was mimicked, although not potentiated, by repeated COC pretreatment. Dephosphorylation of Na^+ channels by PP1 also enhanced I_{Na}, but this effect of PP1 on I_{Na} was not apparently affected by repeated COC administration. Western blot analysis indicates that the protein levels of CaN and DARPP-32 were significantly decreased and increased, respectively, while the PP1 levels were unchanged, in the COC-withdrawn NAc as compared to saline-pretreated controls. Combined with previous findings, our results indicate that both CaN and PP1 modulate the increase in I_{Na} via enhancing dephosphorylation, while p-Thr.34-DARPP-32 reduces I_{Na} by inhibiting PP1-induced dephosphorylation, thereby stabilizing the phosphorylation state, of Na^+ channels in NAc neurons. They also suggest that chronic COC-induced I_{Na} reduction may be attributed to a reduction in Ca^{2+} signaling, which disrupts the physiological balance of phosphorylation and dephosphorylation of Na^+ channels.
Neuropsychopharmacology (2005) **30,** 916–926, advance online publication, 23 February 2005; doi:10.1038/sj.npp.1300654

Keywords: cocaine (COC); calcineurin (CaN); DARPP-32 (dopamine- and cAMP-regulated phosphoprotein; $M_r = 32$ kDa); Ca^{2+}; nucleus accumbens (NAc); voltage clamp; Western blot

INTRODUCTION

The nucleus accumben (NAc) is a forebrain region functionally involved in addiction and withdrawal effects of cocaine (COC) (Hyman, 1996; White and Kalivas, 1998). Repeated COC administration reduces the membrane excitability of NAc neurons via suppressing I_{Na} and I_{Ca} but enhancing I_K (Zhang *et al*, 1998, 2002; Hu *et al*, 2004). However, the mechanism underlying COC-induced changes in ion channel function is unknown. Recent findings suggest that alterations in phosphorylation and dephosphorylation of voltage-sensitive Na^+ channels may contribute to the COC-induced decrease in I_{Na}. It is well established that Na^+ channels are under dynamic control of phosphorylation/dephosphorylation (Greengard *et al*, 1999; Schiffmann *et al*, 1998), in which calcineurin (CaN), a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase (also known as protein phosphatase 2B, PP2B), plays a significant role (see Catterall, 1997; Yakel, 1997; Herzig and Neumann, 2000; Greengard, 2001; Shibasaki *et al*, 2002 for a review). For instance, while cAMP-dependent protein kinase A (PKA) phosphorylates Na^+ channels, which leads to a decrease in I_{Na} (Li *et al*, 1992; Cantrell *et al*, 1999; Smith and Goldin, 1997), CaN dephosphorylates specific serine sites in the intracellular loop of type II-Aα subunit of Na^+ channels (Murphy *et al*, 1993) and enhances I_{Na} (Hu *et al*, 2005). Although PKA-activated p-Thr.34-DARPP-32 reduces I_{Na}, CaN inhibits this action of p-Thr.34-DARPP-32 (Greengard *et al*, 1999; Schiffmann *et al*, 1998). However,

*Correspondence: Dr X-T Hu, Department of Cellular and Molecular Pharmacology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, IL 60064-3095, USA, Tel: +1 847 578 8663, Fax: +1 847 578 3268, E-mail: xiu-ti.hu@rosalindfranklin.edu
Received 3 August 2004; revised 12 November 2004; accepted 15 November 2004
Online publication: 17 November 2004 at http://www.acnp.org/citations/NPP111704040352/default.pdf

the functions of PKA and CaN are enhanced and may be reduced, respectively, after chronic exposure to COC (Terwilliger *et al*, 1991; Zhang *et al*, 2002; Hu *et al*, 2004).

Previous investigations have demonstrated that chronic COC treatment decreases I_{Ca} (Zhang *et al*, 2002; Hu *et al*, 2004). On the other hand, both PKA and CaN regulate Ca^{2+} signaling via phosphorylation and dephosphorylation of inositol 1,4,5-triphosphate receptors (IP_3Rs), which would induce a decrease (Supattapone *et al*, 1988; Ferris *et al*, 1991; Quinton and Dean, 1992; Tertyshnikova and Fein, 1998; but also see Volpe and Alderson-Lang, 1990; Tang *et al*, 2003), and possible increase (Cameron *et al*, 1995, 1997), in IP_3R-mediated Ca^{2+} release, respectively. In contrast, inhibition of CaN not only facilitates PKA-induced phosphorylation of Na^+ channels (Chen *et al*, 1995) but also abolishes dephosphorylation and expression of IP_3Rs (Genazzani *et al*, 1999). These findings suggest that chronic COC may suppress Ca^{2+} signaling via reducing both I_{Ca} and intracellular released Ca^{2+}, thereby reducing CaN function and eventually decreasing I_{Na} in NAc neurons.

An investigation to determine the possible interruption of Ca^{2+} signaling and the physiological balance between PKA-induced phosphorylation and CaN-induced dephosphorylation of Na^+ channels after chronic exposure to psychostimulants has recently begun. Besides the increased PKA activity and attenuated Ca^{2+} signaling after chronic COC treatment (Terwilliger *et al*, 1991; Zhang *et al*, 2002; Hu *et al*, 2004), the protein levels of DARPP-32 and CaN are also increased and decreased, respectively, after repeated administration of methamphetamine (Lin *et al*, 2002). Although these changes in PKA activity and Ca^{2+} signaling appear to be associated with decreased I_{Na} found in COC-withdrawn NAc neurons, whether and how CaN- and PKA-activated p-Thr.34-DARPP-32 are functionally involved in chronic COC-induced I_{Na} reduction requires determination.

As the activity of Na^+ channels is regulated by phosphorylation/dephosphorylation and inhibition of CaN markedly increases the levels of p-Thr.34-DARPP-32 (Nishi *et al*, 1999b, 2002; Greengard *et al*, 1999), we hypothesized that both CaN and p-Thr.34-DARPP-32 modulate chronic COC-induced I_{Na} reduction. An attenuated CaN action would be associated with increased DARPP-32 function. Moreover, since PP1 also dephosphorylates Na^+ channels and is inhibited by p-Thr.34-DARPP-32 (Greengard *et al*, 1998, 1999; Greengard, 2001), its effects on I_{Na} were examined concurrently. Voltage-clamp recordings were performed to determine whether CaN (and PP1) enhances, and p-Thr.34-DARPP-32 suppresses, I_{Na} in NAc neurons, and whether repeated COC administration alters the effects of CaN, PP1, and p-Thr.34-DARPP-32 on voltage-sensitive sodium currents (VSSCs). Western blot analysis was used to determine whether repeated COC administration alters the protein levels of CaN, PP1, and DARPP-32 in the NAc, and whether the possible changes in the protein levels are correlated with the COC-reduced I_{Na}.

MATERIALS AND METHODS

Animals and Pretreatments

Young male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) at 3–4 weeks of age ($\sim$60 g) were used for pretreatment in all studies. Rats were allowed 3 days to acclimate to the environment. They were group housed (3–4 per cage) in a colony room maintained at constant temperature (21–23°C) and humidity (50–65%) on a 12-h/12-h light/dark cycle and provided access to water and food *ad libitum*. Rats were given daily injections of COC (15 mg/kg i.p.) or 0.9% saline (1 ml/kg i.p.) for 5 days in their home cage. NAc tissue was collected after 3 days of withdrawal for the following experiments. After the pretreatment with saline or COC, the age of these rats was approximately 5–6 week old. In electrophysiological experiments, technical limitations usually restrict voltage-clamp recordings in freshly dissociated neurons from rats older than 6 weeks. A prolonged time period for tissue digestion and increased physical force for cell dissociation from older tissues with increased density of fibers could cause damage in dissociated cells that would make the recording extremely difficult.

Preparation of Brain Slices

All procedures were performed in strict compliance with the National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and were approved by our Institutional Animal Care and Use Committee. Rats were decapitated under halothane anesthesia, and brain tissues containing the NAc were rapidly excised and dissected into blocks. The thickness of brain blocks for cell dissociation was 3–4 mm before slicing, and those reserved for Western blot analysis were cut at 1 mm.

Whole-Cell Voltage-Clamp Recordings

The blocks of tissues were immersed in ice-cold high sucrose solution (in mM: NaCl 25, KCl 2.5, HEPES 5, D-glucose 11, sucrose 210, $CaCl_2$ 2, $MgSO_4$ 2; pH value was adjusted to 7.40 with 10 N and 1 N NaOH) and sectioned in the coronal plane (350 μm) with a motorized vibrating microtome. Brain slices were transferred to a stir chamber and incubated in oxygenated (95% O_2:5% CO_2) holding solution (in mM: NaCl 126, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 2, $NaH_2PO_4 + H_2O$ 1.25, $NaHCO_3$ 26, D-glucose 10, pyruvic acid 1, phenol red 0.03; pH value was adjusted to 7.20 with 6 N HCl at room temperature (20–22°C)) for at least 60 min. Slices were then transferred to an oxygen-gassed stir chamber containing protease (Type XIV) in HEPES-buffered HBSS (HEPES: 2.6 g/1 l; pH was adjusted to 7.35 with 10 N NaOH; 300–310 mOsm/l) at 35°C for digestion. After 25–30 min of enzyme digestion, tissues were rinsed three times in the low Ca^{2+}, HEPES-buffered saline (in mM: Na-isethionate 140, glucose 23, HEPES 15, KCl 2, $MgCl_4$ 2, $CaCl_2$ 0.01) and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then placed into a Petri dish mounted on the stage of an inverted microscope containing 1 ml HEPES-buffered HBSS. After the cells were allowed to settle, the solution bathing the cell was changed to our normal recording external solution (see below).

Whole-cell recordings using standard voltage-clamp techniques as applied previously in our laboratory (Zhang *et al*, 1998) were performed in this study. Electrodes were

pulled from Corning 7056 glass capillaries and fire-polished before use. Sodium current was isolated by using the following solution: internal (pipette solution, in mM)—CsF 120, NaCl 10, HEPES 10, sucrose (to replace EGTA 10), Na_2ATP 2, pH 7.2–7.3 (adjusted with 1 M CsOH), 280–285 mOsm/l; external (background solution, in mM)—choline choloride 110, NaCl 30, CsCl 5, $MgCl_2 + 6 \cdot H_2O$ 1, $CaCl_2$ 1, $CdCl_2$ 0.4, glucose 10, HEPES 10, pH 7.3–7.4 (adjusted with 5 M CsOH), 300–305 mOsm/l. To determine the effects of Ca^{2+} signaling on modulation of VSSCs, cytosolic-free Ca^{2+} was preserved in NAc neurons with the absence of Ca^{2+} chelators in the internal solution. In addition, the relatively low concentration of NaCl (30 mM) in the external solution was used to minimize voltage-clamp error. Electrodes filled with this solution had a resistance of 1.8–2.5 MΩ when tested in the bath solution. The junction potential of −5 mV was measured between the electrode and bath solution and was not compensated. Recordings were obtained with an Axon Instruments 200B patch-clamp amplifier and controlled and monitored with a PC running pCLAMP7 with a 2 kHz filter. Step depolarizing pulses were applied at intervals of 5–10 s to allow enough time for Na^+ channels to recover from inactivation. After seal rupture, series resistance (<10 MΩ) was compensated (70–80%) and periodically monitored. All recorded currents were leak-subtracted. Adequate voltage control was determined by standard methods (Colatsky and Tsien, 1979). Recordings were made only from NAc neurons (7–15 μm somal diameter) that had only a few short proximal dendrites. All experiments were performed at room temperature (20–22°C) since voltage-clamp recordings in freshly dissociated medium spiny (dorsal and ventral) striatal neurons under 'body temperature' (~37°C) appear to be problematic due to some unrevealed reasons. The numbers of rats used for whole-cell recordings were usually five to eight per each experimental group.

Drug Applications

Several enzymes and their specific inhibitors were applied either internally or externally to NAc neurons. Preactivated CaN (recombinant CaN-Aα and calcineurin B (CaN-B) coexpressed with yeast myristoyl-CoA:protein N-myristoyltransferase, 100 U), PP1 (50 nM), and p-Thr.34-DARPP-32 phosphorylated by PKA (200 nM) were dissolved in and internally dialyzed from the pipette solution. The selective CaN inhibitor cyclosporin A (CysA, 200 μM) was externally applied in the bath through a gravity-fed system.

Western Blot Analysis

Brains from saline- or COC-pretreated rats ($n = 18$/group) were rapidly removed from the skull, 1 mm thick coronal slices were made using a brain matrix, and were dissected on ice. The NAc was collected from slices corresponding to 2.7–1.7 mm anterior to bregma (Paxinos and Watson, 1986). All collected tissue samples were frozen with liquid nitrogen and transferred into 125 μl of ice-cold homogenization buffer (20 mM Tris-HCl (pH 7.4), 10% sucrose, 1 mM EDTA, 5 mM EGTA, and protease inhibitors (Roche Diagnostics, Mannheim, Germany)). The samples were then sonicated on ice for three times (3 s/each time) and spun down at 14 000 *g* at 4°C for 30 min. The supernatant was collected and used as the soluble fraction. The pellet was resuspended in 50 μl of homogenization buffer for analysis and used as the particulate fraction. Tissue samples were not pooled for analysis. All samples were stored at −80°C.

After determination of protein content with a modified Lowry assay (Bio-Rad, Hercules, CA), the concentrations of all fractions were adjusted to 1 μg protein/μl using homogenization buffer. At this point, some samples were excluded due to low protein concentration. The samples were mixed with appropriate amounts of 4× SDS loading buffer (Invitrogen, Carlsbad, CA) and boiled for 5 min before electrophoresis. Duplicate samples were loaded in quantities of 10 μg of total protein for DARPP-32 detection, or 2 μg of total protein for all other immunoblotting, and were fractionated using 4–12% gradient NuPAGE Bis–Tris gels with MOPS electrophoresis buffer (Invitrogen, Carlsbad, CA). The gels were then transferred to Invitrolon PVDF membranes (Invitrogen, Carlsbad, CA). The membranes were blocked for 60 min with a mixture of 5% nonfat dry milk and TBS-T (Tris-buffered saline with 0.05% Tween-20), and then incubated at 4°C overnight with specific antibodies diluted in blocking solution. Samples were incubated with goat-anti-DARPP-32 sera (1 : 500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse-anti-actin sera (1 : 20 000 dilution) together in the same solution. Mouse antisera for CaN-A (61 kDa, the catalytic subunit of PP2B, which has a CaN-B-binding domain, a CaM-binding domain, and an autoinhibitory domain), CaN-B (19 kDa, the regulatory subunit of PP2B, which has four Ca^{2+}-binding sites, and is bound to CaN-A), PP1 (all from Sigma, St Louis, MO), and actin (Calbiochem, La Jolla, CA) were mixed together in the same incubation solution. Soluble samples were incubated with antibodies diluted to 1 : 10 000 for mouse-anti-CaN-A sera, 1 : 20 000 for mouse-anti-CaN-B sera, 1 : 500 for mouse-anti-PP1 sera, and 1 : 8000 for mouse-anti-actin sera. Particulate samples were incubated with antibodies diluted to 1 : 7500 for mouse-anti-CaN-A sera, 1 : 15 000 for mouse-anti-CaN-B sera, 1 : 500 for mouse-anti-PP1 sera, and 1 : 8000 for mouse-anti-actin sera. After the primary incubation, samples were incubated with the appropriate secondary antibodies diluted in blocking solution for 1 h at room temperature. Immunoreactive bands were visualized with enhanced chemiluminescence (ECL) detection solutions and ECL Hyperfilm (Amersham Biosciences Corp., Piscataway, NJ). Resulting films were scanned with a Scanjet 5400c (Hewlett-Packard, Greeley, CO) and analyzed using Phoretix TotalLab software (Nonlinear Dynamics, Durham, NC). All scanned images were calibrated with a scanned step wedge of known optical densities (Stouffer Graphic Arts, South Bend, IN). In order to adjust for any variations in loading, all detected protein bands were normalized to actin levels. Some data were excluded at this point due to irregularities caused by small air bubbles in the transfer step. Densitometric data from the COC-pretreated group were expressed as a percent change from the saline-treated group. Outlying data points were identified and discarded if they fell outside the range of 2 SD from the mean.

Statistical Significance

An unpaired *t*-test was used to estimate the significance of the difference (*$p<0.05$ and **$p<0.01$) between saline- and COC-pretreated groups. In addition, comparisons of the CaN- or PP1-induced alterations in I_{Na} between SAL- and COC-pretreated NAc neurons during a 5-min period of recording time were made with a two-way analysis of variance (ANOVA) with repeated measures on one variable (time).

RESULTS

Dephosphorylation of Na^+ Channels by Exogenous CaN Enhances Whole-Cell I_{Na} in Freshly Dissociated NAc Neurons

Freshly dissociated medium spiny NAc neurons without or only with a few short dendritic processes were selected for recording. These neurons exhibited whole-cell capacitance of 3–8 pF as we previously reported (Zhang *et al*, 1998, 2002). Whole-cell I_{Na} recordings were elicited by depolarizing pulses from a holding potential of −70 mV. After seal rupture, Na^+ currents were gradually increased along with the formation of whole-cell configuration. Without influence of any external or internal application of drugs, the peak Na^+ currents were stabilized within approximately 1–2 min in NAc neurons of saline-withdrawn rats. However, with internally dialyzed proteins from the recording pipette, the period of time for I_{Na} stabilization was a few minutes longer. When the basal I_{Na} was necessarily stabilized, six traces of I_{Na} were recorded and averaged as the initial control. For all NAc neurons recorded in this study, the initial control I_{Na} was compared with the altered peak I_{Na} following continuous application of proteins or drugs. In addition, in a group of control neurons ($n=7$) from saline-pretreated rats, sodium currents were recorded as a basal control for approximately 5–6 min without application of any drugs (SAL/control) (Figure 1a). In contrast to the response to CaN (see below), a small change (<10%) was observed in the relative current levels during this period of recording time (Figure 1a). Previous studies have demonstrated that CaN induces a rapid and extensive dephosphorylation of voltage-sensitive Na^+ channels in neurons of the rat brain via counteracting the effects of cAMP-dependent phosphorylation on Na^+ channel activity (Murphy *et al*, 1993; Chen *et al*, 1995). Dephosphorylation of Na^+ channels by preactivated CaN (PP2B, 100 U) markedly enhanced whole-cell I_{Na} in medium spiny NAc neurons of saline-pretreated rats (Figure 1a). The CaN-induced I_{Na} enhancement was completely reversed by bath application of the selective CaN inhibitor CysA (200 μM, $n=8/8$ cells) (Figure 1b).

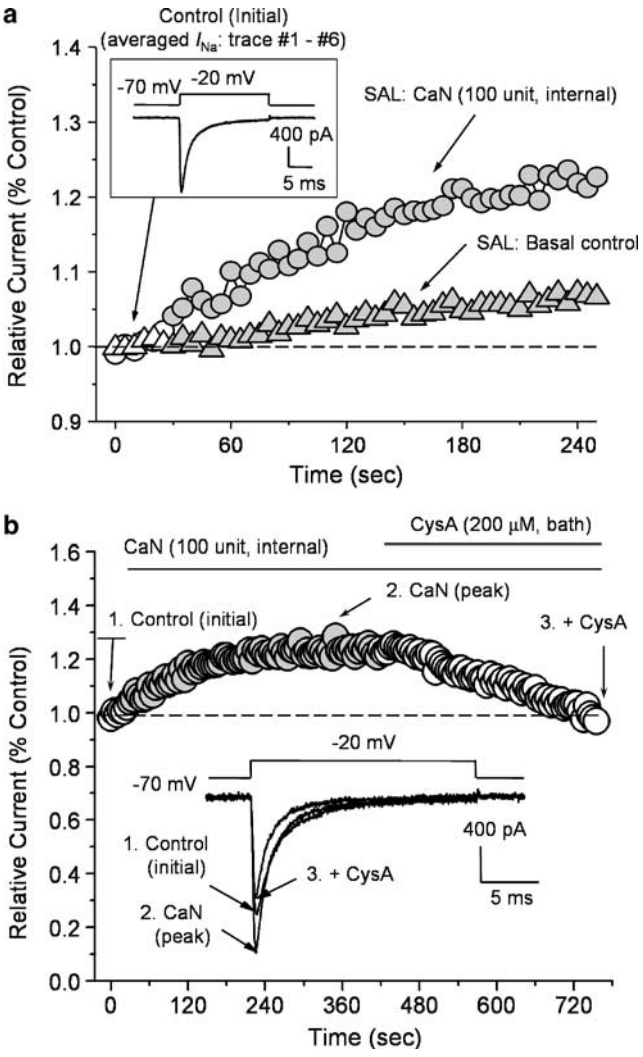

Figure 1 Dephosphorylation of sodium channels and inhibition of p-Thr.34-DARPP-32 by exogenous CaN (preactivated CaN, PP2B) enhances whole-cell I_{Na} in freshly dissociated NAc neurons. (a) The time–relative current (% control) response curves show that cytosolic application of CaN (100 U) gradually enhanced I_{Na} in a medium spiny NAc neuron (circle) as compared to another one without CaN (triangle) recorded in rats after repeated saline pretreatment. The 'open' circles represent the first six relative currents, which were recorded at different time points under a condition in which the I_{Na} levels were stabilized. These currents were also averaged and used as the initial control (inset) to compare with CaN-enhanced I_{Na} (the 'solid' circles) at its maximal levels. As a basal control, the relative currents marked with triangles were recorded from a NAc neuron without application of CaN. (b) Representative traces showing that CaN markedly enhanced I_{Na}, and the CaN-induced I_{Na} enhancement was completely reversed by bath application of the CaN inhibitor CysA (200 μM, $n=8/8$ cells).

Dephosphorylation of Na^+ Channels by CaN Reverses I_{Na} Reduction Induced by Repeated COC Pretreatment

Our previous study has demonstrated that repeated COC administration significantly reduces whole-cell I_{Na} in medium spiny NAc neurons of rats (Zhang *et al*, 1998). The COC-induced I_{Na} reduction in NAc neurons was further investigated in the present study to determine whether it could be reversed via dephosphorylation of Na^+ channels by CaN. As described above, CaN enhanced I_{Na} from its initial control levels to an enhanced new peak level in NAc neurons of saline-pretreated rats (Figure 2a, the left traces). In contrast, the initial control levels of I_{Na} were markedly reduced and the efficacy of CaN on enhancing I_{Na} was attenuated in NAc neurons following repeated COC administration (Figure 2a, the right traces). The bar graphs indicate that CaN induced a significant enhancement in the peak I_{Na} in NAc neurons of saline-pretreated

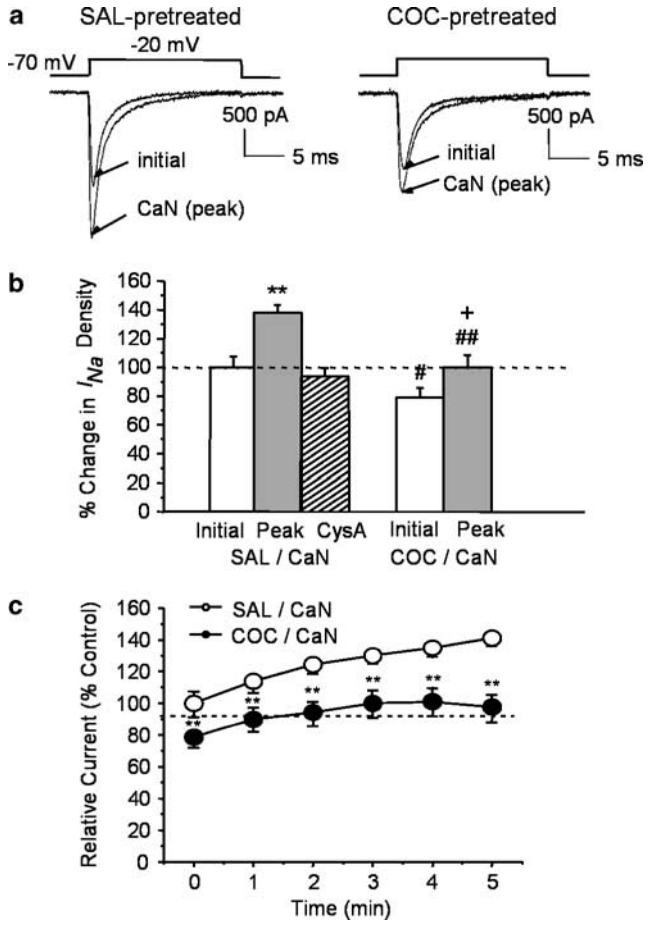

Figure 2 Dephosphorylation of sodium channels and inhibition of p-Thr.34-DARPP-32 by CaN reverses chronic cocaine-induced reduction of I_{Na}. (a) Representative traces showing that CaN enhanced the I_{Na} from its initial control levels to a new peak level in a NAc neuron of saline (SAL)-pretreated rat. However, the initial control levels of I_{Na} in NAc cells were markedly reduced in COC-pretreated rats. The efficacy of CaN for enhancing I_{Na} was also attenuated. (b) Bar graphs indicating that CaN-induced enhancement in the peak I_{Na} ($n=18$ cells, $^{**}p<0.01$) was blocked by CysA (200 μM) ($n=8$ cells, $^{**}p<0.01$). Repeated COC administration significantly reduced peak I_{Na} in NAc neurons as compared to saline-pretreated control ($n=18$ *vs* 17 cells, $^{\#}p<0.05$), but CaN reversed COC-induced I_{Na} reduction to the levels that were comparable to saline-pretreated initial control. Thus, there was a significant I_{Na} enhancement with application of CaN in NAc neurons of COC-pretreated rats ($n=17$ cells, $^{\#\#}p<0.01$). However, the maximal I_{Na} enhancement in NAc neurons induced by CaN was significantly lower in COC-pretreated as compared to saline-pretreated rats ($n=18$ *vs* 17 cells, $^{+}p<0.05$). The percent enhancement induced by CaN was also significantly smaller in COC-pretreated than that in saline-pretreated rats ($^{+}p<0.05$). (c) The relative current (% control) curves also indicate that the effects of CaN on increasing I_{Na} during a 5-min recording period were significantly reduced in NAc cells of COC-pretreated rats as compared to saline-pretreated rats (MANOVA, $^{*}p<0.02$; *post hoc* Neuman–Keuls test, $^{**}p<0.01$).

rats (SAL/CaN/initial *vs* SAL/CaN/peak: 248.9 ± 18.5 *vs* 326.3 ± 28.1 nA/pF; or 100 ± 7.5 *vs* 138.0 ± 5.6%, $n=18$ cells, paired *t*-test, $t=5.8273$, $^{**}p<0.01$), which was completely blocked by CysA (200 μM) (SAL/CaN/peak *vs* SAL/CaN/CysA: 326.3 ± 28.1 *vs* 196.6 ± 16.4 nA/pF, paired *t*-test, $t=11.3854$, $^{**}p<0.01$) (Figure 2b). Therefore, there was no significant difference in the I_{Na} between SAL/CaN/initial control and SAL/CaN/CysA.

In contrast, repeated COC administration significantly reduced the peak I_{Na} in NAc neurons as compared to saline-pretreated control (SAL/initial *vs* COC/initial: 248.9 ± 18.5 *vs* 197.1 ± 15.7 nA/pF, $n=18$ *vs* 17 cells, *t*-test, $t=2.1194$, $^{\#}p<0.05$). Dephosphorylation of Na^+ channels by cytosolic application of CaN reversed chronic COC-suppressed I_{Na} to the levels similar to the initial controls observed in saline-pretreated rats (COC/initial *vs* COC/peak: 197.1 ± 15.7 *vs* 248.8 ± 21.4 nA/pF, $n=17$ cells, paired *t*-test, $t=7.1132$, $^{\#\#}p<0.01$) (Figure 2b). However, the maximal I_{Na} enhancement in NAc neurons induced by exogenous CaN was significantly lower in COC-pretreated rats as compared to saline-pretreated rats (COC/CaN/peak *vs* SAL/CaN/peak: 248.8 ± 21.4 *vs* 326.3 ± 28.1 nA/pF, *t*-test, $t=2.214$; or 25.6 ± 2.6 *vs* 38.0 ± 5.6%, *t*-test, $t=2.0453$, $n=18$ *vs* 17 cells, $^{+}p<0.05$), suggesting that the efficacy of CaN in modulating dephosphorylation of Na^+ channels was reduced after repeated COC administration. Normalized relative current curves recorded during a 5-min time course indicate that the effects of CaN on enhancing I_{Na} were significantly reduced in NAc neurons of COC-pretreated rats as compared to saline-pretreated rats (MANOVA, $F_{1,33}=6.280$, $^{*}p<0.02$, *post hoc*, Neuman–Keuls test, $^{**}p<0.01$) (Figure 2c). These effects of chronic COC on suppressing the function of Na^+ channels and CaN are in agreement with that observed in previous current-clamp studies, in which the activities related to VSSCs and voltage-sensitive Ca^{2+} currents (VSCCs) were significantly decreased in more matured rats (8–10 weeks) after repeated COC administration (Zhang *et al*, 1998; Hu *et al*, 2004). These findings indicate that chronic COC-induced alterations in ion channel function should not be attributed to the relatively young age of rats.

Dephosphorylation of Na^+ Channels by PP1 also Reverses Chronic COC-Induced I_{Na} Reduction

Previous findings have indicated that Na^+ channels are also dephosphorylated by other protein phosphatases, including PP1 (Murphy *et al*, 1993; Chen *et al*, 1995). Similar to the effects of CaN on I_{Na}, dephosphorylation of Na^+ channels by cytosolic application of PP1 (50 nM) also increased the peak I_{Na} in NAc neurons recorded from saline-pretreated rats (Figure 3a, the left traces). Although I_{Na} was decreased following repeated COC administration, it was also enhanced by exogenous PP1 (Figure 3a, the right traces). The bar graphs indicate that PP1 significantly increased the peak I_{Na} in NAc neurons of saline-pretreated rats (SAL/PP1/initial *vs* SAL/PP1/peak: 295.5 ± 16 *vs* 392.6 ± 27.1 nA/pF; or 100 ± 5.4 *vs* 133.0 ± 4.9%, $n=17$ cells, paired *t*-test, $t=6.2597$, $^{**}p<0.01$), while repeated COC administration significantly decreased the peak I_{Na} in NAc neurons (SAL/initial *vs* COC/initial: 295.5 ± 16 *vs* 235.2 ± 21.6 nA/pF, $n=17$ *vs* $n=17$ cells each, *t*-test, $t=2.2452$, $^{\#}p<0.05$) (Figure 3b). In COC-pretreated rats, however, exogenous PP1 not only reversed the reduction of I_{Na} in NAc neurons but also enhanced I_{Na} as it did in saline-pretreated rats (COC/PP1/initial *vs* COC/PP1/peak: 235.2 ± 21.6 *vs* 364.7 ± 37.2 nA/pF, $n=17$ cells, paired *t*-test, $t=6.0838$, $^{\#\#}p<0.01$). The PP1-induced maximal enhancement in I_{Na} recorded from both groups was comparable, and there was no significant difference in the peak I_{Na} values between

saline- and COC-pretreated rats (SAL/PP1/peak *vs* COC/PP1/peak, $p>0.05$) (Figure 3b). Nevertheless, the percent I_{Na} enhancement by PP1 was significantly greater in NAc neurons of COC-pretreated rats as compared to saline-pretreated rats (SAL/PP1/peak *vs* COC/PP1/peak: 33.0 ± 4.9 *vs* $56.9\pm10\%$, $t=2.1098$, $^{+}p<0.05$). In contrast to the effects of CaN, although cytosolic application of PP1 enhanced I_{Na} currents in NAc neurons recorded from both saline- and COC-pretreated rats during a 5-min period, there was no significant difference in the relative current curves between saline- and COC-pretreated rats (MANOVA, $F_{1,28}=2.32$, $p>0.05$) (Figure 3c).

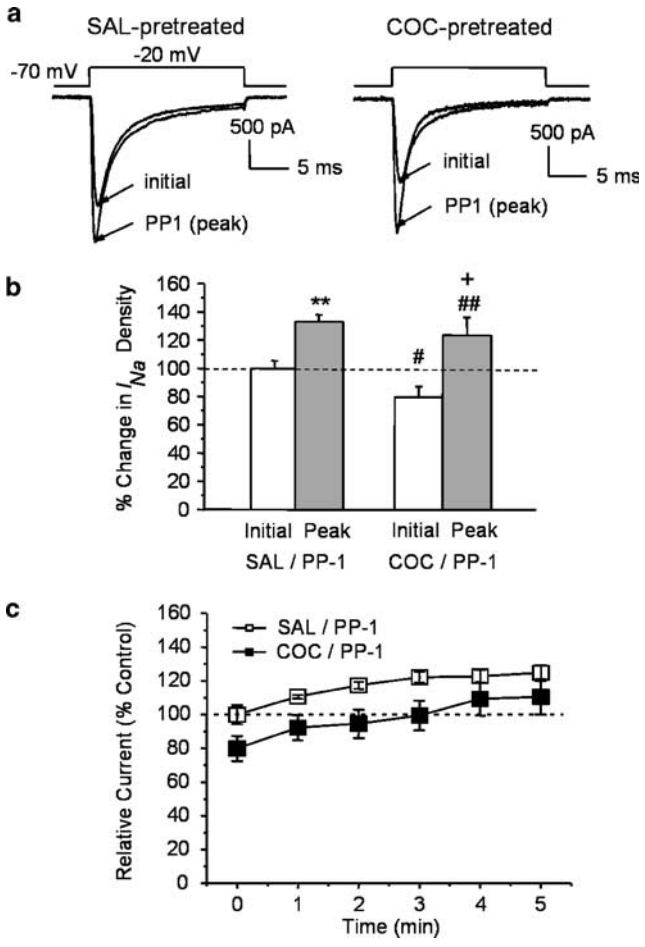

Figure 3 Dephosphorylation of sodium channels by exogenous PP1 also reverses chronic COC-induced reduction of I_{Na}. (a) Representative traces showing that cytosolic application of PP1 (50 nM) increased whole-cell I_{Na} in NAc neurons either saline- or COC-pretreated rats. The maximal effects of PP1 on enhancing I_{Na} in NAc neurons were comparable in saline- and COC-pretreated rats. (b) Bar graphs indicating that PP1 significantly enhanced peak I_{Na} in NAc neurons of saline-pretreated rats ($n=17$ cells, $^{**}p<0.01$). Repeated COC administration significantly reduced the peak I_{Na} as observed previously ($n=17/17$ cells, $^{\#}p<0.05$). However, exogenous PP1 reversed the COC-induced reduction of I_{Na} to the levels that were comparable to saline-pretreated rats ($n=17$ cells, $^{\#\#}p<0.01$). Thus, there was no significant difference in the peak I_{Na} values induced by PP1 between saline- and COC-pretreated rats ($p>0.05$). Nevertheless, the percent enhancement of I_{Na} induced by PP1 was significantly greater in NAc neurons of COC-pretreated as compared to saline-pretreated rats (SAL/PP1/peak *vs* COC/PP1/peak: $^{+}p<0.05$). (c) There was no significant difference in the PP1-induced changes in relative current curves between saline- and COC-pretreated NAc rats during a 5-min recording period (MANOVA, $p>0.05$).

Inhibition of PP1 by Exogenous DARPP-32 Suppresses I_{Na} in NAc Neurons of Saline- but not COC-Pretreated Rats

A recent study reveals that p-Thr.34-DARPP-32, a potent inhibitor of PP1, reduces VSSCs in cultured striatal neurons of rats by stabilizing a phosphorylated state of the sodium channel or an associated regulatory protein (Schiffmann *et al*, 1998). This phenomenon was also observed in the present study. In striking contrast to the effects of CaN and PP1 on I_{Na}, cytosolic application of p-Thr.34-DARPP-32 (200 nM) markedly suppressed I_{Na} in NAc neurons of saline-pretreated rats (Figure 4a). It was noted that application of p-Thr.34-DARPP-32 slightly prolonged the period of time that was necessary for stabilizing the peak I_{Na}. Repeated COC administration also caused a marked decrease of I_{Na} in NAc neurons without exogenous applied p-Thr.34-DARPP-32 (Figure 4a). The I_{Na} reduction in NAc neurons induced by exogenous p-Thr.34-DARPP-32 in saline-pretreated rats was comparable to that induced by repeated COC administration. However, the suppressing effects of p-Thr.34-DARPP-32 on I_{Na} observed in saline-pretreated rats appeared to be occluded in COC-pretreated rats. The bar graphs indicate that although exogenous p-Thr.34-DARPP-32 significantly reduced I_{Na} in NAc neurons

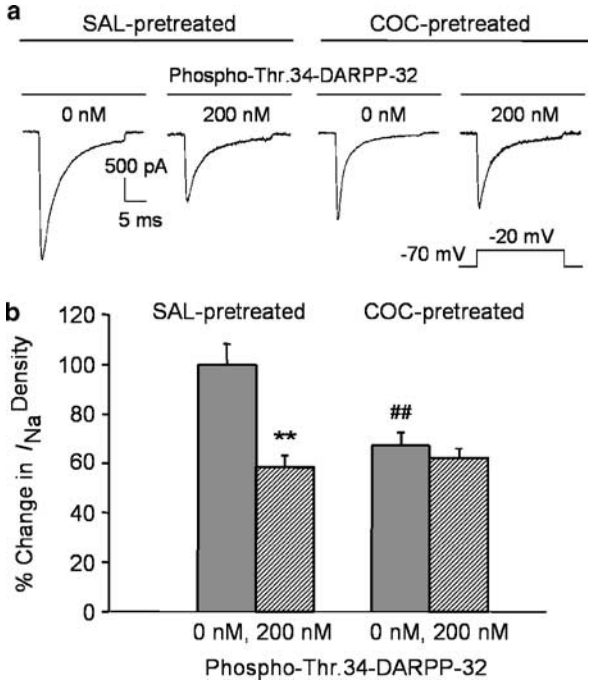

Figure 4 Inhibition of PP1 by exogenous p-Thr.34-DARPP-32 suppresses I_{Na} in NAc neurons of saline- but not COC-pretreated rats. (a) Representative traces showing that internally dialyzed p-Thr.34-DARPP-32 (200 nM) markedly suppressed I_{Na} in NAc neurons of saline-pretreated rats, while repeated COC administration mimicked the decreased I_{Na} in NAc neurons. However, chronic COC-induced I_{Na} reduction was not potentiated by exogenous p-Thr.34-DARPP-32 (200 nM). (b) Bar graphs indicating that p-Thr.34-DARPP-32 significantly reduced I_{Na} in NAc neurons of saline-pretreated rats ($n=7/15$ cells, $^{**}p<0.01$). Although I_{Na} was also reduced in NAc neurons of COC-pretreated rats in the absence of exogenous p-Thr.34-DARPP-32 ($n=7/13$, $^{\#\#}p<0.01$), application of exogenous p-Thr.34-DARPP-32 (200 nM) failed to potentiate the effects of repeated COC treatment on I_{Na} ($n=13/8$, $p>0.05$).

of saline-pretreated rats (SAL: 0 *vs* 200 nM, 100 ± 8 *vs* 58.5 ± 4.6%, $n = 7/15$, $t = 4.664$, $^{**}p < 0.01$), and repeated COC pretreatment significantly decreased I_{Na} in the absence of p-Thr.34-DARPP-32 (SAL:0 *vs* COC:0 nM, 100 ± 8 *vs* 67.4 ± 5.2%, $n = 7/13$, $t = 3.5646$, $^{\#\#}p < 0.01$) (Figure 4b), the reduction of I_{Na} induced by chronic COC pretreatment was not potentiated by cytosolic application of exogenous p-Thr.34-DARPP-32 (COC: 0 *vs* 200 nM, 67.4 ± 5.2 *vs* 62.3 ± 3.8%, $n = 13/8$, $t = 0.6933$, $p > 0.05$).

Repeated COC Administration Decreases the Levels of CaN but not PP1 in the Rat NAc

In order to determine whether the alterations in the effects of exogenous CaN, PP1, and DARPP-32 on the I_{Na} were correlated to the possible changes in the levels of those proteins, Western blot analysis was used to measure the levels of CaN-A (61 kDa), CaN-B (19 kDa), and PP1 (37.5 kDa) within the NAc following repeated saline or COC administration. Figure 5 indicates the NAc region of the brain from where the tissue samples were collected (shaded area). The proteins were measured in both the soluble and particulate fractions. Probably due to the relatively low concentration of CaN in the cytosol (up to 70% of CaN is bound to the membrane or cytoskeleton elements in neurons; Shibasaki *et al*, 2002), there was no significant difference in the soluble fractions of CaN, neither CaN-A nor CaN-B, within the NAc of saline- *vs* COC-pretreated rats (CaN-A: SAL *vs* COC = 100 ± 26.69 *vs* 93.58 ± 13.75%, $n = 20/20$; CaN-B: SAL *vs* COC = 100 ± 30.58 *vs* 90.22 ± 23.96%, $n = 17/18$; PP1: SAL *vs* COC = 100 ± 9.96 *vs* 103.82 ± 11.56%, $n = 19/21$; DARPP-32: SAL *vs* COC = 100 ± 13.44 *vs* 93.79 ± 12.69%, $n = 21/22$; all $p > 0.05$) (figures not shown).

However, there was a small but significant decrease (approximately 13–15%) of CaN in the particulate fractions obtained from COC-pretreated NAc. Both CaN-A and CaN-B immunoreactive bands were significantly reduced in the NAc following repeated COC administration (CaN-A: SAL *vs* COC = 100 ± 5.66 *vs* 82.92 ± 5.08%, $n = 35/33$; $t = 2.238$, $^{*}p < 0.03$; and CaN-B: SAL *vs* COC = 100 ± 4.79 *vs* 84.87 ± 5.35%, $n = 37/38$, $t = 2.105$, $^{*}p < 0.04$) (Figure 6a and b). There was, however, no significant change in PP1 immunoreactive bands between NAc tissues obtained from saline- and COC-pretreated rats (SAL *vs* COC: 100 ± 11.21 *vs* 101.73 ± 9.54%, $n = 27/25$; $p > 0.05$) (Figure 6b). Box plots indicate the alterations in the protein levels with the mean (dashed line), median (solid line), distribution (boxes, indicating the 25th and 75th percentiles and showing 50% of sample distribution), and range ('whiskers', indicating the 10th and 90th percentiles and showing 80% of sample distribution) for the levels of CaN-A, CaN-B, and PP1 measured in the NAc of saline- or COC-pretreated rats. The outlying dots indicate the 5th and 95th percentiles (showing 90% of sample distribution).

Repeated COC Administration also Increases the Levels of DARPP-32 in the NAc

Western blot analysis was also performed to determine the changes in the total protein levels of DARPP-32 in the NAc

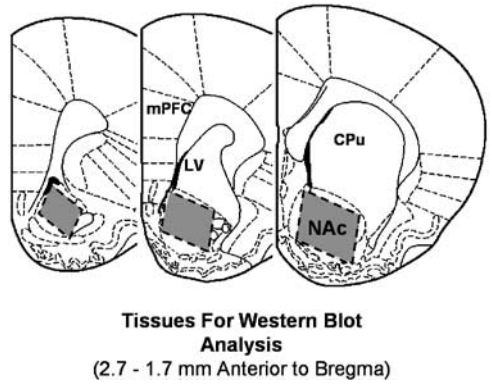

Figure 5 Collection of NAc tissues for Western blot analysis. Brain tissues were collected from the NAc (shadowed areas, 1.7–2.7 mm anterior to the bregma) of saline- and COC-pretreated rats. The protein levels of CaN-A, CaN-B, PP1, and p-Thr.34-DARPP-32 in the NAc were measured by conducting Western blot analysis. NAc: nucleus accumbens; CPu: caudate Putamen; mPFC: medial prefrontal cortex; LV: lateral ventricle.

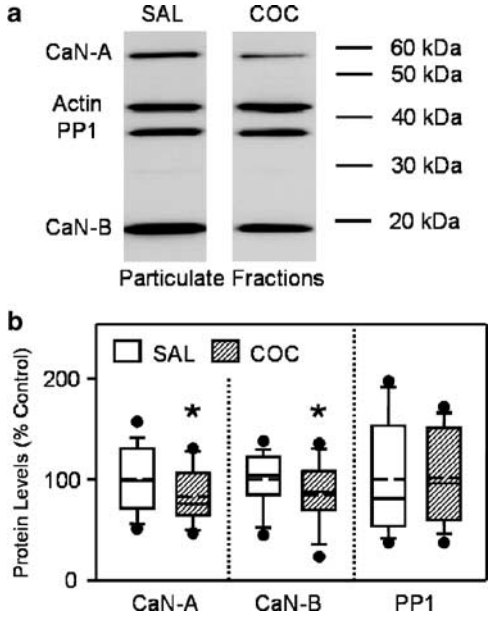

Figure 6 Repeated COC administration decreases the protein levels of CaN, but not PP1, in the rat NAc. Western blot analysis was used to measure the protein levels of CaN-A (61 kDa), CaN-B (19 kDa), and PP1 (37.5 kDa) in the NAc following repeated saline or COC administration. (a) Representative immunoreactive bands indicating a marked reduction in the levels of both CaN-A and CaN-B. The reduction of protein levels was found only in the particulate, but not the soluble, fractions of COC-pretreated NAc. (b) Box plots indicating that there were significant decreases in the levels of both CaN-A and CaN-B in the NAc of COC-pretreated rats as compared to saline-pretreated rats (CaN-A: SAL *vs* COC, $n = 35/33$, $^{*}p < 0.03$; CaN-B: SAL *vs* COC, $n = 37/38$, $^{*}p < 0.04$). However, there was no significant difference in the PP1 levels between saline- and COC-pretreated rats ($n = 15/15$, $p > 0.05$). Box plots showing the mean (dashed line), median (solid line), distribution (boxes, indicating the 25th and 75th percentiles and showing 50% of sample distribution), and range ('whiskers', indicating the 10th and 90th percentiles and showing 80% of sample distribution) of the relative protein levels of CaN-A, CaN-B, and PP1 measured in the NAc of saline- or COC-pretreated rats. The outlying dots indicate the 5th and 95th percentiles (showing 90% of sample distribution).

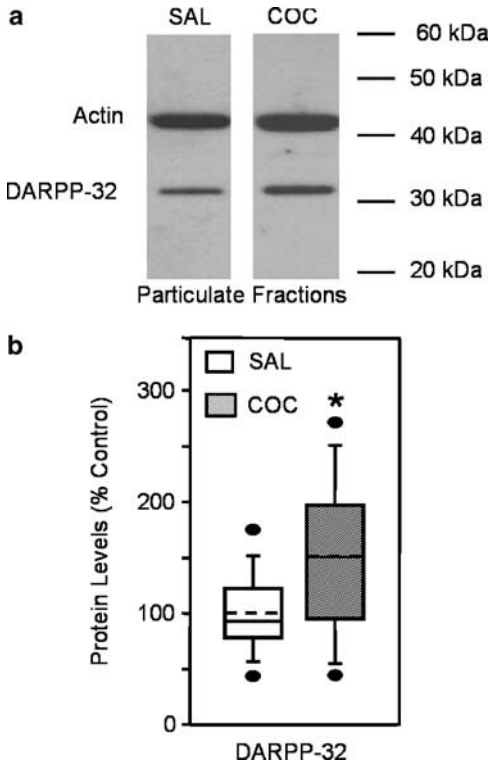

Figure 7 Repeated COC administration increases the levels of DARPP-32 in the rat NAc. Western blot analysis was also used to determine the alterations in the protein levels of total DARPP-32 in the NAc following repeated COC administration. (a) Representative immunoreactive bands indicating that in contrast to reduced CaN-A and CaN-B, the levels of total DARPP-32 were increased in the NAc after repeated COC pretreatment. This increase in the DARPP-32 levels was also found in the particulate fractions only. (b) Box plots indicating that there was a significant increase in the levels of total DARPP-32 in the COC-pretreated NAc ($n = 15/15$, $^*p < 0.05$).

after repeated administration of saline or COC. In contrast to the decreased CaN, COC pretreatment significantly increased the levels of total DARPP-32 in the rat NAc. Thus, DARPP-32 immunoreactive bands obtained from the particulate fractions, but not the soluble fractions, of the NAc were significantly increased by approximately 56% in COC-pretreated rats as compared to saline-pretreated rats (SAL *vs* COC: 100 ± 11.31 *vs* $156.07 \pm 18.53\%$, $n = 13/17$, $t = 2.393$, $^*p < 0.05$) (Figure 7a and b).

DISCUSSION

The present findings indicate that cytosolic application of CaN, PP1, and p-Thr.34-DARPP-32 alters VSSCs in medium spiny NAc neurons of rats. Dephosphorylation of Na^+ channels by CaN and PP1 enhances VSSCs, but inhibiting the activity of PP1 with p-Thr.34-DARPP-32 decreases I_{Na}. Repeated COC administration reduces whole-cell I_{Na} in NAc neurons. This effect of chronic COC on I_{Na} was associated with a reduction in CaN-induced I_{Na} enhancement and occlusion of p-Thr.34-DARPP-32-induced suppression of I_{Na}. Moreover, it was also associated with a decrease in the protein levels of CaN-A and CaN-B, but an increase in the levels of DARPP-32, in the COC-withdrawn NAc. Combined

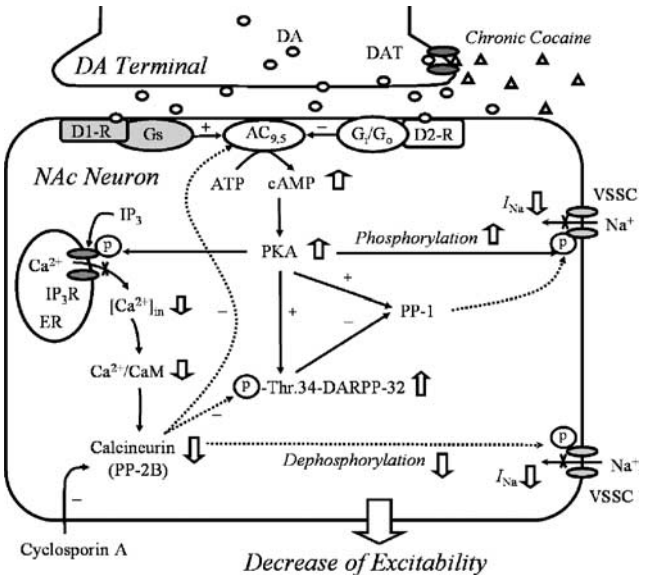

Figure 8 Repeated COC administration suppresses Na^+ channel function in the NAc: increased phosphorylation by PKA and decreased dephosphorylation by CaN (PP2B). Stimulation of D1- and D2-class DA receptors acts to increase and decrease the activity of the cAMP/PKA cascade, respectively. Phosphorylation of voltage-sensitive Na^+ channels by PKA decreases I_{Na}, while dephosphorylation of the channel by activated CaN and PP1 increases I_{Na}. CaN also inhibits the activity of p-Thr.34-DARPP-32, thereby disinhibiting PP1 from suppression of p-Thr.34-DARPP-32. Moreover, CaN also regulates the activity of Ca^{2+}-inhibited $AC_{9.5}$ in NAc neurons. Besides the Na^+ channel, PKA also phosphorylates the IP_3R, thereby decreasing Ca^{2+} release, and activates PP1. The solid line/arrows indicate the alterations in the intracellular pathways or ion channel functions mediated by the cAMP/PKA cascade. The dotted lines/arrows indicate the dephosphorylating effects of CaN and PP1 on their substrates. The sign of '+' indicates a stimulatory/active effect, while '−' indicates an inhibitory effect. The 'upward' and 'downward' open arrows indicate an enhanced and decreased response, respectively. Repeated COC administration enhances and prolongs DA action on both D1- and D2-class receptors, leading to an increased D1R but decreased D2R modulation in the cAMP/PKA cascade. The 'open' arrows indicate the COC-induced alterations. Chronic COC-induced increase in PKA activity leads to an enhancement in phosphorylation of Na^+ channels and IP_3Rs. On the other hand, repeated COC administration decreases free $[Ca^{2+}]_{in}$ by reducing whole-cell I_{Ca} and IP_3R-mediated Ca^{2+} release, probably via disrupting a reciprocal $Ca^+/IP^3R/CaN$ interaction in Ca^{2+} signaling. As a result, the potency of CaN in dephosphorylating Na^+ channels and inhibiting p-Thr.34-DARPP-32 are diminished. However, the mechanism underlying the decreased CaN levels has not been identified. In addition, the modulatory effects of CaN on inhibiting AC activity are also attenuated after chronic COC treatment, which may be related to the increase in DARPP-32 levels. All of these changes result in reduction of VSSCs. The effects of PP1 on the activity of Na^+ channels depend on the combined action of PKA (+) and p-Thr.34-DARPP-32 (−), and is apparently not affected by repeated COC administration. These findings suggest that the I_{Na} reduction in COC-withdrawn NAc neurons may be attributed to a suppression in intracellular Ca^{2+} signaling, which modulates both the increased phosphorylation and decreased dephosphorylation of Na^+ channels by PKA and CaN, respectively.

with previous findings, our results suggest that chronic COC-induced I_{Na} reduction in NAc neurons should be attributed to both an attenuated dephosphorylation and enhanced phosphorylation of Na^+ channels. The mechanism involved in chronic COC-induced I_{Na} reduction is summarized in Figure 8.

Dephosphorylation of Na^+ Channels by CaN was Reduced after Repeated COC Administration

It has been proposed that CaN plays a significant role in regulating VSSCs via Ca^{2+} signaling (see Introduction). The present study further determines that CaN-induced dephosphorylation facilitates the activity of Na^+ channels and subsequently increases I_{Na} in NAc neurons. However, this effect of CaN on I_{Na} is disrupted by repeated COC administration, which decreases the protein levels and diminishes potency of CaN for dephosphorylation of Na^+ channels. The exact mechanism underlying COC-induced attenuation in CaN activity remains unknown. However, these changes in CaN activity are likely related to a significant decrease in $[Ca^{2+}]_{in}$, resulting from a reduced VSCCs (Zhang *et al*, 2002; Hu *et al*, 2004), and/or a possible disruption of intracellular Ca^{2+} release. Although an I_{Na} reduction observed in COC-withdrawn NAc neurons might not be directly linked to decreased $[Ca^{2+}]_{in}$ caused by reduced I_{Ca} (since VSCCs were blocked during the recording), alterations in a reciprocal interaction among cytosolic-free Ca^{2+}, IP_3Rs, and CaN in regulating IP_3R-mediated Ca^{2+} release should be considered. Previous studies have demonstrated that reduced $[Ca^{2+}]_{in}$ diminishes the activity of both CaN and IP_3Rs, while inhibition of CaN suppresses the dephosphorylation and expression of IP_3Rs (see Introduction), which could also decrease Ca^{2+} release and $[Ca^{2+}]_{in}$. In contrast, increased $[Ca^{2+}]_{in}$ activates CaN and enhances I_{Na} (Hu *et al*, 2005). These findings, combined with the present results, suggest that the reciprocal interaction among Ca^{2+}/IP_3R/CaN in regulating Ca^{2+}/CaN signaling and subsequently dephosphorylation of Na^+ channels are functionally disrupted after repeated COC administration. Thus, a reduced I_{Na} in COC-withdrawn NAc neurons should be attributed to, at least partially, a decreased Ca^{2+} signaling.

Phosphorylation of Na^+ Channels was Increased in COC-Withdrawn NAc Neurons

The effects of repeated COC administration on suppressing I_{Na} depend not only on a decreased dephosphorylation of Na^+ channels by CaN but also on an enhanced phosphorylation of the channel by PKA. Previous studies have determined that repeated COC administration increases the activity of the cAMP/PKA cascade via enhancing the D1R-mediated but decreasing the D2R-mediated signaling, leading to an increase in PKA activity in striatal cells (Terwilliger *et al*, 1991; Nestler, 1997; Nestler *et al*, 1990). As PKA-induced phosphorylation of Na^+ channels decreases I_{Na} (see Introduction), the chronic COC-induced increase in PKA activity would cause a further reduction in VSSCs as compared to control (Zhang *et al*, 1998; the present study). Moreover, recent investigations have also demonstrated that PKA activates DARPP-32 by phosphorylating its threonine 34 sites, while CaN functions as a potent inhibitor of p-Thr.34-DARPP-32 by dephosphorylating those sites (Greengard *et al*, 1999; Nishi *et al*, 1997, 1999a; Lindskog *et al*, 1999). As p-Thr.34-DARPP-32 inhibits PP1 (which stabilizes phosphorylation states of Na^+ channels) and CaN is a potent inhibitor of this phosphoprotein (Greengard *et al*, 1999; Schiffmann *et al*, 1998), the effect of p-Thr.34-DARPP-32 on PP1 (and I_{Na}) would be attenuated by CaN. In the present study, application of CaN enhances I_{Na} in NAc neurons, revealing that a tonic inhibition in the activity of Na^+ channels by PKA and p-Thr.34-DARPP-32 was diminished following an enhanced dephosphorylation. Furthermore, because inhibition of CaN significantly increases the levels of p-Thr.34-DARPP-32 in striatal neurons (Nishi *et al*, 1999b, 2002), a marked increase in the levels of DARPP-32 after repeated COC administration could be attributed to, at least partially, a decrease in the CaN levels. Whether the increased levels of total DARPP-32 result from an augmented concentration of p-Thr.34-DARPP-32 requires further determination. In addition, an increase in PKA activity after repeated COC administration would also result in an enhanced phosphorylation of IP_3Rs, which might further decrease free $[Ca^{2+}]_{in}$ through reducing Ca^{2+} release. Given all the above, our findings suggest that the decreased dephosphorylation and increased phosphorylation of Na^+ channels by CaN and PKA, respectively, plus an enhanced activity of p-Thr.34-DARPP-32 in stabilizing the phosphorylation state of the Na^+ channel, play an integrated and critical role in I_{Na} reduction in NAc neurons after chronic COC pretreatment.

The observed increase in the levels of DARPP-32 within the COC-withdrawn NAc is similar to that found in behaviorally sensitized rats after repeated treatment with methamphetamine (Lin *et al*, 2002). These findings suggest a possible common mechanism underlying the increase of DARPP-32 following chronic exposure to psychostimulants. Nevertheless, associated with the increased levels of DARPP-32, the effects of exogenous p-Thr.34-DARPP-32 on suppressing I_{Na} were occluded in COC-withdrawn NAc cells. Although an I_{Na} reduction induced by exogenous p-Thr.34-DARPP-32 in NAc neurons of saline-pretreated rats was mimicked by repeated COC treatment, exogenous p-Thr.34-DARPP-32 failed to potentiate the I_{Na} reduction induced by chronic exposure to COC. These findings suggest that repeated COC administration might have produced a 'ceiling effect' on p-Thr.34-DARPP-32-mediated I_{Na} reduction, in which the effects of p-Thr.34-DARPP-32 on stabilizing the phosphorylation state of Na^+ channels had reached (or were near to) their maximal levels.

A Regulatory Effect of CaN on Dephosphorylating Adenylyl Cyclase (AC) may also be Diminished after Chronic Exposure to COC

Besides a decrease in dephosphorylation of Na^+ channels and increase in the DARPP-32 levels, a diminished dephosphorylation of AC by CaN should also be considered for I_{Na} reduction in COC-withdrawn cells. It is well known that there are at least nine different isotypes of AC (AC_{1-9}), which are broadly subclassified into Ca^{2+}-stimulated, Ca^{2+}-inhibited, and protein kinase C-activated ACs (Antoni *et al*, 1998a, b; Simonds, 1999). The activity of Ca^{2+}-inhibited ACs is regulated by free Ca^{2+} and CaN, through which the cAMP signal is dynamically tuned. For instance, the activity of AC_9, the most abundant cerebral AC at the mRNA level (Paterson *et al*, 1995; Premont *et al*, 1996), is inhibited by Ca^{2+} and this effect of Ca^{2+} on AC_9 is blocked selectively by inhibition of CaN (Antoni *et al*, 1998a, b). Moreover, significant levels of AC_5, another Ca^{2+}-inhibited AC, are

found only in the striatum and the mesolimbic DA system (Glatt and Snyder, 1993; Mons and Cooper, 1995). These findings indicate that the Ca^{2+}-inhibited AC is predominant in medium spiny neurons within the dorsal and ventral striatum of rats. Based on these findings, we propose that a decrease in the CaN activity after repeated COC administration would result in a disinhibition (increase) in the function of Ca^{2+}-inhibited $AC_{9,5}$ and PKA, thereby facilitating phosphorylation of Na^{+} channels by PKA, which also leads to a reduction in I_{Na}.

Although the increase in I_{Na} has been associated with the inhibitory effects of Ca^{2+}/CaN on AC, it is noted that inhibition of PKA by fluoride (F^{-}) (Vargas *et al*, 1999), a component of the internal solution commonly used in previous voltage-clamp recordings, may also play a role in this event. However, we have determined that the F^{-}-induced changes in I_{Na} during a 5–6 min period of recording time were insignificant (usually $<10\%$) as compared to that induced by either a relatively high concentration of cytosolic-free Ca^{2+} (Hu *et al*, 2005) or internally applied CaN. Moreover, we also found that I_{Na} enhancement induced by Ca^{2+} and CaN was more rapid (2–4 min) than that in control cells, which might be affected by F^{-}. These findings are apparently in agreement with the study of Vargas *et al* (1999), and therefore suggest that, during a relatively short period of treatment time, fluoride-induced inhibition in PKA activity may not have a significant impact in Ca^{2+}/CaN-modulated I_{Na} enhancement in rat NAc neurons.

Dephosphorylating Effects of PP1 on I_{Na} was not Affected Following COC Withdrawal

In contrast to CaN and p-Thr.34-DARPP-32, the maximal effects of PP1 on regulating I_{Na} and the protein levels of PP1 appeared to be unchanged in COC-withdrawn NAc neurons. These results, particularly the unchanged effects of PP1 on I_{Na}, were not expected since p-Thr.34-DARPP-32 is a potent PP1 inhibitor (Bibb *et al*, 1999; Greengard *et al*, 1999), and the levels of DARPP-32 were significantly increased in COC-withdrawn NAc neurons. The underlying mechanism regarding this phenomenon is unknown. However, earlier studies have proposed that PP1 is activated by PKA in striatal neurons (Surmeier *et al*, 1995). It is possible that the increased inhibitory effects of p-Thr.34-DARPP-32 on PP1 were associated with and compensated by enhanced activation of PP1 induced by PKA. Therefore, no significant changes in I_{Na} could be detected in response to applied PP1 in COC-withdrawn NAc neurons.

Functional Implications

Our novel findings indicate that both CaN and PP1 enhance whole-cell VSSCs, but p-Thr.34-DARPP-32 reduces I_{Na} in NAc neurons of rats. They also suggest that chronic COC-induced I_{Na} reduction should be attributed to both the increased phosphorylation and decreased dephosphorylation of sodium channels by PKA and CaN, respectively. More importantly, our findings reveal that the chronic COC-induced alterations in the intracellular signal transduction are not only limited in the cAMP/PKA cascade but also in the Ca^{2+} signaling, including the Ca^{2+} signaling-related proteins. A reduction in I_{Na} (and I_{Ca}) decreases the membrane excitability and reduces the ability of NAc neurons to relay essential cognitive and motivational commands to their target neurons. This disconnection (or attenuated connection) may contribute to the mechanism underlying the addiction and withdrawal effects of COC. Taken together, the global depression in the neuronal activity is likely to disrupt the ability of the NAc to integrate motivational and reward-associated learning processes and this could contribute to the pathophysiology of drug addiction.

ACKNOWLEDGEMENTS

We acknowledge Carolyn Grevers for her excellent technical assistance. We also thank Drs Pastor P Couceyro, Ann K Snyder, and Judy Potashkin for their helpful suggestions in Western blot analysis. This study was supported by USPHS Grant DA-04093 and Research Scientist Development Award DA 00456.

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