Published in final edited form as:
Psychopharmacology (Berl). 2008 September ; 200(1): 129–139. doi:10.1007/s00213-008-1189-5.

Administration of the calcineurin inhibitor cyclosporine modulates cocaine-induced locomotor activity in rats

Nii A. Addy[1,2], **Amine Bahi**[2], **Jane R. Taylor**[1,2], and **Marina R. Picciotto**[1,2,*]

1*Interdepartmental Neuroscience Program, Yale University School of Medicine, New Haven, CT 06508*

2*Department of Psychiatry, Yale University School of Medicine, New Haven, CT 06508*

Abstract

Rationale—Cocaine administration in rats increases locomotor activity as a result of underlying changes in neurotransmitter dynamics and intracellular signaling. The serine/ threonine phosphatase, calcineurin, is known to modulate several signaling proteins that can influence behavioral responses to cocaine.

Objective—This study aimed to determine whether calcineurin plays a role in locomotor responses associated with acute and repeated cocaine exposure. Second, we examined cocaine-mediated changes in intracellular signaling in order to identify potential mechanism underlying the ability of calcineurin to influence cocaine-mediated behavior.

Methods—Locomotor activity was assessed over 17 days in male Sprague-Dawley rats (n=48) that received daily administration of cocaine (15 mg/kg, s.c.) or saline in the presence or absence of the calcineurin inhibitor, cyclosporine (15 mg/kg, i.p.). Non-cocaine treated animals from this initial experiment (n=24) also received an acute cocaine challenge on day 18 of testing.

Results—Daily cyclosporine administration potentiated the locomotor response to repeated cocaine 5 mins after cocaine injection and attenuated the sustained locomotor response 15 to 40 mins after cocaine. Further, cyclosporine pretreatment for 17 days augmented the acute locomotor response to acute cocaine 5 to 30 mins after cocaine injection. Finally, repeated exposure to either cocaine or cyclosporine for 22 days increased synapsin I phosphorylation at the calcineurin sensitive Ser 62/67 site, demonstrating a common downstream target for both calcineurin and cocaine.

Conclusion—Our results suggest that calcineurin inhibition augments locomotor responses to cocaine and mimics cocaine-mediated phosphorylation of synapsin I.

Keywords

cocaine; calcineurin; synapsin; locomotor activity; sensitization; nucleus accumbens

Introduction

Cocaine is a psychostimulant that is addictive and readily self-administered by humans and various animal species (Ettenberg et al. 1982; Koob 1992; Ritz et al. 1988). Cocaine decreases dopamine reuptake by inhibiting the dopamine transporter in the mesolimbic system, an effect thought to be critical for self-administration and other behaviors associated with cocaine reinforcement (Goeders and Smith 1983; Karler et al. 1989; Ritz et al. 1987; 1988; Rodd-Henricks et al. 2002). In addition, cocaine administration can increase extracellular glutamate,

*To whom correspondence should be addressed: 34 Park Street, 3rd Floor Research, New Haven, CT 06508, Ph: (203) 737-2042, Fax: (203) 737-2043, E-mail: marina.picciotto@yale.edu.

serotonin and norepinephrine levels in various brain regions (Di Chiara and Imperato 1988; Kalivas and Duffy 1995; 1998; Reid et al. 1997; Reith et al. 1997). Cocaine-mediated increases in levels of dopamine and other neurotransmitters can alter the activity of downstream signaling proteins such as the dopamine- and cAMP regulated phosphoprotein-32 (DARPP32), cAMP response element binding protein (CREB), and extracellular signal-regulated protein kinase (ERK) (Carlezon et al. 1998; Greengard et al. 1999; Mattson et al. 2005; Miserendino and Nestler 1995; Self et al. 1998; Terwilliger et al. 1991). These signaling proteins are known to influence synaptic plasticity in the brain (Ahmed and Frey 2005; English and Sweatt 1997; Hotte et al. 2007; Impey et al. 1988) and are likely to be important for cocaine-mediated plasticity within the mesolimbic dopamine system (Borgland et al. 2004; Liu et al. 2005; Saal et al. 2003; Ungless et al. 2001).

One protein that can potentially modulate several cocaine-mediated processes in the brain is the serine/ threonine phosphatase, calcineurin. When activated by calcium and calmodulin, calcineurin dephosphorylates target proteins such as synapsin I, DARPP32, and CREB, as well as a number of other proteins important for neuronal function including calcium channels, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, and N-methyl-D-aspartate (NMDA) receptors (Greengard et al. 1999; Jovanovic et al. 2001; Winder and Sweatt 2001). Thus, calcineurin activity could modulate cocaine-mediated neurotransmitter release and intracellular signaling through actions at several different pre-synaptic and post-synaptic targets.

For instance, calcineurin-mediated dephosphorylation of synapsin I could potentially influence cocaine's ability to modulate neurotransmitter release. Synapsin I normally tethers synaptic vesicles to the pre-synaptic cytoskeleton to limit vesicular neurotransmitter release (Greengard et al. 1993). When phosphorylated, synapsin I has a decreased affinity for both actin and synaptic vesicles, shifting these vesicles to the readily releasable pool and allowing for greater neurotransmitter release upon stimulation (Bahler and Greengard 1987; Greengard et al. 1993; Huttner et al. 1983). In addition, the synapsin family of proteins is thought to contribute to cocaine-mediated dopamine release in the striatum as this release is decreased in synapsin knockout mice (Venton et al. 2006). Thus, through regulation of synapsin I phosphorylation, calcineurin could serve to either limit or augment cocaine-mediated neurotransmitter release which, in turn, could alter cocaine-mediated behaviors.

In the current study we hypothesized that calcineurin plays an important role in behaviors associated with cocaine exposure and we further hypothesized that cocaine may regulate the phosphorylation of calcineurin target proteins, such as synapsin I. To test these hypotheses, we examined the role of calcineurin in cocaine-mediated locomotor activation and sensitization, both of which result from cocaine-induced neurotransmitter effects in the mesolimbic dopamine system (Cabib et al. 1991; Kalivas and Alesdatter 1993; McCreary and Marsden 1993). Furthermore, we used western blot analysis to determine whether regulation of calcineurin or treatment with cocaine could regulate the phosphorylation state of calcineurin targets, such as synapsin I, *in vivo*. Our results demonstrate that calcineurin inhibition can potentiate locomotor responses to cocaine and that regulation of synapsin I could contribute to this behavioral effect.

Materials and Methods

Subjects

Male Sprague Dawley rats weighing between 200-250 grams upon arrival were supplied by Charles River Laboratories (Wilmington, MA). Animals were placed on *ad libitum* food and water and were housed 2 to 3 per cage on a 12 hour light/dark cycle with lights on at 7 am. All

experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and were approved by the Yale Animal Care and Use Committee.

Drug administration and locomotor activity testing

Open field locomotor activity was assessed in standard plastic cages without bedding (45×24×20 cm) using the automated Omnitech Digiscan Micromonitor system (Columbus, OH, USA) equipped with 16 photocell array (2 cm space between each 2 cells). Horizontal locomotor activity, defined as consecutive beam breaks, was collected by counting photocell beam interruption in 5 min intervals using Micro Pro version 1.3 software (Columbus, OH, USA). Stereotypic activity was defined as short movements that occurred in the absence of ambulatory activity, in which the animal must cross 2 photocells within 500 ms. Thus, stereotypic counts occurred in a maximum area of 2 photocells.

Drug treatment was performed daily and alternated between home cage drug administration (on non-test days) and locomotor chamber administration (on test days) where locomotor activity was assessed in a distinct environment (Fig 1A, 2A, Supp Fig1A). All drugs were injected in a volume of 2 ml/kg body weight. For cyclosporine experiments, animals received intraperitoneal injections of 5, 15 or 20 mg/kg cyclosporine (Sandimmune ® Oral Solution, Novartis, East Hanover, NJ) or olive oil vehicle (ICN Biochemicals, Cleveland, OH). The 15 mg/kg cyclosporine dose, in olive oil vehicle, has been previously demonstrated to increase cyclosporine plasma levels (Donatsch and Ryffel 1986) and to alter brain calcineurin activity (Addy et al. 2007) in rats, while the 5 and 20 mg/kg doses were employed in order to demonstrate a dose-response relationship. The initial injection of cyclosporine or vehicle was administered 24 hours prior to the first locomotor session with an additional injection 1 hr before locomotor testing in order to increase plasma cyclosporine levels during locomotor assessment. This time course of administration was chosen based on previous pharmacokinetics studies demonstrating increased cyclosporine plasma levels in rats on the second day of administration at multiple time points throughout the day (Donatsch and Ryffel 1986). The 20 mg/kg dose of cyclosporine resulted in significant toxicity and 4 animals did not complete the experiment. In rapamycin experiments, animals received 15 mg/kg rapamycin (LC Laboratories, Woburn, MA, USA) or 5 % dimethyl-sulfoxide “DMSO” vehicle (Sigma, St. Louis, MO, USA). For both cyclosporine and rapamycin experiments, each locomotor session began with a 30 min habituation period immediately followed by subcutaneous administration of 15 mg/kg cocaine (NIDA, Bethesda, MD) or saline vehicle (0.9% Sodium Chloride, Hospira, Lake Forest, IL) and a 60 min assessment of locomotor activity. The first experiment was performed over 17 days and included the following treatment groups (n=12 per group); control (saline + oil vehicle), cocaine (15 mg/kg cocaine + oil vehicle), cyclosporine (saline + 15 mg/kg cyclosporine), and cocaine + cyclosporine (15 mg/kg cocaine + 15 mg/kg cyclosporine). In the subsequent experiment, non-cocaine treated animals (control and cyclosporine groups, n=12 per group) were exposed to an acute cocaine challenge (15 mg/kg, s.c.) on day 18 of testing. These experiments were also repeated with a second dose of 10 mg/kg cocaine (NIDA, Bethesda, MD) with the following treatment groups (n=5/6 per group); control (saline + oil vehicle), cocaine (10 mg/kg cocaine + oil vehicle), cyclosporine (saline + 15 mg/kg cyclosporine), and cocaine + cyclosporine (10 mg/kg cocaine + 15 mg/kg cyclosporine). Experimental procedures were identical to those listed above in the 15 mg/kg cocaine study. In the cyclosporine dose-response experiment, treatment groups (n=8 per group) consisted of animals that received 0, 5, 15 and 20 mg/kg cyclosporine treatment for 17 days followed by an acute cocaine challenge on day 18. The fourth experiment consisted of two treatment groups (n=8 per group) of animals that received 0 or 15 mg/kg rapamycin for 17 days and were then exposed to an acute cocaine challenge on day 18. In all of these experiment, animals received two injections per day: an intraperitoneal injection of vehicle, cyclosporine or rapamycin along with a subcutaneous injection of either saline or cocaine.

Tissue dissection and western blot analysis

At the completion of behavioral studies, animals were sacrificed by rapid decapitation 30 mins after subcutaneous injection with cocaine or saline and the head was immediately frozen in chilled 2-methyl butane (VWR International, West Chester, PA). Each brain was later removed and sliced into 1 to 2 mm sections using a chilled matrix (Zivic Labs, Pittsburgh, PA). Region specific dissections of prefrontal cortex, striatum, NAc, and VTA were performed using micro punch tools made from 16 or 18 gauge needles.

Tissue samples were prepared for western blot analysis and run on a 10% polyacrylamide gel at a protein concentration of 10 μg per sample (7.5 μg per VTA sample) as previously described (Brunzell et al., 2003). Nitrocellulose blots were then briefly washed in Tris-Buffered Saline (TBS, 20 mM Tris Base, 137 mM NaCl adjusted to pH 7.6), blocked in TBS with 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) and 5% skim milk, then incubated overnight at 4° C with primary antibodies against the following proteins: calcineurin (BD Biosciences, San Jose, CA 1:1000), pSynapsin I (Ser 62/ Ser 67, courtesy of Angus Nairn, Yale University, 1:500), synapsin I (Cell Signaling, Danvers, MA 1:1000), or GAPDH (Advanced Immunochemical, Long Beach, CA 1:30,000). Blots were then briefly washed in TBS with 0.05% Tween followed by a 1 hr incubation at room temperature with one of the following secondary antibodies: anti-rabbit IgG Rockland IR Dye™ 780 (Rockland Immunochemicals, Gilbertsville, PA 1:5000) or anti-mouse IgG Alexa Fluor ® 680 (Invitrogen, Carlsbad, CA 1:5000). After incubation, blots were washed three times with TBS-Tween and were scanned using a LI-COR Odyssey scanner (LI-COR Biosciences, Lincoln, NE). Protein bands were analyzed and quantified using Odyssey version 1.2 software (LI-COR Biosciences, Lincoln, NE).

Data Analysis

Total locomotor activity and total stereotypy counts were analyzed using a 2 X 2 repeated measures analysis of variance (ANOVA) with testing day as the repeated measure. The variables for the 2 X 2 ANOVA were subcutaneous injection (saline or cocaine) and intraperitoneal injection (oil vehicle or cyclosporine). Additional analysis of daily locomotor activity in 5 min time bins was performed using a 2 X 2 ANOVA with time bin as the repeated measure. Independent samples t-tests, with Bonferroni corrections for multiple comparison, were also performed to assess between group differences in specific time bins. Analysis of the locomotor response to acute cocaine was assessed using a paired samples t-test to compare saline activity on day 17 to cocaine activity on day 18 in the same animals. In addition, an independent samples t-test was performed in order to compare acute cocaine responses in animals that had received exposure to cyclosporine (0,5,15 or 20 mg/kg) or rapamycin (0 or 15 mg/kg) for 17 days prior to acute cocaine exposure on day 18. For western blot analysis, band intensity for each protein was normalized to that of GAPDH, which served as a protein loading control. In addition, all quantifications were normalized to control animals set at 100%. Data were then analyzed using a 2 X 2 Univariate ANOVA, with subcutaneous injection (saline or cocaine) and intraperitoneal injection (oil vehicle or cyclosporine) as the two variables. Statistical significance was assigned to analyses revealing a p value of less than 0.05. All statistical tests were performed using SPSS 14.0 and SPSS 15.0 Software (SPSS Inc, Chicago, IL).

Results

Biphasic modulation of cocaine-mediated locomotor sensitization by chronic systemic cyclosporine administration

Locomotor activity in animals exposed to repeated saline or cocaine (15 mg/kg, s.c.) was examined in combination with cyclosporine (15 mg/kg, i.p.) or oil vehicle (Fig 1A) to determine the role of calcineurin in cocaine-mediated locomotor activation. Repeated measures analysis

of locomotor activity after cocaine/saline co-administered with cyclosporine/oil vehicle revealed a significant effect of testing day ($F_{8,352} = 8.005$, $p < 0.001$, Fig 1C) Cocaine-treated animals showed increased locomotor activity over the 17 day testing period as revealed by a main effect of cocaine on locomotor activity ($F_{1,44} = 327.058$, $p < 0.001$, Fig 1C), with maximal effects observed on day 3 of treatment (Fig 1C), which has been previously observed in other studies as well (Bahi et al. 2004;Dunn et al. 2005). While cyclosporine administration (15 mg/kg/day, i.p.) did not alter the initial development of cocaine-mediated locomotor sensitization on treatment days 1 and 3, it did attenuate the sustained locomotor response to cocaine observed over the entire testing period, as revealed by a cocaine × cyclosporine interaction ($F_{1,44} = 6.205$, $p < 0.05$, Fig 1C). However, given the dramatic increase in locomotor activity on testing days 1 and 3 in both cocaine groups, a ceiling effect may also have contributed to these results.

To determine whether stereotypic movements may have competed with locomotor activity responses to cocaine, we analyzed stereotypic counts over the 17 day testing period (Supp Fig 3A). The repeated measures ANOVA revealed a main effect of test day ($F_{8,352} = 7.975$, $p < 0.001$, Supp Fig 3A) as well as a main effect of cocaine ($F_{1,44} = 334.86$, $p < 0.001$, Supp Fig 3A), but no effect of cyclosporine ($F_{1,44} = 1.778$, $p > 0.05$, Supp Fig 3A). Similar to locomotor activity responses, peak stereotypy activity occurred on day 3 of testing and declined slightly on subsequent testing days. Thus, the observed decline in locomotor activity after peak responding on day 3 (Fig 1C) was not due to an increase in stereotypic movement. Further, while the cyclosporine co-administration with cocaine led to a greater decline in locomotor activity after day 3 (Fig 1C), cyclosporine administration did not alter stereotypic movements as there was no cocaine X cyclosporine interaction ($F_{1,44} = 1.700$, $p > 0.05$, Supp Fig 3A).

In order to characterize the role of calcineurin in cocaine-mediated locomotor responses further, we examined the time course of locomotor activation on each test day by analyzing activity in 5 min bins over the entire 60 min testing session. Analysis by this method revealed a biphasic effect of cyclosporine administration on cocaine-mediated locomotor activity. While cyclosporine (15 mg/kg, i.p.) did not alter the acute response to cocaine on day 1 or the sensitized response to cocaine on day 3, it altered the time course of peak locomotor responding to cocaine on testing days 5 through 17. In animals exposed to repeated cocaine alone, peak locomotor responses were typically observed 10 to 20 mins after subcutaneous cocaine injection (Fig 1D). In cyclosporine treated animals, however, peak locomotor activation was observed 5 mins after subcutaneous cocaine injection (Fig 1D) and led to a potentiated initial response to cocaine on test day 11 ($p < 0.05$, Independent samples t-test with a Bonferroni correction). In contrast, cyclosporine treated animals exhibitied a decreased response to cocaine after 15 mins or more on test days 5 through 17 leading to a cocaine X cyclosporine interaction on test days 9, 15, and 17 ($p < 0.05$, Fig 1D). Thus, cyclosporine administration shifted the time course of the peak locomotor response to cocaine administered at 15mg/kg.

Due to the fact that 15 mg/kg cocaine administration led to peak locomotor responses on day 3 of testing, we performed a second experiment with a 10 mg/kg cocaine dose and 15 mg/kg cyclosporine dose in an attempt to avoid potential ceiling effects of cocaine (Supp Fig 1A). Consistent with the 15 mg/kg cocaine experiment, we observed a main effect of locomotor activity over testing days ($F_{18,136} = 4.682$, $p < 0.001$, Supp Fig 1C) as well as a main effect of cocaine ($F_{1,17} = 69.53$, $p < 0.001$, Supp Fig 1C) and a trend towards a main effect of cyclosporine ($F_{1,17} = 3.559$, $p < 0.08$, Supp Fig 1C). Similar to the previous experiment with 15 mg/kg cocaine, the animals in the 10 mg/kg cocaine experiment showed peak locomotor activity at day 3 and a slight decline in locomotor activity on subsequent test days (Supp Fig 1C). In addition, we observed a trend towards a cocaine X cyclosporine interaction ($F_{1,17} = 3.863$, $p < 0.07$, Supp Fig 1C), similar to the effects observed with the 15 mg/kg cocaine dose (Fig 1C). We also examined locomotor activity on each testing day in 5 min bins over the entire testing period which revealed that cyclosporine co-administration with cocaine (10 mg/kg)

attenuated the locomotor response to cocaine throughout the testing period on Day 11 and 13 ($p < 0.05$, cocaine X cyclosporine interaction) and gave a trend towards a significant attenuation on day 17 ($p < 0.07$, cocaine X cyclosporine interaction); however, in contrast to the effects observed in the 15 mg/kg cocaine study, cyclosporine co-administration with 10 mg/kg cocaine did not alter the locomotor response 5 minutes after injection ($p > 0.05$, independent samples t-test, Supp Fig 1D). Further, stereotypic activity in response to the 10 mg/kg cocaine dose did not appear to compete with locomotor responses, as stereotypy activity peaked on day 3 and subsequently declined (Supp Fig 3B). Analysis of weight gain throughout the duration of both cocaine experiments demonstrated main effects of cyclosporine ($F_{1,44}= 20.816$, $p < 0.001$, Supp Fig 4A; $F_{1,17} = 15.928$, $p < 0.005$, Supp Fig 4B), illustrating that cyclosporine treated animals showed decreased weight gain compared to non-cyclosporine treated animals. However, analysis of activity in the 30 mins prior to administration of 10 mg/kg or 15 mg/kg cocaine revealed no significant effect of cyclosporine ($F_{1,17} = 4.067$, $p > 0.05$ for 10 mg/kg cocaine study, Supp Fig 1B; $F_{1,44} = 3.241$, $p > 0.05$ for 15 mg/kg cocaine study, Fig 1B). Thus, while cyclosporine administration altered weight gain in these animals, it did not alter baseline locomotor activity.

Systemic cyclosporine pretreatment leads to a sensitized locomotor response to acute cocaine administration

In order to determine whether chronic calcineurin inhibition altered the locomotor effects of acute cocaine administration, non-cocaine treated animals (saline + oil vehicle or saline + 15 mg/kg cyclosporine) from the initial experiment (n=24) were exposed to an acute injection of cocaine (15 mg/kg, i.p.) on day 18 of testing (Fig 2A). This design allowed for comparison of acute locomotor response to cocaine in animals that had received either cyclosporine (15 mg/kg, i.p.) or vehicle administration for 17 days prior to cocaine administration. While both groups showed increased locomotor activity in response to an acute challenge with cocaine (paired samples t-test, $p < 0.005$ Fig 2B) animals pretreated with 15 mg/kg cyclosporine showed a potentiated locomotor response compared to vehicle pretreated animals ($p < 0.001$, Fig 2B, Independent samples T-test). In addition, the cyclosporine-induced potentiation was observed at multiple time bins from 5 to 30 mins after cocaine injection ($p < 0.05$, independent samples t-test with a Bonferroni correction for multiple comparisons, Fig 2C). Thus, while daily co-administration of 15 mg/kg cocaine and 15 mg/kg cyclosporine augmented the sensitized locomotor response to cocaine only 5 mins after injection (Fig 1D), cyclosporine pretreatment potentiated the locomotor response across the first 30 minutes of the testing session (Fig 2C). Further, cyclosporine pretreatment resulted in a trend towards a potentiation of the locomotor response to acute cocaine at a 10 mg/kg challenge dose ($p < 0.10$, independent samples t-test, Supp Fig 2A). While analysis of locomotor activity in 5 min bins with a 10 mg/kg cocaine challenge showed effects similar to those in the 15 mg/kg study, no significant difference was observed between the control and cyclosporine pretreated groups ($p > 0.05$, independent samples t-test with a Bonferroni correction for multiple comparisons), likely due to the small sample size (Supp Fig 2B).

In order to further characterize the effects of cyclosporine pretreatment in the locomotor response to acute cocaine, we also performed a cyclosporine dose response in a separate set of rats (n=8, Fig 2D). In this experiment, animals in all 4 cyclosporine treatment groups (0, 5, 15 or 20 mg/kg, i.p. for 17 days) showed increased locomotor activity in response to an acute cocaine challenge on day 18 ($p < 0.05$, paired samples t-test, Fig 2D) while animals pretreated with cyclosporine at 15 mg/kg showed a potentiated response to cocaine as seen previously ($p < 0.05$ independent samples t-test, Fig 2D). Although our results revealed a sharp dose response curve, this was likely due to peripheral side effects associated with the 20 mg/kg cyclosporine dose that may have competed with the locomotor response to cocaine. Specifically, 4 animals in the 20 mg/kg group did not complete the study due to toxicity and sickness behavior. Despite

these complications with the higher cyclosporine dose, our data suggest that the calcineurin inhibition associated with administration of 15 mg/kg cyclosporine is sufficient to alter the locomotor response to cocaine. The fact that calcineurin is expressed ubiquitously throughout the brain (Kincaid et al. 1987), however, complicates the interpretation of these results. In addition, since cyclosporine is an immunophilin which must bind to cyclophilin in order to inhibit calcineurin (Liu et al. 1991), it is possible our results could be due to the immunophilin effects of cyclosporine that are independent of calcineurin inhibition. To address this possibility, we performed an additional experiment with rapamycin, an immunophilin that does not inhibit calcineurin (Liu et al. 1991;Yu et al. 2006). While control animals showed enhanced locomotor activity upon acute cocaine injection (Rap 0 mg, $p < 0.05$, independent samples t-test, Fig 2D), pretreatment with rapamycin (15 mg/kg) did not affect cocaine-induced locomotor activity ($p > 0.05$, independent samples t-test, Fig 2D). Together, these data provide further evidence that calcineurin may be involved in regulation of locomotor responses to cocaine.

Regulation of synapsin I phosphorylation by chronic cocaine or chronic cyclosporine administration

Since we have previously demonstrated that calcineurin inhibition with cyclosporine regulates phosphorylation of the calcineurin target protein, synapsin I (Addy et al. 2007), we used western blot analysis to determine whether cyclosporine administration in the current study altered synapsin I phosphorylation. While chronic cyclosporine administration(15 mg/kg/day, i.p. for 22 days) did not alter calcineurin protein levels (Fig 3A), western blot analysis revealed a main effect of cyclosporine administration on levels of phosphorylated synapsin I at the calcineurin sensitive serine 62/67 site (S62/67) in the NAc ($F_{1,38}= 5.248$, $p < 0.05$, Fig 3B), striatum ($F_{1,42}= 7.619$, $p < 0.01$, Fig 3B) and VTA ($F_{1,36}= 7.971$, $p < 0.01$, Fig 3B) with no effect on total synapsin I levels in any of these regions (Fig 3C). These results demonstrate that cyclosporine administration altered calcineurin activity in sub-cortical regions of the brain, consistent with our previous findings (Addy et al. 2007). Our western blot experiments also revealed a main effect of cocaine administration on pSynI (S62/67) levels in the NAc ($F_{1,38}= 4.290$, $p < 0.05$, Fig 3B), and striatum ($F_{1,42}= 5.911$, $p < 0.05$, Fig 3B), and a strong trend towards a main effect in the VTA ($F_{1,36}= 3.746$, $p < 0.07$, Fig 3B); however, further analysis revealed no significant interaction between cyclosporine and cocaine administration on pSynI levels in the NAc ($F_{1,38}= 0.259$, $p > 0.05$, Fig 3B), striatum ($F_{1,42}= 0.154$, $p > 0.05$, Fig 3B) or VTA ($F_{1,36}= 0.722$, $p > 0.05$, Fig 3B). Indeed, administration of either cyclosporine or cocaine alone for 22 days increased pSynI (S62/67) levels to the same extent with no difference between the two groups (Fig 3B). In contrast, acute cocaine administration did not alter pSynI or total synapsin I levels in any of the regions tested (Fig 3D, 3E). Thus, the fact that repeated administration of cocaine or cyclosporine led to increased pSyn I (S62/67) suggests that synapsin I may be a molecular target of both drugs. While DARPP32 is another calcineurin target that has been shown to be regulated by cocaine exposure (Svenningsson et al. 2005), examination of striatal tissue revealed no significant differences in levels of phosphorylated DARPP32 at a calcineurin sensitive site (threonine 34) after repeated cocaine or cyclosporine administration (data not shown).

Discussion

The current study presents evidence that calcineurin activity modulates cocaine-mediated locomotor behavior. The ability of the calcineurin inhibitor, cyclosporine, to potentiate both the locomotor response to acute cocaine exposure and the early response to repeated cocaine exposure suggests that calcineurin is part of a feedback pathway that limits the effects of cocaine. This is consistent with previous work showing decreased calcineurin expression in the nucleus accumbens of rats after repeated cocaine exposure and withdrawal (Hu et al.

2005). Similarly, calcineurin over-expression in the mouse forebrain disrupts locomotor sensitization and the conditioned place preference associated with the administration of amphetamine, another psychostimulant (Biala et al. 2005), suggesting that increased calcineurin activity may have more general effects that limit psychostimulant action. It should be noted, however, that the conclusion that calcineurin activity is involved in cocaine-induced hyperactivity depends on the specificity of the effect of cyclosporine. While the fact that rapamycin is ineffective in these assays supports this conclusion, off-target effects cannot be ruled out.

The fact that cyclosporine administration mimicked cocaine's actions at synapsin I and potentiated locomotor responses to cocaine suggests synapsin I phosphorylation may play a role in the locomotor responses to cocaine. While repeated cyclosporine administration led to a leftward shift in the locomotor response to repeated cocaine at a 15 mg/kg dose, this was likely due to a potentiated initial locomotor response that led to a ceiling effect and subsequently decreased responding during the remainder of the testing session (15-40 minutes after injection). In addition, repeated cyclosporine administration appeared to preferentially potentiate the initial locomotor response of higher cocaine doses, as these effects were not observed with 10 mg/kg cocaine dose. However, repeated cyclosporine administration led to a robust potentiation of locomotor response to acute cocaine at 15 mg/kg, where ceiling effects were not a factor. The ability of cyclosporine to increase acute cocaine-induced hyperactivity while attenuating the development of cocaine sensitization is somewhat puzzling but is likely due to a differential role for calcineurin signaling in distinct cell types at different time points in these tasks. For example, cocaine sensitization effects may be mediated through activation of calcineurin in DA neurons in the VTA, thus cyclosporine treatment leads to decreased sensitization. In contrast, the activation of calcineurin in medium spiny neurons in the NAc may normally attenuate the acute effects of cocaine on DA responses, and therefore blockade of the enzyme leads to an apparent increase in this acute effect. These are speculations at this point, but future studies using local infusion, or genetic techniques to target calcineurin in specific cell types, could help answer these questions more definitively.

The fact that repeated administration of cocaine also increased synapsin I phosphorylation further suggests that synapsin I may be an important effector protein contributing to cocaine's long term effects in the brain. While phosphorylation of another calcineurin target protein, DARPP32, has also been implicated in cocaine-mediated behavior (Svenningsson et al. 2005), repeated cocaine or cyclosporine administration did not alter DARPP32 phosphorylation in the present study. Although calcineurin inhibition has also been shown to increase DARPP-32 phosphorylation (Nishi et al. 2002), these demonstrations have been performed in slices preparations that are isolated from the rest of the mesolimbic dopamine system. Thus, the lack of a DARPP-32 effect after calcineurin inhibition in our work is likely due to the actions of cyclosporine at several sites within the mesolimbic dopamine circuit. In contrast, the results of the current work provide the first demonstration of increased synapsin I phosphorylation at the serine 62/67 site in response to repeated cocaine exposure.

Given the role of synapsin I in the transition of neurotransmitter vesicles to the readily releasable pool (Greengard et al. 1993; Hilfiker et al. 2005), these data further suggest that cocaine-mediated locomotor activation and sensitization could be due, in part, to cocaine's ability to promote synapsin-dependent neurotransmitter release. Thus, we conclude that calcineurin's ability to modulate synapsin I (S62/67) phosphorylation could serve to regulate the locomotor response to cocaine and may also play a role in other cocaine-associated behaviors. Specifically, cocaine administration led to a robust increase in synapsin I phosphorylation in the striatum and nucleus acumens (Fig 3B) and we hypothesize that regulation of synapsin I at these loci plays an important role in the cocaine effects observed in this study. Further, regulation of synapsin I activity can potentially alter the release of several

neurotransmitters in the striatum and nucleus accumbens which have been implicated in the behavioral responses associated with cocaine exposure. For instance, cocaine-mediated phosphorylation of synapsin I could potentially influence dopamine availability as synapsin I, II and III triple knockouts show decreased cocaine-mediated dopamine release in the striatum compared to wild-type mice (Venton et al. 2006), an effect which would also limit cocaine-induced locomotor responses. While we observed increased synapsin I phosphorylation after repeated cocaine exposure, similar effects have also been demonstrated with amphetamine. For instance, striatal synaptosome preparations from rats previously exposed to repeated amphetamine show increased synapsin I phosphorylation and an enhancement of amphetamine-mediated dopamine release (Iwata et al. 1997). In addition, repeated amphetamine also leads to increases in synapsin I phosphorylation *in* vivo in the striatum of rats (Iwata et al. 1997). Thus, decreases in calcineurin activity and increases in synapsin phosphorylation, may be a necessary molecular consequence of psychostimulant administration, and may be important for modulating the behavioral consequences of these drugs.

The data in the current study also suggest a potential mechanism for regulation of both cocaine-mediated synapsin I phosphorylation and cocaine-mediated locomotor behavior by calcineurin. Specifically, calcineurin acts to dephosphorylate synapsin I at the S62/67 site (Jovanovic et al. 2001) and can thus oppose cocaine-mediated phosphorylation of this target site. Furthermore, dephosphorylated synapsin I decreases vesicular neurotransmitter release (Nichols et al. 1992). Thus, calcineurin-mediated dephosphorylation of synapsin I could potentially regulate changes in neurotransmitter release resulting from cocaine administration. Given the opposing effects of cocaine and calcineurin on synapsin I phosphorylation and on cocaine-mediated locomotor activity, the balance between synapsin I phosphorylation and dephosphorylation may be an important mechanism underlying cocaine-mediated locomotor activation. We therefore propose a model in which cocaine increases the number of vesicles in the readily releasable pool, through actions at synapsin I (Fig 4), which in turn supports cocaine-mediated locomotor responses. In the current study, calcineurin inhibition by cyclosporine removed this regulation, which led to increased synapsin I phosphorylation and this could increase the number of synaptic vesicles in the readily releasable pool. Thus, in cyclosporine treated animals, the increased number of vesicles in the readily releasable pool could allow for increased neurotransmitter release to potentiate the locomotor response to cocaine. Indeed, calcineurin inhibition has been demonstrated to increase both amphetamine-induced locomotor behavior and place conditioning (Borlongan et al. 1995; Gerdjikov and Beninger 2005), suggesting that similar mechanisms may also contribute to amphetamine-induced behavior. Together, the data presented here demonstrate that synapsin I serves as an important cocaine-regulated target protein that may mediate the locomotor responses to acute and chronic cocaine administration. This work also suggests that repeated cocaine exposure could increase extracellular neurotransmitter availability not only by inhibiting reuptake transporters, but by altering the activity of a presynaptic protein, synapsin I, to allow for increased neurotransmitter availability in the presynaptic cleft. Finally, we illustrate that calcineurin plays a critical role in regulating the activity of synapsin I, which may underlie the ability of calcineurin to limit locomotor responses to cocaine administration. While this work highlights the potential importance of synapsin I in cocaine-mediated behavior, additional experiments will be necessary to determine the role of synapsin I in specific brain regions and to delineate the neurotransmitter systems that are influenced by cocaine-mediated regulation of synapsin I.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by DA14241 and DA11717. NAA was supported by MH18882 (American Psychological Association) and DA021459. AB was supported by Fellowship for advanced researchers from the Swiss National Science Foundation (PA00A-117453). MRP was supported by DA00436.

Literature Cited

Addy NA, Fornasiero EF, Stevens TR, Taylor JR, Picciotto MR. Role of calcineurin in nicotine-mediated locomotor sensitization. J Neurosci 2007;27:8571–80. [PubMed: 17687035]

Ahmed T, Frey JU. Plasticity-specific phosphorylation of CaMKII, MAP-kinases and CREB during late-LTP in rat hippocampal slices in vitro. Neuropharmacology 2005;49

Bahi A, Boyer F, Kafri T, Dreyer J. CD81-induced behavioural changes during chronic cocaine administration: in vivo gene delivery with regulatable lentivirus. Eur J Neurosci 2004;19:1621–33. [PubMed: 15066158]

Bahler M, Greengard P. Synapsin I bundles F-actin in a phosphorylation-dependent manner. Nature 1987;326:704–7. [PubMed: 3104800]

Biala G, Betancur C, Mansuy I, Giros B. The reinforcing effects of chronic D-amphetamine and morphine are impaired in a line of memory-deficient mice overexpressing calcineurin. Eur J Neurosci 2005;21:3089–96. [PubMed: 15978018]

Borgland SL, Malenka RC, Bonci A. Acute and chronic cocaine-induced potentiation of synaptic strength in the ventral tegmental area: electrophysiological and behavioral correlates in individual rats. J Neurosci 2004;24:7482–90. [PubMed: 15329395]

Borlongan CV, Freeman TB, Scorcia TA, Sherman KA, Olanow WC, Cahill DW, Sanberg PR. Cyclosporine-A increases spontaneous and dopamine agonist-induced locomotor behavior in normal rats. Cell Transplant 1995;4:65–73. [PubMed: 7728335]

Cabib S, Castellano C, Cestari V, Filibeck U, Puglisi-Allegra S. D1 and D2 receptor antagonists differently affect cocaine-induced locomotor hyperactivity in the mouse. Psychopharmacology (Berl) 1991;105:335–9. [PubMed: 1839178]

Carlezon WA Jr, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ. Regulation of cocaine reward by CREB. Science 1998;282:2272–5. [PubMed: 9856954]

Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A 1988;85:5274–8. [PubMed: 2899326]

Donatsch P, Ryffel B. Pharmacokinetics of Cyclosporine in Toxicological Studies. Transplantation Proceedings 1986;28

Dunn J, Inderwies B, Licata S, Pierce R. Repeated administration of AMPA or a metabotropic glutamate receptor agonist into the rat ventral tegmental area augments the subsequent behavioral hyperactivity induced by cocaine. Psychopharmacology 2005;179:172–180. [PubMed: 15580483]

English JD, Sweatt JD. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J Biol Chem 1997;272:19103–19106. [PubMed: 9235897]

Ettenberg A, Pettit HO, Bloom FE, Koob GF. Heroin and cocaine intravenous self-administration in rats: mediation by separate neural systems. Psychopharmacology (Berl) 1982;78:204–9. [PubMed: 6296898]

Gerdjikov T, Beninger R. Differential effects of calcineurin inhibition and protein kinase A activation on nucleus accumbens amphetamine-produced conditioned place preference in rats. Eur J Neurosci 2005;22:697–705. [PubMed: 16101751]

Goeders NE, Smith JE. Cortical dopaminergic involvement in cocaine reinforcement. Science 1983;221:773–5. [PubMed: 6879176]

Greengard P, Allen P, Nairn A. Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron 1999;23:435–447. [PubMed: 10433257]

Greengard P, Valtorta F, Czernik AJ, Benfenati F. Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 1993;259:780–5. [PubMed: 8430330]

Hilfiker S, Benfenati F, Doussau F, Nairn A, Czernik A, Augustine G, Greengard P. Structural domains involved in the regulation of transmitter release by synapsins. J Neurosci 2005;25:2658–2669. [PubMed: 15758176]

Hotte M, Thuault S, Dineley KT, Hemmings HC Jr, Nairn AC, Jay TM. Phosphorylation of CREB and DARPP-32 during late LTP at hippocampal to prefrontal cortex synapses in vivo. Synapse 2007;61:24–28. [PubMed: 17068779]

Hu X, Ford K, White F. Repeated cocaine administration decreases calcineurin (PP2B) but enhances DARPP-32 modulation of sodium currents in rat nucleus accumbens neurons. Neuropsychopharmacology 2005;30

Huttner WB, Schiebler W, Greengard P, De Camilli P. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. J Cell Biol 1983;96:1374–88. [PubMed: 6404912]

Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR. Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron 1988;21:869–883. [PubMed: 9808472]

Iwata SI, Hewlett GH, Ferrell ST, Kantor L, Gnegy ME. Enhanced dopamine release and phosphorylation of synapsin I and neuromodulin in striatal synaptosomes after repeated amphetamine. J Pharmacol Exp Ther 1997;283:1445–52. [PubMed: 9400020]

Jovanovic J, Sihra T, Nairn A, Hemmings HJ, Greengard P, Czernik A. Opposing changes in phosphorylation of specific sites in synapsin I during Ca2+-dependent glutamate release in isolated nerve terminals. The Journal of Neuroscience 2001;21:7944–7953. [PubMed: 11588168]

Kalivas PW, Alesdatter JE. Involvement of N-methyl-D-aspartate receptor stimulation in the ventral tegmental area and amygdala in behavioral sensitization to cocaine. J Pharmacol Exp Ther 1993;267:486–95. [PubMed: 8229779]

Kalivas PW, Duffy P. D1 receptors modulate glutamate transmission in the ventral tegmental area. J Neurosci 1995;15:5379–88. [PubMed: 7623160]

Kalivas PW, Duffy P. Repeated cocaine administration alters extracellular glutamate in the ventral tegmental area. J Neurochem 1998;70:1497–502. [PubMed: 9523566]

Karler R, Calder LD, Chaudhry IA, Turkanis SA. Blockade of “reverse tolerance” to cocaine and amphetamine by MK-801. Life Sci 1989;45:599–606. [PubMed: 2671566]

Kincaid R, Balaban C, Billingsley M. Differential localization of calmodulin-dependent enzymes in rat brain: evidence for selective expression of cyclic nucleotide phosphodiesterase in specific neurons. Proc Natl Acad Sci U S A 1987;84:1118–22. [PubMed: 3029762]

Koob GF. Neural mechanisms of drug reinforcement. Ann N Y Acad Sci 1992;654:171–91. [PubMed: 1632582]

Liu J, Farmer J, Lane W, Friedman J, Weissman I, Schreiber S. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 1991;66:807–15. [PubMed: 1715244]

Liu QS, Pu L, Poo MM. Repeated cocaine exposure in vivo facilitates LTP induction in midbrain dopamine neurons. Nature 2005;437:1027–31. [PubMed: 16222299]

Mattson BJ, Bossert JM, Simmons DE, Nozaki N, Nagarkar D, Kreuter JD, Hope BT. Cocaine-induced CREB phosphorylation in nucleus accumbens of cocaine-sensitized rats is enabled by enhanced activation of extracellular signal-related kinase, but not protein kinase A. J Neurochem 2005;95:1481–94. [PubMed: 16219028]

McCreary AC, Marsden CA. Cocaine-induced behaviour: dopamine D1 receptor antagonism by SCH 23390 prevents expression of conditioned sensitisation following repeated administration of cocaine. Neuropharmacology 1993;32:387–91. [PubMed: 8497339]

Miserendino MJ, Nestler EJ. Behavioral sensitization to cocaine: modulation by the cyclic AMP system in the nucleus accumbens. Brain Res 1995;674:299–306. [PubMed: 7796110]

Nichols RA, Chilcote TJ, Czernik AJ, Greengard P. Synapsin I regulates glutamate release from rat brain synaptosomes. J Neurochem 1992;58:783–5. [PubMed: 1345942]

Nishi A, Bibb JA, Matsuyama S, Hamada M, Higashi H, Nairn AC, Greengard P. Regulation of DARPP-32 dephosphorylation at PKA- and Cdk5-sites by NMDA and AMPA receptors: distinct roles of calcineurin and protein phosphatase-2A. J Neurochem 2002;81:832–41. [PubMed: 12065642]

Reid MS, Hsu K Jr, Berger SP. Cocaine and amphetamine preferentially stimulate glutamate release in the limbic system: studies on the involvement of dopamine. Synapse 1997;27:95–105. [PubMed: 9266771]

Reith ME, Li MY, Yan QS. Extracellular dopamine, norepinephrine, and serotonin in the ventral tegmental area and nucleus accumbens of freely moving rats during intracerebral dialysis following systemic administration of cocaine and other uptake blockers. Psychopharmacology (Berl) 1997;134:309–17. [PubMed: 9438681]

Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 1987;237:1219–23. [PubMed: 2820058]

Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ. Cocaine self-administration appears to be mediated by dopamine uptake inhibition. Prog Neuropsychopharmacol Biol Psychiatry 1988;12:233–9. [PubMed: 3387585]

Rodd-Henricks ZA, McKinzie DL, Li TK, Murphy JM, McBride WJ. Cocaine is self-administered into the shell but not the core of the nucleus accumbens of Wistar rats. J Pharmacol Exp Ther 2002;303:1216–26. [PubMed: 12438546]

Saal D, Dong Y, Bonci A, Malenka R. Drugs of Abuse and Stress Trigger a Common Synaptic Adaptation in Dopamine Neurons. Neuron 2003;37:577–582. [PubMed: 12597856]

Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, Nestler EJ. Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. J Neurosci 1998;18:1848–59. [PubMed: 9465009]

Svenningsson P, Nairn AC, Greengard P. DARPP-32 mediates the actions of multiple drugs of abuse. Aaps J 2005;7:E353–60. [PubMed: 16353915]

Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res 1991;548:100–10. [PubMed: 1651140]

Ungless MA, Whistler JL, Malenka RC, Bonci A. Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. Nature 2001;411:583–7. [PubMed: 11385572]

Venton BJ, Seipel AT, Phillips PE, Wetsel WC, Gitler D, Greengard P, Augustine GJ, Wightman RM. Cocaine increases dopamine release by mobilization of a synapsin-dependent reserve pool. J Neurosci 2006;26:3206–9. [PubMed: 16554471]

Winder D, Sweatt J. Roles of serine/threonine phosphatases in hippocampal synaptic plasticity. Nature Reviews Neuroscience 2001;2:461–474.

Yu D, Luo J, Bu F, Zhang W, Wei Q. Effects of cyclosporin A, FK506 and rapamycin on calcineurin phosphatase activity in mouse brain. IUBMB Life 2006;58:429–33. [PubMed: 16801218]

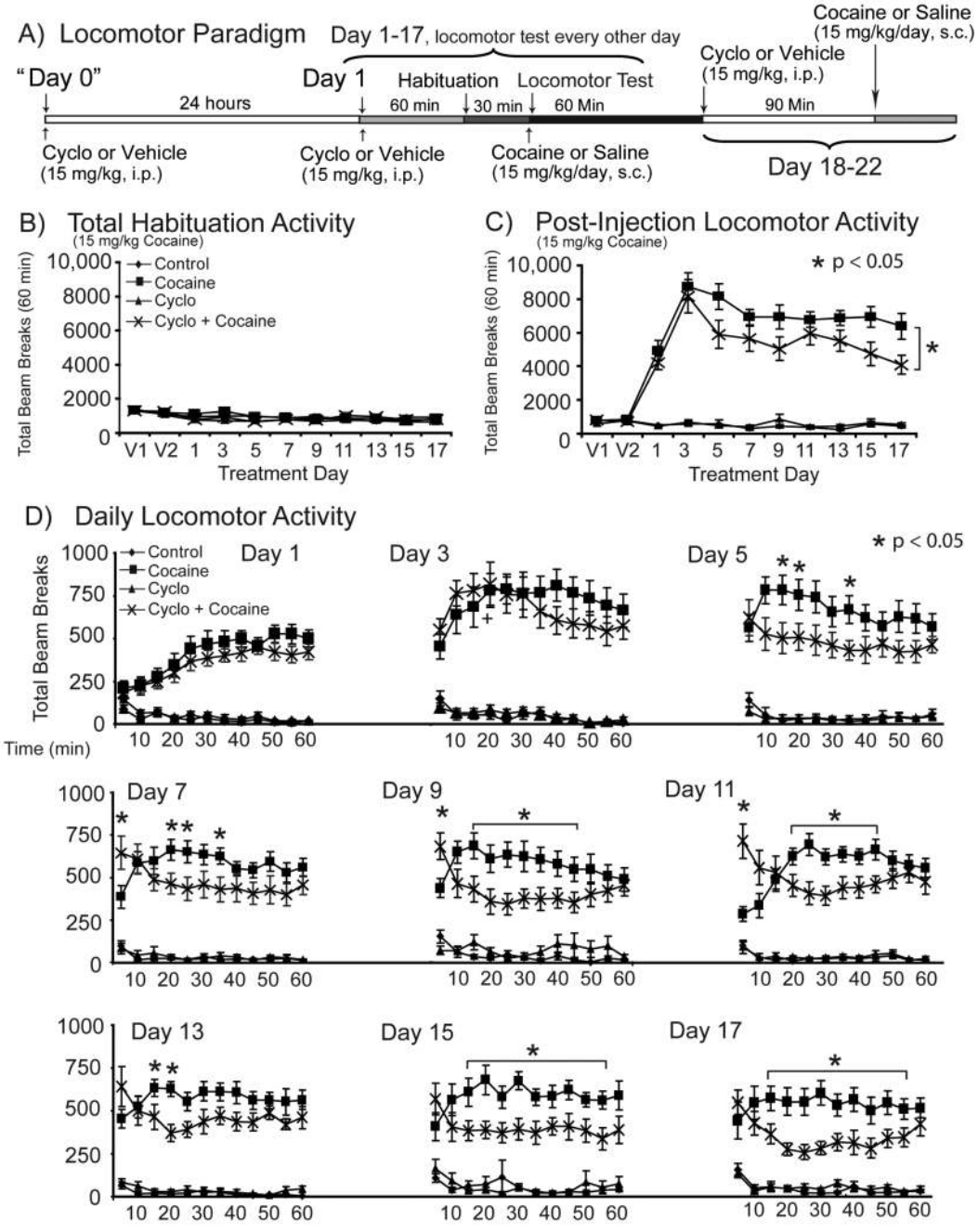

Figure 1. Repeated cyclosporine administration leads to biphasic modulation of cocaine-mediated locomotor sensitization

(A) Schematic of the locomotor testing paradigm for male Sprague-Dawley rats (n=48). **(B)** Chronic systemic cyclosporine administration did not alter baseline habituation activity during the 30 mins before cocaine administration. **(C)** Daily administration of cocaine at 15 mg/kg (s.c.) led to a sensitized and sustained locomotor response (main effect of cocaine, $p < 0.001$) that was attenuated by daily co-administration of cyclosporine (15 mg/kg, i.p, cocaine X cyclosporine interaction, $p < 0.05$). **(D)** Repeated cyclosporine administration (15 mg/kg, i.p.) led to peak LM activity 5 mins after injection and potentiated the locomotor response to repeated cocaine ($p < 0.05$, independent samples t-test with a Bonferroni correction) 5 mins

after injection on day 11 of testing. In contrast, repeated cyclosporine (15 mg/kg, i.p.) administration attenuated the sustained response to repeated cocaine as revealed by a cocaine X cyclosporine interaction on test days 9, 13 and 17 ($p < 0.05$). All data are displayed as the mean ± SEM.

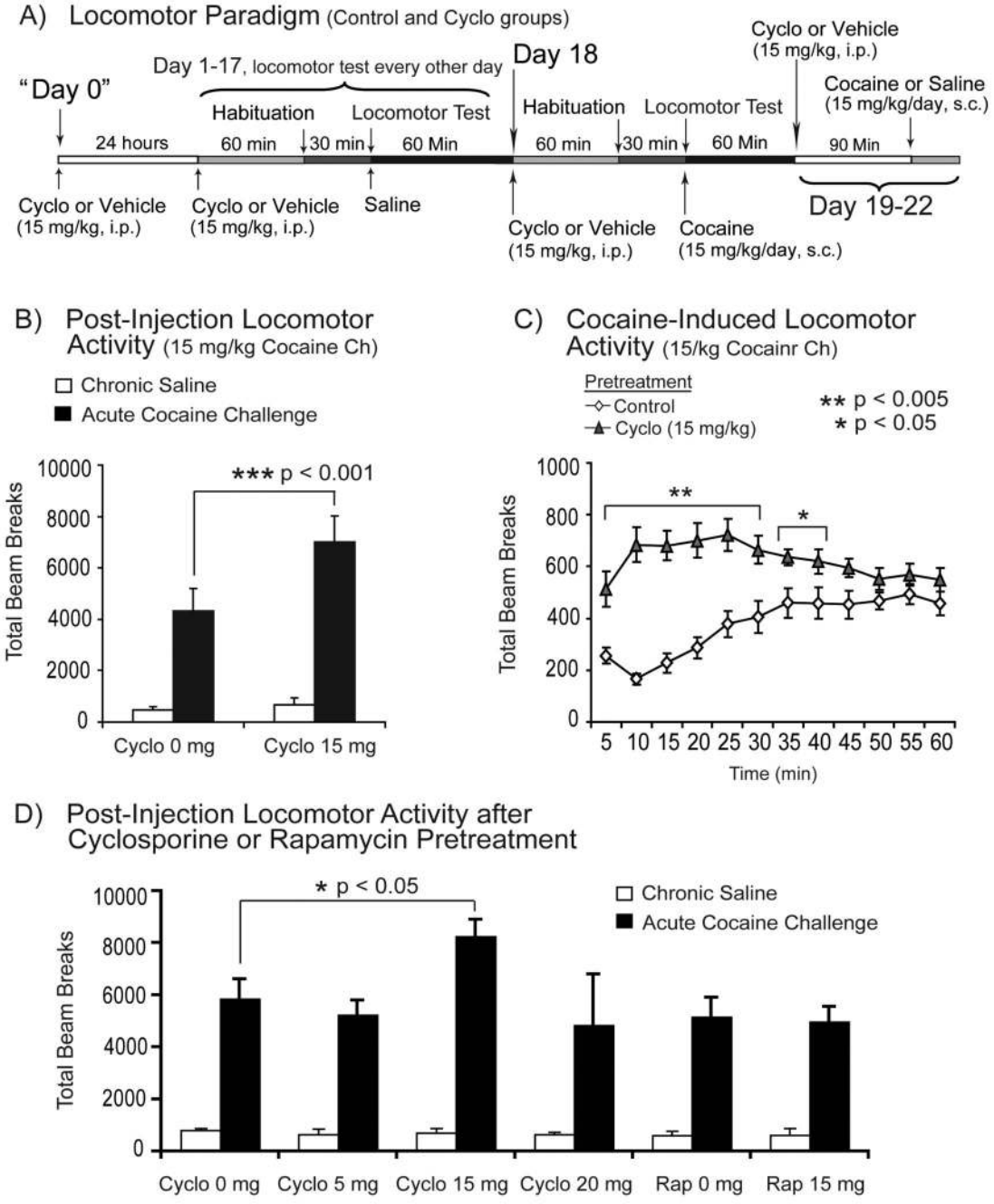

Figure 2. Chronic cyclosporine, but not rapamycin, pretreatment leads to a sensitized locomotor response to acute cocaine administration

(A) Schematic of locomotor testing paradigm for male Sprague-Dawley rats (n=24). **(B)** Acute cocaine administration on day 18 (15 mg/kg, s.c.) led to increased locomotor activity ($p < 0.005$, paired samples t-test), while animals pretreated with chronic cyclosporine showed potentiated locomotor activation in response to cocaine ($p < 0.001$, independent samples t-test) compared to saline vehicle pretreated animals. **(C)** Chronic cyclosporine pre-treatment led to a potentiated locomotor response to cocaine (15 mg/kg, s.c.) in the first 30 mins after injection ($p < 0.05$, independent samples t-test with a Bonferroni correction for multiple comparisons). **(D)** Chronic pre-treatment with 5 or 20 mg/kg cyclosporine or the immunophilin rapamycin

(15 mg/kg) did not affect the locomotor response to acute cocaine challenge. In contrast, animals pretreated with 15 mg/kg cyclosporine for 17 days showed a potentiated response to an acute 15 mg/kg cocaine challenge on day 18 ($p < 0.05$, independent samples t-test), consistent with results in the previous experiment (2B)Cyclo cyclosporine; Rap: rapamycin. All data are presented as the mean± SEM.

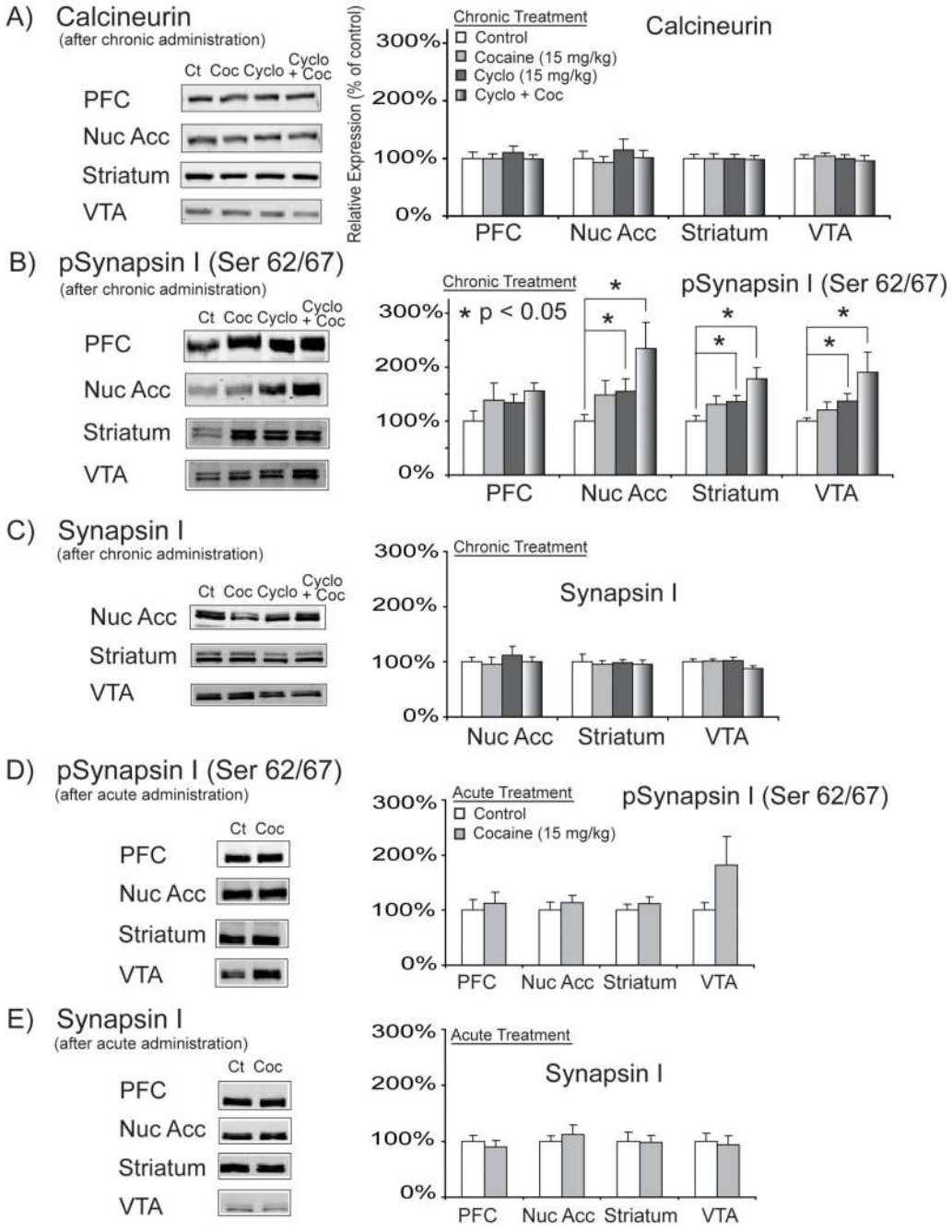

Figure 3. Chronic administration of cyclosporine or cocaine regulates synapsin I phosphorylation in subcortical brain structures

(A) Calcineurin levels in the PFC, NAc, striatum and VTA were not altered by chronic administration of cocaine (15 mg/kg, s.c.) or cyclosporine (15 mg/kg, i.p.). **(B)** Analysis of pSynapsin I levels in the NAc, ST and VTA revealed a main effect of cyclosporine administration as well as a main effect of cocaine administration ($p < 0.05$). In addition, animals that received either cyclosporine alone or co-administration of cocaine and cyclosporine showed increased pSynI (in the NAc, ST and VTA) compared to control treated animals ($p < 0.05$, independent samples t-test). **(C)** Chronic cocaine and cyclosporine administration did not alter total synapsin I levels in the NAc, striatum or VTA. **(D and E)** Acute cocaine

administration (15 mg/kg, s.c.) did not alter pSynI (S62/67) or total synapsin I in the PFC, NAc, striatum or VTA. All data are presented as mean± SEM.

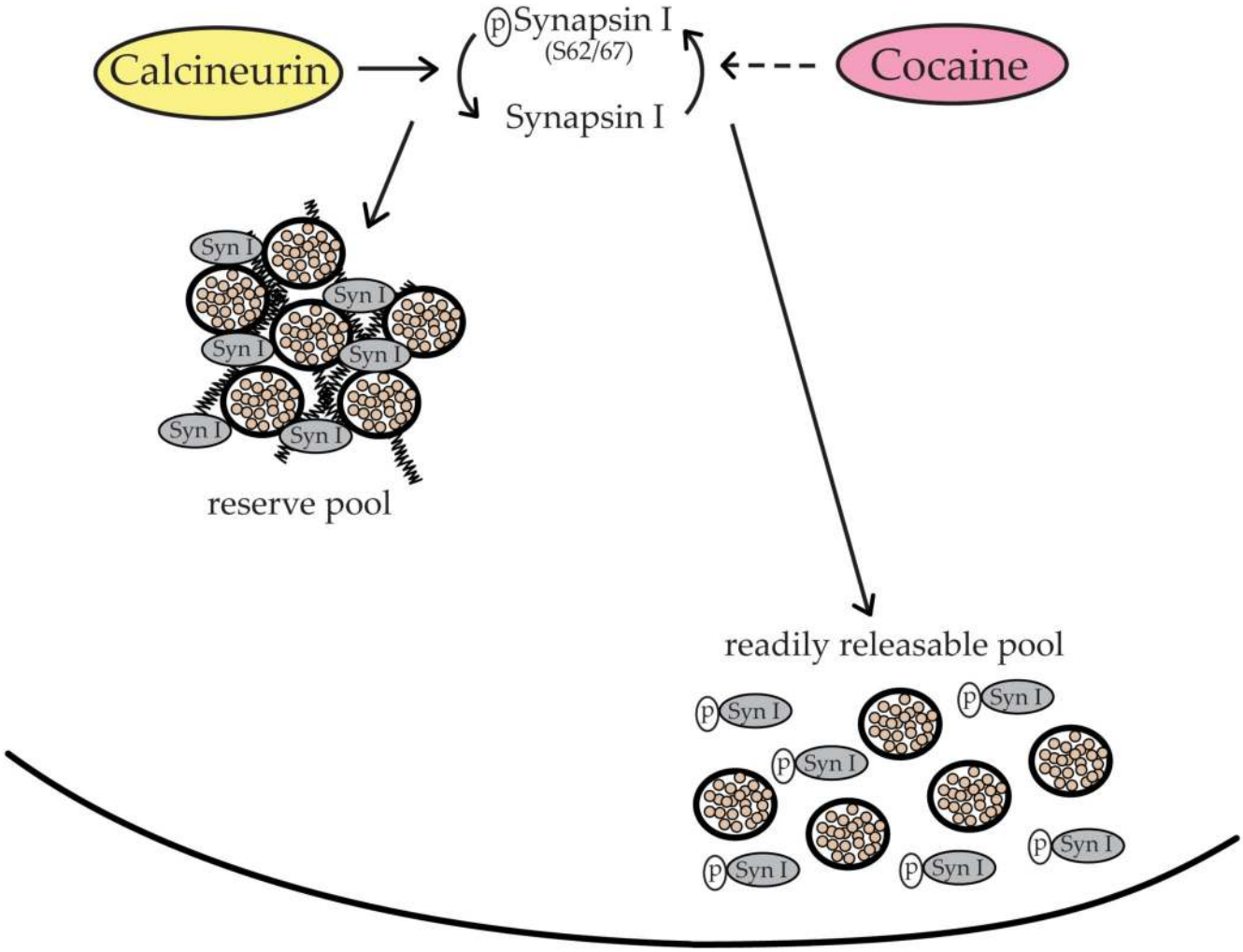

Figure 4. Model of a potential mechanism underlying calcineurin and cocaine effects on readily releasable pool

Calcineurin activity leads to dephosphorylation of the pre-synaptic protein, synapsin I at the calcineurin sensitive S62/67 site (Jovanovic et al. 2001). In its dephosphorylated form, synapsin I binds to both synaptic vesicles and the actin cytoskeleton (Bahler and Greengard 1987; Huttner et al. 1983). In the current work, we demonstrate that repeated cocaine exposure leads to increased synapsin I phosphorylation (Fig 3B), which in turn, would be expected to decrease its affinity for both actin and synaptic vesicles, thus releasing these vesicles from the actin cytoskeleton and allowing them to move to the readily releasable pool (Bahler and Greengard 1987; Greengard et al. 1993; Huttner et al. 1983).