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Augmented behavioral response and enhanced synaptosomal calcium transport induced by repeated cocaine administration are decreased by calcium channel blockers

K. Mills^a, T.A. Ansah^{a,*}, S.F. Ali^{a,b}, S. Mukherjee^a, and D.C. Shockley^a

^a Department of Pharmacology, Meharry Medical College, 1005 D.B. Todd Boulevard, Nashville, Tennessee 37208, USA

^b Neurochemistry Laboratory, Division of Neurotoxicology, National Center for Toxicological Research/FDA, 3900 NCTR Drive, Jefferson, Arkansas 72079-9502, USA

Abstract

Recent studies suggest that calcium influx via L-type calcium channels is necessary for psychostimulant-induced behavioral sensitization. In addition, chronic amphetamine upregulates subtype Cav1.2-containing L-type calcium channels. In the present studies, we assessed the effect of calcium channel blockers (CCBs) on cocaine-induced behavioral sensitization and determined whether the functional activity of L-type calcium channels is altered after repeated cocaine administration. Rats were administered daily intraperitoneal injections of either flunarizine (40 mg/kg), diltiazem (40 mg/kg) or cocaine (20 mg/kg) and the combination of the CCB's and cocaine for 30 days. Motor activities were monitored on Day 1, and every 6th day during the 30-day treatment period. Daily cocaine administration produced increased locomotor activity. Maximal augmentation of behavioral response to repeated cocaine administration was observed on Day 18. Flunarizine pretreatment abolished the augmented behavioral response to repeated cocaine administration while diltiazem was less effective. Measurement of tissue monoamine levels on Day 18 revealed cocaine-induced increases in DA and 5-HT in the nucleus accumbens. By contrast to behavioral response, diltiazem was more effective in attenuating increases in monoamine levels than flunarizine. Cocaine administration for 18 days produced increases in calcium-uptake in synaptosomes prepared from the nucleus accumbens and frontal cortex. Increases in calcium-uptake were abolished by flunarizine- and diltiazem-pretreatment. Taken together, the augmented cocaine-induced behavioral response on Day 18 may be due to increased calcium uptake in the nucleus accumbens leading to increased dopamine (DA) and serotonin (5-HT) release. Flunarizine and diltiazem attenuated the behavioral response by decreasing calcium uptake and decreasing neurochemical release.

Keywords

cocaine; sensitization; calcium channel blockers; motor activity; synaptosomes; rat

*Corresponding author: Department of Pharmacology, Meharry Medical College, 1005 D.B. Todd Blvd, Nashville, TN 37208, Tel: 615 327 6295, Fax: 615 327 6632, tansah@mmc.edu (T.A. Ansah).

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INTRODUCTION

Cocaine is a powerful psychostimulant known to be one of the most strongly reinforcing drugs of abuse. Cocaine binds to dopamine (DA), serotonin (5-HT) and norepinephrine (NE) transporters, blocking the reuptake and subsequent increase in extracellular monoamine levels in the neuronal terminal structures (Ritz et al., 1990; Andrews and Lucki, 2001). In humans, cocaine induces feelings of intense euphoria which often leads to dependence. In rodents, repeated cocaine administration produces the phenomenon called behavioral sensitization, manifested as progressive and enduring augmentation of locomotor activity in response to intermittent drug administration (Kalivas et al., 1998; Robinson and Berridge, 2003). Behavioral sensitization is believed to reflect drug-induced paranoia, craving and relapse (Kalivas et al., 1998; Robinson and Berridge, 2001). We and others have reported that L-type calcium channel blockers (CCBs) modulate a variety of cocaine-induced behaviors. For example, parenteral administration of CCBs impairs cocaine-stimulated locomotor activity (Mills et al., 1998; Pani et al., 1990a), cocaine self-administration (Kuzmin et al., 1992; Martellotta et al., 1994), cocaine-induced conditioned place preference (Pani et al., 1991; Calcagnetti et al., 1995) and behavioral sensitization to repeated cocaine administration (Karler et al., 1991; Reimer and Martin-Iverson 1994). In addition, it has been shown that direct infusion of CCBs into the ventral tegmental area (VTA) attenuates the development of psychostimulant-induced behavioral sensitization (Licata and Pierce 2003) and repeated microinjection of the calcium channel agonist BayK 8644 into the VTA resulted in an augmentation of the behavioral response to cocaine (Licata et al., 2000). L-type calcium channels also play a role in the augmented DA release observed during the expression of sensitized behavior (Pierce and Kalivas, 1997). These data suggest that calcium influx via L-type calcium channels is necessary for psychostimulant-induced behavioral sensitization (Licata and Pierce 2003). Consistent with this is the fact that chronic amphetamine upregulates subtype Cav1.2-containing L-type calcium channels, and the ability of the CCB, diltiazem to increase amphetamine-mediated phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in the DA neurons of the VTA (Rajadhyaksha et al., 2004). It is not known whether the upregulation of the Cav1.2-containing L-type calcium channels is accompanied by increase in the functional activity of the channels. In the present studies, we assessed the functional activity of L-type calcium channels by measuring synaptosomal Ca^{2+} transport after repeated cocaine administration and the effect of the CCB's flunarizine and diltiazem on $^{45}\text{Ca}^{2+}$ -uptake into synaptosomes. We also determined the effects of the CCB's on the augmented motor activity produced by subchronic cocaine administration. Moreover, monoamine analysis was done to determine if the augmented behavioral response to cocaine is accompanied by increases in monoamine levels in the nucleus accumbens, caudate nucleus and frontal cortex and whether the increased monoamine levels can be prevented by pretreatment with CCB's.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing between 250–300 grams were used. All animals were maintained on a 12 hour light/dark cycle. The number of animals used per group in the locomotor activity studies is 7–12. For monoamine analysis and $^{45}\text{Ca}^{2+}$ -uptake studies, 4 animals per group were used. All animal experiments were conducted in accordance with guidelines of the Institutional Care and Use Committee at Meharry Medical College, provided by the National Institutes of Health.

Behavioral Analysis

Motor activity (expressed as Total Distance) was measured using commercially available activity monitoring chambers (AccuScan Instruments, Columbus, OH). The chambers (41 ×

41 × 41 cm) which consisted of transparent Plexiglas cages with photocells and detectors interfaced to an IBM computer was used to determine distance traveled. Each animal was individually habituated to the activity cage for 30 minutes before a 60 minute test session. The calcium channel blockers (CCB) flunarizine (40 mg/kg) and diltiazem (40 mg/kg) or vehicle (1 ml/kg) were administered prior to the habituation period and cocaine (20 mg/kg) or saline (1 ml/kg) was injected before the test session. The treatment groups are: (1) DMSO + Saline; Flunarizine + Saline; DMSO + Cocaine; Flunarizine + Cocaine (2) Saline + Saline; Diltiazem + Saline; Saline + Cocaine; Diltiazem + Cocaine. All drugs were injected intraperitoneally (i.p.). Drug or vehicle was administered daily for a period of 30 days. Activity was monitored every 6th day during the 30 day period. The frequent monitoring of locomotor activity allowed us to monitor the development and progression of sensitization. At Day 18 and 30 time points, animals were sacrificed for neurochemical assays and ⁴⁵Ca²⁺-uptake. The doses of cocaine and CCB's were selected based on previous studies in our laboratory (Mills, 1998).

Monoamine Analysis

At the end of behavioral assessment, animals were decapitated and the brains were removed. The nucleus accumbens, caudate nucleus and frontal cortex were dissected and weighed. Neurochemical analyses were performed as described previously (Ali et al., 1996; Mills et al., 1998). The respective tissues were individually sonicated with cell disruptor (Smith Kline) in 20 times volume of 0.2N perchloric acid. The homogenate was centrifuged at 4°C for 7 minutes at 13,000 × g. A 150-μL aliquot of the supernatant was placed in a centrifugal 0.45-μM nylon-66 filter (Rainin Instrument, Woburn, MA) and recentrifuged at 4°C for 3.5 minutes. Twenty-five microliters of the sample was injected on the column for analysis using a high-performance liquid chromatography electrochemical detector (HPLC-ED; Bioanalytical System, W. Lafayette, IN.).

Preparation of Synaptosomes

Animals were pretreated with either vehicle or CCB as described in behavioral analysis. Sixty minutes after either cocaine (20mg/kg, i.p.) or vehicle administration, animals were decapitated and the brains were removed. The nucleus accumbens, caudate nucleus and frontal cortex were dissected and weighed. Tissues from the nucleus accumbens and caudate nucleus were combined and pooled with tissue from two animals. Tissues from the frontal cortex were similarly pooled from two animals. Synaptosomes were prepared according the method of Dodd and coworkers with minor modifications (Dodd et al., 1981). In brief, tissues were homogenized in 10 mL of ice cold buffer (0.25 M sucrose, 5 mM HEPES, 10 mM KCl, 1 mM MgCl₂, pH 7.05) using a glass homogenizer with a teflon pestle. All subsequent steps were done at 0–4°C. The crude homogenate was centrifuged at 1000g for 10 minutes. The pellet was discarded and the supernatant was taken and recentrifuged at 9000g. The pellet was suspended in 10 ml of 0.32 M sucrose and layered on 8 ml of 1.2 M sucrose. The suspension was centrifuged for 20 minutes at 45,000 rpm (Sorvall Ti 70 rotor, Dupont Co., Wilmington, DE.) and the interface layer was collected and diluted with 0.32 M sucrose to a final volume of 10 ml. The suspension was then layered on 8 ml of 0.8 M sucrose and centrifuged for 20 minutes at 45,000 rpm. The synaptosomal pellet was then resuspended in a total volume of 1 ml of 0.32 M sucrose and divided into two 0.5 ml aliquots for ⁴⁵Ca-uptake assay.

Calcium Uptake

Aliquots (0.1 ml) of the synaptosomal suspension were added to glass tubes containing 0.35 ml of NaCl buffer (136 mM NaCl, 5.6 mM KCl, 1.3 mM MgCl₂, 20 mM Tris HCl, 11 mM glucose, pH 7.4). The tubes were pre-incubated for 20 minutes on ice and for 10 minutes at 37°C in a shaking water bath. Uptake was initiated by adding 0.025 ml of ⁴⁵CaCl₂ (0.5 Ci/assay), in 1 M NaCl for non-depolarizing conditions and in 1 M KCl for depolarizing

conditions. Cold CaCl_2 (0.025 ml of 1.28 mM) was added concurrently and incubated for 40 seconds. Uptake was terminated by rapid filtration through GF-B fiber filters and washed with 5 ml of cold buffer (145 mM KCl, 1.2 mM CaCl_2 , 20 mM Tris HCl, pH 7.4). The amount of radioactivity on the filter disc was determined by liquid scintillation spectrometry. Protein concentrations were determined by the method of Lowry et al., (1951). Calcium uptake was expressed as cpm per mg protein.

Drugs

$^{45}\text{Ca}^{2+}$ ($^{45}\text{CaCl}_2$) was purchased from ICN Biomedicals, Inc. (Irvine, CA.). Cocaine hydrochloride, flunarizine hydrochloride, diltiazem hydrochloride and dimethylsulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO.).

Statistical Analysis

Data were expressed as the group means $\pm$ the standard error of the mean (S.E.M.). Temporal data of total distance were analyzed by a two-way Analysis of Variance (ANOVA) with repeated measures on drug treatment and time. A significant drug $\times$ time interaction was followed by Bonferroni post hoc test (GraphPad Software, San Diego, CA). Monoamine levels and calcium uptake data were subjected to a one-way ANOVA followed by Bonferroni post hoc test. Data were analyzed by using analysis of variance (ANOVA). A value of $p < 0.05$ indicates significance.

RESULTS

Behavioral analysis

In a separate set of experiments we determined that acute or subchronic treatment with the vehicle for flunarizine, DMSO had no effect on basal motor behavior. "Subchronic" refers to treatment period between a week (acute) and 90 days to a year (chronic). As shown in figure 1A, main effect of treatment was observed for locomotor activity ($F_{23,215} = 19.333$; $p < 0.0001$). Bonferroni post-hoc analysis revealed that daily pretreatment with flunarizine (40 mg/kg, i.p.) significantly attenuated basal locomotor activity (Fig. 1A). Flunarizine decreased basal locomotor activity on day 6 ($p < 0.001$), day 12 ($p < 0.01$), day 18 ($p < 0.01$), day 24 ($p < 0.01$) as compared to vehicle plus saline control (Fig. 1A). Cocaine (20 mg/kg, i.p.) produced increases in locomotor activity on all days tested (Fig. 1A). When compared to day 1, 18 days of cocaine administration produced maximal responses ($p < 0.001$) to locomotor behavior (Fig. 1A). Administration of cocaine for 18 days increased the total distance traveled from 3392 ± 1334 (day 1, cocaine treatment) to 16994 ± 2109 (day 18, cocaine treatment) (Fig. 1A). Pretreatment with flunarizine significantly attenuated cocaine-induced locomotor activity on day 6 ($p < 0.05$), day 12 ($p < 0.001$); day 18 ($p < 0.001$); and day 30 ($p < 0.05$); as shown in Fig. 1A. Pretreatment with flunarizine on day 18 was most effective in attenuating cocaine-induced activity. Pretreatment with flunarizine on day 18 attenuated cocaine-induced total distance traveled from a peak of 16994 ± 2109 to 4491 ± 1369 . Flunarizine appears to block cocaine-induced behavioral sensitization because flunarizine pretreatment decreased cocaine-induced locomotor activity to pre-sensitization cocaine response (Day 1). The effect of flunarizine on basal activity cannot account for its effects on cocaine-induced sensitization because flunarizine had no effect on cocaine-induced locomotor activity on Day 1 when sensitization to cocaine had not developed.

In fig 1B, the main effect of treatment was evident for locomotor activity ($F_{23, 213} = 13.337$; $p < 0.0001$). Diltiazem (40 mg/kg, i.p.) pretreatment decreased basal locomotor activity on day 12, ($p < 0.001$) as compared to respective day 12 saline controls (Fig. 1B). Cocaine administration produced a peak effect ($p < 0.05$) on day 18 of cocaine treatment (Fig. 1B).

Monoamine analysis

Figure 2A and 2B show the levels of DA, 5-HT, and the metabolites DOPAC, HVA and 5-HIAA in the nucleus accumbens on day 18. Cocaine treatment produced significant changes in the levels of monoamines ($F_{19,60} = 97.69$; $p < 0.0001$) when compared to saline treated animals. The levels of DA in the nucleus accumbens increased from 357.4 ± 29.7 to 496.3 ± 45.3 after cocaine (20 mg/kg, i.p.) administration ($p < 0.05$). Similarly, the levels of 5-HT increased from 66.8 ± 4.19 to 116 ± 4.4 ($p < 0.001$) after cocaine administration. Flunarizine (40 mg/kg, i.p.) pretreatment decreased basal DA ($p < 0.001$) and DOPAC levels ($p < 0.05$). Figure 2A also shows that flunarizine inhibited the increase in 5-HT ($p < 0.001$) but not DA levels induced by cocaine administration. On the other hand, diltiazem (40 mg/kg, i.p.) pretreatment attenuated ($F_{19,60} = 97.69$; $p < 0.0001$) cocaine-induced increases in both DA ($p < 0.001$) and 5-HT ($p < 0.01$) as depicted in Fig 2B.

Basal monoamine levels in the frontal cortex were not affected by either flunarizine or diltiazem pretreatment. Cocaine increased DA levels in the frontal cortex in both the flunarizine ($F_{19,60} = 13.864$; $p < 0.05$) (Fig. 3A) and the diltiazem ($F_{19,60} = 90.723$; $p < 0.0001$) (Fig. 3B) treated groups. Neither flunarizine nor diltiazem pretreatment significantly affected cocaine-induced increase in DA levels in the frontal cortex, although flunarizine treatment exhibited a decreasing trend toward cocaine-induced DA levels (Fig. 3A). As shown in Fig. 3B, diltiazem inhibited ($p < 0.05$) levels of 5-HIAA after cocaine treatment. Similarly, pretreatment with either flunarizine or diltiazem produced no significant changes in either basal or cocaine-evoked monoamine levels in the caudate nucleus (data not shown).

Calcium uptake studies

There was main effect of treatment ($F_{7,24} = 11.175$; $p < 0.0001$). Acute administration of flunarizine (40 mg/kg, i.p.) on Day 1 decreased ($p < 0.05$) Ca^{2+} -uptake into synaptosomes prepared from nucleus accumbens/caudate nucleus (Fig. 4A). Acute administration of cocaine (20 mg/kg, i.p.) on Day 1 did not affect $^{45}\text{Ca}^{2+}$ -uptake into synaptosomes prepared from nucleus accumbens/caudate nucleus (Fig. 4A). On Day 18, there was an increase ($p < 0.001$) in basal $^{45}\text{Ca}^{2+}$ -uptake in the nucleus accumbens/caudate nucleus (Fig. 4A). The increase in basal $^{45}\text{Ca}^{2+}$ -uptake could be attributed to the effect of DMSO since this effect was not observed when saline was used as the vehicle (Fig 4B). However, this assertion is not in agreement with the observation that in human erythrocytes, DMSO inhibits calcium transport (Romero, 1992). It is possible that the effects of DMSO are dependent on the cell type. Cocaine administration potentiated $^{45}\text{Ca}^{2+}$ -uptake on Day 18 ($p < 0.001$) (Fig. 4A). Flunarizine (40 mg/kg, i.p.) was effective in attenuating ($p < 0.001$) the increase in $^{45}\text{Ca}^{2+}$ -uptake produced by cocaine on Day 18 (Fig. 4A). Flunarizine pretreatment reduced $^{45}\text{Ca}^{2+}$ -uptake induced by cocaine in the nucleus accumbens/caudate nucleus by 83 % (Fig. 4A)

There was main effect of treatment in the diltiazem treatment group ($F_{7,24} = 43.843$; $p < 0.0001$). As shown in Fig. 4B, diltiazem (40 mg/kg, i.p.) pretreatment reduced ($p < 0.01$) $^{45}\text{Ca}^{2+}$ -uptake in the nucleus accumbens/caudate nucleus as compared to the saline controls on Day 1 and Day 18. Cocaine administration potentiated $^{45}\text{Ca}^{2+}$ -uptake on Day 18 ($p < 0.01$). Diltiazem pretreatment also effectively blocked (98% decrease) $^{45}\text{Ca}^{2+}$ -uptake ($p < 0.001$) induced by cocaine in synaptosomes prepared from nucleus accumbens/caudate nucleus.

Main effect of treatment was evident in the frontal cortex as well ($F_{7,24} = 24.437$ $p < 0.001$ Fig. 5A and $F_{7,24} = 67.116$; $p < 0.001$ Fig. 5B respectively). Cocaine administration significantly increased ($p < 0.01$) $^{45}\text{Ca}^{2+}$ -uptake in the frontal cortex on Day 18. Both flunarizine ($p < 0.05$) and diltiazem ($p < 0.01$) reduced cocaine-induced Ca^{2+} -uptake on Day 18 (Fig. 5A and 5B).

DISCUSSION

Our data provide three lines of evidence to suggest that voltage-dependent calcium channels play a role in the enhanced locomotor response to repeated cocaine administration. Firstly, using augmented behavioral response to subchronic cocaine on day 18 of daily administration as a behavioral end-point, the CCB flunarizine attenuated cocaine-induced behavioral effects. Secondly, increases in nucleus accumbens monoamine levels induced by cocaine was blocked by the CCB diltiazem. Thirdly, $^{45}\text{Ca}^{2+}$ -uptake was enhanced in synaptosomes prepared from the striatum and frontal cortex of rats treated for 18 days with cocaine. This enhanced Ca^{2+} -uptake was abolished by pretreatment with the CCB's. The major contribution of this study is that we demonstrated that repeated cocaine administration produces behavioral sensitization and increases $^{45}\text{Ca}^{2+}$ -uptake, both of which are sensitive to the CCB flunarizine. The observed cocaine-induced increase in functional activity of the L-type Ca^{2+} channels is in agreement with studies showing upregulation of Cav1.2-containing L-type calcium channels after chronic amphetamine administration (Rajadhyaksha et al., 2004). The doses of cocaine and CCBs were selected based on previous studies in our laboratory (Mills, 1998) and are in the same order of magnitude as others used by other investigators (Karler et al., 1991; Pani et al., 1990a). However the authors acknowledge the limitations of using single doses of drugs in this study. Differences between the cocaine-induced increases in DA levels in the DILT group and the FLU group (Fig 2A and 2B) as well as the huge differences in cocaine-induced Ca^{2+} -uptake in the nucleus accumbens in the FLU experiment compared to the DILT experiment (Fig 4A and 4B) are a concern and may have made our findings much less compelling. However, we do not think they had a major impact on our overall finding because the changes in the respective groups were qualitatively similar despite the obvious differences in magnitude of the effects.

Behavioral sensitization is a phenomenon whereby responses increase in magnitude to repeated administration of a drug at the same dose (Kalivas et al., 1998; Robinson and Berridge, 2003) and has been reported to be commonly induced by administration of single daily doses of cocaine followed by a withdrawal period and then a challenge phase in which a single "challenge" dose of cocaine is administered. There are many studies which report that repeated administration of cocaine produces behavioral sensitization (Beyer and Steketee, 1999; Bhargava et al., 1997; Bohn et al., 2003; Henry et al., 1995; Izenwasser and French, 2002; Martin-Iverson et al., 1995; Polston et al., 2006; Post et al., 1976). On the other hand continuous infusion of cocaine produces tolerance (Izenwasser and French, 2002; King et al., 1999; Kunko et al., 1998). Although continuous infusion of cocaine has been shown to produce tolerance, behavioral sensitization can develop following intermittent cocaine administration even in absence of a withdrawal period (Ansah et al., 1996; Bhargava et al., 1997; Polston et al., 2006; Post et al., 1976). In our studies, daily administration of a moderate dose of cocaine (20 mg/kg, i.p.) produced behavioral sensitization to locomotor activity on each day that activity was monitored up to day 18, although the increases observed were not sustained throughout the 30 day testing paradigm. Similarly, Polston et al (2006) induced behavioral sensitization in rats by administration of 10 and 20 mg/kg, i.p. cocaine daily for 12 consecutive days and testing for sensitization on Day 13. Bhargava and Kumar (1997) also reported inducing behavioral sensitization in mice after subchronic administration of cocaine. The pattern of motor activity observed is consistent with that generated previously in our laboratory after daily intermittent cocaine (20 mg/kg, i.p.) administration (Ansah et al., 1996). Sensitization dropped off after Day 18, possibly due to tolerance. This observation is at odds with the general notion that tolerance typically develops after continuous drug infusion (Izenwasser and French, 2002; King et al., 1999; Kunko et al., 1998). Treatment with cocaine over the extended period of time (30 days) in our studies may have contributed to the development of tolerance after Day 18 of treatment.

Flunarizine was more effective in attenuating the augmented locomotor response elicited by cocaine on day 18 (Fig. 1A) than diltiazem (Fig. 1B). Flunarizine inhibited cocaine-induced locomotor activity by 73% on day 18 whereas diltiazem inhibited only 43% of cocaine-induced locomotor activity. On the other hand, the same dose of diltiazem significantly decreased cocaine-induced increases in DA levels (45% decrease) whereas flunarizine was not effective (20% decrease). Thus the CCB's had differential effects on cocaine-stimulated behavior and cocaine-induced DA metabolism. We and others have previously reported that acute administration of L-type CCB's belonging to different groups (i.e. 1–4 dihydropyridine, phenylalkylamine, benzothiazepine) may have differential affects on cocaine-induced behaviors (Ansah et al., 1993; Pani et al., 1990a; 1990b; 1990c). In addition, previous studies in our laboratory have identified differences in the magnitude of inhibition of acute cocaine-induced locomotor behavior by pretreatment with various CCB's (i.e. isradipine, nicardipine, and verapamil) (Mills et al., 1994). Pani and coworkers (Pani et al. 1990a) reported that nimodipine and isradipine inhibited cocaine-induced motor stimulation while flunarizine potentiated motor stimulation induced by cocaine. In contrast to the findings by Pani's group our results found no effects of acute (ie. day 1) flunarizine pretreatment on cocaine-induced motor activity (Fig. 2A and 2B). Flunarizine significantly attenuated the locomotor effects of cocaine on day 18 of a 30 day test period. The differences observed between our findings and those of Pani and coworkers could be due to the route of administration and/or dose of flunarizine tested. For example, Pani's group used a dose of 20 mg/kg of flunarizine administered subcutaneously. In our study, a dose of 40 mg/kg of flunarizine administered intraperitoneally was used. Furthermore Pani's group used a dose of 10 mg/kg cocaine (s.c) while we used 20 mg/kg (i.p.).

Subchronic, 18 day cocaine administration did not increase monoamine levels in the caudate nucleus, but produced significant increases in DA and 5-HT levels in the nucleus accumbens (Fig. 2A and 2B). Similarly, DA levels as well as the metabolites DOPAC, HVA, and 5-HIAA in the frontal cortex were augmented by cocaine administration (Fig. 3A and 3B). Previously we have reported that acute cocaine produces increases in DA and 5-HT in the rat nucleus accumbens and the caudate nucleus although the increases observed were not statistically significant (Mills et al., 1998). It appears that under acute conditions the cocaine-induced increases in monoamine levels are transient and that our previous studies using one time point (ie. Day 1) measurement may have missed the peak effects. We do show however in the present studies that following 18 day treatment with cocaine, the augmented behavioral response is accompanied by increases in DA and 5-HT in the nucleus accumbens – a site critical for the behavioral effects of cocaine (Broderick et al., 1993; Reith et al., 1997). It is well documented that both behavioral modification and addictive properties of cocaine result mainly from its binding to the DA transporter (DAT) and inhibition of DA reuptake, thus elevating extracellular DA concentration and prolonging signaling at synapses (Amara and Sonders, 1998). In vivo imaging studies in humans and in vitro studies in post-mortem human brain have demonstrated that chronic cocaine abuse results in increase in DAT-binding site and DAT function (Malison et al., 1998; Mash et al., 2002). The increased neuroadaptive changes in DAT function following chronic cocaine could alter DA metabolism and DA tissue content.

L-type calcium channels are localized in CNS areas with a high density of synaptic connections (Gould et al., 1984) and are involved in neurotransmitter release (Snyder et al., 1983). Perfusion of the L-type CCB verapamil or nicardipine into the rat striatum has been shown to produce a dose-dependent decrease of DA in the microdialysis perfusate. Previously we have reported that acute administration of isradipine, an L-type CCB decreased cocaine-induced DA and 5-HT in both the nucleus accumbens and the caudate nucleus in rats (Mills et al., 1998). Daily pretreatment with flunarizine for 18 days did not significantly decrease tissue DA levels while significantly attenuating tissue 5-HT levels in the nucleus accumbens after cocaine administration. Diltiazem on the other hand, attenuated both DA and 5-HT levels in the nucleus

accumbens of rats in our study. Neither flunarizine nor diltiazem was effective in attenuating subchronic cocaine-induced increases in DA or 5-HT levels in the caudate nucleus or the frontal cortex. The fact that at Day 18, the CCB's decreased cocaine augmented behavioral response and also blocked increases in tissue monoamine levels suggest that the CCB's act centrally, especially in the nucleus accumbens to modulate behavioral response to cocaine.

Calcium uptake has been shown to be fundamental in the process of release of monoamines. For example, it has been demonstrated in rat neurons that an increase in intracellular calcium results in a subsequent increase in neurotransmitter release (Hurd et al., 1989). Furthermore studies in rat brain synaptosomes have demonstrated that CCB's inhibit calcium uptake (Turner and Goldin, 1985; White and Bradford, 1986; Dunn, 1988) and neurotransmitter release (Blaustein, 1975; Raiteri and Levi, 1978). Cocaine administration for 18 days produced increases in $^{45}\text{Ca}^{2+}$ -uptake in synaptosomes prepared from rat nucleus accumbens/caudate nucleus and frontal cortex. Pretreatment with flunarizine and diltiazem prevented the increases in $^{45}\text{Ca}^{2+}$ -uptake in synaptosomes prepared from both the nucleus accumbens/caudate nucleus and frontal cortex. Taken together, the augmented cocaine-induced behavioral response on Day 18 may be due to increased Ca^{2+} -uptake in the nucleus accumbens leading to increased neurotransmitter release. Flunarizine and diltiazem may attenuate the behavioral response by decreasing calcium uptake and decreasing neurochemical release.

Evidence has accumulated that some classic L-type CCB's may also inhibit T-type calcium channels in neurons (Stengel et al., 1998). Flunarizine and diltiazem have been reported to block both L-type and T-type calcium channels in cultured smooth muscle cells although flunarizine was reported to be a more effective T-type blocker than diltiazem (Kuga et al., 1990). The prevailing view is that L-type calcium channels play an important role in the initiation of behavioral sensitization to cocaine. Systemic injections of CCBs block the initiation of behavioral sensitization to cocaine (Reimer and Martin-Iverson, 1994). Moreover, repeated intra-VTA administration of the L-type calcium channel agonist BayK 8644 cross-sensitizes to a subsequent challenge injection of cocaine (Licata et al., 2000). It has been suggested that calcium influx into VTA neurons via L-type calcium channels is enhanced during the development of behavioral sensitization leading to persistent activation of calcium/calmodulin-stimulated kinases which are known to mediate behavioral plasticity (Licata and Pierce, 2003). The increased Ca^{2+} -uptake following repeated cocaine administration could be due to increased protein level of the L-type calcium channels. For example, it has been shown that chronic amphetamine treatment increases the mRNA and protein expression of the L-type Ca^{2+} channel subunit Cav1.2 (Rajadhyaksha et al., 2004). Our functional data showing that the enhanced synaptosomal calcium transport following repeated cocaine administration is blocked by CCBs may provide additional evidence for the role of CCBs in the behavioral effects of cocaine.

CONCLUSION

In the present studies, we have shown that cocaine-induced motor behavior and synaptosomal calcium uptake are increased following repeated cocaine administration. These effects are attenuated by treatment with CCBs and provide further evidence that L-type calcium channels play an important role in the initiation of behavioral sensitization to cocaine.

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Figure 1.

Effect of daily flunarizine and diltiazem pretreatment on cocaine-induced motor activity in rats during a 30 day treatment period. Total Distance is expressed as mean $\pm$ S.E.M. for $n=7-12$ rats per group. **A.** Animals were pretreated daily with flunarizine (40 mg/kg, i.p.). Thirty minutes after pretreatment animals were given a daily cocaine (20 mg/kg, i.p.) challenge and motor activity was monitored for 60 minutes every 6th day. * represents significance at $p<0.001$ as compared to dmso+coc on day 1. # represents significance at $p<0.05$ as compared to respective daily dmso+coc values, ## represents significance at $p<0.001$ as compared to respective daily dmso+coc values, ^ represents significance at $p<0.01$ as compared to respective daily dmso+sal values, and ^^ represents significance at $p<0.001$ as compared to respective

daily dms+sal values. **B.** Animals were pretreated daily with diltiazem (40 mg/kg, i.p.). Thirty minutes after pretreatment animals were given a daily cocaine (20 mg/kg, i.p.) challenge and motor activity was monitored for 60 minutes every 6th day. *represents significance at $p<0.05$ as compared to sal+coc on day 1, ^represents significance at $p<0.001$ as compared to Day 12 sal+sal value.

NUCLEUS ACCUMBENS

Figure 2.

A. Effect of daily flunarizine pretreatment on tissue monoamine levels in the nucleus accumbens after 18-day cocaine treatment. On each day, animals were either pretreated with flunarizine (40 mg/kg, i.p.) or dmsal vehicle (1 ml/kg, i.p.). After 30 minutes animals were administered either cocaine (20 mg/kg, i.p.) or saline (1 ml/kg, i.p.). One hour after cocaine or saline injection on day 18, the nucleus accumbens was dissected and analyzed for dopamine (DA), serotonin (5-HT) and metabolites DOPAC, HVA, and 5-HIAA. Data are expressed as mean $\pm$ S.E.M. for $n=4$ rats per group. * represents significance at $p<0.05$ as compared to dmsal+sal values, ** represents significance at $p<0.001$, as compared to dmsal+sal values, # represents significance at $p<0.001$ as compared to dmsal+coc values, ^ represents significance at $p<0.05$

as compared to dms0+sal values, and ^{^^}represents significance at $p<0.001$ as compared to dms0+sal values.

B. Effect of daily diltiazem pretreatment on extracellular monoamine levels in the nucleus accumbens produced by 18-day cocaine treatment. On each day, animals were either pretreated with diltiazem (40 mg/kg, i.p.) or saline (1 ml/kg, i.p.). After 30 minutes animals were administered either cocaine (20 mg/kg, i.p.) or saline (1 ml/kg, i.p.). One hour after cocaine or saline injection on day 18, the nucleus accumbens was dissected and analyzed for dopamine (DA), serotonin (5-HT) and metabolites DOPAC, HVA, and 5-HIAA. *represents significance at $p<0.05$ as compared to sal+sal values, **represents significance at $p<0.001$, as compared to sal+sal values, #represents significance at $p<0.01$ as compared to sal+coc values, and ##represents significance at $p<0.001$ as compared to sal+coc values.

Figure 3.

A. Effect of daily flunarizine pretreatment on tissue monoamine levels in the frontal cortex after 18-day cocaine treatment. On each day, animals were either pretreated with flunarizine (40 mg/kg, i.p.) or dmsal vehicle (1 ml/kg, i.p.). After 30 minutes animals were administered either cocaine (20 mg/kg, i.p.) or saline (1 ml/kg, i.p.). One hour after cocaine or saline injection on day 18, the frontal cortex was dissected and analyzed for dopamine (DA), serotonin (5-HT) and metabolites DOPAC, HVA, and 5-HIAA. Data are expressed as mean $\pm$ S.E.M. for $n=4$ rats per group. * represents significance at $p<0.05$ as compared to dmsal+sal values.

B. Effect of daily diltiazem pretreatment on extracellular monoamine levels in the frontal cortex produced by 18-day cocaine treatment. On each day, animals were either pretreated with

diltiazem (40 mg/kg, i.p.) or saline (1 ml/kg, i.p.). After 30 minutes animals were administered either cocaine (20 mg/kg, i.p.) or saline (1 ml/kg, i.p.). One hour after cocaine or saline injection on day 18, the frontal cortex was dissected and analyzed for dopamine (DA), serotonin (5-HT) and metabolites DOPAC, HVA, and 5-HIAA. * represents significance at $p < 0.05$ as compared to sal+sal values, ** represents significance at $p < 0.001$, as compared to sal+sal values, and # represents significance at $p < 0.05$ as compared to sal+coc values.

NUCLEUS ACCUMBENS/CAUDATE NUCLEUS

Figure 4.

A. Synaptosomal $^{45}\text{Ca}^{2+}$ uptake in the nucleus accumbens/caudate nucleus of rats receiving daily treatment with flunarizine or cocaine in the presence of flunarizine for 18 days. On each day, animals were pretreated with either flunarizine (40 mg/kg, i.p.) or dms0 (1 ml/kg, i.p.). After 30 minutes animals were administered either cocaine (20 mg/kg, i.p.) or saline (1 ml/kg, i.p.) and tissue was collected as described in methods. Data are expressed as mean $\pm$ S.E.M for N=4 rats per group. @represents significance at $p<0.001$ as compared to respective Day 1 value, **represents significance at $p<0.001$ as compared to dms0+sal on Day 18, #represents significance at $p<0.001$ as compared to dms0+coc on Day 18, and ^represents significance at $p<0.05$ as compared to dms0+sal on Day 1. **B.** Synaptosomal $^{45}\text{Ca}^{2+}$ uptake in the nucleus

accumbens/caudate nucleus of rats receiving daily treatment with diltiazem or cocaine in the presence of diltiazem for 18 days. On each day, animals were pretreated with either diltiazem (40 mg/kg, i.p.) or saline (1 ml/kg, i.p.). After 30 minutes animals were administered either cocaine (20 mg/kg, i.p.) or saline (1 ml/kg, i.p.) and tissue was collected as described in methods. Data are expressed as mean $\pm$ S.E.M for N=4 rats per group. * represents significance at $p<0.01$ as compared to sal+sal on Day 18, # represents significance at $p<0.001$ as compared to sal+coc on Day 18, and ^ represents significance at $p<0.01$ as compared to sal+sal. Note the difference in scales between the two figures.

Figure 5.

A. Synaptosomal $^{45}\text{Ca}^{2+}$ uptake in the frontal cortex of rats receiving daily treatment with flunarizine or cocaine in the presence of flunarizine for 18 days. On each day, animals were pretreated with either flunarizine (40 mg/kg, i.p.) or dmsal (1 ml/kg, i.p.). After 30 minutes animals were administered either cocaine (20 mg/kg, i.p.) or saline (1 ml/kg, i.p.) and tissue was collected as described in methods. Data are expressed as mean $\pm$ S.E.M for N=4 rats per group. [@]represents significance at $p < 0.01$ as compared to respective Day 1 value, ^{*} represents significance at $p < 0.01$ as compared to dmsal+sal on Day 18 and [#] represents significance at $p < 0.05$ as compared to dmsal+coc on Day 18.

B. Synaptosomal $^{45}\text{Ca}^{2+}$ uptake in the frontal cortex of rats receiving daily treatment with diltiazem or cocaine in the presence of diltiazem for 18 days. On each day, animals were pretreated with either diltiazem (40 mg/kg, i.p.) or saline (1 ml/kg, i.p.). After 30 minutes animals were administered either cocaine (20 mg/kg, i.p.) or saline (1 ml/kg, i.p.) and tissue was collected as described in methods. Data are expressed as mean $\pm$ S.E.M for N=4 rats per group. * represents significance at $p<0.01$ as compared to sal+sal on Day 18, # represents significance at $p<0.01$ as compared to sal+coc on Day 18, and ^ represents significance at $p<0.01$ as compared to sal+sal.