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Increased accumbens Cdk5 expression in rats after short-access to selfadministered cocaine, but not after long-access sessions

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Abstract

Upregulation of cyclin-dependent kinase 5 (Cdk5) after chronic cocaine administration has led to speculation that Cdk5 plays an important role in drug addiction. However, as Cdk5 involvement is implicated in a variety of neural events, including neuronal development, synaptic plasticity and learning, a specific role in drug abuse is yet to be determined. The present study utilized cocaine self-administration and food-reinforced operant procedures to assess possible relationships between cocaine intake, food-reinforced operant responding, behavioral activity, and Cdk5 levels in the nucleus accumbens (NAcc), ventral tegmental area (VTA), and prefrontal cortex (PFC) in rats. In Experiment 1, animals undergoing daily cocaine self-administration (1-hr/30 days) or food-reinforced operant sessions (20-min/30 days) showed significant between-group differences in operant responding and behavioral activity, but no significant differences in NAcc, VTA or PFC Cdk5 levels compared to a handled control group. In Experiment 2, animals that had self-administered cocaine in ten daily 1-hr sessions (Short-Access Cocaine) showed significantly greater NAcc Cdk5 expression compared to an Unhandled Control group, and no evidence of cocaine-induced behavioral sensitization. Animals given 4-hr daily access to cocaine over the same number of sessions (Long-Access Cocaine) showed significantly enhanced cocaine-reinforced responding and locomotor activation by the end of the sessions, but no significant differences in Cdk5 expression compared to Control animals. These findings suggest that overexpression of Cdk5 may be a transient adaptation to cocaine experience that subsides with increased cocaine exposure and does not correspond with measures of cocaine-induced behavioral sensitization.

Keywords

nucleus accumbens; locomotor activity; cocaine self-administration; food-reinforced operant responding; cyclin-dependent kinase 5

INTRODUCTION

Cyclin-dependent kinase 5 (Cdk5) is a serine threonine kinase protein involved in several activities critical for normal brain function, including neuronal migration, synaptic plasticity and learning [2,4,6]. Recently, Cdk5 has received attention for a potential role in drug addiction. Support for this role comes from Cdk5's identification as a downstream target of Δ FosB, a

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transcription factor whose upregulation has been proposed to be an important molecule in addiction [16]. As the transcription factor is elevated with repeated use of drugs of addiction [10], it has been suggested that the regulated gene products have a role in addiction mechanisms [15]. Indeed, Cdk5 has been shown to be upregulated from cocaine administration and Δ FosB over-expression in transgenic mice [3]. Additional *in vivo* evidence implicates Cdk5 involvement in drug reward, including studies showing upregulation in the ventral tegmental area (VTA) of rats [14] and downregulation in other areas of the brain [14,21] after cocaine use. In addition, Cdk5 has been shown to be necessary for plasticity in the nucleus accumbens (NAcc), as cocaine-induced increases in dendritic spine density are blocked by administration of a Cdk5 antagonist [17].

Though there are several mechanistic differences between natural and drug rewards in the brain, there is considerable evidence that drugs of abuse activate endogenous pathways that exist to influence behavior directed towards food and other stimuli of biological salience [13]. For instance, DA antagonists reduce sucrose intake [11], DA agonists increase water intake [22] and male sexual function is dependent upon DA neurotransmission [7,12].

Determining an exclusive role of Cdk5 in drug-induced adaptations would demonstrate a clear divergence between mechanisms of drug and natural rewards, and could establish increased Cdk5 levels as a molecular indication of drug abuse and addiction processes. However, similarities between natural and drug reward raise questions as to whether Cdk5 upregulation is exclusive to the drug experience, or if it's involvement is common to reinforcement and/or learning processes. Indeed, NAcc Cdk5 expression was found to be comparable after withdrawal from either cocaine or sucrose reinforcement [14].

Experiment 1 was undertaken to compare Cdk5 expression between control animals and those with chronic cocaine- or food-reinforced operant experience. Experiment 2 was performed to determine potential relationships between cocaine intake levels and Cdk5 expression.

MATERIALS AND METHODS

Animals

39 male Sprague-Dawley rats (bred at The University of Texas Animal Resource Center) weighing 200-250 g at the start of the experiment were used in this experiment. Animals were housed under a reversed 12-hour light/dark schedule (lights on at 8:00 pm) with food and water available *ad libitum*. Prior to surgery animals were group-housed. After surgery was performed in each experiment, all animals were individually housed.

Apparatus

Operant sessions were conducted in Plexiglas chambers (28 x 22 x 21 cm) within sound-attenuating boxes (Med Assoc., St. Albans, VT). Chambers contained a single retractable lever on the right wall, with a stimulus light directly above the lever and a house light on the opposite wall. Three pairs of photobeams, located in the center of the chamber and 5 cm from each of the chamber walls, were used to record locomotor activity through beam breakages. Cocaine infusions were delivered via 10 ml syringe positioned in a pump and connected by tubing to a single-channel swivel mounted on a counterbalanced arm above each chamber. Infusion tubing from the swivel was connected to the animal using a spring-covered tether that screwed into the jugular catheter endpiece located on the animal's head (Plastics One, Roanoke, VA). A MED P4 Intel computer system using MED-PC Software (Med Assoc, St. Albans, VT) was used to control self-administration program and collect lever response and locomotor activity data.

Handling and food training

With exception of non-handled control group in Exp 2, animals were handled daily to habituate them to experimental procedures over a two-week period for 10 min/day. Following the handling period, animals were food restricted and trained in operant chambers to lever press for 45 mg sucrose pellets (Research Diets, New Brunswick, NJ) on a fixed ratio-1 (FR-1) schedule of reinforcement. Operant training continued until each animal pressed the lever at least 40 times in a 10-min period for one week.

Surgery

A subset of the food-trained animals was implanted with an intravenous silastic jugular catheter as previously described [18] and given at least one week to recover post surgery before any testing procedures were done. (Catheters were checked twice daily for leaks and blockages by infusing a heparin/saline solution through the tubing. If catheter patency was in question, intravenous brexival sodium was infused to test for immediate anesthesia.)

Experiment 1

Groups consisted of Cocaine, Food and a Handled Control. The Food (n=5) and Cocaine (n=5) groups were both operant-trained but only the Cocaine group was surgically prepared, as described above. The Handled Control animals (n=5) were handled for ten min daily for the duration of the experiment, but did not undergo operant conditioning or surgery.

Food and Cocaine-Reinforced Operant Sessions

Cocaine and Food groups underwent daily sessions for a total of 30 days (6 days/week). For each session, animals were placed in the operant chambers and could lever press for intravenous (i.v.) cocaine (0.75 mg/kg/inj; Cocaine) or food (45 mg sucrose pellets; Food) on a FR-1 schedule of reinforcement. For the Cocaine group, each cocaine injection was in a volume of 0.1 ml infused over a 6 sec period, during which time the stimulus lamp was illuminated. After each infusion, there was a 20-s timeout during which the lever was retracted. Cocaine self-administration sessions were 1-hr in duration. Sessions for the Food group were identical to the procedures during food training (see above) except that session duration was 20-min during the 30 sessions. Locomotor activity was recorded daily by assessing the number of photobeam breakages within the operant chamber.

Experiment 2

Groups included Short-Access Cocaine (n=8), Long-Access Cocaine (n=8) and an Unhandled Control condition (n=8). In this experiment, cocaine self-administering animals underwent ten consecutive days of sessions. For the Short-Access Cocaine group, self-administration session procedures were identical to the first experiment. For the Long-Access Cocaine group, all procedures were identical except that session duration was 4 hrs in length and the timeout after each cocaine injection was 40-s.

Tissue Collection

In both experiments, two hours after the last session of handling, food or cocaine self-administration, rats were stunned with carbon dioxide for 1 minute and decapitated. Brains were rapidly removed and punches of 2.5 mm diameter and 1 mm thickness were collected from both the left and right brain hemispheres from the NAcc and VTA. For the prefrontal cortex (PFC), a razor blade was used to make a one mm thick transverse cut (see Fig 1). The samples were placed alone in 400 µl centrifuge tubes and placed in liquid nitrogen, later to be transferred to a -80 degree Celsius freezer.

Western blotting

Measurement of the relative protein levels of Cdk5 was accomplished by Western blot analysis. NAcc, VTA and PFC tissue samples from food, cocaine, handled and unhandled rats were dissected onto an icechilled plate and frozen at -80°C . Samples were sonicated in 320 mM sucrose, 5 mM N-2-Hydroxyethyl piperazine-N-2-ethanesulfonic acid (HEPES), 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin, and 1 $\mu\text{g}/\text{ml}$ pepstatin. Homogenates were centrifuged at 5,000 $\times g$ for 5 min (4°C), and the supernatant fraction subsequently centrifuged at 30,000 $\times g$ for 30 min (4°C). The resulting pellet (P2) was re-suspended and protein concentrations were determined with a Bio-Rad protein assay. Since Cdk5 is widely expressed in all rat brain subcellular fractions with no obvious concentration in any one fraction [20], the P2 pellet is considered to be representative of total Cdk5 expression. Protein samples (10 μg) were resolved in an SDS-PAGE system (NuPAGE® Bis-Tris SDS-PAGE, Invitrogen) after adding a 4X loading dye (200 mM Tris, 400 mM dithiothreitol, 8% glycerol, 0.4% bromophenol blue). Proteins were transferred to polyvinylidene difluoride membranes at 30V for 45 min. Membranes were blocked overnight at 4°C in 5% nonfat dry milk in Tris-buffered saline (TTBS) (135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween-20, pH 7.4) with a primary rabbit polyclonal antibody against Cdk5 (1:1000; Santa Cruz Biotechnology). After washing with TTBS, membranes were incubated with a goat anti-rabbit antibody for 1 hour (Santa Cruz Biotechnology, 1:5000). For Experiment 1, the membranes were incubated with SuperSignal® West Dura Extended Duration Substrate (Pierce Chemical) for 2 minutes and placed in plastic sheet prior to exposure to Hyperfilm™ ECL (Amersham Biosciences). The autoradiographs were scanned and analyzed using a KODAK Image station 440 (Kodak Digital Sciences) and KDS-1-D software (Eastman Kodak). Blots were then stripped for 20 minutes at 75°C (8 M urea, 100 mM 2-mercaptoethanol, and 62.5 mM Tris, pH 6.8) and re-probed with an antibody to actin (Santa Cruz Biotechnology) to ensure equal loading.

For Experiment 2, the membranes were incubated with SuperSignal® West Dura Extended Duration Substrate (Pierce Chemical) for 2 minutes and placed in plastic sheet prior to exposure with the ChemiImager 4000 system (Alpha-Innotech Corporation). Blots were then stripped for 20 minutes at 75°C (8 M urea, 100 mM 2-mercaptoethanol, and 62.5 mM Tris, pH 6.8) and re-probed with an antibody to actin (Santa Cruz Biotechnology) to ensure equal loading.

For both experiments, actin levels were assayed with Santa Cruz Actin HRP conjugated goat polyclonal antibody (1:5000 dilution) to confirm equal loading of protein in all lanes. Cdk5 levels were corrected according to actin content.

Statistical Analyses

Cocaine intake, food reinforcement and locomotor activity data during operant sessions were analyzed using one- and two-way repeated measures ANOVA. Post hoc analyses (Fisher's LSD) were used to determine specific differences when warranted by significant Main effects. In Experiment 2, 1-hr cumulative totals of cocaine responses and locomotor activity in Long-Access and Short-Access groups were compared (e.g., 1st hr for Long-Access Cocaine group and entire session for Short-Access group) using two-sample t-tests. Densometric data from western blot findings were converted to percent of their respective controls in each experiment (e.g., Exp. 1 Handled Controls; Exp 2 Unhandled Controls) and analyzed using two-sample t-tests. Significant levels were set at $p < 0.05$.

RESULTS

Experiment 1

Cocaine Intake Levels—In the Cocaine condition, mean ($\pm$ SEM) intake values of self-administered cocaine ranged from 6.0 ($\pm$ 1.88) – 13.5 ($\pm$ 1.8) mg/kg/session. A one-way ANOVA performed on daily intake across the 30 sessions showed no significant differences in intake levels as sessions progressed [$F(4,29)=1.0$; n.s.]

Operant Responses—A two-way ANOVA (Treatment X Session) performed on the number of lever presses/session in the Cocaine and Food Reinforcement groups showed Group Effects [$F(1,8)=14.16$; $p=0.006$], but no significant effects of Session or Treatment by Session Interaction effects [$F(29,232)=1.37$ and $F(29,232)=1.44$; respectively, both n.s.]. Posthoc tests showed that the Food Reinforcement group responded significantly more times per every 20-min session than the Cocaine group during 1-hr sessions (see Fig 2, Panel A, top).

Locomotor Activity—A two-way ANOVA (Treatment X Session) performed on locomotor activity between the Cocaine and Food condition showed significant Group effects [$F(1,8)=30.8$; $p=0.0005$], but no significant Session or Interaction effects [$F(29,232)=1.236$ and $F(29,232)=1.237$, respectively, both n.s.]. Posthoc tests showed that locomotor activity was significantly higher during cocaine selfadministration than during food-reinforced operant sessions (see Fig 2, Panel A, bottom).

Cdk5 Expression—Two-sample t-tests were used to compare densitometric data obtained by Western blot analysis of Cdk5 levels in the PFC, VTA and the NAcc of control groups versus cocaine self-administration and food reinforcement groups. No significant differences in NAcc, VTA nor PFC Cdk5 protein expression levels in the Food [$t(7)=-0.12$; $t(6)=-0.38$ and $t(5)=0.63$, respectively; all n.s.], or Cocaine groups [$t(8)=.049$; $t(8)=0.65$ and $t(4)=0.79$, respectively; all n.s.] when compared to the Handling Control (see Fig 3A).

Experiment 2

Cocaine Intake/Operant Responses—In the Short-Access Cocaine condition, mean ($\pm$ SEM) intake values (mg/kg/session) of self-administered cocaine ranged from 4.1 ($\pm$ 1.24) – 11.06 ($\pm$ 2.11). Intake values for the Long-Access Cocaine group ranged from 21.28 ($\pm$ 3.79) – 47.06 ($\pm$ 3.71) mg/kg/session. A two-way repeated measures ANOVA (Group X Session) on the number of lever responses showed significant Group, Session and Group X Session Interaction effects [$F(1,14)=44.38$, $p<0.0001$; $F(9, 126)=5.5$, $p<0.0001$ and $F(9,126)=2.16$, $p=0.029$, respectively]. Post hoc analyses revealed the Long-Access Cocaine group responded significantly more during the last three, compared to the first three sessions ($p < 0.01$). Lever responses for the Short-Access Cocaine group remained comparable across the 10 self-administration sessions (see Fig 2, Panel B, top). No significant differences in the total number of cocaine-reinforced responses during 1 hr of daily sessions were revealed between the Long-Access Cocaine group (1st hr of each 4 hr session) and the Short-Access Cocaine group [$t(14)=0.74$; n.s.; see insert, Fig 2B, top].

Locomotor Activity—A two-way repeated measures ANOVA performed on locomotor activity across self-administration sessions (Group X Session) showed significant Group [$F(1,14)=29.24$, $p<0.0001$], but no overall Session or Interaction effects [$F(9,126)=1.10$; $F(9,126)=0.60$, respectively, both n.s.]. Post hoc analyses revealed that locomotor activity in Long-Access Cocaine group was significantly greater than the Short-Access Cocaine group in every session. In addition, the Long-Access Cocaine group showed significantly enhanced activity during the last session (Session 10) compared to Sessions 2-5 (see Fig 2, Panel B, bottom).

However, no significant differences in the total amount of locomotor activity during the first hr of daily sessions in the Long-Access Cocaine group compared to the Short-Access Cocaine sessions [$t(14)=0.99$; n.s.; see insert, Fig 2B, bottom].

Cdk5 Expression—Two-sample t-tests were used to compare densitometric data obtained by Western blot analysis of Cdk5 in the PFC, VTA and the NAcc in the Cocaine/Short Access, Cocaine/Long Access and the Unhandled Control group. Compared to the Unhandled Control group, NAcc Cdk5 levels were significantly greater in the Cocaine/Short Access animals [$t(13) = 2.74$; $p=0.017$], but not in Long-Access Cocaine rats [$t(14) = 1.22$; n.s.]. Significant differences from the Unhandled Control group were not detected in VTA or PFC Cdk5 levels in Short-Access Cocaine [$t(11) = 0.62$; $t(11) = -0.33$, respectively, both n.s.], or in Long-Access Cocaine rats [$t(9) = 0.46$; $t(13) = -0.63$, respectively, both n.s.; see Fig 3B].

DISCUSSION

Cdk5 has been previously shown to be upregulated with chronic cocaine administration [3], though some studies have not demonstrated such effects. For instance, a report of Cdk5 levels after tolerance- or sensitization-producing cocaine regimens found no differences in Cdk5 expression in either condition [9]. Others have found Cdk5 to decrease below basal levels with increasing amounts of cocaine intake [21].

In the present study, no differences in Cdk5 expression in the NAcc, VTA or PFC was observed after 30 days of cocaine self-administration, food-reinforced operant sessions or daily handling. However, animals with less cocaine experience (10 days of 1-hr sessions; Short-Access Cocaine) showed significantly greater NAcc Cdk5 expression than Unhandled controls in the absence of behavioral sensitization or increased levels of cocaine intake. Conversely, rats self-administering cocaine over the same number of days, though during longer sessions (4-hr sessions; Long-Access Cocaine), showed enhanced cocaine intake and behavioral activity by the last sessions, but Cdk5 content was comparable to Unhandled Controls. These data are consistent with the notion that Cdk5 upregulation during cocaine administration may be a transient adaptation to cocaine use that subsides with increasing cocaine intake. Indeed, the Short-Access group self-administered approximately 82.0 mg/kg of cocaine over 10 days, and the Long-Access group self-administered approximately 357.0 mg/kg over the same time period. Similarly, previous reports have shown that Cdk5 was increased after repetitive experimenter-delivered daily injections of 20.0 mg/kg (160 mg/kg total) [3], but not when animals self-administered over cocaine over 10 days, with an intake range of 77.0 - 80.0 mg/kg/day over the last 3 sessions [14]. However, it should be noted that the entire striatum rather than just the NAcc was sampled in the former study [3], while only the NAcc region was examined in studies that did not observe cocaine-enhanced Cdk5 levels [9,14]. In addition, it is also important to point out that experimenter-delivered cocaine can cause different neurochemical [8] and behavioral [1] effects from self-administered cocaine. Therefore, differences between reports regarding Cdk5 expression may also be attributable to mode of cocaine delivery.

In Experiment 1, no observed differences in Cdk5 expression were detected between rats given daily 1-hour access to cocaine or food compared to Handled controls. In addition, behavioral sensitization to cocaine did not develop in the cocaine-reinforced group. Though this finding does not provide evidence that cocaine- or food-reinforced training have an impact on Cdk5 levels, it is consistent with a previous report showing similar levels of NAcc Cdk5 after withdrawal of self-administered cocaine and sucrose reward [14]. However, it is possible that handling the control group may have increased Cdk5 levels, and thus obscured detection of potential cocaine or food-induced enhancement of Cdk5 expression. For instance, handling

can be stressful to rats [5], and stress upregulates Cdk5 in cell lines [19]. Therefore, as it is conceivable that handling-induced stress could increase Cdk5 levels, the question as to whether natural reinforcement influences Cdk5 expression cannot be ruled out based on the present findings.

The current report and related literature indicate that Cdk5 expression can be altered following cocaine use. However, the nature of expression may vary considerably depending upon methodological differences, including dose, mode of cocaine delivery, level of cocaine intake and experience, control conditions and the particular brain regions sampled. Though the present study does not provide evidence that natural reward affects Cdk5 expression, the combination of factors necessary to show cocaine-induced upregulation might also be applied toward natural reinforcement in future investigations. Given the similarities between drug and natural reward and the diverse roles that Cdk5 plays in many neuronal activities, it would not be surprising if Cdk5 affects reward substrates in manners not specific to cocaine use.

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Fig 1. Coronal (A) and sagittal (B) sections of the rat brain sections isolated for western blot analyses with distances from bregma. **A.** NAcc and VTA regions collected using a 2.5 mm tissue biopsy punch. **B.** PFC region removed using a razor blade cut.

Fig 2.

Lever presses and locomotor activity during operant sessions. **Panel A/Experiment 1.** *Top:* Rats responded significantly more for sucrose pellets during 20-min operant sessions than for cocaine in 1-hr self-administration sessions. *Bottom:* Significantly higher locomotor activity during cocaine-compared to during food-reinforced sessions. **Panel B/Experiment 2.** *Top:* Rats responded significantly more for cocaine during 4-hr sessions (Long-Access) than during 1-hr sessions (Short-Access). Long-Access rats showed significantly greater response rates during Days 8-10 (** = $p < 0.01$) than during Days 1-3. *Insert:* Cumulative 1-hr response totals (10 sessions) for Long-Access Cocaine (1st hr of 4-hr session; white) and Short-Access Cocaine (black) were not significantly different. *Bottom:* Total locomotor activity counts were significantly higher during 4-hr compared to 1-hr cocaine self-administration sessions. At Day 10, locomotor activity was significantly greater than during Days 2-5 (* = significantly greater than Days 2-5 at $p < 0.05$). *Insert:* Cumulative 1-hr locomotor activity totals for Long-Access Cocaine (white) and Short-Access Cocaine (black) were not significantly different.

Fig 3.

Cdk5 expression in the NAcc, VTA and PFC. Bars show mean protein content ($\pm$ SEM) expressed as a percentage of controls. Representative Western blots of NAcc Cdk5 and actin content. **A/Experiment 1.** Lanes 1, 2 and 3 correspond to Handled Control, Cocaine and Food groups, respectively. No group differences in Cdk5 protein content were detected in any brain region sampled between rats that were handled for 30 days and those with 30 cocaine self-administration or food-reinforced operant sessions. **B/Experiment 2.** Lanes 1, 2 and 3 correspond to Unhandled Control, Short-Access Cocaine and Long-Access Cocaine, respectively. Significant differences in NAcc Cdk5 protein content were detected between Unhandled Control and Short-Access Cocaine (* = $p < 0.05$). No other group differences in Cdk5 expression were detected in any other brain regions sampled.