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Inactivation of the central nucleus of the amygdala reduces the effect of punishment on cocaine self-administration in rats

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Abstract

Continued cocaine use despite the negative consequences is a hallmark of cocaine addiction. One such consequence is punishment that is often used by society to curb cocaine use. Unfortunately, we know little about the mechanism involved in regulation by punishment of cocaine use. The fact that cocaine addicts continue cocaine use despite potential severe punishment suggests that the mechanism may be impaired. Such impairment is expected to critically contribute to compulsive cocaine use. This study aimed to test the hypothesis that the central nucleus of amygdala (CeN) plays a critical role in such regulation. To this end, rats were trained to press a lever to self-administer cocaine under a chained schedule: a response on one lever (cocaine-seeking lever) led to access to the other lever (cocaine-taking lever) on which a response was reinforced by cocaine and cues. Thereafter, responses on the seeking lever were punished by footshock with a probability of 0.5. Cocaine self-administration (SA) was significantly suppressed by punishment in an intensity-dependent manner. Interestingly, rats trained with daily 6-h (extended access) but not 2-h (limited access) sessions showed resistance to the lower intensity of punishment. Inactivation of the CeN induced a robust anti-punishment effect in both groups. These data provided evidence that the CeN is a critical neural substrate involved in regulation by punishment of cocaine SA. Rats with a history of extended cocaine SA appeared to be less sensitive to punishment. The decreased sensitivity could result from the neuroplastic changes induced by extended cocaine SA in the CeN.

Keywords

cocaine; self-administration; punishment; GABA

Introduction

Compulsive cocaine use, operationally defined as continued cocaine use despite severe adverse consequences, is a hallmark of cocaine addiction. Cocaine has powerful rewarding and reinforcing effects and these effects play a critical role in regulation of cocaine use (Goldberg et al., 1971; Wilson et al., 1971; Johanson et al., 1976; Bedford et al., 1978; Spyra et al., 1982; Schenk et al., 1986). Thus, tremendous efforts have been made to understand the neurobiological mechanisms underlying the rewarding and reinforcing processes associated with cocaine use and how dysregulation of these processes contributes to compulsive cocaine use. The mesocorticolimbic dopamine (DA) circuitry, originating from DA neurons in the ventral tegmental area (VTA) and including their targeted forebrain regions emerges as a critical neural substrate involved in rewarding/reinforcing effects of

cocaine (Wise, 1998; Hyman and Malenka, 2001; Nestler, 2001; Koob and Le Moal, 2008; Kalivas et al., 2009). Research has been focused on how dysregulation of the function of the DA circuitry might contribute to compulsive cocaine use (Robinson and Berridge, 1993; Koob and Le Moal, 1997; Everitt et al., 2001; Hyman et al., 2006). Besides the positive consequences, cocaine use is often associated with negative consequences. One such negative consequence is punishment that is often used by society to control cocaine use. The fact that cocaine addicts continue cocaine use despite being fully aware of the potential harsh punishment suggests that the mechanisms involved in regulation by punishment of cocaine use may be impaired in cocaine addicts. Indeed, there is preclinical evidence that rats developed resistance to the inhibitory effect of punishment on cocaine SA after a history of extended cocaine self-administration (SA) (Deroche-Gamonet et al., 2004; Pelloux et al., 2007; Kasanetz et al., 2010; Belin et al., 2011). Thus, impairment of the mechanism involved in the punishment-induced inhibition of cocaine SA is likely another critical factor contributing to compulsive cocaine use. Unfortunately, we have little knowledge regarding the neural mechanism underlying the inhibitory effect of punishment on cocaine SA. There is evidence that the amygdala is implicated in punishment-induced inhibition of behavior reinforced by natural rewards. For example, norepinephrine microinjected into the medial part of the amygdala including the central and medial nuclei has an anti-punishment effect on responding reinforced by milk in rats (Margules, 1968). A detailed study on the anatomical sites responsible for the effect indicates that the dorsomedial part of the amygdala including the central nucleus (CeN) is involved (Margules, 1971). This is consistent with the report that benzodiazepines microinjected into the CeN have anti-punishment effects in rats (Shibata et al., 1982). There are conflicting reports on the role of the BLA in the effect of punishment on reward-reinforced behavior (Margules, 1971; Scheel-Krüger and Petersen, 1982; Shibata et al., 1982; Hodges et al., 1987; Moller et al., 1997). This study aimed to determine whether the CeN plays a critical role in punishment-induced inhibition of cocaine SA.

Methods and Materials

Animals

Male Wistar rats (300–350 g) (Harlan Industries, Indianapolis, IN) were housed individually in plastic home cages in a temperature- and humidity-controlled colony room on a 12-h reverse light-dark cycle (lights off at 08:00). One week before operant training for food SA, rats were placed on a restricted diet to reach 85–90% free-feeding weight. After training, free access to food was available for one week before and after surgery. Food restriction was then reinstated to maintain 85–90% of free-feeding weight throughout the experiments and water was freely available at all times. The experiments were conducted during the dark cycle (between 09:00 and 18:00). All procedures followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by University of Tennessee Health Science Center Animal Care and Use Committee.

Operant Training

Rats were first trained to press either of two levers for sucrose solution (10%) with a fixed-ratio 1 (FR 1) schedule in a standard operant chamber (Med Associates Inc., East Fairfield, VT). The left lever was first inserted into the chamber and pressing the lever resulted in delivery of 0.1 ml sucrose solution and retraction of the lever. The right lever was then inserted 2 s later and pressing the right lever resulted in delivery of the same amount of sucrose solution and retraction of the right lever. The sequence started again 5 s later. The training continued until rats obtained 50 reinforcers on each lever within 1 h. Then, rats were trained under a modified chained schedule (Vanderschuren and Everitt, 2004; Pelloux et al., 2007). Under this schedule, the two levers were assigned as the seeking and taking levers,

respectively; pressing the seeking lever led to access to the taking lever 2 s later and pressing the taking lever was reinforced with sucrose solution. The seeking-taking cycle started again 5 s later. Training continued until rats obtained 100 reinforcers within 60 min.

Surgery

Rats were anesthetized with a mixture of ketamine and xylazine (80 and 10 mg/kg, respectively, intramuscular). Details of catheterization of the jugular vein and brain implantation of guide cannulas are described elsewhere (Caine et al., 1999; Sun et al., 2005). The coordinates for the CeN were: AP, -2.8 mm, ML, ± 4.2 mm, and DV, -7.5 mm (relative to bregma, midline and skull surface, respectively). The coordinates for the area dorsal to the CeN were the same except that the DV was -6.0 mm. Guide cannulas were bilaterally implanted into the area 2 mm dorsal to the targets. An obturator (Plastics One, Roanoke, VA) was inserted into each guide cannula to prevent blockage. After surgery, all rats were allowed to recover for one week during which time 0.1 ml of gentamicin (10 mg/ml, Biowhitaker, Walkersville, MD) was injected through the catheter daily, and the catheter was also flushed twice a day with heparinized physiological saline (30 U/ml). Catheter patency was evaluated by injecting 0.1 ml Brevital (1%) through the catheter. Loss of muscle tone within 5 s after injection indicated a patent catheter.

Cocaine SA training and punishment under the chained schedule

Rats were similarly trained to self-administer intravenous (i.v.) cocaine under the chained schedule. Pressing the seeking lever resulted in retraction of the lever and insertion of the taking lever 2 s later. Pressing the taking lever resulted in an i.v. infusion of cocaine (0.25 mg) in a volume of 0.05 ml over a 1-s period, 20-s presentation of a compound stimulus (two flashing cue lights above the levers and tone), and retraction of the lever. On the offset of the stimulus, the seeking-lever was inserted and the next seeking-taking cycle started. Rats were first screened for the levels of cocaine SA in the first session. Based on the number of cocaine infusions, rats were divided into two groups so that the average of the infusions between groups was similar. Two groups were assigned as the limited and extended access groups. The limited access group was trained in daily 2-h sessions whereas the extended access group in daily 6-h sessions. To avoid potential overdose, the maximum number of cocaine infusions that could be obtained was 40 and 100 for the limited and extended access groups, respectively. Previous studies showed that the method is reliable in balancing the levels of cocaine SA among groups (Ahmed et al., 2002; Ahmed et al., 2003; Ahmed et al., 2005; Lenoir and Ahmed, 2007). Rats were trained for 5–6 days per week for two weeks. By the end of training, the number of cocaine infusions for each rat varied by $< 20\%$ in three consecutive training sessions. Punishment was then added to the chained schedule: responses on the seeking lever resulted in immediate delivery of electric footshock with a probability of 0.5 and other consequences were the same as described above. The probability was chosen to simulate the situation that drug addicts encounter: punishment does not happen after each episode of drug-seeking and drug-taking behavior. The footshock lasted for 0.5 s with a current intensity of 0.2 or 0.4 mA.

Microinjection Procedures

Before the microinjection, the dummy cannulas were removed from the guide cannulas. The drug or drug vehicle was bilaterally microinjected into the brain regions by an infusion pump (Harvard Apparatus, MA) in a volume of 0.3 μ l through 33 ga injection cannulas (Plastics One, Roanoke, VA), which extended 2 mm below the guide cannulas. The volume was delivered over a 1-min period and the injection cannulas stayed in place for another minute to ensure drug diffusion. After microinjection, the dummy cannulas were put back into the guide cannulas.

Drugs

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Bethesda, MD) and dissolved in physiological saline to prepare a solution with a concentration of 5 mg/ml (salt). Muscimol and baclofen were purchased from Tocris (Ellisville, MO), were dissolved in physiological saline to prepare a cocktail containing 0.01 and 0.2 mg/ml, respectively. The pH of the solution was adjusted to ~7.0 with sodium hydroxide.

Experiment 1: effects of inactivation of the CeN on punishment-induced inhibition of cocaine SA under the limited access condition—To determine whether the CeN is involved in the effect of punishment on cocaine SA, the effect of reversible inactivation of the CeN with a mixture of baclofen and muscimol (B/M), a GABA_B and GABA_A agonist, respectively, on the punishment-induced inhibition of cocaine SA under the 2-h access condition was determined. Rats first received three daily consecutive punishment sessions with a footshock intensity of 0.2 mA. Before the fourth punishment session the next day, a mixture of B/M (60 and 3 ng/side) was microinjected into the CeN and the session started 5 min later. The dose was based on our pilot study and published studies (McFarland and Kalivas, 2001; McFarland et al., 2004) showing that this dose combination blocked cocaine- and stress-induced reinstatement of extinguished cocaine-seeking behavior in several brain regions. The next day, rats received another punishment session and thereafter, resumed cocaine SA training without punishment. After the number of cocaine infusions returned to the pre-test level or stabilized, rats were again tested for the effect of inactivation of the CeN on punishment under the exact procedure mentioned above except that the footshock intensity was 0.4 mA. Note that the lower intensity of punishment was tested before the higher intensity. This was done to avoid potential insensitivity of rats to the lower intensity from previous exposure to the higher intensity.

Experiment 2: effects of microinjection of saline into inactivation of the CeN on cocaine SA—To determine whether the anti-punishment effect of inactivation of the CeN was due to microinjection-induced mechanical damage to the CeN, the effect of saline (0.3 µl/side) microinjected into the CeN on punishment (0.4 mA) was also determined in the same rats after Experiment 1.

Experiment 3: effects of inactivation of the CeN on cocaine SA—There is a concern that the increase in punished cocaine SA after inactivation of the CeN could result from either inhibition of punishment or an increase in the reinforcing effect of cocaine. Thus, the effect of inactivation of the CeN on cocaine SA in the absence of punishment was determined in the same rats used in Experiment 1. Rats were first re-trained for cocaine SA under the 2-h access condition in the absence of punishment until the level of cocaine SA returned to the pretest level or stabilized. Then the same doses of B/M were microinjected into the CeN and 5 min later the SA session started in the absence of punishment.

Experiment 4: effects of inactivation of the area dorsal to the CeN on punishment—To determine whether the effect of B/M microinjected into the CeN was due to upward diffusion of the drugs along the shaft of injection cannula, the effect of microinjection of B/M into the area 1.5 mm dorsal to the CeN on punishment was similarly determined in another group of rats trained under the 2-h access condition.

Experiment 5: effects of extended cocaine SA on sensitivity to punishment—To determine whether cocaine SA under the extended access condition can reduce the sensitivity to punishment, an indicator of increased compulsive behavior, the effect of punishment on cocaine SA in rats trained under the 6-h access condition was determined.

The effect of the lower (0.2 mA) and higher (0.4 mA) intensities of punishment were determined in the same order as in Experiment 1. In addition, the effect of inactivation of the CeN on the punishment-induced inhibition of cocaine SA was also determined as in Experiment 1.

Experiment 6: effects of different cocaine access conditions on motivation—

To determine whether the decreased sensitivity to punishment under the 6-h access condition was due to an increase in the level of motivation compared with the 2-h access condition, rats trained under 6-h and 2-h access conditions were also tested to determine the breakpoints under the progressive ratio (PR) schedule of reinforcement. The response requirement after each reinforcement was based on the literature (Loh and Roberts, 1990; McGregor et al., 1994). The ratio of responses to reinforcement follows the sequence of 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603..., which is generated from the function of $(5e^{\text{infusions} \times 0.2}) - 5$. The session ended when no response occurred for a period of 30 min or 6 h passed, whichever occurred first.

Histology

After the experiments, rats were anesthetized with ketamine (100 mg/kg, i.p.) and transcardially perfused with physiological saline followed by formalin (5%). The brains were removed, soaked in formalin for at least 24 h and sliced at 100 μ m thickness with a vibratome. The sections were mounted on gelatin-coated slides, and stained with cresyl violet. The positions of the microinjection cannulas were inspected under a light microscope.

Statistics

The number of reinforcers earned and latency of responding on the seeking and taking levers were recorded during the SA and punished SA sessions. The breakpoint under the PR schedule was measured as the number of cocaine infusions earned during the session (McGregor et al., 1994). Control data were obtained from the training sessions before the test sessions. A mixed three-way ANOVA with punishment intensity and session as the within-subject factors and group (2-h vs 6-h) as the between-subject factor was used for analyzing treatment-dependent effects between 2-h and 6-h groups. A repeated two-way ANOVA was used for analyzing the effects of the within-subject factors. Repeated one-way ANOVA was used for analyzing the effects of punishment on latency and comparing the effects of B/M and vehicle. The significance level was set at 0.05.

Results

Histology

The positions of microinjection sites for 2-h CeN, 2-h dorsal control, and 6-h CeN groups are shown schematically in Figure (Fig) 1. Placements for both CeN groups were within or at the top region of the CeN. Placements for the dorsal control group were located ~1–1.5 mm above the CeN.

Effects of inactivation of the CeN on punishment-induced inhibition of cocaine SA

Rats in the 2-h group reached the SA training criteria (infusions varied <20% in three consecutive sessions) on an average of 8 ± 0.4 (mean $\pm$ SEM) days (range: 6–9). The number of cocaine infusions averaged from the last SA sessions was 28 ± 2.1 . To determine whether there were differential effects of session on cocaine SA in the 2-h and 6-h groups, the data from the two groups ($n=8$, respectively) analyzed with a mixed three-way ANOVA (Fig. 2). There was a significant main effect of session ($F_{5, 70} = 46.85$, $P < 0.001$). Bonferroni's tests

revealed that the levels of punished cocaine SA were significantly lower than control ($P<0.01$) except the level after inactivation of the CeN. After inactivation of the CeN, the level of punished cocaine SA was significantly higher than those without the inactivation ($P<0.01$). These results suggest that punishment significantly decreased cocaine SA and inactivation of the CeN reduced the effects of punishment. In addition, there was a significant main effect of intensity ($F_{1,14}=19.14$, $P<0.01$) indicating that the effects of punishment on cocaine SA were intensity-dependent. Moreover, there was a significant interaction between intensity and group ($F_{1,14}=4.83$, $P<0.05$) indicating that punishment had differential effects on the two groups. The interaction among the three factors was also significant ($F_{5,70}=5.02$, $P<0.01$) suggesting that the two groups showed differential responding to punishment across punishment sessions and intensities. To determine how the two groups behaved differently across sessions and intensities, a repeated two-way ANOVA was used to analyze the effects of session and intensity within each group. In the 2-h group, there was not a significant main effect of intensity ($F_{1,7}=4.75$, $P>0.05$) indicating that the inhibitory effects of 0.2 mA and 0.4 mA in the 2-h group were not statistically different when the data collapsed across the sessions. In contrast, there was a significant main effect of session ($F_{5,35}=30.62$, $P<0.001$) and a significant interaction between session and intensity ($F_{5,35}=3.87$, $P<0.001$). The latter result indicates that the effects of 0.2 and 0.4 mA in different sessions were significantly different. In addition, the inhibitory effect of punishment of 0.2 mA was significant in the second and third punishment session compared with control whereas punishment with 0.4 mA in the first, second, third and fifth session (Bonferroni's tests, $P<0.05$). These results suggest that lack of the main effect of intensity was probably due to the significant decreases of cocaine SA after both 0.2 mA and 0.4 mA. In the 6-h group, there were significant main effects of session ($F_{5,35}=24.42$, $P<0.001$) and intensity ($F_{1,7}=14.39$, $P<0.001$). In addition, there was a significant interaction between session and intensity ($F_{5,35}=19.16$, $P<0.001$) indicating that the effects of 0.2 mA and 0.4 mA were different in different sessions. Cocaine SA was significantly decreased with punishment intensity of 0.4 mA in the second, third, and fifth punishment session (Bonferroni's tests, $P<0.05$). Punishment of 0.2 mA did not significantly decrease cocaine SA.

Given that punishment decreased the rate of cocaine SA, this could result from increased response latencies on either the seeking or taking lever. To clarify this issue, the response latencies on both levers were also measured in the 2-h group. A repeated one-way ANOVA revealed a significant main effect of session on the seeking latency with the shock intensity of 0.2 mA (Fig. 3, the left panel in top row; $F_{5,35}=4.47$, $P<0.001$). Bonferroni's tests revealed that the effect was due to the significant difference between the third and B/M treatment sessions ($P<0.05$). Although there was an increasing trend of the seeking latency in the first, second, third, and fifth punishment sessions compared with control, the differences did not reach statistical significance. With the shock intensity of 0.4 mA, one rat did not respond and another had only two responses on the seeking lever. Under such conditions the seeking latency cannot be reliably determined. Thus, the data analysis on the seeking latency was based on six rats. A repeated one-way ANOVA revealed that there was a significant main effect of session (Fig. 3, the right panel in top row; $F_{5,25}=6.86$, $P<0.0001$). Bonferroni's tests revealed a significant difference between the third session and control ($P<0.05$). In addition, there were significant differences between B/M treatment and the second, third, and fifth sessions ($P<0.05$). The difference was not significant between control and B/M treatment sessions.

There was an increasing trend of the taking latency in the first four punishment sessions and such a trend disappeared in the fifth punishment session with the shock intensity of 0.2 mA. However, a repeated one-way ANOVA did not reveal a significant main effect of session (Fig. 3, the left panel in bottom row, $F_{5,35}=0.77$, $P>0.05$). With the punishment intensity

of 0.4 mA, there was a decreasing trend of the taking latency in the first, second, third, and fifth punishment sessions. However, the differences did not reach statistical significance (Fig. 3, the right panel in bottom row; $F_{5, 25} = 2.49$, $P > 0.05$). Because 50% of responses on the taking lever followed footshock due to the punishment probability of 0.5, there might be difference in the taking latencies following footshock compared with those not following footshock. However, a repeated two-way ANOVA analysis did not reveal significant differences in the taking latencies after footshock compared with those after no footshock (Fig. 4, $F_{1, 40} = 2.05$, $P > 0.05$).

Because the microinjection procedure could cause mechanical damage to the CeN, such damage may contribute to the reduced impact of punishment on responding. If so, we would expect that such irreversible damage produces a long-lasting effect. If the effect was due to the pharmacological action of B/M, we would expect that such an effect is reversible. To this end, data from the third punishment session (0.4 mA) and control were divided into six 20-min bins and the rates of cocaine SA during the 20-min bins were compared with a repeated two-way ANOVA. As shown in Fig. 5, there were main effects of bin ($F_{5, 70} = 5.02$, $P < 0.0001$) and B/M treatment ($F_{1, 70} = 20.05$, $P < 0.0001$). Bonferroni's tests revealed that there were significant differences in the first four bins ($P < 0.01$) but such differences disappeared in the fifth and sixth bins.

Effects of microinjection of saline into the CeN on the punishment-induced inhibition of cocaine SA

To further rule out involvement of the mechanical damage in the effect of B/M, seven of eight rats used in the previous experiment were tested for the effect of microinjection of saline into the CeN on punishment-induced inhibition of cocaine SA. To compare the effect of saline and B/M treatment, data from the punishment sessions before and after saline or B/M treatment as well as controls for the two treatments were averaged. As shown in Fig. 6, a repeated one-way ANOVA revealed a significant main effect of session ($F_{4, 24} = 9.93$, $P < 0.0001$). Bonferroni's tests revealed that there was no significant difference between control and B/M but the difference between control and saline was significant ($P < 0.05$). More importantly, there was a significant difference between saline and B/M ($P < 0.05$). These results indicate that microinjection of B/M but not saline into the CeN had an anti-punishment effect.

Effects of inactivation of the CeN on cocaine SA

The amygdala is also implicated in regulation of cocaine SA, it is possible that inactivation of the CeN may change the strength of the reinforcing effect of cocaine and such a change could contribute to the anti-punishment effect of B/M. Thus, the effect of inactivation of the CeN on cocaine SA was determined in six rats of 2-h group that were used in Experiment 1. Following Experiment 1, these rats were returned to regular SA training until SA behavior was stabilized. Then, B/M was microinjected into the CeN 5 min before the test session. The data from cocaine SA sessions before and after B/M treatment were used as controls. Levels (mean $\pm$ SE) of cocaine SA in the control and B/M treatment sessions were 32 ± 2.0 , 31 ± 2.6 , and 44 ± 3.9 , respectively. A repeated one-way ANOVA revealed that there was a significant main effect of session ($F_{2, 10} = 16.39$, $P < 0.0001$). Bonferroni's tests revealed that there were significant differences between B/M treatment and SA sessions before and after the treatment.

Effects of microinjection of B/M into the area dorsal to the CeN on punished cocaine SA

To determine whether the effect of microinjection of B/M into the CeN was due to upward diffusion of the drugs along the shaft of the injection cannula, the effect of B/M microinjected into an area 1.5mm dorsal to the CeN was studied in another group of rats

($n=7$). As shown in Fig 7, there was a significant main effect of session ($F_{5,30}=11.91$, $P<0.0001$). Bonferroni's tests revealed that there were significant differences between control and the punishment sessions ($P<0.05$). However, there were no differences between B/M treatment and other punishment sessions.

Effects of different cocaine access conditions on motivation

The difference in the level of motivation for cocaine could contribute to the different sensitivities to punishment between 2-h and 6-h groups. Thus, the breakpoints were determined for the two groups. Six rats from the 2-h group and five rats from the 6-h group were tested under the PR schedule after the punishment experiments. The breakpoints of 2-h and 6-h groups were 18 ± 1.4 and 15 ± 1.1 , respectively. An unpaired t -test revealed no significant difference between the two groups ($t=1.67$, $df=9$, $P>0.05$).

Discussion

The results showed that footshock as a negative consequence (punishment) of cocaine-seeking behavior inhibited cocaine SA in an intensity-dependent manner. The effect of punishment also depended on the cocaine access condition. Low intensity punishment appeared to decrease cocaine SA in rats trained under the limited but not extended cocaine access condition. The effect was probably not a result of freezing behavior induced by footshock because the latency on the taking lever was not significantly increased. Reversible inactivation of the CeN of the amygdala with local administration of B/M robustly reduced the punishment-induced inhibition of cocaine SA. Such an anti-punishment effect cannot be attributed to the upward diffusion of B/M to the area dorsal to the CeN because direct microinjection of the drugs into this region had no effect. Although we cannot totally rule out the possibility that the effect may be partially due to diffusion of B/M into the basolateral nucleus (BLA) of the amygdala, the rapid onset of the effect (within 20 min after microinjection) suggests that the effect was mainly mediated by the CeN. In addition, several lines of evidence argue against the possibility that the effect may be due to mechanical damage to the CeN by the microinjection procedure. First, the tip of the injection cannulas was located at the top border area of the CeN and histology did not reveal any consistent damage to the CeN. Second, the effect of B/M was reversible as evidenced by the fact that the anti-punishment effect disappeared in the last 40 min of the 2-h session. Moreover, punishment robustly inhibited cocaine SA the next day in the absence of B/M indicating that if there was any mechanical damage, the function of the CeN related to punishment was not significantly impaired. Third, direct microinjection of saline into the CeN had no effect on the punishment-induced inhibition of cocaine SA. Together, these data strongly support that the anti-punishment effect of B/M was mediated by specific inactivation of the CeN.

Note that manipulation of the amygdala could alter motor activity and such an effect could confound the effect of B/M on cocaine SA. For example, blockade of D_1 -like receptors decreases spontaneous locomotion while increasing the rate of cocaine SA (McGregor and Roberts, 1993). Because locomotion is decreased rather than increased, the motor effect cannot explain the increase in the rate of cocaine SA after D_1 -like receptor blockade in the amygdala. In addition, lesion of the amygdala does not cause enhanced motor response to cocaine but increased the rate of cocaine SA (Whitelaw et al., 1996) indicating that the motor effect is not involved in the effect. We observed here that although inactivation of the CeN decreased the seeking latency in the presence of punishment, the taking latency was not significantly changed. The motor effect cannot explain such a differential effect. Together, these data suggest that it is unlikely that the motor-related effect is primarily responsible for the anti-punishment effect of inactivation of the CeN.

The effect of punishment on cocaine SA depends on the strength of the reinforcing effect of cocaine. For example, in a two-lever choice task where each lever is associated with a different dose of cocaine, responding on the low-dose lever can be shifted to the high-dose lever associated with punishment (Johanson, 1977). Thus, it is possible that inactivation of the CeN may increase the rate of cocaine SA by increasing the reinforcing effect of cocaine rather than decreasing the effect of punishment. Indeed, the amygdala including both CeN and BLA has been implicated in cocaine SA (McGregor and Roberts, 1993; Caine et al., 1995; Hurd et al., 1997). Consistent with these observations, we observed here that inactivation of the CeN also increased the rate of cocaine SA in the absence of punishment. Note, however, that the rate of cocaine SA could be changed via other mechanisms including a decrease in the rate-limiting effect of cocaine. It is well established that the rate of cocaine SA depends on the dose of cocaine and the relationship follows an inverted U-shaped curve (Yokel and Wise; Pickens and Thompson, 1968). When the dose is located in the descending part of the curve, an increase in the dose decreases the rate of cocaine SA. The rate-limiting effect may, at least partially contribute to such a decrease. Because the dose used here is on the descending part of the curve (Whitelaw et al., 1996), the increased rate of cocaine SA after inactivation of the CeN could result from a reduction in the rate-limiting effect of cocaine. Consistent with this idea, blockade of D₁-like receptors in the amygdala fails to increase the breakpoint under the PR schedule of reinforcement (McGregor and Roberts, 1993) although such blockade increases the rate of cocaine SA under the fixed-ratio 1 schedule (McGregor and Roberts, 1993; Caine et al., 1995; Hurd et al., 1997). The breakpoint supposedly indicates the level of motivation and is closely associated with the strength of the reinforcing effect or dose of cocaine (Roberts et al., 1989). Thus, failure to decrease the breakpoint after D₁-like receptor blockade indicates that D₁-like receptors in the amygdala are not involved in the reinforcing effect per se. In addition, excitotoxic lesion of the amygdala does not change the threshold dose of cocaine for initiating and maintaining SA behavior (Whitelaw et al., 1996). If the amygdala is directly involved in the strength of the reinforcing effect of cocaine and inactivation or lesion of the amygdala increases the reinforcing effect of cocaine, we would expect a decrease in the threshold dose of cocaine. Together, these data suggest that it is unlikely that the anti-punishment effect of inactivation of the CeN can be fully explained by the increase in the reinforcing effect of cocaine.

Besides the reinforcing effects, cocaine also has anxiogenic effects. For example, cocaine has been shown to produce conditioned place aversion when conditioning occurs 15 min after i.v. administration of cocaine (Ettenberg and Bernardi, 2007). In addition, rats trained to run a straight alley to self-administer cocaine show approach-withdrawal behavior: running from the start box to the goal box and then stop at the front of the goal box and return to the start box (Ettenberg and Geist, 1991). This retreat behavior is interpreted as a result of the anxiogenic effects of cocaine. Consistent with this idea, the anxiolytic drug diazepam dose-dependently reduces such approach-withdrawal behavior (Ettenberg and Geist, 1991). It is possible that the anxiogenic effects of cocaine may inhibit the rate of cocaine SA. It is well established that the amygdala plays a critical role in fear and anxiety (Margules, 1968, 1971; Davis, 1986; Hodges and Green, 1987; LeDoux, 2000). The involvement of the CeN in the anxiogenic effect of cocaine is supported by a recent study that inactivation of the CeN reduces retreat frequency during the alley-running cocaine SA (Wenzel et al., 2011). Thus, anxiolytic effect of inactivation of the CeN could partially contribute to the increased rate of cocaine SA by reducing the anxiogenic effect of cocaine.

Impaired regulation by negative consequences of cocaine-seeking behavior could be an important mechanism involved in compulsive cocaine use. Indeed, extended cocaine SA reduces the inhibitory effect of punishment on cocaine SA (Deroche-Gamonet et al., 2004; Pelloux et al., 2007; Kasanetz et al., 2010). Consistent with these data, we observed here that

rats trained under the 6-h access condition were insensitive to punishment with the shock intensity of 0.2 mA that significantly decreased cocaine SA in rats trained under the 2-h access condition. In addition, punishment with the shock intensity of 0.4 mA significantly decreased the rate of cocaine SA in the first punishment session in rats trained under the 2-h but not 6-h access condition. It is possible that such a difference could result from a difference in the motivational level in the two groups. There are conflicting reports regarding whether cocaine SA under the extended access condition increases the breakpoint compared to the short access condition (Paterson and Markou, 2003; Liu et al., 2005). In addition, rats demonstrating resistance to punishment after a long period of cocaine SA do not show significant increases in breakpoint compared with rats with a limited period of cocaine SA (Pelloux et al., 2007). Even in the cases where an increase in the breakpoint is observed after a long period of cocaine SA, the time course of such an increase does not correlate with the time course of resistance to punishment (Deroche-Gamonet et al., 2004). In the present study we did not observe a difference in the breakpoint between the two groups. Thus, it is unlikely that the increased motivation could fully explain the differences observed here.

Alternatively, footshock may be less aversive in rats with extended cocaine SA and consequently, cause less inhibition of cocaine SA. There is evidence, however, that punishment-resistant rats after extended cocaine SA show normal Pavlovian fear conditioning (Vanderschuren and Everitt, 2004; Pelloux et al., 2007). In addition, punishment-resistant rats show normal anxiety-like behavior measured in the elevated plus maze procedure (Deroche-Gamonet et al., 2004). These data suggest that the aversive property of footshock or emotional response to aversive stimuli is similar in punishment-resistant and punishment-sensitive rats. Consistent with this idea, our direct observation of behavior during cocaine SA in the presence of punishment revealed that there were no obvious differences in shock-evoked jumping and backpedaling behaviors in the two groups. In addition, both groups showed approach to and withdrawal from the seeking lever during punished cocaine SA. Together, these observations indicate that it is unlikely that a reduced aversive response to punishment can explain the difference.

Given that punishment appeared to be as aversive in the 6-h group as in the 2-h group, the reduced sensitivity of the 6-h group to punishment could be due to a deficit in using emotional information evoked by punishment to inhibit cocaine-seeking behavior. It has been proposed that a deficit in behavioral inhibition is associated with compulsive drug use (Jentsch and Taylor, 1999). For example, cocaine addicts show a deficit in a stop-signal task that requires addicts to respond to go-signals and inhibit such a response when stop-signals occasionally occur (Fillmore and Rush, 2002). In addition, they also show perseverative responding to a previously rewarded stimulus (Ersche et al., 2008). These data suggest that a deficit in the mechanisms involved in behavioral inhibition may be a critical factor in compulsive cocaine use. Although such a deficit could be a pre-addiction trait, preclinical studies provide evidence that chronic cocaine indeed induces such a deficit. For example, perseverative responding to a previously rewarded stimulus is observed in rats after cocaine SA and repeated cocaine injections (Jentsch et al., 2002; Schoenbaum et al., 2004; Calu et al., 2007; Stalnaker et al., 2007). Moreover, chronic cocaine induces a deficit in updating the information related to the association between a stimulus and reward in the amygdala and such a deficit may play a critical role in the deficit of reversal learning (Stalnaker et al., 2007). Given these considerations, the amygdala could be a critical part of the neural network involved in behavioral inhibition. One hypothesis is that extended cocaine SA may induce neuroplastic changes in the CeN that are either quantitatively or qualitatively different from those induced by limited cocaine SA and such differences may contribute to the reduced sensitivity to punishment. Identifying these neuroplastic changes will likely provide novel insights into the mechanism underlying compulsive cocaine use.

In summary, our results provide evidence that the CeN is critically involved in regulation by negative consequences of cocaine SA. Rats trained to self-administer cocaine under the extended access condition were less sensitive to punishment. These data suggest that the neural mechanisms involved in regulation by punishment may be impaired after extended cocaine SA. Future studies on the role of the CeN in such impairment are warranted.

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Abbreviations

B/M	baclofen and muscimol
BLA	basolateral amygdala
CeN	central nucleus of amygdala
DA	dopamine
FR	fixed-ratio
PR	progressive ratio
SA	self-administration
VTa	ventral tegmental area

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Fig. 1. Schematic depiction of microinjection sites in three groups of rats: 2-h CeN, 2-h dorsal, and 6-h CeN. Coronal brain section images were adapted from the atlas of Paxinos and Watson (Paxinos G. and Watson C., 1998). Filled circles, triangles, and squares represent the microinjection sites in the 2-h CeN, 2-h dorsal to the CeN, and 6-h CeN groups, respectively.

Fig. 2.

Effects of reversible inactivation of the CeN on punishment-induced inhibition of cocaine SA in rats trained under the 2-h and 6-h access condition. Data are presented as mean $\pm$ SEM. **Ctrl** indicates the data from the cocaine SA training sessions before the punishment sessions. The numbers indicate the order of the punishment sessions. **B/M** indicates the cocktail of baclofen and muscimol. *Upper panel*: effect of inactivation of the CeN on punished cocaine SA in the 2-h group. *Lower panel*: effect of inactivation of the CeN on punished cocaine SA in the 6-h group.

Fig. 3. Effect of reversible inactivation of the CeN on the seeking and taking latencies in rats trained under the 2-h access condition. Data are presented as mean $\pm$ SEM. *Top row*: effect of punishment and B/M treatment on the seeking latency. *Bottom row*: effect of punishment and B/M treatment on the taking latency. * and # indicate significant differences from **Ctrl** and **B/M** treatment, respectively.

Fig. 4. Effect of reversible inactivation of the CeN on the taking latency following footshock vs no footshock in rats trained under the 2-h access condition. Data are presented as mean $\pm$ SEM. The data were from the third punishment session with the intensity of 0.4 mA. **NoShock** and **Shock** indicate punishment and no punishment, respectively.

Fig. 5. Time-course effect of reversible inactivation of the CeN on punishment-induced inhibition of cocaine SA. Data are presented as mean $\pm$ SEM. * indicates significant differences from Ctrl.

Fig. 6. Effect of microinjection of saline (**Sal**) and **B/M** into the CeN on punishment-induced inhibition of cocaine SA. Data are presented as mean $\pm$ SEM. * and # indicate significant differences from **Ctrl** and **B/M**, respectively.

Fig. 7. Effect of reversible inactivation of the area dorsal to the CeN on punishment-induced inhibition of cocaine SA in rats trained under the 2-h access condition. Data are presented as mean $\pm$ SEM. * and ‡ indicate significant differences from **Ctrl** and the first punishment sessions, respectively.