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Corticotropin-Releasing Factor Within the Central Nucleus of the Amygdala and the Nucleus Accumbens Shell Mediates the Negative Affective State of Nicotine Withdrawal in Rats

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Tobacco addiction is a chronic disorder that is characterized by a negative affective state upon smoking cessation and relapse after periods of abstinence. Previous research has shown that an increased central release of corticotropin-releasing factor (CRF) at least partly mediates the deficit in brain reward function associated with nicotine withdrawal in rats. The aim of these studies was to investigate the role of CRF in the central nucleus of the amygdala (CeA), the lateral bed nucleus of the stria terminalis (BNST), and the nucleus accumbens shell (Nacc shell) in the deficit in brain reward function associated with precipitated nicotine withdrawal. The intracranial self-stimulation procedure was used to assess the negative affective aspects of nicotine withdrawal. Elevations in brain reward thresholds are indicative of a deficit in brain reward function. In all experiments, the nicotinic receptor antagonist mecamylamine (3 mg/kg) elevated the brain reward thresholds of the nicotine-dependent rats (9 mg/kg per day of nicotine salt) and did not affect the brain reward thresholds of the saline-treated control rats. The administration of the nonspecific CRF1/2 receptor antagonist D-Phe $CRF_{(12-41)}$ into the CeA and the Nacc shell prevented the mecamylamine-induced elevations in brain reward thresholds in the nicotine-dependent rats. Blockade of CRF1/2 receptors in the lateral BNST did not prevent the mecamylamine-induced elevations in brain reward thresholds in the nicotine-dependent rats. These studies indicate that the negative emotional state associated with precipitated nicotine withdrawal is at least partly mediated by an increased release of CRF in the CeA and the Nacc shell.
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INTRODUCTION

Tobacco addiction is a chronic disorder that is characterized by loss of control over smoking, the appearance of withdrawal symptoms upon smoking cessation, and relapse after periods of abstinence (American Psychiatric Association, 2000; McLellan *et al*, 2000; O'Brien, 2003). Abrupt cessation of smoking typically mediates negative affective symptoms such as depressed mood, anxiety, irritability, and difficulty concentrating (American Psychiatric Association, 2000). It has been hypothesized that the negative affective aspects of tobacco withdrawal provide a powerful motivation for the continuation of smoking (Koob *et al*, 1997; Markou *et al*, 1998). Experimental evidence suggests that nicotine is one of the main components of tobacco smoke that leads to and maintains the tobacco addiction (Bardo *et al*, 1999; Crooks and Dwoskin, 1997; Stolerman and Jarvis, 1995). The positive reinforcing effects of nicotine are at least partly mediated by the activation of neuronal nicotinic acetylcholine receptors (nAChRs). Blockade of nAChRs decreases the self-administration of nicotine in rats (Corrigall *et al*, 1994; Corrigall and Coen, 1989; Donny *et al*, 1999; Watkins *et al*, 1999). In addition, mice that lack the β2-subunit of the nAChR self-administer less nicotine than wild-type controls (Picciotto *et al*, 1998). Nicotine withdrawal is associated with a deficit in brain reward function and somatic withdrawal signs in rats (Bruijnzeel and Markou, 2004; Epping-Jordan *et al*, 1998; Harrison *et al*, 2001). Epping-Jordan and colleagues reported that systemic administration of the nAChR antagonist dihydro-β-erythroidine induces an elevation in brain reward thresholds (decrease in the reinforcing properties of intracranial self-stimulation, ICSS) and an increase in somatic withdrawal signs in rats chronically treated with nicotine. Similarly, abrupt cessation of nicotine administration mediates an elevation in brain reward thresholds and an increase in somatic withdrawal signs (Epping-Jordan *et al*,

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1998). The administration of nicotine after the discontinuation of chronic nicotine administration has been shown to mitigate somatic nicotine withdrawal signs (Malin *et al*, 1992). These findings indicate that nicotine stimulates the brain reward system and discontinuation of chronic nicotine administration leads to a negative emotional state in rats.

Accumulating evidence suggests that a hyperactivity of brain stress systems may lead to a deficit in brain reward function, which is one of the core symptoms of drug withdrawal and depression (Barr and Markou, 2005; Bruijnzeel and Gold, 2005). Clinical studies indicate that brain corticotropin-releasing factor (CRF) systems are hyperactive in patients with depressive disorders (Nemeroff *et al*, 1984; Zobel *et al*, 2000). Preclinical research indicates that intracerebroventricular (i.c.v.) administration of CRF induces an elevation in brain reward thresholds in rats, which is indicative of a deficit in brain reward function (Macey *et al*, 2000). The observation that increased CRF transmission is important in negative affective states has stimulated research into the function of CRF in drug addictions. These studies have provided evidence for a function of CRF in drug withdrawal-induced anxiety-like behavior. Blockade of CRF receptors has been shown to decrease anxiety-like behavior associated with withdrawal from alcohol, cocaine, and other drugs of abuse (Baldwin *et al*, 1991; Basso *et al*, 1999; Overstreet *et al*, 2004; Rassnick *et al*, 1993; Sarnyai *et al*, 1995). Moreover, in a recent study we demonstrated that the i.c.v. administration of the nonspecific CRF1/2 receptor antagonist D-Phe $CRF_{(12-41)}$ prevents the elevations in brain reward thresholds associated with precipitated nicotine withdrawal in rats (Bruijnzeel *et al*, 2007).

Although extensive progress has been made into the understanding of the function of CRF in drug withdrawal, it is not known through which specific brain sites CRF mediates the deficit in brain reward function associated with nicotine withdrawal. Experimental evidence points toward a function for the central nucleus of the amygdala (CeA) and the lateral bed nucleus of the stria terminalis (BNST) in the negative emotional state associated with nicotine withdrawal. Spontaneous alcohol and cocaine withdrawal and precipitated cannabinoid and nicotine withdrawal have been shown to elevate extracellular CRF levels in the CeA (George *et al*, 2007; Merlo Pich *et al*, 1995; Richter and Weiss, 1999; Rodriguez de Fonseca *et al*, 1997). In addition, alcohol withdrawal increases the release of CRF in the BNST, which can be reversed with subsequent alcohol intake (Olive *et al*, 2002). Another brain site that has been suggested to be important in drug addiction is the nucleus accumbens shell (Nacc shell). It has been suggested that the negative emotional state associated with drug withdrawal is at least partly mediated by a hypodopaminergic function in the Nacc shell (Barr *et al*, 2002; Rada *et al*, 2001). Although CRF and its receptors have been detected in the Nacc shell, there is no experimental evidence suggesting the importance for CRF in the Nacc shell during drug withdrawal (De Souza *et al*, 1985; Swanson *et al*, 1983). The administration of CRF into the lateral ventricles has been shown to induce behavioral activation in rats in a familiar environment, which is indicative of increased arousal and anxiety-like behavior, and a similar effect has been observed after the administration of CRF into the Nacc shell (Holahan *et al*, 1997). This suggests that the release of CRF into the Nacc shell could contribute to negative emotional states.

Taken together, the above-discussed studies suggest that increased CRF transmission may be important in the negative mood state associated with nicotine withdrawal. In addition, the CeA, BNST, and Nacc shell have been suggested to have an important function in drug withdrawal and/or relapse to drug seeking behavior (Koob, 2008). The aim of these experiments was to investigate the function of CRF in the CeA, lateral BNST, and Nacc shell in the deficit in brain reward function associated with precipitated nicotine withdrawal in rats. The effect of the administration of the nonspecific CRF1/2 receptor antagonist D-Phe $CRF_{(12-41)}$ (CRF1 receptor, $Ki = 56$ nM; CRF2 receptor, $Ki = 5.2$ nM) into the CeA, lateral BNST, or Nacc shell on the elevations in brain reward thresholds associated with precipitated nicotine withdrawal was investigated by using a discrete trial ICSS procedure (Gulyas *et al*, 1995; Perrin *et al*, 1999). This procedure was used to assess the negative affective aspects of nicotine withdrawal as it provides a quantitative measure of the emotional aspects of drug withdrawal (Bruijnzeel *et al*, 2006; Schulteis *et al*, 1995; Wise and Munn, 1995). In all of the experiments, response latencies were assessed to determine if the administration of the CRF receptor antagonist into the specific brain sites would affect motor output (Markou and Koob, 1992). Experiments that provide insight into the specific function of brain stress systems in the negative affective aspects of nicotine withdrawal may contribute to the development of nonaddictive pharmacotherapies that reduce tobacco withdrawal symptomatology and improve relapse rates.

MATERIALS AND METHODS

Subjects

Male Wistar rats (Charles River, Raleigh, NC) weighing 250–300 g at the beginning of the experiments were used. Animals were group-housed (two per cage) in a temperature- and humidity-controlled vivarium and maintained on a 12 h light–dark cycle (lights off at 1800 hours). All testing occurred at the beginning of the light cycle. Food and water were available *ad libitum* in the home cages. All subjects were treated in accordance with the National Institutes of Health guidelines regarding the principles of animal care. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care and approved by the University of Florida Institutional Animal Care and Use Committee.

Drugs

Nicotine hydrogen tartrate salt, mecamylamine hydrochloride, and pentobarbital sodium salt were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA) and dissolved in sterile saline (0.9% sodium chloride). The CRF1/2 receptor antagonist [D-Phe^{12}, $Nle^{21,38}$,$C^{\alpha}Me$ Leu^{37}]r/h$CRF_{(12-41)}$ (D-Phe $CRF_{(12-41)}$) was synthesized by The Clayton Foundation Laboratories for Peptide Biology and kindly provided by Dr Jean Rivier (Salk Institute for Biological

Studies, La Jolla, CA). D-Phe $CRF_{(12-41)}$ was dissolved in distilled water and kept on ice until being used in the behavioral experiments. The CRF receptor antagonist was administered within 1 h after being dissolved.

Surgical Procedures

Cannulae and electrode implantations. At the beginning of all the intracranial surgeries, the rats were anesthetized with an isoflurane/oxygen vapor mixture (1–3% isoflurane) and placed in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the incisor bar set 3.3 mm below the interaural line (flat skull). The rats were prepared with 11 mm stainless steel 23-gauge cannulae above the CeA, lateral BNST, or Nacc shell using flat skull coordinates according to Paxinos and Watson, 1998, and a previous study by Koob and colleagues (Funk *et al*, 2006). Bilateral cannulae were implanted 2.5 mm above the CeA (anterior–posterior (AP) −2.3, medial–lateral (ML) ± 4.0 mm, dorsal–ventral (DV) −4.7 from dura), 2.0 mm above the lateral BNST (AP −0.6, ML ± 3.7 and 15° vertical tilt, DV −4.6 from dura), or 2.5 mm above the Nacc shell (AP + 1.7 mm, ML ± 1.0, DV −5.1 from dura). At the end of the surgery, 11 mm removable 30-gauge wire stylets were inserted in the cannulae to maintain patency. For the electrode implantations, the incisor bar was set 5 mm (CeA and lateral BNST groups) above the interaural line or at the interaural line (Nacc shell group). The position of the incisor bar had to be adjusted for the electrode implantations in the Nacc shell group to accommodate the cannulae and the electrode. The rats were prepared with stainless steel bipolar electrodes (model MS303/2; Plastics One, Roanoke, VA) 11 mm in length in the medial forebrain bundle at the level of the posterior lateral hypothalamus (AP −0.5 mm, ML ± 1.7 mm, DV −8.3 mm from dura). The electrodes and cannulae were permanently secured to the skull using dental cement anchored with four skull screws.

Osmotic minipump implantations. The rats were prepared with osmotic minipumps (model 2ML4, 28 day pumps; Durect Corporation, Cupertino, CA) filled with either saline or nicotine hydrogen tartrate dissolved in saline. The pumps were implanted subcutaneously under isoflurane/oxygen (1–3% isoflurane) anesthesia. The nicotine concentration was adjusted to compensate for differences in body weight and to deliver a dose of 9 mg/kg per day of nicotine salt (3.16 mg/kg per day nicotine base).

Apparatus

The experimental apparatus consisted of 12 Plexiglas chambers (30.5 × 30 × 17 cm; Med Associates, Georgia, VT), each housed in a sound-attenuating melamine chamber (Med Associates). The operant conditioning chambers consisted of a metal grid floor and a metal wheel (5 cm wide) centered on a sidewall. A photobeam detector was attached next to the response wheel and recorded every 90° of rotation. Brain stimulation was delivered by constant current stimulators (Model 1200C; Stimtek, Acton, MA). Subjects were connected to the stimulation circuit through bipolar leads (Plastics One) attached to gold-contact swivel commutators (model SL2C; Plastics One). A computer controlled the stimulation parameters, data collection, and all test session functions.

Intracranial Self-Stimulation Procedure

Rats were trained on a modified discrete-trial ICSS procedure (Kornetsky and Esposito, 1979), as described previously (Markou and Koob, 1992). The subjects were trained initially to turn the wheel on a fixed ratio 1 schedule of reinforcement. Each quarter turn resulted in the delivery of a 0.5 s train of 0.1 ms cathodal square-wave pulses at a frequency of 100 Hz. After the successful acquisition of responding, defined as 100 reinforcements within 10 min, the rats were gradually trained on a discrete-trial current-threshold procedure. Each trial began with the delivery of a noncontingent electrical stimulus, followed by a 7.5-s response window within which the animal could respond to receive a second contingent stimulus identical to the initial noncontingent stimulus. A response during this 7.5-s response window was labeled as a positive response and the lack of a response was labeled as a negative response. During a 2-s period immediately after a positive response, additional responses had no scheduled consequences. The intertrial interval (ITI), which followed either a positive response or the end of the response window, had an average duration of 10 s (7.5–12.5 s). Responses that occurred during the ITI resulted in a further 12.5 s delay of the onset of the next trial. During training on the discrete-trial procedure, the duration of the ITI and delay periods induced by time-out responses were gradually increased until animals performed consistently at standard test parameters. The rats were subsequently tested on the current-threshold procedure in which stimulation intensities varied according to the classical psychophysical method of limits. A test session consisted of four alternating series of descending and ascending current intensities starting with a descending series. Blocks of three trials were presented to the subject at a given stimulation intensity, and the intensity was altered systematically between blocks by 5 μA steps. The initial stimulus intensity was set 40 μA above the baseline current-threshold for each animal. Each test session typically lasted 30–40 min and provided two variables: brain reward thresholds and response latencies. The brain reward threshold for a descending series was defined as the midpoint between stimulation intensities that supported responding (ie positive responses on at least two of the three trials), and current intensities that failed to support responding (ie positive responses on fewer than two of the three trials for two consecutive blocks of trials). The threshold for an ascending series was defined as the midpoint between stimulation intensities that did not support responding and current intensities that supported responding for two consecutive blocks of trials. Four threshold estimates were recorded and the mean of these values was taken as the brain reward threshold for a specific subject. The response latency was defined as the time interval between the beginning of the noncontingent stimulus and a positive response. The response latency for each test session was defined as the mean response latency on all trials during which a positive response occurred.

Intracerebral Microinjections

The bilateral injections were administered by using 30-gauge stainless steel injectors that extended 2.5 mm (length of injector tip, 13.5 mm; CeA and Nacc shell) or 2.0 mm (length of injector tip, 13.0 mm; lateral BNST) beyond the guide cannulae. The injection volume was 0.5 μl per side and this was infused over 66 s. The infusion speed was regulated by a Harvard Apparatus syringe pump (model 975) and the pump was equipped with 10 μl syringes (Hamilton, Rena, NE). The syringes were connected to the injectors with Tygon microbore PVC tubing (0.25 mm ID × 0.76 mm OD). The injectors were left in place for 30 s postinjection to allow diffusion from the injector tip. The dummy stylets, 11 mm, were re-inserted immediately after the injectors were removed.

Histology

At the end of the experiments, the rats were killed with an overdose of sodium pentobarbital (150 mg, intraperitoneally) and perfused through the ascending aorta with physiological saline (100 ml) followed by a 10% phosphate buffered formalin solution (150 ml). Brains were postfixed for 24 h and cryoprotected in 10% sucrose in phosphate buffered saline for 48 h. Sections were cut on a Leica CM3050 S cryostat (coronal sections of 40 μm at −15°C). The sections were mounted on Fisher Superfrost Plus slides and stained with cresyl violet. The locations of the guide cannulae and injections sites were verified with light microscopy and with reference to a stereotaxic atlas of the rat brain (Paxinos and Watson, 1998).

Experimental Design

Effect of D*-Phe CRF$_{(12-41)}$ administered into the CeA, lateral BNST, or Nacc shell on precipitated nicotine withdrawal.* Naïve rats were used for all the experiments. After recovery from the electrode implantations, the rats were trained on the ICSS procedure. When stable baseline brain reward thresholds were achieved (defined as less than 10% variation within a 5-day period), the rats were prepared with 28-day osmotic minipumps containing either saline or nicotine dissolved in saline (CeA: saline $n=8$, nicotine $n=8$; lateral BNST: saline $n=9$, nicotine $n=9$; Nacc shell: saline $n=9$, nicotine $n=9$). Brain reward thresholds and response latencies were assessed daily throughout the experiment between 0900 and 1200 hours. The nAChR antagonist mecamylamine (3 mg/kg, s.c.) was used to precipitate nicotine withdrawal. Mecamylamine injections started at least 6 days after the implantation of the minipumps to allow time for the development of nicotine dependence. The CRF1/2 receptor antagonist D-Phe CRF$_{(12-41)}$ (5–500 ng per brain site, unilateral dose) was administered 10 min before treatment with mecamylamine. The rats were placed in the ICSS test chambers 5 min after mecamylamine administration. It was ensured that the minimum time interval between the mecamylamine injections was at least 72 h to reestablish/maintain nicotine dependence. The serum elimination half-life of mecamylamine is approximately 1 h (Debruyne *et al*, 2003). At the end of the experiment, the rats were perfused under pentobarbital anesthesia and the brains were removed for histological verification of the cannulae placements.

Statistical Analyses

For all the experiments, brain reward thresholds and response latencies were expressed as percentages of the values obtained on the day before each test day. Percent changes in brain reward thresholds and response latencies were analyzed using a two-way repeated-measures analyses of variance (ANOVA) with the dose of D-Phe CRF$_{(12-41)}$ as the within-subjects factor and pump content (saline or nicotine) as the between-subjects factor. For all experiments, statistically significant interactions in the ANOVA were followed by the Newman–Keuls *post hoc* test.

RESULTS

Effect of D-Phe CRF$_{(12-41)}$ Administered into the CeA on Precipitated Nicotine Withdrawal

Mean (±SEM) absolute brain reward thresholds before pump implantation for the saline- and nicotine-treated rats were 87.14 ± 3.39 and 88.23 ± 6.66 μA ($t(14)=0.15$, NS), respectively. Mean (±SEM) absolute response latencies for the saline- and nicotine-treated rats were 2.90 ± 0.10 and 3.08 ± 0.15 s ($t(14)=1.00$, NS), respectively. Mecamylamine elevated the brain reward thresholds of the nicotine-treated rats, 146%, and did not affect the brain reward thresholds of the control rats (Figure 1a; Treatment: F1,14 = 57.46, $P<0.0001$). Mecamylamine did not affect the response latencies of the nicotine- or saline-treated rats (Figure 1b; Treatment: F1,14 = 3.49, NS). The administration of D-Phe CRF$_{(12-41)}$ into the CeA prevented the mecamylamine-induced elevations in brain reward thresholds in the nicotine-dependent rats and did not affect the brain reward thresholds of the rats that were chronically treated with saline (Figure 1a; Dose × Treatment interaction: F3,42 = 4.21, $P<0.011$). Newman–Keuls *post hoc* comparisons indicated that 500 ng of D-Phe CRF$_{(12-41)}$ (unilateral dose) completely prevented the elevations in brain reward thresholds associated with precipitated nicotine withdrawal. D-Phe CRF$_{(12-41)}$ did not affect the response latencies (Figure 1b; Dose: F3,42 = 1.23, NS; Dose × Treatment interaction: F3,42 = 0.17, NS). See Figure 2a for a histological reconstruction of the injections sites.

Effect of D-Phe CRF$_{(12-41)}$ Administered into the lateral BNST on Precipitated Nicotine Withdrawal

Mean (±SEM) absolute brain reward thresholds before minipump implantation for the saline- and nicotine-treated rats were 118.84 ± 8.78 and 118.93 ± 14.17 μA ($t(16)=0.01$, NS), respectively. Mean (±SEM) absolute response latencies for the saline- and nicotine-treated rats were 3.28 ± 0.16 and 2.99 ± 0.10 s ($t(16)=1.61$, NS), respectively. Similar to the first experiment, mecamylamine elevated the brain reward thresholds of the nicotine-treated rats, 147%, and did not affect the brain reward thresholds of the saline-treated rats (Figure 3a; Treatment: F1,16 = 17.33, $P<0.0007$). Mecamylamine did not affect the response latencies of the nicotine- or saline-treated rats (Figure 3b; Treatment: F1,16 = 3.21, NS). The administration of D-Phe CRF$_{(12-41)}$ into the lateral BNST did not affect the brain reward thresholds (Dose: F3,48 = 0.64, NS; Dose × Treat-

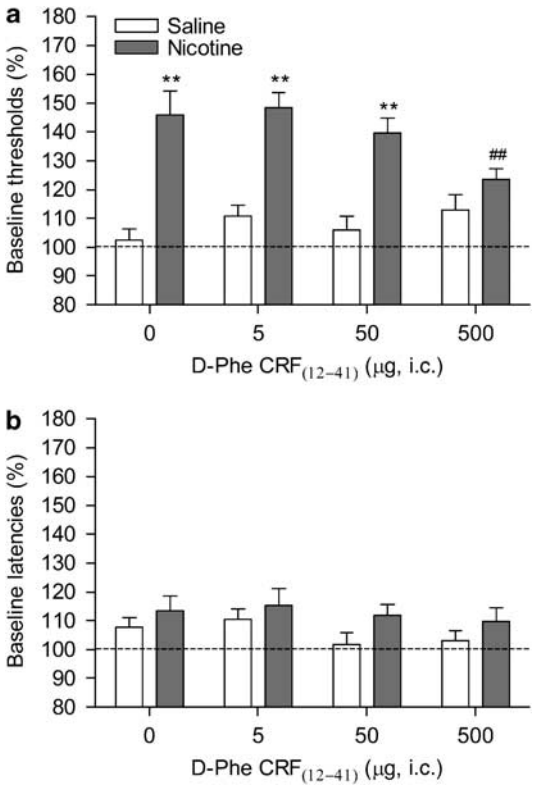

Figure 1 Effect of D-Phe $CRF_{(12-41)}$ (saline, $n=8$; nicotine, $n=8$) administered into the central nucleus of the amygdala (CeA) on the elevations in brain reward thresholds associated with mecamylamine-precipitated nicotine withdrawal (a). Effect of D-Phe $CRF_{(12-41)}$ on the response latencies of rats chronically treated with saline ($n=8$) or nicotine ($n=8$) and acutely treated with mecamylamine (b). Brain reward thresholds and response latencies are expressed as a percentage of the pretest day values. D-Phe $CRF_{(12-41)}$ was administered bilaterally and the figure depicts the unilateral dose. Asterisks (**$P<0.01$) indicate elevations in brain reward thresholds compared to those of the corresponding saline-treated control group. Symbols (## $P<0.01$) indicate lower brain reward thresholds compared to those of rats chronically treated with nicotine and acutely treated with mecamylamine and vehicle (0 μg of D-Phe $CRF_{(12-41)}$). Abbreviation: i.c., intracranial.

ment: $F3,48=0.61$, NS) or the response latencies (Dose: $F3,48=0.92$, NS; Dose × Treatment: $F3,48=2.40$, NS) of the saline- or nicotine-treated rats. See Figure 2b for a histological reconstruction of the injections sites.

Effect of D-Phe $CRF_{(12-41)}$ Administered into the Nacc Shell on Precipitated Nicotine Withdrawal

Mean (±SEM) absolute brain reward thresholds before minipump implantation for the saline- and nicotine-treated rats were 139.21 ± 14.54 and 136.99 ± 15.18 μA ($t(16)=0.11$, NS), respectively. Mean (±SEM) absolute response latencies for the saline- and nicotine-treated rats were 3.61 ± 0.17 and 3.93 ± 0.17 s ($t(16)=1.33$, NS), respectively. Mecamylamine elevated the brain reward thresholds of the nicotine-treated rats, 151%, and did not affect the brain reward thresholds of the saline-treated rats (Figure 4a; Treatment: $F1,16=59.44$, $P<0.0001$). Mecamylamine did not affect the response latencies of the nicotine- or saline-treated rats (Figure 4b; Treatment: $F1,16=0.77$, NS). The administration of D-Phe $CRF_{(12-41)}$ into the Nacc shell prevented the mecamylamine-induced elevations in brain reward thresholds in the nicotine-dependent rats and did not affect the brain reward thresholds of the rats that were chronically treated with saline (Figure 4a; Dose × Treatment interaction: $F3,48=3.48$, $P<0.023$). *Post hoc* analysis indicated that the highest dose of D-Phe $CRF_{(12-41)}$, 500 ng per site, completely prevented the mecamylamine-induced elevations in brain reward thresholds in the nicotine-treated rats. The administration of D-Phe $CRF_{(12-41)}$ into the Nacc shell did not affect the response latencies of the saline- or nicotine-treated rats (Dose: $F3,48=0.35$, NS; Dose × Treatment: $F3,48=1.07$, NS). See Figure 2c for a histological reconstruction of the injections sites.

DISCUSSION

These results demonstrate that the nAChR antagonist mecamylamine elevates the brain reward thresholds of rats that are chronically treated with nicotine and does not affect the brain reward thresholds of saline-treated control rats, which is in line with previous studies (Bruijnzeel and Markou, 2004; Epping-Jordan *et al*, 1998; Watkins *et al*, 2000). The administration of D-Phe $CRF_{(12-41)}$ (500 ng per site, unilateral dose) into the CeA and the Nacc shell prevented the mecamylamine-induced elevations in brain reward thresholds. In contrast, the administration of D-Phe $CRF_{(12-41)}$ into the lateral BNST did not prevent the elevations in brain reward thresholds associated with precipitated nicotine withdrawal. Our findings extend and corroborate previous findings by demonstrating that antagonism of CRF1/2 receptors in the CeA and Nacc shell, but not in the lateral BNST, prevents the negative affective state of precipitated nicotine withdrawal in rats (Bruijnzeel *et al*, 2007; Epping-Jordan *et al*, 1998).

These studies focused on investigating the importance of CRF in the CeA, lateral BNST, and Nacc shell in nicotine withdrawal as all of these structures are considered part of the extended amygdala, are highly interconnected, and have overlapping afferent and efferent connections (Alheid and Heimer, 1988; Heimer *et al*, 1991). In addition, it has been suggested that the extended amygdala has an important function in the negative affective state associated with drug withdrawal (Koob and Le Moal, 2005). Extensive evidence points toward a function for CRF in the CeA in drug addictions. Withdrawal from alcohol (Merlo Pich *et al*, 1995), cannabis (Rodriguez de Fonseca *et al*, 1997), cocaine (Richter and Weiss, 1999), and nicotine (George *et al*, 2007) has been shown to induce an increased release of CRF in the CeA. It has also been shown that alcohol withdrawal-induced anxiety-like behavior in the elevated plus maze test can be reversed by the administration of the nonspecific CRF1/2 receptor antagonist α-helical $CRF_{(9-41)}$ in the lateral ventricles (Baldwin *et al*, 1991) or the CeA (Rassnick *et al*, 1993). Koob and colleagues demonstrated that alcohol intake in rats is increased after chronic exposure to alcohol vapor (Roberts *et al*, 2000) and the increased alcohol intake in the alcohol-dependent animals can be prevented by the administration of D-Phe $CRF_{(12-41)}$ into the CeA before the alcohol self-administration sessions (Funk *et al*, 2006). The results of our study demonstrated that antagonism of CRF

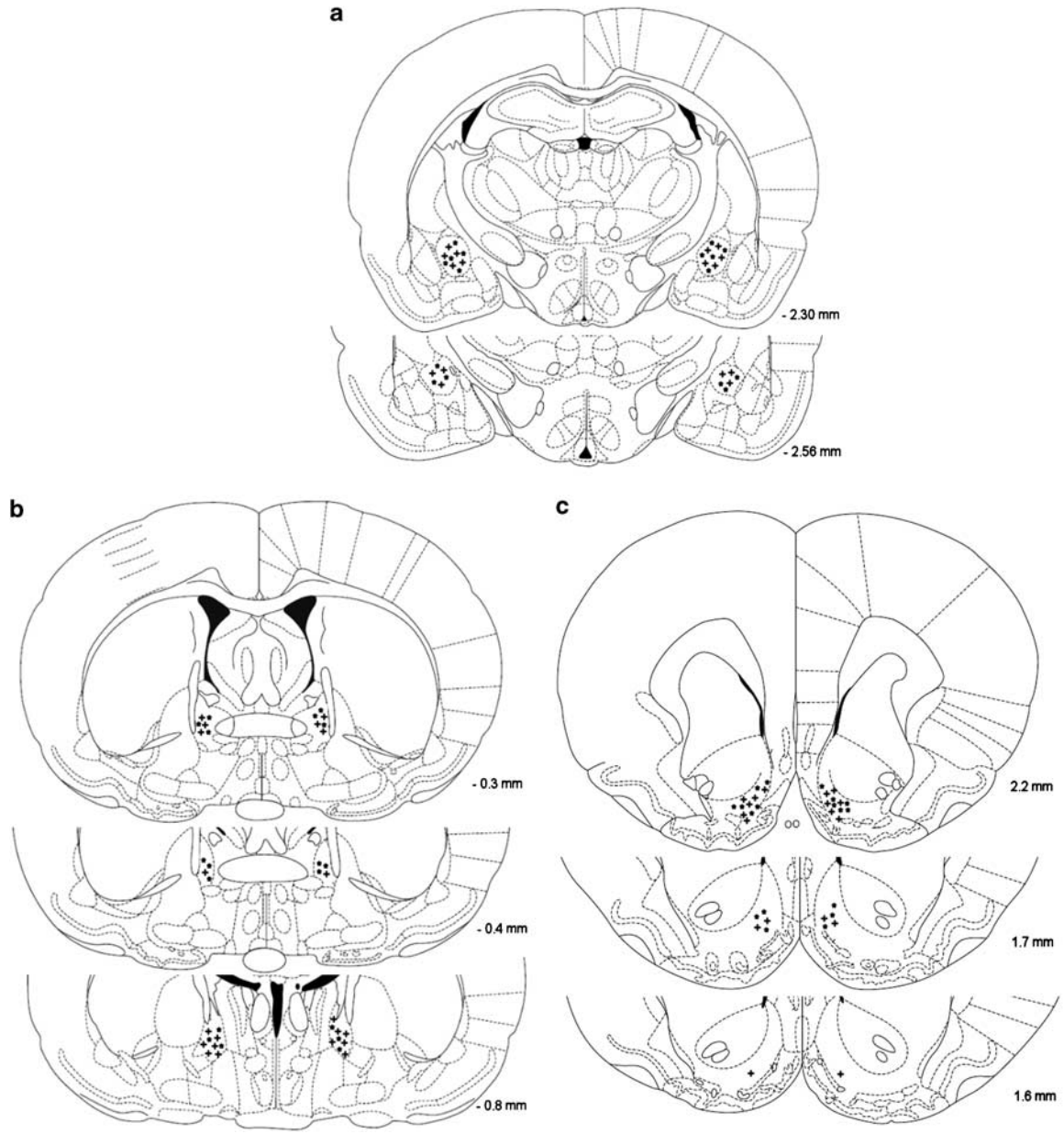

Figure 2 Histological reconstruction of bilateral injection sites in the central nucleus of the amygdala (CeA) (a) of rats chronically treated with nicotine (+, $n=8$) or saline (*, $n=8$). Reconstruction of bilateral injection sites in the lateral bed nucleus of the stria terminalis (BNST) (b) of rats chronically treated with nicotine (+, $n=9$) or saline (*, $n=9$). Reconstruction of bilateral injections sites in the nucleus accumbens shell (Nacc shell) (c) of rats chronically treated with nicotine (+, $n=9$) or saline (*, $n=9$). The figures are copies from the Paxinos and Watson brain atlas (Paxinos and Watson, 1998).

receptors in the CeA prevented the elevations in brain reward thresholds associated with nicotine withdrawal. This suggests that the release of CRF into the CeA may at least partly mediate the deficit in brain reward function associated with nicotine withdrawal. Drug intake during the withdrawal phase could possibly diminish negative affective states by decreasing the release of CRF in the CeA.

The intra-CeA dose of D-Phe $CRF_{(12-41)}$ that prevented the elevations in brain reward thresholds was about 20 times lower than the i.c.v. dose required to prevent the elevations in brain reward thresholds associated with nicotine withdrawal (1 μg intra-CeA [total bilateral dose] *vs* 20 μg i.c.v.) (Bruijnzeel *et al*, 2007). This suggests that the current total bilateral dose, 1 μg of D-Phe $CRF_{(12-41)}$, would not have prevented the deficit in brain reward function associated with nicotine withdrawal when administered into the lateral ventricles. This rules out the possibility that in these studies D-Phe $CRF_{(12-41)}$ diffused into the lateral ventricles and then mediated its effects by acting on other brain sites. It is also unlikely that D-Phe $CRF_{(12-41)}$ prevented the elevations in brain reward thresholds by activating brain reward systems. The i.c.v. administration of D-Phe $CRF_{(12-41)}$ does not alter brain reward thresholds in drug-free rats (Macey *et al*, 2000). In addition, in this study we showed that intra-CeA, lateral BNST, or Nacc shell administration of D-Phe $CRF_{(12-41)}$ does not affect the brain reward thresholds of the chronic saline/acute mecamylamine control group.

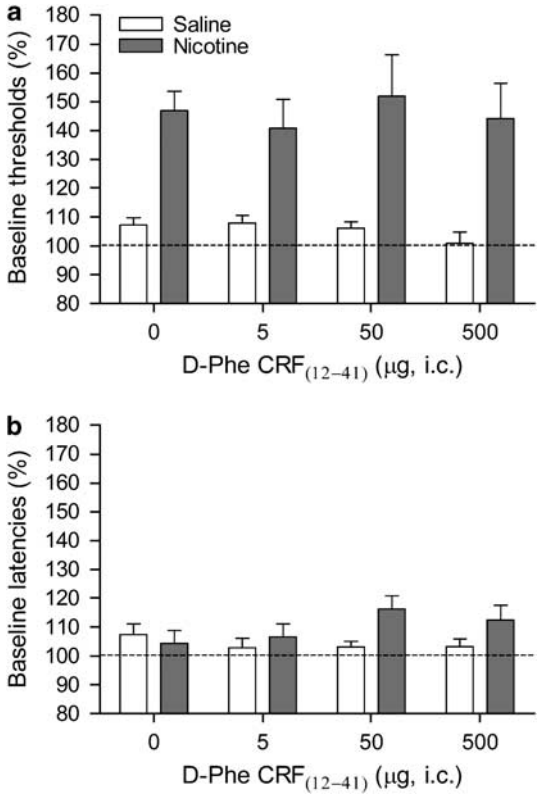

Figure 3 Effect of D-Phe $CRF_{(12-41)}$ (saline, $n=9$; nicotine, $n=9$) administered into the lateral bed nucleus of the stria terminalis (BNST) on the elevations in brain reward thresholds associated with mecamylamine-precipitated nicotine withdrawal (a). Effect of D-Phe $CRF_{(12-41)}$ on the response latencies of rats chronically treated with saline ($n=8$) or nicotine ($n=8$) and acutely treated with mecamylamine (b). Brain reward thresholds and response latencies are expressed as a percentage of the pretest day values. D-Phe $CRF_{(12-41)}$ was administered bilaterally and the figure depicts the unilateral dose. Abbreviation: i.c., intracranial.

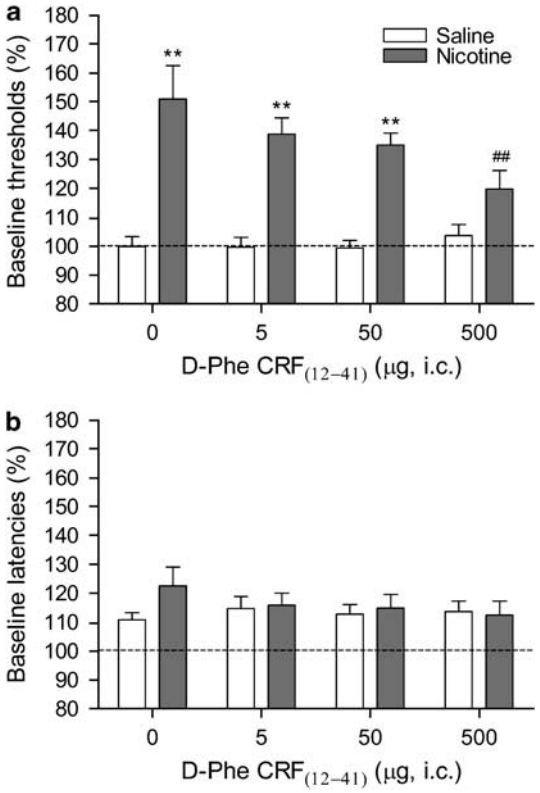

Figure 4 Effect of D-Phe $CRF_{(12-41)}$ (saline, $n=9$; nicotine, $n=9$) administered into the nucleus accumbens shell (Nacc shell) on the elevations in brain reward thresholds associated with mecamylamine-precipitated nicotine withdrawal (a). Effect of D-Phe $CRF_{(12-41)}$ on the response latencies of rats chronically treated with saline ($n=9$) or nicotine ($n=9$) and acutely treated with mecamylamine (b). Brain reward thresholds and response latencies are expressed as a percentage of the pretest day values. D-Phe $CRF_{(12-41)}$ was administered bilaterally and the figure depicts the unilateral dose. Asterisks (**$P<0.01$) indicate elevations in brain reward thresholds compared to those of the corresponding saline-treated control group. Symbols (## $P<0.01$) indicate lower brain reward thresholds compared to those of rats chronically treated with nicotine and acutely treated with mecamylamine and vehicle (0 μg of D-Phe $CRF_{(12-41)}$). Abbreviation: i.c., intracranial.

Some studies suggest that the BNST may be involved in specific aspects of drug addictions. Discontinuation of chronic alcohol administration increases extracellular CRF levels in the BNST, which subsides with subsequent alcohol intake (Olive *et al*, 2002). In addition, naloxone induces a dose-dependent increase in c-fos mRNA in the BNST of morphine-dependent animals (Frenois *et al*, 2002). Evidence for a function of the BNST in negative affective states is provided by Aston-Jones and colleagues (Delfs *et al*, 2000). They reported that the blockade of β-noradrenergic receptors or activation of α2-adrenergic receptors in the BNST prevents opioid withdrawal-induced conditioned place aversion. At this point in time, we are not aware of any studies which reported that blockade of CRF receptors in the lateral BNST prevents the negative affective state associated with drug withdrawal. The results of our study suggest that the activation of CRF receptors in the lateral BNST does not have a function in the negative emotional state associated with nicotine withdrawal. This is in agreement with a previous study showing that blockade of CRF receptors in the BNST does not reduce alcohol intake in alcohol-dependent rats (Funk *et al*, 2006). It is unlikely that the doses of D-Phe $CRF_{(12-41)}$ were too low in this study as the same doses prevented the negative emotional state associated with nicotine withdrawal when administered into the CeA or Nacc shell. In addition, a previous study reported that the intra-BNST administration of doses of D-Phe $CRF_{(12-41)}$, which are within the current dose–response range (10 or 50 ng per side administered bilaterally), prevent stress-induced reinstatement of cocaine seeking behavior (Erb and Stewart, 1999).

Emerging evidence suggests that CRF in the CeA and BNST may have a distinct function in various aspects of drug addictions and anxiety and fear responses (Erb and Stewart, 1999; Walker *et al*, 2003). These studies suggest that CRF in the CeA, but not in the lateral BNST, is important in the negative affective state associated with nicotine withdrawal. CRF in the CeA, but not in the BNST, has also been suggested to be important in the increased alcohol intake in alcohol-dependent animals (Funk *et al*, 2006). Furthermore, the administration of the CRF1/2 receptor antagonist α-helical $CRF_{(9-41)}$ into the CeA, but not into the BNST, reduced the severity of naloxone-precipitated somatic morphine withdrawal signs (McNally and Akil, 2002). The aforementioned studies suggest that

CRF signaling in the CeA mediates acute drug withdrawal signs such as elevations in brain reward thresholds, somatic signs, and increased drug intake. In contrast, CRF transmission in the BNST may at least partly mediate protracted drug withdrawal signs such as stress-induced reinstatement of drug seeking behavior. For example, Erb and Stewart (1999) demonstrated that the BNST, but not the CeA, has an important function in stress-induced reinstatement of cocaine seeking behavior.

Neurochemical changes in the Nacc shell have been implicated in the negative affective state associated with drugs withdrawal. For example, nicotine withdrawal has been associated with a decrease in dopamine levels and an increase in acetylcholine levels in the Nacc shell in rats (Rada *et al*, 2001). The Nacc shell contains CRF-immunoreactive cells and moderate levels of CRF1 and CRF2 receptors have been detected in this brain site (De Souza *et al*, 1985; Merchenthaler *et al*, 1982; Rominger *et al*, 1998; Swanson *et al*, 1983). However, very little research has been conducted to investigate the function of CRF in the Nacc shell in drug addiction. The results of this study indicate that the administration of the CRF1/2 receptor antagonist D-Phe $CRF_{(12-41)}$ into the Nacc shell prevents the elevations in brain reward thresholds associated with precipitated nicotine withdrawal. Thus, this suggests that the negative affective state associated with nicotine withdrawal is at least partly mediated by the release of CRF in the Nacc shell. Research by Koob and colleagues suggests that CRF in the Nacc shell does not have a function in alcohol intake in alcohol-dependent rats (Funk *et al*, 2006). However, emerging evidence suggests that CRF in the Nacc shell has behavioral effects and could be important in psychiatric disorders. The i.c.v. administration of CRF has been shown to increase locomotor activity in rats in a familiar environment and to increase anxiety-like behavior in a novel environment (Sutton *et al*, 1982; Takahashi *et al*, 1989). Kelley and colleagues demonstrated that the administration of CRF into the Nacc shell also increases locomotor activity in rats in a familiar environment (Holahan *et al*, 1997), which suggests that CRF might mediate some of its behavioral effects by stimulating CRF receptors in the Nacc shell. In a recent study it was demonstrated that the administration of CRF into the Nacc shell increases cue-induced motivation to obtain sucrose pellets (Pecina *et al*, 2006). It was suggested that the CRF release in the Nacc shell may also increase the motivation to obtain other positive reinforcers such as drugs of abuse and therefore have an important function in the reinstatement of drug seeking behavior.

D-Phe $CRF_{(12-41)}$ is a nonspecific CRF receptor antagonist and therefore these studies did not distinguish between CRF1 and CRF2 receptor subtypes. Extensive evidence suggests that the CRF1 receptor has a pivotal function in drug withdrawal and relapse. Systemic administration of the small-molecule nonpeptide CRF1 receptor antagonist MPZP has been shown to decrease nicotine withdrawal-induced anxiety-like behavior and to prevent increased nicotine intake after a period of abstinence (George *et al*, 2007). Furthermore, the nonpeptide CRF1 receptor antagonist MTIP blocks alcohol withdrawal-induced anxiety-like behavior, excessive alcohol self-administration in alcohol-dependent rats, and stress-induced reinstatement of alcohol seeking behavior (Gehlert *et al*, 2007). Conflicting findings have been reported with regard to the function of the CRF2 receptor in drug withdrawal. Stimulation of CRF2 receptors decreases alcohol intake in alcohol-dependent animals and decreases alcohol withdrawal-induced anxiety-like behavior (Funk and Koob, 2007; Valdez *et al*, 2004). In contrast, CRF2 receptor knockout mice display decreased somatic morphine withdrawal signs, which suggests that activation of CRF2 receptors contributes to drug withdrawal (Papaleo *et al*, 2008). The above-discussed studies would suggest that the activation of the CRF1 receptor may have an important function in the negative affective state of nicotine withdrawal. At this point in time, additional studies are needed before firm conclusions can be drawn about the function of the CRF2 receptor in drug withdrawal.

In these studies, the function of CRF in nicotine withdrawal was investigated in rats passively exposed to nicotine. It should be noted that passive exposure to nicotine and nicotine self-administration may have different effects on brain chemistry and brain reward function (Epping-Jordan *et al*, 1998; Jacobs *et al*, 2003; Kenny and Markou, 2006). Therefore, additional studies are warranted to investigate the function of CRF in changes in brain reward function after discontinuing chronic nicotine self-administration. Previous research has shown that extended access to nicotine self-administration, 23 h per day, leads to nicotine dependence as indicated by precipitated somatic withdrawal signs (O'Dell *et al*, 2007). Somatic withdrawal signs were not recorded in this study. However, future studies may investigate the effect of the administration of CRF receptor antagonists in specific brain sites on affective and somatic withdrawal signs as this may help to delineate the neuronal substrates underlying the negative affective and somatic withdrawal signs.

Taken together, these findings indicate that blockade of CRF1/2 receptors in the CeA and Nac Shell, but not in the lateral BNST, prevents the elevations in brain reward thresholds associated with precipitated nicotine withdrawal. These studies point toward an important function for CRF in the CeA and Nacc shell in the negative affective state associated with smoking cessation. Further studies are warranted to investigate the function of specific CRF receptor subtypes in the extended amygdala in the negative affective state associated with smoking cessation.

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DISCLOSURE/CONFLICT OF INTEREST

The author(s) declare that except for income received from primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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