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$GABA_A$ and Opioid Receptors of the Central Nucleus of the Amygdala Selectively Regulate Ethanol-Maintained Behaviors

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The present study tested the hypothesis that $GABA_A$ and opioid receptors within the central nucleus of the amygdala (CeA) regulate ethanol (EtOH), but not sucrose-maintained responding. To accomplish this, βCCt, a mixed benzodiazepine (BDZ) agonist–antagonist with binding selectivity at the α1 subunit-containing $GABA_A$ receptor, and the nonselective opioid antagonist, naltrexone, were bilaterally infused directly into the CeA of alcohol-preferring rats. The results demonstrated that in HAD-1 and P rat lines, βCCt (5–60 μg) reduced EtOH-maintained responding by 56–89% of control levels. On day 2, βCCt (10–40 μg) continued to suppress EtOH maintained responding in HAD-1 rats by as much as 60–85% of control levels. Similarly, naltrexone (0.5–30 μg) reduced EtOH-maintained responding by 56–75% of control levels in P rats. βCCt and naltrexone exhibited neuroanatomical and reinforcer specificity within the CeA. Specifically, no effects on EtOH-maintained responding were observed following infusion into the caudate putamen (CPu), a locus several millimeters dorsal to the CeA. Additionally, responding maintained by sucrose, when presented concurrently with ethanol (EtOH) or presented alone, was not altered by βCCt. Naltrexone reduced sucrose-maintained responding only under the 5 μg dose condition when sucrose was presented alone, however, it did not alter sucrose responding when given concurrently with EtOH. These results support the hypothesis that $GABA_A$ and opioid receptors within the CeA can selectively regulate EtOH-maintained responding. The CeA may represent a novel target site in the development of prototypical $GABA_A$ and opioidergic receptor ligands, which selectively reduce alcohol abuse in humans.
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INTRODUCTION

A key objective of alcoholism research is to understand the neuromechanism(s) regulating alcohol addiction and dependence in humans. Research on the neuroanatomical basis of alcohol reward has shown that the nucleus accumbens (NACC), ventral tegmental area (VTA), ventral pallidum (VP), central amygdala (CeA), and hippocampus are involved in GABAergic regulation of ethanol (EtOH) reinforcement (Hyytiä and Koob, 1995; Nowak *et al*, 1998; June *et al*, 1998a, b, 2001; Harvey *et al*, 2002). Opioidergic neurons within the NACC, CeA, and basolateral amygdala (BLA) have also been shown to regulate EtOH reinforcement (Heyser *et al*, 1999; Hyytiä and Kiianmaa, 2001). Of the alcohol-reward substrates, the CeA has emerged as an interesting candidate site for investigation of both GABA and opioid mediation of EtOH reinforcement. This is primarily due to: (1) colocalization of both GABA and opioidergic neurons within the CeA (Oertel *et al*, 1983; Honkaniemi, 1992; Veinante *et al*, 1997), and (2) evidence demonstrating that the CeA contains both efferent and afferent projections to a number of putative alcohol reward loci (Veinante *et al*, 1997; Koob *et al*, 1998a, b; McBride and Li, 1998; Sun and Cassell, 1993; Freedman and Cassell, 1994; Sun *et al*, 1994). These findings suggest the possibility that the CeA may work in conjunction with other loci to regulate alcohol-reinforced behaviors. Thus, pharmacological, neurochemical, histochemical, and connectional evidence led us to hypothesize that both GABA and opioid neuronal systems within the CeA may play an important role in regulating alcohol-motivated behaviors.

Recent evidence suggests both a regional and a receptor subtype specificity for $GABA_A$-containing receptors in regulating alcohol-motivated behaviors. Specifically, the α1 subtype in the anterior and medial VP (Harvey *et al*, 2002)

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and the α5 isoform in the CA1 and CA3 hippocampus (HPC) (June *et al*, 2001) have both been shown to be important mediators of alcohol reinforcement. Unlike the VP and HPC, equivocal research exists on the primary type of isoform within the CeA. For example, while some investigators contend that the CeA comprises of primarily receptors of the α1 subtype (Araki and Tohyama, 1992), others suggest the α2 and α3 receptors are the most predominant isoforms (Wisden *et al*, 1992; Turner *et al*, 1993; Fritschy and Möhler, 1995). Thus, given the proposed subunit composition of the GABA receptors within the CeA, pharmacological compounds capable of exploiting the α1, α2, and α3 subunit-containing GABA$_A$ receptors represent optimal tools to evaluate the role of the GABA$_A$ receptors in alcohol reinforcement.

β-carboline-3-carboxylate-*t*-butyl ester (βCCt) is a mixed benzodiazepine agonist–antagonist ligand with binding selectivity at the α1 receptor (Cox *et al*, 1995; Carroll *et al*, 2001; Harvey *et al*, 2002). However, βCCt also exhibits some affinity (albeit lower) for both the α2 and α3 receptors (Cox *et al*, 1995; Carroll *et al*, 2001; Harvey *et al*, 2002) (for a review, see Harvey *et al*, 2002). Behavioral studies in several species (eg, rats, mice, primates) show that βCCt is a BDZ antagonist exhibiting competitive binding site interaction with BDZ agonists over a broad range of doses (Shannon *et al*, 1984; Griebel *et al*, 1999; Cox *et al*, 1998; Carroll *et al*, 2001, 1991; Rowlett *et al*, 2001; Paronis *et al*, 2001). Other studies show that βCCt produces anxiolytic effects in rodents (Carroll *et al*, 2001) and potentiates the anticonflict response induced by α1 subtype agonists in primates (Paronis *et al*, 2001). Thus, βCCt displays an agonist or antagonist profile depending on the behavioral task, species, and dose employed. Further, recent work in our laboratory (Lüddens, June, and Cook, in preparation) employing human HEK cells has shown that βCCt's *in vivo* agonist activity may be explained by its potent agonist activity at the α2 and α3 receptor subtypes. Hence, it is possible that βCCt might reduce alcohol responding by increasing GABAergic activity/substituting for alcohol's positive reinforcing effects. A number of researchers have demonstrated that positive GABAergic modulators are readily substituted for EtOH in drug discrimination studies (Bowen and Grant, 1998; Hodge and Cox, 1998).

To date, a review of the literature reveals that only one study has *directly* (ie central administration) implicated GABAergic (Hyytiä and Koob, 1995) receptors of the CeA in mediating alcohol reinforcement. Hyytiä and Koob (1995) reported that microinjection of the competitive GABA$_A$ receptor antagonist, SR 95531, in the CeA led to marked reductions in EtOH-maintained responding, while water responding was not affected. A cumulative response analysis revealed that SR 95531 did not suppress EtOH responding during the first few minutes of the operant session. Rather, it produced a brief delay until about the 5-min interval, suggesting that the antagonist did not disrupt the initiation of responding (Hyytiä and Koob, 1995).

In contrast to the magnitude of GABA receptors within the CeA, both *in situ* hybridization and *in vitro*/quantitative autoradiographic studies reveal that very low, or no detectable levels of the μ and δ opioid receptors exist within the CeA (Mansour *et al*, 1987; Paden *et al*, 1987; McBride and Li, 1998; Mansour *et al*, 1995). However, despite these neuroanatomical findings, one study has *directly* implicated the opioid receptors of the CeA in mediating alcohol reinforcement (Heyser *et al*, 1999). Specifically, Heyser *et al* (1999) demonstrated that microinjections of methynaloxonium, an opiate antagonist, into the CeA produced significant reductions in alcohol responding. In contrast, methynaloxonium failed to alter water responding. In cumulative time course analyses, the data revealed that the opiate antagonist did not block the onset (0–3 min) of responding, but produced a delayed effect (5–10 min) on responding. These findings are similar to the results obtained by Hyytiä and Koob (1995). Thus, time course analyses and cumulative record profiles are useful, as they may shed light on the magnitude and duration of antagonism produced by a pharmacological agent across an operant session. In addition, they may provide useful information about neuroregulatory processes (eg onset, maintenance, termination), and their potential neuromechanisms of action (Hodge *et al*, 1995; June, 2002).

Taken together, the above studies suggest a role for the GABA$_A$ and opioid receptor of the CeA in regulating alcohol reward. Further, activational (Morales *et al*, 1998; Porrino *et al*, 1998) and lesion studies (Möeller *et al*, 1997) also indirectly support a role for GABA and opioid neurotransmission in alcohol reinforcement. However, the current literature shows a very limited amount of behavioral research directly implicating GABA and opioid systems within the CeA in alcohol reinforcement (McBride, 2002). In addition, neuroanatomical data reveal a paucity of opioid receptors within the CeA. Thus, it could be argued that a direct role for opioid, and GABA to a lesser extent, in regulating alcohol reinforcement via the CeA can be questioned. Hence, additional studies are warranted to further elucidate the functional role of both opioid and GABA receptors in regulating alcohol reinforcement.

In addition to the scarcity of research studies on opioid and GABAergic regulation of alcohol drinking within the CeA, none of the previous studies have investigated the role of GABA and opioid receptors in regulating alcohol-maintained responding compared with other solutions that have intrinsic reinforcing properties (eg sucrose, saccharin). Further, little research is available comparing pharmacological manipulations of the CeA with similar infusions to other neuroanatomical sites. Therefore, the present study had two goals. The first was to determine whether the GABA$_A$ and opioid receptors within the CeA are selectively involved in EtOH-seeking behavior by comparing the effects of antagonist microinfusions on lever-press responding when EtOH or sucrose were the sole reinforcer, to when both were presented concurrently. To accomplish this, we used βCCt, a mixed BDZ agonist–antagonist with binding selectivity at the α1 receptor, albeit it also exhibits some affinity for the α2 and α3 receptors (Cox *et al*, 1998). To investigate the role of the opioid receptors, we used naltrexone, a nonselective opioid antagonist. The second goal was to determine if these effects were specific to the CeA. This was accomplished by comparing the effects of these agents in the caudate putamen (CPu), a control substrate several millimeters dorsal to the CeA. The CPu has not been reported to play a role in alcohol reinforcement (McBride and Li, 1998; Koob *et al*, 1998a, b; Koob and Le Mol, 1997).

MATERIALS AND METHODS

Subjects

The alcohol-preferring (P) (49th generation) and high-alcohol drinking rats (replicate line #1) (HAD-1) (30th generation) were used to model the human condition of alcohol abuse. Both rat lines have been suggested to be acceptable animal models of the 'acute-reinforcing properties' of alcohol-seeking behavior in humans to the satisfaction of the alcohol research community (Cloninger, 1987; Lumeng *et al*, 1995; McBride and Li, 1998; Murphy *et al*, 2002). Male ($N=30$) and female ($N=19$) alcohol-preferring (P) rats and male ($N=3$) and female ($N=4$) high-alcohol drinking 1 (HAD-1) rats were used in the study. No effects of estrous cycle on EtOH drinking patterns in P (McKinzie *et al*, 1996) and HAD (unpublished data) rats have been observed. Further, previous research has revealed that both male and female HAD and P rat lines are equally sensitive to the antagonizing effects of opiate and $GABA_A$ receptor agents on EtOH-maintained responding, following both systemic and microinfusion studies (June *et al*, 1998c; June, 2002). Thus, to extrapolate the results of our study to alcoholics of both genders, we use both male and female P and HAD rats.

Previous work has suggested that the P and HAD lines are similar in terms of the expression of GABAergic terminals in putative EtOH-reward loci (Hwang *et al*, 1990); however, no evaluation of amygdaloid nuclei has been carried out. Hence, the functional significance of none of the amygdaloid nuclei in P and HAD rats in regulating alcohol-motivated behaviors is known.

At the beginning of the study, the P and HAD rats weighed between 200 and 220 g. The animals were individually housed in plastic cages in a vivarium at 21°C on a 12 h light/dark cycle. Food and water were provided *ad libitum* for the animals, except for the conditions noted under the training phase.

Apparatus

Animals were tested in 15 standard operant chambers (Coulbourn Instruments, Inc, Lehigh Valley, PA) equipped with two levers and two dipper assemblies. Red, yellow, and green cue lights were used to indicate the presence of a reinforcer. The lights were illuminated for 2.5 s. Each reinforced response delivered 0.1 ml of the reinforcer. The reinforcer was presented for a duration of 3 s. Operant sessions were 60 min in length. The operant chamber parameters used to assess the various reinforcers are well established in our laboratory (see June, 2002).

Drugs and Solutions

The EtOH (USP) (10% v/v) and sucrose (Fisher Scientific) solutions (2% w/v and 10% w/v) were prepared in deionized water for the operant chamber as previously described for oral self-administration (June *et al*, 1998a, b; 2001). βCCt and naltrexone hydrochloride (Sigma-Aldrich, St Louis, MO) were mixed immediately before the operant sessions in artificial cerebrospinal fluid as previously described (see June *et al*, 2001 for details). βCCt was synthesized by one of the authors (JC).

The doses depicted in the graphs represent the total injected dosages (ie the doses summed from bilateral injections of both the left and right hemispheres).

Behavioral Training Paradigms

Three different fluid consumption paradigms were used in the present study: (1) EtOH (10% v/v) presented as the sole reinforcer available; (2) sucrose (10% w/v) presented as the sole reinforcer; and (3) EtOH (10% v/v) and sucrose (2% w/v) concurrently available. The training procedure used for each paradigm is briefly discussed below.

EtOH as the Sole Reinforcer

Rats were trained to lever press for EtOH (10% v/v), using a modified version of the sucrose fading technique (June *et al*, 2001; June, 2002; Harvey *et al*, 2002). For all training protocols, animals were water-deprived on the first 3 days of training, using a 23 h fluid deprivation schedule to facilitate lever-pressing. Initially, the rats lever pressed for sucrose (10% w/v) under a Fixed-Ratio 1 (FR1) schedule for 5–7 days. Each lever press delivered 0.1 ml of the reinforcer. During the second phase of the training, the animals lever-pressed for an EtOH and sucrose cocktail mixture under an FR1 schedule. The concentration of sucrose was decreased in a stepwise manner (10, 8, 6, 4, 2, and 0%), and the EtOH was increased in a similar manner (2, 4, 6, 8, and 10%) over 5–7 days. Subsequently, the rats were responding under an FR4 schedule for EtOH (10% v/v) on both the right and left levers. The animals continued to lever-press for EtOH (10% v/v) until their responses stabilized, which was defined as having daily responses within $\pm$ 20% of the average responses for 5 consecutive days.

Sucrose as the Sole Reinforcer

Initially, the rats were responding for sucrose (10% w/v) under an FR1 schedule for 5–7 days. Subsequently, the rats were responding under an FR4 schedule for sucrose (10% w/v) on both the right and left levers. The rats continued to respond for sucrose on the designated schedule until their responding stabilized.

Concurrent Schedule: EtOH and Sucrose

The rats were trained to lever-press for EtOH (10% v/v) using the same procedure used in the paradigm, where EtOH (10% v/v) was presented as the sole reinforcer. When the responding for EtOH (10% v/v) was stabilized, sucrose (2% w/v) was then presented concurrently with the EtOH solution. Subsequently, the rats were responding under an FR4 schedule for sucrose (2% w/v) on one lever and EtOH (10% v/v) on the second lever. To avoid the establishment of a position preference, the solution trays were rotated daily (see June, 2002). In an attempt to equate baseline (BL) responding for both EtOH (10% v/v) and sucrose, a 2% w/v concentration was selected. The rats continued to respond for both EtOH (10% v/v) and sucrose (2% w/v), until their responses were stabilized.

Blood Alcohol Concentration (BAC) Measurement

To ensure that animals were consuming pharmacologically relevant amounts of EtOH during operant sessions, BACs were collected in a subset of animals on days that animals did not receive drug treatment. After the first 20 min of an operant session, approximately 100 μl of whole blood was collected from the rats' tail tip into a heparin-coated microsample tube. The BAC samples were collected at the 20 min time point, since the majority of EtOH-maintained responding typically occurs during the first half of the operant session (June *et al*, 2001; Harvey *et al*, 2002; June, 2002). In addition, an attempt was made to determine the rising phase of the BAC since it has been demonstrated that the rising, but not the falling phase of the blood alcohol curve, correlated significantly with the euphoric properties of EtOH (Lewis and June, 1990). After collection, the whole blood was immediately centrifuged for 5 min at 1100 rpm. Plasma samples of 5 μl were collected with a Gilson Microman M-25 Pipette and injected directly into a GL-5 Analyzer (Analox Instruments; Luxenburg, MA). Micro-analysis consisted of measuring the oxygen consumption in the reaction between the sample of alcohol and alcohol oxidase (AOD), using a Clark-type amperometric oxygen electrode. Alcohol reagent buffer solutions (pH 7.4) and alcohol oxidase enzymes were used in all the samples tested. The results were calculated in units of mg/dl and printed within 20 s of each trial. To assure the accuracy of the measurement, each BAC sample was calibrated twice. The mean of the two samples was used as an index of the level of BAC content for a given rat. Single point calibrations were carried out using 5 μl of aqueous 100 mg/dl (21.7 mmol/l) standard.

Surgery

The animals were anesthetized using sodium pentobarbital (40 mg/kg). After the animals were anesthetized and placed into the stereotaxic apparatus, 22-gauge guide cannulas were surgically implanted bilaterally in the CeA or CPu. The coordinates for the CeA were as follows: AP: −2.0; ML: ± 3.6; DV: −8.5. The coordinates for the CPu were as follows: AP: −2.0; ML: ± 4.9; DV −6.5. In experimental and control animals, the cannulas were aimed 1 mm above the intended brain locus. Cannulas were attached to the skull with anchor screws and dental cement. The animals were allowed 7 days to recover before the continuation of the operant conditioning phase.

Infusion Procedures and Drug Treatments

βCCt and naltrexone were administered 5 min before the 1 h operant session to allow for optimal absorption and CNS distribution. Both agents were bilaterally infused into the target brain loci at a rate of 0.1 μl/min for 5 min, using a Harvard Model C infusion pump (Harvard Instruments, South Natick, MA). Thus, the infusion volume per hemisphere was 0.5 μl, and the total infusion volume was 1 μl. The pH of the drug was determined prior to infusion into the brain loci. The pH was adjusted to 7.4 ± 0.2. During the infusion procedure, the rats were allowed to freely move about their homecage. The microinjector tips were left in place for an additional minute to allow for the entire dose of the drug to reach the target brain loci. βCCt was tested at doses of 1–60 μg, while naltrexone was tested at doses of 1–30 μg. All doses were administered in a randomized design along with the aCSF control infusion. The doses of βCCt were selected based on prior work in our laboratory, showing that they were effective in reducing lever-pressing for EtOH in the VP (Yin *et al*, 2001; June *et al*, 2003). The doses of naltrexone used were selected based on prior work showing that they were effective in reducing the lever-pressing for EtOH in the NACC and VTA (Cummings *et al*, 1999). The range of βCCt and naltrexone doses employed in the present study depended on the contingencies of the operant session. For example, it is well established that a leftward dose–response shift occurs with agents that reduce EtOH-maintained responding (eg opioid antagonists, GABA/BDZ antagonists) when alcohol is presented concurrently with a highly palatable solution (eg saccharin, sucrose) compared with when EtOH is presented alone (June, 2002). Hence, the range of βCCt and naltrexone doses for a given rat depended on the experimental group (eg EtOH alone, sucrose alone, EtOH + sucrose). A minimum of 4 and a maximum of 5 days were allocated between drug treatments to permit animals to return to control levels (eg BL, aCSF). This period prevented confounding of drug treatments due to residual effects. Previously, we observed that βCCt was effective 24 h postdrug administration in reducing EtOH responding in HAD, but not P rats within the VP (Yin *et al*, 2001; June *et al*, 2003). Based on these findings, we hypothesized that βCCt would produce a similar effect in the CeA. In the present study, when residual drug treatment effects were observed, these data were recorded and evaluated relative to the control conditions. Each animal received $\leqslant 7$ infusions in an attempt to minimize damage to the brain tissue. A maximum of two infusions were given per week. All drug infusions were given in a randomized design. The doses depicted in the graphs represent the total rat dose (ie the dose summed from bilateral injections).

Histology

After the behavioral component of the experiment was completed, the animals were killed using CO_2 inhalation. Prior to decapitation, cresyl violet was infused into the injection site at a rate of 0.1 μl/min for 30 s. This helps to identify the placement of the cannulae. The brains were immediately removed and frozen. Within 48 h, the brains were sliced at 45-μm sections using a cryostat. The slides were then stained with cresyl violet and the placement of the cannulae was verified using the Paxinos and Watson (1998). The placements of the cannulae were independently determined by two individuals who were blind to the behavioral data for the rats. Rats with misplacements were excluded from the statistical analysis.

Data Analysis

Four experiments were conducted in the present study. In experiments 1–4, a repeated-measures ANOVA for consumption type and drug dose was conducted. All drug treatment comparisons were made against the no-injection

BL condition and aCSF control condition (see June, 2002; June *et al*, 2001; Harvey *et al*, 2002). The BL condition comprised an average of 6 days. Three of these days were determined before the animals received *any* drug treatment, while the other 3 days were taken 3–4 days after animals had received a particular drug treatment. For example, if βCCt was given at 60 μg on Monday, the fourth BL day condition was taken on Thursday or Friday. On Saturday, βCCt would then be administered at 20 μg, while the fifth BL day would be obtained on Tuesday or Wednesday. Finally, on Thursday βCCt would be infused at 40 μg, and on Sunday or Monday, the sixth BL day would be obtained. The BL condition ensured two things: first, a shift in the BL over the course of the experiment did not affect the data analysis, and secondly, no residual drug treatment effects were present prior to the administration of a subsequent treatment. The criterion for accepting/defining a self-administration day as a 'true' BL day was that daily responses were within $\pm$ 20% of the average responses for five consecutive days prior to any drug treatment (for details see June, 2002). A BL2 was also calculated in all experiments; however, it was not used in the final data analyses. The BL2 condition assessed residual effects 1 week after the *final* drug treatment in all experiments. The BL2 condition comprised an average of 5-days 1-week post-administration of the final drug treatment. When the aCSF condition was compared against the BL1 and BL2 conditions and the BL1 and BL2 conditions were compared against each other, this design permitted an evaluation of the effectiveness of our drug treatments, as well as the sensitivity/robustness of our operant training procedures across the entire duration of the study (see June *et al*, 2001; June, 2002). The aCSF condition consisted of data from a *single* session, in which vehicle was randomly infused into the targeted brain loci. With the exception of the sucrose-alone study (Experiment 2), none of the control conditions were significantly different from each other.

RESULTS

Experiment 1 (EtOH alone in P rats)

Histology. Figure 1a shows a reconstruction of serial coronal sections of the rat brain, illustrating the location of the bilateral microinjection cannulas in the CeA for the P rats in the EtOH self-administration group ($N = 13$). The cannula tracks were well localized in the CeA. The reconstructions in these and subsequent coronal sections represent the placements for all injection sites in a particular experiment. Figure 2a–b depicts the actual bilateral placements for two of the 13 CeA rats in separate photomicrographs, illustrating the extent of the lesion sustained as a result of the bilateral guide cannula. Three rats from the EtOH CeA group were excluded from the final data analyses due to improper placements.

Blood EtOH Content (BAC) Determination

Body weights of the P ($N = 5$, EtOH-only group (males); $N = 5$, concurrent EtOH and sucrose group (males) and HAD-1; and $N = 6$, EtOH-only group (males, $n = 3$; females, $n = 3$)) rats used for BAC determination ranged from 210 to 220 g. BACs were collected on days that no drug treatments were administered in both rat lines. EtOH responding for P rats yielded intakes of 0.77–2.81 g/kg of absolute EtOH. Consumption in milliliters was 1.56–5.43. BACs ranged from 18 to 88 mg/dl. BACs correlated significantly with EtOH responding ($r = 0.84$, $p < 0.01$) and intake ($r = 0.89$, $p < 0.01$). For the HAD-1 rats, alcohol responding yielded intakes of 0.56–1.91 g/kg of absolute EtOH. EtOH consumption in milliliters was 0.92–5.23. BACs ranged from 23 to 82 mg/dl. BACs correlated significantly with EtOH responding ($r = 0.88$, $p < 0.01$) and intake ($r = 0.86$, $p < 0.01$).

βCCt drug treatments. Figure 3a shows the rate of responding maintained by EtOH following bilateral microinfusions of βCCt (20.0–60.0 μg) into the CeA. All doses of βCCt (20.0–60.0 μg) suppressed EtOH-maintained responding. Compared with the control conditions (ie BL, aCSF), responding was reduced by 45–91%, yielding a significant main effect of drug treatment ($F(4, 48) = 16.274$, $p < 0.0001$). The Newman–Keuls *post hoc* test confirmed that all doses (20.0–60.0 μg) significantly reduced EtOH-maintained responding ($p < 0.01$). As our previous research showed that βCCt was effective in reducing EtOH responding 24-h postdrug administration in the VP (June *et al*, 2003), we also evaluated βCCt's action in the present study 24-h postdrug administration following CeA infusions. In contrast to the VP, no effects were observed 24-h postdrug administration in the CeA with any of the drug doses ($F(4, 48) = 1.074$, $p > 0.05$).

Cumulative response profile. Figure 3b illustrates the cumulative time course profile across the 60-min interval for responding maintained by EtOH. The cumulative time course data and all subsequent time course analyses were evaluated using a two-way ANOVA with repeated measures over both time interval and treatment. The cumulative 6×5 analyses for EtOH yielded significant main effects of interval ($F(5, 72) = 3.882$, $p < 0.0036$) and treatment ($F(4, 288) = 80.4$, $p < 0.00001$). Under the control conditions, approximately 41% of the total EtOH-maintained responding occurred during the initial 10 min of the operant session, and 81% occurred by the end of the 30-min interval. In comparison with the control conditions, the 40 and 60 μg doses of βCCt suppressed responding for EtOH during the initial 10-min interval ($p < 0.05$), and this suppression was maintained throughout the remainder of the session ($p < 0.05$). Little, if any, additional suppression responding was observed. The 20.0 μg dose also suppressed EtOH-maintained responding during the initial 10 min ($p < 0.01$). This suppression continued via the remainder of the session ($p < 0.05$), but was attenuated from the 20 to 60-min intervals.

Naltrexone drug treatments. Figure 3c shows the effect of bilateral microinfusions of naltrexone (0.5–5.0 μg) into the CeA on EtOH-maintained responding. Due to the loss of cannulae, the number of subjects in the 5 μg treatment condition was substantially decreased ($N = 7$), while the number of subjects in the other treatment conditions was the same as reported in the βCCt condition ($N = 13$). As a result, two separate repeated measures ANOVA were performed on the data. One for the 5 μg treatment condition

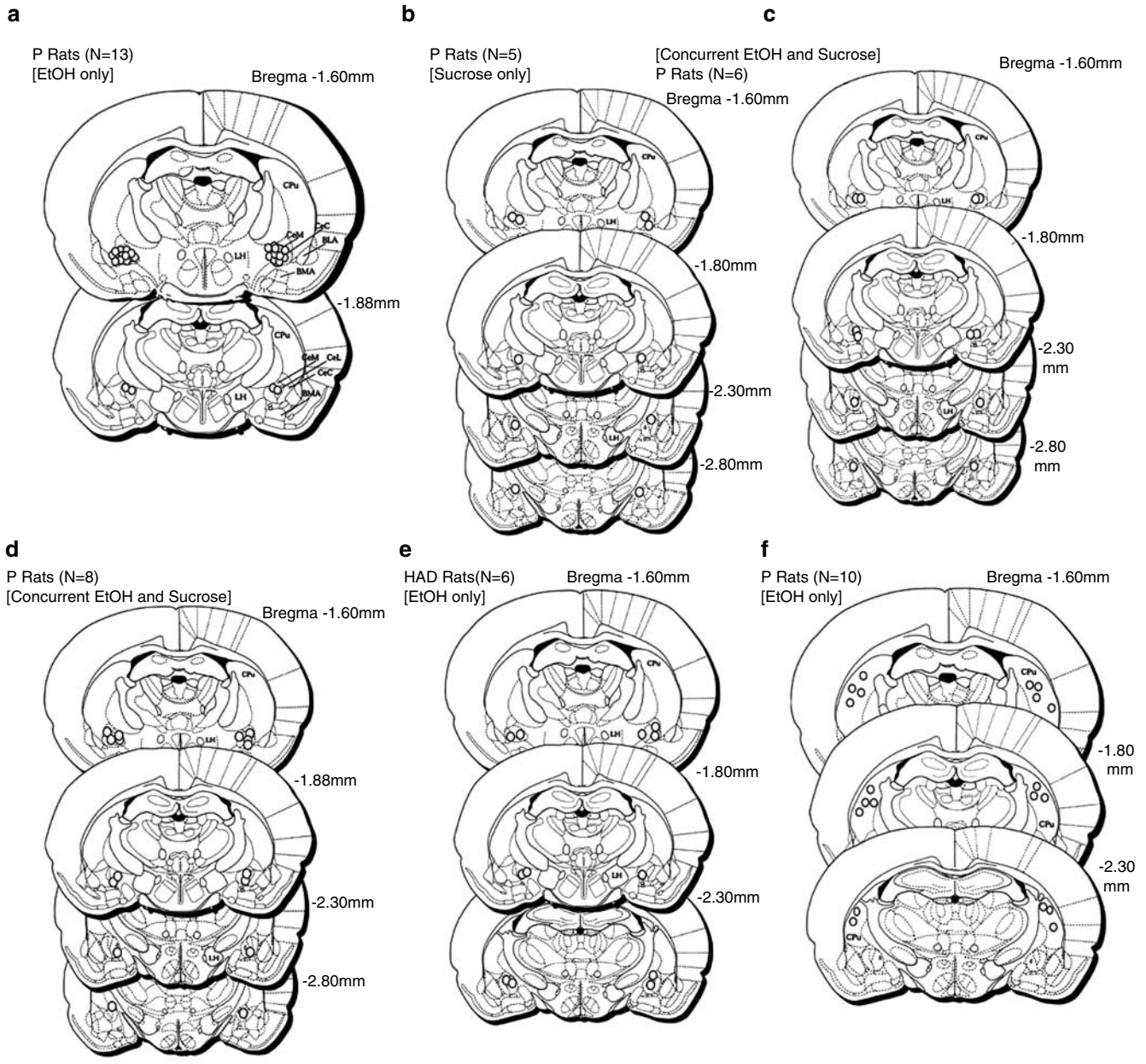

Figure 1 (a–e) Reconstruction of serial coronal sections of the rat brain illustrating the bilateral guide cannula tips of the CeA (central to medial division) and (f) caudate putamen (CPu). Each rat is represented by two open circles: one in the left, and one in the right hemisphere. CPu = caudate putamen; CeM = central amygdala, median division; CeC = central amygdala, central division; BLA = basolateral amygdala; BMA = basomedial amygdala; LH = lateral hypothalamus. Coronal sections are adapted from the rat brain atlas of Paxinos and Watson (1998) for this and subsequent figures.

compared with the control conditions, and the second for the 0.5 and 1.5 μg dose conditions compared with the control conditions. Bilateral microinfusion of the 5.0 μg dose of naltrexone produced significant reductions in EtOH-maintained responding. Compared with the control conditions, responding was reduced with the 5.0 μg dose by 56%. A significant main effect for drug treatment emerged from these data ($F(1,6) = 6.946$, $p < 0.05$). Similarly, compared with the control conditions, the 0.5 and 1.5 μg doses reduced responding by 56–60%, resulting in a significant main effect for drug treatment ($F(3,36) = 7.316$, $p < 0.0006$). However, the maximum suppression was observed with the 0.5 μg dose, insofar as no further suppression was observed as the dose was increased. The Newman–Keuls *post hoc* test confirmed that all doses of naltrexone significantly reduced EtOH-maintained responding in comparison with the two control conditions ($p < 0.05$).

Cumulative response profile. Figure 3d illustrates the cumulative time course profile across the 60-min operant session for responding maintained by EtOH. The 5.0 μg dose ($N = 7$) cumulative time course analysis was conducted in a separate analysis from the other treatment conditions ($N = 13$). The cumulative 6×2 analysis for the 5.0 μg dose yielded significant main effects of interval ($F(5,36) = 2.830$, $p < 0.05$) and treatment ($F(1,36) = 25.822$, $p < 0.0001$).

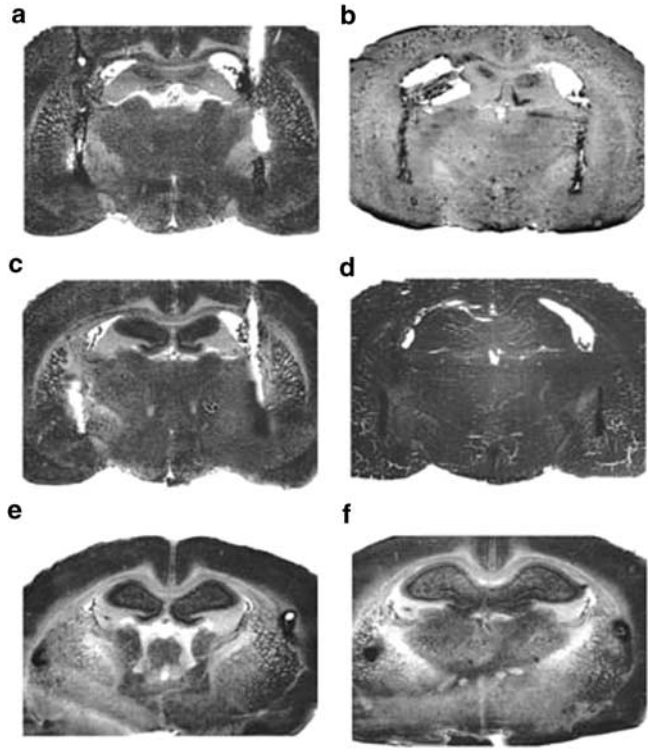

Figure 2 (a–f) shows examples of representative histological photomicrographs for the six groups illustrating the guide cannulae tracks and the magnitude of cellular damage caused by the bilateral cannula implantation.

The cumulative 6×4 analyses for the BL, aCSF, 0.5, and 1.5 μg conditions yielded significant main effects for interval ($F(5, 71) = 3.983$, $p < 0.0031$) and treatment ($F(3, 213) = 32.44$, $p < 0.0001$). Approximately 44% of the total EtOH-maintained responding occurred during the initial 10 min of the operant session, and 85% occurred by the end of the 30 min of the operant session under control conditions. In comparison with the control conditions, the 0.5–5.0 μg doses of naltrexone suppressed responding during the initial 10 min of the operant session ($p \leqslant 0.05$) and the suppression was maintained throughout the remainder of the session, for all doses ($p < 0.05$).

Experiment 2 (Sucrose Alone in P Rats)

Histology. Figure 1b shows a reconstruction of serial coronal sections of the rat brain, illustrating the location of the bilateral microinjection cannulas in the CeA for the P

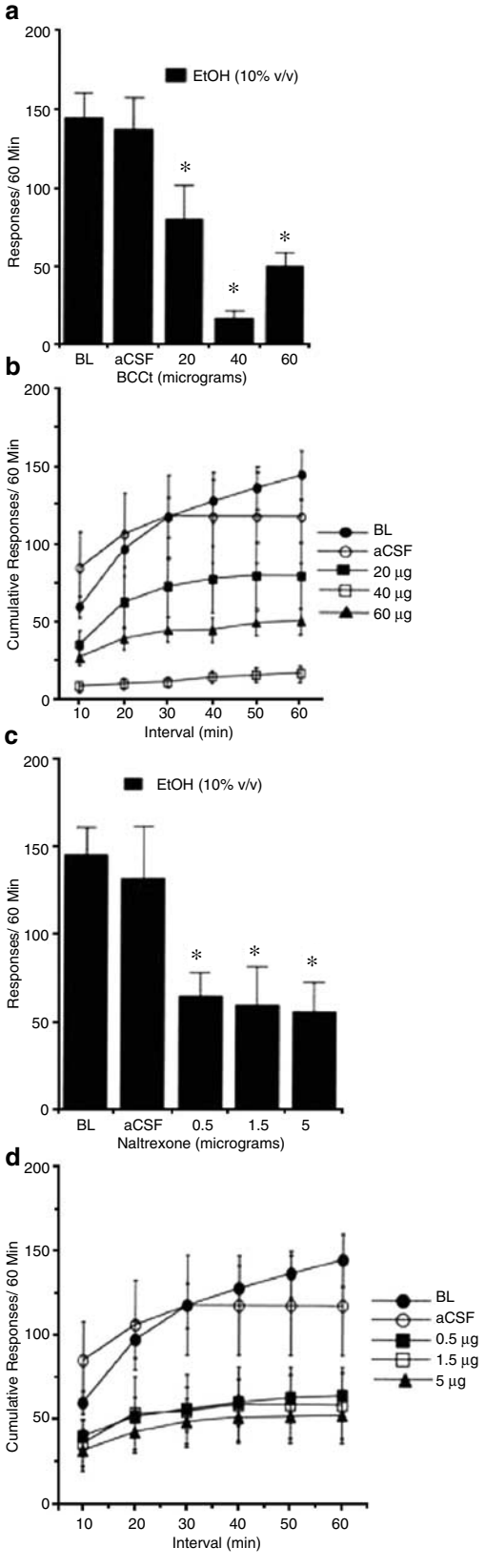

Figure 3 Effects of βCCt (0.0–60.0 μg) following bilateral microinjection in the CeA on responding maintained by EtOH (10% v/v) during a 60-min operant session (a) ($N = 13$). Data are shown as mean (± SEM). The total 60-min operant data were evaluated using a one-way ANOVA with the dose as the factor. *$P < 0.01$ *vs* aCSF, BL by ANOVA and the Newman–Keuls *post hoc* test ($N = 13$). (b) Cumulative time course profile for EtOH (10% v/v) across the six 10-min intervals. The effects of βCCt were observed primarily on the first 10-min interval (ie initiation phase of EtOH responding) ($p < 0.05$) (see methods for additional time course statistics). Effects of naltrexone (0.5–5.0 μg) following bilateral microinjection in the CeA on responding maintained by EtOH (10% v/v) during a 60-min operant session (c) ($N = 13$) for the 0.5 and 1.5 μg conditions, while $N = 7$ for the 5.0 μg condition. Data are shown as mean (± SEM). The total 60-min operant data were evaluated using a one-way ANOVA with the dose as the factor. *$P < 0.01$ *vs* aCSF, BL by ANOVA and the Newman–Keuls *post hoc* test. (d) The cumulative time course profile for EtOH (10% v/v) across the six 10-min intervals. The effects of naltrexone were observed primarily on the first 10-min interval (ie initiation phase of EtOH responding) ($p < 0.05$) (see methods for additional time course statistics).

rats in the sucrose group ($N=5$). The cannula tracks were well localized in the CeA. Figure 2c–d depicts the actual bilateral placements for two of the five CeA rats in separate photomicrographs, illustrating the extent of the lesion sustained as a result of the bilateral guide cannula. Two rats from the CeA sucrose group were excluded from the final data analyses due to improper placements.

βCCt drug treatments. Figure 4a shows the effect of bilateral microinfusions of βCCt (20.0–60.0 μg) into the CeA on sucrose-maintained responding. While βCCt produced elevations in sucrose responding with selected doses, these effects failed to reach statistical significance ($F(4,16)=1.67$, $p>0.08$). Further, no effects were observed 24-h postdrug administration in the CeA with any of the drug doses ($F(4,16)=1.02$, $p>0.05$).

Naltrexone drug treatments. Figure 4b shows the rates of responding maintained by sucrose following bilateral microinjections of naltrexone (0.25–5.0 μg). Naltrexone in doses from 0.25 to 2.5 μg was without effect on sucrose-maintained responding. In contrast, the 5.0 μg dose produced a 50% suppression on responding, contributing to a significant main effect of drug treatment ($F(5,20)=3.120$, $p<0.05$). The *post hoc* test confirmed that the 5.0 μg dose significantly reduced sucrose-maintained responding ($p<0.01$).

Cumulative response profile. Figure 4c illustrates the cumulative time course profile across the 60-min interval for responding maintained by sucrose. The cumulative 6×6 analysis for sucrose yielded significant main effects of interval ($F(5,24)=50.4$, $p<0.0001$) and treatment ($F(5,120)=12.4$, $p<0.00011$). Under the control conditions, approximately 30% of the total sucrose-maintained responding occurred during the initial 10 min of the operant session and 58% occurred by the end of the 30-min interval. The 5.0 μg naltrexone dose suppressed sucrose responding during the 30–60 min of the session ($p<0.01$), but was similar to control levels at the 10–20-min intervals ($p>0.05$).

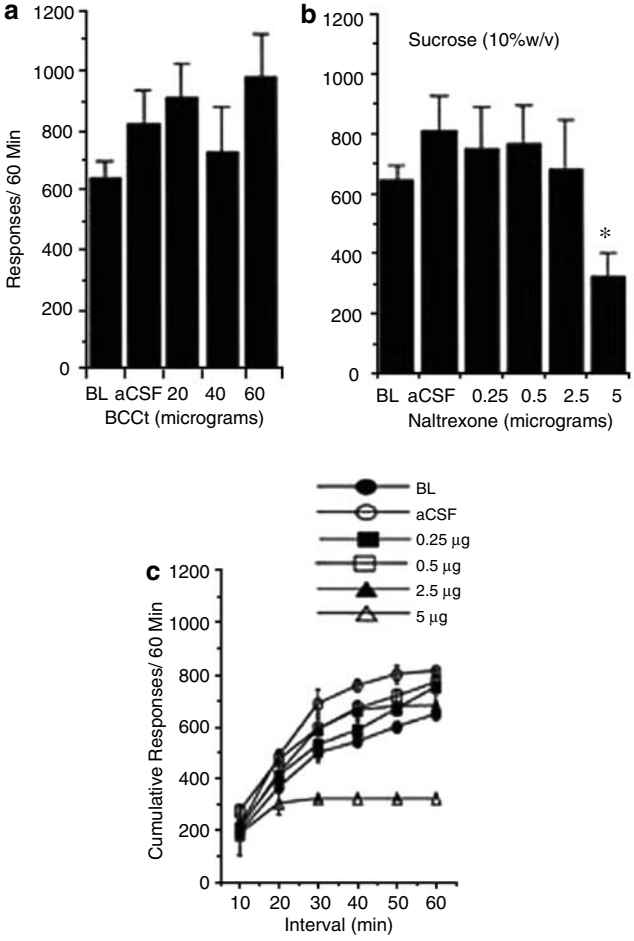

Figure 4 Effects of (a) βCCt (0.0–60.0 μg) and (b) naltrexone (0.0–5.0 μg) following bilateral microinjection in the CeA on responding maintained by sucrose (10% w/v) during a 60-min operant session ($N=5$). Data are shown as mean (± SEM). The total 60-min operant data were evaluated using a one-way ANOVA with the dose as the factor. *$P<0.01$ *vs* aCSF, BL by ANOVA and the Newman–Keuls *post hoc* test. (c) Cumulative time course profile for sucrose (10% w/v) following naltrexone across the six 10-min intervals. The effects of the 5.0 μg naltrexone dose on sucrose responding were not observed until the 30-min interval, and thereafter sustained throughout the remainder of the 60-min session ($p<0.05$) (see methods for additional time course statistics).

Experiment 2 [Concurrent Schedule Presentation of EtOH and Sucrose in P Rats]

Histology. Figure 1c shows a reconstruction of serial coronal sections of the rat brain, illustrating the location of the bilateral microinjection cannulas in the CeA of P rats in the initial concurrent study ($N=6$). The cannula tracks were well localized in the CeA. One rat from the initial concurrent CeA group was excluded from the final data analyses due to improper placement.

βCCt drug treatments. Figure 5a shows the rate of responding for EtOH and sucrose following microinjections of βCCt (1.0–5.0 μg) into the CeA. All doses produced a marked suppression of EtOH-maintained responding; however, the effects were not dose related. That is, the 5.0 μg dose was no more effective than the 1.0 μg dose. Compared with the control conditions, responding for EtOH was reduced by 63–83%. A significant main effect for drug treatment emerged from these data ($F(4,20)=5.359$, $p<0.0042$). The Newman–Keuls *post hoc* test confirmed that βCCt significantly reduced EtOH-maintained responding with all doses ($p<0.01$). However, no effects were seen on responding maintained by sucrose ($F(4,20)=0.757$, $p<0.57$). The 5.0 μg dose of βCCt approached, but did not reach statistical significance for the 60-min session data (but, see time course data, Figure 5c). In addition, no effects were observed 24-h postdrug administration with any of the drug doses on EtOH ($F(4,20)=0.137$, $p<0.05$) or sucrose ($F(4,20)=0.896$, $p<0.05$) responding.

Cumulative response profile. Figure 5b and c illustrates the cumulative time course profile for EtOH and sucrose-maintained responding, respectively, across the 60-min interval. The cumulative 6×5 analysis for EtOH yielded significant main effects for interval ($F(5,30)=2.973$, $p<0.0269$) and drug treatment ($F(4,120)=26.4$,

$p<0.00001$). Approximately 37% of the total EtOH-maintained responding occurred during the initial 10 min of the operant session, and 69% occurred by the end of the 30-min interval under the control conditions. In comparison with the control conditions, all doses of βCCt (1.0–5.0 μg) suppressed responding for EtOH during the initial 10-min interval ($p<0.05$), and this suppression was maintained throughout the remainder of the session ($p<0.05$). The cumulative analyses for sucrose also yielded significant main effects for interval ($F(5,30)=4.97$, $p<0.01$) and drug treatment ($F(4,120)=15.6$, $p<0.001$). Compared with the control condition, the 1 μg dose elevated responding at the 30–60-min interval ($p<0.05$), while the 2.5 μg dose elevated responding at the 50–60-min intervals ($p<0.01$). In contrast, the 5.0 μg markedly suppressed sucrose responding at the 30–60-min intervals ($p<0.01$).

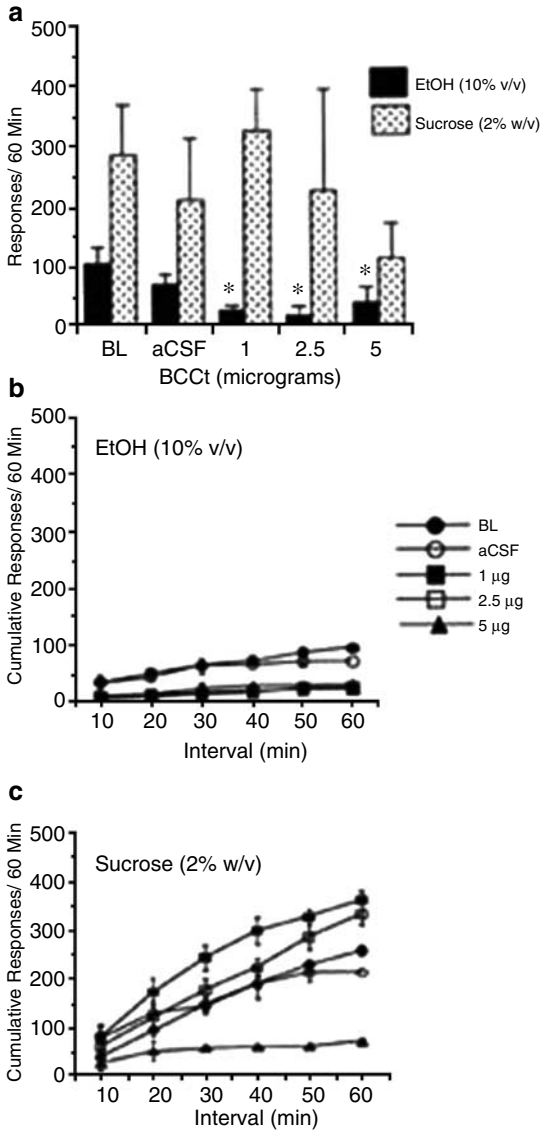

Figure 5 Effects of βCCt (0.0–5.0 μg) (a) on a concurrent fixed-ratio (FR4) schedule for EtOH (10% v/v) and sucrose (2% w/v)-maintained responding during a 60-min operant session ($N=6$). Data are shown as mean (± SEM). The total 60-min operant data were evaluated using a one-way ANOVA with the dose as the factor. *$P<0.01$ *vs* aCSF, BL by ANOVA and the Newman–Keuls *post hoc* test. The cumulative time course profile for EtOH (10% v/v) (b) and sucrose (2% w/v) (c) following βCCt (0.0–5.0 μg) across the six 10-min intervals. The suppressant effects of βCCt on EtOH responding were observed during the initial 10-min interval (ie initiation phase of EtOH responding) ($p<0.05$) (see methods for additional time course statistics). Thereafter, the suppression was maintained throughout the remainder of the session ($p<0.05$). In contrast, the suppression seen with the 5.0 μg dose on sucrose responding was not seen until the 30-min interval, albeit once observed, it was sustained for the remainder of the operant session.

Naltrexone drug treatment. Figure 6a shows responding maintained by concurrent presentation of both EtOH and sucrose, following bilateral microinjections of naltrexone (2.5–5.0 μg) into the CeA. Naltrexone produced a marked suppression of EtOH-maintained responding. Responding was reduced by 74–87% compared with the control

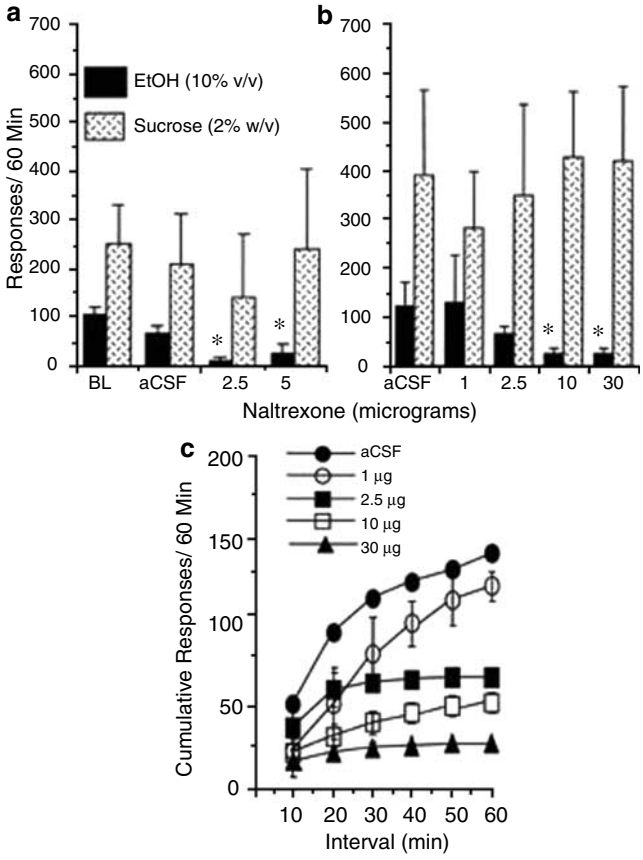

Figure 6 Effects of naltrexone in P rats during the first (0.0–5.0 μg) ($N=6$) (a), and second (0.0–30.0 μg) ($N=8$) study (b), on a concurrent fixed-ratio (FR4) schedule for EtOH (10% v/v) and sucrose (2% w/v) responding during a 60-min operant session. Data are shown as mean (± SEM). The total 60-min operant data were evaluated using a one-way ANOVA with the dose as the factor. *$P<0.01$ *vs* aCSF, BL by ANOVA and the Newman–Keuls *post hoc* test. The overall ANOVA did not reach significance for the EtOH-maintained responding data in the second study; thus correlated *t-tests* (2-tailed) were conducted. The asterisks in panel B reflect these analyses. Panel C depicts the cumulative time course profile for the EtOH (10% v/v) data of panel B following naltrexone (0.0–30.0 μg) across the six 10-min intervals. Naltrexone suppressed responding at the initial 10-min interval (ie initiation phase of EtOH responding) with the 1, 10, and 30 μg doses ($p<0.05$) and by the 30-min interval, all doses except the lowest dose (1 μg) produced a profound reduction in responding ($p<0.01$), which was maintained throughout the 40–60-min intervals ($p<0.01$).

conditions ($F(3,18) = 7.940$, $p < 0.0014$). The Newman–Keuls *post hoc* test confirmed that both doses of naltrexone significantly reduced EtOH-maintained responding ($p < 0.01$). In contrast, naltrexone (2.5–5.0 μg) was without effect on responding for sucrose.

To further examine the effects of naltrexone on concurrent access to EtOH and sucrose, a subsequent study was conducted. This study had two primary objectives: (1) to increase the naltrexone dose response and (2) to increase the sample size with the hope of decreasing the variability among the rats on sucrose-maintained responding to evaluate the effects of our drug treatment more reliably.

Histology. Figure 1d shows a reconstruction of serial coronal sections of the rat brain illustrating the location of the bilateral microinjection cannulas in the CeA for the P rats of the second concurrent study ($N = 8$). The cannula tracks were well localized in the CeA. Only one rat from the second concurrent CeA group was excluded from the final data analyses due to improper placement.

Naltrexone drug treatment. Figure 6b shows the effects of bilateral microinjections of naltrexone (1.0–30.0 μg) into the CeA on responding for EtOH and sucrose in the second study. In contrast to our expectation, basal response rates for the sucrose reinforcer were more varied than the initial study. Nevertheless, naltrexone led to a marked suppression of EtOH-maintained responding. Compared with the control condition, responding was reduced by 47–87%; however, the overall ANOVA failed to reach significance ($F(3,21) = 1.380$, $p > 0.05$) due to the degree of variability in responding for EtOH in the BL condition ($123.88 + 48.92$). However, *post hoc* analyses of the individual drug treatment showed that the 10.0 and 30.0 μg doses did significantly reduce EtOH-maintained responding ($p < 0.05$ and < 0.05, respectively). Further, a correlated *t*-test (two-tailed) of the individual drug treatments also showed that the 10.0 and 30.0 μg doses led to a significant suppression of responding maintained by EtOH relative to the control condition ($t(14) = 2.32$, $p < 0.05$, $t(14) = 2.80$, $p < 0.05$). However, similar to the initial study, sucrose responding was not altered by the naltrexone treatments.

Cumulative response profile. The time course profiles for the two concurrent naltrexone studies were similar. Thus, to avoid redundancy, only data from the second study using the wider dose response will be presented. Figure 6c illustrates the cumulative time course profile for EtOH-maintained responding. The cumulative 6×5 analyses for EtOH yielded a highly significant main effect of dose ($F(4,168) = 10.80$, $p < 0.0001$); however, the interval main effect was not significant ($F(5,42) = 1.10$, $p > 0.398$). Interestingly, the magnitude of EtOH responding at each of the six within-session intervals was relatively similar compared with when EtOH was presented alone ($p > 0.05$) (see Figure 3c). Compared with the control condition, naltrexone suppressed responding at the initial 10-min interval with the 1, 10, and 30 μg doses ($p < 0.05$); and, by the 30-min interval, all doses except the lowest dose (1 μg) led to a profound reduction in responding ($p < 0.01$). This suppression was also maintained throughout the 40–50-min intervals ($p < 0.01$). However, with the 2.5 μg dose, the suppression was delayed. The response reduction occurred at the 20-min interval, and continued throughout the 60-min interval.

Experiment 3 (EtOH Alone in HAD-1 Rats)

Histology. Figure 1e shows a reconstruction of serial coronal sections of the rat brain illustrating the location of the bilateral microinjection cannulas in the CeA of HAD-1 rats ($N = 6$). The cannula tracks were well localized in the CeA. One rat from the HAD-1 group was excluded from the final data analyses due to improper placement.

βCCt drug treatments. Figure 7a shows the effect of bilateral microinjections of βCCt (1.0–40.0 μg) into the CeA on EtOH-maintained responding in HAD-1 rats. βCCt dose-dependently suppressed EtOH-maintained responding. Compared with the control condition, responding was reduced by 52–78%, yielding a significant main effect for drug treatment ($F(4,20) = 3.113$, $p < 0.03$). The Newman–Keuls *post hoc* test confirmed that the three highest doses (10.0, 20.0, 40.0 μg) significantly reduced EtOH-maintained responding ($p < 0.05$).

Cumulative response profile. Figure 7b illustrates the cumulative time course profile across the 60-min interval for EtOH-maintained responding. The cumulative 6×5 analyses for EtOH yielded main effects for interval ($F(5,30) = 2.308$, $p < 0.05$) and drug treatment ($F(4,120) = 19.7$, $p < 0.0001$). Under the control conditions, approximately 22% of the total EtOH-maintained responding occurred during the initial 10 min of the operant session and 64% occurred by the end of the 30-min interval. Compared with the control condition, the 20.0 and 40.0 μg doses of βCCt suppressed responding for EtOH during the 20-min interval ($p < 0.05$), while all three doses suppressed responding at the 30-min interval and was maintained throughout the remainder of the session ($p \leqslant 0.05$).

24 h postdrug treatment. Figure 7c shows the effect of bilateral microinjections of βCCt (10.0–40.0 μg) into the CeA on EtOH-maintained responding 24 h postdrug treatment. All drug treatments that produced an effect on the first day continued to suppress EtOH-maintained responding significantly 24 h postdrug administration ($F(3,15) = 6.419$, $p < 0.0052$). The Newman–Keuls *post hoc* test confirmed that the 10.0–40.0 μg doses of βCCt significantly suppressed responding for EtOH 24 h postdrug administration ($p < 0.01$), albeit the effects were not dose related.

Cumulative response profile. Figure 7d illustrates the cumulative time course profile across the 60-min interval for EtOH-maintained responding 24-h postdrug administration for the 60-min session data. The cumulative 6×4 analyses for EtOH yielded main effects for interval ($F(5,29) = 3.896$, $p < 0.0080$) and treatment ($F(3,87) = 24.802$, $p < 0.0001$). Approximately 27% of the total EtOH-maintained responding occurred during the initial 10 min of the operant session and 65% occurred by

the end of the 30-min interval under the control conditions. In comparison with the control condition, all doses of βCCt (20.0–40.0 μg) suppressed responding for EtOH by the second 10-min interval and this suppression was maintained throughout the remainder of the session ($p<0.01$).

Previously, we demonstrated that when sucrose (3% v/v) was given alone in HAD-1 rats, a dose as high as 60.0 μg of βCCt failed to alter sucrose responding (Yin *et al*, 2001). The HAD-1 rats were not tested in the concurrent paradigm, since it is well established that they do not maintain substantial levels of EtOH-maintained responding (June, 2002) or intake (Lankford and Myers, 1994) in the presence of another palatable reinforcer. Naltrexone was not tested in the HAD-1 rats due to their availability at the time of the present study.

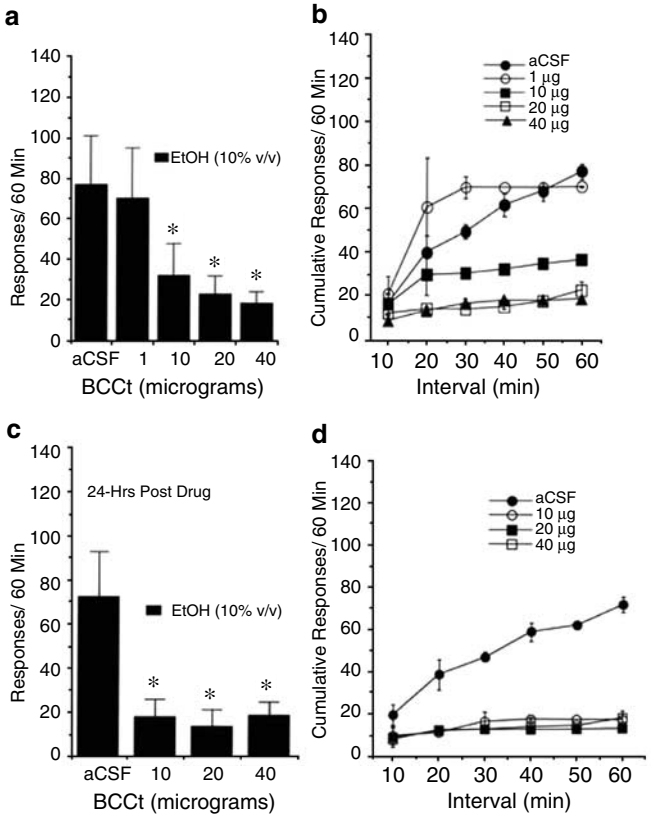

Figure 7 Effects of βCCt (0.0–40.0 μg) in HAD-1 following bilateral microinjection in the CeA on responding maintained by EtOH (10% v/v) during a 60-min operant session (a) Data are shown as mean (± SEM). The total 60-min operant data were evaluated using a one-way ANOVA with the dose as the factor. *$P<0.02$ *vs* aCSF, BL by ANOVA and the Newman–Keuls *post hoc* test, ($N=6$). (b), Cumulative time course profile for EtOH (10% v/v) across the six 10-min intervals. In contrast to the P rats, on the initial day of drug administration, the βCCt-induced suppression was not observed until the 20–30-min intervals ($p<0.05$) (see methods for additional time course statistics). Effects of βCCt (10–40.0 μg) 24 h postdrug administration in HAD-1 rats following bilateral microinjection in the CeA on responding maintained by EtOH (10% v/v) during a 60-min operant session (c). In contrast to the P rats, βCCt continued to induce a marked reduction in EtOH-maintained responding 24 h postdrug administration. Data are shown as mean (± SEM). The total 60-min operant data were evaluated using a one-way ANOVA with the dose as the factor. *$P<0.01$ *vs* aCSF, BL by ANOVA and the Newman-Keuls *post hoc* test ($N=6$). (d) Cumulative time course profile for EtOH (10% v/v) across the six 10-min intervals. At 24 h postdrug administration, the βCCt-induced suppression was observed at the initial 10-min interval with the 10–40.0 μg doses ($p<0.01$) (ie initiation phase of EtOH responding) and was sustained throughout the remainder of the operant session ($p<0.01$).

Experiment 4 (EtOH Alone in CPu Control P Rats)

Histology. Figure 1f shows a reconstruction of serial coronal sections of the rat brain illustrating the location of the bilateral microinjection cannulas in the CPu of the P rats in the EtOH self-administration group ($N=10$). The cannula tracks were well localized in the CPu. All 10 rats were successfully implanted in the CPu. Figure 2e–f depicts the actual bilateral placements for two of the 10 CPu rats in separate photomicrographs illustrating the extent of the lesion sustained as a result of the bilateral guide cannula.

βCct drug treatments. Figure 8a shows the rate of responding following microinjections of βCCt (20.0–60.0 μg) into the CPu, the neuroanatomical control site ($N=6$). βCCt was without effect on EtOH-maintained responding given acutely ($F(3,15)=476$, $p>0.05$) and 24-h postdrug administration ($F(3,15)=389$, $p>0.05$).

Naltrexone drug treatments. Figure 8b shows the rate of responding following microinjections of naltrexone (1.5–30.0 μg) into the CPu on EtOH-maintained responding. Each drug treatment was analyzed separately because of the different number of subjects in each drug treatment condition ($N=8$–10). Nevertheless, the 1.5 μg ($F(1,7)=2.49$, $p>0.05$), 5.0 μg ($F(1,8)=0.616$, $p>0.05$), 10.0 μg ($F(1,9)=0.150$, $p>0.05$), and 30.0 μg ($F(1,8)=0.101$, $p>0.05$) doses all failed to produce significant effects on responding maintained by EtOH.

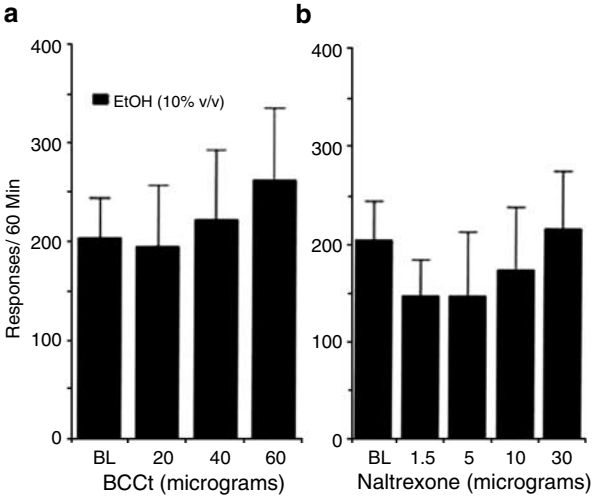

Figure 8 Effects of βCCt (0.0–40.0 μg) ($N=6$) (a) and naltrexone (0.0–30.0 μg) ($N=10$) (b) in P rats following bilateral microinjection in the CPu on responding maintained by EtOH (10% v/v) during a 60-min operant session. Data are shown as mean (± SEM). The total 60-min operant data were evaluated using a one-way ANOVA, with the dose as the factor. The naltrexone drug treatments were analyzed separately because of the different number of subjects in each drug treatment condition ($N=8$–10) (see methods for additional detail). Neither compound altered responding maintained by EtOH.

DISCUSSION

The results of the present study demonstrated that bilateral microinfusions of βCCt, the mixed BDZ agonist–antagonist, into the CeA significantly suppressed EtOH-maintained responding in both P and HAD-1 rats when EtOH was the sole reinforcer available. Suppression was observed during the initial 10 min and maintained for the duration of the 60-min session in P rats, while the suppression was delayed and not detectable until the 20-min interval in the HAD-1 rats. The reduction in responding was also observed 24 h postdrug administration in the HAD-1 rats. The results of the present study are consistent with a recent report from our laboratory, demonstrating that microinfusion of βCCt into the VP produced marked and selective reductions on EtOH-motivated responding in both P and HAD-1 rat lines (June *et al*, 2003). Similar to the present study, βCCt continued to suppress EtOH responding only in the HAD-1 rats 24 h postdrug administration in the VP. Further, we also reported that five- to six-fold lower doses were required to observe maximal suppression on EtOH responding with systemic administration in HAD rats. Taken together, the results of both studies suggest that βCCt is a long-acting drug in HAD-1 rats. It is possible that HAD-1 rats may be innately more sensitive than P rats to the suppressant effects of βCCt; however, the different dosing schedule, particularly on the lower end of the dose response, combined with our failure to employ a concurrent schedule analysis with the HAD line, hinders any unequivocal interpretation on sensitivity differences between the rat lines. However, another explanation (albeit speculative), which may explain the time course of suppression by βCCt on EtOH responding, may be that HAD-1 rats eliminate βCCt at a slower rate in comparison with the P rats. Pharmacokinetic studies on βCCt are currently underway in the two rat lines.

βCCt failed to suppress sucrose-maintained responding in the P rat when sucrose was presented alone at a high concentration of 10% (w/v). βCCt also failed to alter sucrose-maintained responding when it was concurrently available with EtOH at a low concentration of 2% (w/v). Specifically, βCCt doses as high as 60 μg failed to reduce responding for the 10% sucrose. In contrast, a nonsignificant elevation was observed. Such increases are typical of partial and full BDZ agonists on ingestive behaviors (Higgs and Cooper, 1995). As noted previously, βCCt displays agonist effects in anxiolytic paradigms in rodents (Carroll *et al*, 2001; June *et al*, 2003) and primates (Paronis *et al*, 2001). However, behavioral studies also demonstrate that βCCt exhibits competitive binding site interaction with BDZs agonists (Shannon *et al*, 1984; Griebel *et al*, 1999; Carroll *et al*, 2001; Rowlett *et al*, 2001; Paronis *et al*, 2001). Thus, prior research suggests that βCCt is capable of functioning as a partial agonist or a competitive antagonist depending on the dose, behavioral paradigm employed, and subunit composition activated (Lüddens, June, and Cook, in preparation).

Previous research has demonstrated that when sucrose (ie 6–14% w/v) is concurrently presented with EtOH, responding for sucrose will exceed EtOH by at least 50% (Petry and Heyman, 1995; June, 2002). However, if an antagonist reduces EtOH responding despite the fact that responding for the alternative reinforcer is markedly greater than EtOH, this may demonstrate the selectivity of the antagonist (Petry and Heyman, 1995; June, 2002). Research from our laboratory has also shown that a leftward dose–response shift occurs with agents that reduce EtOH-maintained responding (eg opioid antagonists, GABA/BDZ antagonists) when alcohol is presented concurrently with a highly palatable solution (eg saccharin, sucrose) compared with when EtOH is presented alone (June, 2002). Leftward dose–response shift is well established with both naltrexone and nalmefene (June *et al*, 1998c; June, 2002). At present, it is not clearly known why such shifts occur; however, they have been observed with both opioid and GABA agents in both microinjection and systemic injections studies (June *et al*, 1998c; June, 2002). Preliminary studies have also revealed a profile similar to βCCt (unpublished data). It is possible, albeit speculative, that the alternative reinforcer reduces the efficacy of the EtOH such that a lower amount of the antagonist may be required to reduce the EtOH responding.

Given the previous research (June *et al*, 1998c; June, 2002) and our preliminary data, an eight-fold lower dose of βCCt was used in the 'present' concurrent EtOH and sucrose study (1–5 μg *vs* 1–40 μg). In addition, we employed a low sucrose concentration (2% w/v) in the present concurrent study in an attempt to equate basal response rates with the EtOH. However, even at the 2% concentration, the palatability and rewarding efficacy of the sucrose exceeded that of the 10% EtOH solution. Interestingly, while the reward efficacy for the sucrose was greater than the EtOH, the 2% sucrose did not significantly reduce basal alcohol responding in the P rat, as evidenced by the fact that the magnitude of responding was comparable to when EtOH was presented alone. Nevertheless, despite this difference in reward strength, all doses of βCCt (1.0–5.0 μg) selectively reduced EtOH-maintained responding. Thus, these results suggest: (1) as previously reported, even in the presence of a highly palatable reinforcer, EtOH retains its potent reinforcing effects in P rats (for a review, see McBride and Li, 1998) and (2) the βCCt-induced reduction on alcohol-maintained behaviors was not due to a general suppression of consummatory behaviors.

The exact mechanism by which βCCt selectively reduces EtOH-seeking behaviors in the CeA is not known. In addition, the different dose–response profiles between the self-administration studies further impede any clear mechanistic interpretation. However, we hypothesize that βCCt is working as an 'agonist' in the present study based on prior *in vivo* (Carroll *et al*, 2001; Paronis *et al*, 2001; June *et al*, 2003) and recent *in vitro* efficacy data employing human HEK cells (Lüddens, June, and Cook, Lüddens, June, and Cook, in preparation). It should be recalled that some researchers have demonstrated that the α2 and α3 receptors are the most predominant isoforms in the CeA (Wisden *et al*, 1992; Turner *et al*, 1993; Fritschy and Möhler, 1995). However, as noted previously, studies of recombinant receptors show that βCCt exhibits only a >10 fold selectivity for the α1 over the α2 and α3 receptors, and a >110 fold selectivity for the α1 over the α5 receptor subtype (Cox *et al*, 1995). Thus, βCCt exhibits the greatest binding selectivity of the currently available α1 ligands (eg zolpidem, CL 218,872, L-838,417) (Sanger *et al*, 1994; McKernan *et al*, 2000; Harvey *et al*, 2002). Hence, a likely hypothesis is that βCCt produces

its agonist effects via the preponderance of α2 and α3 receptors of the CeA (Wisden *et al*, 1992; Araki and Tohyama, 1992; Turner *et al*, 1993). In contrast, the CeA is essentially devoid, or contains very low levels of the α1, α4, α5, and α6 receptor subtypes (Wisden *et al*, 1992; Araki and Tohyama, 1992; Turner *et al*, 1993).

Another explanation consistent with βCCt's capacity to exhibit an agonist profile pertains to the fact that GABAergic mechanisms involved in normal ingestion may also influence EtOH-motivated responding (Hodge *et al*, 1995). For example, Minano *et al* (1992) reported that microinfusion of GABAergic agonists such as muscimol into the CeA markedly reduced food intake in satiated and fasted rats. Several lines of evidence suggest that βCCt may reduce EtOH responding in a manner consistent with that of muscimol on food intake due to βCCt's ability to exhibit agonist effects. Recently, we reported that βCCt over a range of 0.5–100 μM produced dose-related agonist effects (ie potentiated GABA's actions) in the HEK cell efficacy model at the α2- and α3-containing GABA receptors (Lüddens, June, and Cook, in preparation). Although somewhat speculative, it is possible that the wide βCCt dose–response (1–60 μg) employed across the different EtOH self-administration paradigms of the present study was well within the 0.5–100 μM range (Skolnick, personal communication). Hence, it is further possible that βCCt's GABA-potentiating actions substituted for the pharmacological effects of the ingested EtOH, leading to early termination of responding. Several researchers have demonstrated that positive GABAergic modulators are readily substituted for EtOH in drug discrimination studies (Bowen and Grant, 1998; Hodge and Cox, 1998). In further support of this hypothesis, we have demonstrated that in both the Xenopus oocyte and HEK cell efficacy models (June *et al*, 2003; Carroll *et al*, 2001; Lüddens, June, and Cook, in preparation), βCCt was GABA neutral at the α1 receptor at certain concentrations, or produced very weak inverse agonist effects.

Finally, also consistent with an agonist hypothesis of βCCt's mechanism of action, is the idea that GABAergic neurons within the CeA regulate alcohol's euphoric properties via the involvement of GABA within the mesolimbic dopamine reward circuitry (McBride and Li, 1998). Neuroanatomical evidence has revealed a number of unilateral and reciprocal subcortical GABAergic pathways (Sun and Cassell, 1993) adjacent to putative dopaminergic reward substrates (Koob *et al*, 1998a, b). At certain doses, infusions of βCCt may exhibit a partial agonist profile and may activate GABA in the CeA. Significant increases in GABAergic tone have been shown to disinhibit DA neurons producing an elevation in DA levels (Kalivas, 1993). This increase in DA would substitute for the increase in DA that is normally associated with EtOH administration (Weiss *et al*, 1993), thus causing a subsequent reduction in alcohol drinking. The CeA appears to be in a unique position to serve as a pivotal regulator of both telencephalic and mesencephalic GABAergic and DA input that could control EtOH-motivated behaviors.

Bilateral administration of naltrexone in the CeA suppressed EtOH-maintained responding, when EtOH was the sole reinforcer available in P rats. Furthermore, when a higher concentration of sucrose was given alone, naltrexone doses (eg 0.25–2.5 μg), which were effective in reducing EtOH-maintained responding, was without effect. The 5 μg dose appeared to be the maximally effective dose, as no greater suppression was seen with this dose compared with lower doses when EtOH was presented alone, or concurrently with sucrose (Figure 3c and 5a). However, the 5 μg dose suppressed sucrose responding when presented alone. Interestingly, a dose as high as 30 μg failed to reduce sucrose responding when presented concurrently with EtOH. Thus, minimal occupancy of opioid receptors in the CeA was required to reduce alcohol-motivated behaviors by naltrexone regardless of the behavioral contingencies. In contrast, the degree to which naltrexone reduced responding maintained by sucrose was highly dependent on several contingencies of the operant session. As with the βCCt, the magnitude of suppression of either reinforcers with naltrexone was similar in both male and female rats. This is important in light of the fact that both GABA and opioid systems have been reported to show fluctuations due to gonadal steroid hormones in mature rodents (Wilson and Biscardi, 1992; Wilson, 1992; for a review, see Carroll *et al*, 2002). In any event, these data clearly suggest that EtOH-maintained responding is more sensitive than sucrose responding to the suppressant effects of naltrexone in the CeA. It is possible that the opioid receptors within the CeA, which regulate sucrose-maintained behaviors may be dissociated in part, or totally, from those that control EtOH-maintained responding.

The data of the present study are in agreement with the report of Heyser *et al* (1999). These researchers demonstrated that the nonselective opiate antagonist, methylnaloxoniun (0.125–1.0 μg) microinjected into the CeA dose-dependently reduced EtOH-motivated responding in outbred Wistar rats. Heyser *et al* (1999) further showed that the suppression was delayed with the greatest effects occurring around the 6–8-min intervals. The time course data of the present study indicated that while the greatest suppression by naltrexone was observed during later intervals (ie 20–30 min), some degree of suppression was also observed during the initial 10-min interval. It is likely that these effects were delayed during the first few minutes of the operant session (ie 3–6 min), as the slopes for the dose–response curves for the EtOH alone and the concurrent study (Figure 2b and 3d and 6c) were similar to controls during the initial 10-min interval with the lower naltrexone doses. However, it is difficult to reconcile the findings with the higher doses of the present study with those of Heyser *et al* (1999), as these doses generally tended to suppress responding throughout the entire duration of the operant session. Nevertheless, the findings of both studies strongly suggest that despite the paucity of opioid receptors within the CeA (Mansour *et al*, 1987, 1995; McBride *et al*, 1988), these receptors are highly functional in regulating EtOH-motivated behaviors in outbred rats, and rats with a genetic predisposition to alcoholism. One hypothesis is that efferent and afferent opioid projections exist between adjacent nuclei (eg BLA, basomedial amygdala (BMA), and dorsolateral bed nucleus of the stria terminalis (BST)), and these nuclei may interact to regulate alcohol-reinforced behaviors. The BLA, which contain substantial levels of opioid receptors (Mansour *et al*, 1987, 1995; McBride and Li, 1988), has recently been reported to play a role in both μ and δ opioid receptor

regulation of EtOH-maintained responding (Hyytiä and Kiianmaa, 2001).

The CPu was selected as the control locus based on several factors. First, the CPu is morphologically and neurochemically similar to the CeA. For example, both contain primarily medium-sized spiny GABAergic neurons (Alheid and Heimer, 1988; McDonald, 1982) and both structures possess reciprocal dopaminergic innervation from midbrain tegmental areas (Freedman and Cassell, 1994; Gonzales and Chesselet, 1991). Second, the CPu is devoid of α_1- and α_3-containing GABA$_A$ receptors, but contains substantial levels of the α_2 isoform (Wisden *et al*, 1992; Duncan *et al*, 1995; Fritschy and Möhler, 1995). Third, the CPu contains substantial levels of μ and δ opiate receptors (Mansour *et al*, 1987, 1995; McBride and Li, 1998). Thus, the CPu represents an ideal neuroanatomical control locus to evaluate the specificity of the nonselective opioid antagonist and the selective GABA$_A$ α1 antagonist.

In the present study, moderate to high doses of βCCt (20.0–60.0 μg) and naltrexone (eg 5–30 μg), which were highly effective in reducing EtOH-maintained responding in the CeA, were completely ineffective in the CPu. These data are in agreement with previous research (June *et al*, 2001; Harvey *et al*, 2002), suggesting that the GABA$_A$ subunit composition in the CPu is not sufficient to regulate alcohol-motivated behaviors. Similarly, the data with naltrexone revealed that while high levels of μ and δ opioid receptors exist within the CPu (Mansour *et al*, 1987, 1995), they are not important in regulating alcohol-motivated responding.

Given the proximity of the CeA, BLA, BMA, and BST, it could be argued that diffusion of βCCt or naltrexone from the CeA to one, or several of these bordering nuclei, might have contributed to the effects observed in the present study. Previous autoradiographic studies reveal that using a total unilateral injection volume of 0.3–1.0 μl, radiolabeled opioids were confined to within 0.7 mm of the injection site (Bals-Kubic *et al*, 1993). However, such an explanation is very unlikely since: (1) only animals whose injection were confined to the CeA were included in the final data analyses and (2) animals received only a limited number of infusions (mean = 6), thus sustaining the viability of the tissue. Nevertheless, as Koob and his co-workers have suggested (see Heyser *et al*, 1999), some degree of caution is warranted when contributing drug effects to specific subregions of the amygdala.

Thus far, the discussion related to mechanisms of action has focused on positive reinforcement, but the positive reinforcing properties may not fully explain the mechanism by which βCCt reduces alcohol-seeking behaviors in the P and HAD rats. The CeA is thought to be an important structure in the mediation of emotions, such as anxiety (Sanders and Shekhar, 1995; Koob, 1999). It is possible that βCCt may have reduced anxiety, and therefore reduced alcohol drinking (ie negative reinforcement). The P and HAD rats have been shown to be more anxious than their nonalcohol drinking counterparts (Stewart *et al*, 1993; Salimov *et al*, 1996, but see Baldwin *et al*, 1991). However, this would not be consistent with the tension-reduction hypothesis of EtOH self-administration (Möeller *et al*, 1997; Pohorecky, 1990). Further, anxiety and the antecedents of anxiety have not been strongly supported as a principle motivator, which initiates alcohol drinking in rats selectively bred to consume alcohol (Stewart *et al*, 1993).

In conclusion, the current study demonstrated that GABA and opioid receptors within the CeA can effectively regulate alcohol-motivated behaviors, while producing little, if any, effects on normal ingestive behaviors. In addition to reinforcer specificity, neuroanatomical specificity was also observed, as no reliable suppression on alcohol responding was observed when high doses of the test agents were infused into the CPu, a locus, several mm dorsal to the CeA. The close proximity and coexistence of GABA and opioid receptors, as well as DA receptors, within the reward circuitry (Kalivas *et al*, 1990; Churchill and Kalivas, 1992; Kosaka *et al*, 1987) strongly suggest that these neurotransmitters systems may overlap in the control of key neuronal substrates to regulate the acute reinforcing properties of alcohol (Koob *et al*, 1998a, b; Morales *et al*, 1998; McBride and Li, 1998). The CeA may represent a novel target site in the development of prototypical GABA$_A$ and opioidergic receptor ligands, which selectively reduce alcohol abuse in humans.

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