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Importance of Cocaine- and Amphetamine-Regulated Transcript Peptide in the Central Nucleus of Amygdala in Anxiogenic Responses Induced by Ethanol Withdrawal

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We studied the involvement of cocaine- and amphetamine-regulated transcript peptide (CART) in the central nucleus of amygdala (CeA), lateral bed nucleus of the stria terminalis (BNSTl) and nucleus accumbens shell (AcbSh) in generation of ethanol withdrawal symptoms, with particular focus on anxiety-like behavior using a social interaction test. Administration of CART (54–102) into the lateral ventricle (50 and 100 ng) and bilaterally in the CeA (10 and 20 ng) caused a significant reduction in social interaction, suggesting an anxiogenic action of the peptide. Chronic ethanol treatment for 15 days followed by withdrawal precipitated an anxiogenic response at 24 h that was attenuated by intracerebroventricular (5 μl) and intra-CeA (1 μl) administration of antibodies against CART (1 : 500 dilution). An immunocytochemistry protocol was employed to study the response of the endogenous CART system in the CeA following chronic ethanol withdrawal. At 0 h ethanol withdrawal, CART immunoreactivity was apparent in few fibers and the profile was similar to that in the pair-fed control rats. Twenty-four hours following ethanol withdrawal, a highly significant increase ($P<0.001$) in CART immunoreactivity was noticed in the CeA, which returned to normal 48 and 72 h post-withdrawal. Similar doses of CART or CART antibody injected bilaterally into the BNSTl or AcbSh produced no response in the social interaction test. Furthermore, the CART immunoreactivity profile did not change at the post-withdrawal time points in each of these brain sites. We suggest that CART may mediate the early signs of anxiety-like behavior induced by ethanol withdrawal within the neuroanatomical framework of the CeA.
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INTRODUCTION

A wealth of data suggests that cocaine- and amphetamine-regulated transcript peptide (CART), a new member of the neuropeptide family, has neurotransmitter-like actions in the brain (Kuhar and Dall Vechia, 1999) and increases in regions of the brain in response to the psychostimulants, cocaine and amphetamine (Douglass *et al*, 1995). To date, several CART peptide fragments have been identified (Kuhar and Yoho, 1999; Thim *et al*, 1999). Among them, two biologically active fragments, CART 55–102 and 62–102, have been isolated and sequenced from the rat hypothalamus and pituitary (Gautvik *et al*, 1996; Kristensen *et al*, 1998; Thim *et al*, 1998, 1999; Bannon *et al*, 2001). Within the brain, CART-expressing elements are particularly abundant in the nucleus accumbens (Acb), lateral bed nucleus of stria terminalis (BNSTl), arcuate nucleus (ARC), amygdala, hippocampus, midbrain raphe, locus coeruleus, and pituitary (Couceyro *et al*, 1997; Koylu *et al*, 1997, 1998; Elias *et al*, 1998; Vrang *et al*, 1999).

CART peptides are involved in the regulation of pain, arousal, startle response, regulation of calcium channels, and neuroendocrine hormone secretion (Bannon *et al*, 2001; Yermolaieva *et al*, 2001; Smith *et al*, 2004). A role of CART in the Acb in drug reward and reinforcement has also been suggested (Koylu *et al*, 1998; Smith *et al*, 1999). Salinas *et al* (2006) have recently observed that CART peptide and its mRNA are expressed in the Acb in response to acute ethanol administration, suggesting that CART may be an endogenous mediator of the rewarding and reinforcing properties of ethanol. In support of this concept is the recent identification of a polymorphism in intron 1 of the CART gene that is associated with alcoholism in the Korean male population (Jung *et al*, 2004). CART also plays an important role in anxiety-like behavior and stress-related

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responses (Chaki *et al*, 2003; Stanek, 2006), and dose-dependently enhances anxiety-like behavior in the elevated plus maze (EPM) test in both rats (Kask *et al*, 2000) and mice (Asakawa *et al*, 2001).

Regions of the extended amygdala that include the central nucleus of amygdala (CeA), BNSTl and nucleus accumbens shell (AcbSh), play a key role in drug abuse and dependence (Koob, 2003b), mediation of alcohol addictive processes (Koob, 2003b; Pandey, 2004) and physiological responses associated with anxiety-like behavior (LeDoux *et al*, 1988; Walker and Davis, 1997). The amygdala also plays an important role in processing anxiety-related signals induced by ethanol withdrawal and exhibits changes in the neurotransmitter profile during ethanol dependence and withdrawal (Merlo Pich *et al*, 1995; Koob, 2003a; Zhang and Pandey, 2003). Lesions of the CeA, but not basolateral amygdala, decrease anxiety-like behaviors and voluntary ethanol consumption in rats (Moller *et al*, 1997). Chronic intake of ethanol modifies synaptic and intracellular signaling in the amygdala (Pandey *et al*, 2003), whereas acute ethanol administration stimulates c-*fos* expression in CeA (Criado and Morales, 2000). CART-containing fibers have been demonstrated in the CeA and its role in drug reward and reinforcement has been proposed (Koylu *et al*, 1998). However, the importance of CART in ethanol withdrawal-induced anxiety-like behavior has not been explored.

In the present study, we investigated the role of CART in the extended amygdala in the regulation of anxiety-like behavior following the chronic ethanol treatment and withdrawal in rats. We hypothesized that through actions in the extended amygdala, CART may be involved in ethanol withdrawal-induced anxiety-like behavior. Herein, we administered CART intracerebroventricularly (icv), and also directly into the CeA, BNSTl or AcbSh of the normal rats, and evaluated the anxiety-like behavior by employing a social interaction test. Using the same behavioral paradigm, ethanol withdrawal-induced anxiety-like behavior was tested in rats treated with antibodies against CART given by the icv and intra-CeA, -BNSTl or -AcbSh routes. Furthermore, the effect of chronic ethanol treatment and/or withdrawal on the endogenous CART-containing system in CeA, BNSTl or AcbSh was investigated using immunocytochemistry coupled with morphometric analysis.

MATERIALS AND METHODS

Animals

Adult, male, Sprague–Dawley rats (230–250 g) were group-housed, but after icv and intra-nuclei (CeA, BNSTl or AcbSh) cannulation, they were housed individually. All animals had free access to food (Lipton, India) and drinking water except during the test period or some special experimental protocols. They were maintained on a 12 h dark/light cycle, in controlled temperature ($25 \pm 2^{\circ}$C) and relative humidity (50–70%). The experimental procedures were approved by the Institutional Animal Ethical Committee.

Intracerebral Cannulations

The detailed procedure of cannulation, drug administration, and post-surgical care has been described (Kokare *et al*, 2005, 2006). A stainless steel guide cannula (C316G/Spc; Plastics One, Roanoke, VA) was implanted into the right lateral cerebral ventricle using the stereotaxic coordinates, −0.8 mm posterior, + 1.3 mm lateral to midline, and 3.5 mm ventral with respect to bregma (Paxinos and Watson, 1998). Similarly, the cannulae were implanted bilaterally in the CeA (−2.4 mm posterior, $\pm$4.0 mm lateral to midline and 8.0 mm ventral), BNSTl (−0.3 mm posterior, $\pm$1.6 mm lateral to midline and 6.0 mm ventral) or AcbSh (+ 1.7 mm anterior, $\pm$0.8 mm lateral to midline and 6.5 mm ventral) in other groups. The animals were allowed a recovery period of 7 days and those with any neurological/motor deficits were excluded from the study.

Ethanol Treatment

The rats were given ethanol in a liquid diet for 15 days according to the previously described protocol (Pandey *et al*, 1999; Kokare *et al*, 2006). Briefly, rats were given a nutritionally balanced control liquid diet (Novartis, India) for 2 days for adaptation to the novel food. From day 3 onwards, ethanol was gradually introduced into the liquid diet starting with 4.5% (v/v) ethanol on the first day, 7.5% (v/v) ethanol on the second day and 9% (v/v) ethanol thereafter for 15 days (ethanol-fed group, $n = 27$). The rest of the animals continued on the nutritionally balanced control liquid diet (pair-fed control group, $n = 23$). A fresh aliquot of ethanol diet or control liquid diet (100 ml/rat) was provided each morning. After 15 days, all the animals were placed on an ethanol-free, nutritionally balanced diet and killed at 0, 24, 48, and 72 h post-withdrawal, and their brains processed for CART immunolabeling.

Icv, Intra-CeA, -BNSTl and -AcbSh Administration of CART and CART Antibody

The methods of icv and nuclei-specific injections and preparation of artificial CSF (aCSF) have already been described (Kokare *et al*, 2005, 2006). Cannulated rats were randomly allocated to different groups ($n = 8$ in each). All injections were performed between 0900 and 1200 h in a randomized way and rats, once used, were not re-employed. CART (54–102) was dissolved in aCSF and injected into the right lateral ventricle or bilaterally into the CeA, BNSTl or AcbSh. Various agents like aCSF (5 μl/rat, icv; $n = 7$, and 1 μl each side for intra-CeA, -BNSTl or -AcbSh; $n = 6$, $n = 7$ or $n = 7$ respectively) and CART (25–100 ng/rat, icv; $n = 8$ per group and 2–20 ng/rat for intra-CeA, -BNSTl or -AcbSh; $n = 6$, $n = 8$ or $n = 6$ per group respectively) were given as single injection and subjected to the social interaction test (see below) following an interval of 30 min.

To study the effect of immunoneutralization of endogenous CART on social interaction following ethanol-withdrawal, cannulae were implanted either into the lateral ventricle or bilaterally into the CeA, BNSTl or AcbSh as described above. Twenty-two hours following withdrawal, rats were injected with (i) aCSF (5 μl/rat, icv; $n = 8$ or 1 μl each side for intra-CeA, -BNSTl or -AcbSh; $n = 6$ in each group), (ii) non-immune serum (1 : 500 dilution, 5 μl/rat, icv; $n = 7$ or 1 μl each side for intra-CeA, -BNSTl or -AcbSh; $n = 7$ in each group) or (iii) antibody against CART (1 : 500 dilution, 5 μl/rat, icv; $n = 6$ or 1 μl each side for intra-CeA;

$n = 8$, -BNSTl; $n = 8$ or -AcbSh; $n = 6$). Two hours after the injection, animals were subjected to social interaction test (see below). Earlier studies used the antibodies for immunoneutralization in similar timeframe and dilution range (Scruggs *et al*, 2003). The responses were scored by a trained observer blind to various treatment conditions. In all experiments, CART and CART antibody-treated groups were run in parallel with their respective control group. The placement of the cannula for icv injection was tested for accuracy by injecting dilute India ink and post-mortem examination of the distribution of ink in the ventricles (Kokare *et al*, 2006) and the 52 animals with correct placement were used for statistical analysis.

Following intra-CeA, -BNSTl and -AcbSh injections, the brains (168 rats) were removed, sectioned, stained with cresyl violet, and the position of the tip of each injection site was determined (see Supplementary Figure S1). Only animals with correct cannula placements (45 rats for intra-CeA, 52 rats for intra-BNSTl, and 44 rats for intra-Acb) were used for statistical analysis.

Social Interaction Test for Anxiety

The social interaction test (File and Hyde, 1978) was performed according to Gonzalez *et al* (1998) with slight modification. Two rats naïve to the test were taken. The index rat was implanted with cannula, while the untreated dummy partner was taken from separate cage and placed into the center of a test box (60×60 cm open field with 16 15×15 cm squares marked on the floor) simultaneously for a 10-min period. Reduced social interaction time reflected the increased anxiety-like behavior. Locomotor activity was measured as the number of squares entered during the interaction session. The time that the index rat socially interacted, which includes genital investigation, sniffing, following, grooming, kicking, crawling under or over the partner, and touching or nearly touching their faces with the untreated dummy partner was visually measured. The occurrence of at least one of the behaviors was recorded and the data are shown in Figures 1–5.

Immunocytochemistry

Brains of ethanol-treated as well as pair-fed rats collected at the 0, 24, 48, and 72 h post-withdrawal time points were processed for immunocytochemical labeling. Rats were anesthetized (pentobarbital, 50 mg/kg, intraperitoneally), perfused transcardially with heparinized phosphate-buffered saline (PBS; pH 7.4) for 30 s followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10–15 min. The brains were post-fixed in the same fixative overnight, cryoprotected in 30% sucrose solution, embedded, and serially sectioned on a cryostat (Leica) at 20 μm thickness in the coronal plane and collected in PBS. Sections were processed for CART immunolabeling using the streptavidin-biotin-peroxidase method described earlier (Singru *et al*, 2007). Briefly, sections were incubated in mouse monoclonal primary antibodies against CART (54–102) diluted in 1% bovine serum albumin (Sigma) containing 0.3% Triton X-100 and 0.09% sodium azide at 1 : 5000 dilution for 2 days at 4°C. After rinsing in PBS, the sections were incubated in biotinylated-IgG followed by ABC (Vector) and the immunoreaction product was developed in diaminobenzedine/H_2O_2 in Tris buffer. Sections were dehydrated, cleared in xylene, and mounted with DPX. To ensure reliable comparisons among different groups and maintain stringency in tissue preparation and

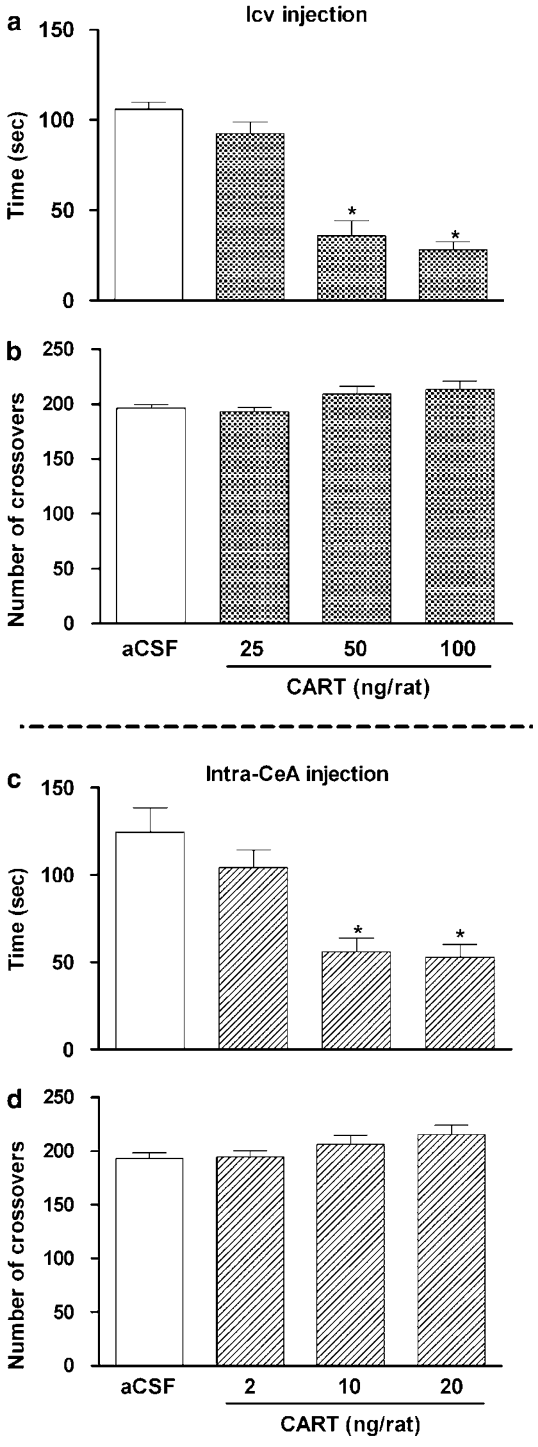

Figure 1 Effect of different doses of CART (54–102) administered via icv or intra-CeA route on social interaction time (a and c) and number of crossovers (b and d) in the social interaction test in normal rats. The data were analyzed by one-way ANOVA with repeated measures on drug treatments followed by *post hoc* Dunnett's test. Each bar is the mean ± SEM for 6–8 rats. *$P < 0.01$ *vs* aCSF-treated group.

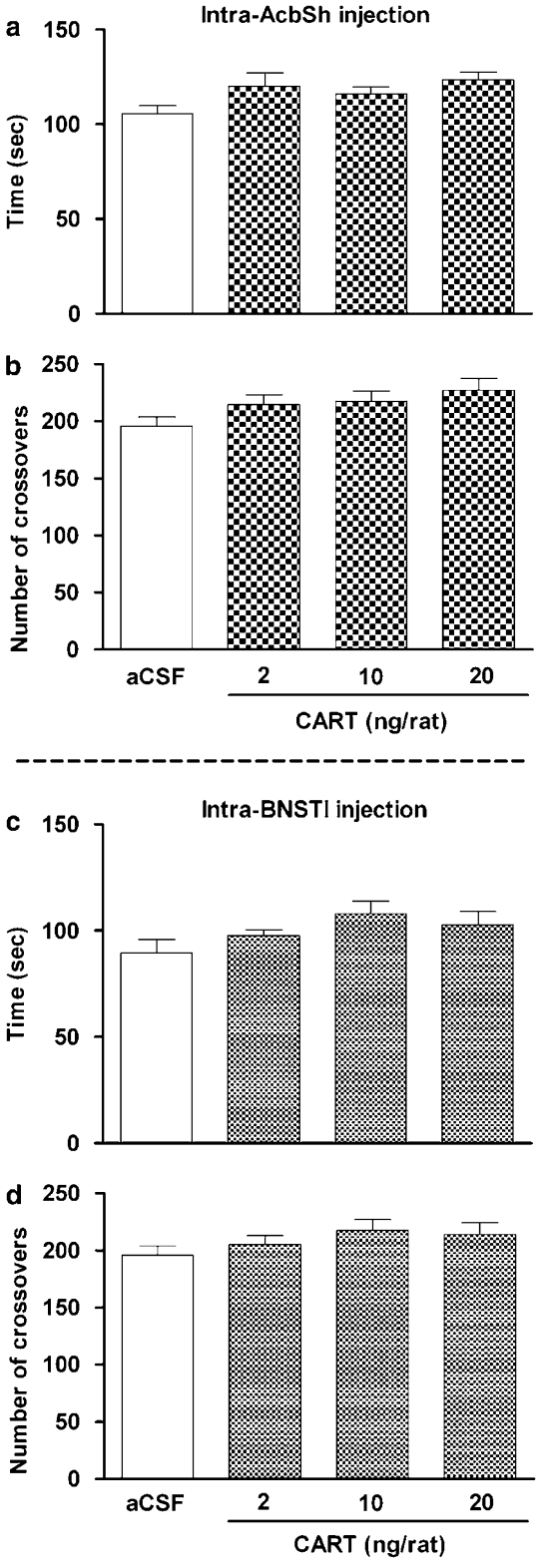

Figure 2 Effect of different doses of CART (54–102) administered bilaterally via intra-AcbSh or -BNSTl route on social interaction time (a and c) and number of crossovers (b and d) in the social interaction test in normal rats. The data were analyzed by one-way ANOVA with repeated measures on drug treatments followed by *post hoc* Dunnett's test. Each bar is the mean ± SEM for 6–8 rats. No significant difference was found at the different doses of CART administered ($P > 0.05$).

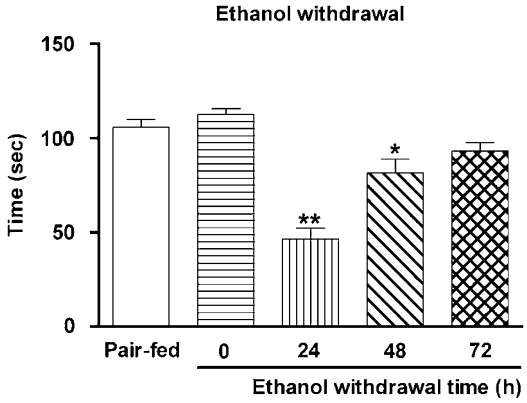

Figure 3 Effect of chronic ethanol treatment followed by withdrawal at 0, 24, 48, and 72 h time points on social interaction time. The data were analyzed by repeated-measure one-way ANOVA followed by *post hoc* Dunnett's test. Each bar is the mean ± SEM for 5–8 rats. $^{*}P < 0.05$ or $^{**}P < 0.001$ *vs* pair-fed group.

staining conditions, sections from the brains of different groups were processed at the same time under identical conditions. The details of generation of CART antibody and specificity have already been described (Thim *et al*, 1998). Omission of primary antibody and replacement with BSA produced no immunoreaction. In preadsorption controls, 1 ml diluted antibody incubated with CART at 10^{-5} M for 24 h before incubation completely blocked the immunoreaction.

Morphometric Analysis

The area occupied by CART-immunoreactive cells/fibers in the CeA, BNSTl and AcbSh was evaluated using microscopic images from predetermined areas in the sections. The images (× 480) were analyzed using Leitz-LaborLux S microscope, CCD video camera system (JVC, Japan) and Leica-Qwin Standard software (version 3). The method has already been standardized in our laboratory (Sakharkar *et al*, 2005).

The area (μm^2) covered by CART immunoreactivity in the cells/fibers was estimated from the transverse sections passing through the CeA, BNSTl, and AcbSh from the pair-fed and ethanol-withdrawn rats. Figures 6 and 7 demarcate the rectangular area in each section from which the cell/fiber area was evaluated. The images of immunoreactive cells/fibers were digitized, the background was considered as threshold, and areas occupied by immunostained cells/fibers were measured based on individual pixel intensity in the pair-fed and rats subjected to ethanol withdrawal. The immunoreactive cells/fibers above the threshold were filled with overlaid color, and the area of the color overlay was automatically obtained using Leica-QWin Standard software. Five measurements from predetermined fields of the CeA, BNSTl and AcbSh on both sides of each brain were taken. The data from all the animals in each group were pooled separately and the mean ± SEM calculated.

Statistical Analyses

For the dose-dependent study of CART in the social interaction test, statistical significance was determined by

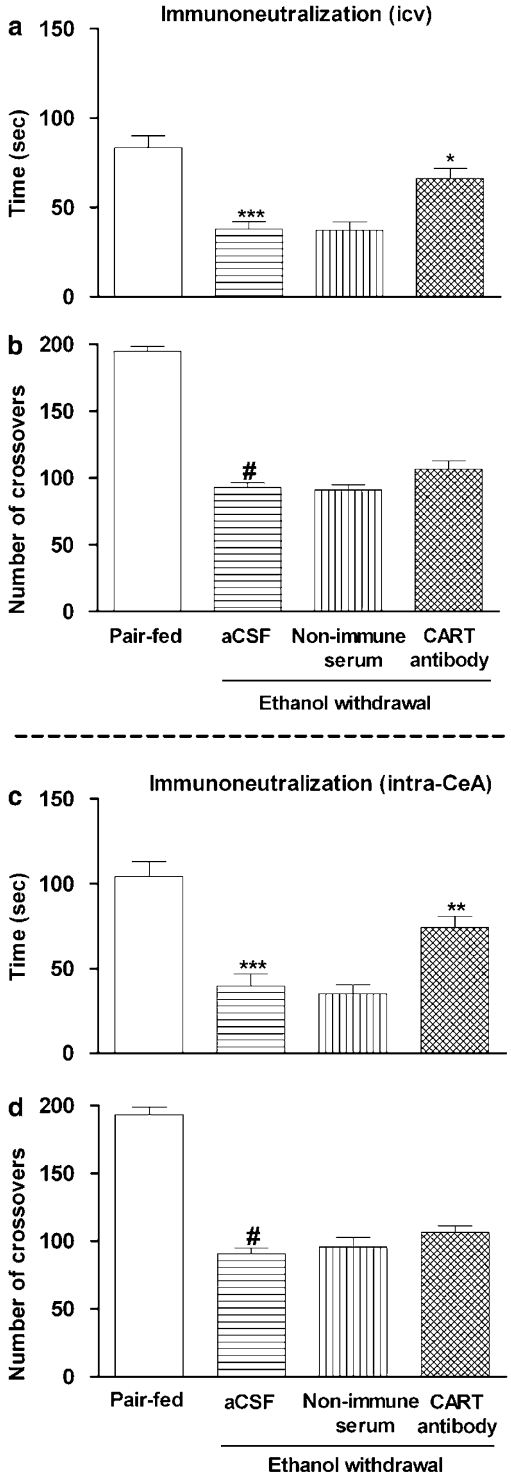

Figure 4 Effect of aCSF, non-immune serum, and CART antibody administered by icv or intra-CeA route on the social interaction time (a and c) and number of crossovers (b and d) in pair-fed and/or 24 h ethanol-withdrawal rats. The agents were administered 2 h before the test. The data were analyzed by unpaired *t*-test or one-way repeated measures ANOVA followed by Student-Newman–Keuls test. Each bar is the mean ± SEM for 6–8 rats. $^{***}P<0.0002$ and $^{\#}P<0.0001$ *vs* pair-fed group; $^{*}P<0.01$ and $^{**}P<0.001$ *vs* non-immune serum-treated group following 24 h ethanol withdrawal.

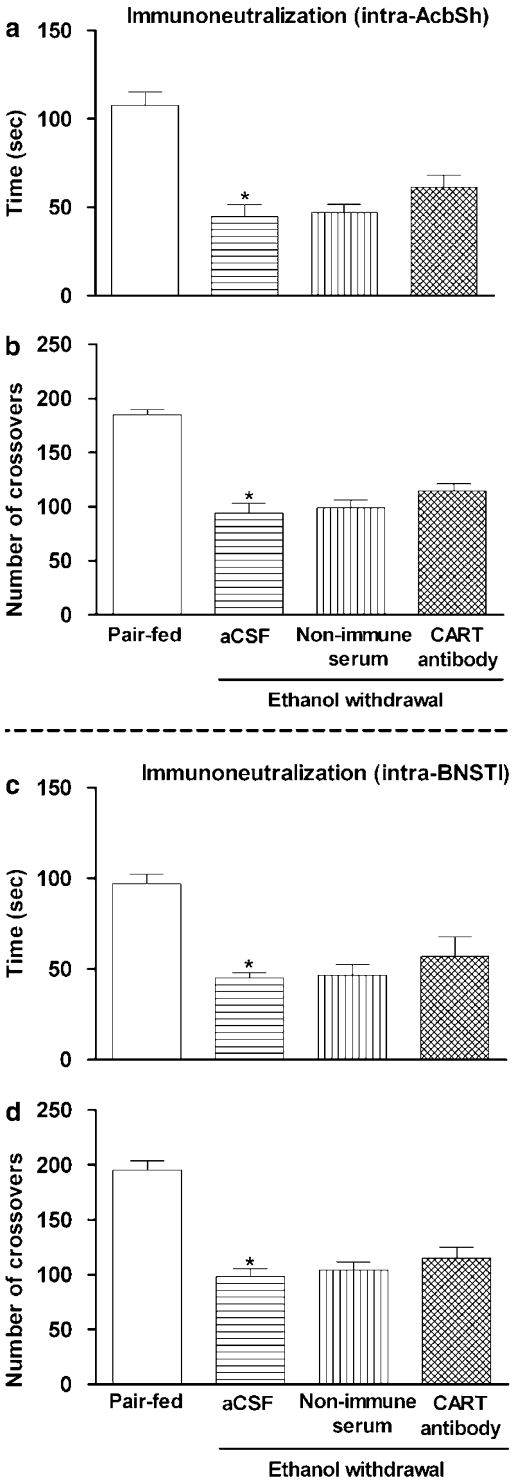

Figure 5 Effect of aCSF, non-immune serum and CART antibody administered by intra-AcbSh or -BNSTl route on the social interaction time (a and c) and number of crossovers (b and d) in pair-fed and/or 24 h ethanol-withdrawal rats. The agents were administered 2 h before the test. The data were analyzed by unpaired *t*-test or one-way repeated measures ANOVA followed by Student-Newman–Keuls test. Each bar is the mean ± SEM for 6–8 rats. $^{*}P<0.0001$ *vs* pair-fed rats. No significant difference was found in the non-immune serum-, and CART antibody-treated groups following 24 h ethanol withdrawal ($P>0.05$).

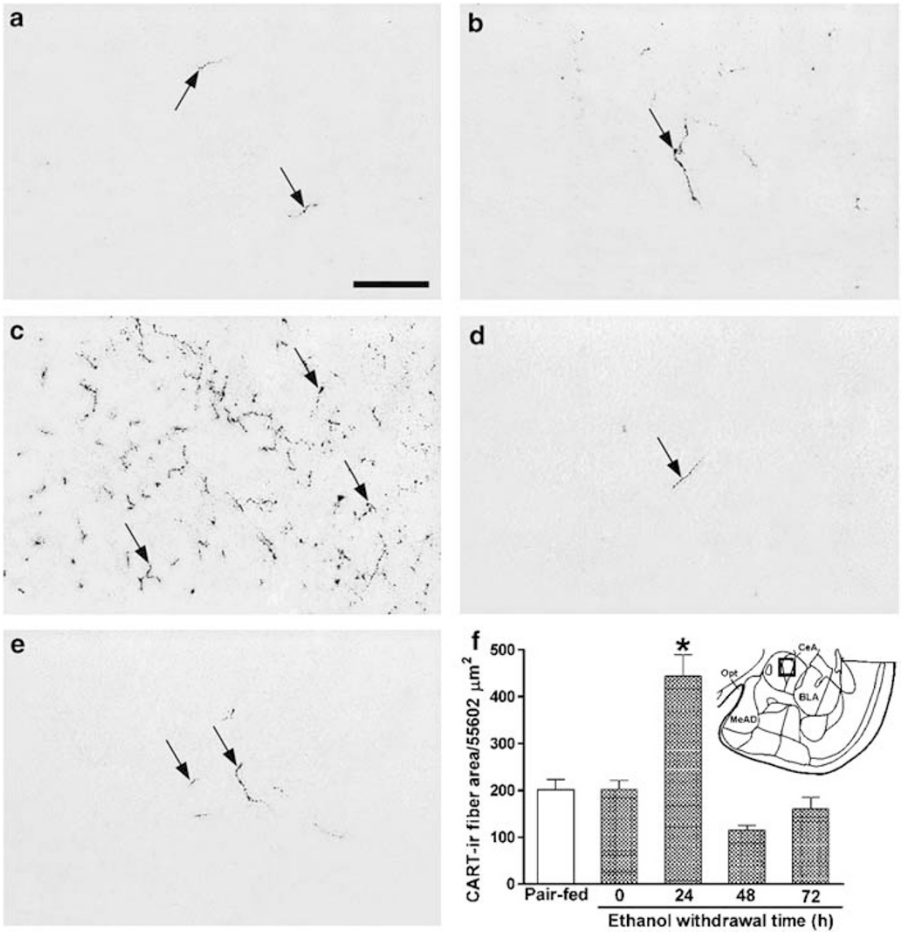

Figure 6 Photomicrographs showing CART-immunoreactive (CART-ir) fibers (arrows) in the CeA of pair-fed (a) and ethanol-withdrawn rats at 0 h (b), 24 h (c), 48 h (d), and 72 h (e). No significant difference in the CART immunoreactivity is seen in the CeA of pair-fed (a) and 0 h (b) ethanol-withdrawn rats. As compared to these animals, a dramatic increase in CART immunoreactivity is seen 24 h (c) following ethanol withdrawal. However, the CART immunoreactivity is considerably reduced in 48 h (d) and 72 h (e) ethanol-withdrawn animals; the immunoreactivity is comparable to that in the pair-fed animals. Diagram (f), represents the semiquantitative morphometric analysis of CART immunoreactivity in the CeA of pair-fed and ethanol-withdrawn animals at different time points. The outline of the transverse section through the brain (coordinates: bregma −2.56 mm, Paxinos and Watson, 1998) indicates the region of the CeA (square not to scale) from which the measurements were collated. BLA, lateral part of basolateral amygdaloid nucleus; MeAD, anterodorsal part of medial amygdaloid nucleus; opt, optic tract. *$P < 0.001$ *vs* pair-fed group. Scale bar = 50 μm.

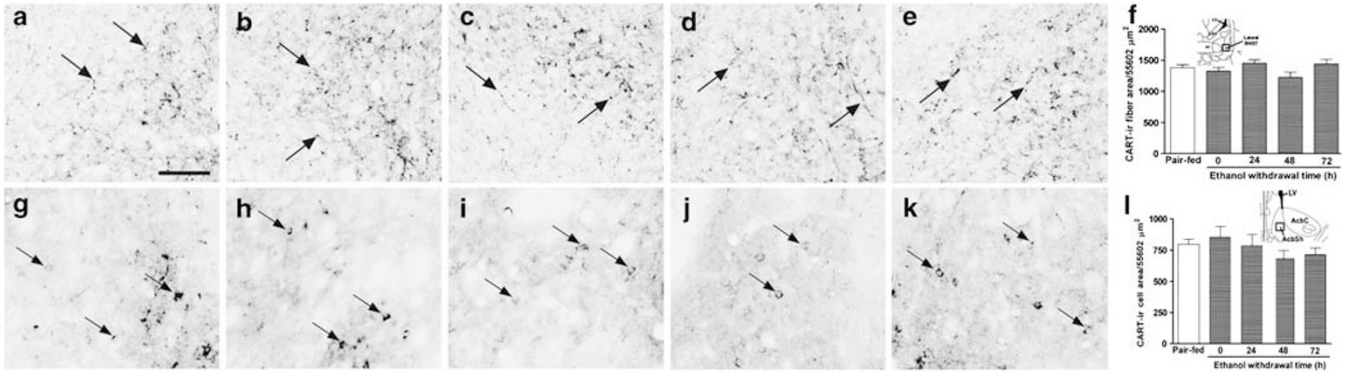

Figure 7 Photomicrographs showing CART-immunoreactive (CART-ir) fibers (arrows) in the BNSTl and CART-ir cells (arrows) in the AcbSh of pair-fed (a, g) and ethanol-withdrawn rats at 0 h (b, h), 24 h (c, i), 48 h (d, j), and 72 h (e, k). CART immunoreactivity profile is similar in the brain sections of the pair-fed (a, g) and the rats at different time points post-ethanol withdrawal (b-e, h-k, $P > 0.05$). Diagrams f and l represents the semiquantitative morphometric analysis of CART immunoreactivity in the BNSTl and AcbSh of pair-fed and ethanol-withdrawn animals at different time points. The outlines of the transverse sections through brain indicate the regions of the BNSTl and AcbSh at the coordinates −0.30 and + 1.70 mm with reference to bregma respectively (Paxinos and Watson, 1998) from which the measurements were collated (square not to scale). ac, anterior commissure; AcbC, nucleus accumbens core; LSV, ventral part of the lateral septal nucleus; LV, lateral ventricle. Scale bar = 50 μm.

one-way analysis of variance (ANOVA) with repeated measures on drug treatments followed by *post hoc* Dunnett's test. The results of ethanol-withdrawn rats at different time points were also analyzed by one-way ANOVA followed by Dunnett's test. The data obtained from ethanol-withdrawn and pair-fed rats were compared with the unpaired *t*-test. The data from immunoneutralization and morphometric studies were analyzed with one-way repeated measures ANOVA, and individual means were compared by the *post hoc* Student-Newman–Keuls test. Differences were considered significant at $P<0.05$.

RESULTS

Effect of CART in the Social Interaction Test

Control rats normally engaged in 110 s of social interaction during the 10-min test period. However, CART, at the dose of 25–100 ng/rat by the icv route, significantly and dose-dependently reduced the time spent in the social interaction ($F(3, 30) = 73.813$; $P<0.0001$), as compared to aCSF-treated rats (Figure 1a). Similarly, at the dose of 2–20 ng/rat by intra-CeA route, the time spent in the social interaction was significantly and dose-dependently reduced ($F(3, 26) = 12.337$; $P<0.0001$) as compared to that in the aCSF-treated rats (Figure 1c). On the other hand, CART at the dose of 2–20 ng/rat by intra-BNSTl or -AcbSh route, did not influence the time spent in the social interaction ($F(3, 24) = 2.111$; $P>0.05$ or $F(3, 30) = 2.103$; $P>0.05$ respectively) as compared to that in the aCSF-treated rats (Figure 2a and c). None of the above treatments caused any effect on the number of line crossings (Figures 1b, d and 2b, d) excluding effects on locomotor activity.

Effect of Ethanol Withdrawal in the Social Interaction Test

Chronic treatment of ethanol for 15 days (0 h ethanol withdrawal) did not influence social interaction time compared to the pair-fed control rats ($P>0.05$, Figure 3). However, ethanol-withdrawn rats at 24 and 48 h time points display a significant decrease ($P<0.001$ and $P<0.05$ respectively, Figure 3) in social interaction time as compared to pair-fed controls. The ethanol-withdrawn rats at 72 h showed slight augmentation in the social interaction time as compared to the 48 h ethanol-withdrawn rats, although the change was not significant ($P>0.05$, Figure 3).

Effect of Immunoneutralization of CART 24 h Following Ethanol Withdrawal in the Social Interaction Test

During the 10 min social interaction test period, ethanol-withdrawn rats displayed the behavior for 35–40 s, while the corresponding pair-fed control rats sustained interaction for approximately 95 s and the differences were found to be highly significant (compared with unpaired *t*-test, $P<0.0002$, Figure 4a and c). Ethanol withdrawal also reduced locomotor activity (Knapp *et al*, 2004; Kokare *et al*, 2006) as reflected by the lower number of crossovers during the social interaction test (compared with unpaired *t*-test, $P<0.0001$, Figures 4b, d and 5b, d). Icv (1 : 500 dilution, 5 μl/rat) and intra-CeA (1 : 500 dilution, 1 μl each side) injection of CART antibody resulted in significant reversal of ethanol withdrawal reduction in the time spent in the social interaction ($F(2, 20) = 17.340$; $P<0.0001$ and $F(2, 20) = 16.091$; $P<0.0001$ respectively), as compared to non-immune serum-treated rats (Figure 4a and c). However, the intra-AcbSh or -BNSTl (1 : 500 dilution, 1 μl each side) injection of CART antibody did not produce changes in the social interaction time ($F(2, 20) = 2.055$; $P=0.153$ or $F(2, 18) = 0.729$; $P=0.498$ respectively) as compared to non-immune serum-treated rats (Figure 5a and c). On the other hand, non-immune serum or CART antibody administered via icv (Figure 4b, $F(2, 20) = 3.354$; $P=0.057$) or directly into the CeA (Figure 4d, $F(2, 20) = 2.136$; $P=0.147$), AcbSh (Figure 5b, $F(2, 20) = 2.054$; $P=0.157$) or BNSTl (Figure 5d, $F(2, 18) = 1.042$; $P=0.376$) did not affect the reduced number of crossovers observed in the ethanol-withdrawn animals.

Effect of Ethanol Treatment and Withdrawal on CART Immunoreactivity; the Central Nucleus of Amygdala

Immunocytochemical application of antibodies against CART revealed few, weakly CART-immunoreactive fibers in the CeA of pair-fed control rats (Figure 6a) as well as in rats treated for 15 days with ethanol, and the difference was found not significant ($P>0.05$, Figure 6b). However, there was a dramatic increase in CART-immunoreactive fibers in the CeA 24 h following ethanol withdrawal ($P<0.001$, Figure 6c) as compared to the pair-fed control animals, as well as animals treated for 15 days with ethanol. Considerable reduction ($P<0.001$) in CART immunoreactivity was noticed in the CeA at 48 h following ethanol withdrawal (Figure 6d) as compared to 24 h of ethanol withdrawal. The CeA of 72 h ethanol-withdrawn rats showed slight augmentation in the immunoreactivity as compared to the 48 h ethanol-withdrawn rats (Figure 6d and e), although the change was not significant ($P>0.05$). The area covered by the immunoreactive fibers in the 72 h ethanol-withdrawn rats was similar to that in the pair-fed rats (Figure 6f).

The Lateral Bed Nucleus of Stria Terminalis and Nucleus Accumbens Shell

Although the BNSTl showed several CART-containing fibers, no cells were detected. Chronic ethanol treatment for 15 days and withdrawal at 0, 24, 48, and 72 h time points did not influence the CART-immunoreactive profile in the BNSTl ($P>0.05$, Figure 7a–f). Some CART-immunoreactive cells were seen in AcbSh area of control rats (Figure 7g). No change in CART immunoreactivity was noticed following ethanol withdrawal at different time points ($P>0.05$, Figure 7h–l).

DISCUSSION

Several studies have suggested an important role of CART in anxiety-like behavior (Kask *et al*, 2000; Asakawa *et al*, 2001; Chaki *et al*, 2003). Administration of CART (89–103) icv to rats results in a decreased ratio of open/total arm entries and reduces time spent in open part of the maze, thus suggesting increased anxiety-like behavior (Kask *et al*, 2000). Using EPM as well as social interaction tests

as behavioral paradigms, Chaki *et al* (2003) reported anxiogenic-like activity in mice following the icv administration of CART (55–102) in the dose range of 0.1–0.3 μg. Recently, the role of the CART in the modulation of anxiogenic-like effects has been reviewed (Stanek, 2006). The present results confirm and extend these findings. Administration of 50 and 100 ng CART (54–102) to normal rats by the icv route produced significant anxiety-like behavior in the social interaction test. The peptide was also given at lower doses (10 and 20 ng/rat) directly into the amygdala because this is believed to be the locus for generation of anxiety-like behavior (Adolphs *et al*, 1994; Kokare *et al*, 2005), and produced a similar response. The data suggest that CeA might be the site of anxiogenic-like action of CART. This interpretation is fully in agreement with immunocytochemistry data demonstrating that the number of CART-immunoreactive fibers in the CeA increased many fold at 24 h post-ethanol withdrawal when the anxiety-like behavior was at its peak. All the above treatments did not influence locomotor activity, and therefore effects observed in the social interaction test cannot be ascribed to alteration in locomotion.

CART is known to produce anxiety-like behavior (Kask *et al*, 2000; Chaki *et al*, 2003), which is also a cardinal sign of ethanol withdrawal, and therefore anxiety-like behavior was selected as a behavioral end point for evaluation. Results following administration of the antibodies against CART to the ethanol-withdrawn rats, icv as well as directly into the CeA, also suggest an anxiogenic role for CART. Treatment with CART antibodies at 22 h post-ethanol withdrawal reversed the peak anxiety-like behavior observed at 24 h following ethanol withdrawal. A period of 24 h was selected because peak anxiety-like behavior was reported at this time point in ethanol withdrawal studies following chronic treatment (Pandey *et al*, 1999). While, ethanol-withdrawn animals show reduction in the locomotor activity, which was in agreement with the previous studies (Knapp *et al*, 2004; Kokare *et al*, 2006), injection of CART antibody did not affect the locomotor counts. Thus, reversal of anxiety-like behavior in 24 h ethanol-withdrawn animals may not be related to the locomotion component.

In the present study, chronic ethanol treatment for 15 days did not result in any change in CART immunoreactivity in the CeA as compared to that in the pair-fed animals. The significance of this observation is not known. While icv as well as intra-CeA injection of CART produced anxiety-like behavior, ethanol withdrawal-generated anxiety-like behavior was attenuated by immunoneutralization of endogenous CART. These data suggest that CART produces its anxiogenic influence via its action in the CeA. This concept is further supported by the direct correlation between the population of CART-immunoreactive fiber in the CeA post-withdrawal at 24, 48, and 72 h with the degree of anxiety-like behavior as revealed in the social interaction test. The fiber population was maximum at 24 h post-withdrawal when the anxiety-like behavior was at its peak. We suggest, therefore, that increased synthesis as well as release of CART at this time point may be responsible for immunoreactivity as well as the behavioral profile. Further, at 48 h, when there was a considerable reduction in anxiety-like behavior, the CART fiber population was drastically reduced. We presume that at this time point, reduced secretion of CART in the CeA contributes to reduced anxiety-like behavior as compared to that at 24 h, allowing the animal to resume normal behavior. Indeed, at 72 h, when the population of the fibers was similar to that of the pair-fed controls, anxiety-like behavior was markedly reduced and not significantly different from the pair-fed animals. While the site of origin of the CART fibers in the CeA is not known, we suspect that these may originate from the hypothalamic ARC as α-melanocyte stimulating hormone (α-MSH)-containing cells of the ARC are known to coproduce CART (Barsh and Schwartz, 2002; Wittmann *et al*, 2005) and project to amygdala (Eskay *et al*, 1979; O'Donohue *et al*, 1979).

It is of interest that CART and α-MSH show a considerable degree of overlap in their distribution and physiological properties. Both peptides are anorectic (Tian *et al*, 2004) and implicated in the regulation of anxiety-like behavior, depression, and stress like conditions (Kuhar *et al*, 2002; Chaki *et al*, 2003; Kokare *et al*, 2005; Goyal *et al*, 2006; Stanek, 2006). In addition, α-MSH has anxiogenic properties (Kokare *et al*, 2005) and increases in association with ethanol withdrawal-induced anxiety-like behavior (Kokare *et al*, 2006). Furthermore, administration of antibodies against α-MSH enhances the anxiolytic effect of ethanol (Kokare *et al*, 2006). Taken together, the data suggest that α-MSH and CART may jointly influence or potentiate the anxiogenic behavior through actions in the amygdala.

In addition to CeA, the BNSTl and AcbSh also showed the presence of CART-immunoreactive elements (Koylu *et al*, 1997, 1998) and are known to play a key role in drug abuse and dependence (Koob, 1992; Koob and Le Moal, 1997; Olive *et al*, 2002; Funk *et al*, 2006). CART-immunoreactive neurons have been reported in the AcbSh of rats (Salinas *et al*, 2006), and numerous studies have demonstrated an involvement of these areas in physiological responses associated with anxiety-like behavior (LeDoux *et al*, 1988; Walker and Davis, 1997). Nevertheless, administration of CART into the BNSTl and AcbSh at the same doses used for intra-CeA injection, did not alter the anxiety-like behavior assessed by the social interaction test. The administration of CART antibody in these brain sites did not influence the time spent in social interaction at 24 h following ethanol withdrawal, and the CART immunoreactivity profile following ethanol withdrawal at 0, 24, 48, and 72 h time points remained unchanged. Taken together, the data indicate that the CeA, and not BNSTl and AcbSh, is the primary locus of action of CART to produce anxiety-like behavior following ethanol withdrawal. Funk *et al* (2006) have similarly observed that the role of corticotropin releasing hormone to enhance ethanol self-administration in ethanol-withdrawn and -dependent rats is mediated in the CeA whereas BNSTl and AcbSh may not be involved.

CART has been extensively implicated in drug reward, reinforcement, and addiction (Kuhar *et al*, 2002; Salinas *et al*, 2006; Vicentic and Jones, 2007) and therefore, the possibility exists that CART may be closely linked with ethanol pharmacology. The present study suggests a possible correlation between ethanol withdrawal-generated anxiety-like behavior and the CART-containing system in the CeA of the rat. We suggest that chronic ethanol exposure may cause functional adaptations in the CART

neuronal circuitries in the CeA, heightening expression following ethanol withdrawal and precipitating anxiety-like symptoms.

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DISCLOSURE/CONFLICT OF INTEREST

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