Ethanol enhancement of cocaine- and amphetamine-regulated transcript mRNA and peptide expression in the nucleus accumbens

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Abstract
Cocaine- and amphetamine-regulated transcript (CART) is a peptide neurotransmitter that has been implicated in drug reward and reinforcement. CART mRNA and peptide expression are highly concentrated in several compartments of the mesolimbic reward pathway. Several lines of evidence suggest that CART peptides may contribute to rewarding behaviors and the addiction liability of psychostimulants; however, there are no reports of basic work concerning CART in relation to alcohol and mechanisms of alcohol dependence development. Therefore, in this study we investigated the response of CART transcript and peptide to acute ethanol administration in vivo. Rats were administered ethanol (1 g/kg or 3.5 g/kg, 1 h, ip) and CART expression was measured by RT-PCR in the nucleus accumbens (NAcc). Ethanol (3.5 g/kg) increased CART transcription markedly. The interactions of dopamine on ethanol-induced CART expression were further evaluated pharmacologically using D1 and D2/D3 receptor antagonists. Both SCH 23390 (0.25 mg/kg) or raclopride (0.2 mg/kg) pre-treatment significantly suppressed ethanol-enhancement of CART mRNA transcription. Confocal immunofluorescence microscopy revealed that CART peptide immunoreactivity was also enhanced in both the core and the shell of the NAcc by ethanol administration. These findings demonstrate that CART mRNA and peptide expression are responsive to acute ethanol administration in vivo and suggests that CART peptides may be important in regulating the rewarding and reinforcing properties of ethanol.

Keywords: accumbens, Cocaine- and amphetamine-regulated transcript, dopamine, ethanol, reward.

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The mesolimbic dopamine system is postulated to be a significant neural network critical for mediating the rewarding effects of drugs of abuse. In particular, ethanol and most drugs of abuse display a common propensity to increase extracellular dopamine in the nucleus accumbens (NAcc), a key component in the neuronal circuitry of the mesolimbic dopamine system (Di Chiara and Imperato 1988; Pontieri et al. 1995; Pontieri et al. 1996; Tanda et al. 1997). The dopaminergic hypothesis of ethanol reward is further supported by several observations including, but not limited to the observations that: (1) increased extracellular dopamine release in the NAcc in rats self-administering ethanol (Weiss et al. 1993; Gonzales and Weiss 1998; Yim et al. 1998; Yim and Gonzales 2000); (2) dopamine receptor D\(^{-1}\) antagonists reduce ethanol operant responding (Hodge et al. 1993; Rassnick et al. 1993; Samson et al. 1993); (3) D1-null and DARPP-32 knock-out mice show a failure in ethanol self-administration (El-Ghundi et al. 1998; Risinger et al. 2001), and (4) ethanol directly excites dopaminergic ventral tegmental area (VTA) neurons (Brodie et al. 1999; Shen 2003). Thus, there exists strong evidence that accumbal dopaminergic systems contribute substantially to the development and/or expression of alcohol addiction.

Given the significance of the mesolimbic dopamine system in drug and alcohol addiction, the identification of novel genes that may regulate or modulate dopaminergic neurotransmission would seem to present a particularly compelling pursuit. One potential novel candidate gene product demonstrated to play a role in the drug addiction process is the recently identified neuropeptide, Cocaine-and Amphetamine-Regulated Transcript (CART) peptide. CART mRNA was originally identified to be up-regulated in the striatum of...
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rats following acute administration of psychostimulants (Douglass et al. 1995). Immunocytochemical studies revealed that CART peptides are expressed in key brain regions associated with drug reward and reinforcement, including the NAcc and VTA (Smith et al. 1997; Koylu et al. 1998; Smith et al. 1999). Taken together, these reports suggest that CART expression may be ethanol responsive.

Considerable evidence exists to suggest that CART peptides have a significant regulatory effect on the mesolimbic dopamine system. In the VTA, CART peptide immunoreactive fibers are located proximal to dopaminergic neurons with some CART peptide-positive terminals forming synapses onto dopamine neurons and GABAergic interneurons (Koylu et al. 1998; Dallvechia-Adams et al. 2002). In the NAcc, CART peptide is present in a subset of GABAergic projection neurons that express substance p and receive a dopamine input (Smith et al. 1997; Koylu et al. 1998; Dallvechia-Adams et al. 2002; Hubert and Kuhar 2005; Yang et al. 2005). In this manner, CART may modulate dopamine activity by two mechanisms: directly via stimulation of dopaminergic neurons or indirectly via disinhibition of GABA release (Dallvechia-Adams et al. 2002). Therefore, in the NAcc, CART could have a direct effect on GABA release and in this manner may regulate GABAergic transmission, thus facilitating dopaminergic transmission. Coincidentally, a recent report from Yang et al. (2005) demonstrated a CART peptide interaction with GABAergic neurotransmission in the NAcc to regulate feeding behavior. It is particularly significant that GABA is thought to be an important component in mechanisms underlying the rewarding and reinforcing properties of ethanol (Chester and Cunningham 2002; Davies 2003; Koob 2004).

Conversely, there is evidence to suggest that dopamine can exert some control over CART peptide synthesis and utilization. Currently, a CART-specific receptor remains to be identified; however, drugs specific for D1 or D3 dopamine receptors have been shown to affect CART transcription in the NAcc (Beaudry et al. 2004; Hunter et al. 2004). Additionally, systemic haloperidol, a D2 receptor antagonist, blocked the effects of CART peptide on locomotor activity following local injection into rat VTA (Kimmel et al. 2000).

There is also evidence for CART in the neuroadaptative effects associated with chronic drug use in humans. Microarray data from two groups found CART transcript up-regulated in mesolimbic dopamine structures from victims of cocaine overdose (Tang et al. 2003; Albertson et al. 2004). Moreover, polymorphisms in the CART gene are associated with alcoholism in a population of Korean males (Jung et al. 2004). Together, with previous animal model data, alterations in CART gene expression may be associated with long-term neuroadaptive changes observed in the chronic-exposed human brain.

Given the strong evidence for CART peptides in rewarding behavior and addiction liability, we were surprised by the paucity of information regarding CART and ethanol interactions. Therefore, in this study, we investigated the response of CART transcript and peptide to ethanol administration in vivo. Furthermore, we examined several possible mechanisms by which ethanol might modulate CART expression using dopamine receptor antagonists. Finally, in this study we focused our investigation on the nucleus accumbens because the role of mesolimbic dopamine in the behavioral response to both natural and drug-associated reward is best established in this brain region.

Materials and methods

Animals
Male Sprague-Dawley rats weighing approximately 100–150 g (30–35 days old) were obtained from the University of Texas Animal Resource Center. Animals were housed communally (3 per cage) with access to food and water ad libitum. All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were habituated to handling and intraperitoneal (ip) injections daily for four days prior to the day the experiment was carried out. The handling process included 15 min of contact with the rats followed by a sham ip injection and then transfer to a novel cage environment for 15 min prior to being returned to their homecage. To assess the dose–response of acute ethanol administration on CART mRNA expression, rats received either 1.0 or 3.5 g/kg ethanol or saline (volume matched) ip. Following drug administration, rats were segregated into the novel cages by treatment group. For the antagonist studies, rats received raclopride (0.25 mg/kg, ip), SCH 23390 (0.2 mg/kg, ip) or volume-matched saline, 25 min prior to injection of 3.5 g/kg EtOH or saline. After one hour, rats were either sacrificed by rapid decapitation for RT-PCR or anesthetized with chloral hydrate for perfusion studies.

Results were calculated from total of 6–7 animals per experimental group.

Drug and antibodies
Raclopride was purchased from Tocris Cookson, Inc (Ellisville, MO, USA). SCH 23390 was from Sigma-Aldrich (St Louis, MO, USA). Molecular grade ethanol and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trizol Reagent, Superscriptase II Reverse Transcriptase (SSII) kit, Taq DNA polymerase and PCR Reagent system were purchased from Invitrogen (Carlsbad, CA). QuantumRNA18S internal standards were purchased from Ambion, Inc. (Austin, TX, USA). Anti-rabbit CART antiserum was purchased from Phoenix Pharmaceuticals (Belmont, CA). Anti-mouse GABA antiserum was purchased from Sigma-Aldrich.

Reverse transcription-PCR
Following decapitation, brains were rapidly removed on ice and tissue from the nucleus accumbens was dissected using a 3 mm micropunch. Tissue was immediately homogenized in 1 mL Trizol Reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated in accordance with manufacturer’s protocol and stored at −20°C until use. First-strand cDNA was reverse transcribed using the Super-
Technology (Skokie, IL, USA). The sequence of the rat CART specific primers were synthesized by Integrated DNA Technologies.

Immunofluorescence
Following the in vivo experimental design described above, male Sprague–Dawley rats were administered ip injections of saline or ethanol and 60 min later transcardially perfused with PBS followed by 4% paraformaldehyde under chloral hydrate anesthesia and according to IACUC guidelines. Frozen coronal sections (25 μm) of the NAcc were prepared using a Leica cryostat (Meyer Instruments, Houston, TX, USA) and were mounted in rostro-caudal sequence on subbed glass slides. Slices were permeabilized for 30 min in 0.2% Triton in PBS and then blocked in 10% goat serum in 0.2% Triton-PBS for 2 h. Tissue sections were simultaneously double-labeled with anti-rabbit CART antiserum (1 : 2000; Phoenix Pharmaceuticals, Belmont, CA) and anti-mouse GABA antiserum (1: 1000; Sigma) in 2.8% goat serum and 0.2% triton-PBS and incubated 24 h at 4°C. After rinses in 0.2% triton-PBS, slides were incubated with AlexaFluor goat anti-rabbit 488 and goat anti-mouse 647 (1 : 1000, Molecular Probes) for 1 h at 23°C. Fluorescence was detected by laser scanning confocal microscopy on an Olympus IX 70 inverted microscope equipped with a Fluoview FVX confocal scanhead (Olympus USA, Farmingdale, NY, USA). The PMT settings for the first section on the slide were determined using the auto-exposure function in the Fluoview program. Subsequent sections on each slide were scanned using the same exposure settings for that slide. The between-slide variability of the PMT settings was less than 10%. Images were captured with a 30 s scan using a 20x UPlanFl Olympus objective. Scans were analyzed with Metamorph image analysis package (v 6.0, Universal Imaging Corporation, Downingtown, PA, USA) and fluorescence intensity was calculated using a grid of 5 μm regions-of-interest transposed over each image under identical conditions for threshold level (for 12 bit images = 1050). Analysis of CART peptide fluorescence was calculated in NAcc slices through-out medial-to-caudal regions (bregma +1.60 to +0.70 mm), where the core and shell were clearly distinguishable (Heimer and Alheid 1991). Specifically, for quantification, two planes were matched for saline and ethanol based on identifiable morphology (rostal; (+ 1.6) anterior commissure is located at the ventrolateral corner of the NAcc; caudal; (+ 1.0) the NAcc surrounds the a. commissure) (Palkovits and Brownstein 1988; Paxinos and Watson 1998). The values for each subregion were then averaged across each subject group (saline vs. ethanol) and the results were reported as the mean optical density of CART fluorescence.

Statistics
The data were analyzed with one-way ANOVA for between group comparisons. Post-hoc analysis was used to determine significance compared to control.

Results
In vivo characterization of ethanol and dopamine receptor pharmacology on CART
mRNA expression
CART mRNA expression was measured by amplification of a 160 bp primer sequence specific for the rat CART 55–105 peptide fragment. Results were calculated as a ratio of CART mRNA to the 18S ribosomal RNA internal standard (488 bp) (Fig. 1a). Acute administration of 3.5 g/kg ethanol produced a significant increase in CART mRNA expression in the NAcc (one-way ANOVA $F_{2,16} = 6.30, \ p < 0.05$). CART transcript in the NAcc was increased 30% from animals dosed with 3.5 g/kg ethanol compared with those dosed with saline or 1 g/kg ethanol (Fig. 1b). In general, all animals dosed with 3.5 g/kg ethanol exhibited signs of physical intoxication within 15 min (i.e. ataxia, sedation, hypnosis) (Majchrowicz 1975).

Next, we assessed the modulation of ethanol-induced CART expression in the presence of systemically active antagonists for dopamine D1 and D2/D3 receptors (Fig. 2). Injection acclimated rats were pre-treated with SCH 23390, a D1 receptor antagonist or raclopride, a D2/D3 receptor antagonist, for 25 min prior to 3.5 g/kg ethanol administration for 1 h. Ethanol-induced CART mRNA expression was significantly decreased by 58% with pre-treatment of SCH 23390 (Fig. 2a). Similarly, pre-treatment with raclopride significantly decreased ethanol-induced CART mRNA expression by 53% (Fig. 2b). It is relevant to note that biological activity of raclopride is more selective for D2 receptors at the dose used in this experiment (Seeman and Van Tol 1994).

Ethanol increases CART immunoreactivity in the nucleus accumbens
CART immunoreactivity was detected in both the core and the shell of the NAcc as previously reported (Koylu et al. 1998; Smith et al. 1999) (Fig. 3). In saline treated animals, a dense network of CART immunoreactive fibers were observed to follow a rostral to caudal gradient (Fig. 3a). Under saline conditions, the mean density of CART peptide (averaged optical density of slices from accumbal sections 1.6–0.7 mm anterior to bregma) was greater in the shell than in the core ($5.7 \pm 1.5$ and $2.4 \pm 0.8$ arbitrary O.D. units, respectively) (Figs 3b, c). Ethanol dose-dependently increased CART immunoreactivity in both the NAcc core and shell. In the shell, the mean optical density increased...
2.4- and 3.4-fold after 1 and 3.5 g/kg ethanol treatment, respectively. In the core, the effect of ethanol on CART peptide immunofluorescence was considerably greater. The mean optical density for CART peptide staining in the core increased 4.5-fold in response to 1 g/kg and 6.4-fold with 3.5 g/kg ethanol. Using double-labeled immunofluorescence, we confirmed that CART and GABA colocalize in a population of neurons in the NAcc (Fig. 4). In rats that received 3.5 g/kg ethanol, numerous CART-labeled neurons were observed (Fig. 4b). Moreover, CART-labeled varicosities were observed in accumbal tissues from animals dosed with 3.5 g/kg ethanol (arrows, Fig. 4b). Cells immunoreactive for both CART and GABA are indicated with arrowheads in Fig. 4.

Discussion

The results of this study demonstrate that (1) acute in vivo administration of an intoxicating dose of ethanol induces CART transcript in the NAcc; (2) both D1 and D2/3 antagonists block the ethanol-induced increase in CART mRNA expression; (3) CART peptide immunoreactivity in the core and the shell is dose-dependently increased by moderate and high ethanol doses; (4) the core of the NAcc displays a greater sensitivity to the effects of ethanol on CART immunoreactivity. The present findings are the first to
demonstrate the modulation by ethanol on CART mRNA and peptide expression.

Our finding that ethanol increased CART expression in the NAcc is similar to the initial report by Douglass et al. (1995) describing the up-regulation of CART transcript in the striatum and nucleus accumbens of rats after acute administration of cocaine and amphetamine. Subsequent studies have demonstrated significant regulatory interactions of CART peptides with the mesolimbic dopamine circuitry (Koylu et al. 1998; Kimmel et al. 2000; Jaworski et al. 2003; Yang et al. 2004). However, while CART peptides may represent an important target underlying addictive processes, little is known about their coupling and regulatory mechanisms.

A CART-specific receptor has yet to be identified; however, several recent reports suggest that CART signaling is mediated through G-protein coupled receptors (GPCR) involving Gi/Go (Yermolaieva et al. 2001; Lakatos et al. 2005). In electrophysiological studies, Yermolaieva et al. (2001) showed CART peptide inhibition of pertussis toxin-sensitive voltage-gated Ca2+ channels in hippocampal neurons. In a pituitary-derived cell line, Lakatos et al. (2005) provided evidence of a CART responsive GPCR that couples to Gi/Go and activates an ERK signaling pathway. The success of a recent binding study by Keller et al. (2005) of CART-GFP binding to HepG2 and dissociated hypothalamic cells may be key for the development of CART-specific antagonists to aid in the identification of CART receptors. In this study, we hypothesized that the effects of ethanol on CART mRNA may be mediated, in part, via complex mechanisms involving dopaminergic signal transmission in the NAcc. There is plausible evidence to suggest that the

Fig. 3 Ethanol enhances CART peptide immunoreactivity in the core and shell of NAcc. (a) Representative confocal images of CART immunofluorescence from medial-to-caudal (bregma 1.6–0.7 mm) serial accumbal sections following systemic administration of saline (top panel) or 3.5 g/kg ethanol (bottom panel) for 1 h (b) and (c) Bar graph represents the quantification of CART peptide immunofluorescence measured in the core and the shell of accumbens following saline, 1 g/kg or 3.5 g/kg ethanol for 1 h. Mean optical density was calculated from sections from the same accumbal region and standardized to control. One-way ANOVA analysis of the shell indicates significant effects, $F_{2,21} = 24.8, p < 0.001$; Dunnett’s test was used for post hoc analysis; *$p < 0.002$; **$p < 0.0001$ compared to control. One-way ANOVA of the core indicates significant effects, $F_{2,16} = 13.5, p < 0.0005$; Post-hoc analysis, *$p < 0.01$; **$p < 0.0003$ compared to control. (scale bar = 200 m). shNAcc = shell NAcc; cNAcc = core NAcc; ac = anterior commissure.
effects of ethanol on CART transcript may be modulated via downstream effectors of dopamine signaling pathways. One such mechanism might involve the cAMP-responsive element binding protein (CREB) (Dudman et al. 2003; Carlezon et al. 2005; Pandey 1998, Dudman et al. 2003). Ethanol-induced changes in CREB phosphorylation and CRE-mediated gene expression are important molecular events thought to contribute to reward and reinforcement (Pandey 1998; Yang et al. 1998; Asher et al. 2002; Newton and Messing 2005; Pandey et al. 2005). Therefore, it is notable that the CART gene promoter contains a CRE consensus site activated via a cAMP/PKA-mediated mechanism (Barrett et al. 2002; Dominguez et al. 2002). In this manner, ethanol activation of dopamine D1 receptors coupled with intracellular signaling pathways regulating CREB function, could induce CART expression in the NAcc. However, since D1 receptor antagonism was only moderately effective on the transcriptional regulation of CART by ethanol, it is obvious other mechanisms must exist.

Indeed, the effectiveness of raclopride in reducing ethanol-induced CART transcript suggests that signaling pathways modulated via dopamine D2 or D3 receptors should also be considered. Dopamine D2 or D3 receptor Gi/Go-protein coupled signal transduction modulates many signaling pathways, including MAPK and CREB (Yan et al. 1999). Striatal D2 and D3 receptors are negatively coupled to adenylate cyclase and inhibit cAMP formation (Missale et al. 1998). Recent evidence from Kuhar et al. (2005) suggests that D3 receptor activation decreases CART transcript and peptide expression in the NAcc, possibly by inhibition of CREB function. In this way, we hypothesize that dopamine receptor activation is a critical early event coupling dopamine stimulation and gene regulation to influence CART expression in the NAcc.

The core and shell subregions of the NAcc have distinct biochemical and functional responses to primary reinforcers (Di Chiara and Imperato 1988; Bassareo and Di Chiara 1999) as well as to drugs of abuse (Ito et al. 2000; Ito et al. 2004). In our study, moderate levels of CART immuno-positive cells and fibers could be detected in both the core and shell of the NAcc under basal conditions. We demonstrate that CART peptide expression is enhanced by ethanol in a similar dose-dependent manner in both the shell and the core of the NAcc. We also detected a greater than 6-fold increase over control
of CART staining in the core following 3.5 g/kg ethanol administration; while in the shell, CART staining increased slightly more than 3-fold with the same ethanol dose. We speculate that functional differences in the core and shell subregions may contribute to differences in the regulation of CART synthesis and/or expression.

The importance of CART peptides in drug reward and reinforcement is a very recent development. The identification of CART-specific receptors and the development of pharmacological regulators will indeed contribute to the significance of these neuropeptides in the mechanisms underlying drug addiction and dependence. In this study, we demonstrate that CART mRNA and peptide expression is sensitive to moderate or high doses of ethanol administered in vivo. While at present, the contributions of CART peptides to the addictive liability of ethanol are unclear, the findings from this study are significant for the identification of novel mechanisms underlying the rewarding and reinforcing properties of drugs of abuse. Furthermore, more in depth biochemical and electrophysiological studies will greatly contribute to the understanding of the intricacies of ethanol, dopamine and CART interactions.

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